Interleukin 17A Exacerbates Atherosclerosis by Promoting Fatty Acid-Binding Protein 4–Mediated ER Stress in Macrophages

Qi Gao1,2,* Yang Jiang,1,3,* Shen Dai1, Bo Wang1, Fei Gao4, Chun Guo1, Faliang Zhu1, Qun Wang1, Xiaoyan Wang1, Jianing Wang1, Yongyu Shi1, Yun Zhang,4 Wanjun Chen5 and Lining Zhang1

1Department of Immunology, Shandong University School Medicine, 2Department of Clinical Laboratory, Provincial Hospital Affiliated to Shandong University, 3Department of Biology Laboratory, the Second Hospital of Shandong University, 4Key Laboratory of Cardiovascular Remodeling and Function Research, Qilu Hospital, Shandong University, Jinan, Shandong, P.R. China; and 5Mucosal Immunology Section, OIIB, National Institute of Dental and Craniofacial Research, NIH, USA.

*Q.G. and Y.J. contributed equally to this work.

Running Title: IL-17A Promotes aP2-Mediated Macrophage ER Stress

Subject codes:
[130] Animal models of human disease
[134] Pathophysiology
[135] Risk Factors
[137] Cell biology/structural biology
[142] Cell biology/structural biology
[90] Lipid and lipoprotein metabolism

Address correspondence to:
Dr. Lining Zhang
Department of Immunology
Shandong University School Medicine
44# Wenhua Xi Road
Jinan 250012
Shandong, P.R. China
Tel: +86-0531-88382038
Fax: +86-0531-88382038
zhanglining@sdu.edu.cn

Dr. Wanjun Chen
Mucosal Immunology Section
Oral Infection and Immunity Branch
National Institute of Dental and Craniofacial Research
US National Institutes of Health
30 Convent Drive
Bethesda, Maryland 20892
Tel: 301-435-7168
Fax: 301-435-7168
wchen@dir.nidcr.nih.gov

In September 2012, the average time from submission to first decision for all original research papers submitted to Circulation Research was 11.5 days.

DOI: 10.1161/CIRCRESAHA.112.272567
ABSTRACT

**Rationale:** Apoptosis and fatty acid-binding protein-4 (FABP4) induced-endoplasmic reticulum (ER) stress in macrophage is an important pathological process in several vascular occlusive diseases, including atherosclerosis, both of which are accelerated by lipids or inflammatory cytokines.

**Objective:** To determine whether interleukin 17A (IL-17A) accelerates atherosclerosis through activating FABP4-mediated ER stress in macrophage.

**Methods and Results:** We show here that IL-17A induced ER stress in both murine and human-derived macrophages in vitro, and in the atherosclerotic lesions of ApoE-/- mice. Treating ApoE-/- mice with a chemical chaperone alleviated IL-17A–mediated ER stress and macrophage apoptosis, which was accompanied by recovered atherogenesis. Mechanistically, IL-17A up-regulated the expression of FABP4 (aP2), a cytosolic lipid chaperone that is able to promote lipid-induced macrophage ER stress, through NF-κB and ERK/p38 mitogen-activated protein kinase (MAPK) pathways in macrophages. The inhibition of aP2 expression with a specific chemical inhibitor significantly blocked IL-17A-accelerated ER stress and apoptosis in plaque, and partially rescued IL17A-induced atherogenesis.

**Conclusions:** The data collectively establish a previously unrecognized link between IL-17A and ER stress through cytosolic lipid chaperone aP2 in macrophages and provide a new insight for understanding the role of IL-17A in atherosclerosis.

**Keywords:** IL-17A, FABP4 (aP2), ER stress, macrophages, atherosclerosis

**Non-standard Abbreviations:**

- ApoE: apolipoprotein E
- aP2: adipocyte lipid-binding protein
- ATF6: activating transcription factor 6
- CHOP: C/EBP homologous protein
- eIF2α: eukaryotic translation initiation factor 2α
- ER: endoplasmic reticulum
- FABP4: Fatty acid-binding protein-4
- GM-CSF: granulocyte/macrophage colony stimulating factor
- HDL: high-density lipoprotein
- IRE1: inositol-requiring enzyme 1
- LDL: low-density lipoprotein
- LPS: lipopolysaccharide
- MAPK: mitogen-activated protein kinase
- NF-κB: nuclear factor kappa B
- PBA: 4-phenyl butyric acid
- PBMC: Peripheral blood mononuclear cells
PERK  protein kinase R-like eukaryotic initiation factor 2 kinase
TCH  total cholesterol
TG  triglycerides
TLR  toll like receptor
TNF-α  tumor necrosis factor α
UPR  unfolded protein response
XBP1  X-box binding protein 1

INTRODUCTION

Atherosclerosis, a lipid-driven, chronic inflammatory disease of large and medium-sized arteries, is the pathological basis for multiple cardiovascular diseases, a leading cause of mortality worldwide.1 Many kinds of immune cells, such as macrophages and CD4+ T cells contribute to formation and advance of lesions.2-4 Recently, Th17 cells producing IL-17A were found involved in the pathogenesis of atherosclerosis.5,8 Although not unanimous,9-11 increasing evidence, including our own studies, suggests that IL-17A is pro-atherogenic.12-14 Increased IL-17A expression and number of IL-17A+ T cells were detected in human15 or murine atherosclerotic lesions and found associated with plaque size.16,17 Furthermore, atherosclerotic lesions were reduced with knockout of IL-17A18,19 or blockade of IL-17A protein with specific neutralizing antibody17,20 or adenovirus-produced IL-17A receptor,21 whereas treatment with exogenous IL-17A aggravated the formation of lesions in ApoE-/-mice.17 In addition, data from humans and a mouse model suggested that IL-17A enhances the vulnerability of plaque.12,15,11 However, the mechanism by which IL-17A accelerates the formation and progression of atherosclerosis remains poorly understood.

Endoplasmic reticulum (ER) is site where newly synthesized secretory and membrane-associated proteins are correctly folded and assembled.22,23 Once the ER function is perturbed by various pathological conditions, newly synthesized unfolded proteins accumulate in the ER, which results in ER stress, also defined as the unfolded protein response (UPR).24,25 The initiation of canonical UPR engages 3 distinct signaling branches mediated by protein kinase R (PKR)-like eukaryotic initiation factor 2α kinase (PERK), inositol-requiring enzyme 1 (IRE1)26,27 and activating transcription factor 6 (ATF6).28 PERK, by phosphorylating eukaryotic translation initiation factor 2 (eIF2α), IRE1 and ATF6, by promoting the expression of their downstream targets such as transcription factor genetic X-box binding protein 1 (XBP-1),29,31 inhibits protein translation, stimulation of protein degradation, and production of chaperone proteins.22,23 The recovery of ER function by the UPR is critical for cell survival, but chronic or unresolved ER stress can lead to apoptosis.32

Accumulating evidence indicates that the UPR is chronically activated in macrophages and endothelial cells in atherosclerotic lesions. Oxidative stress, high levels of intracellular cholesterol and saturated fatty acids in advanced lesions can lead to prolonged activation of the UPR.33 Prolonged ER stress further triggers macrophage apoptosis by up-regulating expression of C/EBP-homologous protein (CHOP) or caspase12,34,37 which in turn leads to plaque necrosis and the progression of atherosclerosis if the apoptotic cells are not rapidly cleared.38 However, improvement of the ER
chaperone function via a chemical chaperone attenuates atherosclerotic lesions.\textsuperscript{39} Blocking IL-17A with IL-17A antibody reduced apoptosis in atherosclerotic plaques.\textsuperscript{20} We recently showed that IL-17A promotes apoptosis of human vascular endothelial cells by a mitochondrial pathway in vitro.\textsuperscript{40} Macrophages, a key player in atherosclerosis formation, are targets of IL-17A and participate in IL-17A–mediated inflammation.\textsuperscript{41} So we make a hypothesis that IL-17A may activate an ER stress pathway, thereby altering the function and survival of macrophages and the course of atherogenesis.

Fatty acid-binding protein-4 (FABP-4) (aP2) is expressed specifically in both adipocytes and macrophages and is a cytosolic lipid chaperone that regulates cellular lipid metabolism and reception of lipid signals.\textsuperscript{42} aP2 promotes chronic metabolic diseases, including atherosclerosis. aP2 deficiency in macrophages protects against atherosclerosis.\textsuperscript{43-45} It is the predominant regulator of lipid-induced macrophage ER stress in vitro and in vivo.\textsuperscript{39} However, whether IL-17A can affect aP2 expression in macrophages to regulate ER stress is unknown.

In this study, we found that IL-17A induced ER stress in macrophages through aP2. Prolongation of IL-17A-induced ER stress promoted the apoptosis of macrophages and atherosclerosis. We established a previously unrecognized link between IL-17A and ER stress through cytosolic lipid chaperone aP2 in macrophages and provide a new insight for understanding the role of IL-17A in atherosclerosis.

METHODS

Animals.
Male C57BL/6 wild-mice and ApoE-/- mice were purchased from Beijing University. Mice were 6–8 weeks old (21–25g) and were housed at a constant temperature (24°C), under a 12-h dark/12-h light cycle room in the Animal Care Facility of Shandong University Medical School according to institutional guidelines. All animal studies were approved by the Animal Care and Utilization Committee of Shandong University, China.

In vivo application of exogenous IL-17A, 4-phenyl butyric acid (PBA) and ap2 inhibitor.
ApoE-/- mice (n=10 for each group) were fed a high-cholesterol diet from 8 weeks of age. Four weeks later, two groups for treatment once a week for 5 weeks: intraperitoneal injection with exogenous recombinant IL-17A, 0.02% of recombinant mouse IL-17A in normal saline (2μg/mouse/time, PMC0175, Invitrogen, Clasbad, CA, USA); and normal saline containing 0.02% albumin as a control.\textsuperscript{9,17} The third group (n=10), mice were pretreated before IL-17A with the chemical chaperone PBA (P21005, Sigma-Aldrich, St. Louis, MO, USA) (100 mg/kg/time) twice a week for 5 weeks\textsuperscript{39} at the same time mice also received IL-17A treatment (2μg/mouse/time) once a week for 5 weeks. The fourth group (n=7) fed with a high-cholesterol diet from 8 weeks of age and two weeks later, mice were treated by oral gavage with vehicle including 10% 1-methyl-2-pyrrolidone and 5% cremophor EL with ethanol in 100μl of water or 20mg/kg/d of the ap2 inhibitor BMS309403 dissolved in the vehicle for 6 weeks\textsuperscript{39,45}. Mice also received IL-17A treatment (2μg/mouse/time) once a week for 5 weeks after four weeks high-cholesterol diet.
Induction and culture of human monocyte-derived macrophages and THP-1-derived macrophages.
Peripheral blood mononuclear cells (PBMCs) from healthy volunteer blood at Shandong University Medical School were isolated by Ficoll density gradient centrifugation and washed twice with serum-free RPMI 1640 medium (Gibco, Invitrogen, Clasbad, CA, USA). PBMCs were then plated at 3 ml/well in 6-well cell culture plates, 3 hours later un-adherent cells were removed and adherent cells cultured with RPMI 1640 complete medium (10% FBS, 1% L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids, 1% penicillin/streptomycin and 50ng/ml rhGM-CSF) (catalog: 300-03, PeproTech, Suzhou, JS, P.R. China). Half of the medium was replaced every 2 days for culture for 7 days. On the day of the experiment, the cells were washed 3 times in warm PBS and incubated as described in the figure legends. THP-1 cell lines were cultivated in 6-well flat-bottom plates with DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and PMA(50ng/ml) for 48 hours, then the cells were washed 3 times in warm PBS and incubated as described in the figure legends.

Supplemental methodology.
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org. For detailed methods related to analysis of lipid profile, Histopathology and immunohistochemistry, TUNEL and macrophage apoptosis assay, Isolation of mouse peritoneal macrophages and culture in vitro, Western blot and RT-PCR analysis, Enzyme-linked Immunosorbent Assay (ELISA), and Statistical analysis see the Online Data Supplement.

RESULTS

IL-17A promotes macrophage apoptosis in vitro.
Accumulated evidence indicates that Th17 cells and its major effector, IL-17A, play a pro-atherogenic role. However, the underlining mechanism is unclear. Nowadays, there is increasing evidence in vitro and in vivo indicating that late apoptosis of intimal cells, notably macrophages, plays an important role in atherosclerotic plaque progression. To study the mechanism by which IL-17A promotes the development of atherosclerosis, we firstly examined the effect of IL-17A on apoptosis of macrophages. We showed that macrophages treated by IL-17A (25ng/ml, 50ng/ml and 100ng/ml) for 48 hours exhibited increase in apoptotic bodies as white arrows point and the number of cells containing apoptotic bodies further arose at 72 hours (Figure 1A). Next, we confirmed the role of IL-17A promoting macrophages apoptosis by flow cytometry. As shown in Figure 1B, C, the number of late apoptotic (Annexin V+ PI+) macrophages increased markedly when cells were treated in IL-17A 25ng/ml for 48 hours and 72 hours. Furthermore, the effect of IL-17A on macrophage was in a dose dependent manner. In addition, we also observed whether IL-17A accelerated ox-LDL induced apoptosis in macrophages. Results from flow cytometry analysis showed that the percentage of PI positive cells was significant higher with ox-LDL and IL-17A stimulation (Supp Figure I). This indicates that prolonged-IL-17A intervention raised significantly macrophage apoptosis and promoted ox-LDL-induced macrophage apoptosis or necrocytosis in vitro.
IL-17A induced ER stress in murine and human macrophages in vitro.

Our previous report demonstrated that IL-17A induced vascular endothelial cell apoptosis. Here we found that IL-17A also promoted macrophage apoptosis. Because ER stress is an important mechanism of macrophage apoptosis, we further investigated the impact of IL-17A on ER stress in murine and human macrophages. IL-17A time-dependently increased levels of phosphorylated PERK (p-PERK) and its downstream molecule p-eIF2α in the murine macrophage cell line (RAW264.7) and primary peritoneal macrophages (Figure 2A,B). Thus, IL-17A could initiate the UPR in ER by activating the PERK pathway. Furthermore, IL-17A increased the splicing of XBP1 (XBP1s), the downstream target molecule of IRE1α and ATF6 pathways, at 1 h and up to 12 h (Figure 2A,B). Thus, IL-17A initiated a UPR by activating p-PERK, p-eIF2α and XBP1s, which induced ER stress in murine macrophages. To investigate whether IL-17A plays a similar role in human macrophages, human peripheral blood mononuclear cells (PBMCs) isolated from healthy donors were induced into macrophages by human granulocyte macrophage-cerebral spinal fluid (GM-CSF), and human monocyte Thp-1 cells were induced into macrophages by phorbol-12-myristate-13-acetate (PMA). Consistent with the results from mice, IL-17A time-dependently increased levels of p-PERK and p-eIF2α, and XBP1s in human macrophages (Figure 2 C,D). Thus, IL-17A promotes ER stress in both murine and human macrophages in vitro.

Exogenous IL-17A induced ER stress in macrophages in atherosclerotic lesions of ApoE-/- mice.

To confirm the impact of IL-17A on ER stress in vivo, we treated ApoE-/- mice that had been fed with high cholesterol diet with recombinant IL-17A and analyzed the expression of the ER stress markers p-PERK, p-eIF2α and XBP-1s in the arterial wall with plaque and in local lesions. Western blot analysis revealed significantly up-regulated levels of p-PERK, p-eIF2α and XBP-1s with IL-17A treatment (Figure 3A,B) and immunohistochemistry revealed increased levels of p-eIF2α (20.59% ±1.35% vs. 30.75%±3.04%, P<0.05)and p-PERK (28.59% ±1.36% v.s. 38.55% ±1.80%, P<0.01) (n=5) mainly in macrophage-dense areas of atherosclerotic lesions (Figure 3C). Of note, IL-17A had no effect on blood lipid levels or body weight (Supp. Table I), which indicates that IL-17A directly induced ER stress responses in macrophages in vivo without affecting lipid metabolism.

Inhibiting ER stress alleviated IL-17A–induced ER stress and reversed IL-17A–aggravated atherogenesis in ApoE-/- mice.

4-phenyl butyric acid (PBA), a chemical chaperone that can mimic endogenous chaperone Bip and alleviate ER stress, provides an experimental opportunity to investigate the role of ER stress in diseases. Previous research has indicated that PBA treatment could reduce the atherosclerosis in ApoE-/- mice with high fat diet. In our early experiment, this result had substantially been reproduced (Supp. Figure II). To clarify the cause of ER stress in IL-17A–aggravated atherogenesis, we treated ApoE-/- mice with recombinant mouse IL-17A (2μg) or IL-17A with PBA (100mg/kg). IL-17A and/or PBA had no significant impact on body weight or blood lipid profiles of mice (Supp. Table 1). However, PBA could markedly block IL-17A–induced ER stress responses: levels of p-PERK, p-eIF2α and XBP-1s protein in vessels with atherosclerotic plaque were lower with PBA than with IL-17A alone (n=5) (Figure 3A,B). Immunohistochemistry staining revealed reduction expression of p-PERK and p-eIF2α in macrophage-rich areas of plaque lesions with PBA than IL-17A alone treated mice (p-PERK: 38.55% ±1.80% vs. 29.33%±1.00%, P<0.01; p-eIF2α:30.75% ±3.04% v.s. 22.79% ± 1.24%, P<0.05, n=5; Figure 3C), with no significant reduction in proportion of

DOI: 10.1161/CIRCRESAHA.112.272567
macrophage to lesion area (IL-17A-treated vs IL-17A+PBA-treated: 31.55% ± 2.79% v.s. 29.55% ± 2.39%, P>0.05). Thus, PBA reduced IL-17A–induced macrophage ER stress in vivo.

To clarify whether blockade of IL-17A–induced ER stress by PBA could reverse IL-17A–aggravated atherogenesis in ApoE--/ mice, we analyzed plaque area in aortic root sections of atherosclerotic mice. Plaque size was lower by 45% with PBA treatment than with IL-17A alone (P<0.001) (Figure 3D), along with suppression of IL-17A–induced ER stress. These data establish a link between ER stress and IL-17A–accelerated atherosclerotic lesions.

*IL-17A–induced ER stress leads to apoptosis of macrophages.*

ER-associated CHOP and caspase 12 pathways are involved in ER stress-mediated apoptosis. To elucidate the association of IL-17A–induced ER stress and macrophage apoptosis, we examined the expression of CHOP and caspase 12 in murine macrophages in vitro. The expression of CHOP was markedly increased (Figure 4A) but that of caspase 12 only slightly up-regulated with IL-17A treatment (data not shown). The expression of cleaved caspase 3 was also increased (Figure 4A), which suggests that IL-17A probably led to macrophage apoptosis by activating the CHOP–caspase 3 axis.

To address this issue in vivo, we examined the expression of CHOP, caspase 12 and caspase 3 in aortic vasculature with atherosclerotic lesions. The protein expression of CHOP and cleaved caspase 3 was markedly higher with IL-17A than control treatment by western blot (Figure 4B,C) and immunohistochemistry analysis (Figure 4D, CHOP: Control vs. IL-17A 13.82% ± 0.68% vs. 22.07% ± 1.63%, P<0.01, n=5). However, caspase 12 level did not differ between IL-17A and control treatment (data not shown). Indeed, atherosclerotic lesions with increased number of macrophages with IL-17A treatment also showed significantly increased number of apoptotic cells as compared with control lesions on TUNEL staining (Control v.s. IL-17A: 45.33 ± 3.0 versus 114.3 ± 14.29, P<0.001) (Figure 4D).

To investigate whether the IL-17A–induced macrophage apoptosis was attributed to ER stress signal pathways, we examined the levels of CHOP and caspase 3 and apoptotic cells with PBA treatment. The expression of CHOP and caspase 3 and number of apoptotic cells was lower with PBA than with IL-17A alone (Figure 4B,C,D), which indicates that IL-17A induces apoptosis in macrophages through the ER stress in mice and probably mainly through activating the CHOP–caspase 3 pathway, which may accelerate the progression of atherosclerosis.

*aP2 is required for IL-17A–induced ER stress in macrophages.*

Recent study has suggested that aP2, an intracellular “lipid chaperone”, mediates saturated fatty acid-induced ER stress and apoptosis in macrophages. Therefore, aP2 might be involved in IL-17A–induced ER stress in macrophages. We first investigated the effect of IL-17A on aP2 expression in macrophages. IL-17A up-regulated aP2 mRNA and protein levels in macrophages in vitro (Figure 5A) and significantly increased the expression of aP2 in atherosclerotic lesions in vivo (Control v.s. IL-17A-treated: 15.03% ± 1.3% versus 23.65% ± 2.11%, P<0.01, n=5) (Figure 5B). To determine the role of increased aP2 level in IL-17A–induced ER stress, we treated macrophages with the aP2 inhibitor (BMS309403) and IL-17A and found that 50 M BMS309403 almost completely inhibited the expression of aP2 (Supp. Figure III). Importantly, inhibition of aP2 markedly reduced...
the levels of p-eIF2α and XBP-1s in macrophages (Figure 5C). Therefore, aP2 expression is required at least in part for IL-17A–induced ER stress in macrophages.

To address this issue in vivo, we treated ApoE-/- mice with recombinant mouse IL-17A (2μg) or IL-17A with aP2 inhibitor (BMS309403)(20mg/kg/d)93,45. IL-17A and/or aP2 inhibitor had no significant impact on body weight or blood lipid profiles of mice (Supp. Table II). However, inhibition of aP2 could markedly block IL-17A–induced ER stress in macrophage. As shown in Figure 5D,5E, levels of p-PERK, p-eIF2α and XBP-1s protein in vessels with atherosclerotic plaque were lower with aP2 inhibitor than with IL-17A alone by Western Western blot or by Immunohistochemistry staining (n=7) (p-PERK: 38.55% ±1.80% v.s. 16.66% ±1.23%, P<0.001; p-eIF2α: 30.75% ± 3.04% v.s. 17.59% ± 1.10%, P<0.01, n=5; Figure 5E), with no significant reduction in proportion of macrophage to lesion area. So, aP2 inhibitor also reduced IL-17A–induced macrophage ER stress in vivo.

To clarify whether blockade of IL-17A–induced ER stress by aP2 inhibitor could reverse IL-17A–aggravated apoptotic macrophages and atherogenesis in ApoE-/- mice, we analyzed the level of CHOP proteins, numbers of apoptotic macrophage and plaque area in aortic root sections of atherosclerotic mice. The expression of CHOP and number of apoptotic cells was lower with aP2 inhibitor than with IL-17A alone (Figure 5D,5E), which indicates that IL-17A induces apoptosis in macrophages through the ER stress in mice could be reversed by aP2 inhibitor. And, plaque size in aortic root (Figure 5F, p<0.05, n=5) and on aortic arch-thoracic-abdominal aorta (Figure 5G, p<0.05, n=3) was lower with aP2 inhibitor treatment than with IL-17A alone, along with suppression of IL-17A–induced ER stress. These data suggest that IL-17A induced ER stress in macrophage through up-regulating the expression of aP2.

**IL-17A up-regulated aP2 by activating NF-κB and extracellular signal-regulated kinase (ERK)/p38 mitogen–activated protein kinase (MAPK) pathways.**

Next, we investigated the underlying molecular mechanisms of IL-17A–mediated aP2 upregulation. IL-17A induced the phosphorylation of IκB (p-IκB) in macrophages within 15 min (Figure 6A) and up-regulated the expression of p-ERK at 15 min, then p-p38 MAPK at 2 h, whereas p-Jun N-terminal kinase (p-JNK) was activated later, at 3 h after IL-17A stimulation (Figure 6B). These data indicate that IL-17A could activate NF-κB and MAPK signal pathways in macrophages in vitro.

To determine the cause of these signal pathways in IL-17A–upregulated aP2 level, we treated mouse macrophages with the inhibitor of NF-κB (PDTC), ERK (PD98059), JNK (SP600125) or p38 (SB203580) for 3, 6, and 9 h. Western blot analysis revealed that inhibition of NF-κB, ERK and p38 but not JNK suppressed the expression of aP2 (Figure 6D). Thus, IL-17A up-regulates the expression of aP2 by activating NF-κB and ERK, p38 MAPK signaling pathway in macrophages.
DISCUSSION

Today, the role of IL-17A in atherosclerosis development still remains controversial. Some research have proposed that IL-17A plays an atheroprotective role, while others have demonstrated IL-17A does a proatherogenic role. In addition, a few of reports have showed that the deficiency of IL-17A or IL-17A blockage by mouse-anti-mouse IL-17A have no effect on plaque burden. Here, we indicate that IL-17A can induce ER stress in both murine and human-derived macrophages in vitro, and in the atherosclerotic lesions of ApoE-/- mice which contribute to IL-17A–caused macrophage apoptosis and aggravation of atherogenesis. Moreover, we reveal a link between IL-17A and ER stress through aP2 in macrophages and small inhibitors of aP2 significantly reduced macrophage apoptosis and atherogenesis in ApoE-KO mice. Altogether, our study provides additional evidence and mechanism for a pro-atherogenic role of IL-17A in atherosclerosis.

Increasing data have demonstrated that elevated ER stress in macrophages accelerates the development of early and advanced atherosclerotic lesions. Atherosclerotic plaques, particularly advanced lesions, contain a large amount of toxic lipids (such as saturated fatty acids or free cholesterol) and pro-inflammatory cytokines (tumor necrosis factor α [TNF-α], interferon γ [IFN-γ], IL-6, IL-12), which provide a pathophysiological environment. Toxic lipid-induced ER stress in macrophages contributes to cell apoptosis, inflammation in plaque and progression of atherosclerosis. However, few studies have investigated the role of cytokines in inducing ER stress. TNF-α induced the UPR in murine fibrosarcoma L929 cells, and lipopolysaccharide (LPS) with IFN-γ activated ER stress in the macrophage RAW264.7 cell line. IL-17A is highly expressed in atherosclerotic lesions, particularly in advanced plaque, in mice and humans and contributes to the development of atherosclerosis. IL-17A can stimulate macrophages to produce a high level of IL-17A-induced ER stress response. However, increased TNF-α level may exacerbate the IL-17A–induced ER stress response. In addition, IL-17A induced ER stress in cultured human and murine-derived macrophages in media containing 10% serum without exogenous toxic lipids (Figure 2). Thus, IL-17A may induce ER stress directly or indirectly by up-regulating TNF-α in macrophages independent of toxic lipid levels.

Previous research has shown the lipid chaperone aP2 as a central modulator of lipid-induced ER stress responses. Toxic lipid up-regulate aP2 expression, which promotes the production of phospholipids rich in monounsaturated fatty acids and bioactive lipids that render macrophages resistant to lipid-induced ER stress. Here we demonstrated that aP2 is also a linker of IL-17A–activated ER stress responses in macrophages. aP2 is highly up-regulated with oxidized low-density lipoprotein stimulation in macrophages and Toll-like receptor (TLR) activation by LPS (a TLR4 ligand), zymosan (a TLR2 ligand) and polyinosine: polycytidylic acid (poly I:C, a TLR3 ligand) even without exogenous lipids. Signaling pathways from TLR and IL-17A receptors are similar. Both IL-17RA and TLRs trigger the MAPK and NF-κB pathways. Both engage TNF receptor-activated factor to trigger inflammatory signaling components such as NF-κB and ERK.
Thus, the pathways may have coevolved common strategies to respond to specific insults. Here, we demonstrated that IL-17A greatly up-regulates the expression of lipid chaperone aP2 mainly by NF-κB and ERK, p38 MAPK pathways. This action of IL-17A on aP2 is independent of lipid supplementation in cultured macrophages and alteration of lipid metabolism in hyperlipidemic mice. Furthermore, blocking aP2 markedly mitigated IL-17A–induced ER stress activation and rescued IL-17A-induced atherogenesis. The data establish a previously unrecognized link between IL-17A and ER stress through the cytosolic lipid chaperone aP2 in macrophages.

IL-17A-elevated ER stress in macrophages is required for its promotion of ER stress-associated macrophage apoptosis and atherosclerotic lesions. Several experimental findings have supported CHOP as a transcriptional target for three UPR signal branches and playing an important role in ER stress-associated macrophage apoptosis, particularly in advanced atherosclerotic lesions. In addition, in certain cell types, species and conditions, such as murine macrophages, ER stress-mediated apoptosis involves caspase 12 activation. Here we found that IL-17A stimulation markedly elevated CHOP expression but had no obvious impact on caspase 12 activation in vitro and in vivo. More importantly, treatment with the ER chemical chaperone PBA and aP2 inhibitor could alleviate IL-17A–induced ER stress and decrease CHOP expression and the number of apoptotic macrophages in lesions; it could reverse the IL-17A–aggravated atherosclerosis in ApoE-/- mice (Figure 3, 5). So we demonstrated that IL-17A–induced ER stress leads to macrophage apoptosis mainly via the CHOP. These data suggest that therapeutic applications targeting ER stress responses may be effective for dyslipidemia and inflammation-promoted atherosclerosis.

We propose that the binding of IL-17A to IL-17A receptor (IL-17RA) in macrophages activates NF-κB and MAPK (ERK, p38 and JNK) signal pathways. Activation of the NF-κB and p38, ERK pathway is responsible for the up-regulation of the lipid chaperone aP2, which mediates the activation of the ER stress response (elevated expression of p-eIF2α and XBP1s) in macrophages. Chronic ER stress leads to high expression of CHOP, whereby IL-17A induces macrophage apoptosis and then aggravates atherosclerosis. In addition, ER stress may directly influence the development of atherosclerosis (Figure 7). These findings illustrate a crucial mechanism of how IL-17A may affect macrophage ER stress and exacerbate the development of atherosclerosis.

SOURCES OF FUNDING
This research was supported by the National “973” program of China (2011CB503906), the National Natural Science Foundation of China (81172863, 81128013, 81072407) and the Intramural Research Program of the National Institute of Dental and Craniofacial Research, US National Institutes of Health.

DISCLOSURES
None.

DOI: 10.1161/CIRCRESAHA.112.272567
REFERENCES

DOI: 10.1161/CIRCRESAHA.112.272567


33. Feng B, Yao PM, Li Y, Devlin CM, Zhang D, Harding HP, Sweeney M, Rong JX, Kuriakose G, RETRACTED ARTICLE

DOI: 10.1161/CIRCRESAHA.112.272567

This article was retracted in April 2013.


47. Suzuki M, Mihara M. Adiponectin induces CCL20 expression synergistically with IL-6 and TNF-a in THP-1 macrophages. *Cytokine* 2012; 58: 344-350.


49. Van Vré EA, Ait-Oufella H, Tedgui A, Mallat Z. Apoptotic Cell Death and Efferocytosis in


This article was retracted in April 2013
**FIGURE LEGENDS**

**Figure 1.** Interleukin 17A (IL-17A) promotes macrophage apoptosis in vitro. Mouse primary macrophages from C57BL/6 mice were stimulated with IL-17A, 25, 50 and 100ng/ml, for 48 or 72 h in vitro. (A) Hoechst staining showed nuclear fragmentation and condensation and cells undergoing apoptosis. (Original magnification 400×); (B) Apoptosis rate of cells with Annexin V and PI staining determined by flow cytometry. (C) Quantification of apoptosis. Data are mean±SEM from 3 representative experiments. * P<0.05, **P<0.01.

**Figure 2.** IL-17A induced endoplasmic reticulum stress (ER) in murine and human-derived macrophages in vitro. (A) The mouse macrophage line (RAW264.7) or (B) mouse primary macrophages from C57BL/6 mice were stimulated with IL-17A (25ng/ml) in vitro for different times and cells cultured in same fresh medium for 6 hours as control. Western blot analysis of the protein levels of p-PERK, p-eIF2α, splicing of XBP-1 (XBP-1s) and β-actin. Data are representative of 3 independent experiments. Human monocyte line Thp-1 was induced into macrophages with phorbol-12-myristate-13-acetate (PMA) (50ng/ml) for 2 days. Peripheral blood mononuclear cells (PBMCs) isolated from healthy human whole blood were induced into macrophages by use of human granulocyte macrophage-cerebral spinal fluid (50ng/ml) for 7 days. Then, Thp-1 and PBMC-derived macrophages were stimulated with human IL-17A (50ng/ml) and cells cultured in same fresh medium for 6 hours as control. Western blot analysis of levels of p-PERK, p-eIF2α, XBP-1s and β-actin is representative of 3 independent experiments.

**Figure 3.** 4-phenyl butyric acid (PBA) can alleviate IL-17A-aggravated atherogenesis and IL-17A–induced ER stress in macrophage-dense areas of atherosclerotic lesions in mice. ApoE-/- mice, 8 weeks old, were fed a high-fo diet for 4 weeks and then treated for 5 weeks with recombinant mouse IL-17A (once a week), IL-17A(once a week) with PBA (twice a week), or the same quantity of mouse albumin (control) (twice a week). (A) Western blot analysis levels of p-PERK, p-eIF2α, XBP1s and β-actin in mouse aortic arch with plaque lesions and (B) quantification. Data are mean±SEM. (n=5/group), (C) Immunohistochemistry of mouse aortic root serial sections for levels of Moma-2 (for macrophages), p-PERK and p-eIF2α (original magnification ×100), and (D) Oil-red O staining for lipid content on the left (original magnification ×40). Quantification of staining from control, IL-17A-treated and IL-17A plus PBA–treated mice (n=5/group) expressed as percentage of total lesion area on the right. Data are mean± SEM. * P<0.05, ** P<0.01, *** P<0.001.

**Figure 4.** IL-17A–induced ER stress in macrophages led to apoptosis. Mouse primary macrophages from C57BL/6 mice were stimulated with IL-17A (25ng/ml) for different times and cells cultured in same fresh medium for 6 hours as control. (A) Western blot analysis the expression CHOP and cleaved caspase 3. Data are representative of 3 independent experiments. (B) Western blot analysis the expression of CHOP and cleaved caspase 3 in mouse aortic arch with IL-17A or IL-17A combined with PBA treatment and (C) quantification. Data are mean±SEM (n=5/group), (D) Immunohistochemistry of mouse aortic roots serial sections for CHOP (original magnification ×40) and Moma-2 staining (original magnification ×200). Number of apoptotic cells in plaque lesions labeled by TUNEL staining (original magnification ×200). Quantification expressed as percentage of macrophage-positive area (n=5/group) on the right. Data are mean± SEM. ** P<0.01, *** P<0.001.
**Figure 5.** IL-17A up-regulated aP2, which is required for IL-17A–induced ER stress in macrophages. (A) RT-PCR and western blot analysis of mRNA and protein levels of aP2 and β-actin in IL-17A (25 ng/ml)-treated mouse primary macrophages. Data are mean±SEM from 3 independent experiments. * P<0.05, **P<0.01, ***P<0.001. (B) Immunohistochemistry of mouse aortic roots for aP2 (original magnification ×40). Quantification of aP2 staining expressed as percentage of total lesion area. Data are mean± SEM (n = 5/group). ** P<0.01. (C) Western blot analysis of aP2, p-eIF2α, XBP-1s and β-actin expression with pretreatment with aP2 inhibitor (BMS309403) (50 μM) for 3 h, then IL-17A (25ng/ml) stimulation for different times and cells cultured in same fresh medium for 6 hours as control. Data are representative of 3 independent experiments. (D) Western blot analysis levels of p-PERK, p-eIF2α, XBP1s,CHOP, cleaved caspase 3 and β-actin in mouse aortic arch with plaque lesions and quantification. Data are mean±SEM. (n=5/group). (E) Immunohistochemistry of mouse aortic root serial sections for levels of Moma-2 (for macrophages), p-PERK and p-eIF2α, CHOP, cleaved caspase 3 and β-actin in mouse aortic arch with plaque lesions and quantification. Number of apoptotic cells in plaque lesions labeled by TUNEL staining (original magnification ×200). (F) Oil-red O staining for lipid content (original magnification ×40). Quantification of staining from control, IL-17A-treated and IL-17A plus aP2 inhibitor–treated mice (n=5/group) expressed as percentage of total lesion area on the right. (G) Oil-red O staining for aortic arch-thoracic-abdominal aorta lipid deposition. Quantification of total lesions area (n=3/group) on the right. Data are mean± SEM. * P<0.05, ** P<0.01, ***P<0.001.

**Figure 6.** IL-17A up-regulated expression of aP2 by activating NF-κB and ERK, p38 pathway. Mouse primary macrophages were stimulated by IL-17A (25ng/ml); (A) Western blot analysis of NF-κB activity detected with p-IκB. (B) Western blot analysis of protein levels of ERK (p-ERK), p38 (p-p38) and JNK (p-JNK) and (C) analysis of aP2 in macrophages pre-treated with NF-κB inhibitor (20 μg/ml) or (D) ERK (30 μM), JNK (30 μM), p38 (10 μM) for 1 h, then IL-17A (25ng/ml). Data are representative of 3 independent experiments.

**Figure 7.** IL-17A exacerbates atherosclerosis by promoting aP2-mediated ER stress in macrophages. IL-17A combines with IL-17 receptor on macrophages, then transmits this signal to activate the NF-κB or MAPK (ERK, p38 and JNK) signal pathway. The NF-κB and p38, ERK pathway is responsible for the up-regulation of lipid chaperone aP2, and aP2 partially mediates the activation of the ER stress response in macrophages. The inhibition of aP2 could diminish the expression of p-eIF2α and XBP1s. Chronic ER stress leads to increased expression of CHOP, which induces macrophage apoptosis and aggravates atherosclerosis. In addition, ER stress can directly influence the development of atherosclerosis. In summary, IL-17A exacerbates the development of atherosclerosis by promoting aP2-mediated ER stress in macrophages.

This article was retracted in April 2013
Novelty and Significance

What Is Known?

- Prolonged ER stress induced by high levels of intracellular cholesterol and saturated fatty acids in macrophage triggers apoptosis and accelerates the progression of atherosclerotic plaque.
- The fatty acid-binding protein-4 (FABP-4) (aP2) is a central modulator of lipid-induced ER stress responses.
- The proinflammatory cytokine IL-17A, an -Th17 T-cells has been shown to be involved in atherogenesis, but the underlying mechanisms remain unclear.

What New Information Does This Article Contribute?

- IL-17A increases the production of FABP-4 (aP2) in macrophage via NF-κB and MAPK-regulated signal transduction pathways.
- The increase in aP2 is required for IL-17A-induced ER stress, macrophage apoptosis, and acceleration of atherogenesis.

Several studies, including our previous work, suggest that IL-17A is pro-atherogenic. Nevertheless, the mechanism by which IL-17A accelerates atherogenesis remains poorly understood. It has been shown that the cytosolic lipid chaperone aP2 is a central modulator of lipid-induced ER stress responses. In this study, we found that IL-17A induces ER stress in macrophages by up-regulating aP2. Inhibition of aP2 reverted IL-17A-induced ER stress, apoptosis of macrophages and atherosclerosis. These findings reveal a previously unrecognized link between IL-17A and ER stress through aP2 in macrophages and provide a new insight in understanding the role of IL-17A in atherosclerosis. In addition, because aP2 regulates lipid metabolism and signaling, it links inflammation to metabolism. Inhibition of aP2 could provide a new approach for inhibiting atherosclerosis-and related inflammatory diseases.
Figure 1

A. IL-17A 0ng/ml 25ng/ml 50ng/ml 100ng/ml

48h

72h

B. IL-17A 0ng/ml 25ng/ml 50ng/ml 100ng/ml

48h

72h

C. IL-17A (ng/ml) 0 25 50 100

Annexin V+PI%
Figure 2

A. RAW264.7

- IL-17A
- p-PERK
- PERK
- p-eIF2α
- eIF2α
- XBP1s
- β-actin

B. Mice primary macrophages

- IL-17A
- p-PERK
- PERK
- p-eIF2α
- eIF2α
- XBP1s
- β-actin

C. Thp-1-derived macrophages

- IL-17A
- p-PERK
- PERK
- p-eIF2α
- eIF2α
- XBP1s
- β-actin

D. PBMC-derived macrophages

- IL-17A
- p-PERK
- PERK
- p-eIF2α
- eIF2α
- XBP1s
- β-actin
A. 

- p-PERK
- PERK
- p-eIF2α
- eIF2α
- XBP1s
- β-actin

B. 

- Graph showing protein expression levels for Control, IL-17A, and IL-17A + PBA.

C. 

- Table showing isotype expression for Control, IL-17A, and IL-17A + PBA.

D. 

- ORO staining for Control, IL-17A, and IL-17A + PBA.
Figure 6

A.

<table>
<thead>
<tr>
<th></th>
<th>IL-17A</th>
<th>0h</th>
<th>15min</th>
<th>30min</th>
<th>1h</th>
<th>3h</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-IκB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IκB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th></th>
<th>IL-17A</th>
<th>0h</th>
<th>16min</th>
<th>30min</th>
<th>1h</th>
<th>2h</th>
<th>3h</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-ERK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-p38</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-p38</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-JNK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JNK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C.

<table>
<thead>
<tr>
<th></th>
<th>IL-17A</th>
<th>3h</th>
<th>6h</th>
<th>9h</th>
<th>3h</th>
<th>6h</th>
<th>9h</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDTC</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>aP2</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D.

<table>
<thead>
<tr>
<th></th>
<th>DMEM</th>
<th>JNK inhibitor (SP600125)</th>
<th>ERK inhibitor (PD98059)</th>
<th>p38 inhibitor (SB203580)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-17A</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>aP2</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 7
Interleukin 17A Exacerbates Atherosclerosis by Promoting Fatty Acid-Binding Protein 4–Mediated ER Stress in Macrophages
Qi Gao, Yang Jiang, Shen Dai, Bo Wang, Fei Gao, Chun Guo, Faliang Zhu, Qun Wang, Xiaoyan Wang, Jining Wang, Yongyu Shi, Yun Zhang, Wanjun Chen and Lining Zhang

Circ Res. published online October 9, 2012;

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2012/10/09/CIRCRESAHA.112.272567

An erratum has been published regarding this article. Please see the attached page for:
/content/112/8/e87.full.pdf

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2012/10/09/CIRCRESAHA.112.272567.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
The authors of the following article, which published Online First on October 9, 2012, have requested that it be retracted from publication in Circulation Research:

After Online First publication, apparent duplication of panels eIF2a in Figure 2B and 2D, XBP1s in Figure 2D and 3A, and caspase 3 in Figure 4A and 4B was discovered. After being informed by the Editors of these errors, the authors immediately provided the original blots for these figure panels to the Editors. On request of the Editors, the Academic Committee of Shandong University School of Medicine conducted an institutional investigation into apparent duplication of at least 3 figure panels. The investigation confirmed these errors. According to the committee, the authors were able to provide new original blots for these panels that supported their conclusions and attribute these errors to careless management of data. In addition, the original blots of p-eIF2a in Figure 2C and p-PERK in Figure 2D provided to the committee were similar but not identical to the corresponding figures in the article. As reported to the journal by the committee, the authors could only provide blots with a similar trend for CHOP in Figure 4B because the original blots were missing.

Although the authors stand by their conclusions of the article, they recognize that the errors in the manuscript have affected the impact of the article. Therefore, the authors have requested to retract the article. The authors apologize for their carelessness, and Editors of Circulation Research agree the article warrants retraction in order to correct the literature.
ONLINE FIGURES

Online Figure I. IL-17A promote ox-LDL-induced macrophage apoptosis
Mouse primary macrophages from C57BL/6 mice were pre-stimulated with ox-LDL for 12 hours and then IL-17A was added for 24 hours. Apoptosis rate of cells with PI staining determined by flow cytometry. Quantification of apoptotic cells on the right. Data are mean±SEM from 3 representative experiments. * P<0.05, **P<0.01.

Online Figure II. 4-phenyl butyric acid (PBA) can alleviate the development of atherosclerosis
ApoE−/− mice, 8 weeks old, were fed a high-fat diet for 4 weeks and then treated for 5 weeks with PBA (100mg/kg/time), or normal saline (control). Oil-red O staining for lipid content and plaque size (original magnification ×40). Quantification of staining from control and PBA−treated mice (n=5/group) expressed as percentage of total lesion area. Data are mean±SEM. * P<0.05.
Online Figure III. Effective concentration and time of aP2 inhibitor (BMS309403)
Mouse primary macrophages were stimulated with aP2 inhibitor at various concentrations. The protein level of aP2 was analyzed by Western blot. Results are representative of 3 independent experiments.

Online Figure IV. IL-17A promotes macrophages secreting TNF-α, IL-6 and IL-1β
Mice macrophage cell line (RAW264.7) and mouse primary macrophages were cultured in vitro and stimulated with IL-17A (20 and 50 ng/ml) for 12 or 24 h. The levels of TNF-α, IL-6 and IL-1β in cultured supernatant were detected by ELISA. Data are mean ±SEM from 3 independent experiments. (A) RAW264.7 cells; (B) Primary macrophages. * P<0.05, **P<0.01, ***P<0.001.
Online Table I Body weight and plasma lipid levels after IL-17A or IL-17A and PBA treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=10)</th>
<th>IL-17A (n=10)</th>
<th>IL-17A+PBA (n=10)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>25.52±1.93</td>
<td>28.30±1.00</td>
<td>28.10±0.39</td>
<td>0.27</td>
</tr>
<tr>
<td>TCH (mmol/l)</td>
<td>42.02±0.67</td>
<td>39.51±2.41</td>
<td>38.27±1.68</td>
<td>0.42</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>2.25±0.26</td>
<td>2.07±0.16</td>
<td>2.02±0.18</td>
<td>0.72</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>11.04±0.34</td>
<td>10.47±0.68</td>
<td>10.84±0.70</td>
<td>0.80</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>29.02±0.47</td>
<td>27.62±1.66</td>
<td>26.49±1.50</td>
<td>0.49</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM.
TCH, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Online Table II Body weight and plasma lipid levels after IL-17A or IL-17A and aP2 inhibitor treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=5)</th>
<th>IL-17A (n=5)</th>
<th>IL-17A+ aP2 inhibitor (n=5)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>27.75±2.10</td>
<td>28.03±1.77</td>
<td>29.52±1.85</td>
<td>0.33</td>
</tr>
<tr>
<td>TCH (mmol/l)</td>
<td>35.56±2.65</td>
<td>31.29±2.58</td>
<td>23.41±2.50</td>
<td>0.08</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>2.41±0.18</td>
<td>2.32±0.22</td>
<td>2.14±0.31</td>
<td>0.76</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>10.69±0.38</td>
<td>10.51±0.87</td>
<td>10.01±0.92</td>
<td>0.81</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>29.36±0.55</td>
<td>26.84±1.89</td>
<td>24.86±2.08</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM.
TCH, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
ONLINE METHODS

Animals
Male C57BL/6 wild-mice and ApoE-/- mice were purchased from Beijing University. Mice were 6–8 weeks old (21–25g) and were housed at a constant temperature (24℃), under a 12-h dark/12-h light cycle room in the Animal Care Facility of Shandong University Medical School according to institutional guidelines. All animal studies were approved by the Animal Care and Utilization Committee of Shandong University, China.

In Vivo Application of Exogenous IL-17A, 4-phenyl butyric acid (PBA) and aP2 inhibitor
ApoE-/- mice (n=10 for each group) were fed a high-cholesterol diet from 8 weeks of age.
Four weeks later, two groups for treatment once a week for 5 weeks: intraperitoneal injection with exogenous recombinant IL-17A, 0.02% of recombinant mouse IL-17A in normal saline (2μg/mouse/time, PMC0175, Invitrogen, Clasbad, CA, USA); and normal saline containing 0.02% albumin as a control.1,2 The third group (n=10), mice were pretreated before IL-17A with the chemical chaperone PBA (P21005, Sigma-Aldrich, St. Louis, MO, USA) (100 mg/kg/time) twice a week for 5 weeks,3 at the same time mice also received IL-17A treatment (2μg/mouse/time) once a week for 5 weeks. The fourth group (n=7) fed with a high-cholesterol diet from 8 weeks of age and two weeks later, mice were treated by oral gavage with vehicle including 10% 1-methyl-2-pyrrolidone and 5% cremophor EL with ethanol in 100μl of water or 20mg/kg/d of the aP2 inhibitor BMS309403 dissolved in the vehicle for 6 weeks.4,5 And mice also received IL-17A treatment (2μg/mouse/time) once a week for 5 weeks after four weeks high-cholesterol diet.

Lipid Profile
Total plasma cholesterol and triglyceride levels were determined by an automated enzymatic technique, and low-density lipoprotein and high-density lipoprotein levels were detected with use of an automated chemically modified technique (Roche Modular DPP System, Roche, Basel, Switzerland).2

Histopathology and Immunohistochemistry
After mice were killed, aortic root vessels were perfused with phosphate buffered saline (PBS), then 4% paraformaldehyde. Aortic root vessels were removed and fixed in 4% paraformaldehyde overnight, then embedded in OCT compound. Serial cryosections of 6μm were cut along the aortic root specimens and were stained with Oil-red O. Corresponding sections on separate slides were stained for macrophages with a rat anti-mouse macrophage-specific antibody (Moma-2, AbD Serotec, UK) or primary antibodies for P-PERK (sc-32577, Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-eIF2α (119A11, Cell Signaling Technology, Danvers, MA, USA), FABP4(aP2) (ab-13979, Abcam, Hong Kong), CHOP (sc-575, Santa Cruz Biotechnology, CA, USA), or caspase 12 (ab-62463, Abcam, Inc. Cambridge, MA, USA). Histological stains were detected by use of an Olympus microscope (IX71), and the area of plaque was measured by use of ImagePro-Plus 6.0 and dp2-bsw (Olympus, Tokyo, Japan).

TUNEL and Macrophage Apoptosis Assay
Apoptotic cells in the intimal area of atherosclerotic lesions were labeled after proteinase K treatment by TUNEL with use of the in situ cell death detection kit Fluorescein (Roche Diagnostics, Basel, Switzerland). For in vitro analysis, macrophages were assayed for early to
mid-stage apoptosis by staining with Alexa Fluor 488–conjugated Annexin V and for late-stage apoptosis by co-staining with propidium iodide (PI) according to the manufacturer’s directions, then were detected by flow cytometry with use of Cytomics FC500 (Beckman Coulter, Brea, CA, USA). Hoechst staining for nuclei was viewed at room temperature by use of an Olympus IX-71 inverted fluorescent microscope.

**Isolation of Mouse Peritoneal Macrophages and Culture in Vitro**

Peritoneal macrophages from male C57BL/6 mice were harvested 3 days after intraperitoneal injection of starch broth by peritoneal lavage. All macrophages were cultivated in 6-well flat-bottom plates with Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen, Clasbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and incubated in humidified 5% CO₂ at 37°C for 2 h to allow macrophage adherence. The nonadherent cells were removed by 3 washes with DMEM. The adherent cells were harvested, and purity of macrophages was detected by flow cytometry after staining with PECy5-conjugated anti-F4/80Ab (BM8; eBioscience, San Diego, CA, USA). Purified macrophages (purity >95%) were incubated as described in figure legends on the day of the experiment.

**Induction and Culture of Human Monocyte-derived Macrophages and THP-1-derived macrophages**

Peripheral blood mononuclear cells (PBMCs) from healthy volunteer blood at Shandong University Medical School were isolated by Ficoll density gradient centrifugation and washed twice with serum-free RPMI 1640 medium (Gibco, Invitrogen, Clasbad, CA, USA). PBMCs were then plated at 3 ml/well in 6-well cell culture plates, 3 hours later un-adherent cells were removed and adherent cells cultured with RPMI 1640 complete medium (10% FBS, 1% L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids, 1% penicillin/streptomycin and 50ng/ml rhGM-CSF) (catalog: 300-03, PeproTech, Suzhou, JS, P.R. China). Half of the medium was replaced every 2 days for culture for 7 days. On the day of the experiment, the cells were washed 3 times in warm PBS and incubated as described in the figure legends. THP-1 cell lines were cultivated in 6-well flat-bottom plates with DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and PMA(50ng/ml) for 48 hours, then the cells were washed 3 times in warm PBS and incubated as described in the figure legends.

**Western Blot Analysis**

Extraction and detection of protein was as described. A 50-µg quantity of protein was separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA), which were blocked with 1% bovine serum albumin in TBST containing 0.1% Tween-20 for 1 hour, then incubated overnight at 4°C with antibodies for p-PERK, PERK, p-eIF2α, eIF2α, CHOP, caspase3, p-p38, p38, p-ERK, ERK, p-JNK, JNK, p-IκB, IκB (all 1:1000; Cell Signaling Technology, Danvers, MA, USA), aP2 (ab-13979, 1:1000; Abcam, Inc. Hong Kong), or β-actin (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), then horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG or murine anti-mice IgG) for 1 hour at room temperature. After a washing, signals were visualized by use of SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA). Western blot was performed at least 3 times for each sample.
RT-PCR Analysis
Total RNA in primary macrophages was isolated by the Trizol reagent method (Invitrogen, Clasbad, CA, USA) and underwent reverse transcription and semi-RT-PCR. The primers for aP2 were sense, 5’-ATG CTTTGTTGGGAACCTG-3’ and antisense, 5’-CCTGTCGTCTGCGGTATT-3’ (230-bp product length).

Enzyme-linked Immunosorbent Assay (ELISA)
Cytokines in culture supernatants quantified using mouse TNF-α, IL-6 and IL-1β ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Statistical Analysis
All analysis involved SPSS v11.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean±SEM. Nonparametric ANOVA and unpaired t tests were used to compare continuous data. A p< 0.05 was considered statistically significant.

REFERENCES