Smooth Muscle Cell–Derived Interleukin-17C Plays an Atherogenic Role via the Recruitment of Proinflammatory Interleukin-17A+ T Cells to the Aorta

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Objective—Atherosclerosis is characterized by frequent communication between infiltrating leukocytes and vascular cells, through chemokine and cytokine networks. Interleukin-17C (IL-17C) is detectable within atherosclerotic lesions; however, the potential involvement of this cytokine has not been examined. Thus, we sought to investigate the role of IL-17C in atherosclerosis.

Approach and Results—The expression of IL-17 cytokines was profiled within aortas of apolipoprotein E double knockout (Apoe−/−) mice, and Il17c expression was elevated. Flow cytometry experiments revealed a major population of aortic IL-17C−producing smooth muscle cells. Next, we generated Il17c−/−Apoe−/− mice and demonstrated that atherosclerotic lesion and collagen content was diminished within Western diet–fed Il17c−/−Apoe−/− aortas and aortic roots in comparison to Apoe−/− controls. Smooth muscle cells and fibroblasts were mainly responsible for the reduced Col1A1 expression in the aorta of Il17c−/−Apoe−/− mice. Importantly, IL-17C–treated Apoe−/− aortas showed upregulated Col1A1 expression ex vivo. Il17c−/−Apoe−/− mice displayed a proportional reduction in aortic macrophages, neutrophils, T cells, T helper 1 cells, and T regulatory cells, without corresponding changes in the peripheral immune composition. Examination of aortic IL-17A+ T-cell receptor γδ T cells and Th17 cells demonstrated a stark reduction in the percentage and number of these subsets within Il17c−/−Apoe−/− versus Apoe−/− mice. Explanted 12-week Western diet–fed Apoe−/− aortas treated with IL-17C resulted in the induction of multiple vascular chemokines and cytokines. Th17 cells demonstrated attenuated migration toward supernatants from cultures of Il17c−/−Apoe−/− smooth muscle cells, and short-term homing experiments revealed diminished recruitment of Th17 cells to the aorta of Il17c−/−Apoe−/− recipients.

Conclusions—Smooth muscle cell–derived IL-17C plays a proatherogenic role by supporting the recruitment of Th17 cells to atherosclerotic lesions. (Arterioscler Thromb Vasc Biol. 2016;36:1496-1506. DOI: 10.1161/ATVBAHA.116.307892.)

Key Words: atherosclerosis ▪ cytokines ▪ inflammation ▪ leukocytes ▪ smooth muscle cells

Cardiovascular diseases are the leading cause of global deaths, and atherosclerosis-associated cardiovascular diseases were responsible for 13.5 million out of 17.3 million cardiovascular diseases worldwide in 2008.1 The formation of atherosclerotic plaques is a chronic arterial inflammatory process that is characterized not only by the formation of lipid-rich plaques2 but also by the regular communication between arterial hematopoietic cells and vascular cells through cytokine and chemokine networks. T cells, as a part of the adaptive immune response, actively participate in regulating local and systemic inflammation during atherogenesis. In this respect, the pro- and antiatherogenic roles of interferon γ (IFNγ)+ T helper 1 (Th1) and T regulatory (Treg) cells are well established. However, the roles of interleukin-17A (IL-17A)+ T cells, including T helper 17 cells (Th17) and IL-17A+ T-cell receptor (TCR) γδ T cells, have been controversial, because of conflicting results on the functions of IL-17A in atherosclerosis.3–8

The IL-17 cytokine family consists of 6 family members, (IL-17A–IL-17F), which vary in their distribution, biological roles, and sequence homology.9,10 The roles and biological functions of IL-17A– and IL-17F–producing cells in inflammation have been well studied. However, the functions of other members of the IL-17 family (IL-17B-D) were unclear until recently.9 Recent work on the biology of IL-17C has revealed that IL-17C signals through a heteromeric receptor consisting of the interleukin-17 receptor E (IL-17RE) subunit and the common interleukin-17 receptor A (IL-17RA) subunit,1,1,12 which is known to be shared with IL-17A, IL-17F, and IL-17B. IL-17C signaling results in the induction of chemokines and proinflammatory cytokines, including Cxcl1, Cxcl2, Ccl20, Tnf, and Il1b within IL-17Aα− and IL-17RE− expressing colonic epithelial cells and keratinocytes.12,13 Th17 cells that express IL-17RA and IL-17RE upregulate IL-17A, IL-17F, and IL-22 production in response to IL-17C.11

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in the mouse, which suggests that the receptor complex for IL-17C signaling is present within the aorta.

Because of the plethora of types of cells that express IL-17RA and IL-17RE, IL-17C is likely to play pleiotropic roles in host defense, autoimmune, or inflammatory pathologies.11,14,15 IL-17C is expressed by colonic epithelial cells and plays key roles in host survival by maintaining mucosal barrier integrity in a Citrobacter rodentium infection model12 and dextran sulfate sodium–induced colitis models.13,16 IL-17C is expressed within psoriatic plaques.13,14,17 Similarly, IL-17C is required for the development of myelin oligodendrocyte glycoprotein–induced experimental autoimmune encephalomyelitis. In the context of atherosclerosis, recent work from our group demonstrated that several IL-17 cytokines are present within atherosclerotic apolipoprotein E double knockout (Apoe−/−) aortas,14 suggesting that the receptor complex might play a role in Th1 differentiation or maintenance. Importantly, IL-17RA and IL-17RE are expressed within Apoe−/− aortas,4 suggesting that the receptor complex for IL-17C signaling is present within the aorta.

In addition to IL-17C, IL-17A is an important proinflammatory cytokine that is required to efficiently control bacterial and fungal infections, and also participates in major autoimmune diseases.9,19 Several studies have reported elevated levels of Th17 and IL-17A+ TCRγδ T cells within atherosclerotic Apoe−/− and Ldlr−/− mice,18,20–22 in patients with coronary artery disease and in endarterectomy patients with vulnerable plaques.3,23,24 However, the precise role(s) that IL-17A plays have been disputed.3–8 Mechanistic studies in murine models of atherosclerosis have yielded at least 2 unifying hypotheses, that IL-17A plays a proatherogenic role by affecting aortic chemokine and cytokine production and myeloid cell recruitment3,4,6,8,18,20,22,25 or an atheroprotective role via regulation of aortic Th1 content, smooth muscle cell (SMC) content, and collagen deposition.5,21,26

In this study, we hypothesized that in addition to IL-17A, other IL-17 family members might participate in the pathobiology of atherosclerosis. We report here that aortic IL-17C is elevated within atherosclerotic Apoe−/− mice in comparison to nonatherosclerotic C57Bl6 controls. Aortic SMCs are, unexpectedly, prominent sources of arterial IL-17C within Apoe−/− and C57Bl6 mice. To study the role of IL-17C in atherosclerosis, Il17c−/−Apoe−/− mice were generated. Deficiency of IL-17C resulted in moderate reductions in atherosclerotic lesions, lesional collagen content, aortic leukocyte, macrophage, neutrophil, Th1 and Treg cells, and robustly reduced aortic Th17 and IL-17A+TCRγδ T cell content, because of defective IL-17C–mediated IL-17A+ T-cell recruitment to the aorta. Importantly, these data underscore the significance of communication between aortic IL-17C+ SMCs and arterial IL-17A–producing T cells to efficiently promote atherogenesis.

Materials and Methods

Animals

Il17c−/− mice (kindly provided by Amgen, Inc) on the C57Bl6 background were crossed with Apoe−/− mice to generate Il17c−/− Apoe−/− mice. Six-week-old male and female Apoe−/− and Il17c−/− Apoe−/− mice were placed on a high-fat Western diet (21% fat and 0.15% cholesterol, TD.88137; Harlan Laboratories, Indianapolis, IN) for 12 weeks and used at 18 weeks of age. For en face preparations, Apoe−/− and Il17c−/−Apoe−/− littersmates from Il17c−/−Apoe−/− breeders were used. Dedicated Il17c−/−Apoe−/− and Il17c−/+Apoe−/− breeders were used subsequently because atherosclerotic lesions were equivalent between Apoe−/− mice from Il17c−/−Apoe−/− and Apoe−/− breeders. For the in vitro chemotaxis assays, 40-week-old Il17a+cre/+R26R tdTomato albumin Apoe−/− mice were used. All animals were kept in specific-pathogen-free conditions, and animal experiments were approved by the Eastern Virginia Medical School Animal Care and Use Committee. Unless noted otherwise, all surgical procedures and organ collections were collected aseptically to prevent potential bacterial or fungal contaminants from affecting the results. An expanded Materials and Methods section is available in the online-only Data Supplement.

Results

IL-17C Is Elevated in Atherosclerotic Apoe−/− Aortas and Is Produced Primarily by Aortic Smooth Muscle Cells

Although several recent studies have examined the role(s) of IL-17A and IL-17F in atherosclerosis, the potential roles of other IL-17 family members have not been explored. To reconfirm18 that IL-17 cytokine family members are present during atherosclerosis, we initially examined the expression of the IL-17 cytokines in 40-week-old Apoe−/− and C57Bl6 aortas. For all experiments presented in this study, the aortas were cleaned of all periaortic adipose tissue but contained the aortic adventitia. The aortas with surrounding adventitia were used for flow cytometric analysis or reverse transcription-polymerase chain reaction (RT-PCR). Several IL-17 cytokines were present and elevated within aged atherosclerotic Apoe−/− aortas, including Il17a, Il17c, and Il17f (Figure 1A). To confirm these results and to determine whether IL-17C expression is altered during atherogenesis, we examined the aortas isolated from 12-week chow diet–fed C57Bl6 and Apoe−/− mice, and the aortas from 12-week WD-fed Apoe−/− mice for IL-17C expression (Figure 1B). IL-17C expression was elevated in chow diet–fed atherosclerotic Apoe−/− aortas and was further enhanced in 12-week WD-fed Apoe−/− mice (Figure 1B). Next, to determine the sources of aortic IL-17C, we examined 12-week WD-fed Apoe−/− aortas for IL-17C expression by flow cytometry.

### Nonstandard Abbreviations and Acronyms

- **Apoe**: apolipoprotein E
- **CD**: cluster of differentiation
- **IL-17A**: interleukin-17A
- **IL-17C**: interleukin-17C
- **IL-17RA**: interleukin-17 receptor A
- **IL-17RE**: interleukin-17 receptor E
- **Ldlr**: low-density lipoprotein receptor
- **PLN**: peripheral lymph node
- **SMC**: smooth muscle cells
- **TCR**: T-cell receptor
- **Th**: T helper
- **Treg**: T regulatory
- **WD**: Western diet

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Aortic Il17c is elevated in Apo−/− mice and interleukin-17C (IL-17C)+ smooth muscle cells (SMCs) and CD45−CD31−αSMA−CD29− vascular cells are the sources of aortic IL-17C within Apo−/− mice. A, mRNA expression of IL-17 cytokine family members. Forty-wk-old C57Bl6 and Apo−/− aortas with aortic adventitia were steriley collected, processed for reverse transcription-polymerase chain reaction, and assessed for Il17a, Il17c, Il17e, and Il17f expression. n=6 mice/genotype, 3 independent experiments. B, mRNA expression of Il17c in steriley collected 12-wk chow diet–fed C57Bl6 mice, Apo−/− mice, and 12-wk Western diet (WD)–fed Apo−/− aortas. n=6 mice/genotype, 3 independent experiments. C, Expression of Il17c in whole Apo−/− aortas (blue bars) and magnetically sorted Apo−/− aortic leukocytes (CD45+, teal bar), Apo−/− vascular cells (CD45− cells, pink bar), and fluorescence activated cell sorting–sorted Apo−/− CD45−CD31−αSMA−CD29− SMCs (red bar). n=12 Apo−/− mice, 3 independent experiments. D, Aortic cell suspensions from 40-wk-old C57Bl6 mice, Apo−/− mice, and 12-wk WD–fed Apo−/− mice were stained with anti-CD45, CD31, αSMA, CD29, and IL-17C antibodies or appropriate isotype controls and assessed by flow cytometry. Representative IL-17C staining and isotype control staining of nonhematopoietic vascular cells, gated endothelial cells, SMCs, fibroblasts, and remaining vascular cells within 12-wk WD–fed Apo−/− aortas. The corresponding isotype control for IL-17C within each gate is shown. E, Quantification of the percentage of different vascular cell subsets and (F) IL-17C positivity within each subset, as a percentage of the entire aorta within 40-wk-old C57Bl6 mice (green bars), Apo−/− mice (blue bars), and 12-wk WD–fed Apo−/− mice (blue bars, right) aortas. The data depicts means±SEM. n=14 mice/genotype, 6 independent experiments. Means±SEM are shown, *P<0.05; **P<0.01.

In agreement with reports on the biology of IL-17C within mucosal epithelial cells and keratinocytes, IL-17C was almost exclusively expressed by CD45− nonhematopoietic cells, but not CD45+ hematopoietic cells, within the aorta of 12-week WD–fed Apo−/− mice (Figure 1D). To determine the nonhematopoietic cellular sources of aortic IL-17C, we developed a scheme for examining and isolating vascular cell components by fluorescence activated cell sorting (Figure 1 in the online-only Data Supplement) with a specific focus at IL-17C production within vascular cell subsets (Figure 1D–1F). Leukocytes were excluded from the analysis based on CD45 expression, and CD45+ vascular cells were subsequently examined for CD31, αSMA, and CD29 positivity (Figure 1 in the online-only Data Supplement). CD45+CD31+ endothelial cells, CD45+CD31+αSMA+ SMCs, CD45+CD31+αSMA+CD29+ fibroblasts, and CD45+CD31+αSMA+CD29+ vascular cells were subgated and examined for IL-17C production (Figure 1D–1F). As expected, SMCs and endothelial cells represented the clear majority of vascular cells recovered from aged C57Bl6, Apo−/−, and 12-week WD–fed Apo−/− aortas (Figure 1E). Interestingly, CD45+CD31+αSMA+ SMCs and CD45+CD31+αSMA+CD29+ vascular cells were the...
primary producers of IL-17C within the aorta, and a higher percentage of SMCs were IL-17C+ within Apoe−/− aortas in comparison to nonatherosclerotic C57Bl6 aortas (Figure 1F). To confirm our results using a complimentary technique, we isolated primary Apoe−/− aortic SMCs using magnetic separation from 12-week WD-fed Apoe−/− mice and assessed Il17c expression (Figure 1C). In confirmation of our flow cytometric results, Il17c expression was detected in nonhematopoietic cells and further significantly enriched in CD45−CD31−CD29+ SMCs (Figure 1C). Collectively, these data demonstrate that the vasculature is an important source of IL-17C in atherosclerosis. In addition, these data are the first to report that IL-17C can be detected by flow cytometry using conventional phorbol 12-myristate 13-acetate (PMA)-based restimulation techniques.

**IL-17C–Deficient Conditions Reduce Atherosclerosis and the Number of Aortic Leukocytes and Myeloid Cell Subsets**

To directly assess the role of IL-17C in atherosclerosis, we generated Il17c−/− Apoe−/− mice and compared atherosclerotic lesions between 12-week WD–fed Apoe−/− and Il17c−/− Apoe−/− mice. Apoe−/− and Il17c−/− Apoe−/− mice showed no difference in body weight and plasma cholesterol, triglyceride, low-density lipoprotein cholesterol, or high-density lipoprotein cholesterol contents between males and females (Table 1). Diet-matched Il17c−/− Apoe−/− mice developed 40% smaller lesions throughout the aorta (Figure 2A and 2B) and a 32% reduction in aortic root lesions (Figure 2C) in comparison with 12-week WD–fed Apoe−/− littermates. Because some reports have suggested that IL-17A might promote collagen deposition by SMCs, and other reports did not observe any effects of IL-17A on lesional collagen content, we next sought to determine whether IL-17C might affect lesional SMC or collagen content within the aortic root. Although we did not detect any difference in the percentage of lesional aortic root SMCs (Figure 2D), we did detect a 40% reduction in total collagen and type 1 collagen fiber contents (Figure 2E; data not shown) in 12-week WD-fed Il17c−/− Apoe−/− mice. To confirm these results and to test whether IL-17C might play a role in promoting collagen deposition, we treated explanted 12-week WD-fed Apoe−/− aortas with or without IL-17C and assessed procollagen expression (Colla1; Figure 2F). In this system, IL-17C promoted procollagen expression. To identify the specific cell type that is responsible for the IL-17C–dependent increased collagen synthesis, we isolated primary Apoe−/− and Il17c−/− Apoe−/− populations of CD45+ aortic leukocytes, CD45+CD31+ endothelial cells, CD45+CD31−CD29+ SMCs, and CD45−CD31−CD29+ fibroblasts by fluorescence activated cell sorting. The primary cells were then assessed for Colla1 expression (Figure 2G). Colla1 expression was enhanced in IL-17C–expressing Apoe−/− SMCs and fibroblasts versus IL-17C–deficient Il17c−/− Apoe−/− SMCs and fibroblasts. These data suggest that IL-17C does play a role in promoting procollagen expression in aortic SMCs and fibroblasts, but IL-17C plays a decidedly proinflammatory role in atherosclerosis.

The development of atherosclerotic plaques requires active and sustained recruitment of leukocytes into the aortic wall.7 As previous work from our group7 demonstrated that the IL-17A/IL-17RA pathway promotes the recruitment of leukocytes to the aorta, notably monocytes and neutrophils, we next sought to characterize the aortic immune composition in Il17c−/− Apoe−/− mice by flow cytometry. In agreement with our en face and aortic root lesion data (Figure 2), Il17c−/− Apoe−/− aortas with the surrounding aortic adventitia displayed a ≥35% reduction in both the percentage and number of aortic CD45+ leukocytes, in comparison to Apoe−/− littermates (Figure 3A and 3B). To determine whether IL-17C–deficient conditions might impact the abundance of aortic or peripheral myeloid cells and myeloid cell subsets, we examined the percentage and numbers of aortic infiltrating and peripheral CD11b+ myeloid cells, CD68+ macrophages, and GR1+ neutrophils. Twelve-week WD-fed Il17c−/− Apoe−/− mice displayed a significant reduction (≥40%) in the percentage and number of aortic CD11b+ myeloid cells, CD11b+CD68+ macrophages, and CD11b+GR1+ neutrophils versus Apoe−/− controls (Figure 3C and 3D). Because IL-17C is known to be produced by colonic epithelial cells and keratinocytes and IL-17C plays important roles in helping to maintain mucosal immunity, we next determined whether IL-17C–deficient conditions might additionally affect the systemic immune composition. Surprisingly, we did not detect differences in the composition of CD11b+ leukocytes, macrophages, neutrophils, Ly6C+ monocytes, or Ly6C+ monocytes within the blood or spleens of 12-week WD-fed Il17c−/− Apoe−/− and Apoe−/− mice (data not shown), suggesting that the effects seen within the aorta are because of the local effects of IL-17C.

**IL-17C Supports the Accumulation of Proatherogenic IL-17A+ T Cells Within the Aortic Wall**

As recent work by Chang et al11 demonstrated that both Th17 cells and IL-17A+ TCRγδ+ T cells express IL-17RA and

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<th>Table 1. Twelve-Wk Western Diet–Fed Apoe−/− and Il17c−/− Apoe−/− Mice Body Weights, Fasting Plasma Triglyceride, HDL Cholesterol, LDL Cholesterol, and Total Cholesterol Levels</th>
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HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; and NS, not significant. Il17c−/− Apoe−/− vs Apoe−/− controls, P>0.05.
IL-17RE, the functional receptor for IL-17C, and that IL-17C helps to regulate proinflammatory IL-17A production in a model of experimental autoimmune encephalitis, we next examined the T-cell composition of 12-week WD-fed Il17c−/−Apoe−/− and Apoe−/− mice (Figure 4). The percentages of aortic CD3+ and TCRαβ+ CD3+ T cells were similar between Il17c−/−Apoe−/− and Apoe−/− mice (Figure 4A and 4B); however, the absolute number of infiltrating aortic CD3+ and TCRαβ+ CD3+ T cells were decreased by 60% in Il17c−/−Apoe−/− aortas (Figure 4B). To determine whether IL-17C might affect the abundance of aortic Th1, Th17, Treg, or cytokine-producing TCRαβ+ T cells (which are mostly TCRγδ+ T cells within the aorta, additional data not shown) in 12-week WD-fed Apoe−/− and Il17c−/−Apoe−/− aortas, the number of these subsets were proportionally lower in Il17c−/−Apoe−/− aortas as a consequence of diminished aortic CD3+ T-cell counts (Figure 4D). In contrast, we detected a stark reduction (≈70%) in both the percentage and number of aortic CD3+ TCRαβ+ T cells (Figure 4E and 4F) within 12-week WD-fed Apoe−/− and Il17c−/−Apoe−/− aortas.

Colonic epithelial cells and keratinocytes are also important sources of IL-17C in vivo; thus, we similarly characterized T-cell subsets within the spleens, periaortic lymph nodes, and peripheral lymph nodes of 12-week WD-fed Apoe−/− and Il17c−/−Apoe−/− mice to determine whether a global deficiency of IL-17C might affect peripheral T-cell subsets. Despite diminished aortic Th1 and Treg cellularity and the strong reduction in both the percentage and number of aortic Th17 and IL-17A+ TCRαβ+ T cells, we did not observe differences...
in the percentage or number of these subsets in the spleens, periaortic lymph nodes (Figure II in the online-only Data Supplement), or peripheral lymph nodes (data not shown) of Apoe−/− and Il17c−/−Apoe−/− mice. Together, these data suggest that a global deficiency of IL-17C results in severely impaired accumulation of aortic IL-17A+ T cells within the arterial wall and proportionally reduced aortic macrophage, neutrophil, Th1, and Treg cellularity in Apoe−/− mice.

**IL-17C Promotes Aortic Chemokine and Cytokine Expression and Helps to Recruit Th17 Cells to Atherosclerotic Plaques**

Several studies have shown that IL-17A promotes arterial vascular chemokine and cytokine expression and supports the recruitment of monocytes and neutrophils to atherosclerotic lesions.3,4,18,20,23,28–31 Thus, we next sought to determine the effects of IL-17C on aortic chemokine and cytokine expression (Figure 5A). Twelve-week WD-fed Apoe−/− aortas were explanted for 24 hours with or without IL-17C and assessed for Ccl2, Ccl7, Ccl20, Cx3cl1, Cxcl1, Cxcl2, Cxcl5, Il6, Il23a, and Il1b expression by RT-PCR. In this system, IL-17C broadly supported the expression of these cytokines and chemokines (Figure 5A), including the pro-Th17 chemokine Ccl20 and cytokines Il6, Il23, and Il1b. Thus, because IL-17C helped to induce aortic pro-Th17 chemokine and cytokine expression and Il17c−/−Apoe−/− mice displayed a specific reduction in aortic Th17 and IL-17A+ TCRαβ+ T-cell content (Figure 4), we hypothesized that IL-17C+ SMCs might support the accumulation of Th17 and IL-17A+ T cells within the arterial wall. To test this hypothesis, we performed IL-17C–dependent Th17 chemotaxis experiments in vitro, in order to clarify whether Th17 cells directly or indirectly migrate to IL-17C. In brief, we generated a new strain of IL-17A lineage tracing Il17aicre/icreR26RtdTomato/tdTomatoApoe−/− mice in our laboratory, in which the expression of IL-17A is detected via R26RtdTomato/tdTomato expression. While IL-17A+tdTomato+CD4+ T cells efficiently migrate to IL-17C–producing Il17c−/+Apoe−/− aortic SMC supernatants, Th17 cell migration to Il17c−/−Apoe−/− aortic SMC supernatants was significantly reduced (Figure 5B).

Thus, IL-17C does play a role in supporting the recruitment of Th17 cells in chemotaxis assays. To further examine the role of IL-17C in the regulation of Th17 cell homing in vivo, we adoptively transferred fluorescently labeled Apoe−/− splenocytes to 12-week WD-fed Apoe−/− and Il17c−/−Apoe−/− mice for 72 hours and tracked the migration of Apoe−/− splenic CD4+ and Th17 cells to recipient spleens (Figure 5C and 5D), periaortic lymph nodes (data not shown), and aortas (Figure 5E and 5F). We found an equivalent percentage and number of donor CD4+ T cells and Th17 cells within the spleens (Figure 5C and 5D) and periaortic lymph nodes (data not shown) of Apoe−/− and Il17c−/−Apoe−/− recipient mice. In
Figure 4. Disruption of interleukin-17C (IL-17C) results in severely diminished aortic IL-17A+ Th17, IL-17A+ CD3+TCRαβ+ T-cell content. A–F, Twelve-wk Western diet (WD)-fed Apoe<sup>−/−</sup> and Il17c<sup>−/−</sup>Apo<sup>−/−</sup> aortas with surrounding aortic adventitia were stained with anti-CD45, CD3, TCRβ, IL-17A, IFNγ, and Foxp3 antibodies and analyzed by flow cytometry. n=7 Apoe<sup>−/−</sup> mice, n=11 Il17c<sup>−/−</sup>Apo<sup>−/−</sup> mice, 4 independent experiments. A, Representative CD3 and TCRβ staining within WD-fed Apoe<sup>−/−</sup> and Il17c<sup>−/−</sup>Apo<sup>−/−</sup> mice CD45+ aortic leukocytes and (B) quantification of the percentage and number of CD3+ and CD3+TCRβ+ T cells/aorta. C, Representative IFNγ and IL-17A staining in CD3+TCRβ+ gated aortic T cells and (D) quantification of the percentages and numbers of IFNγ<sup>+</sup> Th1, IL-17A+ Th17, and Foxp3<sup>+</sup> Tregs/aorta. E, Representative IFNγ and IL-17A staining within CD3+TCRβ+ gated aortic T cells. F, Quantification of the percentage and number of CD3+TCRαβ−IFNγ<sup>+</sup> T cells and IL-17A+ T cells in 12-wk WD-fed Apoe<sup>−/−</sup> (blue) and Il17c<sup>−/−</sup>Apo<sup>−/−</sup> (red) mice aortas. Means±SEM are shown. *P<0.05; **P<0.01.
Figure 5. Interleukin-17C (IL-17C) supports aortic chemokine expression and supports the recruitment of IL-17A+ Th17 cells to the aorta.

A, Twelve-wk Western diet (WD)-fed Apoe−/− aortas were sterilely explanted and untreated (black bars) or cultured with 100 ng/mL IL-17C (gray bars) for 24 h before being collected and processed for reverse transcription-polymerase chain reaction. Fold induction of Ccl2, Ccl7, Cxcl2, Cxcl3, Cxcl5, Cxcl10, Il6, Il23a, and Il1b expression. n=9 Apoe−/− mice, 3 independent experiments.

B, Twelve-wk WD-fed Apoe−/− and I17c−/−Apoe−/− mice aortic smooth muscle cells (CD45−CD31−CD29− aortic cells) were sterilely sorted by fluorescence activated cell sorting and cultured for 1 h in vitro to collect cell supernatants. CD4+ T cells were isolated in parallel from 40-wk chow diet–fed I17aiecre/icreR26RtdTomato/tdTomato Apoe−/− mice. I17aiecre/icreR26RtdTomato/tdTomato Apoe−/− CD4+ T cells migrated toward either a migration media (vehicle control) or 1000 ng/mL rCCL20 or Apoe−/− or I17c−/−Apoe−/− aortic smooth muscle cell supernatants for 2 h. The transmigrated cells were collected and assessed for IL-17AtdTomato+ Th17 cells by flow cytometry and normalized to the percentage of Th17 cells in the starting population. n=5 independent experiments, all assays were performed in triplicate.

C–F, 30×10^6 12-wk WD-fed Apoe−/− mice splenocytes were labeled with cell trace violet (CTV) and adoptively transferred to 12-wk WD-fed Apoe−/− and I17c−/−Apoe−/− mice for 72 h. The migration of CTV+CD4+ T cells and CTV+ Th17 cells within the recipients was assessed by flow cytometry. Representative flow cytometry plots for CTV+ splenocytes and CD4+ Th17 cell migration to recipient spleens (C) and aortas (E) are shown. Quantification of the percentage and number of donor CD4+ T cells and Th17 cells within the spleens (D) and aortas (F) of recipient 12-wk WD-fed Apoe−/− (blue) and I17c−/−Apoe−/− (red) recipients. n=5 recipients/genotype, 4 independent experiments. Bars depict means±SEM. *P<0.05; **P<0.01; ***P<0.001.
SMCs, but not in CD45<sup>+</sup> cells. These results are noteworthy for several reasons. To date, the identification of the cellular sources of IL-17C in vivo and the examination of cytokine production within SMCs have been technically limited to Western blot, histological assays, or PCR-based assays. We report here that with a careful gating strategy, aortic SMCs can be examined for cytokine production ex vivo and that IL-17C can be detected by conventional PMA, ionophore-based cytokine flow cytometry techniques. Additionally, the data presented here represent the first report to demonstrate that aortic SMCs are an important source of IL-17C in vivo. SMCs play critical roles in atherosclerosis, not only in stabilizing atherosclerotic plaques through collagen deposition and the formation of fibrous caps but also in promoting inflammation by producing proinflammatory cytokines. Evidence demonstrate that SMCs may assume a proinflammatory phenotype in sterile inflammation and participate in atherosclerosis. SMCs may produce several cytokines and chemokines in atherosclerosis, including proinflammatory IL-8, CCL2, CXCL1, IL-1β, IL-6, atheroprotective IL-19, and now, IL-17C as well. Although the specific mechanisms of IL-17C induction within SMCs remain to be determined, previous work on IL-17C and SMC biology suggested that several pathways might be involved. SMCs respond to oxidized low-density lipoprotein via the lectin-like oxidized low-density lipoprotein scavenger receptor (LOX-1) in culture by producing MCP-1, CXCL1, TNFα, and VCAM-1, with similar expression in an Nfkb-, Nfat-, and Jnk-dependent manner; suggesting that oxidized low-density lipoprotein or oxidized lipids might promote IL-17C expression within atherosclerosis as well. Additionally, as PMA and Ionomycin C are known to activate T cells in a PKC-, NFκB-, NFAT-, and MAPK-dependent manner, PMA and Ionomycin C stimulation may induce IL-17C expression in aortic SMCs in a PKC-dependent manner. In support of this notion, IL-17C is quickly induced within the gut in response to TLR 2 and TLR 5 in an Nfkb-dependent manner, suggesting that oxidized low-density lipoprotein or oxidized lipids might promote IL-17C expression within atherosclerosis as well. As IL-17C helps to maintain mucosal immunity and is abundant within the inflamed aorta and skin, it was important to examine whether IL-17C might affect the peripheral immune composition. More recent work has further proposed that IL-17C might directly affect activation of vasculature and plaque macrophage functions as well. As Il17c<sup>−/−</sup> Apoe<sup>−/−</sup> mice demonstrated an atheroprotective phenotype, with reduced IL-17A+ T-cell content and Th17 recruitment to the aorta, and a decrease in total lesional collagen, our findings here further support a proatherogenic role of Th17 cells. The recruitment of myeloid cells to nascent plaques plays a critical role in the initiation and progression of atherosclerotic plaques, and IL-17A promotes the accumulation of monocytes and neutrophils within atherosclerotic lesions. Thus, we sought to determine whether a global deficiency of IL-17C might affect the aortic or circulating myeloid cell immune composition. These experiments revealed a proportional 40% to 50% decrease in aortic myeloid cells, including macrophages and neutrophils, highlighting a strong correlation between atherosclerotic plaque lesion size and macrophage and neutrophil content within the aortic wall. As IL-17C helps to maintain mucosal immunity and is abundant within the inflamed aorta and skin, it was important to examine whether IL-17C might affect the peripheral immune system. However, the peripheral immune composition was unchanged, suggesting that the local actions of SMC-derived IL-17C were responsible for the protective phenotype of Il17c<sup>−/−</sup> Apoe<sup>−/−</sup> mice. As specific Il17c<sup>−/−</sup> mice are not currently available, we used global IL-17C-deficient Apoe<sup>−/−</sup> mice in this study. Further studies using a SMC-specific IL-17C-deficient mouse model might reveal additional effects of SMC-derived IL-17C on atherosclerosis.
IL-17C has been reported to play a key role in promoting IL-17A production within IL-17RE–expressing Th17 and IL-17A+ TCRγδ+ T cells and key roles in the pathology of experimental autoimmune encephalitis and psoriasis. We detected here an overall reduction in the total number of aortic infiltrating CD3- and CD3+ TCRβ+ T cells, TH1 cells, and Treg cells within WD Il17c−/−Apoe−/− mice. However, when we examined Th17 and IL-17+ TCRβ+ T cells, we observed a disproportionate decrease in the percentage and number of cells within Il17c−/−Apoe−/− aortas versus Apoe−/− controls. Several mechanisms could be responsible for a diminished aortic IL-17A+ T-cell response, ranging from defective migration to altered differentiation, proliferation, or survival. We reasoned that if changes in Th17 differentiation, proliferation, or survival were responsible for diminished aortic Th17 content, Il17c−/−Apoe−/− mice would display similar defects peripherally. As peripheral T-cell subsets were unaffected between Il17c−/−Apoe−/− and Apoe−/− mice and IL-17C broadly supported pro-Th17 chemokine/cytokine expression in vivo experiments, we hypothesized that aortic IL-17C might support Th17 cell recruitment during atherogenesis. In our adoptive transfer experiments, donor CD4+IL-17A+ Th17 cells did not migrate as well to the aortas of Il17c−/−Apoe−/− versus Apoe−/− recipients. Interestingly, equivalent amounts of donor CD4+IFNγ+ Th1 cells accumulated in Il17c−/−Apoe−/− and Apoe−/− recipient aortas (data not shown).

Thus, these results demonstrate that the reduction of aortic Th17 cells in Il17c−/−Apoe−/− mice are because of the inefficient recruitment of Th17 cells, rather than a defect in T-cell recruitment or peripheral Th17 differentiation or accumulation. Together, these results demonstrate that in sterile conditions, IL-17C is produced and acts locally on the aortic wall to promote proinflammatory chemokines and cytokines and the efficient recruitment of proinflammatory monocytes, neutrophils, and Th17 cells. These observations are important as little is known about the recruitment of Th17 cells in atherosclerosis. Th17 cells are known to express CCR6 and CCR4; however, as Th17 recruitment was unchanged within Ccr6−/− Ldlr−/− mice, CCR6 may not be necessary for the accumulation of aortic Th17 cells. Thus, IL-17C is likely initially produced within the arterial wall and serves to promote the accumulation of Th17 and IL-17A+ TCRγδ+ T cells. Once Th17 cells migrate to the aortic wall, IL-17A may support the production of proinflammatory chemokines and monocyte and neutrophil recruitment to nascent atherosclerotic plaques, a process that is critical for the initiation and progression of atherosclerotic plaques.

Altogether, our data demonstrate a novel proatherogenic role for vascular IL-17C and highlight a dynamic between arterial IL-17C and Th17 and IL-17A+ TCRγδ+ T cells. These data suggest a model in which aortic IL-17C might act in an autocrine/paracrine manner on nearby vascular cells to support the recruitment of IL-17A+ T cells and suggest that in unison, both IL-17A and IL-17C help to recruit additional myeloid cells, thereby affecting the cellularity of atherosclerotic lesions.

Acknowledgments

We thank Amgen, Inc, for providing us with Il17c−/− breeding pairs. We also thank Raaj Talalukker, Chris McGary, and Chih Wu for their technical assistance.

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Disclosures

None.

References


Il17c−/−Apoe−/−
Aortic smooth muscle cells are the major producers of IL-17C in the aortic wall. Interleukin-17C (IL-17C) expression is elevated in atherosclerotic aortas.

Interleukin-17A and IL-17C help to recruit additional myeloid cells, thereby affecting the cellularity of atherosclerotic lesions.
Smooth Muscle Cell–Derived Interleukin-17C Plays an Atherogenic Role via the Recruitment of Proinflammatory Interleukin-17A + T Cells to the Aorta
Matthew J. Butcher, Tayab C. Waseem and Elena V. Galkina

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In the article by Butcher et al, which appeared in the August 2016 issue of the journal *(Arterioscler Thromb Vasc Biol. 2016;36:1496–1506. DOI: 10.1161/ATVBAHA.116.307892)*, corrections were needed.

In the text, 3 supplemental figures were cited; however, there are only 2 supplemental figures. Two changes reflecting this have been made in the text.

The color bars in Figure 2G were not correct. The blue bars should be red (for *Il17c<sup>−/−</sup>* *Apoe<sup>−/−</sup>*), and the red bars should be blue (for *Apoe<sup>−/−</sup>*).

The authors apologize for the errors.

The online version of the article has been corrected and is available at [http://atvb.ahajournals.org/content/36/8/1496](http://atvb.ahajournals.org/content/36/8/1496).
Endothelial Cells
IL-17C: Increased CCL2, CXCL1, CXCL2, CCL5, CCL20, IL-6, IL-1β, & IL-23 expression

Accelerated Atherosclerosis

IL-17C-dependent recruitment

Th17 IL-17A+ T cells

1. IL-17C

2. IL-17C

3. IL-17A

4. IL-17A

5. Accelerated Atherosclerosis
Supplemental Figure I: Aortic vascular cell flow cytometry gating scheme. Representative scheme for gating on vascular cell subsets for the flow cytometry experiments in Figure 1. Single CD45- vascular cells were gated and subdefined as follows: CD45-CD31+: endothelial cells, CD45-CD31-αSMA+: Smooth muscle cells, CD45-CD31-αSMA-CD29+: fibroblasts, CD45-CD31-αSMA-CD29-: remaining vascular cells.
Supplemental Figure II: Peripheral T cells and T cell subsets are not impacted by IL-17C deficient conditions.

(A-D) 12 week WD Apoe<sup>−/−</sup> and Il17c<sup>−/−</sup> Apoe<sup>−/−</sup> spleens and peri-aortic lymph nodes were stained with anti-CD45, CD3, TCRαβ, IL-17A, IFNγ, and Foxp3 antibodies or appropriate isotype controls, and assessed via flow cytometry. (A) Representative CD3 and TCRαβ staining of Apoe<sup>−/−</sup> and Il17c<sup>−/−</sup> Apoe<sup>−/−</sup> splenocytes. (B) Quantification of the percentage of splenic and peri-aortic lymph node CD3<sup>+</sup> T cells and CD3<sup>+</sup>TCRαβ<sup>+</sup> T cells. (C) Representative IL-17A, IFNγ, and Foxp3 staining within Apoe<sup>−/−</sup> and Il17c<sup>−/−</sup> Apoe<sup>−/−</sup> splenic CD3<sup>+</sup> TCRαβ<sup>+</sup> T cells. (D) Quantification of the percentage of splenic and peri-aortic lymph node Th1, Th17, Treg, CD3<sup>+</sup> TCRγδ<sup>+</sup> IL-17A<sup>+</sup> T cell and CD3<sup>+</sup> TCRγδ<sup>+</sup> IFNγ<sup>+</sup> T cell contents. Red – Apoe<sup>−/−</sup>, Blue – Il17c<sup>−/−</sup>Apoe<sup>−/−</sup> mice. n=7-11 mice/genotype, 4 independent experiments, bars depict means±SEM.
Supplemental Materials and Methods section.

Dyes, recombinant proteins, and antibodies. The following antibodies were used: TCRab-FITC (H57-597), Ly6C-FITC (AL-21), Ly6G (Gr1)-APC (RB6-8C5, Ly6G-specific antibody), CD68-PE (FA11), IL-17A-PE or -eFluor 660 (TC11-1810), CD11b-eFluor 450 (M1/70.15), IFNγ-eFluor 450 (XMG1.2), CD3-APC eFluor 780 (17A2), CD4-PerCP eFluor 710 (RM4-5), CD29-PE (eBioHMB1-1), CD31-eFluor 450 (390), (all from eBioscience, Inc), CD45-PE (30-F11, Molecular Probes, Life Technologies, Carlsbad, CA), α-Smooth Muscle Actin-FITC (1A4, Sigma-Aldrich, St. Louis, MO), and CD16/CD32 antibodies (The Lymphocyte Culture Center, University of Virginia).

For IL-17C cytokine flow cytometry experiments, unlabeled Rat anti-mouse IL-17C (IgG2a, 311522) and Rat IgG2a (MAB006, both from R&D Systems, Bio-Technne, Minneapolis, MN) antibodies were labeled with Alexa Fluor 647 dye with Alexa Fluor 647 Antibody Labeling Kits (Molecular Probes, A-20186), following the manufacturer’s protocol. Labeled antibody preparations were dialyzed (Slide-A-Lyzer Dialysis Cassettes, Pierce, Thermo Fisher Scientific) after the labeling procedure and concentrated to 0.2 mg/ml. The degree of labeling for each preparation was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) to be 4.5-5 moles of Alexa Fluor 647 dye/mole of antibody. 0.2μg/1x10^6 million cells of IL-17C-Alexa Fluor 647 or IgG2a-Alexa Fluor 647 antibody was used for each flow cytometry test. Recombinant murine IL-17C (2306-ML-025/CF R&D Systems) was used in the ex vivo explant studies described.

En Face preparations. 12 week WD-fed Apoe−/− and Il17c−/− Apoe−/− aortas were excised and stained for atherosclerotic lesions using Oil Red O as previously described. Photomicrographs were taken and the percent area occupied by lesions was determined using Image J (v1.44). Three independent observers quantified the Oil Red O staining and the aggregate scores are presented (Figure 3).

Histology. Hearts from the 12 week WD-fed Apoe−/− and Il17c−/− Apoe−/− En Face cohorts were perfused with 4% Para-formaldehyde in phosphate buffered saline by cardiac puncture and collected for histology. Sequential 5µm aortic root sections (totaling >300µm of coverage) from the point of the appearance of the aortic valve leaflets were collected. Sections at depths 50, 150, and 250µm were deparaffinized, rehydrated, and stained with antibodies against α-Smooth muscle actin (1A4, Sigma-Aldrich, St. Louis, MO) following antigen retrieval (Vector Laboratories, Burlingame, CA). α-SMA staining was detected using the following biotin-streptavidin reagents: Rabbit-Anti-Mouse-Biotin IgG (B8520, Sigma-Aldrich, St. Louis, MO) and Vectastain ABC kit (Vector Labs, Burlingame, CA). Sections at depths 55, 155, and 255µm were stained for collagen fibers using a pico sirius red staining kit (Polysciences, Inc., Warrington, PA) following the manufacturer’s instructions. Images of the stained sections were acquired by white light microscopy and polarized light microscopy (Picosirius Red stained sections) and quantified by two independent observers using Image J (v1.44). The results are presented as a percentage area of marker positive staining within the total area of the atherosclerotic plaques.
Quantitative real time PCR. Total RNA was isolated from atherosclerotic aortas using Trizol reagent (Invitrogen, Life Technologies). To identify major producers of pro-collagen, we sorted primary Apoe<sup>-/−</sup> and Il17c<sup>-/−</sup> Apoe<sup>−/−</sup> CD45<sup>−</sup> aortic leukocytes, CD45<sup>−</sup>CD31<sup>−</sup> endothelial cells, CD45<sup>−</sup>CD31<sup>−</sup>CD29<sup>−</sup> SMCs, and CD45<sup>−</sup>CD31<sup>−</sup>CD29<sup>−</sup> fibroblasts by FACS. The primary cells were then assessed for Col1a1 expression by RT-PCR. To examine cell-specific expression of IL-17C aortas from 12 week CD C57Bl6, Apoe<sup>−/−</sup>, and 12 week WD-fed Apoe<sup>−/−</sup> mice were collected and expression of Il17c in whole Apoe<sup>−/−</sup> aortas, magnetically sorted Apoe<sup>−/−</sup> aortic leukocytes (CD45<sup>−</sup>, Stemcell Technologies), Apoe<sup>−/−</sup> vascular cells (CD45- cells), and FACS-sorted Apoe<sup>−/−</sup> CD45<sup>−</sup>CD31<sup>−</sup>CD29<sup>−</sup> SMCs was determined. Co-isolated genomic DNA was removed from the preparations by DNase I treatment using RNeasy kits (Qiagen, Valencia, CA) following the manufacturer’s instructions. 1μg of Total RNA was reverse transcribed as described<sup>2</sup> using Moloney murine leukemia virus reverse transcriptase, 10μM dNTPs, and random hexamers (Invitrogen, Life Technologies). RT-PCR was conducted using mouse Th17 PCR profiler arrays (SA Bioscience, Qiagen, Frederick, MD) and gene-specific mouse Taqman probes (Actb (Mm00607939_s1), Gapdh (Mm99999915_g1), Il17c (Mm00521397_m1), Il17a (Mm00439618_m1), Ccl2 (Mm00441242_m1), Ccl7 (Mm00443113_m1), Cx3cl1 (Mm00436454_m1), Cxcl1 (Mm04207460_m1), Cxcl2 (Mm00436450_m1), Cxcl5 (Mm00436451_g1), and Il6 (Mm00446190_m1), Applied Biosystems, Carlsbad, CA). Ct values were determined using an iCycler iQ Real-time detection system (Bio-Rad laboratories, Hercules, CA). All PCR results were normalized to Actb, Gapdh, and Hsp90ab1 as housekeeping gene controls and the data is presented as a fold change compared to Apoe<sup>−/−</sup> or vehicle controls (2<sup>−ΔΔCT</sup> method).

Flow Cytometry. Aortic, splenic, peripheral lymph node, and peri-aortic lymph node single cell suspensions were prepared as previous described<sup>1,3,4</sup>. In brief, mice were anesthetized and their vasculature was perfused by cardiac puncture with 20U/ml sodium heparin in phosphate buffered saline. The aortas with surrounding aortic adventitia were sterilely microdissected, collected, and subsequently digested with the following cocktail of enzymes in PBS for 1 hour at 37C, as described<sup>1,3,4</sup>: 125 U/ml Collagenase type XI, 60 U/ml hyaluronidase type I-s, 60 U/ml DNase1, and 450 U/ml Collagenase type 1 (all from Sigma-Aldrich, St. Louis, MO). For studies with peripheral primary and secondary lymphatic organs, the following organs were collected from each mouse: The spleen, peripheral lymph nodes (superficial and deep cervical, auxiliary, brachial, and inguinal lymph nodes), peri-aortic lymph nodes (sacral, lumbral, renal, and mediastinal lymph nodes), and blood.

For intracellular cytokine staining experiments, aortic, splenic, peripheral lymph node, and peri-aortic lymph node single cell suspensions were cultured for 5 hours in RPMI 1640 supplemented with 10% FBS, 1% Penicillin/Streptomycin, 10ng/ml Phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich), 500ng/ml Ionomycin (Sigma Aldrich), and GolgiStop (BD Biosciences). Intracellular staining for IFNγ, IL-17A, and Foxp3 or appropriate isotype controls was performed using eBioscience Foxp3/Transcription Factor Staining Buffer (eBioscience). In the T cell phenotyping experiments, aortic IL-17A<sup>+</sup> CD3<sup>+</sup>TCRβ<sup>+</sup> T cells were considered to be IL-17A<sup>+</sup> γδ<sup>+</sup> T cells as we demonstrated previously that TCRβ<sup>+</sup> Th17 cells and IL-17A<sup>+</sup> TCRγδ<sup>+</sup> T cells are the major cellular producers of aortic IL-17A in vivo. Intracellular staining for CD68, IL-17C, and α-Smooth muscle actin were conducted using Fix&Perm cell permeabilization reagents (BD Biosciences). For some experiments aortas from 2-3 mice were combined in order to have sufficient number of analyzed cells. For all flow cytometry experiments involving aortas, data from the entire sample was acquired on the flow cytometer. A CytekDXP 8 color
(Cytek Development Inc.) upgraded FACSCalibur (BD Biosciences) was used to acquire samples, and FlowJo (Tree Star Inc., Ashland, OR) was used to analyze the data. For all flow cytometry experiments, the gates were placed based on isotype and fluorescent minus one controls.

**Adoptive Transfer Experiments.**

For adoptive splenocyte transfer experiments, 3-4x 12 week WD Apoe⁻/⁻ spleens were collected steriley and single cell suspensions were prepared. Erythrocytes were lysed using ACK lysis buffer (8.29mg/ml NH₄Cl, 1mg/ml KHCO₃, 0.372 mg/ml EDTA, pH 7.2, all from Sigma Aldrich). Leukocytes were subsequently labeled with 5µM CellTrace Violet (CTV) dye (Molecular Probes, Life Technologies) in PBS at 37°C for 10 minutes and washed twice with PBS containing 1% FBS. 30x10⁶ CTV⁺ splenocytes in 0.25ml of PBS were injected into 12 week WD Apoe⁻/⁻ and Il17c⁻/⁻ Apoe⁻/⁻ recipient mice via tail vein injection (i.v.). As a negative control for the injected CTV⁺ splenocytes, 12 week Apoe⁻/⁻ mice were injected with 0.25ml of PBS. 72 hours post injection, the recipient and sham control aortas, spleens, and peri-aortic lymph nodes were collected and processed for intracellular cytokine flow cytometry experiments. The gates for CTV⁺ donor cells and cytokine⁺ CTV⁺ donor cells were set based on sham negative controls and isotype controls, respectively.

**Transwell migration assays.**

12 week WD-fed Apoe⁻/⁻ and Il17c⁻/⁻ Apoe⁻/⁻ aortic smooth muscle cells (CD45⁻CD3¹CD29⁻ aortic cells) were steriley FACS sorted and cultured for an hour in 10 % of FBS complete RPMI in vitro to collect cell supernatants. CD4⁺ T cells were isolated in parallel from 40 week CD Il17a⁺/⁺ R26RtdTomato/tdTomato Apoe⁻/⁻ mice. 0.3x10⁶ Il17a⁺/⁺ R26RtdTomato/tdTomato Apoe⁻/⁻ CD4⁺ T cells were cultured in 200µl of migration media in the top wells of transwell plates and allowed to migrate towards 600µl of: a migration media vehicle control, 1000 ng/ml recombinant mouse CCL20, Apoe⁻/⁻ or Il17c⁻/⁻ Apoe⁻/⁻ aortic smooth muscle cell supernatants for 2 hours. The transmigrated cells were collected and assessed for IL-17A tdTomato⁺ Th17 cells by flow cytometry and normalized to the percentage of Th17 cells in the starting population. All assays were performed in triplicate.

**Statistical Analysis.**

Statistical comparisons between two groups were conducted using unpaired Student’s T Tests and the data reported as mean±SEM. For statistical comparisons between three or more groups, a one way ANOVA with Tukey HSD post-hoc tests were used. Statistical significance was set at p<0.05.
Reference List


