A Critical Function of Th17 Proinflammatory Cells in the Development of Atherosclerotic Plaque in Mice

Qi Gao, Yang Jiang, Tian Ma, Faliang Zhu, Fei Gao, Pin Zhang, Chun Guo, Qun Wang, Xiaoyan Wang, Chunhong Qi Gao, Yang Jiang, Tian Ma, Faliang Zhu, Fei Gao, Pin Zhang, Chun Guo, Qun Wang, Xiaoyan Wang, Chunhong Qi Gao, Yang Jiang, Tian Ma, Faliang Zhu, Fei Gao, Pin Zhang, Chun Guo, Qun Wang, Xiaoyan Wang, Chunhong Ma, Yun Zhang, Wanjun Chen and Lining Zhang

J Immunol published online 15 October 2010
http://www.jimmunol.org/content/early/2010/10/15/jimmunol.1000116

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/10/15/jimmunol.1000116.DC1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
A Critical Function of Th17 Proinflammatory Cells in the Development of Atherosclerotic Plaque in Mice

Qi Gao,*† Yang Jiang,*† Tian Ma,* Faliang Zhu,* Fei Gao,† Pin Zhang,‡ Chun Guo,* Qun Wang,* Xiaoyan Wang,* Chunhong Ma,* Yun Zhang,‡ Wanjun Chen,‡ and Lining Zhang*

Considerable evidence supports that the CD4+ T cell-mediated immune response contributes to the development of atherosclerotic plaque. However, the effects of Th17 cells on atherosclerosis are not thoroughly understood. In this study, we evaluated the production and function of Th17 and Th1 cells in atherosclerotic-susceptible ApoE−/− mice. We observed that the proportion of Th17 cells, as well as Th1, increased in atherosclerotic ApoE−/− mice compared with nonatherosclerotic wild-type littermates. In ApoE−/− mice with atherosclerosis, the expression of IL-17 and retinoic acid-related orphan receptor γt was substantially higher in the arterial wall with plaque than in the arterial wall without plaque. Increased Th17 cells were associated with the magnitude of atherosclerotic plaque in ApoE−/− mice. Importantly, treatment of ApoE−/− mice with neutralizing anti–IL-17 Ab dramatically inhibited the development of atherosclerotic plaque, whereas rIL-17 application significantly promoted the formation of atherosclerotic plaque. These data demonstrate that Th17 cells play a critical role in atherosclerotic plaque formation in mice, which may have implications in patients with atherosclerosis.

Atherosclerosis is a chronic inflammatory process within the arterial wall, which affects millions of people worldwide (1, 2). Accumulated evidence suggests that CD4+ T cells are involved in the development of atherosclerotic lesions. Th1 response promotes plaque development (3, 4). Deficiency in IFN-γ or its receptor reduces the formation of lesions, and exogenous administration of IFN-γ enhances it (5–7). In line with this, apolipoprotein E-deficient (ApoE−/−) mice lacking IL-12p40 showed less plaque development (8, 9). The role of Th2 immune response in the development of atherosclerosis remains controversial. In mouse models that are relatively resistant to atherosclerosis, a Th2 response protects against early fatty streak development (10, 11). However, in models of LDLR−/− mice, deficiency in IL-4 was associated with a decrease in atherosclerotic lesion formation, suggesting a potentially proatherogenic effect (12).

Th17 cells have been described as a new subset, in addition to Th1 and Th2 cells (13–17). Th17 cells produce IL-17 and several other proinflammatory cytokines and are differentiated from Ag-activated CD4+CD25− T cells in the presence of TGF-β and proinflammatory cytokines, such as IL-6 and IL-21 (16–20). Furthermore, the orphan nuclear receptor retinoic acid-related orphan receptor γt (RORγt) has been identified as the key transcription factor that determines the differentiation of the Th17 lineage (21). Th17 cells were shown to play a role in chronic inflammation and organ-specific autoimmune diseases in animals, such as rheumatoid arthritis, experimental autoimmune encephalomyelitis, and colitis, which have historically been associated with Th1 responses (22, 23). Recent studies showed that Th17 cells and their major effector cytokine IL-17 are increased in the peripheral blood of patients with coronary atherosclerosis. T cells infiltrated in the atherosclerotic plaque express IL-17 in humans and in mouse models (24–26), but whether IL-17 plays a causative role in the development of artherosclerosis remains a subject of debate (27, 28).

In this study, we provide evidence that Th17/IL-17+ cells promote the formation of atherosclerotic plaque in ApoE−/− mice, suggesting Th17/IL-17+ cells as potential therapeutic targets for atherosclerosis.

Materials and Methods

Mice

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

Induction of atherosclerosis in ApoE−/− mice

The male ApoE−/− and C57BL/6 mice received a high-cholesterol diet (0.25% cholesterol and 15% cocoa butter) for 8–16 wk, starting from 8 wk of age, to induce atherosclerotic plaque, as in standard atherosclerotic mouse models. To establish the rapid atherosclerotic mouse model, mice were anesthetized with pentobarbital sodium (40 mg/kg) by i.p. injection, and carotid atherosclerotic lesions were induced by perivascular constrictive

Abbreviations used in this paper: HDL, high-density lipoprotein; LDL, low-density lipoprotein; Mac, macrophage; n.s., not significant; RORγt, retinoic acid-related orphan receptor γt; SMC, smooth muscle cells; SOCS3, suppressor of cytokine signaling 3; TCH, total cholesterol; TG, triglyceride; WT, wild-type.
silica collars placed on the left common carotid artery of 10-wk-old mice (29). Carotid atherosclerotic plaque was detected 6–14 wk after surgery.

**Histopathology and immunohistochemistry or immunofluorescence**

After mice were sacrificed, vessels were perfused with PBS, followed by 4% parafomaldehyde. The left common carotid arteries and the aortic root vessels were removed and fixed in 4% parafomaldehyde overnight and then embedded in OCT compound. Serial cryosections of 5 μm were cut along the carotid artery or the aortic root specimens and were routinely stained with H&E and Oil Red O. Corresponding sections on separate slides were stained for macrophages using a rat anti-mouse macrophage–specific Ab (MoMa-2; AbD Serotec, Oxford, U.K.), for smooth muscle cells by rabbit polyclonal to α smooth muscle actin (ab5694; Abcam, Hong Kong), and for CD4+ T cells by rat anti-mouse CD4 (H129.19; BD Pharmingen, San Diego, CA). The expression of IL-17 and IFN-γ in plaques was detected by rabbit polyclonal anti-mouse IL-17 Ab (H-132, Santa Cruz Biotechnology, Santa Cruz, CA) and rat anti-mouse IFN-γ Ab (XMGI1.2; BD Pharmingen, San Diego, CA). The nucleus was stained by Hoechst. The different histological stains were observed using an Olympus microscope (IX71; Olympus Corporation, Tokyo, Japan), and the area of plaque was measured using Image ProPlus 6.0 and DP2-BSW.

**Lipid profile**

Total plasma cholesterol and triglycerides (TGs) were determined with an automated enzymatic technique, and low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels were determined with an automated chemically modified technique (Roche Modular DPP System, Roche, Switzerland).

**RT-PCR analysis for cytokines and transcript factors**

Total RNAs in the arterial walls of the carotid artery, proximal aortic arch, and the aortic branches were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), and semi-RT-PCR were performed. The sequences of the sense and antisense primers were as follows: RORγt, sense: 5'GCCGGACAGACATCATCCT-3', antisense: 5'-TTGGCAAAACTCCACCACATA-3' (582-bp product length); T-bet, sense: 5'-TTCCCATTCCTGCTCTCATC-3', antisense: 5'-CCCTCTGCCTCTTCTATTAC-3' (346-bp product length); IL-17A, sense: 5'-CTCTGACAGACTCTTACACGA-3', antisense: 5'-CTCTTCTTCTAGGACGAGCG-3' (187-bp product length); and IFN-γ, sense: 5'-TACTGCCCACGCGCACAGTCATTGAA-3', antisense: 5'-GCAGCGACTCTTTCGCTTCCT-3' (405-bp product length).

**Intracarotid cytokine staining**

Splenocytes were prepared from ApoE−/− mice at 8, 16, and 24 wk of age (n = 5 for each group) and were stimulated with 25 ng/ml PMA (Sigma-Aldrich, St. Louis, MO), 1 μg/ml ionomycin (Sigma-Aldrich), and 10 μg/ml brefeldin A (eBioscience, San Diego, CA) for 4 h at 37°C under 5% CO2 environment. The cells were washed with washing buffer and then stained with PE-conjugated anti-CD4 Ab (H129.19; BD Pharmingen). Then, cells were fixed and perforated by the fixation and permeabilization kit (eBioscience, San Diego, CA) and then stained intracellularly with PE-conjugated anti-IL-17 (eBio17B7; eBioscience) and FITC-conjugated anti–IFN-γ Ab (XMGI1.2; BD Pharmingen). Flow-cytometric analysis was performed with a CytoFins FC500 (Beckman Coulter, Brea, CA).

**In vivo blockage of IL-17 or application of exogenous IL-17**

ApoE−/− mice were fed a high-cholesterol diet from 8 wk of age. The carotid atherosclerotic lesions were induced by high-cholesterol diet alone (HDL) or high-cholesterol diet plus perivascular placement of constrictive collars at 10 wk, as previously reported (29). For blockage of IL-17 with anti–IL-17A Ab, at the end of 10 wk, mice were treated with anti–IL-17A neutralization mAb (50 μg/mouse/time, clone: 50104, rat IgG2A, R&D Systems, Minneapolis, MN) and the isotype Ab (rat IgG2A, clone: 54447; R&D systems) on the day of challenge and once a week for 4 wk. For application of exogenous rIL-17, 0.02% mouse rIL-17 in normal saline (2 μg/mouse/time; PMC0175; Invitrogen) and normal saline containing 0.02% albumin as control were used with the same interventional strategy as Ab treatment. During this interventional period, carotid artery plaque sizes were evaluated weekly by microultrasound imaging after mice were anesthetized, and plaque morphology was detected by histopathology after sacrifice, 1 wk following the last challenge.

**Microultrasound imaging**

Baseline ultrasound imaging parameters of the left carotid artery were measured with the Vevos770 System (Visualsonics, Toronto, Ontario, Canada) at the beginning of the experiment with 8-wk-old mice. Microultrasound imaging of the carotid plaques was analyzed 1 wk after the last challenge with anti–IL-17A Ab treatment. During all experiments, mice were anesthetized with 0.8% pentobarbital sodium, which resulted in heart rate reaching 350 beats/min. Temperature was monitored with a rectal probe and regulated using a heating pad. An optimal freeze-frame image was taken manually to visualize and measure the external elastic membrane area, plaque area, and maximal and minimal intimal thickness. The vascular lumen–intimal interface was selected as the internal measurement site and the media–adventitial interface as the external limit. External elastic membrane area derived from reference segments was also measured. All of the measurements were repeated twice at the same site (30).

**Statistical analysis**

All analysis was done by SPSS 11.0 (SPSS, Chicago, IL). Data are expressed as mean ± SEM. The linear regression analysis was used to test correlation between the percentage of Th subsets and lesion area. Non-parametric ANOVA and unpaired t tests were used for comparison of continuous data. A p value <0.05 was considered significant.

**Results**

**High levels of IL-17 and IFN-γ in the atherosclerotic plaque of ApoE−/− mice**

To explore the effect of Th17 cells on the development of atherosclerosis, we first assessed the dynamic change in Th17 percentage throughout the entire experimental period (Fig. 1A). Because IL-17 is the major effector molecule for Th17 cells, we examined the expression of IL-17 in the carotid vascular wall by immunohistochemistry staining. As shown in Fig. 1B, no IL-17 was detected in the carotid vascular wall in 8-wk-old ApoE−/− mice or age-matched WT control mice fed a normal common diet. However, substantial expression of IL-17 was detected in the tissues of the carotid vascular wall in early (16 wk) and late (24 wk) atherosclerotic plaques in ApoE−/− mice but not within the carotid vascular wall in age-matched control mice. In addition, IFN-γ was detected in the plaques (Fig. 1B). To confirm the cellular sources of IL-17, we costained IL-17 with CD4+ T cells, macrophages, endothelial cells, or vascular smooth muscle cells with immunofluorescent staining. IL-17 was mainly expressed in CD4+ T cells and macrophages infiltrated into the atherosclerotic plaque (Fig. 1C) but not in endothelial cells or vascular smooth muscle cells (data not shown).

Consistent with the results from immunohistochemistry and immunofluorescence staining, the expression of IL-17 and IFN-γ mRNA in the vascular wall of ApoE−/− mice was also markedly higher than in WT control mice (Fig. 2). Notably, the expression of RORγt and T-bet, the transcription factors controlling Th17 and Th1 cell differentiation, respectively, was significantly higher in the vascular wall of ApoE−/− mice than in control mice as early as 8 wk (Fig. 2), suggesting that the inflammatory programs started even before obvious plaque formation. The levels of RORγt and T-bet continued to increase and were maintained at higher levels in ApoE−/− mice at the late stages of atherosclerotic plaque (16–24 wk). The data suggest that IL-17 and IFN-γ were involved in the development of atherosclerotic inflammation.
Increased Th17 and Th1 cells in the spleen of ApoE<sup>−/−</sup> mice with atherosclerotic plaque

We next examined whether the increase in IL-17 and IFN-γ in the vascular wall was due to systemic inflammation or restricted to local inflammatory processes. To this end, we analyzed the percentage of Th17 (IL-17<sup>+</sup>IFN-γ<sup>+</sup>) and Th1 (IL-17<sup>+</sup>IFN-γ<sup>+</sup>) cells in the spleen of ApoE<sup>−/−</sup> mice by flow cytometry. As shown in Fig. 3, the presymptomatic ApoE<sup>−/−</sup> mice (8 wk old) had minimal numbers of Th17 (<1%) cells with no statistically significant difference compared with control mice (0.48 ± 0.04% versus 0.4 ± 0.14%, respectively; n = 5 mice; p > 0.05). Although the number of Th1 cells was higher than Th17 cells, there was no statistically
significant difference between ApoE<sup>−/−</sup> and control mice (7.9 ± 0.44% versus 5.36 ± 1.47%, respectively; n = 5 mice; p > 0.05). However, the frequencies of Th17 and Th1 cells in 16-wk-old ApoE<sup>−/−</sup> mice with plaque were significantly higher than in control mice (Th17 cells: 1.47 ± 0.33% versus 0.32 ± 0.05%, n = 5 mice, p < 0.05; Th1 cells: 18.1 ± 1.54% versus 4.58 ± 1.43%, n = 5 mice, p < 0.05), which were further increased in the ApoE<sup>−/−</sup> mice with advanced atherosclerotic plaque (24 wk old) (Th17 ApoE<sup>−/−</sup> versus control: 2.44 ± 0.49% versus 1.0 ± 0.19%, n = 5 mice, p < 0.05; Th1 ApoE<sup>−/−</sup> versus control: 27.18 ± 2.59% versus 15.24 ± 1.75%, n = 5 mice, p < 0.05). The results suggest that the increase in Th17 and Th1 cells in the plaque is attributable to a systemic upregulation of Th17 and Th1 responses.

In addition to IL-17<sup>+</sup>IFN-γ<sup>+</sup> (Th17) or IL-17<sup>+</sup>IFN-γ<sup>+</sup> (Th1) cells in CD4<sup>+</sup> splenocytes, we observed a population of CD4<sup>+</sup> T cells expressing IL-17<sup>+</sup> and IFN-γ<sup>+</sup> (Fig. 3). The frequency of these IL-17<sup>+</sup>IFN-γ<sup>+</sup> double-positive CD4<sup>+</sup> T cells was negligible in the nonatherosclerotic ApoE<sup>−/−</sup> mice (0.14 ± 0.02%) and even in the mice with early-stage atherosclerotic plaque (0.23 ± 0.03%), which had no significant differences compared with age-matched WT control mice (8 wk: 0.12 ± 0.05%, 16 wk: 0.14 ± 0.05%, n = 5 mice, p > 0.05). However, the CD4<sup>+</sup>IL-17<sup>+</sup>IFN-γ<sup>+</sup> T cells significantly increased in the late-stage atherosclerotic ApoE<sup>−/−</sup> mice (24 wk: 1.28 ± 0.24%) compared with age-matched control

**FIGURE 3.** Th1, Th17, and Th17/Th1 in mice with or without atherosclerotic plaque. A. The percentages of Th1, Th17, and Th17/Th1 in splenocytes of C57BL/6 and ApoE<sup>−/−</sup> mice were detected by flow cytometry. Splenocytes from nonatherosclerotic and atherosclerotic mice or C57BL/6 mice were stimulated with PMA (25 ng/ml), ionomycin (1 μg/ml), and blocking reagent-brefeldin A (10 μg/ml), incubated at 37°C, 5% CO<sub>2</sub> for 4 h, and then assayed for assessment of the number of CD4<sup>+</sup>IFN-γ<sup>+</sup> (Th1), CD4<sup>+</sup>IL-17<sup>+</sup> (Th17), and CD4<sup>+</sup>IL-17<sup>+</sup>IFN-γ<sup>+</sup> (Th1/Th17) T cells by flow cytometry. B. Changes in Th1, Th17, and Th17/Th1 cells in spleen of C57BL/6 and ApoE<sup>−/−</sup> mice at 8, 16, and 24 wk of age (n = 5 mice per group). *p < 0.05; **p < 0.01; ***p < 0.001.

**FIGURE 4.** Correlation between the proportion of Th1, Th17, and Th17/Th1 cells and the area of atherosclerotic plaque or the level of plasma lipids. Correlations of Th1 (A), Th17 (B), or Th17/Th1 (C) with the area of plaque. Correlations of Th1 or Th17 with TCH (D, G), TG (E, H), and LDL/HDL (F, I).
mice (0.46 ± 0.08%, n = 5 mice, p < 0.05). These results revealed an abnormal increase in the IL-17+IFN-γ+ cell subset within the CD4+ population that may be correlated with the development of atherosclerotic plaque in ApoE/−/− mice.

Positive correlation between percentage of Th17 or Th1 cells and development of atherosclerotic plaque or serum levels of total cholesterol in ApoE/−/− mice

We next analyzed whether there was a correlation between the percentages of Th17, Th1, or IL17+IFN-γ+ cells and the size of plaques. Through simple linear-regression analysis, we observed that the area of plaque correlated with the proportion of Th1 cells (Fig. 4A, r = +0.86, n = 15, p < 0.01), and it was also closely associated with Th17 cell subset (Fig. 4B, r = +0.91, n = 15, p < 0.01). In addition, the numbers of IL17+IFN-γ+ cells correlated with the size of plaque (Fig. 4C, r = +0.7, p < 0.01). This further strengthens the notion that Th17 and/or Th1 is associated with the development of atherosclerosis.

It is known that the cholesterol levels in plasma affect the development of atherosclerosis. We then assessed the level of lipids in plasma (Table I) and analyzed the correlation of Th17 and Th1 cells with the plasma lipid levels. As shown in Fig. 4D and 4G, Th1 and Th17 cells were highly correlated with the levels of total cholesterol (TCH) in the plasma. However, there was no significant correlation between Th17 or Th1 cells and TGs (Fig. 4E, 4H). Furthermore, Th17 and Th1 cells did not correlate with the HDL/LDL ratio (Fig. 4F, 4I). These results demonstrate that the increases in Th17 and Th1 cells were closely related to the levels of TCH.

**Table I. Plasma lipid levels (mmol/l) in C57BL/6J and ApoE/−/− mice**

<table>
<thead>
<tr>
<th>Mice (n = 5)</th>
<th>TCH</th>
<th>TGs</th>
<th>HDL Cholesterol</th>
<th>LDL Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57 (8 wk)</td>
<td>2.09 ± 0.16</td>
<td>1.5 ± 0.16</td>
<td>1.51 ± 0.09</td>
<td>0.53 ± 0.07</td>
</tr>
<tr>
<td>ApoE/−/− (8 wk)</td>
<td>14.94 ± 4.42</td>
<td>2.33 ± 0.6</td>
<td>4.16 ± 0.29</td>
<td>6.05 ± 0.8**</td>
</tr>
<tr>
<td>C57 (16 wk)</td>
<td>2.12 ± 0.33</td>
<td>0.79 ± 0.06</td>
<td>2.48 ± 0.17</td>
<td>0.76 ± 0.29</td>
</tr>
<tr>
<td>ApoE/−/− (16 wk)</td>
<td>40.1 ± 1.12*</td>
<td>2.02 ± 0.05*</td>
<td>12.24 ± 0.4*</td>
<td>6.73 ± 2.80**</td>
</tr>
<tr>
<td>C57 (24 wk)</td>
<td>4.08 ± 0.72</td>
<td>1.14 ± 0.22</td>
<td>3.43 ± 0.15</td>
<td>1.41 ± 0.10</td>
</tr>
<tr>
<td>ApoE/−/− (24 wk)</td>
<td>28.3 ± 2.26*</td>
<td>2.50 ± 0.43*</td>
<td>10.5 ± 0.73*</td>
<td>23.3 ± 3.36*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM.

*p < 0.05; **p < 0.01, versus the age-matched C57BL/6J littermates.
Treatment of ApoE<sup>−/−</sup> mice with IL-17–specific Ab markedly suppresses the development of atherosclerotic plaque

To confirm the causative role of Th17/IL-17 in the development of atherosclerosis, we treated the ApoE<sup>−/−</sup> mice systemically with neutralizing anti-mouse IL-17 Ab. ApoE<sup>−/−</sup> mice were fed a high-cholesterol diet from 8 wk of age. The carotid atherosclerotic lesions were rapidly induced by perivascular placement of constrictive collars at week 10, as described in Materials and Methods. Five days later, mice were randomly divided into two groups (10 mice/per group) and treated with anti–IL-17A Ab (50 μg/mouse/week, i.p.) or isotype control Ab (rat IgG, 50 μg/mouse/week, i.p.) for 4 wk. During interventional periods, the change in plaque size was evaluated by microultrasound imaging. Micro-ultrasonography showed that the carotid artery of all mice (10/10) in the isotype-control Ab group developed larger plaques, with surface areas of 187,900 ± 31,230 μm<sup>2</sup>, whereas only 10% (1/10) of mice treated with anti–IL-17A Ab developed plaques and with smaller surface areas (68,200 μm<sup>2</sup>; p < 0.0001) (Fig. 5A). Furthermore, we measured the size of plaque in continuous sections of carotid artery by histopathology. Consistent with the result from micro-ultrasonography, 9 of 10 mice treated with anti–IL-17A Ab had no detectable plaque in the carotid artery, and only 1 mouse developed a small plaque (7,093 μm<sup>2</sup>), whereas the control group had large plaques (41,320 ± 4,286 μm<sup>2</sup>, n = 10, p < 0.0001) (Fig. 5B). Furthermore, we confirmed the effects of anti–IL-17A–neutralizing Ab on the development of atherosclerosis in the standard ApoE<sup>−/−</sup> model. Consistent with the results for the carotid artery, the plaque size within the aortic root in the anti–IL-17A Ab-treated group (10,320 ± 6,789 μm<sup>2</sup>, n = 5) was markedly smaller than that of the control group (38,030 ± 8,964 μm<sup>2</sup>, n = 5, p < 0.05) (Fig. 5C). To determine the impact of neutralization of IL-17 on cell composition within the plaque, we examined the number of macrophages and smooth muscle cells as indicators of disease condition (31–35). As shown in Fig. 5D, there were many macrophages and some smooth muscle cells in the intima of isotype-control mice, whereas no detectable macrophages or smooth muscle cells were found in anti–IL-17A Ab-treated mice. In addition, body weight and the lipid levels in sera of mice showed no significant difference (16 wk old) between control and anti–IL-17A–treated groups (Table II). Our data demonstrated that blockage of IL-17 with IL-17–specific Ab markedly inhibited the development of atherosclerotic plaque in standard and rapid atherosclerotic mice models.

**Treatment with exogenous IL-17 exacerbates plaque formation in ApoE<sup>−/−</sup> mice**

To further confirm the role of IL-17 in the development of atherosclerotic plaque, we injected exogenous mouse rIL-17 weekly (2 μg/mouse) into ApoE<sup>−/−</sup> mice for 5 wk. IL-17 injection resulted in no obvious adverse effects in mice; body weight and lipid levels in sera of mice were comparable in the treated and control groups (Table II). However, injection of IL-17 significantly exacerbated plaque formation in the aortic root (plaque size: 110,700 ± 15,760 μm<sup>2</sup> in IL-17–treated group versus 38,030 ± 8,964 μm<sup>2</sup> in control group, n = 5, p < 0.001) (Fig. 6A). Furthermore, we examined macrophages and smooth muscle cells in the plaque. Although IL-17 treatment had no significant effect on the number of macrophages, it decreased the numbers of smooth

**Table II.** Body weight and plasma lipid levels after anti–IL-17Ab or rIL-17 treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Isotype (n = 15)</th>
<th>Anti–IL-17Ab (n = 15)</th>
<th>p Value</th>
<th>Control (n = 5)</th>
<th>IL-17 Treatment (n = 5)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>30.3 ± 0.25</td>
<td>29.5 ± 0.27</td>
<td>0.06</td>
<td>31.2 ± 0.94</td>
<td>29.36 ± 0.49</td>
<td>0.12</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>2.09 ± 0.16</td>
<td>1.98 ± 0.24</td>
<td>0.42</td>
<td>2.17 ± 0.34</td>
<td>2.31 ± 0.29</td>
<td>0.50</td>
</tr>
<tr>
<td>TCH (mmol/l)</td>
<td>36.7 ± 3.42</td>
<td>32.5 ± 2.6</td>
<td>0.06</td>
<td>36.7 ± 3.4</td>
<td>35.8 ± 2.9</td>
<td>0.44</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>6.12 ± 1.33</td>
<td>5.79 ± 0.56</td>
<td>0.60</td>
<td>8.12 ± 1.73</td>
<td>6.48 ± 0.67</td>
<td>0.08</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>10.12 ± 1.46</td>
<td>11.79 ± 1.56</td>
<td>0.12</td>
<td>10.12 ± 1.46</td>
<td>10.68 ± 1.63</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM.
muscle cells in plaques, suggesting increased instability of the plaques. These data further confirm that IL-17A promotes the development of atherosclerosis in vivo.

Discussion

In this study, we showed that Th17/IL-17 cells promoted the formation of atherosclerotic plaque in ApoE<sup>−/−</sup> mice. First, we found that the proportion of Th17 cells increased in atherosclerotic ApoE<sup>−/−</sup> mice compared with nonatherosclerotic WT littermates. The increased Th17 cells were associated with the magnitude of atherosclerotic plaque formation in ApoE<sup>−/−</sup> mice. Furthermore, the expression of IL-17 and RORγt was enhanced significantly in arterial walls with plaque compared with arterial wall tissue without plaque; in addition, Th17 cells could be detected within the atherosclerotic plaques. More importantly, we confirmed that treatment with neutralizing anti–IL-17A Ab markedly inhibited the development of atherosclerotic plaque, whereas rIL-17 injection significantly promoted the formation of atherosclerotic plaque. Our data demonstrate that Th17/IL-17+ cells play a critical role in the pathogenesis of atherosclerosis and suggests IL-17 as a therapeutic target for atherosclerosis.

Accumulated evidence indicates that CD4<sup>+</sup> T cells are involved in the development of atherosclerosis. Transfer of CD4<sup>+</sup> T cells from atherosclerotic ApoE<sup>−/−</sup> mice into ApoE<sup>−/−</sup>iscid/iscid mice enhances atherosclerotic lesion development (5, 6, 36). In addition to the previous studies demonstrating a role for Th1 cells in atherosclerosis (37), we found in this study that Th1 cells also play a critical role in the development of atherosclerosis. We found that the number of Th17 cells in spleens of young nonatherosclerotic ApoE<sup>−/−</sup> mice (8 wk) was comparable to that in the control C57BL/6 mice. However, the proportion of Th17 cells was significantly higher in the ApoE<sup>−/−</sup> mice with early-stage atherosclerosis (16 wk) and further increased in the late stage (24 wk) compared with control mice. The increased Th17 cells were associated with the area of atherosclerotic plaque. Furthermore, we observed that the expression of IL-17 and RORγt were also enhanced markedly in vascular wall tissue with atherosclerotic plaque in ApoE<sup>−/−</sup> mice, and Th17 cells were detected in atherosclerotic plaques (Fig. 1D). The results strongly suggest that Th17 cells participate in the formation of plaque, which was supported by several recent reports (24–26). To confirm the causative role of Th17 in atherosclerosis, ApoE<sup>−/−</sup> mice were treated with the neutralizing anti-mouse IL-17A mAb and with exogenous IL-17 before they produced atherosclerotic plaque. Indeed, neutralization of IL-17A in vivo dramatically inhibited the development of plaque, whereas IL-17A significantly exacerbated atherosclerosis, validating a critical role for IL-17 in the formation of atherosclerotic plaque. Although CD4<sup>+</sup> T cells are a major source of IL-17, they are not the only source of this cytokine. Neutrophils, as well as CD8<sup>+</sup> and γδ<sup>+</sup> T cells, were shown to produce IL-17 in response to IL-15 (38). In the current study, we observed that some CD4<sup>+</sup> T cells in the spleen also produced IL-17. However, the number of CD4<sup>+</sup>IL-17<sup>+</sup> cells increased in late atherosclerotic ApoE<sup>−/−</sup> mice but not in early atherosclerotic ApoE<sup>−/−</sup> mice ( Supplemental Fig. 1). In addition, consistent with the results from Eid et al. (24), we found that Th17/Th1 cells producing IL-17 and IFN-γ only enhanced in late atherosclerotic ApoE<sup>−/−</sup> mice but not in early atherosclerotic ApoE<sup>−/−</sup> mice. These data indicate that IL-17 in early atherosclerotic ApoE<sup>−/−</sup> mice is mainly derived from Th17 cells. Collectively, our results demonstrate that Th17 cells promote the formation of early atherosclerotic plaques in ApoE<sup>−/−</sup> mice, which is consistent with a more recent report about IL-17A activity (24–27). The early targeting Th17 cells provide an attractive therapeutic means to control atherosclerotic development. In contrast, the data from Taleb et al. (28) showed that IL-17A inhibited the formation of plaque in the LDLR<sup>−/−</sup> mice model in a suppressor of cytokine signaling 3 (SOCS3)-dependent manner. They showed that neutralization of IL-17 accelerated atherosclerosis in SOCS3-conditional knock-out LDLR<sup>−/−</sup> mice, but it had no effect on lesion development in SOCS3-WT LDLR<sup>−/−</sup> mice. However, treatment of rIL-17 significantly inhibits lesion development in SOCS3-WT LDLR<sup>−/−</sup> mice. This discrepancy suggests that the role for IL-17A in the development of atherosclerotic plaque may be affected by multiple factors, such as differences in the mice model used (LDLR<sup>−/−</sup> mice versus ApoE<sup>−/−</sup> mice), cytokine profile (IFN-γ level), and expression status of certain regulatory factor (SOCS3). However, detailed reasons for this discrepancy remain to be elucidated.

Although Th17 and Th1 cells are proinflammatory cells, the relationship between the two subsets in atherosclerosis is unclear. There is evidence that the Th1 cytokine IFN-γ inhibits IL-17 production in vitro and in vivo (20). The addition of anti–IFN-γ enhances Th17 proliferation, whereas rIFN-γ inhibits Th17 proliferation during T cell activation in vitro. In a type 1 diabetes model, treatment with Ig-glutamic acid decarboxylase protected against disease by inducing IFN-γ, which inhibited IL-17–producing T cells, and neutralization of IFN-γ enhanced IL-17 production (39). In the experimental Ag-induced arthritis model, arthritis was exacerbated in IFN-γ<sup>−/−</sup> mice as the result of increased Th17 responses (40). In addition, IL-17 may negatively regulate IFN-γ production. Treatment with anti–IL-17 enhanced IFN-γ production in mice with fungal infections (41). However, we showed that Th17 and Th1 cells are enhanced in ApoE<sup>−/−</sup> mice mice with atherosclerotic plaques compared with WT littermates. The increase in Th17 and Th1 cells is associated with the area of atherosclerotic plaque in ApoE<sup>−/−</sup> mice. Furthermore, the expression of Th1 and Th17 cytokines (IFN-γ and IL-17, respectively) is elevated in vascular walls with atherosclerotic plaques in ApoE<sup>−/−</sup> mice. More importantly, the percentage of Th1 cells in the spleen had a significant positive correlation with that of Th17 cells (Supplemental Fig. 2). Our results suggest that Th1 and Th17 cells may be involved in the development of atherosclerotic plaques in ApoE<sup>−/−</sup> mice, but the relationship between them remains to be elucidated. Nevertheless, a role for Th17/IL-17<sup>+</sup> cells in the pathogenesis of atherosclerosis is revealed.

Acknowledgments

We thank the Key Laboratory of Cardiovascular Remodeling and Function Research, Qilu Hospital, Shandong University, for expert technical assistance. We thank Brian Zamarro, National Institute of Dental and Craniofacial Research, National Institutes of Health, for critical reading of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References

8

Th17 Cells in Atherosclerosis in Mice


Supplement figure 1

The change of CD4⁻IL-17⁺ cells with the formation of atherosclerotic plaque

Splenocytes from young nonatherosclerotic and old atherosclerotic mice or C57BL/6 mice were stimulated with phorbol myristate acetate (PMA, 25ng/ml), ionomycin (1ug/ml) and plus blocking reagent-Brefeldin A (10μg/ml) incubated in 37°C, 5% CO₂ condition for 4 h, then assayed for assessment of the number of CD4⁻IL-17⁺T cells. The changes of CD4⁻IL-17⁺T cells at the different age points were analyzed. Statistic results represent data from 5 mice in each group, * P<0.05.

Supplement figure 2

The correlation of Th1 and Th17 cells

The correlations of Th1 with Th17 were analyzed using SPSS 11.0.
Supplement Figure 1

The graph shows the percentage of CD4+ IL-17+ cells over time for C57 and ApoE-KO groups. Treatment for 24 weeks compared to 8 weeks shows a significant increase in CD4+ IL-17+ cells for both groups, with ApoE-KO showing a steeper increase.