Role of Interleukin 17 in Inflammation, Atherosclerosis, and Vascular Function in Apolipoprotein E–Deficient Mice

Meena S. Madhur, Samuel A. Funt, Li Li, Antony Vinh, Wei Chen, Heinrich E. Lob, Yoichiro Ikawura, Yelena Blinder, Ayaz Rahman, Arshed A. Quyyumi, David G. Harrison

Objective—Interleukin 17A (IL17A) is involved in many inflammatory processes, but its role in atherosclerosis remains controversial. We examined the role of IL17A in mouse and human atherosclerosis.

Methods and Results—Atherosclerosis was induced in apolipoprotein E (ApoE)−/− and IL17A/ApoE−/− mice using high-fat feeding, angiotensin II infusion, or partial carotid ligation. In ApoE−/− mice, 3 months of high-fat diet induced interferon-γ production by splenic lymphocytes, and this was abrogated in IL17A/ApoE−/− mice. IL17A/ApoE−/− mice had reduced aortic superoxide production, increased aortic nitric oxide levels, decreased aortic leukocyte and dendritic cell infiltration, and reduced weight gain after a high-fat diet compared with ApoE−/− mice. Despite these favorable effects, IL17A deficiency did not affect aortic plaque burden after a high-fat diet or angiotensin II infusion. In a partial carotid ligation model, IL17A deficiency did not affect percentage of stenosis but reduced outward remodeling. In this model, neutralization of the related isoform, IL17F, in IL17A/ApoE−/− mice did not alter atherosclerosis. Finally, there was no correlation between IL17A levels and carotid intima-media thickness in humans.

Conclusion—IL17 contributes to vascular and systemic inflammation in experimental atherosclerosis but does not alter plaque burden. The changes in plaque composition caused by IL17 might modulate plaque stability. (Arterioscler Thromb Vasc Biol. 2011;31:1565–1572.)

Key Words: atherosclerosis • cytokines • nitric oxide • superoxide • vascular biology

Atherosclerosis is a complex inflammatory disease characterized by derangements in the vascular, metabolic, and immune systems. Activated T cells, particularly CD4+ T helper cells, are found in atherosclerotic lesions, and their role in plaque development varies depending on the subset. In 2005, a novel T helper subset that produces the unique cytokine, interleukin 17 (IL17), designated Th17 cells, was described.1 There are 6 known isoforms of IL17, designated A through F, of which IL17A and IL17F are produced by Th17 cells.2 IL17A is the most widely studied and mediates many autoimmune and inflammatory diseases (reviewed in Tesner et al). Other sources of IL17 include CD8+ T cells, γδ T cells, natural killer T cells, natural killer cells, and neutrophils.3

See accompanying article on page 1465

IL17 has been detected in human atherosclerotic lesions.4 Eid et al5 found that human coronary artery infiltrating T cells produce IL17, interferon-γ (IFN-γ), or both and that IL17 and IFN-γ act synergistically to induce proinflammatory responses in vascular smooth muscle cells. Patients with acute myocardial infarction and unstable angina have increased peripheral Th17 cells and IL17 levels.6 We previously showed that IL17A is required for the maintenance of angiotensin II–induced hypertension and vascular dysfunction, both of which are risk factors for atherosclerosis.7 One might therefore predict that IL17A is proatherogenic. In prior studies, IL17 seemed to reduce, increase, or have no effect on atherosclerosis depending on the experimental model and method of inhibition.8–16 Unfortunately, most of the approaches used in these prior reports could be complicated by nonspecific/off-target effects of the interventions or incomplete IL17 suppression.

We therefore sought to accurately examine the role of IL17 in atherosclerosis by generating IL17A/apolipoprotein E (ApoE)−/− double-deficient mice and to inducing lesions using 3 separate models (high-fat diet, angiotensin II infusion, and partial carotid ligation). We also compared carotid intima-media thickness, a surrogate marker of early atherosclerosis, with serum levels of IL17A in a population of relatively healthy humans aged 50 to 69 years. Our results indicate that although IL17A modulates some aspects of systemic and vascular inflammation and vascular function, inhibition of IL17A is insufficient to decrease atherosclerotic plaque burden.

Materials and Methods

Animals and Induction of Atherosclerosis

The Institutional Animal Care and Use Committee at Emory University approved all animal protocols. IL17A−/− mice were generated as described in Nakae et al17 and back-crossed to the C57BL/6J background.
ground. ApoE/−/− mice on a C57BL/6J background were obtained from the Jackson Laboratory and crossed to IL17A/−/− mice to generate homozygous IL17/ApoE/−/− mice. At 8 to 11 weeks of age, male mice were started on a diet composed of 35 kcal% fat, 1.25% cholesterol, and 0.5% cholate (catalog no. D12336, OpenSource Diets) for 12 weeks. Other mice underwent implantation of osmotic minipumps (model 2004, Alzet Corp) for infusion of angiotensin II (A2900, Sigma-Aldrich) at a dose of 1000 ng/kg per minute for 4 weeks. In separate mice, partial left carotid ligation was performed as previously described.18 These animals were fed a high-fat diet for the next 2 weeks. To examine a potential role of IL17F, IL17A/ApoE/−/− were treated with anti-IL17F neutralization polyclonal antibody (100 μg/mouse per injection, catalog no. AF2057, R&D Systems) or its isotype control (goat IgG, 100 μg/mouse per injection, catalog no. AB-108-C, R&D Systems) once a week for 3 weeks starting 1 week before carotid ligation.

Human Carotid Intima-Media Thickness Evaluation and IL17A Determinations

Carotid intima-media thickness measured by B mode ultrasound was compared with IL17A levels (measured using reagents from R&D Systems and a Luminex platform) in 16 healthy subjects aged 50 to 69 years.

Statistics

Data are expressed as mean±standard error of the mean. A probability value ≤0.05 was considered significant.

See supplemental material, available online at http://atvb.ahajournals.org, for an expanded Materials and Methods section.

Results

IL17A Is Upregulated in Response to Diet-Induced Atherosclerosis and Promotes IFN-γ Production

To determine the role of IL17A in atherosclerosis, we first examined the effect of high-fat feeding on T cell production of IL17A. Splenic lymphocytes were isolated from ApoE/−/− mice fed a high-fat diet or normal chow (regular) diet for 3 months and cultured on anti-CD3 plates, and the culture supernatants were analyzed for IL17A using ELISA (A) or a cytokine bead array (B to D) (n=4 to 7 per group). Body weight (in grams) was measured at baseline and after 3 months of HF diet in ApoE/−/− and IL17/ApoE/−/− mice (E) (n=12 to 15 per group). Aortic superoxide production was measured by dihydroethidium–high-performance liquid chromatography (F) (n=5 to 7 per group). Aortic nitric oxide levels after 3 months of high-fat diet feeding were measured by electron spin resonance (ESR). Example ESR spectra (G) and summary data (H) are shown (n=4 to 5 per group). Data in A and H were analyzed using the Student’s t test. Data in E were analyzed using 2-way repeated-measures ANOVA. Other statistical data were analyzed using 1-way ANOVA with the Neuman-Keuls post hoc test.
shown in Figure 1A, a high-fat diet markedly increased release of IL17A by these cells.

We then examined how IL17A affects production of the Th1 cytokines IFN-γ and tumor necrosis factor-α and the Th2 cytokines IL4 and IL5. IL17A-deficient mice were crossed with ApoE-deficient mice to generate homozygous IL17/ApoE−/− mice. IFN-γ and IL5 production by splenic lymphocytes, as detected by cytokine bead array, were markedly increased by a high-fat diet in ApoE−/− mice (Figure 1B and 1D). The increase in IFN-γ, but not IL5, was significantly abrogated in IL17/ApoE−/− mice, indicating that IL17A is necessary for IFN-γ but not IL5 production in response to a high-fat diet (Figure 1B and 1D). Tumor necrosis factor-α production was not altered by a high-fat diet and was similar between ApoE−/− mice and IL17/ApoE−/− mice (Figure 1C). IL4 production was undetectable in mice fed a regular diet and was increased in response to a high-fat diet to a similar degree in ApoE−/− mice and IL17/ApoE−/− mice (data not shown). Thus, production of both Th1 (IFN-γ) and Th2 (IL4 and IL5) cytokines increased in response to a high-fat diet, but only the increase in IFN-γ was IL17A dependent.

**Effect of IL17A on Weight Gain and Plasma Lipids in Response to High-Fat Diet**

Body weight and plasma lipids were not significantly different between ApoE−/− and IL17/ApoE−/− mice at baseline (Figure 1E and Table). High-fat feeding increased weight by 2.6 g in ApoE−/− mice but not in IL17/ApoE−/−, suggesting that IL17A may play a role in weight gain in response to a high-fat diet (Figure 1E).

**IL17A Promotes Superoxide Production and Decreases Nitric Oxide Levels in Response to High-Fat Diet**

Atherosclerosis increases vascular production of reactive oxygen species and decreases nitric oxide production. We determined the effect of IL17A on aortic superoxide production using ApoE−/− and IL17/ApoE−/− mice fed a regular diet or high-fat diet for 3 months. Baseline levels of superoxide were similar between ApoE−/− and IL17/ApoE−/− mice. The high-fat diet, however, increased aortic superoxide production in ApoE−/− mice by approximately 2-fold, and this increase was significantly blunted in IL17/ApoE−/− mice (Figure 1F). Thus, IL17 is necessary for high-fat diet induced elevations in vascular superoxide.

Aortic nitric oxide levels correlate with endothelium-dependent relaxation and improved vascular function. Aortic nitric oxide levels in ApoE−/− and IL17/ApoE−/− mice fed a high-fat diet were measured using electron spin resonance. Nitric oxide production by aortic rings of IL17/ApoE−/− mice were increased compared with aortic rings of ApoE−/− mice (Figure 1G and 1H).

**IL17A Deficiency Has No Effect on Atherosclerotic Plaque Area or Aneurysm Formation but Decreases Aortic Inflammatory Cell Infiltration**

To determine the effect of IL17A on atherosclerotic plaque development, we quantified plaque area in the descending aorta (Figure 2A and 2B) and aortic root (Figure 2C and 2D) in ApoE−/− and IL17/ApoE−/− mice fed a high-fat diet for 3 months. Surprisingly, plaque area in these regions was similar between IL17/ApoE−/− mice and age-matched ApoE−/− controls. We then investigated whether plaque morphology was different using Russell-Movat pentachrome staining (Figure 3A). No major differences were noted among extra-cellular matrix components such as elastin (black), collagen (yellow), and mucins (blue-green). Flow cytometry of whole aortas, however, revealed a modest decrease in total leukocytes (CD45+ cells) and dendritic (CD11b-11c+) cells and a trend toward reduction of aortic macrophages (CD11b+F4/ 80+ cells) and T cells (CD3+ cells) in the IL17A-deficient mice (Figure 3B). Immuno-staining of atherosclerotic plaques

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**Table. Plasma Lipid Profile in Response to High-Fat Diet**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ApoE−/− (n=12), mg/dL</th>
<th>IL17/ApoE−/− (n=11), mg/dL</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>1185±83</td>
<td>1593±241</td>
<td>0.11</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>56±6.6</td>
<td>57±9.6</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Data are expressed as mean±standard error of the mean.
demonstrated a decrease in total macrophage content within lesions (Figure 3C). Thus, although plaque size and extracellular matrix content were similar between ApoE/−/− and IL17/ApoE/−/− mice, the plaques did appear to be less inflammatory in IL17/ApoE/−/− mice.

Angiotensin II enhances atherosclerosis in ApoE/−/− mice.19 Because we previously showed that angiotensin II stimulates T cell production of IL17A,9 we examined the role of IL17A in angiotensin II induced atherosclerosis. Angiotensin II (1000 ng/kg per minute) was infused via osmotic minipump for 4 weeks in ApoE/−/− and age-matched IL17/ApoE/−/− mice. Angiotensin II induced atherosclerosis in ApoE/−/− and IL17/ApoE/−/− mice to a similar extent, indicating that IL17A does not contribute to plaque development in an angiotensin II–induced model of atherosclerosis (Figure 4A). Plasma lipids following 4 weeks of angiotensin II were similar between ApoE/−/− and IL17/ApoE/−/− mice (data not shown).

Infusion of angiotensin II at this dose, 1000 ng/kg per minute, induces aneurysm formation in ApoE/−/− mice.19 We found that aneurysm formation was similar in ApoE/−/− mice and IL17/ApoE/−/− mice (18% and 20%, respectively), suggesting that IL17A does not influence aneurysm development.

IL17F Is Upregulated in IL17A-Deficient Mice and Is Increased by High-Fat Diet

IL17F is closely related to IL17A, can dimerize with IL17A, and binds to the same receptor. Von Viettinghoff and Ley20 showed that IL17F was upregulated in IL17A-deficient mice. Therefore, we examined whether IL17F was also upregulated in our IL17/ApoE/−/− mice and whether IL17F production was influenced by a high-fat diet. Interestingly, IL17F production at baseline (regular diet) was markedly increased in IL17/ApoE/−/− mice. A high-fat diet further increased IL17F production, and IL17F levels were similar in ApoE/−/− and IL17/ApoE/−/− mice after 3 months of high-fat diet (Figure

Figure 3. Effect of IL17 on plaque composition. ApoE/−/− and IL17/ApoE/−/− mice were fed 3 months of high-fat diet. A, Russell-Movat pentachrome staining in the aortic root at magnifications of 4× and 10×. Black indicates elastic fibers; yellow, collagen; blue/green, mucins; red, muscle; intense red, fibrinoid. Figures are representative of 4 mice per group. To analyze inflammatory cell content, flow cytometry of single-cell suspensions of whole aortas were performed as shown in B. Representative scatter plots are shown on the left, and summary data of n=9 to 12 mice per group are shown on the right. Data were analyzed using the Student’s t test. CD45+ cells represent total leukocytes, CD3+ cells represent T cells, CD11b−11c+ cells represent dendritic cells, and CD11b+F4/80+ cells represent macrophages. C, Plaque macrophage content in the aortic root as determined by immunostaining with a Mac3 antibody. Alkaline phosphatase (pink) was used to detect the secondary antibody. Slides were counterstained with hematoxylin. A representative of n=3 per group is shown at magnifications of 4× and 10×.
4B). This elevation in IL17F in our model raised the possibility that IL17F could be assuming a compensatory or redundant role in plaque formation in the absence of IL17A.

Neutralization of IL17F in IL17A-Deficient Mice Has No Effect on Partial Carotid Ligation-Induced Atherosclerosis

We further examined the effect of IL17A with and without concomitant IL17F neutralization in a rapid (2-week) partial carotid ligation model of atherosclerosis described by Nam et al.18 Interestingly, we found that percentage of stenosis of the ligated carotid artery was not affected by the absence of IL17A, but vessel diameter (as measured by internal elastic lamina area) was significantly decreased in the IL17A-deficient mice, indicating that IL17A may play an important role in outward remodeling of vessels under conditions of disturbed flow (Figure 5A and 5B). To determine whether IL17F compensates for the loss of IL17A during the development of atherosclerosis, we administered an IL17F neutralizing antibody weekly beginning 1 week before carotid ligation. IL17F neutralization had no effect on internal elastic lamina area or percentage of stenosis (Figure 5A and 5C), arguing against a significant role of IL17F in atherosclerosis development.

IL17A Levels Do Not Correlate With Carotid Intima-Media Thickness in Humans

Increased carotid intima-media thickness is an early subclinical marker of atherosclerosis and a predictor of future cardiovascular events.21 We examined serum IL17A levels and carotid intima-media thickness in a small population of healthy humans aged 50 to 69 years. As shown in the Supplemental Figure, there was no correlation between IL17A levels and either mean or far wall carotid intima-media thickness, suggesting that IL17A does not participate in human atherosclerotic plaque formation.

Discussion

In the present study, we found that IL17A modulates systemic and vascular inflammation by stimulating IFN-γ production and increasing vascular reactive oxygen species and vascular leukocyte infiltration, and it contributes to weight gain in response to high-fat diet. Despite these favorable effects, IL17A had no effect on gross lesion size in either mouse or human atherosclerosis. Furthermore, neutralization of a related cytokine, IL17F, had no effect on atherosclerosis in a rapid partial carotid ligation model.

To our knowledge, this is the first study to use a genetic deletion of IL17A and ApoE to study the role of IL17A in atherosclerosis. Several recent studies on this topic have provided conflicting results showing that IL17A reduces, increases, or has no effect on atherosclerotic plaque development.10–16 Differences in these results may be due to the methods used for eliminating IL17A, nonspecific effects of the interventions, or lack of complete elimination in some studies.

Despite finding no effect on plaque burden, we did find decreased vascular inflammation in the IL17A-deficient mice. We used 2 independent methods to assess vascular infiltration of inflammatory cells. We first used flow cytometry of whole aortas and found that total leukocytes and dendritic cells were significantly reduced in IL17A-deficient mice, with a trend toward reduced macrophages and T cells. Flow cytometry has the limitation that the entire aorta is analyzed, which could underestimate differences that could be present if only lesions were analyzed. We then performed immunostaining on atherosclerotic plaques and found that plaque macrophage content appeared to be reduced in IL17A-deficient mice. These results are consistent with reports by Erbel et al12 and Smith et al,13 who also found that IL17A blockade reduced plaque macrophage content. A potential mechanism for this finding is that IL17A can act on vascular...
wall cells to induce the production of numerous cytokines and chemokines, such as IL6, CXCL8, CCL5, CXCL1, GCSF3, and monocyte chemoattractant protein-2. Monocyte chemoattractant protein-2 binds to cell surface receptors such as CCR1 and CCR5, expressed on leukocytes, and CXCL1 is important in monocyte recruitment into the artery wall. In addition, monocytes express the IL17RA receptor, and IL17A directly influences monocyte chemotaxis. We and others have shown that IL17A promotes inflammation in a manner similar to that observed in the present study. Shahara et al demonstrated that in rheumatoid arthritis, IL17A promotes monocyte migration into synovial tissue via upregulation of chemokine ligand 2/monocyte chemoattractant protein-1 expression. We previously found that IL17A promotes aortic infiltration of CD45+ leukocytes and CD3+ T lymphocytes in response to angiotensin II–induced hypertension.

We found that in addition to promoting inflammation, IL17A is necessary for increased vascular superoxide (O2•−) production in response to high-fat diet–induced atherosclerosis. This is similar to our previous finding that IL17A promotes vascular O2•− production in response to angiotensin II–induced hypertension. The mechanisms by which IL17A induces vascular O2•− production remain unclear. In preliminary studies, we were unable to demonstrate a direct effect of IL17A on O2•− production in either human endothelial or vascular smooth muscle cells in culture; however, a recent report suggests that IL17A can activate the NADPH oxidase in murine vascular smooth muscle cells. It is also possible that IL17A increases superoxide production via recruiting inflammatory cells, which in turn can produce reactive oxygen species.

It is interesting that IL17A modulates a number of factors thought to be associated with plaque progression, such as
IFN-γ production, vascular inflammation, and superoxide production, and yet we did not find any effect on aortic plaque burden or carotid stenosis. Our finding that atherosclerotic plaque burden was similar despite reduced O$_2^-$ production is in keeping with prior studies showing that mice overexpressing superoxide dismutase are not protected against atherosclerosis.27,28 Moreover, plaque size does not necessarily correlate with clinical outcomes in human coronary atherosclerosis. Plaque stability is more predictive of acute plaque rupture and coronary events. Our findings suggest that IL17A deficiency, through reduced leukocyte infiltration and decreased superoxide production, could stabilize plaques. Thus, IL17A might not be an important determinant of plaque size but rather a modulator of plaque composition and stability. This is in agreement with clinical data showing that Th17 cells and IL17 levels are elevated in patients with unstable angina and acute myocardial infarction compared with patients with stable angina and controls.8

In our carotid ligation model, we observed that IL17A deficiency had no effect on percentage of stenosis in the ligated vessel, but it markedly decreased outward remodeling. Outward remodeling is a compensatory response to lesion development that preserves lumen diameter in the setting of increasing atherosclerosis burden. In this regard, IL17A$^{-/-}$ mice are similar to mice lacking matrix metalloproteinase-9, in which outward remodeling in response to total carotid occlusion is significantly impaired.29 Because reactive oxygen species are known to increase matrix metalloproteinase-9 expression and activation,30,31 it is interesting to speculate that the absence of outward remodeling in the IL17A/ApoE$^{-/-}$ mice might reflect the lower levels of O$_2^-$ observed in these animals compared with ApoE$^{-/-}$ mice.

We also show that IL17A/ApoE$^{-/-}$ mice are resistant to high-fat diet–induced weight gain. A link between IL17A and obesity was first suggested by a small clinical study showing plasma levels of IL17A were elevated in 26 obese women obesity was first suggested by a small clinical study showing that mice overexpressing superoxide dismutase are not protected against atherosclerosis.27,28 Moreover, plaque size does not necessarily correlate with clinical outcomes in human coronary atherosclerosis. Plaque stability is more predictive of acute plaque rupture and coronary events. Our findings suggest that IL17A deficiency, through reduced leukocyte infiltration and decreased superoxide production, could stabilize plaques. Thus, IL17A might not be an important determinant of plaque size but rather a modulator of plaque composition and stability. This is in agreement with clinical data showing that Th17 cells and IL17 levels are elevated in patients with unstable angina and acute myocardial infarction compared with patients with stable angina and controls.8

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We also show that IL17A/ApoE$^{-/-}$ mice are resistant to high-fat diet–induced weight gain. A link between IL17A and obesity was first suggested by a small clinical study showing plasma levels of IL17A were elevated in 26 obese women compared with 20 lean women.32 Similar to our findings, Winer et al.33 showed that diet-induced obesity increases IL17 from splenic lymphocytes. Moreover, IL17A stimulates pro-inflammatory responses in adipocytes.34 Recently, Pini and Fantuzzi35 found that neutrophil-derived IL17A is increased in obese mice during acute inflammation and contributes to inflammatory responses. These findings are in keeping with a role of inflammation in the metabolic syndrome and suggest that IL17 might contribute to this disorder.

In summary, using a genetic approach that avoids potential confounding off-target and nonspecific effects, we found that IL17A is involved in systemic and vascular inflammation and weight gain in response to a high-fat diet. Contrary to several prior reports, however, we showed that IL17A is unlikely to contribute significantly to plaque progression. Nevertheless, through its effects on reactive oxygen species and leukocyte infiltration, IL17A is an important potential mediator of plaque stability, and its effects on plaque stability and outward remodeling warrant further investigation.

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Disclosures
None.

References


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ROLE OF INTERLEUKIN 17A IN INFLAMMATION, ATHEROSCLEROSIS AND VASCULAR FUNCTION IN APOLIPOPROTEIN E-DEFICIENT MICE

Short title: Madhur  IL17A and Atherosclerosis

ONLINE SUPPLEMENT

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T cell isolation and Cytokine Analyses: Spleens were removed and disrupted using forceps to yield single-cell suspensions that were passed through 70 µm sterile filters. RBC lysis buffer (eBioscience) was used to remove red blood cells. Splenic T cells were then isolated using autoMACS and a T cell negative selection kit (both from Miltenyi Biotech) and cultured at a density of 2x10⁵ cells per well in 96-well anti-CD3 antibody-coated plates (BD PharMingen) for 48 hours in the presence of IL2 (R&D Systems, 20 U/ml). Cell culture supernatants were used for IL17A ELISA (Invitrogen), IL17F ELISA (R&D Systems) and Cytometric Bead Array (BD Pharmingen) studies according to the manufacturer’s recommendations. Cytometric Bead Array analysis allowed for simultaneous determination of TNFα, IFNγ, IL2, IL4, and IL5 concentrations. Samples were analyzed on a LSR-II flow cytometer with DIVA software (Becton Dickinson).

Plasma Lipid Analysis: Blood was collected by cardiac puncture and centrifuged. Lipid analysis was performed commercially (Cardiovascular Specialty Laboratories, Atlanta, GA). All lipid determinations were performed using a Beckman CX7 chemistry analyzer and reagents from Beckman Diagnostics (Fullerton, CA) for total cholesterol and triglycerides.
Measurement of vascular superoxide and nitric oxide production: Superoxide production by 2 mm aortic segments was measured by quantifying formation of 2-hydroxyethidium from dihydroethidium by high-performance liquid chromatography (HPLC). This product specifically reflects the reaction of superoxide with dihydroethidium as previously validated. Calcium ionophore stimulated nitric oxide production by 2 mm aortic segments was measured by electron spin resonance using the colloid probe Fe$^{2+}$ diethyldithiocarbamate (Fe[DETC]$_2$) as previously described.

Aortic atherosclerotic plaque and aneurysm analyses: After sacrifice the left ventricle was punctured and the mice were pressure perfused with 0.9% sodium chloride solution followed by pressure fixation with 10% formaldehyde solution. Hearts and aortas were stored in 10% formaldehyde until analysis. The extent of atherosclerotic lesion formation in the thoracic and abdominal aorta was analyzed as follows. The aorta was dissected free of perivascular fat, opened longitudinally, and pinned down at the edges. Areas of plaque were clearly visualized as raised white lesions against the black background of the plate. Digital photographs of the aortas were used to perform planimetry using the software package ImageJ. Lesion area was expressed as percentage of aorta. To analyze plaque area in the aortic root, the hearts and part of the ascending aorta were embedded in paraffin and serial 5\(\mu\)m thick sections containing the aortic valve were obtained. Sections were then stained with hematoxylin and eosin and plaque area was quantified using ImageJ. Data are expressed as square microns. To analyze plaque composition, Russell-Movat pentachrome staining (American MasterTech Scientific, Inc; Item No KTRMP) was performed on paraffin embedded sections of the aortic root according to the manufacturer’s protocol.

Aneurysm incidence (defined as greater than 50% dilation of the aorta) was recorded upon sacrifice and dissection of mice that were infused for 4 weeks with angiotensin II. Occasionally, mice died during angiotensin II infusion. In these cases, autopsy was performed to detect the presence of an abdominal aneurysm or a retroperitoneal hematoma, suggesting rupture of an abdominal aneurysm.

Carotid atherosclerosis analysis and Oil red O staining: Mice were sacrificed and perfused at physiological pressure with saline containing heparin. The left and right carotid arteries were removed en bloc with the trachea and esophagus. For frozen sections, tissue was embedded in Tissue-Tek optimum cutting temperature medium, frozen in liquid nitrogen, and stored at -80°C until stained. Oil red O staining was carried out using frozen sections as previously described and nuclei were stained with hematoxylin. Images were captured with a Zeiss epifluorescence microscope. Images were analyzed with Image J software to quantify lesion size as previously described.

Immunohistochemistry for macrophages: Seven \(\mu\)m frozen sections of the aortic root were prepared. Immunostaining was performed using a rat anti-mouse Mac3 antibody (BD Pharmingen; Cat No 550292) at a 1:50 dilution for 1 hour at room temperature and a secondary biotinylated goat anti-rat IgG (Vector; Cat No BA 9400) at a 1:1000 dilution for 30 min at room temperature. A Vectastain alkaline phosphatase system (Vector AK-5000 and Vector SK-5100) with levamisole (Vector SP-5000) to
inhibit endogenous alkaline phosphatase activity was used to detect the secondary antibody. All slides were counterstained with hematoxylin.

**Flow Cytometry:** A separate set of ApoE−/− and IL17/ApoE−/− mice were sacrificed to analyze cell composition within the vessel wall using flow cytometry. Whole aortas were removed and dissected free of perivascular fat. Harvested aortas were digested using collagenase type IX (125U/ml); collagenase type IS (450U/ml) and hyaluronidase IS (60U/ml) dissolved in 20 mM HEPES-PBS buffer containing calcium and magnesium for 30 minutes at 37°C, while constantly agitated. The dissolved tissue was then passed through a 70 µm sterile filter (Falcon, BD), yielding a single cell suspension. Cells were washed twice with PBS buffer and then stained with a live/dead cell stain (near infrared, Invitrogen). After washing with 1% BSA PBS buffer, cells were blocked for nonspecific Fc binding with anti-mouse CD16/32 (2.4G2) and then stained using the following fluorescent antibodies: FITC-CD45 (total leukocytes, 30-F11), PE-CD3 (T cells, 145-2C11), PerCP-Cy5.5-CD11c (HL3), APC-CD11b (M1/70) and PE-Cy7-F4/80 (macrophages, BM8). Antibodies were purchased from BD Biosciences except for PE-Cy7-F4/80 (eBioscience).

Flow cytometry was performed using a LSR-II flow cytometer with DIVA software (Becton Dickinson). Countbright counting beads (Invitrogen) were used to normalize total cell counts among samples. At least 10,000 bead events were acquired to insure the accuracy of the assay. Absolute cell count (cells/aorta) = events of cells counted/total number of beads counted x input bead number. CD11b+*F4/80*+ cells were defined as macrophages; and CD11b−CD11c+ cells were defined as dendritic cells. Gating was applied using fluorescence minus one (FMO) controls constructed with staining panels where one of the fluorescent markers of interest was replaced with an isotype control. Data were analyzed with Flowjo software 9.1 (Treestar).

**Patient population, analysis of serum IL17A levels, and carotid intima-media thickness (IMT) determinations:** Sixteen study subjects for whom serum samples and carotid IMT data were available were chosen at random from a pre-existing database at Emory University Hospital. Institutional Review Board approval was obtained for the human studies. Of these subjects, 10 were male and 6 were female. The age range was 50-69 years with a mean age of 58 years. The average BMI was 26, average systolic blood pressure 117 mmHg, and average LDL 114 mg/dL.

The Fluorokine® MultiAnalyte Profiling (MAP) Human base Kit A (Catalog #: LUH000) from R&D Systems (Minneapolis, USA) was used to determine the levels of IL17 analyte (Catalog # LUH317) on a Luminex 100 Bio-Plex platform (Bio-Rad, CA USA). All samples are centrifuged before testing and all assays are performed as directed by manufacturer.

Carotid IMT, the distance between the junction of the lumen and intima and that of the media and adventitia, was measured by means of B-mode ultrasound of the carotid arteries. IMT was quantified both on the near and far wall at the distal 1.0 cm of the left and right common carotid arteries proximal to the bifurcation and the bifurcation itself, therefore yielding a total of 8 segments (4 on each side). For each segment, the sonographer used 5 different scanning angles to identify the optimal longitudinal image of IMT (showing the greatest IMT), and 3 images of the latter were frozen and stored on
optic disk. The actual measurements of IMT were performed off-line using a semi-automated computerized analytical software (Carotid tools, MIA Inc., Iowa City, USA), by 2 observers blinded to the test results. The IMT of each of the 3 frozen images for each segment was averaged to determine the mean IMT for that segment at the near and far wall, at each side and both sides combined.

Statistics: Data are expressed as mean ± standard error of the mean. When comparing only 2 groups, Student’s t-test was used. For comparisons involving 3 or more groups, one-way ANOVA followed by Neuman-Keuls post-hoc test was employed. For body weight analysis prior to and after high fat diet, we employed ANOVA for repeated measures. For the interleukin 17F ELISA analysis, Student’s t-test was employed with a Bonferroni correction for 4 comparisons. The p values provided reflect the Bonferroni correction. A p value <0.05 was considered significant.

REFERENCES FOR ONLINE SUPPLEMENTAL MATERIALS AND METHODS


A

Serum IL17A levels (pg/ml)

Mean Carotid Intima-Media Thickness (mm)

B

Serum IL17A levels (pg/ml)

Far Wall Carotid Intima-Media Thickness (mm)
Supplemental Figure: Correlation of Serum IL17A levels with Carotid Intima-Media Thickness in Humans. No correlation was observed between mean (A) and far wall (B) carotid intima-media thickness and serum IL17A levels in 16 healthy humans aged 50-69 years (p=n.s.).
Summary

목적
IL-17A (Interleukin 17A)는 다양한 염증과정에 관여하지만 죽상경화 발생과정에서의 역할은 알려지지 않았다. 따라서 mice 및 사람에게서 죽상경화와의 연관성을 연구하였다.

방법 및 결과
Apolipoprotein E (ApoE)/-와 IL-17A/ApoE/-mice에 대하여 고지방 식이, angiotensin II infusion, 또는 부분적인 carotid ligation을 시행하였다. 3달간의 고지방 식이는 splenic lymphocytes에서 interferon-γ의 생산을 증가시켰으며, 이는 IL-17A 발현 과정에서의 관찰되지 않았다. 또한 이러한 IL-17A/ApoE/-mice에서는 aortic superoxide production이 줄어들었으며, aortic nitric oxide level이 상승되었고, aortic leukocyte 및 dendritic cell infiltration의 감소, 그리고 체중 증가 풍화가 관찰되었다. 이러한 긍정적인 변화에도 불구하고, IL-17A의 결핍을 유도하였을 때 고지방 식이, angiotensin II infusion에 의한 플라크의 부하를 줄이지 못하였다. 부분적인 carotid ligation model에서는 IL-17A 결핍이 협착의 정도를 감소시키지는 못하였으나, 외부로의 remodeling을 감소시켰다. 추가적인 isoform, IL-17F의 결핍 유도 역시 아무런 변화를 유도하지 못하였다. 사람에게서는 IL-17A level과 carotid intima-media thickness의 연관성 역시 관찰되지 않았다.

결론
IL-17이 혈관 및 전반적인 염증의 정도에는 영향을 미칠지 모르나, 플라크의 부하를 감소시키지는 못한다. 관찰된 플라크 성장의 변화는 아마도 플라크의 안정화에는 기여할 것으로 추론된다.

한 기훈 교수
서울아산병원 심장내과
**Commentary**

적상경화의 발전과 면역반응
적상경화의 발전과 연관된 대부분의 기전에는 대식세포의 역할이 관여하며, 이는 소위 즉각적인 면역반응인 innate immunity를 매개로 한다. 논문에도 언급되었듯이 대식세포 이외에도 적상경화 반에서는 T-, B-임파구 및 NK세포 등이 관찰되는데, 이들 세포가 관여하는 지연성 면역반응인 adaptive immunity가 적상경화의 경과에 관여하는 것을 의미한다. 현재 대식세포 안에도 소위 M1, M2와 같은 상반된 작용을 하는 subset이 있다고 기술되고 있지만, CD4+ T-임파구(helper cell) 안에 염증을 조장하는 소위 Th1, 염증을 억제하는 Th2 subset이 존재하여 적상경화의 발전 또는 지연을 이기한다는 가설은 오랫동안 논의되어 왔다.

**IL-17A란?**
Mice의 cytotoxic cells에서 발견이 최초 보고이며, 이후 155개의 염기서열로 이루어진 사람의 IL-17이 보고되었다. 이들은 IL-17A부터 F형까지의 6가지 isoform이 존재하는 family이며, 서로 16-50%의 상동을 가지고 있고 확연히 다른 조직 분포를 보인다. IL-17은 주로 Th1 세포에서 만들어지지만 memory T cell에서도 발현된다.

**Th17 세포란?**
Th1, Th2가 아닌 IL-17을 분비하는 CD4+ T cells의 존재가 최근에야 밝혀졌으며, 이를 Th17 cells로 명명하게 되었다. dendritic cells가 분비하는 IL-6 및 TGF-β에 의하여 naïve CD4+ T cells로부터 분화되고 dendritic cells가 분비하는 IL-23에 의하여 유지된다. Th17세포들은 IL-21을 분비하는데 이는 Th17 세포의 분화 촉진과 함께, Th1 subset의 분화를 억제한다. 또한 IL-6, 23과 같이 stat3를 활성화시켜 IL-17 및 IL-21, IL-23R 유전자의 발현을 유도한다. 최근 mice에서는 IL-1역시 IL-17 발현을 유도한다고 하며, 이들과는 반대로 IL-2, IL-27, IFN-γ, IL-4, 및 retinoic acid 등은 IL-17의 발현을 억제한다고 한다. 최근 mice에서는 IL-1역시 IL-17 발현을 유도한다고 하며, 이들과는 반대로 IL-2, IL-27, IFN-γ, IL-4, 및 retinoic acid 등은 IL-17의 발현을 억제한다.

**IL-17A과 적상경화**
고찰에 언급된 바와 같이 IL-17은 다양한 염증성 cytokines, chemokines의 발현, ROS 생성의 매개 등 기본적으로 적상경화를 증가시킬 수 있는 기전들을 활성화시키므로, 적상경화를 유발할 수 있는 물질로 판단될 수 있다. 한편으로는 Th1 아형의 분화를 억제하는 등, 적상경화를 억제할 수 있는 주전적인 잠재적 역할도 가지고 있다. 이러한 상황에서 IL-17의 결핍으로 적상경화반의 부하자체를 줄이지 못하였다는 것은, 적어도 IL-17은 적상경화를 진행하는 주 기전으로 인정될 수 없음을 의미한다. 플라크의 안정화라는 측면에서 저자들은 플라크의 안정화를 위해 IL-17A 또는 IL-17F의 결핍이 대식세포의 MMP tissue factor의 발현 또는 INF-γ 등의 발현에 어떠한 영향을 미치는지에 대하여 추가적인 기전 연구가 필요할 것으로 기대된다.

**REFERENCE**
Role of Interleukin 17 in Inflammation, Atherosclerosis, and Vascular Function in Apolipoprotein E-Deficient Mice

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Objective—Interleukin 17A (IL17A) is involved in many inflammatory processes, but its role in atherosclerosis remains controversial. We examined the role of IL17A in mouse and human atherosclerosis.

Methods and Results—Atherosclerosis was induced in apolipoprotein E (ApoE)−/− and IL17A/ApoE−/− mice using high-fat feeding, angiotensin II infusion, or partial carotid ligation. In ApoE−/− mice, 3 months of high-fat diet induced interferon-γ production by splenic lymphocytes, and this was abrogated in IL17A/ApoE−/− mice. IL17A/ApoE−/− mice had reduced aortic superoxide production, increased aortic nitric oxide levels, decreased aortic leukocyte and dendritic cell infiltration, and reduced weight gain after a high-fat diet compared with ApoE−/− mice. Despite these favorable effects, IL17A deficiency did not affect aortic plaque burden after a high-fat diet or angiotensin II infusion. In a partial carotid ligation model, IL17A deficiency did not affect percentage of stenosis but reduced outward remodeling. In this model, neutralization of the related isoform, IL17F, in IL17A/ApoE−/− mice did not alter atherosclerosis. Finally, there was no correlation between IL17A levels and carotid intima-media thickness in humans.

Conclusion—IL17 contributes to vascular and systemic inflammation in experimental atherosclerosis but does not alter plaque burden. The changes in plaque composition caused by IL17 might modulate plaque stability. (Arterioscler Thromb Vasc Biol. 2011;31:1565-1572.)

Key Words: atherosclerosis • cytokines • nitric oxide • superoxide • vascular biology

Atherosclerosis is a complex inflammatory disease characterized by derangements in the vascular, metabolic, and immune systems. Activated T cells, particularly CD4+ T helper cells, are found in atherosclerotic lesions, and their role in plaque development varies depending on the subset. In 2005, a novel T helper subset that produces the unique cytokine, interleukin 17 (IL17), was described.1 There are 6 known isoforms of IL17, designated A through F, of which IL17A and IL17F are the most widely studied and produce the most bioactive effects. Other sources of IL17 include CD8+ T cells, γδ T cells, natural killer T cells, natural killer cells, and neutrophils.

IL17 has been detected in human atherosclerotic lesions.6 Eid et al7 found that human coronary artery infiltrating T cells produce IL17, interferon-γ (IFN-γ), or both and that IL17 and IFN-γ act synergistically to induce proinflammatory responses in vascular smooth muscle cells. Patients with acute myocardial infarction and unstable angina have increased peripheral Th17 cells and IL17 levels.8 We previously showed that IL17A is required for the maintenance of angiotensin II-induced hypertension and vascular dysfunction, both of which are risk factors for atherosclerosis.8 One might therefore predict that IL17A is proatherogenic. In prior studies, IL17A seemed to reduce, increase, or have no effect on atherosclerosis depending on the experimental model and method of inhibition.10–16 Unfortunately, most of the approaches used in these prior reports could be complicated by nonspecific/off-target effects of the interventions or incomplete IL17 suppression.

We therefore sought to accurately examine the role of IL17 in atherosclerosis by generating IL17A/apolipoprotein E (ApoE)−/− double-deficient mice and to inducing lesions using 3 separate models (high-fat diet, angiotensin II infusion, and partial carotid ligation). We also compared carotid intima-media thickness, a surrogate marker of early atherosclerosis, with serum levels of IL17A in a population of relatively healthy humans aged 50 to 69 years. Our results indicate that although IL17A modulates some aspects of systemic and vascular inflammation and vascular function, inhibition of IL17A is insufficient to decrease atherosclerotic plaque burden.

Materials and Methods

Animals and Induction of Atherosclerosis

The Institutional Animal Care and Use Committee at Emory University approved all animal protocols. IL17A−/− mice were generated as described in Nakae et al17 and back-crossed to the C57BL/6J back-
Figure 1. Cytokine production, body weight, and vascular reactive oxygen species in Apoe<sup>−/−</sup> and IL17/ApoE<sup>−/−</sup> mice in response to high-fat diet. Splenic lymphocytes from Apoe<sup>−/−</sup> and IL17/ApoE<sup>−/−</sup> mice fed a regular (Reg) diet or high-fat (HF) diet for 3 months were cultured on anti-CD3 plates, and the culture supernatants were analyzed for IL17A using ELISA (A) or a cytokine bead array (B to D) (n = 4 to 7 per group). Body weight (in grams) was measured at baseline and after 3 months of HF diet in Apoe<sup>−/−</sup> and IL17/ApoE<sup>−/−</sup> mice (E) (n = 12 to 15 per group). Aortic superoxide production was measured by dihydrothiadium-high-performance liquid chromatography (F) (n = 5 to 7 per group). Aortic nitric oxide levels after 3 months of high-fat diet feeding were measured by electron spin resonance (ESR). Example ESR spectra (G) and summary data (H) are shown (n = 4 to 5 per group). Data in A and H were analyzed using the Student's t test. Data in E were analyzed using 2-way repeated-measures ANOVA. Other statistical data were analyzed using 1-way ANOVA with the Neuman-Keuls post hoc test.

Human Carotid Intima-Media Thickness Evaluation and IL17A Determinations

Carotid intima-media thickness measured by B mode ultrasound was compared with IL17A levels (measured using reagents from R&D Systems and a Luminex platform) in 16 healthy subjects aged 50 to 69 years.

Statistics

Data are expressed as mean±standard error of the mean. A probability value ≤0.05 was considered significant.

See supplemental material, available online at http://atvb.ahajournals.org, for an expanded Materials and Methods section.

Results

IL17A Is Upregulated in Response to Diet-Induced Atherosclerosis and Promotes IFN-γ Production

To determine the role of IL17A in atherosclerosis, we first examined the effect of high-fat feeding on T cell production of IL17A. Splenic lymphocytes were isolated from Apoe<sup>−/−</sup> mice fed a high-fat diet or normal chow (regular) diet for 3 months and cultured on anti-CD3 plates for 48 hours. As...
shown in Figure 1A, a high-fat diet markedly increased release of IL17A by these cells.

We then examined how IL17A affects production of the Th1 cytokines IFN-γ and tumor necrosis factor-α and the Th2 cytokines IL4 and IL5. IL17A-deficient mice were crossed with ApoE-deficient mice to generate homozygous IL17A/ApoE<sup>−/−</sup> mice. IFN-γ and IL5 production by splenic lymphocytes, as detected by cytokine bead array, were markedly increased by a high-fat diet in ApoE<sup>−/−</sup> mice (Figure 1B and 1D). The increase in IFN-γ, but not IL5, was significantly abrogated in IL17A/ApoE<sup>−/−</sup> mice, indicating that IL17A is necessary for IFN-γ but not IL5 production in response to a high-fat diet (Figure 1B and 1D). Tumor necrosis factor-α production was not altered by a high-fat diet and was similar between ApoE<sup>−/−</sup> mice and IL17A/ApoE<sup>−/−</sup> mice (Figure 1C). IL4 production was undetectable in mice fed a regular diet and was increased in response to a high-fat diet to a similar degree in ApoE<sup>−/−</sup> and IL17A/ApoE<sup>−/−</sup> mice (data not shown). Thus, production of both Th1 (IFN-γ) and Th2 (IL4 and IL5) cytokines increased in response to a high-fat diet, but only the increase in IFN-γ was IL17A dependent.

**Effect of IL17A on Weight Gain and Plasma Lipids in Response to High-Fat Diet**

Body weight and plasma lipids were not significantly different between ApoE<sup>−/−</sup> and IL17A/ApoE<sup>−/−</sup> mice at baseline (Figure 1E and Table). High-fat feeding increased weight by 2.6 g in ApoE<sup>−/−</sup> mice but not in IL17A/ApoE<sup>−/−</sup> mice, suggesting that IL17A may play a role in weight gain in response to a high-fat diet (Figure 1E).

**IL17A Promotes Superoxide Production and Decreases Nitric Oxide Levels in Response to High-Fat Diet**

Atherosclerosis increases vascular production of reactive oxygen species and decreases nitric oxide production. We determined the effect of IL17A on aortic superoxide production using ApoE<sup>−/−</sup> and IL17A/ApoE<sup>−/−</sup> mice fed a regular diet or high-fat diet for 3 months. Baseline levels of superoxide were similar between ApoE<sup>−/−</sup> and IL17A/ApoE<sup>−/−</sup> mice. The high-fat diet, however, increased aortic superoxide production in ApoE<sup>−/−</sup> mice by approximately 2-fold, and this increase was significantly blunted in IL17A/ApoE<sup>−/−</sup> mice (Figure 1F). Thus, IL17A is necessary for high-fat diet induced elevations in vascular superoxide.

Aortic nitric oxide levels correlate with endothelium-dependent relaxation and improved vascular function. Aortic nitric oxide levels in ApoE<sup>−/−</sup> and IL17A/ApoE<sup>−/−</sup> mice fed a high-fat diet were measured using electron spin resonance. Nitric oxide production by aortic rings of IL17A/ApoE<sup>−/−</sup> mice were increased compared with aortic rings of ApoE<sup>−/−</sup> mice (Figure 1G and 1H).

**IL17A Deficiency Has No Effect on Atherosclerotic Plaque Area or Aneurysm Formation but Decreases Aortic Inflammatory Cell Infiltration**

To determine the effect of IL17A on atherosclerotic plaque development, we quantified plaque area in the descending aorta (Figure 2A and 2B) and aortic root (Figure 2C and 2D) in ApoE<sup>−/−</sup> and IL17A/ApoE<sup>−/−</sup> mice fed a high-fat diet for 3 months. Surprisingly, plaque area in these regions was similar between IL17A/ApoE<sup>−/−</sup> mice and age-matched ApoE<sup>−/−</sup>-controls. We then investigated whether plaque morphology was different using Russell-Movat pentachrome staining (Figure 3A). No major differences were noted among extra-cellular matrix components such as elastin (black), collagen (yellow), and mucins (blue-green). Flow cytometry of whole aortas, however, revealed a modest decrease in total leukocytes (CD45+ cells) and dendritic (CD11b+11c+) cells and a trend toward reduction of aortic macrophages (CD11b+F4/80+ cells) and T cells (CD3+ cells) in the IL17A-deficient mice (Figure 3B). Immunostaining of atherosclerotic plaques

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**Table. Plasma Lipid Profile in Response to High-Fat Diet**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ApoE&lt;sup&gt;−/−&lt;/sup&gt; (n=12, mg/dL)</th>
<th>IL17/ApoE&lt;sup&gt;−/−&lt;/sup&gt; (n=11, mg/dL)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol</td>
<td>1185±83</td>
<td>1593±241</td>
<td>0.11</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>56±6.6</td>
<td>57±9.6</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Data are expressed as mean±standard error of the mean.
demonstrated a decrease in total macrophage content within lesions (Figure 3C). Thus, although plaque size and extracellular matrix content were similar between ApoE−/− and IL17/ApoE−/− mice, the plaques did appear to be less inflammatory in IL17/ApoE−/− mice.

Angiotensin II enhances atherosclerosis in ApoE−/− mice.19 Because we previously showed that angiotensin II stimulates T cell production of IL17A,9 we examined the role of IL17A in angiotensin II induced atherosclerosis. Angiotensin II (1000 ng/kg per minute) was infused via osmotic minipump for 4 weeks in ApoE−/− and age-matched IL17/ApoE−/− mice. Angiotensin II induced atherosclerosis in ApoE−/− and IL17/ApoE−/− mice to a similar extent, indicating that IL17A does not contribute to plaque development in an angiotensin II-induced model of atherosclerosis (Figure 4A). Plasma lipids following 4 weeks of angiotensin II were similar between ApoE−/− and IL17/ApoE−/− mice (data not shown).

Infusion of angiotensin II at this dose, 1000 ng/kg per minute, induces aneurysm formation in ApoE−/− mice.19 We found that aneurysm formation was similar in ApoE−/− mice and IL17/ApoE−/− mice (18% and 20%, respectively), suggesting that IL17A does not influence aneurysm development.

**IL17F Is Upregulated in IL17A-Deficient Mice and Is Increased by High-Fat Diet**

IL17F is closely related to IL17A, can dimerize with IL17A, and binds to the same receptor. Von Vietinghoff and Ley20 showed that IL17F was upregulated in IL17A-deficient mice. Therefore, we examined whether IL17F was also upregulated in our IL17/ApoE−/− mice and whether IL17F production was influenced by a high-fat diet. Interestingly, IL17F production at baseline (regular diet) was markedly increased in IL17/ApoE−/− mice. A high-fat diet further increased IL17F production, and IL17F levels were similar in ApoE−/− and IL17/ApoE−/− mice after 3 months of high-fat diet (Figure 4B).
4B). This elevation in IL17F in our model raised the possibility that IL17F could be assuming a compensatory or redundant role in plaque formation in the absence of IL17A.

**Neutralization of IL17F in IL17A-Deficient Mice Has No Effect on Partial Carotid Ligation-Induced Atherosclerosis**

We further examined the effect of IL17A with and without concomitant IL17F neutralization in a rapid (2-week) partial carotid ligation model of atherosclerosis described by Nam et al. Interestingly, we found that percentage of stenosis of the ligated carotid artery was not affected by the absence of IL17A, but vessel diameter (as measured by internal elastic lamina area) was significantly decreased in the IL17A-deficient mice, indicating that IL17A may play an important role in outward remodeling of vessels under conditions of disturbed flow (Figure 5A and 5B). To determine whether IL17F compensates for the loss of IL17A during the development of atherosclerosis, we administered an IL17F neutralizing antibody weekly beginning 1 week before carotid ligation. IL17F neutralization had no effect on internal elastic lamina area or percentage of stenosis (Figure 5A and 5C), arguing against a significant role of IL17F in atherosclerosis development.

**IL17A Levels Do Not Correlate With Carotid Intima-Media Thickness in Humans**

Increased carotid intima-media thickness is an early subclinical marker of atherosclerosis and a predictor of future cardiovascular events. We examined serum IL17A levels and carotid intima-media thickness in a small population of healthy humans aged 50 to 69 years. As shown in the Supplemental Figure, there was no correlation between IL17A levels and either mean or far wall carotid intima-media thickness, suggesting that IL17A does not participate in human atherosclerotic plaque formation.

In the present study, we found that IL17A modulates systemic and vascular inflammation by stimulating IFN-γ production and increasing vascular reactive oxygen species and vascular leukocyte infiltration, and it contributes to weight gain in response to high-fat diet. Despite these favorable effects, IL17A had no effect on gross lesion size in either mouse or human atherosclerosis. Furthermore, neutralization of a related cytokine, IL17F, had no effect on atherosclerosis in a rapid partial carotid ligation model.

To our knowledge, this is the first study to use a genetic deletion of IL17A and ApoE to study the role of IL17A in atherosclerosis. Several recent studies on this topic have provided conflicting results showing that IL17A reduces, increases, or has no effect on atherosclerotic plaque development. Differences in these results may be due to the methods used for eliminating IL17A, nonspecific effects of the interventions, or lack of complete elimination in some studies.

Despite finding no effect on plaque burden, we did find decreased vascular inflammation in the IL17A-deficient mice. We used 2 independent methods to assess vascular infiltration of inflammatory cells. We first used flow cytometry of whole aortas and found that total leukocytes and dendritic cells were significantly reduced in IL17A-deficient mice, with a trend toward reduced macrophages and T cells. Flow cytometry has the limitation that the entire aorta is analyzed, which could underestimate differences that could be present if only lesions were analyzed. We then performed immunostaining on atherosclerotic plaques and found that plaque macrophage content appeared to be reduced in IL17A-deficient mice. These results are consistent with reports by Erbel et al. and Smith et al, who also found that IL17A blockade reduced plaque macrophage content. A potential mechanism for this finding is that IL17A can act on vascular...
The left carotid artery (LCA) of ApoE-/- mice was partially ligated followed by 2 weeks of high-fat feeding to induce accelerated atherosclerosis in the ligated artery. Mice were injected weekly with IL17F or isotype control antibody (Ctrl Ab) for 3 weeks starting 1 week before ligation. Frozen sections of carotid arteries were stained with Oil Red O, hematoxylin, and eosin. A, Representative section of the LCA and right carotid artery (RCA) from each group. Quantification of internal elastic lamina (IEL) area of the LCA (B) and percentage of stenosis of the LCA (C) are shown (n=5 per group). Data were analyzed using 1-way ANOVA.

**Figure 5.** Effect of IL17A and IL17F isoforms on partial carotid ligation–induced atherosclerosis. The left carotid artery (LCA) of ApoE-/- and IL17A/ApoE-/- mice was partially ligated followed by 2 weeks of high-fat feeding to induce accelerated atherosclerosis in the ligated artery. Mice were injected weekly with IL17F or isotype control antibody (Ctrl Ab) for 3 weeks starting 1 week before ligation. Frozen sections of carotid arteries were stained with Oil Red O, hematoxylin, and eosin. A, Representative section of the LCA and right carotid artery (RCA) from each group. Quantification of internal elastic lamina (IEL) area of the LCA (B) and percentage of stenosis of the LCA (C) are shown (n=5 per group). Data were analyzed using 1-way ANOVA.

We found that in addition to promoting inflammation, IL17A is necessary for increased vascular superoxide (O$_{2}^-$) production in response to high-fat diet–induced atherosclerosis. This is similar to our previous finding that IL17A promotes vascular O$_{2}^-$ production in response to angiotensin II–induced hypertension. The mechanisms by which IL17A induces vascular O$_{2}^-$ production remain unclear. In preliminary studies, we were unable to demonstrate a direct effect of IL17A on O$_{2}^-$ production in either human endothelial or vascular smooth muscle cells in culture; however, a recent report suggests that IL17A can activate the NADPH oxidase in murine vascular smooth muscle cells. It is also possible that IL17A increases superoxide production via recruiting inflammatory cells, which in turn can produce reactive oxygen species.

It is interesting that IL17A modulates a number of factors thought to be associated with plaque progression, such as...
IFN-γ production, vascular inflammation, and superoxide production, and yet we did not find any effect on aortic plaque burden or carotid stenosis. Our finding that atherosclerotic plaque burden was similar despite reduced O₂− production is in keeping with prior studies showing that mice overexpressing superoxide dismutase are not protected against atherosclerosis.27,28 Moreover, plaque size does not necessarily correlate with clinical outcomes in human coronary atherosclerosis. Plaque stability is more predictive of acute plaque rupture and coronary events. Our findings suggest that IL17A deficiency, through reduced leukocyte infiltration and decreased superoxide production, could stabilize plaques. Thus, IL17A might not be an important determinant of plaque size but rather a modulator of plaque composition and stability. This is in agreement with clinical data showing that Th17 cells and IL17 levels are elevated in patients with unstable angina and acute myocardial infarction compared with patients with stable angina and controls.8

In our carotid ligation model, we observed that IL17A deficiency had no effect on percentage of stenosis in the ligated vessel, but it markedly decreased outward remodeling. Outward remodeling is a compensatory response to lesion development that preserves lumen diameter in the setting of increasing atherosclerosis burden. In this regard, IL17A−/− mice are similar to mice lacking matrix metalloproteinase-9, in which outward remodeling in response to total carotid occlusion is significantly impaired.29 Because reactive oxygen species are known to increase matrix metalloproteinase-9 expression and activation,30,31 it is interesting to speculate that the absence of outward remodeling in the IL17A/ApoE−/− mice might reflect the lower levels of O₂− observed in these animals compared with APOE−/− mice.

We also show that IL17A/ApoE−/− mice are resistant to high-fat diet-induced weight gain. A link between IL17A and obesity was first suggested by a small clinical study showing that plasma levels of IL17A were elevated in 26 obese women compared with 20 lean women.32 Similar to our findings, Winer et al33 showed that diet-induced obesity increases IL17 from splenic lymphocytes. Moreover, IL17A stimulates proinflammatory responses in adipocytes.34 Recently, Pini and Fantuzzi35 found that neutrophil-derived IL17A is increased in obese mice during acute inflammation and contributes to inflammatory responses. These findings are in keeping with a role of inflammation in the metabolic syndrome and suggest that IL17 might contribute to this disorder.

In summary, using a genetic approach that avoids potential confounding off-target and nonspecific effects, we found that IL17A is involved in systemic and vascular inflammation and weight gain in response to a high-fat diet. Contrary to several prior reports, however, we showed that IL17A is unlikely to contribute significantly to plaque progression. Nevertheless, through its effects on reactive oxygen species and leukocyte infiltration, IL17A is an important potential mediator of plaque stability, and its effects on plaque stability and outward remodeling warrant further investigation.

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**Disclosures**

N one.

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