The Lamina Adventitia Is the Major Site of Immune Cell Accumulation in Standard Chow-Fed Apolipoprotein E–Deficient Mice

Michael P.W. Moos, Nicole John, Rolf Gräßner, Silke Noßmann, Bernd Günther, Rüdiger Vollandt, Colin D. Funk, Brigitte Kaiser, Andreas J.R. Habenicht

Objective—Cells of adaptive immunity have been implicated in atherogenesis. Though substantial information is available on immune cells in atherosclerotic lesions of the lamina intima, cells in the lamina adventitia have received less attention.

Methods and Results—The composition of immune cells in the innominate artery and abdominal aorta was examined in young, adult, and old apolipoprotein (apo) E/− mice and wild-type mice on standard mouse chow. In the innominate artery of apoE/− mice, adventitial T cells increased at 32, 52, and 78 weeks exceeding those of the intima by 6-, 24-, and 85-fold. Single T cells dominated in young mice, later T/B cell clusters emerged, and lymphoid-like structures reminiscent of inflammatory follicles formed preferentially in the abdominal aorta of old mice. Follicles contained organized sets of immune response-regulating cells: Interdigitating dendritic cells, T cell effectors, proliferating B cells, and plasma cells. Adventitial T cell inflammation was associated with a marked increase in transcripts of the chemokine MIP-1α in the aorta but not in spleen or liver.

Conclusions—Adventitial lymphocyte infiltration and formation of inflammatory follicle-like structures in the abdominal aorta of old apoE/− mice point to the adventitia as a site of local adaptive immune reactions during atherogenesis in hyperlipidemic mice. (Arterioscler Thromb Vasc Biol. 2005;25:2386-2391.)

Key Words: adventitia ■ atherosclerosis ■ leukocytes ■ immune system ■ inflammation

Atherosclerosis is an inflammatory condition of the arterial wall involving cells of innate and adaptive immunity.1–5 The predominant leukocytes in atherosclerotic plaques are heterogeneous populations of macrophages/macrophage-derived foam cells and T cells6–10 and, to a much lesser extent, B cells11–14 and natural killer T cells.15 However, identification of the role of distinct leukocytes in atherogenesis remains a considerable challenge.7 Although macrophages are well-recognized to play a proatherogenic role, the impact of lymphocyte subpopulations remains to be fully understood. Studies in hyperlipidemic mice support a proatherogenic role of Th1 T cells and an antiatherogenic role of Th2 T cells and B cells.10–14,16–20 The majority of studies of arterial wall leukocyte characterization have focused on intima lesions of young animals that were fed cholesterol-rich diets. Although these studies yielded substantial information, they have precluded systematic studies of other aspects of atherogenesis. Experiments spanning the entire life until old age may help to delineate age-related mechanisms of atherogenesis.21,22

Most studies related to adaptive immunity of atherosclerosis focused on characterization of intima lesions though adventitial inflammation may participate in arterial wall pathology3,7,23,24 and a role of the adventitia in atherogenesis has also received attention.25–27 However, little information is available on adventitia inflammation and immunity in hyperlipidemic mice as a function of age.28 In earlier studies we observed that the adventitial tissue of atherosclerotic arteries of apolipoprotein E-deficient (apoE/−) mice is a major site of T cell accumulation.29 We now report on systematic studies using cross-sections of the innominate artery where atherosclerotic plaques closely mimic those found at the human carotid bifurcation,30 and of the subdiaphragmatic portion of the abdominal aorta, a predilection site for aneurysm formation in apoE/− mouse aorta.31 We used chow-fed mice to avoid excessive hyperlipidemia and studied mice up to 78 weeks to address several questions: Where in the arterial wall do lymphocytes reside? Is the extent of lymphocyte inflammation associated with age and lesion size? Does the composition of adventitial immune cells changes relative to the...
duration of hyperlipidemia and during aging? Our data indicate that the major T cell compartment of the arterial wall is the lamina adventitia rather than the lamina intima, that adventitial T cells greatly expand throughout life, and that T cells form clusters with B lymphocytes during aging. Moreover, T and B cell-containing lymphoid follicle-like structures emerge in the abdominal aorta of old apoE−/− mice that contain dendritic cells, several types of T cells, proliferating B cells, and plasma cells. Thus, in the connective tissue of apoE−/− mouse arteries lymphoid structures develop which are reminiscent of tertiary follicles previously observed in other autoimmune diseases.32

Methods
Animals
ApoE−/− and wild-type (wt) mice on the C57BL/6J background were purchased from The Jackson Laboratories (Bar Harbor, Me). Mice were housed in a specific pathogen-free environment on a 12-hour light–dark cycle and fed a standard rodent chow (Altromin, Lage, Germany). Animal procedures were approved by the Animal Care Committee of Thuringia.

Preparation of Mouse Aortas and Atherosclerotic Lesion Analysis
At 16, 32, 52, and 78 weeks of age mice were euthanized by CO2 inhalation. The aorta was perfused in situ, dissected, embedded in Tissue Tec (Sakura Finetek, Zoeterwoude, the Netherlands) exactly at its position in situ, and stored at −80°C. Cross-sections of different parts of the aorta were prepared without disrupting the adventitial tissue. Because of the curvature of the aortic arch cryosections of the innominate artery immediately above the bifurcation from the aorta also contained the adjacent section of the aortic arch and the intact adventitial tissue between the two vessels. For analysis of the adventitial aorta, the segment localized near the renal artery was used.

Atherosclerosis was quantified by arteria innominata analysis.33

Serial 10-μm cryosections from the innominate artery above the bifurcation from the aortic arch were collected and mounted on Polysine glass slides (Menzel Gmbh & Co. KG, Braunschweig, Germany). Every tenth section was stained with oil red O. Areas from 8 consecutive oil red O-stained sections were measured using Leica Q500/W software and both the surface area of lesions34; the density of CD68+ macrophages and the fraction area of lesions (FA)35 and the fraction area of lesions (FA)36

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Statistical Analyses
Measurements are expressed as means±SEM. Results were analyzed by Student t test. Morphometric data were analyzed by multiple regression analysis and Pearson’s correlation coefficients were calculated by using SPSS software (SPSS, Chicago, Ill).

Results
T Cells Reside in the Lamina Adventitia of Hyperlipidemic Mice and Accumulate With Increasing Age
In the innominate artery/aortic arch section of 16-, 32-, 52-, and 78-week-old mice maintained on normal mouse chow the density of CD3+ T cells/mm² tissue area showed age- and strain-dependent differences. In young (ie, 16 weeks) wt and apoE−/− mice, the majority of T cells resided in the adventitia (Figure 1). Few T cells or macrophages were observed in the lamina intima of either strain and lesion formation was negligible at 16 weeks. In wt mice, T cells remained constant throughout most of their life; only at the age of 78 weeks a small but significant increase in adventitial T cells was observed (Figure 1a). In contrast, in 32-week-old apoE−/− mice T cells in the adventitia were elevated 4-fold compared with 16-week-old (P<0.001) and progressively increased thereafter, amounting to a 6-fold increase at 52 weeks (P<0.001) and a 10-fold increase at 78 weeks (P<0.001) (Figure 1a). In the intima of wt mice there were only sporadic T cells but they were readily observed in apoE−/− mice. However, despite marked increases in adventitial T cells, no comparable changes in intimal T cells were observed in apoE−/− mice (Figure 1b). Lesion T cells in apoE−/− mice peaked at 32 weeks, decreased at 52 weeks, and further declined thereafter (Figure 1b). T cells in the adventitia versus intima of apoE−/− mice showed a reciprocal relationship with 6-, 24-, and 85-fold increases at 32, 52, and 78 weeks (Figure 1c). Thus, atherosclerosis in apoE−/− mice is associated with a large adventitial but not intimal T cell accumulation. To determine whether adventitial T cells were part of an overall perivascular inflammation, the number of total adventitial cells was determined by quantification of DAPI+ cells. This density did not yield significant changes at any time point (Table I, available online at http://atvb.ahajournals.com). The T cell response was also not accompanied by similar increases in adventitial macrophages; the density of CD68+ cells remained stable in either strain and yielded only a small decrease in 78-week-old apoE−/− mice (Table I).
also identified that T cell density was largely dependent on age. When similar calculations were performed on adventitia of 32-week-old apoE<sup>−/−</sup> mice, nearly identical significances were obtained when atherosclerosis formation was measured by either the lesion surface or the fraction area of lesion. The decisive factor was age as revealed by a partial correlation analysis using age as the control parameter. Similar results were obtained using regression analysis when the estimated model was: T cells = B<sub>0</sub> + B<sub>1</sub> × I/M ratio + B<sub>2</sub> × age. The goodness of fit test showed that at least one of the 2 regression coefficients B<sub>1</sub> or B<sub>2</sub> was significantly different from 0 (P<0.001). The coefficient of determination (R<sup>2</sup> = 0.763; when R<sup>2</sup> is the proportion of variation in the dependent variable explained by the regression model) revealed that the extent of T cell accumulation was largely described by the regression equation. The Wald test also identified that T cell density was largely dependent on age. When similar calculations were performed on adventitial macrophages or total adventitial cells no significant correlations could be obtained. These data indicate that hyperlipidemia leads to an early T cell recruitment into the lamina adventitia that is selective for this leukocyte lineage, that T cell accumulation depends on age, and that adventitial T cell accumulation correlates with I/M ratio largely through its common dependence on age (Figure 2).

### Adventitial T Cell Inflammation Correlates With Age and Lesion Size

In statistical analyses of the morphometric data determination of the Pearson correlation coefficient revealed strong relationships between I/M ratio versus age: r<sub>p</sub> = 0.831, P<0.001; adventitial T cells versus I/M ratio: r<sub>p</sub> = 0.706, P<0.001; and adventitial T cells versus age: r<sub>p</sub> = 0.873, P<0.001. Nearly identical significances were obtained when atherosclerosis formation was measured by either the lesion surface or the fraction area of lesion. The decisive factor was age as revealed by a partial correlation analysis using age as the control parameter. Similar results were obtained using regression analysis when the estimated model was: T cells = B<sub>0</sub> + B<sub>1</sub> × I/M ratio + B<sub>2</sub> × age. The goodness of fit test showed that at least one of the 2 regression coefficients B<sub>1</sub> or B<sub>2</sub> was significantly different from 0 (P<0.001). The coefficient of determination (R<sup>2</sup> = 0.763; when R<sup>2</sup> is the proportion of variation in the dependent variable explained by the regression model) revealed that the extent of T cell accumulation was largely described by the regression equation. The Wald test also identified that T cell density was largely dependent on age. When similar calculations were performed on adventitial macrophages or total adventitial cells no significant correlations could be obtained. These data indicate that hyperlipidemia leads to an early T cell recruitment into the lamina adventitia that is selective for this leukocyte lineage, that T cell accumulation depends on age, and that adventitial T cell accumulation correlates with I/M ratio largely through its common dependence on age (Figure 2).

### Single T Cells Predominate in Young ApoE<sup>−/−</sup> Mice, T/B Cell Clusters, and Lymphoid Follicle-Like Structures Emerge in Older Mice

In the innominate artery of wt mice at all time points, single T cells or small T cell aggregates and no B cells were noted consistent with previous reports. By contrast, in the adventitia of 32-week-old apoE<sup>−/−</sup> mice singular T cells accumulated. At 52 weeks, T cell-restricted clustering was evident and at 78 weeks cell clusters were found containing both T and B cells. Approximately 2% of all adventitial DAPI<sup>+</sup> cells were identified as B lymphocytes.

At 78 weeks, in all apoE<sup>−/−</sup> mice scattered T/B cell clusters were found throughout the arterial tree. In 5 of 7 animals, large cell aggregates with lymphoid follicle-like structure had emerged in the adventitia of the abdominal aorta (Figure 3), whereas somewhat smaller leukocyte aggregates were observed in the remaining 2 mice. Follicles with very high cell density (>600 cells/mm<sup>2</sup>) were preferentially located at aorta segments with severe circular lesions (Figure 3c). Large B cell clusters centered in areas of unencapsulated follicles

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**Figure 1.** The lamina adventitia is the major artery T cell compartment in wt and apoE<sup>−/−</sup> mice. Age-dependent changes in the density of T cells (CD3<sup>+</sup> cells/mm<sup>2</sup>) in the laminae adventitia (a) and intima (b) determined in the innominate artery of wt and apoE<sup>−/−</sup> mice at 16 (open columns), 32 (striped columns), 52 (hatched columns), and 78 (black columns) weeks and ratio of T cell density adventitia/intima in apoE<sup>−/−</sup> mice (c). Means ± SEM; number of mice analyzed at 16, 32, 52, 78 weeks: wt 9, 12, 13, 7; apoE<sup>−/−</sup> 11, 8, 13, 7; n.d. = not detectable. Student t test: †P<0.005 and ††P<0.05 vs mice at other ages; *P<0.001 vs 16 weeks, P<0.005 vs wt mice of the same age; **P<0.05 vs 32 weeks, P<0.001 vs 52 weeks, P<0.001 vs 52 weeks, P<0.001 vs mice at other ages; ***P<0.05 vs 32 weeks, P<0.001 vs 32 weeks, P<0.001 vs 16 weeks, P<0.005 vs wt mice of same age. Paired t test for ratio T cell density adventitia/intima: §P<0.005; §§P<0.001.

**Figure 2.** Relationships between adventitial T cell number, age, and lesion size in individual innominate artery segments. ApoE<sup>−/−</sup> innominate arteries were prepared and analyzed at 16, 32, 52, and 78 weeks (n=27) as described in the Method section. Pearson correlation coefficients revealed strong relationships between all variables (intima/media [I/M] ratio vs age: r<sub>p</sub> = 0.831, P<0.001; I/M ratio vs adventitial T cells: r<sub>p</sub> = 0.706, P<0.001; age vs adventitial T cells: r<sub>p</sub> = 0.873, P<0.001).
reminiscent of tertiary lymphoid tissues (Figure 3d). These areas contained T cells, macrophages (Figure 3e) and endothelial cells (Figure 3f). CD3ε+/CD4+ cells comprised the majority of T cells, but CD3ε+/CD8+, CD3ε+/CD25+ T cells, and CD3ε+/CD8+ cells were also observed (Figure 4a through 4c). Furthermore, CD3ε+/CD4+ cells probably representing follicle inducer cells (Figure 4a) and few large NK cells were evident (Figure 4d). Follicles located adjacent to the external elastic lamina (Figure 4e) contained CD138+ plasma cells revealing local B cell maturation (Figure 4f). A considerable proportion of B cells stained positive for Ki67 (Figure 4g) indicating B cell expansion, and large CD11c+/MHC II+ dendritic cells were prominent (Figure 4h).

**Atherosclerotic Aortae Express High Levels of MIP-1α**

MIP-1α transcripts were determined by quantitative reverse-transcription polymerase chain reaction in whole aorta extracts of 16-, 32-, and 52-week-old mice. Compared with wt aortae (n = 5), apoE−/− aortae (n = 10) showed a significant elevation of MIP-1α transcripts, which were increased 5-fold at 16, 20-fold at 32, and >50-fold at 52 weeks (Figure I, available online at http://atvb.ahajournals.org). However, MIP-1α transcripts did not show a significant increase in spleen or liver of 52-week-old apoE−/− versus wt mice (Figure I). These data revealed a marked, age-dependent, and arterial wall-specific increase of this Th1 chemokine in chow-fed apoE−/− mice.

**Figure 3.** Follicles form in old apoE−/− mouse aorta adventitia in areas inflicted with severe and advanced circular atherosclerosis. a to c, Oil red O/hematoxylin-stained sections of aortae at 78 weeks. a, wt control; b, apoE−/− aorta with advanced lesion; the lamina adventitia harbors a dense leukocyte infiltrate; c, apoE−/− aorta; lymphoid follicle-like structures (*); T cell aggregates (#); erosion of lamina adventitia (→); d, B cells in follicle-like structures; e, CD3ε+ T cells and CD68+ macrophages and/or dendritic cells; f, follicles contain vasa vasorum. Phase contrast to visualize tissue structure. Dotted lines indicate external elastic lamina. Bar 100 μm.

**Figure 4.** Cell phenotypes in adventitia follicles of old apoE−/− mice. Follicles in adventitial tissue of the abdominal aorta of a 78-week-old apoE−/− mouse. a, CD4+ T cells (colocalization of CD3 and CD4 yields yellow); note the presence of CD4+/CD3+ inducer cells (arrows, green); b, CD8α+ T cells (CD3ε, CD8α), arrow indicates double positive cell; c, CD25+ T cells (CD3ε, CD25); d, Ly49-G2 natural killer cells (CD3ε separate from Ly49-G2); e, B cells (CD3ε separate from B220); f, CD138+ plasma cells; g, proliferating B cells (double B220+/Ki67+ cells); h, CD11c+/MHC-II+ dendritic cells (CD11c colocalizes with MHC-II). Phase contrast to visualize tissue structure. DAPI stains nuclei; dotted lines designate external elastic laminae. Bar 50 μm.
Discussion

The salient finding of this study is the age-dependent emergence of lymphocyte-containing cell infiltrates in the lamina adventitia of normal mouse and apoE−/− mice and the formation of lymphoid follicle-like structures in the subdiaphragmatic portion of the abdominal aorta of old apoE−/− mice with advanced atherosclerosis. The data demonstrate that in atherosclerotic arteries the lamina adventitia is a major compartment of arterial wall inflammation associated with lymphocyte infiltration and lymphoid follicle-like organogenesis in response to hyperlipidemia and old age.

Because previous studies in both humans and animals focused on intima lesions, immune cell infiltrates in adventitial tissue were only incidentally noticed and a possible role of the adventitia in atherogenesis has not yet been extensively elucidated.25–27 We observed that in the aortic adventitia of old apoE−/− mice with moderate levels of hyperlipidemia lymphoid follicle-like structures that resemble tertiary follicles arise. Fully developed follicles contained a complete set of adaptive immune cells ranging from antigen-presenting CD11c+/MHCII+ dendritic cells and proliferating B cells to numerous antibody-producing CD138+ plasma cells that should allow an entire cellular and humoral immune response possibly directed toward an autoantigen. Our results indicate that lymphocyte-mediated immunity of the lamina adventitia may play a crucial role in atherosclerosis development. However, it is not yet clear whether adventitial immune cells promote disease pathology or whether they are beneficial for the outcome of the disease.

The age-dependent changes that we found in the apoE−/− aorta are reminiscent of lymphoid neogenesis, as described for autoimmune thyroiditis or rheumatoid arthritis.32 Moreover, formation of tertiary lymphoid organs and an association between allograft immunity and lymphoid neogenesis in the target organ were recently demonstrated in a murine model of chronic cardiac allograft rejection.39 Inflammasome-associated nonencapsulated lymphoid structures in nonlymphoid organs are assumed to be a key determinant in the pathogenesis of autoimmune and allograft diseases. They are characterized by antigen-driven immune responses which first lead to a nonorganized infiltrate predominantly composed of T cells and sporadic B cells. With disease progression cell aggregates are formed, which also contain interdigitating dendritic cells and few proliferating B cells. Finally, lymphoid follicles with germinal centers and follicular dendritic cell networks emerge.32 Our own data showing the consecutive emergence of adventitial T and B lymphocytes as well as the formation of large follicles containing CD11c+/MHCII+ dendritic cells and numerous plasma cells could indicate that inflammatory infiltrates promote autoimmunity to oxidized low-density lipoprotein and other hyperlipidemia- or atherosclerosis-dependent autoantigens. The adventitial follicles may derive their antigen from the foam cell-rich lesions as we observed their antigen from the foam cell-rich lesions as we observed dependent autoantigens. The adventitial follicles may derive from the foam cell-rich lesions as we observed dependent autoantigens. The adventitial follicles may derive from the foam cell-rich lesions as we observed dependent autoantigens.

In conclusion, we demonstrated that the aortic lamina adventitia is a major site of arterial wall inflammation associated with lymphocyte infiltration and lymphoid follicle-like organogenesis in response to hyperlipidemia and old age. The lamina adventitia may be an important new arterial wall tissue compartment that deserves further attention to study the relation between hyperlipidemia, aging, immunity, and atherogenesis.

References

Moos et al  Adventitial Cells and Mouse Atherosclerosis  2391


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Short title: Moos Adventitial Cells and Mouse Atherosclerosis

Authors: Michael P.W. Moos, Nicole John, Rolf Gräbner, Silke Noßmann, Bernd Günther*, Rüdiger Vollandt†, Colin D. Funk§, Brigitte Kaiser, and Andreas J.R. Habenicht

Data Supplement:

Figure I. Ratios of MIP-1α transcripts in aorta, spleen and liver of apoE⁻/⁻ vs wt mice.
MIP-1α transcripts were determined by QRT-PCR analysis of RNA extracts from apoE⁻/⁻ and wt mouse aortae at 16, 32 and 52 weeks and from spleen and liver at 52 weeks and the ratios for MIP-1α transcripts per 1000 GAPDH of apoE⁻/⁻ vs wt mice were calculated. Number of mice analyzed: wt = 5; apoE⁻/⁻ = 10.
Student’s t test for number of transcripts/1000 GAPDH in apoE⁻/⁻ vs wt mice: * p < 0.05; ** p < 0.001; n.s. = not significant
### Table I. Number of T cells and macrophages in the adventitia of wt and apoE<sup>-/-</sup> mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age (weeks)</th>
<th>Cell density* (cells/mm&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>T cells and macrophages (% of DAPI&lt;sup&gt;+&lt;/sup&gt; cells)</th>
<th>n</th>
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<tr>
<td></td>
<td></td>
<td>Total cells†</td>
<td>T cells</td>
<td>Macrophages</td>
</tr>
<tr>
<td>Wt</td>
<td>16</td>
<td>2681 ± 264</td>
<td>42.3 ± 7.6</td>
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<tr>
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<td>78</td>
<td>3040 ± 227</td>
<td>108.3 ± 12.1</td>
<td>637.1 ± 66.8</td>
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<tr>
<td>ApoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
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<td>2530 ± 206</td>
<td>28.7 ± 6.0</td>
<td>669.1 ± 67.4</td>
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<tr>
<td></td>
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<td>115.4 ± 16.9</td>
<td>823.0 ± 80.9</td>
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<td>2936 ± 172</td>
<td>174.1 ± 17.1</td>
<td>638.8 ± 31.3</td>
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<td>78</td>
<td>3360 ± 307</td>
<td>283.5 ± 49.4</td>
<td>562.8 ± 80.1</td>
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</table>

*Cell densities were assessed by determination of DAPI<sup>+</sup> cells (total cells), CD3ε<sup>+</sup> T cells, and CD68<sup>+</sup> macrophages per tissue area of the innominate artery as described in Methods. † Percentage of CD3ε<sup>+</sup> T cells and CD68<sup>+</sup> macrophages per DAPI<sup>+</sup> cells. Data are means ± SEM of n mice. Student’s t test: ‡ for significances see Figure 1; § p < 0.05 vs mice at other ages; ¶ p < 0.001 vs 16 weeks, < 0.05 vs wt mice of the same age. # p < 0.05 vs 32 weeks, < 0.001 vs 16 weeks and vs wt mice of the same age; ** p < 0.001 vs 16 weeks, < 0.05 vs 52 weeks, < 0.005 vs 32 weeks and vs wt mice of the same age; *** p < 0.05 vs 32 and 52 weeks, < 0.005 vs 16 weeks.