The IL-17A/IL-17RA Axis Plays a Proatherogenic Role via the Regulation of Aortic Myeloid Cell Recruitment

Matthew J. Butcher, Breanne N. Gjurich, Tracy Phillips, Elena V. Galkina

Rationale: Atherosclerosis is a disease of large- and medium-sized arteries that is characterized by chronic vascular inflammation. While the role of Th1, Th2, and T-regulatory subsets in atherogenesis is established, the involvement of IL-17A-producing cells remains unclear.

Objective: To investigate the role of the IL-17A/IL-17RA axis in atherosclerosis.

Methods and Results: We bred apolipoprotein-E-deficient (ApoE−/−) mice with IL-17A-deficient and IL-17 receptor A-deficient mice to generate Il17a−/−ApoE−/− and Il17ra−/−ApoE−/− mice. Western diet fed Il17a−/−ApoE−/− and Il17ra−/−ApoE−/− mice had smaller atherosclerotic plaques in the aortic arch and aortic roots, but showed little difference in plaque burden in the thoracoabdominal aorta in comparison with ApoE−/− controls. Flow cytometric analysis of Il17a−/−ApoE−/− and Il17ra−/−ApoE−/− aortas revealed that deficiency of IL-17A/IL-17RA preferentially reduced aortic arch, but not thoracoabdominal aortic T cell, neutrophil, and macrophage content in comparison with ApoE−/− aortic segments. In contrast to ubiquitous IL-17RA expression throughout the aorta, IL-17A was preferentially expressed within the aortic arch of WD-fed ApoE−/− mice. Deficiency of IL-17A or IL-17RA reduced aortic arch, but not thoracoabdominal aortic TNFα and CXCL2 expression. Aortic vascular IL-17RA supports monocyte adherence to explanted aortas in ex vivo adhesion assays. Short-term homing experiments revealed that the recruitment of adoptively transferred monocytes and neutrophils to the aortas of Il17a−/−ApoE−/− mice is impaired in comparison with ApoE−/− recipients.

Conclusions: The IL-17A/IL-17RA axis increases aortic arch inflammation during atherogenesis through the induction of aortic chemokines, and the acceleration of neutrophil and monocyte recruitment to this site. (Circ Res. 2012;110:00-00.)

Key Words: atherosclerosis ■ inflammation ■ leukocytes ■ chemokines ■ migration
Deficiency of IL-17A and IL-17RA Reduces Atherosclerosis in Aortas of Apoe<sup>−/−</sup> Mice

To directly assess the role of the IL-17A/IL-17RA axis in atherosclerosis, we generated Il17a<sup>−/−</sup>Apoe<sup>−/−</sup> and Il17ra<sup>−/−</sup>Apoe<sup>−/−</sup> mice. Il17a<sup>−/−</sup>Apoe<sup>−/−</sup>, Il17ra<sup>−/−</sup>Apoe<sup>−/−</sup>, and age- and diet-matched Apoe<sup>−/−</sup> mice showed no difference in body weight, total cholesterol, and triglyceride levels (data not shown). The aortas of Il17a<sup>−/−</sup>Apoe<sup>−/−</sup> mice fed a Western diet (WD) for 15 weeks developed 35% smaller aortic lesions in comparison with Apoe<sup>−/−</sup> mice (Figure 1A). We also examined the aortic arches (Figure 1B) and thoracoabdominal aortas (Figure 1C) of Il17a<sup>−/−</sup>Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup> mice separately. Plaque development was diminished within the aortic arch (Figure 1B), but not in the thoracoabdominal aortas (Figure 1C) of Il17a<sup>−/−</sup>Apoe<sup>−/−</sup> mice. We also detected a 19% reduction in aortic root plaque burden within Il17a<sup>−/−</sup>Apoe<sup>−/−</sup> mice in comparison with Apoe<sup>−/−</sup> mice (Figure 1D).

To further delineate the role of the IL-17A/IL-17RA axis in atherosclerosis, we examined atherogenesis in IL-17RA-deficient Apoe<sup>−/−</sup> mice. Il17ra<sup>−/−</sup>Apoe<sup>−/−</sup> mice developed 25% smaller lesions within whole aortas (Figure 2A) and aortic roots (Figure 2D) in comparison with Apoe<sup>−/−</sup> mice. IL-17RA deficiency resulted in reduced lesions within the aortic arch (Figure 2B), but not in the thoracoabdominal aortas (Figure 2C) of Il17ra<sup>−/−</sup>Apoe<sup>−/−</sup> mice. We also assessed the cross-sectional area of Il17ra<sup>−/−</sup>Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup> aortic arch and thoracoabdominal aortic plaques. In agreement with the en face data, deficiency of IL-17RA yielded a 41% reduction in aortic arch lesions and had no effect on thoracoabdominal lesions (Figure 3A). Interestingly, aortic plaque burden throughout the aorta and within the aortic roots of Il17a<sup>−/−</sup>Apoe<sup>−/−</sup>, Il17ra<sup>−/−</sup>Apoe<sup>−/−</sup>, and Apoe<sup>−/−</sup> mice fed a 20-week chow diet were not significantly altered (4.3%±0.2, 4.2%±0.2, and 4.7%±0.5, n=12–14 mice/per group, P=0.3).

To further characterize the impact of the IL-17A/IL-17RA axis on lesion composition, we examined the collagen fiber content of the aortic arch and thoracoabdominal aortas of Il17ra<sup>−/−</sup>Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup> mice using picrosirius red staining. The immature, mature, and total collagen fiber content was similar between Il17ra<sup>−/−</sup>Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup> mice (Figure 3B and 3C). These results suggest that the IL-17A/IL-17RA pathway does not affect the maturation of aortic collagen after a 15-week WD regimen. To test the impact of the IL-17A/IL-17RA axis on aortic smooth muscle cell (SMC) content, we performed immunostaining for α-smooth muscle actin. The aortic arch and thoracoabdominal...
nal aortic SMC content of Il17ra−/− Apoe−/− and Apoe−/− mice were similar (aortic arch: 1.02±0.02-fold Il17ra−/− Apoe−/− versus Apoe−/−, 1.00±0.03-fold Apoe−/− versus Apoe−/−, P=0.80; thoracoabdominal aortas: 0.99±0.04-fold Il17ra−/− Apoe−/− versus Apoe−/−, 1.00±0.02-fold Apoe−/− versus Apoe−/−, P=0.89), suggesting that IL-17A does not significantly affect total aortic SMC content.

Disruption of the IL-17A/IL-17RA Axis Decreases the Cellularity of Atherosclerotic Aortas

The development of atherosclerosis is accompanied by marked recruitment of leukocytes into the aortic wall.19 As IL-17A supports the induction of epithelial6,20 and vascular11–13,21 chemokines, we assessed the effects of IL-17A/IL-17RA on the cellularity and immune cell composition...
of the aorta during atherogenesis. We detected a 50% reduction in CD45 leucocytes and CD3 T cells within the whole aortas of 15-week WD-fed Il17a−/− Apoe−/− and Il17ra−/− Apoe−/− mice in comparison with Apoe−/− mice (Figure 4A). Next, to determine if disruption of the IL-17A/IL-17RA axis alters the aortic T cell response, we examined Th1 and Th17 cells within the aortas of Il17a−/− Apoe−/−, Il17ra−/− Apoe−/−, and Apoe−/− mice. While the relative percentage of IFNγ+ cells was unaltered (Figure 4C), the overall number of Th1 cells were significantly diminished in Il17ra−/− Apoe−/− and Il17a−/− Apoe−/− aortas (Figure 4D). In agreement with these data, we detected a significant reduction in aortic Ifng, Il6, and Tnfα mRNA expression (Figure 4B). Interestingly, deficiency of IL-17RA in Il17ra−/− Apoe−/− mice had no effect on the percentage (Figure 4C), total number of aortic IL-17A+ T cells (Figure 4D), or the percentage of IL-17A+ T cells within the spleen, peripheral lymph nodes, and blood (data not shown) in comparison with Apoe−/− mice.

To determine if the reduction of aortic leukocytes, like the reduction of plaques, occurred specifically in the aortic arch, we determined the total CD45 leucocyte cellularity of...
II\textsubscript{17}a\textsuperscript{−/−} Apoe\textsuperscript{−/−} and II\textsubscript{17}ra\textsuperscript{−/−} Apoe\textsuperscript{−/−} aortic segments. The number of CD45\textsuperscript{+} leukocytes was significantly reduced in II\textsubscript{17}a\textsuperscript{−/−} Apoe\textsuperscript{−/−} and II\textsubscript{17}ra\textsuperscript{−/−} Apoe\textsuperscript{−/−} aortic arches in comparison with Apoe\textsuperscript{+/-} controls (1.5±0.1×10\textsuperscript{5}, 1.2±0.1×10\textsuperscript{5}, 2.4±0.2×10\textsuperscript{5} cells/aortic arch, respectively, P<0.01). Deficiency of II\textsubscript{17}A or II\textsubscript{17}RA significantly reduced the T cell content of II\textsubscript{17}a\textsuperscript{−/−} Apoe\textsuperscript{−/−} and II\textsubscript{17}ra\textsuperscript{−/−} Apoe\textsuperscript{−/−} aortic arches (Figure 4E and 4F). In contrast, we found no difference in the numbers of T cells isolated from the thoracoabdominal aortas of II\textsubscript{17}a\textsuperscript{−/−} Apoe\textsuperscript{−/−} and II\textsubscript{17}ra\textsuperscript{−/−} Apoe\textsuperscript{−/−} mice in comparison with Apoe\textsuperscript{+/-} controls (1.5±0.2×10\textsuperscript{5}, 1.4±0.2×10\textsuperscript{5}, 1.3±0.1×10\textsuperscript{5} cells/thoracoabdominal aorta, respectively, P=0.7, Figure 4E and 4F).

To further understand the role of II\textsubscript{17}A/II\textsubscript{17}RA in the regulation of aortic leukocyte numbers, we next examined the myeloid cell content of the aortas, PLNs, spleens, and blood of WD-fed II\textsubscript{17}a\textsuperscript{−/−} Apoe\textsuperscript{−/−}, II\textsubscript{17}ra\textsuperscript{−/−} Apoe\textsuperscript{−/−}, and Apoe\textsuperscript{+/-} mice. CD45\textsuperscript{+}CD11b\textsuperscript{+} cells were notably reduced in II\textsubscript{17}a\textsuperscript{−/−} Apoe\textsuperscript{−/−} and II\textsubscript{17}ra\textsuperscript{−/−} Apoe\textsuperscript{−/−} aortas in comparison with Apoe\textsuperscript{+/-} aortas (2.6±0.4×10\textsuperscript{5}, 2.3±0.4×10\textsuperscript{5}, and 4.1±0.8×10\textsuperscript{5} cells, respectively, P<0.05). To examine myeloid cell subsets, we performed staining for CD68 and the neutrophil/myelocyte marker Gr-1, which recognizes the Ly6C and Ly6G sites. We found 3 major populations: CD68\textsuperscript{+}Gr-1\textsuperscript{−}, MΦ, CD68\textsuperscript{+}Gr-1\textsuperscript{+} neutrophils, and a subset of CD68\textsuperscript{+}Gr-1\textsuperscript{−} leukocytes within the aortas (Figure 5). To better characterize CD68\textsuperscript{+}Gr-1\textsuperscript{+} cells, we analyzed the expression of CCR1, L-selectin, and Ly6C. CD68\textsuperscript{+}Gr-1\textsuperscript{+} cells were found to express CCR1, L-selectin, and intermediate levels of Ly6C (Online Figure 1). While the 2 major subsets of monocytes—proinflammatory (Ly-6C\textsuperscript{hi} Gr1\textsuperscript{−}CCR2\textsuperscript{+} CX3CR1\textsuperscript{low}) and patrolling (Ly-6C\textsuperscript{lo} Gr1 CCR2 CX3CR1\textsuperscript{hi}) monocytes—are well characterized, little is known about Ly6C\textsubscript{int} cells. Our results suggest that Ly6C\textsubscript{int} cells are CD68\textsuperscript{+}Gr-1\textsuperscript{+} with some features (L-selectin\textsuperscript{+}CCR2\textsuperscript{+}) of proinflammatory monocytes (Online Figure 1). Although the relative percentages of aortic MΦ and neutrophils were unaltered between II\textsubscript{17}a\textsuperscript{−/−} Apoe\textsuperscript{−/−} and Apoe\textsuperscript{+/-} mice (Figure 5A), we found a significant decrease in the number of aortic CD68\textsuperscript{+}Gr-1\textsuperscript{−} MΦ, CD68\textsuperscript{+}Gr-1\textsuperscript{+} leukocytes and CD68\textsuperscript{−}Gr-1\textsuperscript{+} neutrophils in II\textsubscript{17}ra\textsuperscript{−/−} Apoe\textsuperscript{−/−} aortas (Figure 5B). There was no difference in splenic or peripheral blood myeloid cell subsets in II\textsubscript{17}a\textsuperscript{−/−} Apoe\textsuperscript{−/−} and Apoe\textsuperscript{+/-} mice (unpublished data).

To determine if the II\textsubscript{17}A/II\textsubscript{17}RA dependent-reduction of MΦ and neutrophils occurred predominantly in the aortic arch, we assessed the aortic arches and thoracoabdominal aortas of II\textsubscript{17}a\textsuperscript{−/−} Apoe\textsuperscript{−/−}, II\textsubscript{17}ra\textsuperscript{−/−} Apoe\textsuperscript{−/−}, and Apoe\textsuperscript{+/-} mice by flow cytometry. We found distinct effects of II\textsubscript{17}A and II\textsubscript{17}RA deficiency on the myeloid cell content in the aortic arch, but not in the thoracoabdominal aortas of II\textsubscript{17}a\textsuperscript{−/−} Apoe\textsuperscript{−/−} and II\textsubscript{17}ra\textsuperscript{−/−} Apoe\textsuperscript{−/−} mice. While the relative percentage of MΦ and neutrophils were unaltered (Figure 5C), the total number of aortic arch MΦ and neutrophils were
Figure 4. Disruption of the IL-17A/IL-17RA axis decreases the overall T cell content of the aortic arch, but not the thoracoabdominal aorta. 

A. Aortic cell suspensions from 15-week WD-fed Il17a<sup>−/−</sup> Apoe<sup>−/−</sup>, Il17ra<sup>−/−</sup> Apoe<sup>−/−</sup>, and Apoe<sup>−/−</sup> mice were stained and analyzed by flow cytometry (n=7 mice/genotype, 5 independent experiments; white bars, Il17a<sup>−/−</sup> Apoe<sup>−/−</sup>, gray bars, Il17ra<sup>−/−</sup> Apoe<sup>−/−</sup>, and black bars, Apoe<sup>−/−</sup> mice).

B. The mRNA expression of Il6, Ifnγ, and Tnf from the aortas Il17a<sup>−/−</sup> Apoe<sup>−/−</sup>, Il17ra<sup>−/−</sup> Apoe<sup>−/−</sup>, and Apoe<sup>−/−</sup> mice (n=12 aortas/genotype, 3 independent experiments).

C. Representative flow cytometry plots of IFNγ<sup>+</sup> CD3<sup>+</sup> and IL-17A<sup>+</sup> CD3<sup>+</sup> cells in the aortas of 15-week WD-fed Il17a<sup>−/−</sup> Apoe<sup>−/−</sup>, Il17ra<sup>−/−</sup> Apoe<sup>−/−</sup>, and Apoe<sup>−/−</sup> mice. Plots are gated on CD45<sup>+</sup> CD3<sup>+</sup> T cells (n=5 mice/genotype, 4 experiments).

D. Total number of IFNγ<sup>+</sup> CD3<sup>+</sup> and IL-17A<sup>+</sup> CD3<sup>+</sup> T cells from Apoe<sup>−/−</sup>, Il17a<sup>−/−</sup> Apoe<sup>−/−</sup>, and Il17ra<sup>−/−</sup> Apoe<sup>−/−</sup> aortic arch and thoracoabdominal aortas. 

E, F. Total number of aortic arch and thoracoabdominal aortic TCR<sup>+</sup> T cells from Apoe<sup>−/−</sup>, Il17a<sup>−/−</sup> Apoe<sup>−/−</sup>, and Il17ra<sup>−/−</sup> Apoe<sup>−/−</sup> mice. The data depict means±SEM. *P<0.05, **P<0.01, ***P<0.001.
reduced by ≈44% in Il17ra−/−Apoe+/− and Il17a−/−Apoe−/− mice (Figure 5D). These findings further implicate the IL-17A/IL-17RA axis as a regulator of aortic myeloid cell content. Interestingly, despite the prevalent effects of IL-17RA deficiency within the aortic arch, the reduction of MΦs in the aortic arch (≈44%, Figure 5D) was smaller in comparison with MΦ reduction in the whole aorta (≈63%, Figure 5B) of Il17ra−/−Apoe−/− mice, suggesting that other proatherogenic factors, in addition to MΦs, are regulated by the IL-17A/IL-17RA axis within the aortic arch.

IL-17A/IL-17RA Supports TNFα and Aortic Chemokine Production Within the Aortic Arch of Apoe−/− Mice

As IL-17A induces the production of reactive oxygen species (ROS), proinflammatory chemokines, and cytokines at sites of inflammation, including the vasculature, we hypothesized that IL-17A may potentially regulate the homing of leukocytes to atherosclerotic aortas. In keeping with these findings, we evaluated aortic chemokine expression in Il17ra−/−Apoe+/−, Il17a−/−Apoe−/−, and Apoe−/− mice (Figure 6A).
Cc12, Ccl20, Cx3cl1, Cxcl1, Cxcl12, Cxcl2, and Cxcl5 mRNA expression was reduced in WD-fed Il17a−/−Apoe−/− and Il17ra−/−Apoe−/− aortas in comparison with Apoe−/− aortas (Figure 6A), indicating that the IL-17A/IL-17RA axis regulates the expression of several chemokines within the aorta. As CCL2 and CX3CL1 support monocyte migration,23 and CXCL1, CXCL2, and CXCL5 participate in neutrophil recruitment into the aorta,24 these data implicate the IL-17A/IL-17RA axis as a strong potential regulator of monocyte and neutrophil migration via aortic chemokine induction.

Monocytes express IL-17RA and IL-17RC and chemotactically migrate toward IL-17A both in vitro and in vivo models of rheumatoid arthritis.25,26 We previously found that IL-17A supported monocyte adherence to explanted Apoe−/− aortas in an ex vivo adhesion assay.13 To further determine the role of vascular IL-17RA in monocyte adhesion, we explanted whole aortas from WD-fed Il17ra−/−Apoe−/− mice and coincubated them with CFSE-labeled Apoe−/− monocytes in the presence or absence of IL-17A (Figure 6B) to assess the contribution of aortic IL-17RA to IL-17A/IL-17RA-dependent monocyte adherence. Consistent with our prior data,13 IL-17A increased the adhesion of Apoe−/− monocytes to Apoe−/− aortas by 48±4% (Figure 6B1 and 6B2). Cocultures of Apoe−/− monocytes and Il17ra−/−Apoe−/− aortas resulted in a 13±5% (P<0.05) increase in adhesion in response to IL-17A (Figure 6B3 and 6B4). Thus as IL-17A-induced adhesion was significantly reduced when IL-17RA-deficient Apoe−/− aortas were used (Figure 6B), vascular IL-17RA likely supports monocyte adherence to the aortic lumen.

To further understand the potential effects of the IL-17A/IL-17RA axis in leukocyte recruitment into the aorta, we next examined the expression of IL-17A, IL-17RA, and several chemokines within the aortic arch and thoracoabdominal aortas of Il17ra−/−Apoe−/− or Il17a−/−Apoe−/− and Apoe−/− mice. While IL-17RA mRNA was expressed throughout the aorta with no significant differences between the aortic arch and thoracoabdominal aorta (Figure 7A), IL-17A was preferentially detected within the aortic arch of Apoe−/− mice (Figure 7B). These data highlight the distinct
arch-specific expression of IL-17A and suggest a reason for the preferential effects of IL-17A/IL-17RA deficiency on the aortic arch at this time point. Additionally, we detected elevated levels of TNFα and CXCL2 in \textit{Apo}^{-/-} aortic arches in comparison to \textit{Apo}^{-/-} thoracoabdominal aortas (Figure 7C and D, respectively).

Next, we investigated the effects of IL-17A or IL-17RA deficiency on the expression of TNFα and several chemokines within the aortic arch and thoracoabdominal aorta. Deficiency of IL-17A or IL-17RA led to reduced TNFα protein expression in the aortic arch, without affecting the thoracoabdominal aorta, suggesting that the IL-17A/IL-17RA axis is a regulator of TNFα synthesis in atherosclerosis-prone sites of the aorta (Figure 7C). Deficiency of IL-17A or IL-17RA also reduced \textit{Cxcl2} expression within the aortic arch in comparison with \textit{Apo}^{-/-} mice (Figure 7D). In contrast, no difference in \textit{Cxcl2} expression was detected among \textit{Il17a}^{-/-}, \textit{Il17ra}^{-/-}, and \textit{Apoe}^{-/-} thoracoabdominal aortas (Figure 7D). Together, these data highlight an important role of the IL-17A/IL-17RA axis in the up-regulation of TNFα and several chemokines within the aortic arch during atherogenesis. Interestingly, we also found that not all chemokines were regulated by the IL17A/IL-17RA axis exclusively in the aortic arch. \textit{Cxcl1}
mRNA expression, but not CXCL1 protein expression, was attenuated in the aortic arches of Il17a−/− Apoe−/− and Il17ra−/− Apoe−/− mice in comparison with Apoe−/− mice (Figure 7E).

Recruitment of Neutrophils and Monocytes to Atherosclerotic Aortas Is Reduced in IL-17A- or IL-17RA- Deficient Apoe−/− Mice

To investigate the effects of the IL-17A/IL-17RA axis on in vivo monocyte and neutrophil homing into aortas, we performed short-term adoptive transfer experiments. CFSE-labeled peripheral blood leukocytes were adoptively transferred into Il17a−/− Apoe−/− and Il17ra−/− Apoe−/− mice to directly investigate the migration of monocytes and neutrophils in the recipients. To identify subsets of CD45+CFSE+CD11b+ cells containing CD68+Gr-1+ monocytes, Gr-1+CD68+ leukocytes, and Gr-1+CD68− neutrophils, circulating blood was used to characterize myeloid cell populations and analyze the distribution of emigrating cells in the aortas (Figure 8A). Although the percentage of emigrated CFSE+CD11b+ monocytes and neutrophils was unaltered between Il17ra−/− Apoe−/− and Apoe−/− aortas (Figure 8A), the absolute number of CD45+CD11b+CFSE+CD68+Gr-1+ MΦ, Gr-1+CD68− neutrophils, and Gr-1+CD68+ leukocytes was significantly lower in the aortas of Il17ra−/− Apoe−/− recipients (Figure 8B). Interestingly, while the absence of IL-17RA reduced monocyte and neutrophil homing into the aortas, it did not alter myeloid cell trafficking into the spleen and PLN (unpublished data). The number of circulating CFSE+ monocytes in the blood were unaltered between the recipients; however, the number of circulating CFSE+ Gr-1+CD68+ neutrophils and Gr-1+CD68− leukocytes were slightly decreased in Il17ra−/− Apoe−/− recipients (P<0.05, Figure 8B). To assess the role of IL-17RA in the migration of myeloid cells to the aorta, we performed competitive homing experiments to examine the migration of IL-17RA-deficient and IL-17RA-sufficient monocytes and neutrophils to Apoe−/− aortas. We found no difference between the migration of Il17ra−/− Apoe−/− and Apoe−/− monocytes and neutrophils to the aortas of Apoe−/− recipients (unpublished data). Overall, these in vitro and in vivo results suggest that the IL-17A/IL-17RA axis plays a proinflammatory role during atherogenesis through the induction of aortic chemokines and the recruitment of neutrophils and monocytes to atherosclerotic plaque.

Discussion

The development and persistence of atherosclerosis depends on chronic inflammation mediated by both the innate and adaptive immune responses. Several recent publications have convincingly shown elevated levels of circulating and arterial Th17 and IL-17A+ T cells in atherosclerosis-prone Apoe−/− and Ldlr−/− mice and CAD patients. Despite a clear correlation between elevated levels of IL-17A and atherosclerosis, to date, the functions of IL-17A-producing cells and IL-17A in atherosclerosis remain poorly defined.
In this study, we sought to delineate the role of IL-17A\textsuperscript{+} cells and the IL-17A/IL-17RA pathway in atherosclerosis through the use of newly generated \textit{Il17a}\textsuperscript{−/−} \textit{Apoe}\textsuperscript{−/−} and \textit{Il17ra}\textsuperscript{−/−}\textit{Apoe}\textsuperscript{−/−} mice. We previously reported a reduction in atherosclerotic plaques in soluble IL-17RA-blockaded \textit{Apoe}\textsuperscript{−/−} mice.\textsuperscript{15} In the present study, we expanded our focus and investigated the impact of IL-17A or IL-17RA deficiency on atherosclerosis. We found a significant decrease in the aortic lesions of WD, but not chow diet-fed \textit{Il17a}\textsuperscript{−/−} \textit{Apoe}\textsuperscript{−/−} and \textit{Il17ra}\textsuperscript{−/−}\textit{Apoe}\textsuperscript{−/−} mice in comparison with \textit{Apoe}\textsuperscript{−/−} mice. To further dissect the roles of the IL-17A/IL-17RA axis, we examined several potential sites of atherosclerosis including the aortic arch, thoracoabdominal aorta, and aortic roots of mice fed WD. IL-17A/IL-17RA deficiency had striking inhibiting effects on atherosclerotic plaque burden in the aortic arch, but only minor effects within the thoracoabdominal compartment. The observation that IL-17A exerts limited effects on the descending aorta is in line with the findings of Madhur et al that IL-17A-deficient \textit{Apoe}\textsuperscript{−/−} mice display slight nonsignificant reductions in thoracoabdominal aortic plaques in comparison with \textit{Apoe}\textsuperscript{−/−} controls.\textsuperscript{18} There are several examples of the site-specific development and progression of atherosclerosis.\textsuperscript{26} Deficiency of IL-4 within the bone marrow resulted in no change in aortic-root lesion area, despite reduced \textit{en face} lesion areas.\textsuperscript{29} The deficiency of p47 NADPH oxidase subunit II in \textit{Apoe}\textsuperscript{−/−} mice reduced aortic atherosclerosis to a greater extent than the aortic root.\textsuperscript{30} In the present study, we characterized the aortic arch-specific influence of the IL-17A/IL-17RA axis.

Histological examination of the aortic arch and thoracoabdominal aortic collagen content surprisingly revealed no differences between \textit{Il17ra}\textsuperscript{−/−} \textit{Apoe}\textsuperscript{−/−} and \textit{Apoe}\textsuperscript{−/−} mice at this time point. Interestingly, we also found no difference in SMC content between \textit{Il17a}\textsuperscript{−/−} \textit{Apoe}\textsuperscript{−/−} and \textit{Apoe}\textsuperscript{−/−} mice, indicating that the IL-17A/IL-17RA axis does not significantly influence SMC proliferation and migration within the atherosclerotic aorta. While several recent studies have suggested that IL-17A may support SMC proliferation in vitro,\textsuperscript{11,31} Danzaki et al demonstrated elevated aortic root SMC content in 8-week WD-fed \textit{Il17a}\textsuperscript{−/−} \textit{Apoe}\textsuperscript{−/−} mice.\textsuperscript{32} Further examination of the effects of the IL-17A/IL-17RA axis on aortic SMC and endothelial cell functions are ultimately necessary.

One of the striking observations of this study was the differences in atherosclerotic lesions between the aortic arch and thoracoabdominal aorta of 15-week WD-fed mice. To assess the potential mechanisms for this phenomenon, we analyzed the expression of IL-17A and IL-17RA at these different anatomic sites of the aorta. IL-17RA was expressed ubiquitously in the aorta, whereas IL-17A expression was preferentially found within the aortic arch. Interestingly, we detected no difference in the expression of IL-17A in the TA and AA in 5-week-old \textit{Apoe}\textsuperscript{−/−} mice, indicating that the specific up-regulation of IL-17A does not occur in relatively healthy aortas. Altogether, our findings suggest that the aortic expression of IL-17A may depend on the stage of lesion development, which varies at different anatomic locations. The stage of lesion development also depends on the local inflammation that accompanies atherogenesis. Indeed, elevated expression of the proinflammatory cytokine TNFα and several chemokines that are involved in aortic leukocyte migration were preferentially detected in the aortic arches of 15-week WD-fed \textit{Apoe}\textsuperscript{−/−} mice. Further temporal studies will be required to determine if IL-17A-producing cells may be similarly recruited into advanced thoracoabdominal aortas.

To date, the exact role of IL-17A\textsuperscript{+} cells in atherosclerosis is unclear because of conflicting results from neutralizing antibody and bone marrow transfer experiments.\textsuperscript{12–14,16–18,27} However, some of the discrepant results may be attributable to differences in the experimental design, diets, or other confounding factors. In addition, most of these studies have examined aortic root atherosclerosis, but only a few studies directly examined whole aortic plaque burden by \textit{en face} analysis. The results reported here clearly demonstrate the aortic arch-specific effects of IL-17A/IL-17RA, and at least partially shed light on the controversy of reported effects of IL-17A/IL-17RA axis blockade during atherogenesis.

Th17 cells play an important role in the immune response, and are major contributors to autoimmune diseases such as multiple sclerosis, inflammatory bowel disease, and arthritis.\textsuperscript{5} There are several pathways by which IL-17A-producing T cells might affect local inflammation. IL-17A supports the production of IL-6 and IL-8, and the chemokines CCL5, CCL2, CXCL1, and CXCL10 in several cell types, including endothelial and vascular smooth muscle cells (VSMCs).\textsuperscript{33,34} Fibroblasts, and epithelial cells.\textsuperscript{6,20} The results reported here clearly demonstrate that the IL-17A/IL-17RA axis affects the expression of multiple aortic chemokines, including Ccl2, Ccl20, Cx3cl1, Cxcl1, Cxcl12, Cxcl2, and Cxcl5, thus accelerating leukocyte recruitment to atherosclerotic vessels.

In support of this notion, we detected reduced numbers of T cells, CD68\textsuperscript{+} Gr-1\textsuperscript{+} MΦ, CD68\textsuperscript{−} Gr-1\textsuperscript{+} myeloid cells, and CD68\textsuperscript{−} Gr-1\textsuperscript{−} neutrophils in WD-fed \textit{Il17ra}\textsuperscript{−/−} \textit{Apoe}\textsuperscript{−/−} and \textit{Il17a}\textsuperscript{−/−} \textit{Apoe}\textsuperscript{−/−} aortas at steady state conditions. Interestingly, the phenotype of reduced aortic MΦ content was also reported when anti-IL-17A Abs were used to block IL-17A functions in vivo.\textsuperscript{12,16} Importantly, additional separate examination of the aortic arch and thoracoabdominal segments of \textit{Il17ra}\textsuperscript{−/−} \textit{Apoe}\textsuperscript{−/−}, \textit{Il17a}\textsuperscript{−/−} \textit{Apoe}\textsuperscript{−/−}, and \textit{Apoe}\textsuperscript{−/−} aortas by flow cytometry revealed diminished numbers of T cells, MΦ, and neutrophils specifically within the aortic arches of \textit{Il17ra}\textsuperscript{−/−} \textit{Apoe}\textsuperscript{−/−} and \textit{Il17a}\textsuperscript{−/−} \textit{Apoe}\textsuperscript{−/−} mice. Thus, these results clearly emphasize a distinct role for the IL-17A/IL-17RA axis in the regulation of the number of T and myeloid cells within the aortic arch, and to a lesser extent, the thoracoabdominal aorta. It is interesting to note that the reduction in leukocyte cellularity within the aortic arch was relatively smaller in comparison with the reduction in plaque burden, suggesting that other IL-17/IL-17RA-dependent factors in addition to the total cellularity are involved in atherogenesis.

Monocytes express IL-17RA, and recent data suggest that IL-17A can also directly affect monocyte chemotaxis in vivo and in vitro.\textsuperscript{25} Antibody blockade of IL-17A in the synovial fluid of rheumatoid arthritis patients inhibited in vitro monocyte chemotaxis, and MΦ accumulation in the bronchoalveo-
lar lavage fluid during allergic airway inflammation. Le-thally irradiated low-density lipoprotein receptor-deficient (Ldlr−/−) mice reconstituted with IL-17RA-deficient bone marrow resulted in reduced aortic root lesions, neutrophil, and mast cell content in comparison with Il17ra−/− recipients, suggesting a potential role of IL-17RA on hematopoietic populations during atherogenesis. Evidence has also demonstrated that vascular ECs and SMCs express IL-17RA, and are able to respond to IL-17A. To address the extent to which IL-17RA expression by vascular cells impacts IL-17A-induced adhesion to atherosclerotic aortas, we performed ex vivo adhesion assays. Supplementation of explanted aortas with rIL-17A strongly supported monocyte adherence in a manner that depended on vascular IL-17RA.

The recruitment of leukocytes into the aortas during the initial and established stages of atherosclerosis is one of the key components of the progression of atherosclerosis. To further establish the role of IL-17A in the regulation of leukocyte content in the aortas, we determined whether the IL-17A/IL-17RA axis affects the migration of monocytes and neutrophils to the aortas in short-term adoptive transfer experiments. We detected significant reductions of CFSE+ emigrated Apo−/− monocytes and neutrophils within the aortas of Il17ra−/− mice when compared to control Apo+/− recipients, highlighting an essential role of vascular IL-17RA in supporting myeloid cell migration. Of note, we found no difference in the migration of Il17ra−/−Apo−/− and Apo+/− monocytes and neutrophils to Apoe−/− aortas in the short-term homing experiments. These findings suggest that the reduction of monocyte and neutrophil content of the aorta in steady state conditions is due to impaired capacity of myeloid cells to migrate into atherosclerotic aortas in the absence of IL-17A or vascular IL-17RA. Interestingly, as studies involving chemokine or chemokine receptor-deficient mice display distinct anatomic alterations in atherosclerotic plaque formation, the overt phenotype of Il17a−/−Apo−/− and Il17ra−/−Apo−/− mice can be partially attributed to the regulation of multiple aortic chemokines by IL-17A.

While several studies have suggested a proatherogenic role of IL-17A on the basis of neutralizing strategies and bone marrow transfers, Taleb et al and Danzaki et al recently proposed an atheroprotective role for IL-17A+ T cells through cross-regulation of Th1 cells and IFNγ. Danzaki et al demonstrated elevated Th1 cell content and IFNγ production in 8-week WD-fed Il17a−/−Apo−/− splenocytes. Although initial in vitro differentiation assays have implicated cross-regulation of Th17 cell differentiation by Th1 cells, recent data have shown that Th17 cells are induced in parallel to Th1 cells in some pathological conditions. Indeed, while Th1 cells represent the major T helper subset in atherosclerosis, Th17, Th1, and Th1/Th17 cells are present in both murine and human atherosclerotic arteries. Therefore, we tested whether the deficiency of IL-17A or IL-17RA would alter the Th1 response during atherogenesis. While the percentage and number of splenic and peripheral blood Th1 cells were unaltered, the numbers of aortic Th1 cells were diminished within Il17a−/−Apo−/− and Il17ra−/−Apo−/− mice. These data indicate that the deficiency of IL-17A or IL-17RA has no effect on the generation and maintenance of Th1 cells in atherosclerosis. Altogether, our data suggest that while the IL-17A/IL-17RA axis does not affect the percentage of Th1 cells, it does influence the number of aortic T cells and, therefore, the total levels of T cell–derived IFNγ.

In summary, using IL-17A- and IL-17RA-deficient Apoe−/− mice, we demonstrate that the IL-17A/IL-17RA pathway plays a proinflammatory role during atherogenesis preferentially within the aortic arch. On atherogenesis, Th17 and other IL-17A-producing cells accumulate within the aortas and release IL-17A, which in turn produces the induction of TNFα and various chemokines, resulting in accelerated monocyte and neutrophil homing and further development of atherosclerosis preferentially within the aortic arch of the aorta.

Acknowledgments

We thank Dr. Iwakura for providing breeding pairs of Il17a−/− mice and Amgen Inc. for providing breeding pairs of Il17ra−/− mice.

Sources of Funding

This work was supported by the NHLBI HL107522 (to E.V. Galkina) and AHA Predoctoral Fellowship grant 11PRE7520041 (to M.J. Butcher).

Disclosures

None.

References

The role of IL-17A in atherosclerosis is currently unclear because of conflicting data obtained from IL-17A neutralization studies and differences in measurements of atherosclerosis. In other pathological states, IL-17A promotes leukocyte recruitment to sites of inflammation by supporting the production of stromal chemokines. In the present study, we sought to clarify the role of IL-17A in atherosclerosis using both IL-17A- and IL-17A-deficient atherosclerosis-prone ApoE−/− mice. We demonstrate that IL-17A and IL-17A-deficient mice display reductions in aortic arch but not in extent of thoracoabdominal aortic plaques, numbers of leukocytes, nor levels of chemokines. Additionally, examination of aortic and thoracoabdominal aortic expression of Il17a and Il17a within WD-fed mice revealed the exclusive expression of IL-17A within the aortic arch, which corresponded with elevated levels of proinflammatory TNFα, CCL2, and CCL1. Adoptive transfer experiments demonstrate reduced recruitment of monocytes and neutrophils to the aortas of IL-17A-deficient ApoE−/− mice. These findings suggest that IL-17A plays a site-specific proinflammatory role and that it specifically stimulates chemokine and cytokine production, as well as monocyte and neutrophil recruitment in the aortic arch.
The IL-17A/IL-17RA Axis Plays a Proatherogenic Role via the Regulation of Aortic Myeloid Cell Recruitment
Matthew J. Butcher, Breanne N. Gjurich, Tracy Phillips and Elena V. Galkina

Circ Res. published online February 2, 2012;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2012/02/01/CIRCRESAHA.111.261784

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2012/02/02/CIRCRESAHA.111.261784.DC1

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

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

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Materials and Methods.

Dyes, recombinant proteins and antibodies. The antibodies used were as follows: TCR\(\alpha\beta\)-FITC (H57-597), Ly6C-FITC (AL-21), CD68-PE (FA11), IL-17A-PE (TC11-18H10), ROR\(\gamma\)t-APC (AFKJS-9), CD45-PerCP or PO (30-F11), CD11b-PB (M1/70.15), IFN\(\gamma\)-eFluor 450 (XMG1.2), CD4-APC (L3T4), Gr-1-Alexa Fluor 647 or APC-Alexa Fluor 750 (RB6-8C5), Foxp3-PE or Alexa Fluor 647 (MF23), CD3-FITC or APC-CY7 (17A2), and anti-mouse CD16/CD32 (The Lymphocyte Culture Centre, UVA). To distinguish between live and dead cells in flow cytometry experiments, a LIVE/DEAD® Aqua Dead Cell Stain Kit (Invitrogen) was used. Recombinant mouse rIL-17A was purchased from R&D Systems.

Preparation of mouse tissues and histology.

En Face. Aortas were excised and analyzed for atherosclerotic lesions using Oil Red O as previously described.\(^1\) In some experiments, the aortic arch and thoracoabdominal portions of en face aortas were analyzed separately using image J(v1.44).

Aortic Roots. For immunohistochemistry, mice were perfused by cardiac puncture with 4% PFA and hearts were collected. Sequential 5\(\mu\)m aortic root sections were cut from the point of appearance of the aortic valve leaflets. Three 5\(\mu\)m aortic root sections >300\(\mu\)m were collected and analyzed by Russell modified-Movat staining as previously described.\(^1\)

Cross-sectional analysis of aortic regions. 12 week WD fed Apoe\(^{-}\) and \(\text{Il17ra}^{-}\)Apoe\(^{-}\) aortas (n=8 mice/group) were perfusion fixed with 4% PFA by cardiac puncture, excised, and subdivided into three major segments: the proximal half of the aortic arch (from the origin to the left common carotid artery), the distal half of the aortic arch (from the left common carotid artery to the end of the curvature), and the thoracoabdominal aorta (from the end of the curvature to the origin of the common iliac arteries). The aortic segments were paraffin embedded and sequential 4\(\mu\)m sections through the proximal and distal aortic arch were collected. Thirty sequential 4\(\mu\)m sections were collected from the thoracoabdominal aorta. Three-five proximal, distal, thoracoabdominal aortic sections/mouse were deparaffinized, rehydrated, endogenous peroxidase activity destroyed, and followed by antigen retrieval with antigen unmasking solution (Vector Laboratories) as previously described.\(^2\) Sections were blocked with normal horse serum (Invitrogen, Life Technologies), avidin/biotin blocking kit (Vector Laboratories), and stained with monoclonal anti-actin \(\alpha\)-smooth muscle (Clone 1A4, Sigma) using biotinylated horse anti-mouse IgG (BA-2001, Vector) and the avidin-biotin peroxidase method (Vector Laboratories). Collagen maturation was determined using a picrosirius red stain kit (Polysciences Inc., Warrington, PA) following the manufacturer’s protocol. Two independent observers quantified the staining using Image J (v1.44).

Quantitative real time PCR. Total RNA was extracted from aortas and corresponding aortic arch and thoracoabdominal aortic segments using Trizol® reagent (Invitrogen). Contaminating genomic DNA was removed by DNase I treatment using RNeasy kits (Qiagen, Valencia, CA) according to the manufacturer’s instructions. 1\(\mu\)g of total RNA was reverse transcribed in cDNA synthesis reactions containing Moloney murine leukemia virus reverse transcriptase, 10\(\mu\)m dNTPs, and random hexamers (all from Invitrogen). RT-PCR was performed using mouse Th17 PCR profiler arrays (SA Bioscience, Qiagen, Frederick, MD) or Taqman probes from Applied Biosystems (Carlsbad, CA). Ct values were determined using an iCycler iQ Real-time detection system (Bio-Rad laboratories, Hercules, CA). The results were normalized to Actb, Gapdh, and Hsp90ab1 as housekeeping genes. Data is presented as a fold change compared to a reference (1 fold) as determined by the 2\(^{-}\Delta\Delta\text{Ct}\) method.
Flow Cytometry. Single cell suspensions from the aorta and surrounding adventitia were prepared as described. Briefly, mice were anesthetized and their vasculature was perfused by cardiac puncture with PBS containing 20 U/ml of Heparin. Aortas were microdissected and subsequently 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 (Sigma-Aldrich, St. Louis, MO) in PBS for 1 hour at 37°C as previously described. In some experiments, separate cell suspensions were prepared from the aortic arch and thoracoabdominal aorta by microdissecting the aorta prior to enzymatic digestion. For intracellular cytokine staining, splenic, peripheral lymph node (PLN), and aortic single cell suspensions were cultured for 5 hours with RPMI 1640 supplemented with 10% FBS, 1% Penicillin/Streptomycin, 10 ng/ml PMA, 500 ng/ml calcium ionophore and GolgiStop. Intracellular staining for IFNγ, IL-17A, and CD68 was performed using Fix&Perm® cell permeabilization reagents (BD Biosciences). A Cytek DXP 8 Color 488/637/407 (Cytek Development Inc.) upgraded FACSCalibur™ (BD Biosciences) was used with FlowJo (Tree Star Inc., Ashland, OR.) to collect and analyze data. For all flow cytometry experiments, gates were set based on isotype and fluorescent minus one controls.

Detection of cytokine production within aortic segments. Aortic supernatants were prepared as previously described. Briefly, 12 week WD fed Il17a−/−Apoe−/−, Il17ra−/−Apoe−/−, and Apoe−/− aortas (n=6/genotype) were excised and subdivided into the aortic arch and thoracoabdominal aortic segments. Aortic supernatants were prepared by chopping the aortic segments into small pieces in 50μl of plain RPMI-1640 and incubating for 20 min at 37°C. The supernatants were subsequently collected and cytokines were detected using TNF-α, CXCL1, and CXCL2 Flow Cytomix assays (eBioscience) following the manufacturer's instructions.

Ex vivo monocyte adhesion and co-incubation assays. The assay was performed as described previously. Briefly, aortas from 15 week WD fed Il17ra−/−Apoe−/− and Apoe−/− mice were explanted in RPMI-1640 containing 10% FBS, 1% Penicillin/Streptomycin, with or without 100ng/ml of rIL-17A overnight at 37°C. Aortas were subsequently opened longitudinally and pinned to sterile agarose in conditioned media. In parallel, monocytes were isolated from Apoe−/− peripheral blood by positive selection via anti-CD115-biotin Abs and streptavidin-MACS microbeads (Miltenyi Biotec), and labeled with 2 μM Carboxyfluorescein diacetate-succinimidyl ester (CFSE) as earlier described. CFSE-labeled monocytes were co-incubated with pinned aortas in conditioned media for an hour. Unbound monocytes were washed away with PBS and the number of monocytes adhered to the aorta was counted using fluorescent microscopy.

Measurement of plasma lipids. Plasma triglyceride and total cholesterol levels were determined using Cholesterol E kits (Wako Diagnostics, Wako Chemicals USA Inc, Richmond, VA) following the manufacturer’s instructions.

Adoptive Transfer Experiments. For experiments involving the adoptive transfer of peripheral blood leukocytes, heparinized blood was pooled from 5-10 WD fed Apoe−/− mice. Erythrocytes were lysed using ACK lysis buffer (8.29mg/ml NH₄CL, 1mg/ml KHCO₃, 0.372mg/ml EDTA, all from Sigma-Aldrich). Leukocytes were labeled with 0.1 μM CFSE (Invitrogen) in PBS at 37°C for 10 minutes and washed twice in PBS containing 1% FCS. 5x10⁶ labeled cells in 0.2 ml of PBS were injected into WD fed Apoe−/− and Il17ra−/−Apoe−/− recipients via tail vein injection (i.v.). Aortas, blood, spleens and PLNs were collected 12 hrs after adoptive transfer for flow cytometry analysis.

Statistical Analysis. Differences in lymphocyte homing in the adoptive transfer experiments were compared using paired Student’s test. Other comparisons were made using unpaired Student’s test with the data expressed as mean±SEM. Comparisons of three or more groups
were conducted using multiple unpaired t tests and the Bonferroni-Holm correction for multiple comparisons. Statistical significance was set at \( p < 0.05 \).
Supplemental Figure I. CD68<sup>+</sup>Gr-1<sup>+</sup> cells express CD62L, CCR2, CCR1, and intermediate levels Ly6C. (A-B) Representative CD68<sup>+</sup>Gr-1<sup>+</sup> splenocyte FACS plot from 12 week WD-fed Apoe<sup>−/−</sup> mice. Apoe<sup>−/−</sup> splenocytes were stained with anti-CD45, CD11b, CD68, Gr-1, Ly6C, CCR1, CD62L, and CCR2-specific abs and analyzed by flow cytometry (n=5 mice, two independent experiments). The representative flow cytometry plots gated on CD45<sup>+</sup>CD11b<sup>+</sup> cells (top left) and sub-gated on CD45<sup>+</sup>CD11b<sup>+</sup>CD68<sup>+</sup>Gr-1<sup>+</sup> cells (top right and bottom row).
References


