Inhibition of IL-17A Attenuates Atherosclerotic Lesion Development in ApoE-Deficient Mice


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Inhibition of IL-17A Attenuates Atherosclerotic Lesion Development in ApoE-Deficient Mice


The importance of an (auto)immune response in atherogenesis is becoming increasingly well understood. IL-17A-expressing T cells modulate immune cell trafficking, initiating inflammation and cytokine production in (auto)immune diseases. In human carotid artery plaques, we previously showed the presence of IL-17A-producing T cells and IL-23; however, IL-17A effects on atherogenesis have not been studied. Aortic root sections from 8-wk-old apolipoprotein E-deficient mice fed a standard chow diet were examined after 12 wk for lesion area, plaque composition, cellular infiltration, cytokine expression, and apoptosis. The treatment group (n = 15) received anti-IL-17A Ab and the controls (n = 10) received irrelevant Abs. Inhibition of IL-17A markedly reduced atherosclerotic lesion area (p < 0.001), maximal stenosis (p < 0.001), and vulnerability of the lesion. IL-17A mAb-treated mice showed reduced cellular infiltration, down-regulation of activation markers on endothelium and immune cells (e.g., VCAM-1), and reduced cytokine/chemokine secretion (e.g., IL6, TNFα, CCL5). To investigate possible mechanisms, different atherogenic cell types (e.g., macrophages, dendritic cells, HUVECs, vascular smooth muscle cells) were stimulated with IL-17A in addition to TNF-α, IFN-γ, or LPS to induce cellular activation or apoptosis in vitro. Stimulation with IL-17A induced proinflammatory changes in several atherogenic cell types and apoptotic cell death in murine cells. Functional blockade of IL-17A reduces atherosclerotic lesion development and decreases plaque vulnerability, cellular infiltration, and tissue activation in apolipoprotein E-deficient mice. The present data support a pathogenic role of IL-17A in the development of atherosclerosis by way of its widespread proinflammatory and proapoptotic effects on atherogenic cells. The Journal of Immunology, 2009, 183: 8167–8175.

A chronic inflammatory response of the arterial wall is the critical mechanism in the development of atherosclerosis (1, 2). The disease process is associated with local formation of modified autoantigens that are targeted by both the innate and adaptive immune system (1, 3). Inflammatory cells such as macrophages and T lymphocytes are believed to be primarily involved in the initiation and progression of atherogenesis (3, 4). Extravasation of these inflammatory cells is stimulated by adhesion molecules induced by proinflammatory cytokines and chemokines (1). Once the blood cells have attached, chemokines produced in the underlying intima promote the migration into the subendothelial space (5). Plaque-residing APCs such as dendritic cells (DC)4 then can augment T cell stimulation through the expression of the costimulatory ligands and provide optimal stimulation conditions for T lymphocytes (6).

Numerous proinflammatory cytokines are involved in the pathogenesis of atherosclerosis. IL-17A, a recently discovered proinflammatory cytokine, is mainly produced by Th17 cells, a separate CD4+ T cell subset distinct from Th1, Th2, and Treg polarization via involvement of TGF-β, IL-6, and the retinoic acid-related orphan receptor γt. The IL-17A receptor is ubiquitously expressed and the cytokine has pleiotropic activities, including induction of the production of TNF-α, IL-1β, and MCP-1 as well as adhesion molecules like ICAM-1 (7–9). An association between IL-17A and numerous (auto)immune diseases and inflammatory processes such as experimental autoimmune encephalomyelitis and acute myocarditis has been described (10, 11). However, the role of IL-17A in atherogenesis is currently unknown. To test the hypothesis that IL-17A accelerates atherogenesis, we blocked IL-17A with an anti-IL-17A Ab (mAb) in apolipoprotein E-deficient (apoE(−/−)) mice, a well established mouse model for atherosclerosis (12). In addition, we investigated possible mechanisms of IL-17A on atherogenic cells (vascular smooth muscle cells (VSMC), monocytes/macrophages, HUVECs, DCs, and CD4+ T cells) in vitro.

Materials and Methods

Animals

Female apoE(−/−) mice 8 wk of age (strain B6.129P2) on a C57BL/6J background were kept within the animal care facility of the University of Heidelberg (Heidelberg, Germany). Mice were fed a normal chow diet. Blocking anti-mouse IL-17A mAb (100 μg; R&D Systems) (n = 15 mice) or 100 μg of irrelevant IgG (n = 10 mice) were administered i.p. once a week for 12 wk. The housing and care of animals and all the procedures done in the study were in accordance with the guidelines and regulations of the local

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2 C.E. and L.C. contributed equally to this article.

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4 Abbreviations used in this paper: DC, dendritic cell; apoE(−/−), apolipoprotein E-deficient; C57, cycle threshold; EMSA, electrophoretic mobility shift assay; HDL, high-density lipoprotein; LDL, low-density lipoprotein; oxLDL, oxidized LDL; qPCR, quantitative PCR; TF, tissue factor; Treg, regulatory T cell; VSMC, vascular smooth muscle cell.

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Animal Care Committee (Institutional Review Board approval info: AZ 35–9185.81/G-136/05).

Tissue processing

For RNA isolation, the aortic arch was dissected and snap-frozen as described previously (13). The entire aortic root, a predilection site for lesion development in apoE−/− mice (14), was serially sectioned and, beginning with the first slide containing all three valves, a section was stained every 75 μm.

Immunohistochemistry

Cryosections from aortic root were fixed in acetone, air-dried, and incubated with mAbs as described previously (13). The detection of apoptosis was performed using an in situ cell death detection kit (Roche) according to the manufacturer’s protocols.

Determination of plasma lipid concentration, plasma cell composition, and IL-17A serum levels

Total serum cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides, and blood count were analyzed with a standard method by the Department of Clinical Chemistry of University of Heidelberg. Serum IL-17A levels were measured on the day of tissue harvesting with IL-17A ELISA (Hoezel Biotech) according to the manufacturer’s protocols.

Real-time PCR

Total RNA was isolated using RNeasy kit (Qiagen) and the Boehringer cDNA kit (Roche Diagnostics). For quantitative PCR (qPCR), Roche real-time PCR kit with SYBR Green (Roche Diagnostics) was used as described previously (13). Data were analyzed on the basis of the relative expression method with the formula relative expression 2−ΔΔCq, where ΔCq is the difference in threshold cycle (Cq) between the gene of interest and the housekeeping gene (β-actin) as a control. cDNA transcript levels of indicated cytokines/chemokines were measured at the end of the experimental period. For primer sequences see the online data supplement.5

Flow cytometry

Isolated cells of each spleen and peripheral lymph node as well as HUVECs were harvested and labeled with the appropriate Ab (CD3, CD4, CD25, VCAM-1, ICAM-1, and E-selectin; BD Pharmingen) to perform flow cytometry as described previously (13). For intracellular staining, the cells were incubated in permeabilization working solution with saponin 0.1% together with FoxP3 or IFN-γ Ab (anti-mouse, BD Pharmingen). The detection of apoptosis was performed using an annexin V-FITC apoptosis detection kit (BD Pharmingen) according to the manufacturer’s protocols.

Cell isolation, culture, and in vitro experiments

HUVECs were isolated from human umbilical cord as described previously (15). Murine macrophages were elicited of the peritoneum using 3% thioglycolate (Brewer’s thioglycolate medium). Four days after the thioglycolate injection, cells were isolated out of the peritoneum by lavage with cold PBS and 1 mL/kg 1% HCl. At day of tissue harvesting with IL-17 mAb-treated mice and controls. All values are shown as mean ± SD. n.s., Not significant.

Table I. Blood and baseline parametersa

<table>
<thead>
<tr>
<th>Parameter</th>
<th>IL-17 mAb-treated (n = 15) ± S.D.</th>
<th>Control (n = 10) ± S.D.</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes (/μl)</td>
<td>3.08 ± 0.89</td>
<td>3.83 ± 2.19</td>
<td>n.s.</td>
</tr>
<tr>
<td>Erythrocytes (/μl)</td>
<td>7.92 ± 1.09</td>
<td>7.01 ± 1.14</td>
<td>n.s.</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>12.14 ± 1.51</td>
<td>12.49 ± 3.18</td>
<td>n.s.</td>
</tr>
<tr>
<td>Thrombocytes (μl)</td>
<td>394.20 ± 201.33</td>
<td>460.67 ± 208.07</td>
<td>n.s.</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>101.67 ± 38.87</td>
<td>78.50 ± 24.04</td>
<td>n.s.</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>334.44 ± 54.94</td>
<td>315.33 ± 36.20</td>
<td>n.s.</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>293.83 ± 52.60</td>
<td>307.50 ± 36.10</td>
<td>n.s.</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>5.40 ± 2.50</td>
<td>98.33 ± 2.50</td>
<td>n.s.</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>28.80 ± 2.40</td>
<td>29.30 ± 2.10</td>
<td>n.s.</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>9.50 ± 1.12</td>
<td>9.30 ± 1.12</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

a Body weight and length, lipid profile, and hematological parameters were measured on the day of tissue harvesting (20 wks) of IL-17 mAb-treated mice and controls. All values are shown as mean ± SD. n.s., Not significant.

Results

Unstimulated cells served as controls. Cells were harvested at the end of the experimental period and subjected to RNA/DNA extraction or flow cytometry analysis as described above. Protein levels in the supernatant of IL-17A-stimulated and -unstimulated cells were assayed by ELISA (SA Biosciences) according to the manufacturer’s protocols.

Preparation of nuclear extracts

Endothelial cells and macrophages were prepared for electrophoretic mobility shift assay (EMSA) as described previously (16). In brief, cells were homogenized in 400 μl of hypotonic buffer (10 mmol/L HEPES, pH 7.9; 10 mmol/L KCl; 0.1 mmol/L EDTA; 0.1 mmol/L EGTA; 2 mmol/L DTT) supplemented with proteinase and phosphatase inhibitors (5 mg/ml E64; 1 mmol/L NaF; 0.2 mmol/L Na3VO4; 0.5 mg/ml Pefabloc), afterward supplemented with 25 ml of 10% Nonidet P-40, and were stored at −80°C until used.

EMSA

The Bradford method was used to measure the protein concentrations as described previously (16). The oligonucleotides (1.75 pmol/μl) were labeled with (γ32P)-ATP by using T4 polynucleotide kinase. Specific activities used in each assay were around 10,000 cpm. One hundred-fold excess of unlabelled oligonucleotides were used for cold inhibition. Binding reactions were resolved on 4% native polyacrylamide gel and exposed to x-ray film.

Statistical analysis

Figure data are presented as means ± SEM and tables as means ± SD. Nonparametric Mann-Whitney U test was used to compare groups of animals. A level of p < 0.05 was considered statistically significant.

Results

Animal/blood analysis

Dosing and subsequent sufficient systemic Ab levels were established in pilot experiments evaluating escalating doses of IL-17A mAb and serum levels 1, 3, and 7 days after injection (data not shown). Based on these results, mice were treated with 100 μg anti-IL-17A mAb by intraperitoneal injection once a week for 12 wk. No animal died during Ab application and IL-17A mAb-treated mice showed no growth retardation during the experimental period.

Body weight and length of experimental animals, serum concentrations of total cholesterol, HDL and LDL cholesterol, triglycerides, and whole blood counts did not differ on the day of tissue harvesting (20 wk of age) between both groups (Table I). Systemic IL-17A levels (serum) were significantly reduced in IL-17A mAb-treated compared with control mice (Table II). Notably, serum levels of oxidized LDL (oxLDL) Abs showed no difference between both groups (Table II).

Using flow cytometry to investigate systemic changes, the proportion of CD3+ T cells isolated from the spleen was mildly reduced in IL-17A mAb-treated mice compared with control mice.
(p = 0.04; Table III). The number of IL-17A-expressing CD4+ cells remained unchanged (~1% in both groups; data not shown).

Effects of IL-17A mAb treatment on atherosclerotic plaques in apoE−/− mice

Treatment with IL-17A mAb resulted in significantly reduced atherosclerotic lesions (Fig. 1). The aortic root of IL-17A mAb-treated mice showed a reduced mean lesion area with 1586 ± 723 mm2 (mean ± SD) compared with control mice with 2716 ± 1187 mm2 (~52%; p < 0.001; Fig. 1). The fractional stenosis was also markedly reduced in IL-17A mAb-treated mice compared with controls (~53%; 15 ± 10% vs 34 ± 15%; p < 0.001; Fig. 1). Maximum stenosis was reduced accordingly in IL-17A mAb-treated vs control mice (~51%; 16.3 ± 8.7% vs 32.5 ± 12.2%; p < 0.001, Fig. 1).

Staining with Movat to evaluate plaque composition in IL-17A mAb-treated mice did not show higher amounts of collagen in the lesion (p = 0.004; Table IV), but also that collagen was more prominent in the fibrous cap (Fig. 2, A and B). Immunolabeling of α-smooth muscle actin showed no difference between groups (Fig. 2, C and D), but in the region of the fibrous cap IL-17A mAb-treated mice showed an accumulation of α-smooth muscle actin compared with control mice (p = 0.02; Table IV).

Cellular infiltration/activation of plaque

Immuno-histochemistry staining revealed a reduction of the amount of CD3+ T cells in atherosclerotic lesions (total plaque) of IL-17A mAb-treated mice compared with control mice (p = 0.01; Fig. 2, K and L). In addition, T cell density (cell number/plaque area (mm2)) was also significantly reduced (p = 0.028; Table IV). qPCR results corroborated the immunohistochemistry findings. Tissue levels of cDNA transcripts for CD3ε, part of the CD3 receptor on T cells, were distinctly lower in IL-17A mAb-treated mice than in controls (p < 0.001; Table V). LCK-specific sequences, as a marker of the CD4 TCR, were less abundant in tissues from IL-17A mAb-treated mice (p = 0.01; Table V). IL4, a cytokine largely expressed by Th2 cells, was significantly higher expressed in IL-17A mAb-treated mice (p = 0.01). No significant difference of IFNγ could be observed between groups (Table V). In IL-17A, mAb-treated mice a significant reduction of FOXP3, a marker for Treg, compared with control mice (Table V). However, when FOXP3 was assessed in relation to CD3ε or LCK (i.e., CD4) signals, no change in relative expression was seen.

Lesional macrophages were identified by immuno-histochemistry with a MAC-2 Ab and showed reduced numbers per total lesion (384 ± 155 vs 209 ± 106; p = 0.01; Fig. 2, E and F) as well as reduced density of macrophages (cell number/plaque area (mm2)) in IL-17A mAb-treated mice compared with controls (p = 0.04; Table IV). Tissue levels of cDNA transcripts for IL1β, IL6, and TNFα, mainly expressed by macrophages and endothelial cells, showed reduced numbers in IL-17A mAb-treated animals compared with controls (Table V), whereas no difference in the qPCR levels of MCP-1 (CCL2), macrophage inflammatory protein-1α, or endothelial NO synthase expression were observed (Table V).

Leukocyte influx into atherosclerotic lesions was investigated by measuring some chemokines (CCL5, CX3CL1, CXCL1, CXCL1, and CCL20) and their receptors (CCR2, CCR5, CX3CR1, and CXCR6). Although the expression of CCL5 was significantly reduced in mice treated with IL-17A mAb, reduction of CCR5 did not reach statistical significance (Table V). No difference was seen for CXCL1, CX3CL1, and CXCL16 or for the chemokine receptors CCR2, CX3CR1, and CXCR6 (not shown).

Expression of TNF receptor family member (TR2) TNFRSF14, predominantly found on endothelial cells, was significantly down-regulated in IL-17A mAb-treated mice compared with control mice (p = 0.03; Table V). IL-17A mAb-treated mice also showed a decrease of adhesion molecule VCAM-1 expression in atherosclerotic lesions, measured by qPCR as well as by immunohistochemistry staining (p < 0.001 and p = 0.02, respectively; Fig. 2, G–I, and Table V). By semiquantitative scoring, VCAM-1 expression was also reduced when analyzing the endothelial cell compartment separately (not shown).

To investigate apoptotic cell death in the lesion, TUNEL+/−, diamidino-2-phenylindole staining was performed on frozen sections. Whereas TUNEL-positive stained cells were observed in both groups, blocking IL-17A led to reduced apoptosis in atherosclerotic plaques (p = 0.04; Table IV). Lesions of IL-17A mAb-treated mice typically showed occasionally single TUNEL-positive cells distributed throughout the lesion or were negative for apoptosis (Fig. 2M). In controls, large TUNEL-positive areas were present in the lesion, mainly in the area of the necrotic core (Fig. 2N).

In vitro effects of IL-17A on atherogenic cells

To identify the mechanisms by which IL-17A may promote atherosclerosis, we studied the specific effects of this cytokine on different atherogenic cell types.

Endothelial cells. HUVEC cells, stimulated with 10–1000 ng/ml IL17A, underwent activation and expressed higher levels of CXCL8 (Fig. 3A) as well as IL1β, ICAM1, VCAM1, Eselectin, tissue factor (TF), MCP1 (CCL2), and IL6 (not shown) by qPCR.

<table>
<thead>
<tr>
<th>Variable</th>
<th>IL-17 mAb-treated (n = 15) ± SD</th>
<th>Control (n = 10) ± SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-17 (baseline)</td>
<td>154.64 ± 54.20</td>
<td>157.44 ± 57.65</td>
<td>n.s.</td>
</tr>
<tr>
<td>IL-17 (20 wks)</td>
<td>41.23 ± 20.19</td>
<td>173.76 ± 52.66</td>
<td>0.002</td>
</tr>
<tr>
<td>oxLDL-IgG</td>
<td>0.11 ± 0.03</td>
<td>0.12 ± 0.03</td>
<td>n.s.</td>
</tr>
<tr>
<td>oxLDL-IgM</td>
<td>0.48 ± 0.07</td>
<td>0.39 ± 0.19</td>
<td>n.s.</td>
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</tbody>
</table>

* On the day of tissue harvesting (20 weeks) serum levels of IL-17 (pg/ml) as well as IgG and IgM oxLDL antibody concentrations in the serum were determined by ELISA. Similar baseline IL-17 serum concentrations in both groups are shown for comparison. All values are shown as mean ± SD, n.s., Not significant.

<table>
<thead>
<tr>
<th>Variable</th>
<th>IL-17 mAb-treated (n = 15) ± SD</th>
<th>Control (n = 10) ± SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>36.29 ± 6.18</td>
<td>44.43 ± 7.68</td>
<td>0.04</td>
</tr>
<tr>
<td>CD3/CD25</td>
<td>6.21 ± 1.47</td>
<td>9.02 ± 1.88</td>
<td>0.01</td>
</tr>
<tr>
<td>CD4/IFN-γ</td>
<td>14.93 ± 10.06</td>
<td>26.10 ± 29.58</td>
<td>0.03</td>
</tr>
<tr>
<td>CD4/CD25/FoxP3</td>
<td>7.46 ± 1.06</td>
<td>10.25 ± 1.95</td>
<td>0.006</td>
</tr>
<tr>
<td>IL-4</td>
<td>6.15 ± 3.59</td>
<td>2.84 ± 1.57</td>
<td>0.04</td>
</tr>
<tr>
<td>IL-10</td>
<td>3.99 ± 1.22</td>
<td>2.50 ± 1.59</td>
<td>0.02</td>
</tr>
</tbody>
</table>

* Splenocytes of experimental animals were isolated to determine the percentage of CD3+, CD4+, CD25+, CD4+ IFN-γ+, and CD4+ CD25+ FoxP3+ T cells relative to total spleen cells, using flow cytometry (percentage of positive cells/total spleen cells). Further, the cDNA transcript levels of IL-4 and IL-10 (expressed by Th2 cells) were measured, using qPCR and normalized to β-actin. Data are shown expressed as cDNA copies/1000 copies of β-actin. All values are shown as mean ± SD, n.s., Not significant.
VCAM-1 surface expression caused by TNF-α

Flow cytometry analysis of ICAM-1 expression showed that IL-17A ± TNF-α stimulation of HUVECs for 1 or 3 h did not result in any changes between the groups (not shown). Interestingly, HUVECs stimulated with IL-17A for 8 h showed an increased ICAM-1 surface expression compared with unstimulated cells. The ICAM-1 up-regulation on HUVECs by IL-17A stimulation was still detectable after 24 h, but was not seen after 48 or 72 h. Furthermore, addition of IL-17A to TNF-α led to a potentiation of the increased ICAM-1 surface expression induced by TNF-α after 8 h (not shown). However, the effect was limited to the 8-h time point.

Flow cytometry analysis of E-selectin showed that only after 3 h there was a increase of surface E-selectin expression on HUVECs stimulated with IL-17A (not shown). Addition of IL-17A to TNF-α, however, did not further increase E-selectin surface expression.

EMSAs of nuclear extracts and subsequent densitometric evaluation showed an increase of DNA binding activity of the transcription factor NF-κB in IL-17A-stimulated cells as compared with unstimulated cells at 30 min, 1 h, and 2 h (not shown). Stimulation with IL-17A in addition to TNF-α led to a potentiation of the effects, showing marked up-regulation of the binding activity of the transcription factors NF-κB 1–2 h after stimulation.

**Macrophages.** RT-PCR analysis of cytokine production was performed in RAW cells after stimulation with 10–100 ng/ml IL-17A (Fig. 3C). Stimulation with IL-17A resulted in a dose- and time-dependent activation of RAW cells with an up-regulation of TNFα (Fig. 3D) as well as IL1β, IL6; adhesion molecules VCAM1, ICAM1, and Eselectin; chemokines MCP1, and CCL5; and the prothrombic protein TF (not shown) at different time points (1, 2, 4, 8, and 24 h). Stimulation with IL-17A in addition to 1 mg/ml LPS led to a potentiation and prolongation of the effect of LPS, extending expression of VCAM1, IL6, Eselectin, and TF beyond 24 h.

To confirm the findings of the macrophage cell line, peritoneal macrophages were stimulated with IL-17A. IL-17A induced an

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**FIGURE 1.** Effects of IL-17A mAb treatment on atherosclerotic plaques in apoE−/− mice. Morphometric quantification of lesion area [μm²] (A), fractional stenosis [%] (B), and maximum stenosis [%] (C) of early atherosclerotic lesions were compared. Representative Oil Red O immunohistostainings from aortic root of control (D) and 17 mAb-treated (E) mice are shown. Data represent the median ± 75th and 25th percentile.

<table>
<thead>
<tr>
<th>Table IV. Quantification of immunohistochemistry analysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
</tr>
<tr>
<td>Collagen</td>
</tr>
<tr>
<td>VSMC-lesion</td>
</tr>
<tr>
<td>VSMC-fibrous cap</td>
</tr>
<tr>
<td>VCAM-1</td>
</tr>
<tr>
<td>MAC-2</td>
</tr>
<tr>
<td>CD3</td>
</tr>
<tr>
<td>TUNEL</td>
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</table>

*Immunohistochemistry analysis of lesion composition of IL-17 mAb-treated mice compared to controls. Collagen was calculated as percentage of Movats®-stained plaque area to total lesion area (%) in IL-17 mAb-treated and control mice. Number of positive cells were related to lesion area (VCAM-1, CD3, MAC-2), to fibrous cap (VSMCs), to DAPI® cells (TUNEL), and per plaque (VCAM-1) in IL-17 mAb-treated and control mice. All values are shown as mean ± SD. n.s., Not significant.
Cellular composition of lesions and plaque characterization in atherosclerotic lesions of apoE−/− mice. Representative photomicrographs of immunohistochemistry stainings of collagen (Movat; A and B), VSMCs (α-smooth muscle actin; C and D), macrophages (MAC-2; E and F), grade of activation of the tissue (VCAM-1; G and I), and of activated endothelial cells (VCAM-1; H and J), T cells (CD3; K and L) and apoptosis (TUNEL; M and N). A–G, I, and K–N show 4× and H and J 10× magnification.

Table V. Quantitative tissue PCR results for different cytokines, chemokines, adhesion molecules and transcription factors from the thoracic aortaα

<table>
<thead>
<tr>
<th>Variable</th>
<th>IL-17 mAb-treated (n = 15) ΔCT ± SD</th>
<th>Control (n = 10) ΔCT ± SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3α</td>
<td>3.11 ± 2.02</td>
<td>8.58 ± 2.47</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Endothelial NO synthase</td>
<td>0.35 ± 0.18</td>
<td>0.24 ± 0.10</td>
<td>n.s.</td>
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<tr>
<td>LCK</td>
<td>3.26 ± 2.10</td>
<td>6.75 ± 4.09</td>
<td>0.01</td>
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<tr>
<td>IL4</td>
<td>0.33 ± 0.19</td>
<td>0.18 ± 0.09</td>
<td>0.01</td>
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<td>FOXP3</td>
<td>1.06 ± 0.43</td>
<td>0.67 ± 0.41</td>
<td>0.006</td>
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<tr>
<td>IL1β</td>
<td>2.76 ± 1.93</td>
<td>1.37 ± 1.27</td>
<td>0.01</td>
</tr>
<tr>
<td>TNFα</td>
<td>3.24 ± 2.18</td>
<td>4.28 ± 4.43</td>
<td>0.04</td>
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<tr>
<td>IL6</td>
<td>78.46 ± 51.39</td>
<td>157.27 ± 193.99</td>
<td>0.04</td>
</tr>
<tr>
<td>MCP-1(CCL2)</td>
<td>63.44 ± 24.65</td>
<td>64.36 ± 28.74</td>
<td>n.s.</td>
</tr>
<tr>
<td>MIP-1α(CCL3)</td>
<td>2.62 ± 0.94</td>
<td>2.54 ± 0.86</td>
<td>0.03</td>
</tr>
<tr>
<td>TR2 (TNFRSF14)</td>
<td>0.02 ± 0.02</td>
<td>0.35 ± 0.85</td>
<td>0.03</td>
</tr>
<tr>
<td>VCAM1</td>
<td>22.19 ± 0.87</td>
<td>64.30 ± 28.02</td>
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<td>IFNγ</td>
<td>2.01 ± 1.35</td>
<td>1.41 ± 0.85</td>
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<td>CCL5</td>
<td>30.08 ± 18.24</td>
<td>52.57 ± 24.96</td>
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<td>CCR5</td>
<td>18.95 ± 16.79</td>
<td>57.53 ± 45.67</td>
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α Values are normalized to β-actin and expressed as cDNA copies/1000 β-actin copies. All values are shown as mean ± SD. n.s., Not significant.

Discussion

Atherosclerosis is a chronic inflammatory disease of the arterial wall in which both innate and adaptive immunity are involved. Inflammation is central to all stages of atherosclerosis. It is already implicated in the formation of early fatty streaks in which the endothelium is activated and expresses chemokines and adhesion molecules leading to monocyte/lymphocyte recruitment and infiltration into the subendothelium (1). Evidence from human and experimental studies suggests a dominant role of the CD4+ T cell immune response, especially Th1 cells, in atherogenesis (17, 18).
Our study demonstrates that blocking IL-17A, mainly produced by a newly described CD4^+ T cell subset, Th17, cells, with IL-17A mAb leads to a reduction in early atherosclerotic lesion area/frac-tional stenosis/maximum stenosis by \(50\%\) in apoE \(-/-\) mice, a validated mouse model of atherosclerosis (12). In addition, reduced plaque vulnerability is indicated by increased VSMC accumulation and collagen content of the fibrous cap as well as reduced apoptosis in the lesion. IL-17A mAb treatment results in reduced inflammatory cellular infiltration with attenuation of (proinflammatory) cytokine/chemokine expression and less tissue activation. These findings are reproduced in vitro. IL-17A stimulation leads to activation of several atherogenic cells involved in atherogenesis including HUVECs, VSMCs, macrophages, CD4^+ T cells, monocytes, and DCs, as well as an induction of apoptotic cell death in VSMCs, thus representing possible mechanisms of IL-17A on atherogenesis.

The present findings show that inhibition of IL-17A by block- ing Ab successfully reduced systemic levels of IL-17A without affecting the proportion of Th17 cells in the circulation. In the aortic root, apoE \(-/-\) mice show most rapid development of lesions between 15 and 30 wk of age on regular diet (19). IL-17A mAb-treated mice show a significant deceleration of arterial disease progression during this interval, with a significant reduction in the extent of atherosclerotic lesions at 20 wk of age/at the end of the 12 wk observation period. The extent of protection afforded by IL-17A inhibition in the aortic root is similar to that reported in combined IFN-\(\gamma\) receptor-deficient/ apoE \(-/-\) mice or apoE \(-/-\) mice fed a high-fat (Western) diet treated with IL-18 binding protein for 12 wk (18, 20). Despite modestly higher plasma cholesterol levels, IFN-\(\gamma\) receptor \(-/-\)/apoE \(-/-\) mice showed a 59% reduction in plaque size compared with apoE \(-/-\) controls (19).

FIGURE 3. In vitro effects of IL-17A on various cell types. A, HUVECs were stimulated with IL-17A in a time- and dose-dependent manner and expression of indicated cytokines was analyzed by qPCR. Results were normalized to \(\beta\)-actin. B, Measurement of surface VCAM-1 expression on HUVECs treated for 3 h with 10 ng/ml IL-17A (solid black line), 5 ng/ml TNF-\(\alpha\) (dotted light gray line), 10 ng/ml IL-17A plus 5 ng/ml TNF-\(\alpha\) (solid dark gray line), or unstimulated cells (shaded histogram) by flow cytometry. Solid light gray line represents staining with an isotype-matched control Ab. Representative data from one of three independent experiments with similar results is shown. C, Time-dependent VCAM-1 surface expression level in unstimulated HUVECs (light short dashes) and HUVECs stimulated with IL-17A (long bold dashes), TNF-\(\alpha\) (light solid line) and IL-17 plus TNF-\(\alpha\) (dotted and dashed line). D, RAW cells were stimulated with IL-17A in a time- and dose-dependent manner, indicated cytokines were analyzed by qPCR, and results were normalized to \(\beta\)-actin. E, Electromobility shift assay of nuclear extracts and subsequent densitometric evaluation of IL-17A alone or in combination with LPS-stimulated RAW cells (60 min) are shown for indicated dosages. F, VSMCs were treated with IL-17A in a time- and dose-dependent manner, expression of indicated cytokines was assayed by qPCR, and results were normalized to \(\beta\)-actin. G, Apoptosis in VSMCs was induced by 100 ng/ml IL-17A in addition to 500 U/ml INF-\(\gamma\) and 5 ng/ml TNF-\(\alpha\), which led to a potentiation of the effect of IFN-\(\gamma\) in addition to IL-17A or TNF-\(\alpha\). H, DCs stimulated with IL-17A in a time- and dose-dependent manner were analyzed for the proinflammatory cytokine IL-12 and indicated molecules, analyzed by qPCR, and results were normalized to \(\beta\)-actin.
Previous pathological studies showed that vulnerable plaques are rich in inflammatory cells and exhibit a substantial loss in smooth muscle cells, mainly by apoptosis and collagen content (21, 22). All these signs of increased plaque instability are markedly attenuated in IL-17A mAb-treated mice. Together with the finding that IL-17A induces expression of matrix metalloproteinases MMP1 and MMP9 as well as apoptosis in VSMC in vitro, these observations suggest that IL-17A might be a major determinant of plaque vulnerability. The relevance of these results obtained in apoE−/− mice to human disease is strengthened by our finding of increased IL-17A expression in symptomatic carotid atherosclerotic plaques (our unpublished observations).

The biological actions of IL-17A are predominantly proinflammatory (7). Induction of cytokines like IL-6, chemokines such as CXCL8 (23), MCP-1 (7, 24), and GROα (25) is reported, thereby enhancing the inflammation and promoting the recruitment of monocytes and neutrophils (26, 27). Further, IL-17A synergizes with other proinflammatory cytokines like TNF-α for IL-6, CXCL8, and GM-CSF induction in other cell types (23, 28). We report that the inhibition of IL-17A and its proinflammatory effects causes a down-regulation of the inflammatory compound with reduced cellular infiltration and activation (e.g., T cell/macrophage infiltration). The diminished cell activation was reflected in reduced expression of several cytokines like IL6 and TNFα, adhesion molecules such as VCAM-1, and prothrombic molecule TF on the tissue level. Notably, inhibition of IL-17A does not seem to influence the levels of IFN-γ, which is in line with previous findings which also found no significant difference of IFN-γ levels between mice treated with anti-IL-17A Abs and controls (29). The findings of reduced transcript levels of various cytokines/chemokines by inhibition of IL-17A were then reproduced in different atherogenic cell types involved in atherosclerosis including HUVECs, macrophages, VSMCs, T cells, DCs, and monocytes in vitro. Similar findings concerning the stimulatory capacity of IL-17A in endothelial and vascular smooth muscle cells were recently published (29). By inducing the production of multiple cytokines, chemokines, and adhesion molecules in different atherogenic cell types, IL-17A might directly control both mononuclear cell accumulation and cell death within the plaque, important determinants not only of early atherogenesis, but also of plaque disruption and thrombosis.

It can be speculated that upon activation of HUVECs and murine macrophages via IL-17A, further degradation of IκB and subsequent activation of NF-κB is triggered, thereby inducing NF-κB-dependent gene transcription as shown in recent publications for several other cell types (8, 30). This may lead to enhanced inflammation by triggering the induction of additional adhesion molecules, cytokines, and growth factors.

T cells participate in atherosclerotic lesion generation as early as monocytes (3). It has been previously shown that most of the T cells in atherosclerotic lesions bear the CD3 and CD4 markers and these T cell subsets seem to be potent players in atherosclerotic plaque progression (17). Whereas Th1 cells seem to strongly induce the proatherogenic inflammatory process, Th2 responses seem to be atheroprotective (3, 18, 31). A switch in T polarization toward a Th2 response in IL-17A mAb-treated mice is suggested in the present study, indicated by reduced Th1 cytokine expression but increased expression of IL4, which is seen in mice on high fat diet (32) but not on normal chow diet. The shift in Th1/Th2 polarization due to inhibition of IL-17A may partly account for the reduced extent of atherosclerosis in IL-17A mAb-treated mice, but needs further investigation.

The proportion of spleen cell-derived total CD3+ T cells (and CD4 subset) was mildly reduced in IL-17A mAb-treated mice compared with controls. Although the absolute proportion of different T cell types was likewise reduced, the proportion of these three T cell populations (Th2, CD25+ T cells, and IFN-γ+ T cells) relative to CD3 or CD4 T cell numbers was not altered. Similar changes were noted in T cells extracted from peripheral lymph nodes. The moderate lowering of CD3+ T cells and proportional reduction of different T cell subsets of IL-17A-treated mice (compensated by increased B cell numbers) could represent a generalized alteration of immune cell homeostasis or proliferation, possibly by IL-17A inhibition, the exact basis of which remains to be elucidated.

The chemokine/chemokine receptor network is essential for direction of leukocyte migration in homeostatic and inflammatory conditions. In addition, leukocyte influx into atherosclerotic lesions mediated by chemokine receptors is thought to be an important mechanism promoting atherosclerosis. Numerous reports described the important role of chemokines and their receptors in the regulation of leukocyte recruitment during atherosclerosis (33). Our study showed that the functional blockade of IL-17A led to reduced expression of CCL5 without significantly affecting the levels of CCR5. In addition, CCL5 expression was inducible by IL-17A stimulation in VSMCs and macrophages. However, IL-17A was only able to induce CCR5 transcript levels in VSMCs, not in monocytes or T cells.

The chemokine CCL5 is expressed by various cell types. It is chemotactic for T cells and monocytes, thus increasing the adherence of monocytes to endothelial cells and playing an active role in recruiting leukocytes. It has been further observed that Abs to CCL5 dramatically inhibit the cellular infiltration into inflammatory sites. Previously published data also shows that the deposition of CCL5 by platelets triggers shear-resistant monocyte arrest on inflamed or atherosclerotic endothelium and thus may epitomize a novel principle relevant to inflammatory or atherogenic monocyte recruitment from the circulation (34).

**Table VI. Cytokine and chemokine production by VSMCs and macrophages (RAW cells) in response to IL-17A**

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<th>TNF-α</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IL-12</th>
<th>IL-17</th>
<th>SDF-1</th>
<th>CCL5</th>
<th>MCP-1</th>
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<td>+ IL-17A (24 h)</td>
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<td>82.63</td>
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<td>Unstim (4 h)</td>
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<tr>
<td>+ IL-17A (24 h)</td>
<td>13.95</td>
<td>0.16</td>
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<td>0</td>
<td>82.17</td>
<td>1.28</td>
<td>0.90</td>
<td>24.95</td>
<td>0.12</td>
<td>0.19</td>
<td>0.51</td>
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*The relative levels of 11 cytokines and chemokines were determined in the supernatant of VSMCs and macrophages (RAW cells) treated with IL-17 at 50 ng/mL for 4 to 24 h using a multi-analyte ELISA kit (SA Biosciences) pre-coated with capture Abs. Unstim, Unstimulated.
Following these results, IL-17A seems to support the chemoc-tactant, the recruitment and the adhesion of leukocytes such as monocytes and T cells by influencing CCL5, and adhesion mole-cule expression in atherosclerotic lesions. Thus, this may be one of the underlying mechanisms of IL-17A in atherogenesis.

IL-17A mAb-treated mice exhibited normal fertility, develop-ment, and growth. Plasma cholesterol and triglycerides as well as oxLDL Ab levels were equivalent between both groups. Previous studies investigating administration of IL-12 (35) or IL-18 (36) to apoE−/− mice also found no effects of these proin-flammatory cytokines on plasma cholesterol levels. We report that IL-17A mAb-treated mice have reduced atherosclerotic le-sions in the presence of unchanged serum cholesterol and HDL/ LDL ratio. These findings suggest that IL-17A is unlikely to affect atherogenesis via changes in hyperlipidemia or produc-tion of oxLDL Abs.

A limitation of this study is that blocking IL-17A is not equivalent to inhibiting Th17 cell generation as IL-17A is also affect atherogenesis via changes in hyperlipidemia or produc-tions in the presence of unchanged serum cholesterol and HDL/ LDL ratio. These findings suggest that IL-17A is unlikely to affect atherogenesis via changes in hyperlipidemia or production of oxLDL Abs.

We greatly appreciate the expert technical assistance of Nadine Wambgsans.

Disclosures
The authors have no financial conflict of interest.

References


### Primer sequences
Primer sequences (murine (m) and human (h)), forward (FP) and reverse (RP) primer, are shown.

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