Blockade of Interleukin-17A Results in Reduced Atherosclerosis in Apolipoprotein E–Deficient Mice

Emily Smith, PhD; Konkal-Matt R. Prasad, PhD; Matthew Butcher, BS; Anca Dobrian, PhD; Jay K. Kolls, MD; Klaus Ley, MD; Elena Galkina, PhD

Background—T cells play an important role during the immune response that accompanies atherosclerosis. To date, the role for interleukin (IL)-17A in atherogenesis is not well defined. Here, we tested the hypothesis that atherosclerosis-prone conditions induce the differentiation of IL-17A–producing T cells, which in turn promote atherosclerosis.

Methods and Results—IL-17A was found to be elevated in the plasma and tissues of apolipoprotein E–deficient ($Apoe^{-/-}$) mice. IL-17A–expressing T cells were significantly increased in the aortas, spleen, and lamina propria of aged $Apoe^{-/-}$ mice compared with age-matched *C57BL/6* mice. IL-17A$^+$ T cells resided in both adventitia and aortas of aged $Apoe^{-/-}$ mice fed a chow diet. Elevated levels of IL-17A$^+$ T cells were also detected in the aortas of 21-week-old $Apoe^{-/-}$ mice fed a Western diet for 15 weeks. IL-17A$^+$ T cells were characterized as predominantly CD4$^+$ T helper 17 (Th17) cells and $\gamma\delta^+$ T cells. Blockade of IL-17A in $Apoe^{-/-}$ mice by use of adenovirus-produced IL-17 receptor A reduced plaque burden in $Apoe^{-/-}$ mice fed a Western diet for 15 weeks. In addition, the treatment diminished circulating IL-6 and granulocyte colony-stimulating factor levels and limited CXCL1 expression and macrophage content within the aortas. Conversely, IL-17A treatment of whole aorta isolated from $Apoe^{-/-}$ mice promoted aortic CXCL1 expression and monocyte adhesion in an ex vivo adhesion assay.

Conclusions—These results demonstrate that atherosclerosis-prone conditions induce the differentiation of IL-17A–producing T cells. IL-17A plays a proatherogenic inflammatory role during atherogenesis by promoting monocyte/macrophage recruitment into the aortic wall. **(*Circulation*. 2010;121:1746-1755.)**

Key Words: atherosclerosis ■ immune system ■ inflammation ■ lymphocytes ■ aorta ■ interleukin-17

Atherosclerosis is the leading cause of cardiovascular disease worldwide. Defined as chronic inflammation of the artery wall, its progression from fatty streaks to more complex lesions and plaque rupture involves a complicated interplay between many different cell types and cytokine networks. Both innate and adaptive immune responses have been shown to regulate local and systemic inflammation during atherogenesis.[1,2] T cells are found within the adventitia of normal/noninflamed vessels as a result of a constitutive T-cell homing into the aorta.[3] Atherosclerosis-prone conditions accelerate T-cell recruitment into the aorta of apolipoprotein E–deficient ($Apoe^{-/-}$) mice in both the early and advanced stages of atherosclerosis.[3] The majority of aortic T cells are T-cell receptor $\alpha\beta^+$CD4$^+$ cells, with few CD8$^+$ and $\gamma\delta^+$ T cells present.[1,4] Of the CD4$^+$ T cells, T helper 1 (Th1) cells predominate over T helper 2 (Th2) cells during early lesion formation and respond with an elevated production of interferon (IFN)-γ and interleukin (IL)-6. In the later stages of the disease, a switch to a Th2 response and IL-4 production is evident in the atherosclerotic lesions of $Apoe^{-/-}$ mice.[5]

Clinical Perspective on p 1755

IL-17A is a member of the IL-17 family, which includes IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F.[6] Many lymphocyte subsets secrete IL-17A in response to cytokine or monoclonal antibody stimulation, including CD4$^+\alpha\beta^+$ (Th17 cells) CD8$^+$, CD4$^-$CD8$^-\alpha\beta^{low}$, natural killer T cells, and $\gamma\delta^+$ T cells.[7] The expression of IL-17A is low under normal/noninflamed conditions, in which $\gamma\delta^+$ T cells are the largest IL-17A–producing T-cell subset.[6] In several murine models of autoimmune diseases, including multiple sclerosis, inflammatory bowel disease, and arthritis, serum IL-17A levels are elevated, and the T helper 17 (Th17) cell population is expanded and plays a highly pathogenic role.[8] Conversely, IL-17A is a protective cytokine in host responses against extracellular pathogens through the induction of proinflammatory cytokines such as IL-6, tumor

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From the Robert M. Berne Cardiovascular Research Center (E.S.), University of Virginia, Charlottesville, Va; Department of Biomedical Engineering (K.-M.P.), University of Virginia, Charlottesville, Va; Department of Microbiology and Molecular Cell Biology, Department of Physiology, Eastern Virginia Medical School (M.B., A.D., E.G.), Norfolk, Va; Louisiana State University Health Sciences Center (J.K.K.), Department of Genetics, New Orleans; and Division of Inflammation Biology, La Jolla Institute for Allergy and Immunology (K.L.), La Jolla, Calif.

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Correspondence to Elena Galkina, PhD, Assistant Professor, Department of Microbiology and Molecular Cell Biology, Eastern Virginia Medical School, 700 W Olney Rd, Norfolk, VA 23507-1696. E-mail galkinev@evms.edu

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necrosis factor-α, and granulocyte colony-stimulating factor (G-CSF) and the chemokines CXCL8, CCL2, CXCL1, and CXCL2 in infected tissues.[6] These downstream mediators of IL-17A are involved in granulopoiesis and induce the recruitment of neutrophils, eosinophils, and monocytes into sites of inflammation.[9]

To date, the contribution of IL-17A to atherogenesis remains controversial, with different studies proposing either a proatherogenic or an atheroprotective role for IL-17A. In patients with unstable angina, levels of plasma IL-17A and the Th17-related cytokines IL-6 and IL-23 are elevated.[10] Cultured human T cells isolated from atherosclerotic coronary arteries also produce a unique combination of IL-17A and IFN-γ after polyclonal stimulation compared with T cells extracted from nondiseased vessels.[11] In murine models of atherosclerosis, deletion of IL-18 in *Apoe*$^{-/-}$mice results in an increase in Th17 cells within the aorta and exacerbated atherosclerosis.[12] Additionally, the absence of IL-17 receptor A (IL-17RA) on bone marrow–derived cells in lethally irradiated LDL receptor (*Ldlr*)–deficient mice also reduces atheroma formation.[13] Together, these studies suggest a proatherogenic role for IL-17A and IL-17A–producing cells. More recently, a study[14] using intravenous administration of recombinant IL-17A reduced atherosclerosis in young chimeric *Ldlr*$^{-/-}$ mice, which suggests atheroprotection by IL-17A; however, the administration of anti-IL-17A antibodies had no effects on atherosclerosis in this model.

Here, we report that *Apoe*$^{-/-}$ mice have elevated plasma levels of IL-17A and show increased numbers of IL-17A–expressing T cells, in particular Th17 and IL-17A^{+} $\gamma\delta^{+}$ T cells, within the aorta (adventitia and intima) and spleen. Blockade of IL-17A by soluble IL-17 receptor A (sIL-17RA) causes a significant decrease in the plasma levels of IL-6 and G-CSF, diminishes aortic macrophage content, reduces aortic CXCL1 expression, and leads to reduced plaque burden in *Apoe*$^{-/-}$ mice. IL-17A treatment of isolated *Apoe*$^{-/-}$ aortas increases CXCL1 expression and monocyte adhesion. Taken together, these results demonstrate a proatherogenic role of IL-17A and highlight a new role for IL-17A–producing T-cell subsets in the progression of atherosclerosis.

Methods

Animals

Female and male *Apoe*$^{-/-}$ mice on a *C57BL/6* background and control *C57BL/6* mice (Jackson Labs, Bar Harbor, Me), were kept on a chow diet and were used between 40 and 55 weeks of age. Alternatively, *Apoe*$^{-/-}$ mice were fed a Western diet (21% fat and 0.15% cholesterol, Harlan Teklad, Harlan Laboratories, Indianapolis, Ind) for 15 weeks and used at 21 weeks of age. All animals were kept in specific-pathogen–free conditions, and animal experiments were approved by the Animal Care and Use Committees of the University of Virginia and Eastern Virginia Medical School. Blood counts were taken via tail bleed and analyzed with an automatic analyzer (Hemavet 850, CDC Technologies Inc, Oxford, Conn). Additional Methods can be found in the online-only Data Supplement.

Statistical Analysis

For unpaired *t* tests, data were expressed as mean±SEM. Statistical significance between *Apoe*$^{-/-}$ and *C57BL/6* mouse groups was set at $P<0.05$.

Results

Levels of IL-17A Are Elevated in *Apoe*$^{-/-}$ Mice

To test the role of IL-17A in the immune response that accompanies advanced atherosclerosis, we analyzed IL-17A messenger RNA (mRNA) within the aorta, spleen, peripheral lymph nodes (including inguinal, axillary, brachial, and cervical nodes), mesenteric lymph nodes, and lamina propria of 40- to 55-week-old female and male *Apoe*$^{-/-}$ and *C57BL/6* mice. We chose to study aged *Apoe*$^{-/-}$ mice because they have been shown to develop advanced fibrous plaques at 40 to 45 weeks of age.[15] We found a significant increase in IL-17A expression in the aorta, spleen, peripheral lymph nodes, and lamina propria of *Apoe*$^{-/-}$ mice but not in the mesenteric lymph nodes (Figure 1A). Because the IL-17 family consists of 5 additional members (IL-17B, C, D, E, and F), we also examined the mRNA expression of IL-17C, D, E, and F in *Apoe*$^{-/-}$ aortas. IL-17A mRNA expression was found to be approximately 5-fold greater than that of the other IL-17 family members examined (Figure 1B). No IL-17E expression was found in aortas isolated from *C57BL/6* or *Apoe*$^{-/-}$ mice. Importantly, circulating levels of IL-17A were also elevated in the plasma of aged *Apoe*$^{-/-}$ compared with *C57BL/6* mice (Figure 1C). Because circulating neutrophil levels correlate closely with plasma IL-17A and G-CSF levels,[9] we analyzed neutrophil numbers in the blood of *Apoe*$^{-/-}$ mice. Circulating neutrophil counts (polymorphonuclear leukocytes) were also significantly elevated in *Apoe*$^{-/-}$ mice compared with *C57BL/6* mice (Figure 1D); however, no significant difference was seen in the numbers of white blood cells, peripheral blood lymphocytes, or monocytes (Figure 1D).

IL-17A induces the release of many chemokines, including CXCL1 and CCL2, as well as tumor necrosis factor-α, IL-1β, and IL-6 from monocytes, epithelial cells, and fibroblasts.[6] Circulating levels of tumor necrosis factor-α and IL-2 were significantly elevated in *Apoe*$^{-/-}$ mice (Figure 1C). Both Th1 (IL-12) and Th2 (IL-4, IL-5, IL-10, and IL-13) cytokines were also elevated in the plasma of *Apoe*$^{-/-}$ mice compared with *C57BL/6* mice (online-only Data Supplement Figure IA through IE). We next explored whether atherosclerosis-prone conditions induced elevated IL-17A levels through the SOCS-3 (suppressor of cytokine signaling-3)–dependent pathway. We found no difference in aortic mRNA SOCS-3 expression in 21-week-old *Apoe*$^{-/-}$ mice fed a Western diet or 40-week-old *Apoe*$^{-/-}$ mice fed a chow diet compared with *C57BL/6* mice (data not shown).

Th17 and $\gamma\delta^{+}$T Cells Are the Major Producers of IL-17A During Atherosclerosis

As IL-17A was elevated at the RNA and circulating protein level in aged *Apoe*$^{-/-}$ mice, we attempted to identify the cellular source behind its production. Under normal conditions, the number of Th17 cells found in *C57BL/6* mice is very small, with the main source of IL-17A–producing T cells being located in the lamina propria.[16,17] Here, we demonstrate elevated numbers of CD45^{+}IL-17A^{+} cells in the aorta, lamina propria, and spleen of aged female and male *Apoe*$^{-/-}$ mice compared with *C57BL/6* mice (Figure 2). These IL-

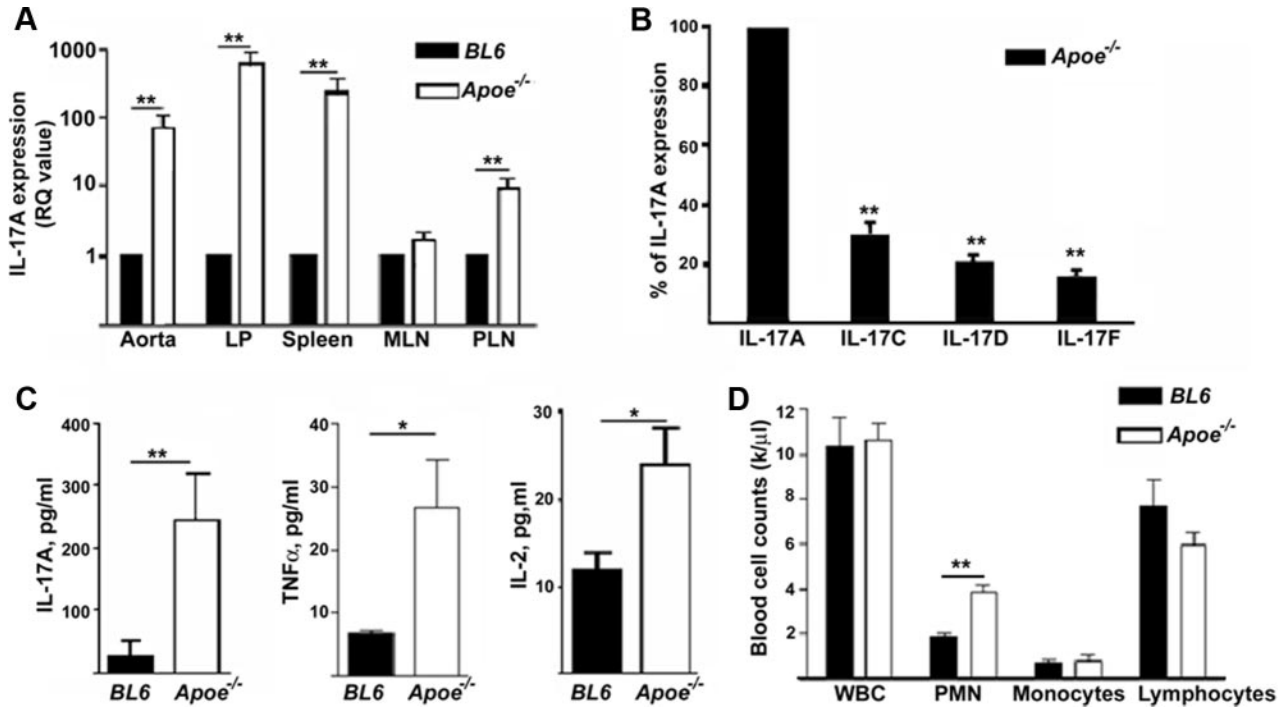

Figure 1. Basal levels of IL-17A were elevated in female and male *Apoe*$^{-/-}$ mice. A, IL-17A mRNA was elevated in aorta, spleen, lamina propria (LP), and peripheral lymph node (PLN) of *Apoe*$^{-/-}$ mice (open bars, n=6) compared with *C57BL/6* mice (solid bars, n=6). B, mRNA expression of IL-17C, IL-17D, and IL-17F was normalized compared with IL-17A (set as 100%) in the aortas of *Apoe*$^{-/-}$ mice (n=8). C, Serum IL-17A, tumor necrosis factor-α (TNF-α), and IL-2 were analyzed in the plasma of *Apoe*$^{-/-}$ (open bars, n=10 to 11) and *C57BL/6* mice (solid bars, n=10 to 11). D, Neutrophil (PMN) counts were elevated in *Apoe*$^{-/-}$ mice (open bars, n=8) compared with *C57BL/6* mice (solid bars, n=5). WBC indicates white blood cells. *$P<0.05$, **$P<0.01$ by unpaired Student *t* test. RQ value indicates relative quantification.

17A$^+$ cells were CD45$^+$ and CD3^{int} (CD3-intermediate) or CD3$^+$, which indicates they were of T-cell origin (Figure 2A and 2B). The percentage of T cells within the atherosclerosis-prone aortas consisted of 35% to 40% from the leukocyte population.[3] Here, we demonstrated that approximately 5% to 7% of those cells in aortas were IL-17A–producing T cells. Further characterization of the IL-17A$^+$ T cells revealed the presence of IL-17A–producing $\gamma\delta^+$ T cells and CD4$^+$ (Th17) cells in the aorta and spleen of both *Apoe*$^{-/-}$ and *C57BL/6* mice (Figure 2C, 2D, and 2E). Together, these data demonstrate a large elevation of IL-17A–producing T cells under atherosclerosis-prone conditions and suggest that both Th17 and $\gamma\delta^+$ T cells may be responsible for the elevated levels of IL-17A seen systemically in *Apoe*$^{-/-}$ mice. Large numbers of CD3$^+$IL-17A$^+$ leukocytes were also present in the aortas and spleen of *Apoe*$^{-/-}$ mice fed a Western diet for 15 weeks compared with CD3$^+$IL-17A$^+$ cells in *C57BL/6* mice (Figure 2B), although the number of IL-17A$^+$ T cells was not significantly different in *Apoe*$^{-/-}$ mice fed a chow or Western diet, which suggests that the atherosclerosis-prone background, rather than diet, is responsible for the induction of IL-17A$^+$ cells in vivo. We confirmed these data by reverse-transcription polymerase chain reaction, which also showed a significant increase in IL-17A mRNA expression in aortas isolated from *Apoe*$^{-/-}$ mice fed a Western diet for 15 weeks compared with *C57BL/6* aortas (data not shown).

There are several reports that have demonstrated lymphocyte accumulation in the aortic adventitia in normal[3] and atherosclerotic[3,18] vessels. To further analyze the location of IL-17A$^+$ T cells within the artery, we isolated the surrounding adventitia from the aortas (Figure 2F; online-only Data Supplement Figure III). Flow cytometry on the isolated adventitia and aortas from aged *Apoe*$^{-/-}$ mice showed that IL-17A$^+$ cells were present in both the adventitia and, to a lesser extent, the vessel wall (Figure 2G).

Blockade of IL-17A Diminishes Atherosclerosis in Ad-IL-17RA:Fc–Treated Apoe$^{-/-}$ Mice

To identify the role for IL-17A in atherosclerosis, we used a fusion protein composed of the IL-17RA extracellular domain fused with the murine IgG1 CH2 and CH3 domains (Ad-IL-17RA:Fc[19]) to block circulating IL-17A in Apoe$^{-/-}$ mice. To express IL-17RA:Fc in vivo, an E1-deleted recombinant adenovirus (Ad-IL-17RA:Fc) was generated.[19] In vivo, these adenoviruses are typically expressed in the liver, and soluble products are detected in the plasma of recipient mice.[20] Ad-IL-17RA:Fc significantly inhibits recombinant IL-17A–induced production of IL-6 by 3T3 fibroblasts.[19] Released sIL-17RA blocks the activities of IL-17A but not IL-17F and acts as a soluble receptor for IL-17A.[21] Importantly, Ad-IL-17RA:Fc does not induce leukocyte activation via surface Fc receptors (data not shown).

We experimentally developed a protocol that allows maintenance of the expression of Ad-IL-17RA:Fc for 15 weeks (online-only Data Supplement Figure II). To accelerate atherosclerosis development, mice were fed a Western diet.[15] To promote tolerance to the adenovirus and mini-

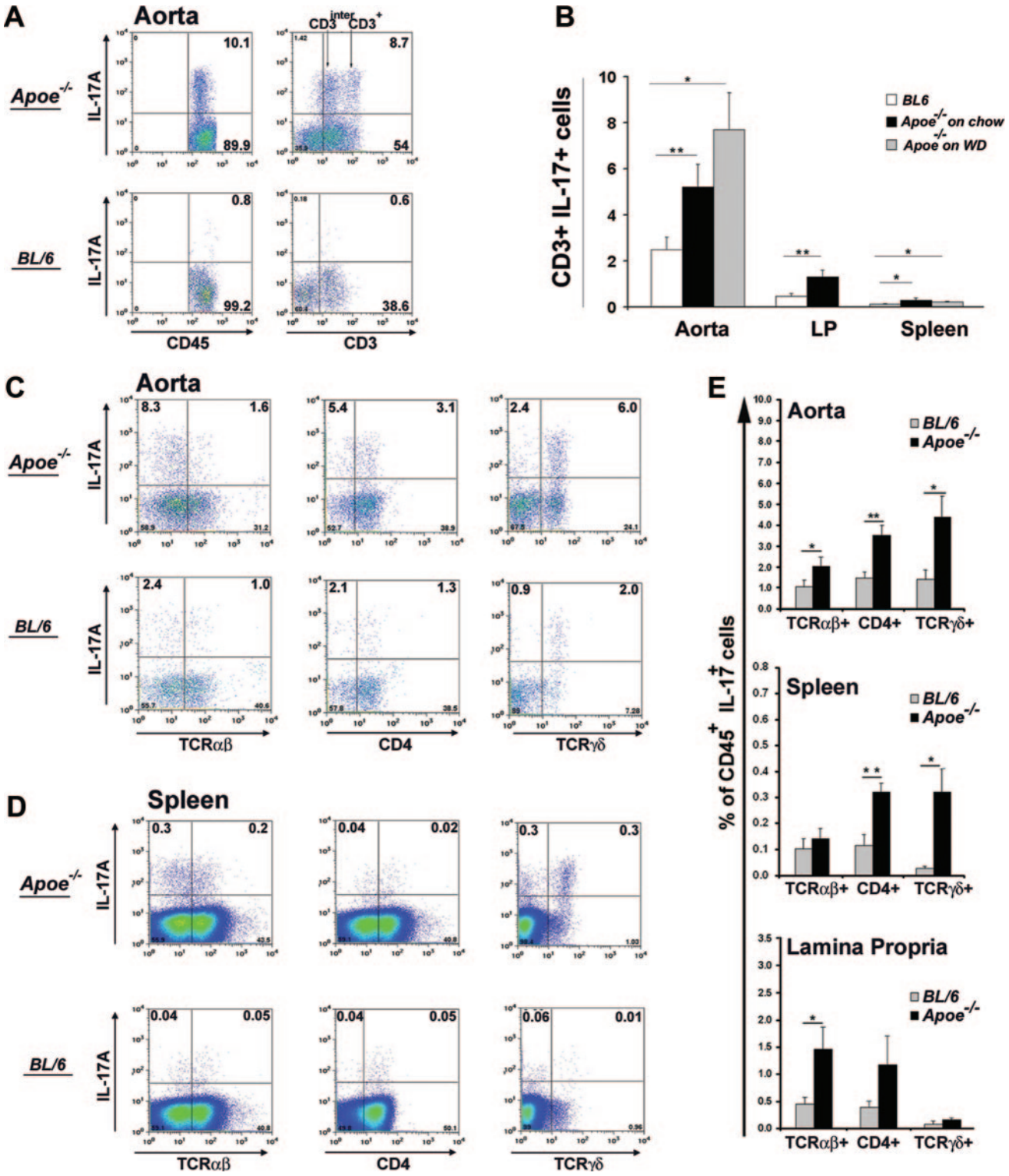

Figure 2. IL-17A$^+$CD3$^+$ and IL-17A$^+$CD3^{int} T cells were elevated in female and male *Apoe*$^{-/-}$ mice. To examine leukocyte population within the analyzed tissues, a gate was set on CD45$^+$ cells. A, Representative flow cytometry dot plot showing elevation of IL-17A$^+$CD45$^+$CD3$^+$ and IL-17A$^+$CD45$^+$CD3^{int} T cells in the aortas of aged *Apoe*$^{-/-}$ mice compared with *C57BL/6* mice. B, IL-17A$^+$CD3$^+$ T cells were elevated in the aortas, spleen, and lamina propria of aged *Apoe*$^{-/-}$ (black bars, n=12) mice and 21-week-old *Apoe*$^{-/-}$ mice fed a Western diet (WD; gray bars, n=4) compared with *C57BL/6* mice (white bars, n=10). *$P<0.05$, **$P<0.01$ by unpaired Student *t* test with Bonferroni-Holm correction for multiple comparisons (aorta and spleen). C and D, Representative flow cytometry dot plot of an aorta (C) and spleen (D) showing elevated IL-17A$^+$ cells. E, The phenotype of IL-17A-expressing cells in the aorta and spleen of *C57BL/6* (gray bars) and *Apoe*$^{-/-}$ (black bars) mice. Results show mean±SEM, n=6 mice from at least 3 independent experiments. F, Representative image of isolated murine aortic adventitia and aorta. G, Representative flow cytometry dot plot showing the presence of IL-17A$^+$ T cells in the adventitia and aortic wall of 45-week-old *Apoe*$^{-/-}$ mice. TCR indicates T-cell receptor; SSC, side scatter.

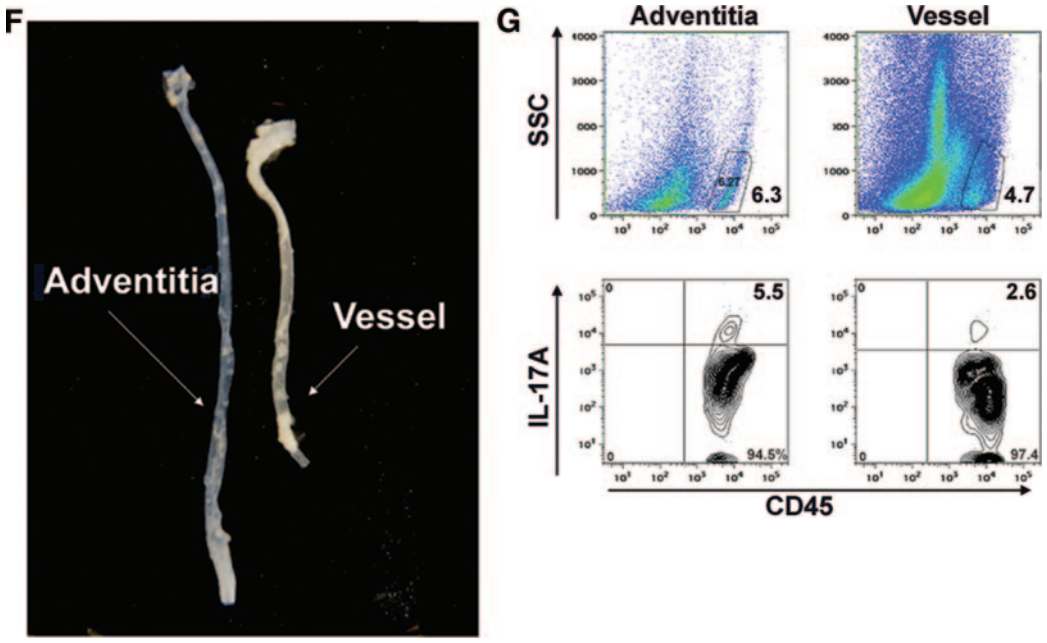

Figure 2 (Continued).

mize possible adenovirus-mediated immune responses, we performed retro-orbital intravenous injections of Ad-IL-17RA:Fc or Ad-Lu (which encodes firefly luciferase) in 4-day-old *Apoe*$^{-/-}$ mice. This was followed by booster injections of Ad-IL-17RA:Fc or Ad-Lu at days 42 and 104 (online-only Data Supplement Figure II). To track the expression of Ad-IL-17RA:Fc over time in vivo, we performed Western blots using plasma from Ad-IL-17RA:Fc and, as negative controls, plasma from Ad-Lu–injected mice (Figure 3A, lane 3) and IL-17RA–deficient (*Il-17ra*$^{-/-}$) mice (Figure 3A, lane 4). Figure 3A shows the detection of plasma sIL-17RA in 6-week-old *Apoe*$^{-/-}$ mice 7 days after the second injection of Ad-IL-17RA:Fc (Figure 3A, lanes 1 and 2). To confirm the expression of control Ad-Lu, we analyzed Ad-Lu expression by bioluminescence imaging (data not shown). sIL-17RA was also detectable in the plasma of Ad-IL-17RA:Fc–recipient mice on the day of termination (Figure 3B). To confirm that tolerance was maintained in the adenovirus-treated mice, we analyzed IFN-γ concentration in peripheral blood of recipient mice and found that plasma levels of IFN-γ did not reach more than $\approx$22 ng/mL in Ad-IL-17RA:Fc– and Ad-Lu–treated recipients (Figure 4B). These results suggest that an acute immune response did not occur in the adenovirus-recipient mice cohorts. *Apoe*$^{-/-}$ mice in both groups had comparable levels of triglycerides (186$\pm$28 and 190$\pm$40 mg/dL for Ad-IL-17R-Fc– and Ad-Lu–injected *Apoe*$^{-/-}$ mice, respectively). Interestingly, *Apoe*$^{-/-}$ mice that received Ad-IL-17RA:Fc showed an increase in total plasma cholesterol levels compared with *Apoe*$^{-/-}$ mice that received control Ad-Lu (591$\pm$61 and 350$\pm$40 mg/dL, respectively, $P<0.001$).

After the adenovirus treatments, the aortas of 21-week-old *Apoe*$^{-/-}$ mice were analyzed for plaque burden with en face Oil Red O staining. *Apoe*$^{-/-}$ mice that received Ad-IL-17RA:Fc developed on average 54% smaller lesions (5.7$\pm$0.4%) throughout the aorta compared with mice that received control Ad-Lu construct (11.0$\pm$0.8%; Figure 3C and 3D). The percentage of plaque area within the aortic roots was decreased by 18% in Ad-IL-17R:Fc–injected *Apoe*$^{-/-}$ mice compared with control Ad-Lu–injected *Apoe*$^{-/-}$ mice (45.8$\pm$2.1% and 54.5$\pm$1.4%, respectively; $P<0.05$). These results strongly implicate IL-17A as a proatherogenic cytokine in atherosclerosis.

To investigate the mechanisms by which the blockade of circulating IL-17A may lead to diminished atherosclerosis, we examined the circulating levels of key proinflammatory cytokines in the plasma of treated *Apoe*$^{-/-}$ mice. We found a significant decrease in IL-6 plasma levels in Ad-IL-17RA:Fc–treated compared with Ad-Lu–treated *Apoe*$^{-/-}$ mice (Figure 4A). Previous studies have shown that IL-17A drives the production of G-CSF, which is directly responsible for the differentiation and proliferation of neutrophil precursors in the bone marrow.[9] Therefore, we hypothesized that inhibition of IL-17A may lead to decreased levels of G-CSF in Ad-IL17-RA:Fc–treated mice. As shown in Figure 4C, we detected diminished levels of G-CSF in the plasma of Ad-IL17-RA:Fc–treated compared with Ad-Lu–treated *Apoe*$^{-/-}$ mice. These data confirm that sIL-17RA neutralized plasma IL-17A activity and as a consequence decreased G-CSF production, which in turn may have lowered blood neutrophil numbers. Ad-IL-17RA:Fc treatment did not affect circulating IL-17F plasma levels in *Apoe*$^{-/-}$ recipient mice compared with *Apoe*$^{-/-}$ mice that received Ad-Lu (Figure 4D).

IL-17A Regulates Macrophage Accumulation Within Aortas of Apoe$^{-/-}$ Mice via a CXCL1–Dependent Mechanism

IL-17A promotes inflammation by the induction of proinflammatory chemokines such as CCL2 and CXCL1, which

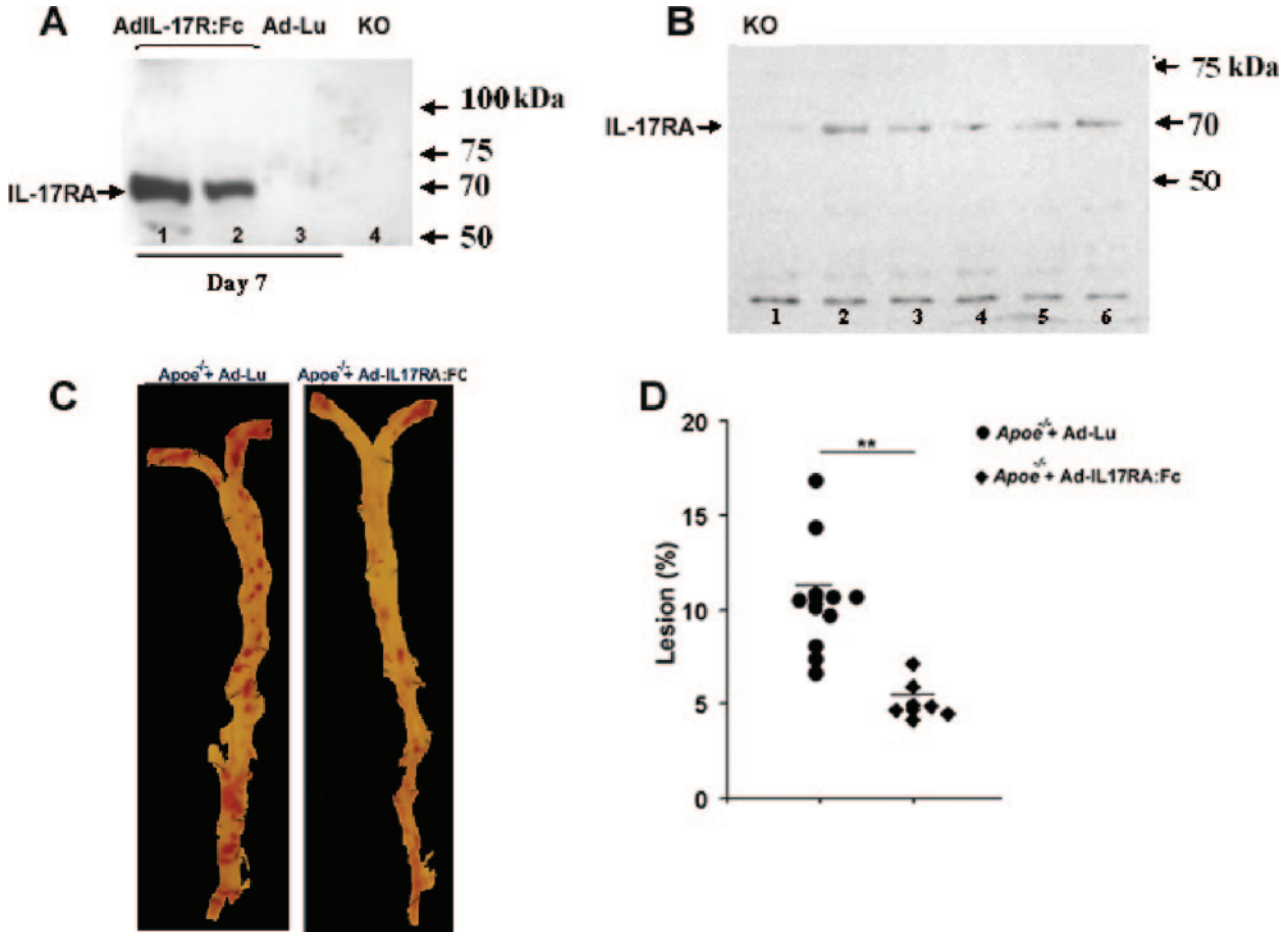

Figure 3. *Apoe*$^{-/-}$ mice treated with Ad-IL-17RA:Fc showed a reduction in plaque burden over the entire aorta. A, Detection of sIL-17RA in plasma of *Apoe*$^{-/-}$ mouse 7 days after Ad-IL-17RA:Fc (lanes 1 and 2) and Ad-Lu (lane 3) treatment by immunoprecipitation and Western blot; lane 4, knockout (KO; plasma from *Il17ra*$^{-/-}$ mice). B, Western blot demonstrating detection of sIL-17RA in plasma of *Apoe*$^{-/-}$ mice injected with Ad-IL-17RA:Fc on day of termination (21-week-old *Apoe*$^{-/-}$ mice). Lane 1, KO indicates negative control (*Il17ra*$^{-/-}$ plasma); lanes 2 to 6, experimental *Apoe*$^{-/-}$ mice. C, Representative en face staining of aortas excised from 21-week-old (15 weeks on Western diet) *Apoe*$^{-/-}$ mice treated with Ad-IL-17RA:Fc (right) or Ad-Luc (left). D, Lesion sizes for *Apoe*$^{-/-}$ mice treated with Ad-IL-17RA:Fc (n=7) or Ad-Luc (n=11; % of entire aorta). Each symbol represents 1 animal; horizontal bars represent means. $^{**}P<0.01$.

enables the recruitment of neutrophils and monocytes into inflamed tissue. Therefore, we examined the presence of CXCL1 and CCL2 in the aortas of Ad-IL-17RA:Fc–treated or Ad-Lu–treated mice. A significant reduction in CXCL1 expression but not CCL2 expression (not shown) was seen in aortic roots of Ad-IL-17RA:Fc–treated *Apoe*$^{-/-}$ mice compared with control Ad-Lu–treated *Apoe*$^{-/-}$ mice (Figure 5A and 5C). A significant decrease in Mac-2^{+} area staining for macrophages was also found within the aortic roots in Ad-IL-17RA:Fc–treated *Apoe*$^{-/-}$ mice (Figure 5B and 5D). The decrease in the total number of macrophages in Ad-IL-17RA:Fc–treated *Apoe*$^{-/-}$ mice was also detected when we evaluated the number of plaque macrophages (78±7 per 10^4 μm^2 and 285±10 per 10^4 μm^2 macrophages for Ad-IL-17RA:Fc–treated *Apoe*$^{-/-}$ and Ad-IL-17Lu–treated *Apoe*$^{-/-}$ mice, respectively). These results suggest that IL-17A–producing T cells might promote atherogenesis by inducing monocyte migration into the atherosclerotic lesion.

To further test the hypothesis that IL-17A is involved in the regulation of monocyte adhesion/migration, we performed ex vivo monocyte adhesion assays using IL-17A–treated or vehicle-treated isolated *Apoe*$^{-/-}$ aortas. We detected increased adhesion of CFSE-labeled monocytes to the IL-17A–treated luminal aortic wall in the presence of IL-17A compared with vehicle-treated aortas (Figure 5E and 5F). Interestingly, elevated monocyte adhesion was also detected when both monocytes and isolated aortas were treated with IL-17A (Figure 5E and 5F). To determine whether the differences in monocyte adhesion could be explained by the induction of CXCL1, we further analyzed the aortas for CXCL1 expression by reverse-transcription polymerase chain reaction and detected a significant increase in CXCL1 expression in both IL-17A–treated monocytes/aortas and IL-17A–treated aortas compared with vehicle-treated aortas (Figure 5G). Together, these results suggest that IL-17A can activate vascular cells, induce CXCL1 production, and further accelerate monocyte recruitment into the aortic wall. In addition to this effect, IL-17A elevates monocyte adhesion through activation of monocytes in peripheral circulation.

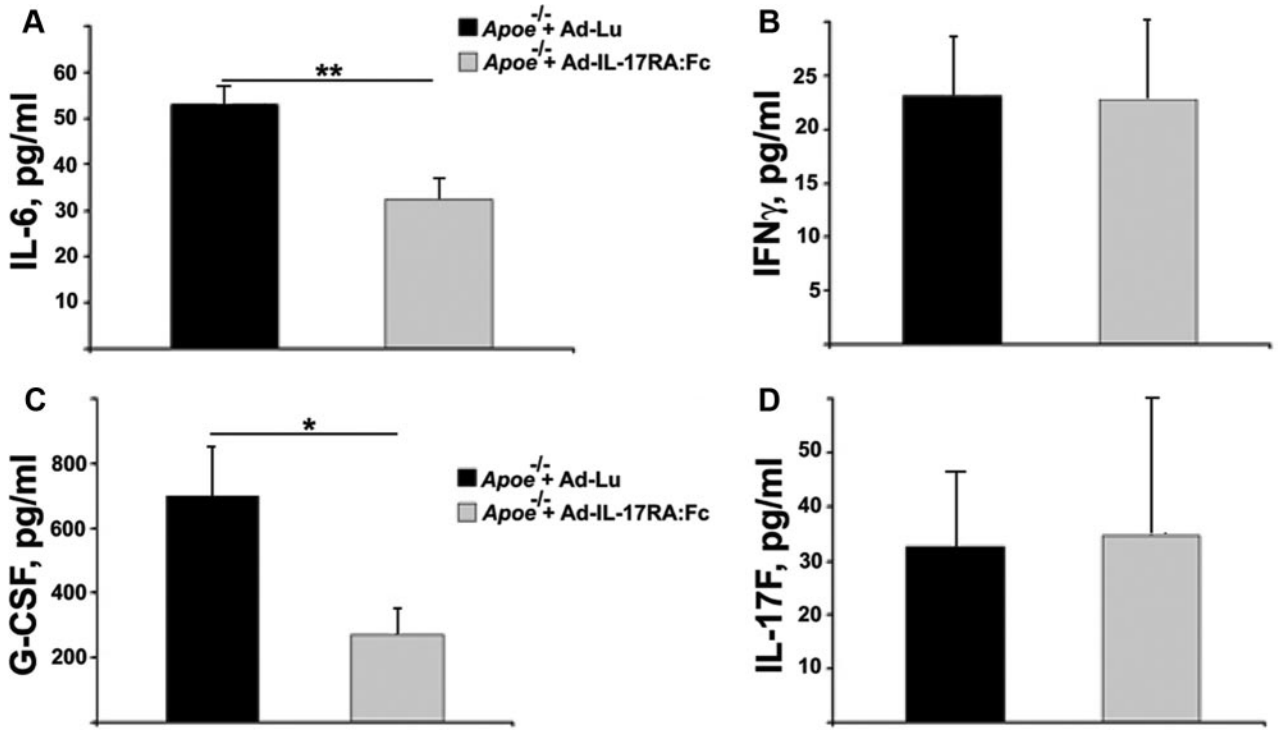

Figure 4. Decreased plasma levels of IL-6 and G-CSF in *Apoe*$^{-/-}$ mice treated with Ad-IL-17RA:Fc. Collected plasma supernatants from *Apoe*$^{-/-}$ mice treated with Ad-IL-17RA:Fc or with control Ad-Lu were analyzed for the presence of (A) IL-6, (B) IFN-γ, (C) G-CSF, and (D) IL-17F. Results show mean±SE from 5 to 10 mice. *$P<0.05$, **$P<0.01$.

Discussion

The present study reveals that IL-17A–producing T cells are present in the aortas of *C57BL/6* mice, and their numbers are greatly elevated under the atherosclerosis-prone conditions found in *Apoe*$^{-/-}$ mice fed either a chow or Western diet. Interestingly, not only Th17 but also γδ^{+} T cells are a source of IL-17A within atherosclerosis-prone aortas. The adenovirus-mediated blockade of IL-17A resulted in reduced plasma levels of IL-6 and G-CSF and diminished plaque burden, with a concomitant reduction of aortic macrophages and CXCL1. Moreover, IL-17A elevated CXCL1-dependent monocyte adhesion to isolated aortas ex vivo. Thus, we demonstrate a proatherogenic role for IL-17A in the development of atherosclerosis in mice.

The association of IL-17A with human autoimmune diseases has been shown extensively in patients with rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, and psoriasis.[8] A growing body of evidence also suggests that IL-17A might be involved in the immune response during atherosclerosis.[10,11,14,22] Soluble levels of IL-17A are elevated during the onset of acute coronary syndrome compared with stable angina[10] and in a subset of aged patients with coronary atherosclerosis and referent outpatients.[11] Recently, Xie et al[23] reported elevated levels of IL-17A in the plasma of *Apoe*$^{-/-}$ mice at 8 to 16 weeks of age. Here, we have confirmed and extended these results and demonstrated elevated levels of plasma IL-17A in 40- to 50-week-old *Apoe*$^{-/-}$ mice with a concomitant increase in IL-2, IL-6, and tumor necrosis factor-α. We also found that IL-17A was the most prevalent cytokine of the IL-17 family in *Apoe*$^{-/-}$ mice. Indeed, the number of IL-17A^{+} T cells was increased in the artery wall, spleen, and lamina propria of aged *Apoe*$^{-/-}$ mice, which suggests that systemic inflammation found in *Apoe*$^{-/-}$ mice may initiate the differentiation of IL-17A–producing T cells in secondary lymphoid and nonlymphoid tissues. Recent reports have indicated an important role of adventitia in atherogenesis.[1,24] Most CD3^{+} cells are located within the aortic adventitia of atherosclerosis-prone *Apoe*$^{-/-}$ mice.[3,18] The present study clearly identified IL-17A–producing T cells by flow cytometry and reverse-transcription polymerase chain reaction on preparations of total aorta that included artery wall and surrounding adventitia. Using flow cytometry on the aortic adventitia, we also demonstrated that IL-17A^{+} T cells are located in the adventitia and within the aortic intima of *Apoe*$^{-/-}$ mice. The roles of leukocytes that reside in different locations during atherogenesis are not well understood, and further studies will identify the specific role of IL-17A^{+} cells in these 2 different sites.

Recently, a study by Eid et al[11] showed that CD4^{+} cells isolated from human atherosclerotic coronary arteries produce IL-17A and IFN-γ in response to polyclonal stimulation in vitro. In the present report, we further examined the phenotype of IL-17A^{+} cells and demonstrated that not only Th17 cells but also IL-17A^{+}γδ^{+} T cells were significantly elevated in atherosclerosis-prone murine aortas. The mechanisms behind human and murine Th17 cell differentiation are well established in vitro; however, little information is available about the induction of IL-17A^{+} cells during chronic inflammation in vivo. SOCS-3 is involved in the preferential promotion of Th17 cell induction.[25] Interestingly, we found no significant changes in SOCS-3 expression between the aortas of *C57BL/6* and *Apoe*$^{-/-}$ mice. These preliminary

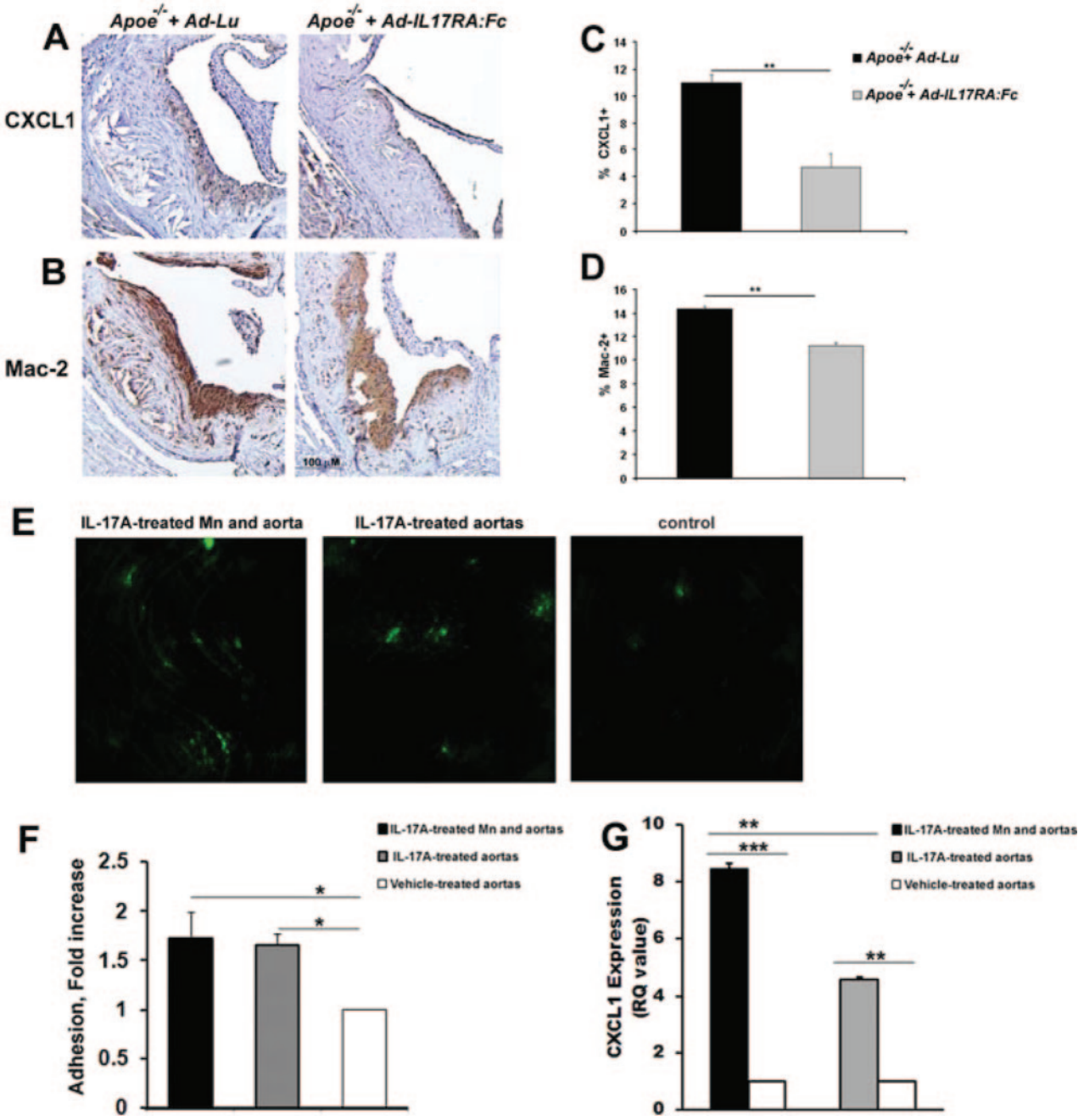

Figure 5. IL-17A increases monocyte adhesion to aortic wall via CXCL1. A, Paraffin-embedded sections of aortic roots from 21-week-old *Apoe*$^{-/-}$ mice that received either Ad-IL-17RA:Fc or Ad-Lu adenoviruses were stained with antibodies against CXCL1 (A; brown) and Mac-2 (B; brown) and counterstained with hematoxylin (blue). Representative staining from 1 mouse is shown. Percentage of (C) CXCL1- and (D) Mac-2–positive staining relative to total area of aortic root. Values represent mean±SEM of 5 to 10 mice per group. E, IL-17A–treated aortas show increased monocyte adhesion. Aortas were harvested from 25- to 30-week-old *Apoe*$^{-/-}$ mice and treated with IL-17A as described in Methods. Fluorescently labeled monocytes (green) were added to aortas for an adhesion assay, and adherent monocytes were counted by blinded observers using a fluorescent microscope. F, Data represent mean±SE of 3 to 4 counted aortas per group from 4 independent experiments. G, Aortic mRNA expression. Total cellular RNA from vehicle-treated (white bars), IL-17A–treated (gray bars), and unwashed IL-17A–treated (black bars) aortas were examined for CXCL1 by quantitative reverse-transcription polymerase chain reaction. *$P<0.05$, **$P<0.01$, ***$P<0.001$ by Student *t* test with Bonferroni-Holm correction for multiple comparison (for Figure 5F and 5G). Data are from 3 independent experiments using 3 to 4 aortas per group. RQ value indicates relative quantification; Mn, monocyte.

findings suggest that IL-17A is likely upregulated independently of SOCS-3 in atherosclerosis-prone conditions. Further studies are necessary to determine the mechanisms behind IL-17A induction in atherosclerosis. Recently, CD27 was identified as a thymic determinant of the balance between IFN-γ and IL-17A–producing $\gamma\delta^+$ T-cell subsets.[26] $\gamma\delta^+$ T cells also express ROR$\gamma\tau$ and produce IL-17A, IL-21, and IL-22 in response to IL-1β and IL-23, without T-cell receptor engagement.[27] The existence of an expanded IL-17A$^+\gamma\delta^+$ T-cell population in *Apoe*$^{-/-}$ mice suggests at least

partial T-cell receptor–independent upregulation of IL-17A during atherosclerosis. Further studies will be necessary to investigate the mechanisms of induction of IL-17A$^+$ T cells during atherogenesis.

There are several pathways by which IL-17A might affect local inflammation. IL-17A induces the production of cytokines and chemokines in several cell types, including endothelial and vascular smooth muscle cells.[28,29] IL-17A and IFN-γ synergistically initiate production of IL-6, CXCL8, CCL5, CXCL1, and CXCL10 in cultured vascular smooth muscle cells.[11] CXCL1 is a chemokine that triggers monocyte arrest on endothelium during atherogenesis and thus plays a critical role in monocyte recruitment into artery wall.[30] Monocytes express IL-17RA on their surface, and recent data suggest that IL-17A can also directly affect monocyte chemotaxis in vivo and in vitro.[31,32] Antibody blockade of IL-17A in the synovial fluid of rheumatoid arthritis patients inhibits monocyte migration in vitro, as well as macrophage accumulation in the bronchoalveolar lavage fluid during allergic airway inflammation.[31,32]

The role of IL-17A and IL-17A–expressing T cells in atherosclerosis remains unclear. Several studies with conflicting results have been published, and more research on the role of the IL-17A–dependent response in atherosclerosis is warranted. The present study for the first time characterizes the subpopulations of T cells that express IL-17A in atherosclerosis and highlights a possible role of IL-17A in the regulation of monocyte migration into aortas. We report that blockade of IL-17A in vivo leads to decreased CXCL1 expression within atherosclerosis-prone aortas and diminished aortic macrophage content. We also demonstrate the proinflammatory role of IL-17A in the regulation of monocyte migration into the aortas via ex vivo monocyte adhesion assay. Together, these results indicate that IL-17A contributes to the pathogenesis of atherosclerosis at least through the regulation of monocyte recruitment to the aortic wall via CXCL1 expression on the vessel lumen. It remains to be determined whether IL-17A also activates monocytes/macrophages within the plaque to induce the synthesis of tumor necrosis factor-α, IL-1β, IL-6, CXCL2, and granulocyte macrophage colony-stimulating factor and further perpetuate inflammation.

IL-17A regulates levels of G-CSF in circulation and thus affects neutrophil numbers in the blood. We detected decreased levels of G-CSF in the plasma of *Apoe*$^{-/-}$ mice that received Ad-IL-17RA:Fc. It would be important to investigate the role of IL-17A–dependent involvement of neutrophils in atherogenesis. To further understand the effect of the cytokine profile on blockade of IL-17A, we also analyzed plasma levels of IL-17F, a cytokine that can compensate for a deficiency in IL-17A in *Il-17a*$^{-/-}$ mice.[33] We found no difference between Ad-IL-17RA:Fc– and Ad-Lu–treated groups of mice, which suggests that neutralization of IL-17A is not enough to induce compensatory elevation of IL-17F in *Apoe*$^{-/-}$ mice.

Regulation of Th17 cell differentiation by IFN-γ has been shown in vitro,[8] and implicates the Th1 cytokines as potent inhibitors of Th17 cell differentiation; however, a population of cells that secrete both IFN-γ and IL-17A can be detected among cells isolated from inflammatory conditions in vivo.[34–36] To date, it is unclear whether IL-17A can regulate the Th1 response in vivo. The results of the present study demonstrate that blockade of IL-17A with sIL-17RA has no effect on levels of plasma IFN-γ in *Apoe*$^{-/-}$ mice. Further studies will be necessary to explore the complex relationship between Th1 and Th17 cell differentiation programs in vivo.

In summary, the discovery of IL-17A–producing Th17 and $\gamma\delta^+$ T cells and their proatherogenic properties challenges the Th1 and Th2 paradigm thought to be involved in the adaptive immune response of cardiovascular disease. The present findings suggest a new IL-17A–dependent pathway by which the immune system may influence the development and progression of atherosclerosis.

Acknowledgments

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Disclosures

None.

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CLINICAL PERSPECTIVE

Atherosclerotic lesion progression depends on chronic inflammation within the artery wall, and T cells are involved in the immune response that accompanies atherogenesis. Interleukin (IL)-17A is a recently discovered cytokine that plays a protective role in host defenses against extracellular pathogens and a pathogenic role in several autoimmune diseases, including multiple sclerosis, inflammatory bowel disease, and arthritis. In the present study, we showed that plasma levels of IL-17A and aortic IL-17A–producing $\gamma\delta^+$ and $CD4^+$ (Th17) T cells were significantly elevated in the atherosclerosis-prone conditions found in apolipoprotein E–deficient ($Apoe^{-/-}$) mice. We confirmed a proatherogenic role of IL-17A using adenovirus-delivered soluble IL-17 receptor against IL-17A, which caused a significant decrease in plasma levels of IL-6 and granulocyte colony-stimulating factor, diminished aortic macrophage content and CXCL1 expression, and led to a reduction in plaque burden in treated $Apoe^{-/-}$ mice. Conversely, the treatment of isolated $Apoe^{-/-}$ aortas with recombinant IL-17A increased CXCL1 expression and monocyte adhesion to vessel wall. Our findings highlight a proatherogenic role for IL-17A in coronary atherosclerosis and suggest that future therapies targeting IL-17A could potentially reduce vascular wall infiltrates and lesion size and attenuate atherosclerosis and other forms of vascular disease.

Online supplemental materials.

Recombinant Proteins and Antibodies. Abs used were as follows (all from BD-Pharmingen, San Diego, CA): Per-CP or PE-Texas Red-CD45, PE-IL-17A, Pacific blue-CD3ε, APC-Cy7-CD4, FITC or APC-TCR β chain, biotinylated or FITC-γδ TCR, LIVE/DEAD® Aqua Dead Cell Stain Kit (Invitrogen) and CD16/CD32 (The Lymphocyte Culture Centre**,** UVA).

Flow cytometry. Single cell suspensions from the aorta and surrounding adventitia were prepared as previously described.[1] Briefly, mice were anesthetized, and their vasculature was perfused by cardiac puncture with phosphate-buffer saline (PBS) containing 20 units/ml of heparin. Harvested aortas were microdissected and digested with 125 U/ml collagenase type XI, 60 U/ml hyaluronidase type I-s, 60 U/ml DNAse1 and 450 U/ml collagenase type I (all enzymes Sigma, St. Louis, MO) in PBS at 37^{o}C for 1 hour.[1] Single cell suspensions from spleen, mesenteric lymph nodes (MLN), peripheral LN (PLN), lamina propria (LP)[2] and aortas were treated with 10 ng/ml PMA, 500 ng/ml calcium ionophore and GolgiStop for 5 h. Intracellular staining for IL-17A and CD4 was performed using Fix&Perm® cell permeabilization reagents. In some experiments, 1-2 aortas were pooled for the intracellular staining. To determine the location of IL-17-expressing cells, harvested aortas with surrounding adventitia were digested with 312.5 U/ml collagenaseII and 5.625 U/ml elastase (Worthington Biochemical Corp., Lakewood, NJ) for 1 hr at 37°C, and adventitia was carefully removed. Aortas and the adventitia were further digested separately for 30 min at 37°C. Cell suspensions from pooled 3-4 aortas were treated as described above. Flow cytometry analysis was performed on a FACSCalibur^{TM} or CyanADP^{TM}, data analyzed using FlowJO (Tree Star Inc., Ashland, OR) software. Gates were set by isotype controls or fluorescent minus one control.

Preparation of Mouse Aortas and histochemistry. Aortas were excised as previously described[1] and stained with Oil Red O. Images were scanned, and the percent of surface areas occupied by lesions were determined with Image-ProPlus (Media Cybernetics, Inc). For immunohistochemistry, mice were perfused by cardiac puncture with 4% PFA and hearts were collected. Sequential 5 μm thick sections of the aortic root were cut from the point of the appearance of aortic valve leaflets. Tissues were stained with Abs against Mac-2 (Cedarlane Lab) and CXCL1 (R&D Systems) with avidin-biotin technology. Images were scanned and analyzed by Image-ProPlus. The percent of the surface area occupied by the staining was determined as a ratio of positively stained area to the total cross-sectional area of the aortic root. Alternatively, the surface area of the plaque and the total number of Mac-2^+ macrophages within the plaques was determined. The total numbers of macrophages were then normalized to the total area of the plaques to determine the number of macrophages per $10^4 \mu m^2$.

mRNA quantification. To analyze IL-17A expression, RNA was extracted from the aorta, small intestines, spleen, MLN and PLN following homogenization in Trizol® (Invitrogen), according to manufacturer's instructions. Reverse transcription and PCR steps were performed using QuantiTect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA). 1 μg of total RNA was used for all tissues. Values were determined using iCycler iQ Real-Time Detection System Software (Qiagen, Valencia, CA). The corresponding values were normalized to Ribosomal RNA control reagent and then normalized to individual *C57BL/6* mouse organs as the calibrator control (always equal to 1), thereby expressing the values as relative quantification (RQ) values. Alternatively, to determine the expression of the different members of IL-17 family, mouse Th17 PCR profiler array (SABioscience) was used according to the manufacturer's instructions.

Adenovirus transfer and western blotting. To test the role for IL-17A in atherosclerosis, *Apoe*$^{-/-}$ mice were injected retro-orbitally with $0.5x10^9$ PFU of Ad-IL-17RA:Fc[3] at 4 day old, then with $1x10^9$ PFU of Ad-IL-17RA:Fc at 52 and 104 day (Supplemental Fig.2). As a control, Ad-Lu[3] was injected into the control group of *Apoe*$^{-/-}$ mice at the same time points. *Apoe*$^{-/-}$ mice were fed chow diet for 6 weeks and then WD for 15 weeks. Immunoprecipitation was carried out by pre-absorbing rat monoclonal anti-mouse IL-17RA Ab (R &D systems) to protein A/G agarose beads (PIERCE). Plasma soluble IL17-RA was detected by western blot analysis using rabbit anti mouse IL-17RA Ab (Santa Cruz Biotechnology, Inc, CA) followed by incubation with HRP conjugated anti-rabbit IgG (Zymed, Invitrogen). After 5 minute incubation with super signal west pico chemiluminescent substrate (PIERCE, IL) membranes were exposed to film for detection of the soluble IL-17RA protein.

Measurements of cytokines and chemokines. Mouse IL-17A was measured using mouse multi-cytokine detection kit (Millipore, MA). Plasma IFN-γ and IL-6 were measured by BD Cytometric Bead Array (BD Pharmingen, CA). G-CSF and IL-17F were quantified by ELISA (R&D Systems and eBioscience, respectively).

Measurements of plasma lipids. Plasma triglyceride and total cholesterol levels were determined via an automated enzymatic technique (Boehringer Mannheim GmbH, CA).

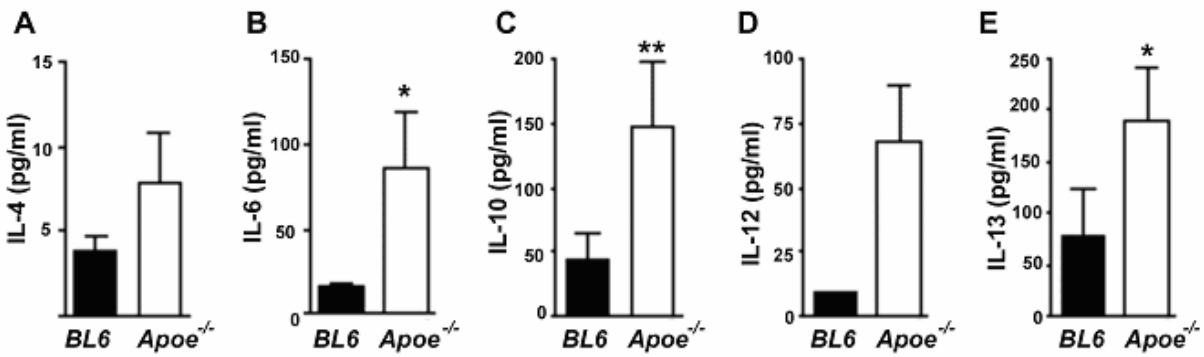
Ex vivo monocyte adhesion assay. Assay was performed as described previously[4]. Briefly, aortas were isolated from 27-30 week old *Apoe*$^{-/-}$ mice, and incubated overnight in RPMI-1640

media containing 10% FBS with or without 10ng/ml of rIL-17A (R&D Systems). Next, aortas were washed either with media or kept with media containing IL-17A, opened longitudinally and pinned to sterile agar. Monocytes were isolated from peripheral blood by positive selection with anti-CD115-bio Abs and streptavidin-specific microbeads (Miltenyi Biotec) and labeled with 2 μM carboxyfluorescein diacetate-succinimidyl ester (CFSE) as previously described.[1] CFSE-labeled monocytes were incubated with the pinned aortas, and 1 hour later, unbound monocytes were washed away with PBS. The number of monocytes adherent to the aorta was counted using fluorescent microscopy. To determine the expression of CXCL-1 in IL-17A-treated aortas, we isolated total RNA and performed real time PCR using CXCL1 and Beta-Actin Taqman primers (Applied Biosystems, CA) as described above.

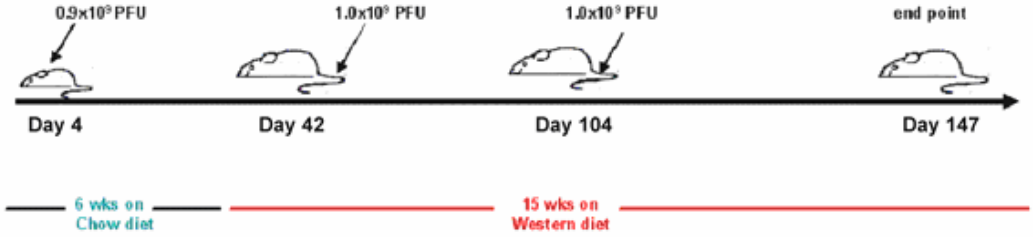
Supplemental Figure S1: Cytokine levels are elevated in *Apoe*$^{-/-}$ mice. Serum levels of (A) IL-4, (B) IL-5, (C) IL-10, (D) IL-12 and (E) IL-15 were analyzed in *C57BL/6* and *Apoe*$^{-/-}$ mice. *P<0.05, **P<0.01 by unpaired Students t test.

Supplemental Figure S2: Scheme of the injection of Ad-IL-17RA:Fc or control Ad-Lu into *Apoe*$^{-/-}$ recipient mice. *Apoe*$^{-/-}$ mice were injected retro-orbitally with $0.5x10^9$ PFU of Ad-IL-17RA:Fc at 4 days-old, then with $1x10^9$ PFU of Ad-IL-17RA:Fc at 52 and 104 days. As a control, Ad-Luc was injected into the control group of *Apoe*$^{-/-}$ mice at the same time points. *Apoe*$^{-/-}$ mice were fed chow diet for 6 weeks and then WD for 15 weeks.

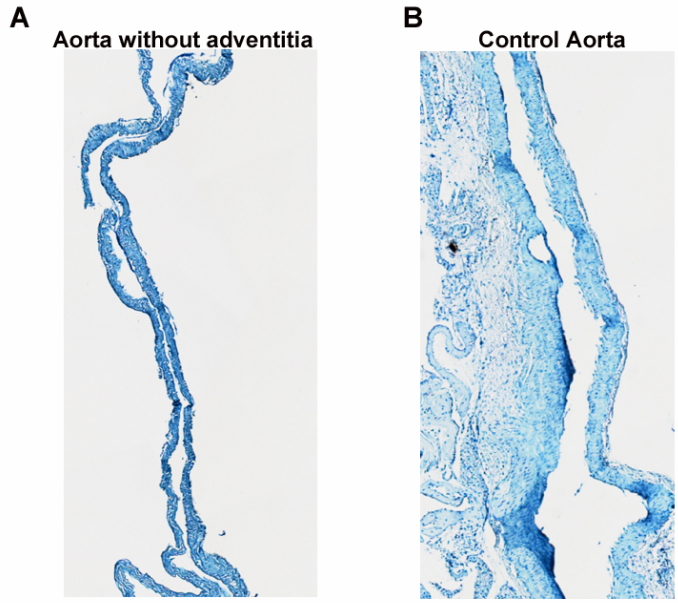
Supplemental Fig.S3: Isolation murine aorta and adventitia. (A) Harvested *Apoe*$^{-/-}$ aortas with surrounding adventitia were digested with 312.5 U/ml collagenaseII and 5.625 U/ml elastase for 1 hr at 37°C, and adventitia was carefully removed. (B) Control aortas with surrounding adventitia were harvested from *Apoe*$^{-/-}$ mice. Longitudinal 5 µm thick sections of the aortas were cut and tissues were stained with hematoxylin.



Supplemental Fig.1S



Supplemental Fig.2S



Supplemental Fig.3S

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