Interleukin-17A Promotes Aortic Endothelial Cell Activation via Transcriptionally and Post-translationally Activating p38 Mitogen-activated Protein Kinase (MAPK) Pathway*

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Interleukin-17 (IL-17)-secreting T helper subset 17 cells were recently identified as a CD4+ T helper subset implicated in various inflammatory and autoimmune diseases. The issues of whether and by what mechanism hyperlipidemic stress induces IL-17A to activate aortic endothelial cells (ECs) and enhance monocyte adhesion remain largely unknown. Using biochemical, immunological, microarray, experimental data mining analysis, and pathological approaches focused on primary human and mouse aortic ECs (HAECs and MAECs) and our newly generated apolipoprotein E (ApoE)−/−/IL-17A−/− mice, we report the following new findings. 1) The hyperlipidemia stimulus oxidized low density lipoprotein up-regulated IL-17 receptor(s) in HAECs and MAECs. 2) IL-17A activated HAECs and increased human monocyte adhesion in vitro. 3) A deficiency of IL-17A reduced leukocyte adhesion to endothelium in vivo. 3) IL-17A activated HAECs and MAECs via up-regulation of proinflammatory cytokines IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokine CXC motif ligand 1 (CXCL1), and CXCL2. 4) IL-17A activated ECs specifically via the p38 mitogen-activated protein kinases (MAPK) pathway; the inhibition of p38 MAPK in ECs attenuated IL-17A-mediated activation by ameliorating the expression of the aforementioned proinflammatory cytokines, chemokines, and EC adhesion molecules including intercellular adhesion molecule 1. Taken together, our results demonstrate for the first time that IL-17A activates aortic ECs specifically via p38 MAPK pathway.

Hyperlipidemia, a major risk factor for cardiovascular disease and stroke, is defined as pathologically elevated plasma concentrations of cholesterol and other lipids (1). We and others have previously reported that hyperlipidemia, hyperglycemia, hyperhomocysteinemia, and other risk factors promote vascular inflammation and cardiovascular disease via several chronic inflammatory mechanisms, which include endothelial cell (EC) activation (2–4), EC dysfunction (5, 6) and injury (7, 8), increasing monocyte recruitment and differentiation (9, 10), and decreasing regulatory T cell population (11–13).

In 2003, a new lineage of CD4+RORt+ (retinoid-related orphan receptor) T cells was identified and defined by its production of proinflammatory cytokine interleukin-17 (IL-17) and hence named T-helper 17 cells (14, 15). IL-17A is the signature cytokine of T-helper 17 cells (15), but it was reported later that other immune cells including macrophages (16), neutrophils (17), dendritic cells (18), CD8+ T cells (19), αβ T cells (20), γδ T cells (21), and natural killer T cells (22) also produce IL-17A. However, whether IL-17A plays an imperative role in aortic EC activation remains poorly defined.

Aortic ECs, which line the inner surface of the aortic artery, are the first type of cells exposed to plasma proinflammatory cytokines during the host immune response to inflammatory stimuli including hyperlipidemia. As we pointed out recently, ECs are conditional innate immune cells (23). Therefore, endothelial activation is considered as a part of their innate immune function, which is the initial event that is responsible for monocyte adhesion and recruitment into arteries (24). However, whether hyperlipidemia-elevated proinflammatory cytokine IL-17A in plasma promotes aortic EC activation and, if so, the underlying mechanisms remain largely unknown.

Although controversial (25–27), the concept that IL-17A plays a proatherogenic role is prevailing. To improve our understanding of the role of this important cytokine, we focused our efforts on testing the hypothesis that hyperlipidemia-mediated elevation of IL-17A promotes aortic EC activation. Using flow cytometry, EC biology array, intravital

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**Materials and Methods**

**Mice—** C57BL/6 mice and apolipoprotein E knock-out (ApoE−/−) mice in a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-17−/− mice in a C57BL/6 background were generously provided by Dr. Yoichiro Iwakura’s laboratory (28) (University of Tokyo, Tokyo, Japan). ApoE−/− mice and IL-17−/− mice were cross-bred to establish ApoE−/−/IL-17−/− mice. Age-matched male mice were used for all experiments as we described previously (29). All procedures in animals were performed in accordance with the approval of the Temple University Institutional Animal Care and Use Committee.

**Genotyping—** Murine genomic DNA was genotyped as described previously (11).

**Mouse Endothelial Cell Isolation and Primary Culture—** Mouse aortic ECs (MAECs) were isolated and cultured as we described previously (8).

**Human Aortic Endothelial Cell (HAEC) Culture—** HAECs (Clonetics Corp., San Diego, CA) were cultured as described previously (8). For inhibition of p38 MAPK experiments, HAECs were serum-starved for 6 h. After serum starvation, the cells were pretreated for 1 h with p38 inhibitor (SB203580; 10 μM) (Selleckchem, Houston, TX) in serum-free medium. Prior to pretreatment, HAECs were treated for the times indicated in figures with recombinant human IL-17 (100 ng/ml; R&D Systems, Minneapolis, MN) in serum-free medium.

**Flow Cytometry and Data Analysis—** The treated cells were stained with allophycocyanin-conjugated ICAM-1 antibody (BD Biosciences) for 30 min. Then cells were analyzed by flow cytometry using a BD FACSCalibur or LSRII flow cytometer. Data were analyzed using FlowJo software (Ashland, OR). The uncompensated data were collected from the flow cytometer (either BD FACSCalibur or LSRII flow cytometer). Forward and side scatter gates were used to select the live cell population from clumps and debris. The positive gate was determined by its matched IgG control, and single staining was used to determine the compensation parameter.

**Monocyte-EC Static Adhesion Assay—** HAECs (passage 9) were cultured in 24-well plates and treated with human IL-17 (100 ng/ml; R&D Systems). THP-1 cells (human monocytic cell line; American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium with 10% FBS, 1% penicillin/streptomycin, and 2 mM l-glutamine. Human peripheral blood monocytes (HPBMCs) were freshly isolated. All procedures were performed in accordance with the approval of the Temple University Institutional Review Board. THP-1 cells or HPBMCs (5 × 10⁶ cells/ml) were stained with 2 μg/ml calcein AM (Invitrogen) fluorescence dye for 30 min at 37 °C. THP-1 cells or HPBMCs (1 × 10⁶ cells/ml) were suspended in complete RPMI 1640 medium and complete M199 medium (1:1) or DME monkey culture medium and complete M199 medium (1:1), respectively, and added to the HAEC monolayer. Prior to adding THP-1 cells/HPBMCs, the HAECs were washed twice with PBS to remove the traces of IL-17. After 1-h incubation at 37 °C, unattached THP-1 cells or HPBMCs were removed by careful washing with phosphate-buffered saline (PBS) supplemented with CaCl₂ and MgCl₂. Fluorescence was measured as reported previously (30) with a microplate fluorescence reader (FLx800, Bio-Tek, Winooski, VT) at 494/517 nm. The percentage of adherent THP-1/HPBMC cells to the HAEC monolayer was determined as a percentage of the no-treatment control group. For assays with blocking antibodies, 100 ng/ml anti-GM-CSF, 50 ng/ml anti-CXCL1/2, and 10 ng/ml anti-IL-6 were added to treated ECs 30 min prior to the adhesion assay.

**Protein Extraction and Western Blotting Analysis—** Protein extraction from mouse aorta and Western blotting analysis were performed as we described previously (31). Protein concentrations were determined by the bicinchoninic acid (BCA) assay with BSA standards (Pierce/Thermo). Proteins were separated on 8% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked by 5% nonfat milk in Tris-buffered saline containing 0.01% Tween 20. Membranes were probed overnight at 4 °C or at room temperature for 1–2 h (all the primary antibodies were purchased from Cell Signaling Technology and the catalogue numbers are as follows: phospho-p38 MAPK, 9215s; p38 MAPK, 9212s; phospho-MKK3, 12280s; MKK3, 5674s; phospho-JNK, 9251s; JNK, 9252s; phospho-MKK4, 9156s; and MKK4, 9152s). Membranes were then washed extensively with TBST and incubated with the appropriate horseradish peroxidase-labeled secondary antibodies for 1 h at room temperature. Afterward, membranes were incubated with enhanced chemiluminescence (ECL) substrate for horseradish peroxidase (Pierce/Thermo), and the ECL intensity was detected by x-ray film exposure in a dark room. The x-ray films were developed by the SRX-101A medical film processor (Konica Minolta, Mississauga, Ontario, Canada). The expression levels of proteins as indicated by the ECL intensity were measured with ImageJ software (National Institutes of Health, Bethesda, MD).

**RNA Extraction and Real Time PCR—** Total RNA was isolated as we described previously (32) from HAECs with TRIzol® reagent (Invitrogen). HAECs grown in 60-mm culture dishes were rinsed with iced-cold PBS and then lysed with 1 ml of TRIzol reagent. Lysed cells were incubated for 5 min at room temperature for complete dissociation of nucleoprotein complex. Then 200 μl of chloroform was added to each sample, and the mixture was shaken vigorously for 15 s and centrifuged at 13,000 rpm for 15 min at 4 °C. The upper aqueous phase was
collected, and RNA was precipitated by addition of an equal volume of isopropanol. After centrifugation, the RNA pellet was washed twice with 75% ethyl alcohol in nuclease-free water and collected, and RNA was precipitated by addition of an equal volume of isopropanol. After centrifugation, the RNA pellet was washed twice with 75% ethyl alcohol in nuclease-free water (Qiagen, Valencia, CA). The RNA was solubilized in 30 μl of nuclease-free water. RNA quality and concentration were determined by the NanoDrop 2000 (Thermo Scientific, Wilmington, DE). Two micrograms of total RNA was reverse transcribed to generate complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription Kit (Invitrogen) on the StepOnePlus PCR System (Applied Biosystems, Foster City, CA) and used following the directions of the Mouse Endothelial Cell RT2 Profiler PCR Array (SA Biosciences). Data were analyzed with the SA Biosciences PCR Array Data Analysis software.

The mRNA expression levels of genes were determined by quantitative real time PCR (qRT-PCR) with SYBR Green dye (Invitrogen) using the StepOnePlus PCR System (Applied Biosystems, Foster City, CA). The primers used are listed in Table 1.

### Table 1: List of primers used in qRT-PCR

| Gene      | Sequence (5’–3’) |
|-----------|-----------------|
| **Human**<br>β-Actin | AACTTCTTCAACATGAGGCTGGA<br>CTGAGAATTCCAGCCTACAAGTGG |
| GM-CSF    | ACTTTCTCGCTGTCTCACCC<br>CCAATCGTTGACCTGCTTTTTG |
| CXCL1     | TCTCTCTTCTCTTCTGAG<br>GAATTCTGCTCTTCAACTTCTG |
| CXCL2     | AAGCGAAGTACATAGCAACAGC<br>AGGAGAACCATCAATGAGG |
| IL-6      | CAATCTCATCTCTGGAGA<br>CCATCTCTGAGGTTTCAAGTGG |
| ICAM-1    | AAGTGGATTCTTACAACTGCCC<br>CAATGTGCTATTCAAACTGCCC |
| VCAM-1    | TCTACAATGCAAAGTAAAACCTG<br>AGGCGCTACCTAAAGTAACTC |
| E-selectin | AAGTTCCGCTTCTCTGAG<br>CAAAAGATCCTGACATCAAAGG |
| **Mouse**<br>β-Actin | CTGTATTCCCCTCAATCTG<br>GCTCTGCCTCACCAGAATAG<br>GGTTTTCCTTCAGCCACTTTG |
| IL-17RA   | TCTTCTACCTTGTTGCTGC |

RNA was converted to cDNA with the RT2 First Strand Kit (SA Biosciences, Valencia, CA) and used following the directions of the Mouse Endothelial Cell RT2 Profiler PCR Array (SA Biosciences). Data were analyzed with the SA Biosciences PCR Array Data Analysis software.

The mRNA expression levels of genes were determined by quantitative real time PCR (qRT-PCR) with SYBR Green dye (Invitrogen) on the StepOnePlus PCR System (Applied Biosystems, Foster City, CA). The primers used are listed in Table 1.

### Transcriptome Microarray Analysis—Total RNA was extracted from the aortas of mice fed a high fat diet for 3 weeks using the RNeasy kit (Qiagen). RNA quantity was determined by the RNA 28S/18S ratio using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Then samples were labeled and hybridized to the Affymetrix GeneChip Mouse Gene 2.0 ST Arrays (Santa Clara, CA) following the manufacturer’s instructions at the Expression Microarray Core Facility in the Fox Chase Cancer Center, Temple University. Scanned microarray images were analyzed using the Affymetrix Gene Expression Console with the robust multiarray average normalization algorithm.

### Microarray Data Analysis—Affymetrix data analysis was done in the R statistical environment using “oligo” and “limma” packages. Heat maps and scatter plots were generated using the statistical tools provided by the R and Bioconductor projects. Database mining analysis was performed by downloading the Gene Expression Omnibus (GEO) microarray data set from the National Institutes of Health NCBI GEO database and examining the expression levels of genes of interest and housekeeping genes as we reported (33).

### Intravital Microscopy—These studies were carried out according to our previous publication (2). Male mice were anesthetized with 50 mg/kg pentobarbital via intraperitoneal injection and maintained on a thermostatically controlled rodent blanket at 37 °C. The cremaster muscle was exteriorized. Briefly, a midline incision was made through the scrotal skin, and the cremaster muscle was dissected from the associated fascia and connective tissue. The distal end of the exposed muscle was pinned gently on a silicone pedestal, and the cremaster muscle was cauterized longitudinally. The testis and epididymis were separated from the underlying muscle and gently pushed back into the abdominal cavity. The cremaster muscle was spread out on a Plexiglas microscope stage pedestal and superfused continuously with a bicarbonate-buffered saline (127 mM NaCl, 4.7 mM KCl, 2 mM CaCl2, 2H2O, 1.2 mM MgSO4, 28 mM NaHCO3, and 5 mM glucose) (34) using a peristaltic pump. The buffer was aerated continuously with a 95% N2 and 5% CO2 gas mixture to maintain a pH of 7.35–7.45. The temperature of the buffer was maintained at 35 °C. The muscle was allowed to equilibrate for 15 min prior to observing leukocyte rolling and adhesion. Venules and arterioles that were 40–50 μm in diameter as measured by CellSens Dimension software (Olympus, Tokyo, Japan) were observed on a microscope (Olympus BX51WI) with a mounted digital camera (Olympus DP80). Leukocyte rolling was determined as the number of cells that rolled past an imaginary line that is perpendicular to the vessels in 1 min. Adhesion was determined as cells that adhered to the vessel wall for 1 min on a 100-μm length of endothelium along the vessel. Rolling and adhesion in five venules and two arterioles were observed in each mouse.

### Atherosclerotic Lesion Analysis—Aortas were extracted after perfusion with PBS. Then the tissues were fixed overnight with 4% paraformaldehyde, and the tissues were kept in a 20% sucrose solution for 24 h. After fully removing all adipose and connective tissues, the aortas were stained with Sudan IV (5 mg/ml in 70% isopropanol) for 40 min at 37 °C followed by a 5-min incubation in 70% isopropanol. A longitudinal cut was made to expose the internal surface of the vessel and pinned open on a black silicone tray for imaging. The images of the aorta were captured with an AxioCam camera mounted to a Stemi 2000-C microscope (Carl Zeiss Inc.). The lesion area in the aorta was defined as the red area stained with Sudan IV. The summated lesion area was then divided by the total aorta area to tabulate the atherosclerotic lesion area.

To determine the level of atherosclerotic lesion in the aortic root, the hearts were extracted, and the lower ventricular portion was embedded in optimal cutting temperature compound (OCT) medium. Then 10-μm sections were obtained where the aortic valves first appear and stained with Oil Red O and hematoxylin. The stained sections were mounted and imaged with an AxioCam camera mounted to an Axioskop 2 microscope. The atherosclerotic lesion area within the aortic sinus was defined as the area stained with Oil Red O and measured with ImageJ. The percentage of the total sinus area was calculated by dividing the lesion area by the total sinus area.
Data Analysis—Data are expressed as means of replicate measurements or mean normalized values of multiple experiments ± S.E. For comparisons between two groups, two-tailed Student’s t test (α = 0.05) was used for evaluation of statistical significance. For comparison across multiple groups, one-way analysis of variance with Bonferroni post-test was used.

Results

Hyperlipidemia Stimuli Induced IL-17 Receptor Subunit Expressions in Aortic ECs—A previous study from Galkina and co-workers (35) showed that plasma IL-17A was significantly elevated by more than 7-fold in hyperlipidemic ApoE−/− mice in comparison with that in wild-type (WT) mice. Our ELISA experiments also confirmed this finding and showed that hyperlipidemia significantly increased plasma IL-17A levels from undetectable levels (<5 pg/ml) seen in WT mice to 36.6 ± 6.17 pg/ml in ApoE−/− mice fed with normal chow diet and 146.0 ± 7.50 pg/ml in ApoE−/− mice fed a high fat diet for 8 weeks (Fig. 1A). Because ECs are the first cells exposed to elevated IL-17A in plasma, we tested the hypothesis that the pro-inflammatory lipid stimulus oxidized low density lipoprotein (ox-LDL) could stimulate the IL-17A receptor pathway. To examine this hypothesis, we isolated and cultured MAECs from WT mice. We used CD31 staining and Dil-Ac-LDL functional uptake in our MAEC culture to ensure the specificity of our cultured ECs (data not shown). We treated MAECs with ox-LDL and used RT-PCR to determine the expression levels of IL-17 receptor A (IL-17RA) mRNA transcript. We found that...
after the treatment with ox-LDL (50 μg/ml) for 24 h MAECs had an increased expression of IL-17RA transcripts compared with the untreated cells (Fig. 1B). This suggested that MAECs were equipped with up-regulated receptor to receive the stimulation of elevated plasma IL-17 in hyperlipidemic conditions (Fig. 1A). Similarly, we used flow cytometry to detect IL-17A receptor expression in HAECs after ox-LDL treatment with LDL and vehicle as controls. Ox-LDL treatment for 24 h significantly increased the expression of IL-17RA on HAECs relative to the LDL control, which did not induce IL-17RA expression (Fig. 1C). As our recent study (15) and review (36) pointed out, the functional receptor complex for IL-17A is a heterodimer containing IL-17RA and IL-17 receptor C (IL-17RC). Thus, we also examined the expression of IL-17RC under stimulation by ox-LDL. Similarly, ox-LDL significantly increased the expression of IL-17RC on HAECs compared with LDL-treated or untreated cells (Fig. 1D). These results suggested that hyperlipidemia stimuli induced IL-17 receptor subunit expression in mouse and human aortic ECs.

Monocyte Adhesion Was Enhanced to IL-17-treated Aortic ECs—Next, we hypothesized that hyperlipidemia-induced IL-17 and its receptors might contribute to EC activation. We examined this hypothesis by using the monocyte-EC adhesion assays. HAEC monolayer was treated with IL-17A (100 ng/ml) for various time periods in vitro, and untreated fluorescent calcine AM-labeled THP-1 monocytes were allowed to adhere to the EC monolayers. We found that there was a gradual increase in the number of THP-1 monocytic cells that adhere to the IL-17A-treated endothelial monolayer up to 24 h. This effect was first detected in the shortest treatment time of 2 h. The most dramatic increase in adhesion was seen in the 12-, 24-, and 36-h treatment periods. After 48 h of IL-17A treatment, the percentages of cell adhesion began to recede to the levels of the controls (Fig. 2A). Furthermore, to confirm our result seen with the human monocyctic cell line THP-1 and demonstrate the pathophysiological relevance of the finding, we isolated primary human peripheral blood mononuclear cells from healthy individuals and allowed untreated peripheral blood mononuclear cells to adhere to HAECs that were treated with human IL-17A for 24 h. We found that the adhesion of primary human peripheral blood mononuclear cells to IL-17A-treated HAECs was significantly enhanced when compared with the control group (Fig. 2, B and C). These in vitro results suggested that IL-17A activated ECs and increased the monocyte adhesion to the endothelium.

Deficiency of IL-17A in ApoE−/− Mice Reduced the Leukocyte Adhesion to the Endothelium in Vivo—Next, we sought to determine whether endogenous IL-17A contributes to EC activation during early hyperlipidemia in vivo. To examine this hypothesis, we generated ApoE−/−/IL-17A−/− mice by cross-breeding IL-17A−/− mice with hyperlipidemic ApoE−/− mice. We reasoned that if IL-17A plays an important role in activating ECs then we should be able to observe less leukocyte rolling and adhesion in the endothelium in vivo in IL-17A-deficient mice. To visualize and quantify in vivo leukocyte rolling and adhesion to the endothelium in early hyperlipidemia, we used intravital microscopy (Fig. 3A) to examine vessels of the cremaster muscle of ApoE−/− mice and ApoE−/−/IL-17A−/− mice fed with a high fat diet for 3 weeks. Of note, the cholesterol level and triglycerides level were increased to 600 and 300 mg/dl in ApoE−/− mice, respectively, as we reported previously (3). As shown previously, we were not able to detect any leukocyte rolling or adhesion in the arterioles of the cremaster muscle (not shown) due to the rapid movement of blood cells within these vessels (34). Similarly, the high blood flow speeds and high pulse vibrations in middle and aortic arteries make the current technology of intravital microscopy unsuitable in determining in vivo leukocyte rolling and adhesion to the endothelium in these arteries (37). In the cremaster venules, we were able to measure leukocyte rolling and adhesion to the endothelium. There were no differences in the venule diameters (Fig. 3B) and detected numbers of rolling leukocytes on the endothelium of the cremaster muscle venules between ApoE−/− mice and ApoE−/−/IL-17A−/− mice (Fig. 3C), suggesting that the depletion of IL-17A did not cause significant changes in venule diameter or leukocyte rolling potential. However, we did find that there was a 63.8% decrease in the number of leukocytes adhered to the endothelium of the ApoE−/−/IL-17A−/− mice (5.142 cells/100 μm (in distance)/min) compared with ApoE−/− mice (8.425 cells/100 μm (in distance)/min) and that this decrease in leukocyte adhesion was statistically significant (p < 0.05) (Fig. 3D). These results suggested that the hyperlipidemia-induced endogenous IL-17A elevation in plasma promotes leukocyte adhesion to ECs in vivo in ApoE−/− mice.
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Inducing Proinflammatory Cytokines and Chemokines Including IL-6, GM-CSF, CXCL1, and CXCL2—Activation of the endothelium is characterized by a few features, which include enhanced proinflammatory cytokine and chemokine production, increased cell surface expression of adhesion molecules, and augmented leukocyte adhesion. To dissect the molecular mechanism underlying the role of IL-17A in aortic EC activation, we isolated and cultured primary MAECs to determine whether IL-17A induces the expression of cytokines and chemokines. The Mouse Endothelial Cell Biology RT2 Profiler PCR Array (Qiagen), which simultaneously profiles the expression of 84 genes related to EC biology, was used (Fig. 4A). We found that after treatment with IL-17A (100 ng/ml) for 24 h four proinflammatory chemokine and cytokine genes that are associated with EC activation were significantly up-regulated (at least by 4-fold) in MAECs (Fig. 4B). The four genes that were up-regulated were CXCL1, CXCL2, IL-6, and CSF2 (also known as GM-CSF). To further corroborate the data we obtained from the PCR array, we conducted gene expression studies for the aforementioned four genes in MAECs treated with IL-17 for 24 h and observed a significant up-regulation in all four (Fig. 4C). Furthermore, we conducted additional gene expression studies to determine whether these four genes were also up-regulated in HAECS after treatment with human IL-17A. We found that the CXCL1, CXCL2, IL-6, and GM-CSF mRNA transcripts were indeed up-regulated in HAECS after treatment with IL-17A for 12 h. The changes of the four genes
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FIGURE 4. IL-17 induces the gene expression of four proinflammatory cytokines and chemokines, namely IL-6, GM-CSF, CXCL1, and CXCL2, in mouse and human aortic endothelial cells. The expressions of 84 genes related to EC biology were profiled by using the Mouse Endothelial Cell Biology RT² Profiler PCR Array. MAECs were treated with IL-17 for 24 h and used in the Mouse Endothelial Cell Biology RT² Profiler PCR Array. A, 84 genes assessed by the array. B, scatter plot of the changes of gene expression in IL-17-treated MAECs. Expressions of four genes were dramatically induced by IL-17 treatment; blue lines indicate a -fold change of 4. C, IL-17-mediated increases in the expressions of CXCL1, CXCL2, IL-6, and CSF2 (GM-CSF) were further confirmed in MAECs by quantitative PCR. Data are presented as means ± S.E. (n = 5). *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus control. D, human IL-17 treatment significantly enhanced the expressions of CXCL1, CXCL2, IL-6, and CSF2 in HAECs treated with IL-17 (100 ng/ml) for 12 h as detected by qRT-PCR. Data were normalized to β-actin expression and are presented as means ± S.E. (n = 3). *, p < 0.05 versus control. E, the blockage of proinflammatory cytokines IL-6 and GM-CSF and chemokines CXCL1 and CXCL2 with specific antibodies decreased non-treated monocyte adhesion to IL-17-stimulated endothelial cells. Endothelial cells were treated with IL-17, and the blocking antibodies against CXCL1/2, IL-6, and/or GM-CSF were added 30 min prior to the adhesion assay. Antibodies against CXCL1/2, IL-6, and GM-CSF reversed the effect of IL-17 on increasing monocyte adhesion to endothelial cells relative to the IgG control. F, fluorescence microscopic image of labeled non-treated THP-1 cells adhered to endothelial cells. Data are presented as means ± S.E. (error bars) (n = 3). *, p < 0.05 versus control; #, p < 0.05 versus IL-17-treated group.
in IL-17A-treated HAECs were significant, although the induction of these four genes in HAECs was not as great as that observed in MAECs (Fig. 4D). These results suggested that IL-17A-induced up-regulation of these four proinflammatory cytokines and chemokines in aortic ECs is specific and conserved throughout biological evolution. In addition, we acknowledge that the difference in the duration of the IL-17A treatment in these two independent experiments might also have contributed to the differential magnitude of the changes observed.

To determine whether enhanced monocyte adhesion to IL-17A-treated ECs is functionally dependent on these four up-regulated proinflammatory cytokines and chemokines, we used blocking antibodies against CXCL1/2, IL-6, and GM-CSF in our monocyte-EC adhesion assay. We found that blocking antibodies against CXCL1/2, IL-6, and GM-CSF abrogated the increased THP-1 monocytic cell adhesion to aortic ECs that were treated with IL-17A for 24 h. IgG isotype control was used as a nonspecific antibody control, which was ineffective in reducing monocyte adhesion to activated ECs, suggesting the specificities of these cytokine/chemokine blocking antibodies (Fig. 4, E and F). Of note, the antibody that blocks IL-6 had the highest blockage efficiency among the three antibodies in equal amounts, suggesting the potential dominant role of IL-6 in mediating the role of IL-17 in activating ECs among the IL-17A targets. These results suggested that IL-17A activates aortic ECs via up-regulation of proinflammatory cytokines and chemokines IL-6, GM-CSF, CXCL1, and CXCL2.

Because the up-regulation of prototypic endothelial adhesion molecule ICAM-1 in ECs after stimulation is considered as a key feature of EC activation, we also examined whether IL-17 with or without IL-6 could induce the up-regulation of ICAM-1 in HAECs. To examine this issue, we treated HAECs with IL-17 (50 ng/ml), IL-6 (20 ng/ml), and IL-17 (50 ng/ml) plus IL-6 (20 ng/ml) for 6 h with non-treated cells as controls followed by measuring ICAM-1 expression using a flow cytometer. The results showed that both IL-6 and IL-17 significantly induced ICAM-1 expression (Fig. 5, A and B). Co-administration of IL-17 with IL-6 further increased ICAM-1 expression, suggesting that IL-17 can directly and indirectly activate HAECs via its target cytokine IL-6 (Fig. 5, A and B). This result correlated well with the previous finding of the synergistic effects of IL-17 and IL-6 in astrocytes (38). Taken together, IL-17A activated the ECs by increasing the expression of proinflammatory cytokines and chemokines as well as by enhancing expression of the EC adhesion molecule ICAM-1.

**IL-17A Activated Human Primary Aortic ECs via p38 MAPK Pathway**—To explore the possible mechanisms that lead to IL-17A-induced aortic EC activation, we used microarray analysis to determine the gene expression of certain key inflammatory signaling pathway(s) modulated by IL-17A (Fig. 6, A and B). The results showed that IL-17A modulated aortic EC gene expressions extensively in vivo. Among the changes observed, we found that IL-17A up-regulated IL-17RA expression (Fig. 6C) presumably via a positive feedforward mechanism. These data suggested that hyperlipidemia stimuli-induced IL-17RA expression (Fig. 1) may act via direct ox-LDL-related signaling and indirect hyperlipidemia-induced IL-17A signaling. In addition, IL-17A up-regulated the expressions of EC adhesion molecules such as ICAM-1 and ICAM-2 (Fig. 6). This result correlated well with our results of IL-17A induction of ICAM-1 in HAECs. A previous report showed that IL-17A activated microvascular ECs via p38 MAPK phosphorylation in the lungs (39). However, the issue of whether IL-17A activates aortic ECs via the p38 MAPK pathway remains to be determined. Therefore, based on IL-17A induction of proinflammatory cytokines and chemokines in aortic ECs, we examined whether IL-17A activates aortic arterial ECs via the MAPK pathway by transcriptionally increasing the transcripts of MAPK components. The results showed that deficiency of IL-17A resulted in decreased mRNA transcript expression of p38 MAPK (Mapk14) and two kinases upstream of the p38 MAPK pathway, Map3k1 (Mekk1) and Map2k3 (Mkk3) (40). To further confirm the aortic transcriptome results, we performed Western blotting and probed for total and phosphorylated p38 MAPK levels in mouse aortic protein lysates extracted from IL-17−−/ApoE−− and ApoE−/− mice fed with a high fat diet for 3 weeks (Fig. 7A). Correlated with our microarray results, we found that IL-17−−/ApoE−− mice had slightly decreased expression of total p38 MAPK (Fig. 7B) but not total JNK MAPK (Fig. 7C). In addition, IL-17−−/ApoE−− mice also had significantly lower expression of phospho-p38 MAPK in the aortas relative to ApoE−/− mice (Fig. 7D), suggesting that IL-17A also post-translationally promotes p38 MAPK activation. Moreover, there was no significant change of JNK MAPK phosphorylation, suggesting the specificity of IL-17A in regulating p38 MAPK phosphorylation (Fig. 7E). The integration of our mRNA transcript expression results from the aortic microarray (Fig. 6) and protein phosphorylation results from Western blotting (Fig. 7) suggested that IL-17A activated the p38 MAPK pathway by increasing the mRNA transcript expression of upstream MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K), and p38 MAPK and by phosphorylation of p38. In contrast, IL-17A did not increase the expression of the upstream kinase transcripts of JNK, Map3k10 (Mlk2), Map3k11 (Mlk3), and Map2k4 (Mkk4) or the phosphorylation of JNK, further suggesting the specificity of IL-17A-mediated activation upon p38 MAPK (Fig. 7F). Of note, a-pre
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Previous report showed that p38 MAPK activated endothelial nitric-oxide synthase (eNOS) and phosphorylated eNOS at serine 1177 (Ser-1177) by indirectly phosphorylating estrogen receptor-α Ser-118 (41). Because eNOS plays a critical role in regulating EC response to inflammation (42), we also examined whether IL-17A regulates eNOS phosphorylation at two sites, Ser-1177 and threonine 495 (Thr-495) in the aorta. The results showed that there was a trend of increased phosphorylation of eNOS at Ser-1177 site and less phosphorylation of eNOS at the Thr-495 site (Fig. 7, G and H) in the aortas of IL-17A−/− mice, which correlated well with the report on IL-17-mediated inhibition of eNOS phosphorylation at Thr-495 in

FIGURE 6. Mouse aortic microarray results indicate that IL-17 up-regulates the expressions of many genes in mouse aortas including IL-17RA, endothelial cell adhesion molecules, and MAPK pathway components. A, mouse aortic RNAs were isolated from ApoE−/− mice and IL-17A−/− mice fed with a high fat diet for 3 weeks and subjected to Affymetrix microarray analysis. B, volcano plot (scatter plot) of -fold change versus p value for all gene transcripts determined by the Affymetrix GeneChip Mouse Gene 2.0ST Arrays in mouse aortas from ApoE−/− mice and IL-17A−/− mice fed with a high fat diet for 3 weeks. The volcano plot shows that there are numerous genes that were modified with statistical significance in IL-17−/−/ApoE−/− mouse aortas relative to ApoE−/− mouse aortas. C, the heat map and -fold changes of IL-17 receptor signaling genes. D, heat map and -fold changes of endothelial adhesion molecule genes. E, heat map and -fold changes of p38 MAPK (MAPK14) pathway genes. The values with p < 0.05 are highlighted in red.
Depletion of IL-17 in ApoE−/− mice decreases the phosphorylation of p38 MAPK but not the phosphorylation of JNK MAPK nor the phosphorylation of eNOS in mouse aortas, and IL-17 also induces the phosphorylation of p38 MAPK in vitro. Mouse aortic proteins were isolated from ApoE−/− mice and IL-17−/−/ApoE−/− mice fed with a high fat diet for 3 weeks. Representative Western blotting analyses of phosphorylated and total p38, phosphorylated and total JNK, and phosphorylated and total eNOS, and β-actin expressions are shown.

**A**, quantification of total p38. **B**, quantification of total JNK. **C**, quantification of the phosphorylated p38. **D**, quantification of phosphorylated JNK. **E**, schematic representation of how IL-17 transcriptionally and post-translationally activates the p38 MAPK pathway but not JNK MAPK pathway. **F**, quantitation of phosphorylated eNOS (Ser-1177). **G**, quantification of phosphorylated eNOS (Thr-495). **H**, IL-17 treatment induced the phosphorylation of p38 MAPK and its upstream kinase Map2k3 in HAECs. Representative Western blotting analyses of phosphorylated p38 MAPK, phosphorylated and total Map2k3, and total Map2k3 are shown. **I**, IL-17 treatment did not induce JNK and its upstream kinase Map2k4 in HAECs. Western blotting analyses of phosphorylated JNK, total JNK, phosphorylated Map2k4, and total Map2k4 are represented. Data are presented as means ± S.E. (*error bars*). *p < 0.05 (significant); n.s., not significant.
mouse aorta (43). However, our data showed that there was no statistical significance in these changes, possibly due to the counteracting effects of IL-17A-Rho kinase (43) signaling in inhibition of eNOS and IL-17A-p38 MAPK-estrogen receptor-α (41) signaling in activating eNOS in aortic ECs.

To further confirm the role of IL-17 in activating the p38 MAPK pathway, we tested the expression of p38 MAPK components in IL-17-treated HAECs by immunoblotting. However, we could not observe an increase in protein expression of either p38 MAPK or JNK or their upstream targets Map2k3 and Map2k4, respectively, in HAECs with IL-17 treatment (Fig. 7, I and J). Nevertheless, we observed an increase in phosphorylated p38 MAPK and phosphorylated Map2k3 in IL-17A-treated HAECs (Fig. 7I). Interestingly, we could not observe post-translational modification of JNK and its upstream target Map2k4 with IL-17 treatment (Fig. 7). Our in vitro data further validated that IL-17 specifically activates the p38 MAPK pathway in aortic ECs.

We then hypothesized that p38 MAPK deficiency would decrease IL-17A-induced gene expression of cytokines, chemokines, and endothelial adhesion molecule ICAM-1. To test this hypothesis, we performed an experimental data mining analysis in the National Institutes of Health NCBI GEO microarray data set database. The results of the only available p38α MAPK-deficient embryonic tissue microarray data set (GEO data set accession number GSE7342) in the National Institutes of
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A

Chow diet / high fat Diet  Sacrifice

Age (weeks)  8  11

Normal chow diet  High fat diet

ApoE⁻/⁻  IL-17⁻/⁻ApoE⁻/⁻

B

Body weight

C

LDL

HDL

Heart weight

Triglycerides

Non-esterified FFA

Spleen weight

Total Cholesterol

D

E

ApoE⁻/⁻  IL-17⁻/⁻ApoE⁻/⁻

chow

HF

% Lesion Area
Health NCBI database showed that p38α MAPK deficiency resulted in decreased expressions of IL-6 and (p < 0.05) and ICAM-1 (p < 0.08) (Fig. 8A). Although the regulation of these EC activation markers by p38 MAPK in embryonic tissue may not be directly related to IL-17A signaling, these results at least suggest that p38 MAPK activity may be critical for up-regulation of endothelial activation molecules. To further validate whether p38 MAPK activation mediates IL-17A-induced EC activation, we stimulated HAECs with IL-17A and examined ICAM-1 expression in the presence or absence of p38 MAPK-specific inhibitor SB203580 by flow cytometry. The results showed that the inhibition of p38 MAPK significantly decreased the expression of ICAM-1 in IL-17A-treated HAECs (Fig. 8B). Additionally, we analyzed the gene expression of CXCL1/2 in IL-17A-treated cells in the presence of SB203580 and observed that p38 MAPK inhibition can significantly ameliorate IL-17A-mediated induction of these two genes (Fig. 8, C and D). Moreover, we further demonstrated that untreated monocyte adhesion to IL-17A-treated HAECs can be suppressed by inhibiting p38 MAPK (Fig. 8E). Taken together, these results suggested that IL-17A activates HAECs via a p38 MAPK pathway.

**Deficiency of IL-17A in ApoE \(^{-/-}\) Mice Modulated the Lipid Profile but Did Not Significantly Affect the Atherosclerotic Lesion Formation**—Based on the results found with intravital microscopy of in vivo leukocyte adhesion as well as microarray analysis of in vivo aorta gene expression during early hyperlipidemia, we expected that IL-17 \(^{-/-}\)/ApoE \(^{-/-}\) mice would have decreased early atherosclerotic lesion formation. To test this hypothesis, we fed male ApoE \(^{-/-}\) and IL-17 \(^{-/-}\)/ApoE \(^{-/-}\) mice with either normal chow diet or high fat diet for 3 weeks and then measured the size of atherosclerotic lesions (Fig. 9A). Of note, the weights of body, heart, and spleen as well as lipid profiles including LDL, triglycerides, non-esterified free fatty acids, and total cholesterol were not affected by IL-17 gene deletion in ApoE \(^{-/-}\) mice (Fig. 9, B and C). Surprisingly, we found that the antiatherogenic high density lipoprotein (HDL) levels in the IL-17 \(^{-/-}\)/ApoE \(^{-/-}\) mice were significantly lower than those in ApoE \(^{-/-}\) mice fed with a high fat diet (Fig. 9C). In addition, there was also a slight decrease of LDL levels in IL-17 \(^{-/-}\)/ApoE \(^{-/-}\) mice (Fig. 9C).

Next, we quantified en face atherosclerotic plaque formation in the whole aortas of ApoE \(^{-/-}\) and IL-17 \(^{-/-}\)/ApoE \(^{-/-}\) mice. We found that IL-17 gene deficiency did not significantly affect atherosclerotic plaque buildup in the whole aorta (Fig. 9D). In agreement with results found in the en face staining, the percentages of lesion found in the aortic root were similar between the ApoE \(^{-/-}\) mice and IL-17 \(^{-/-}\)/ApoE \(^{-/-}\) mice (Fig. 9E). Thus, despite its proatherogenic role in promoting EC activation and leukocyte adhesion in vivo, IL-17A did not significantly contribute to early atherosclerotic lesion formation, possibly due to its counteracting effects on lipid metabolism (Fig. 9C).

**Discussion**

Previous studies of the role of IL-17 in atherogenesis have reported contradictory results; therefore, the role of IL-17 in atherogenesis remained controversial (44). These reports have claimed that IL-17 either had no atherosclerosis-regulatory effect (25), had a proatherosclerotic effect (26), or showed an antiatherosclerotic effect (27). However, the prevailing concept is that IL-17A plays a proatherogenic role in a context-dependent manner (45). Atherosclerosis is a complex process in which there is interplay of many cell types and a variety of pro- and antiatherogenic factors in the disease progression. Therefore, to gain insight into the role specifically played by IL-17 in atherosclerosis, we took a mechanistic approach and focused on studying the effects of IL-17 on aortic ECs, the first frontier vascular cells exposed to hyperlipidemia.

EC activation is the early essential step for an immune response, which includes two molecular events: up-regulation of cell surface adhesion molecules to make ECs more adhesive and increased secretion of proinflammatory cytokines and chemokines to attract monocytes and other inflammatory cells for transendothelial recruitment (46). Using biochemical, immunological, microarray, and data mining analyses as well as our newly generated ApoE \(^{-/-}\)/IL-17A \(^{-/-}\) mice, we report the following new findings.

1) The hyperlipidemia stimulus ox-LDL up-regulated IL-17 receptor A and IL-17 receptor C in mouse and human aortic ECs.

2) The deficiency of IL-17A in ApoE \(^{-/-}\) mice reduced leukocyte adhesion to endothelium in vivo, and IL-17A increased the adhesion of human monocytes to human primary aortic ECs in vitro.

3) IL-17A activated mouse and human primary aortic ECs via induction of proinflammatory cytokines IL-6 and GM-CSF and chemokines CXCL1 and CXCL2, which are critical for IL-17A-promoted endothelial adhesiveness.

4) IL-17A activated human primary aortic ECs via p38 MAPK pathway, and the deficiency of p38 MAPK in vitro decreased IL-17A-mediated induction of EC activation molecules including ICAM-1 and CXCL1/2 chemokines, leading to reduced monocyte adhesion to ECs.

5) IL-17A deficiency altered the lipid profile. Plasma HDL levels were lower in ApoE \(^{-/-}\)/IL-17A \(^{-/-}\) mice; this may counteract the protective effects exerted by IL-17 gene deficiency in early atherosclerosis. Taken together, our results demonstrated for the first time that IL-17A activated aortic ECs via a p38 MAPK pathway. Thus,
A previous report showed that IL-17 activated microvascular ECs via p38 MAPK phosphorylation in the lungs (39). IL-17 was also found to induce endothelial dysfunction in resistant (small) arteries and hypertension via a Rhoa/Rho kinase pathway (43) and to activate cultured mouse aortic ECs by increasing MCP-1 and IL-6 secretion (47). However, our current study had distinct advantages that enabled us to demonstrate the following findings for the first time. 1) We utilized an IL-17 gene knock-out mouse model in an ApoE-null background to demonstrate the specific role of IL-17 in endothelial activation and subsequent leukocyte adhesion in the presence of hyperlipidemia stimuli. This provided us a better model to study the distinct function of IL-17 in endothelial activation in vivo than the previous study that had used intracremaster injection of recombinant IL-17 into WT mice to examine this issue (39). 2) Although a previous report showed that IL-17 activated p38 MAPK phosphorylation in lung microvascular ECs, our microarray analysis, GEO p38 MAPK KO microarray data analysis, and p38 MAPK inhibitor experimental results demonstrated that IL-17 specifically activated a p38 MAPK pathway but not a JNK pathway by slightly inducing p38, MAP2K, and MAP3K gene transcription in aortic ECs. Additionally, we demonstrated that IL-17 could increase the kinase activity of p38 MAPK in aortic ECs. Also, we further validated that IL-17 induced the kinase activity of p38 and its upstream target Map2k3 in human primary ECs. 3) Our flow cytometry data also revealed that the hyperlipidemic stimulus ox-LDL up-regulated the protein expression of IL-17A receptor subunits IL-17RA and IL-17RC in HAEcs and IL-17RA in MAECs, whereas others showed a significant increase of IL-17RA and IL-17RC mRNA by qRT-PCR in bronchoscopic biopsy tissues of patients with asthma (39). 4) Also, to the best of our knowledge, we demonstrated for the first time that IL-17A induced the up-regulation of GM-CSF, IL-6, CXCL1, and CXCL2 in both mouse and human aortic ECs that subsequently led to enhanced monocyte adhesion, whereas others showed that IL-17A induced IL-1, IL-6, inducible NOS, COX2, and stromelysin in chondrocytes (48). Thus, our study is the first to provide a novel insight into the role of IL-17A in promoting aortic EC activation in hyperlipidemia.

Nevertheless, we did not observe a profound protection by IL-17 gene deletion in ApoE-null mice against atherosclerotic plaque formation. Our data are comparable with a previous study that had demonstrated that ApoE−/− mice treated with anti-mouse IL-17A had no significant changes in atherosclerotic plaque burden relative to the control mice (44). We believe that this observation is presumably due to alteration of the lipid profiles seen in ApoE−/−/IL-17A−/− mice. We observed a significant reduction in antiatherogenic HDL levels, which implicated a potential decrease in reverse cholesterol transport in ApoE−/−/IL-17A−/− mice that might counteract the protection exerted by IL-17 gene deletion. Similar to our findings in mice, previously published data implicated that plasma IL-17 levels were low in patients with metabolic syndrome with low HDL levels (49). However, our mice were fed with high fat diet for 3 weeks, which represents a model of early atherogenesis as we recently reported (3). Further studies are required to elucidate the long-term effects exerted by IL-17 on atherosclerosis under hyperlipidemia stimuli. Moreover, this study further established the complexity of atherosclerotic disease progression due to the interplay among a myriad of cells, metabolites, chemokines, and cytokines. Insignificant changes of atherosclerotic plaque sizes in the double KO compared with those of ApoE −/−/IL-17A−/− controls do not diminish the role of IL-17 in promoting human aortic endothelial cell activation demonstrated in our report and actually emphasize the significant roles of IL-17 promotion of aortic endothelial cell activation in counteracting potential roles of IL-17 in modulating other cellular events in atherogenesis.

In summary, via multiple approaches, we demonstrated that hyperlipidemia, one of the most prominent metabolic risk factors, promoted IL-17 gene knock-out mice presumably via enhancing T helper 17 subset polarization and that IL-17A induced aortic EC activation, the early phase of hyperlipidemia-induced vascular inflammation. Our current work has shed light onto the role of IL-17 in EC biology and has provided important insights for future development of novel therapeutics for early intervention of cardiovascular diseases and other inflammatory diseases.

**Author Contributions**—J. M. and G. N. carried out most of the experiments, analyzed the results, and wrote most of the paper. J. L.-P., X. L., Y.-F. L., X. W., A. S., A. V., Y. S., H. S., F. L., M. V. A., S. P. K., Y. X. J., and H. W. provided material input and helped revise the manuscript. X.-F. Y. conceived the study and wrote the paper with J. M. and G. N.

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