Role of Toll-Like Receptor 4 in Intimal Foam Cell Accumulation in Apolipoprotein E–Deficient Mice

Mie Higashimori, Jeffrey B. Tatro, Kathryn J. Moore, Michael E. Mendelsohn, Jonas B. Galper, Debbie Beasley

Objective—Atherosclerosis encompasses a conspicuously maladaptive inflammatory response that might involve innate immunity. Here, we compared the role of Toll-like receptor 4 (TLR4) with that of TLR2 in intimal foam cell accumulation and inflammation in apolipoprotein E (ApoE) knockout (KO) mice in vivo and determined potential mechanisms of upstream activation and downstream action.

Methods and Results—We measured lipid accumulation and gene expression in the lesion-prone lesser curvature of the aortic arch. TLR4 deficiency reduced intimal lipid by ≈75% in ApoE KO mice, despite unaltered total serum cholesterol and triglyceride levels, whereas TLR2 deficiency reduced it by ≈45%. TLR4 deficiency prevented the increased interleukin-1α (IL-1α) and monocyte chemoattractant protein-1 mRNA levels seen within lesional tissue, and it also lowered serum IL-1α levels. Smooth muscle cells (SMC) were present within the intima of the lesser curvature of the aortic arch at this early lesion stage, and they enveloped and permeated nascent lesions, which consisted of focal clusters of foam cells. Cholesterol enrichment of SMC in vitro stimulated acyl-coenzyme A:cholesterol acyltransferase-1 mRNA expression, cytoplasmic cholesterol ester accumulation, and monocyte chemoattractant protein-1 mRNA and protein expression in a TLR4-dependent manner.

Conclusion—TLR4 contributes to early-stage intimal foam cell accumulation at lesion-prone aortic sites in ApoE KO mice, as does TLR2 to a lesser extent. Intimal SMC surround and penetrate early lesions, where TLR4 signaling within them may influence lesion progression. (Arterioscler Thromb Vasc Biol. 2011;31:50–57.)

Key Words: atherosclerosis ■ genetically altered mice ■ Toll-like receptors ■ foam cells ■ smooth muscle cells

Atherosclerosis involves a strikingly maladaptive inflammatory response, initially to retained and modified lipoproteins1 and later to apoptotic or necrotic cell debris that accumulates within the arterial wall.2 Toll-like receptors (TLR) elicit innate immune responses and inflammation when activated by either exogenous microbial products or endogenous molecules with similar structural features, and they are candidate mediators of atherogenic inflammation. TLR2 and TLR4 are expressed in arterial lesions of mice and humans,3,4 and exogenous microbial TLR2 and TLR4 agonist ligands promote atherosclerosis in hypercholesterolemic mice,5,6 implicating these receptors as candidate atherogenic mediators. In gene knockout (KO) mouse models, TLR2 deficiency reduced early atherogenic events and later aortic lesion development.7,8 In contrast, deficiency of TLR4, the bacterial lipopolysaccharide (LPS) receptor, produced modest effects9 or no effects10 on aortic lesion burden in studies limited to advanced disease stages. However, prolonged and severe hypercholesterolemia may generate distinct modified lipids or proteins that promote inflammation and atherogenesis via distinct, TLR-independent pathways. Therefore, here we tested the hypothesis that TLR4 promotes atherogenesis during early-stage disease, using a mouse model of shorter-term and less severe hypercholesterolemia, and compared its influence to that of TLR2.

TLR signaling strongly activates proinflammatory genes in multiple cell types found within early atherosclerotic lesions, but little is known about how this promotes atherogenesis. Such atherogenic TLR signaling may not be restricted to hematopoietic cells, because a proatherogenic influence of TLR2 in hypercholesterolemic mice was found to be conferred by nonhematopoietic cells,5 and TLR2-expressing endothelial cells were found at lesion-prone sites.7 Other candidate nonhematopoietic cell types include intimal smooth muscle cells (SMC), which may express both TLR2 and TLR4.11,12 In humans, several findings point to an important role of intimal SMC in early lesion formation. Intimal accumulation of SMC precedes human lesion development, and the earliest histologically identifiable lesions contain extracellular lipids, thought to accumulate because of binding to proteoglycans released by SMC.13 Foam cells of myeloid or SMC origin then accumulate in the intima,14 and some...
evidence suggests that apoptotic death of SMC leads to the appearance of lipid pools.\textsuperscript{15} Thus, TLR2 and TLR4 within SMC could potentially contribute to early atherogenesis by multiple mechanisms, including early proteoglycan synthesis, foam cell accumulation, or release of macrophage-recruiting chemokines and proinflammatory cytokines.\textsuperscript{11,12} A role of SMC in early atherogenesis has not previously been described in mouse models, but mouse aortic SMC are similar to human aortic SMC in their propensity to accumulate intracellular cholesteryl esters in vitro.\textsuperscript{16,17} Therefore, in the present study, we evaluated the potential roles of SMC in early intimal lipid accumulation, using a hypercholesterolemic mouse model of atherogenesis in concert with three-dimensional confocal analysis of SMC involvement in lesions in vivo. We also studied potential mechanisms of TLR action in SMC in vitro.

Our results reveal that early aortic lipid accumulation and expression of proinflammatory mediators are markedly dependent on TLR4, and to a lesser extent TLR2, at lesion-prone sites in apolipoprotein E (ApoE)--deficient mice. Also, intimal SMC showed integral involvement in the microarchitecture of some nascent lesions, enveloping and permeating early foam cell clusters, supporting their potential to modulate lipid accumulation. Furthermore, we found that SMC incubated in cholesterol-enriched medium in vitro express monocyte chemoattractant protein-1 (MCP-1) and the cholesterol-esterifying enzyme acyl-coenzyme A:cholesterol acyltransferase (ACAT1), and they also accumulate intracellular cholesteryl ester in a TLR4-dependent fashion,\textsuperscript{18} raising the possibility that TLR4 expressed by SMC contributes to proinflammatory events and foam cell formation in early atherogenesis.

Methods
A detailed description of the methods is given in the supplemental materials, available online at http://atvb.ahajournals.org.

Mice and Serum Analysis
ApoE KO, TLR2 KO, and TLR4 KO mice were crossbred to obtain double-KO mice. Serum total cholesterol and triglyceride levels were assayed using enzymatic methods. Serum IL-1\textalpha, MCP-1, and soluble vascular cell adhesion molecule-1 (VCAM-1) levels were determined by ELISA, and serum endotoxin levels were determined after heat-inactivating serum proteins.
Expression and Serum IL-1
Promote Lipid Accumulation in the Lesser Curvature of the Aortic Arch
To elucidate the mechanisms whereby TLR4 and TLR2 promote lipid accumulation in the lesser curvature of the aortic arch, we used real-time reverse transcription–polymerase chain reaction to analyze expression in the lesion-prone LCAA. IL-1α gene expression in the LCAA is increased 9-fold in ApoE KO versus those in wild-type mice, and this effect was markedly reduced in both TLR4 DKO and TLR2 DKO mice (Figure 3). TLR4 deficiency also reduced serum IL-1α levels in ApoE KO mice (Table). To determine whether IL-1 receptor signaling has been implicated in lesion development in ApoE KO mice, we tested whether TLR4 contributes to local IL-1α gene expression in the lesion-prone LCAA. IL-1α mRNA levels at this site were increased 9-fold in ApoE KO versus those in wild-type mice, and this effect was markedly reduced in both TLR4 DKO and TLR2 DKO mice (Figure 3). TLR4 deficiency also reduced serum IL-1α levels in ApoE KO mice (Table). TLR4 deficiency reduced serum IL-1α levels in ApoE KO mice (Figure 3).

Table. TLR4 Deficiency Reduces Serum IL-1α (pg/ml)

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<th>Genotype</th>
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<th>36 Weeks</th>
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<td>13 (9, 16)</td>
<td>3 (1, 11)</td>
</tr>
<tr>
<td>ApoE KO</td>
<td>16 (10, 41)</td>
<td>7 (1, 11)</td>
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<td>TLR4 DKO</td>
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Values are median (25th percentile, 75th percentile) (n), for male mice fed a standard chow diet.

*P < 0.05 versus ApoE KO.
(Figure 3). Because IL-1RA is strongly inducible by IL-1 and thus may be indicative of ongoing IL-1 signaling,21,22 the results suggest that both TLR4 and TLR2 may promote IL-1 signaling within aortic lesions.

**TLR4 Deficiency Reduces Lesional VCAM-1, MCP-1, and TLR2 Gene Expression**

VCAM-1 is a key mediator of monocyte adhesion to endothelium during early atherogenesis,23–25 whereas MCP-1 released into the subendothelial space promotes monocyte diapedesis and migration into the intima.26,27 VCAM-1 mRNA levels were not affected at age 12 weeks (data not shown) but were doubled in the aortic arch of 18-week-old ApoE KO mice versus those in wild-type controls (Figure 3), and this increase was abolished in TLR4-deficient mice. MCP-1 mRNA expression increased 2.5-fold in the LCAA of ApoE KO mice versus those seen in wild-type mice, and this increase was also abolished in TLR4 DKO mice (Figure 3). VCAM-1 and MCP-1 mRNA levels appeared lower in TLR2 DKO mice, but the differences were not statistically significant. Serum levels of soluble VCAM-1 (Supplemental Table IV) and MCP-1 (data not shown) were unchanged by TLR2 or TLR4 deficiency.

Because TLR2 and TLR4 expression are increased in human atherosclerotic lesions,3,4 we also tested whether altered TLR2 or TLR4 mRNA expression occurred during early lipid accumulation. TLR2 mRNA levels increased more than 3-fold in LCAA of ApoE KO mice versus those in wild-type mice (Figure 3) but were unaltered in TLR4 DKO mice, indicating that TLR2 upregulation was likewise TLR4 dependent. mRNA levels of myeloid-related protein-8 (Mrp8), a putative endogenous activator of TLR4,28 were also increased in ApoE KO mice, but not significantly so. In contrast, TLR4 mRNA levels were unchanged in early lesions of ApoE KO or TLR2 DKO mice versus those seen in wild-type LCAA segments. These results raise the possibility that TLR4-inducible TLR2 signaling may contribute to lesion formation during early atherogenesis.

Early atherogenesis involves the accumulation of extracellular proteoglycans, which bind lipoproteins and trap them in the subendothelial space, where they become modified.5 Two distinct proteoglycans, biglycan and perlecan, accumulate in early lesions of ApoE KO mice,5,9 but it is unknown whether expression of their corresponding mRNAs is increased locally. The levels of both biglycan and perlecan mRNA were similar in the aortic arch of 12-week-old chow-fed wild-type, ApoE KO, and TLR4 DKO mice, and biglycan mRNA was significantly increased rather than reduced in TLR2 DKO mice (Figure 3). These results thus do not support a role of reduced proteoglycan mRNA expression in the atherogenic effect of TLR4 deficiency.

**Intimal SMC Are Present in the LCAA at the Early Lesion Stage and Are Integral to the Microarchitecture of Nascent Lesions**

To test whether SMC could contribute to early lesion development in ApoE KO mice in vivo, we determined whether intimal SMC are present in the lesion-prone LCAA of 16-week-old mice. Using en face confocal immunostaining analysis, we found SMA⁺ cells within the lesion-prone lesser curvature, but these were sparse or absent within the lesion-resistant greater curvature (Figure 4A and 4B). In LCAA of older (25 weeks) ApoE KO mice, most intimal lipid was intracellular, seen as clusters of lipid-laden foam cells (Figure 4C to 4F). Notably, SMC were associated with a subset (~50%) of nascent foam cell aggregates (Figure 4C and 4F) but were absent from others (Figure 4E). In some cases, spindle-shaped SMC surrounded and permeated spheroidal aggregates of lipid-laden foam cells that made up a develop-
ing lesion (Figure 4C, 4D, and 4F; Supplemental Figure II and III). The spindle-shaped SMC were devoid of lipid, but additional spindle-shaped cells that contained abundant neutral lipid were found directly adjacent to them, raising the possibility that the spindle-shaped foam cells might potentially be derived from SMC. The lesions in TLR4 DKO mice were smaller, as expected, and characterized by small clusters of fewer foam cells that were enveloped by only a few SMC (Figure 4G) or by sparse foam cells with no associated SMC (not shown). In comparison, a nearly complete layer of contiguous SMC, reminiscent of a fibrous SMC cap, enveloped large foam cell aggregates within the LCAA of older ApoE KO mice (44 weeks; Figure 4H). The results show that SMC are integral to the microarchitecture of some developing lesions and are situated to influence foam cell accumulation.

TLR4 Dependence of SMC Cholesterol Ester Accumulation and ACAT1 Gene Expression
To determine whether TLR4-dependent accumulation of intracellular cholesterol ester in SMC might contribute to intimal lipid accumulation in ApoE KO mice, we compared the cholesterol ester content of wild-type and TLR-deficient mouse aortic SMC incubated in cholesterol-enriched medium, an established model of SMC conversion to foam cells.16,17 Mouse aortic SMC uniformly accumulated abundant cytoplasmic neutral lipid droplets following 68 hours of incubation with cyclohexatin-cholesterol (Figure 5A), as previously described. TLR4 deficiency reduced cholesteryl ester accumulation by \( \approx 30\% \) versus that seen in TLR4-expressing SMC (Figure 5B). ACAT1 is the key cholesteryl-esterifying enzyme in most cell types, and its mRNA expression is upregulated during conversion of human SMC to foam cells17 and by nuclear factor-\( \kappa B \),30 a key mediator of TLR4-induced gene transcription. Therefore, we tested whether ACAT1 gene expression is increased in a TLR4-dependent manner in SMC incubated with free cholesterol. ACAT1 mRNA levels increased markedly (5-fold) in cholesterol-enriched SMC, and this increase was abolished in TLR4-deficient SMC (Figure 5C). Similar results were obtained with SMC from ApoE KO mice (data not shown). Exposure to LPS without free cholesterol also induced TLR4-dependent ACAT1 gene expression (Figure 5C), but not foam cell formation (data not shown), suggesting that both cholesterol enrichment and TLR4 signaling are required to induce accumulation of cytoplasmic cholesteryl esters in SMC. The results suggest that cholesterol-enriched aortic SMC accumulate cholesteryl esters via a mechanism involving TLR4-dependent ACAT1 gene expression.

Cholesterol Enrichment Stimulates MCP-1, CD68, and Mrp8 mRNA Expression: Role of TLR4
SMC incubated in cholesterol-enriched media lose expression of SMC-specific proteins and gain expression of macrophage-specific proteins.16,17 In agreement, we found reduced SMA expression in cholesterol-enriched SMC, whereas a subset of the cells expressed cytoplasmic CD68 (Figure 6A). Cholesterol enrichment increased CD68 mRNA levels 7.6-fold in ApoE KO SMC and only 4.3-fold in ApoE/TLR4 DKO SMC. Because TLR4 signaling is a potent inducer of MCP-1 expression in mouse SMC,11 we tested whether cholesterol enrichment increased MCP-1 expression in a TLR4-dependent manner. Cholesterol enrichment increased MCP-1 mRNA levels 4-fold and MCP-1 release 5-fold in ApoE KO SMC, and both responses were markedly reduced in ApoE/TLR4 DKO SMC (Figure 6B). Mrp8 mRNA was increased \( \approx 2\)-fold, but its regulation was TLR4-independent. MCP-1, CD68, and Mrp8 mRNA upregulation were similar in ApoE KO and ApoE/TLR2 DKO SMC (data not shown). These results suggest that cholesterol enrichment promotes SMC expression of proteins thought to be macrophage-specific, by mechanisms that are at least in part TLR4 dependent, consistent with our hypothesis that TLR4 signaling might play a role in SMC conversion to a macrophage-like phenotype.

Discussion
Our findings demonstrate an important role of TLR4, and to a lesser extent TLR2, in early intimal foam cell accumulation in hypercholesterolemic ApoE KO mice. We found that TLR4 deficiency markedly reduced aortic lipid accumulation, by \( \approx 70\% \) to \( 80\% \), within the LCAA of ApoE KO mice fed chow or a short-term high-cholesterol diet, indicating that such lipid deposition is strongly TLR4 dependent. Our results point to an early atherogenic influence of TLR4 that was stronger than had been suggested by previous studies. The latter studies focused on advanced disease in older ApoE KO mice that were fed a high-cholesterol diet for many months, and found only a modest or no role of TLR4.9,10 Such prolonged feeding of ApoE KO mice with a high-cholesterol diet produces severe hypercholesterolemia, which may have limited relevance to human atherogenesis, and may have failed to detect the presently observed critical role of TLR4 in early lesions because of later activation of additional receptors. The TLR4- and TLR2-induced effects reported here occurred in the absence of altered serum cholesterol or triglyceride levels, indicating that signaling via these receptors increases the susceptibility of the artery wall to inflammation and lipid deposition.

Both TLR4 and TLR2 contributed strongly to the upregulation of the IL-1\( \alpha \) and IL-1RA genes seen in lesional tissue of ApoE-deficient mice, and TLR4 deficiency lowered serum IL-1\( \alpha \), providing further evidence for a role of IL-1\( \alpha \) in the proatherogenic effects of TLR4. IL-1 receptor signaling plays a proatherogenic role in ApoE KO mice,20 and IL-1\( \alpha \) promotes multiple cellular responses relevant to early atherogenesis, including MCP-1 expression and SMC proliferation.31,32 IL-1 signaling also disrupts low-density lipoprotein receptor regulation and induces foam cell formation in human coronary artery SMC,33 suggesting a potential mechanism for increased cellular lipid accumulation. Together, these results raise the possibility that TLR-dependent IL-1\( \alpha \) gene expression may contribute to intimal lipid accumulation.

TLR2 gene expression was also upregulated within lesional aortic tissue of ApoE KO mice but not that of TLR4-deficient mice. Local upregulation of TLR2 might contribute to intimal lipid accumulation in ApoE KO mice, as TLR2 deficiency reduced it, albeit somewhat less so than TLR4. TLR4 signaling upregulates TLR2 gene expression in endothelial cells, SMC, and macrophages in vitro,12,34,35 suggesting that multiple cell types might account for the observed TLR4-dependent, local TLR2 gene expression in

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ApoE-deficient mice. In low-density lipoprotein receptor–deficient mice, TLR2 expression was regionally upregulated in the LCAA, specifically within endothelial cells.\(^7\) Thus, endothelial cell-specific expression might similarly account for the TLR2 upregulation seen here in the LCAA of ApoE KO mice.

Our main findings point to a proatherogenic role of TLR4, raising the important question of how TLR4 becomes activated in ApoE KO mice. One possibility is that circulating LPS provides a tonic stimulus to TLR4 signaling, as LPS can be found in the serum of apparently healthy men and laboratory mice, particularly those consuming a high-fat diet.\(^{36,37}\) Although the observed levels of serum LPS in ApoE KO mice were seemingly low (≈100 pg/mL) and similar to those in wild-type mice, ApoE-deficient mice are reportedly more sensitive to LPS than wild-type mice, exhibiting markedly enhanced cytokine production and mortality after systemic LPS administration in vivo.\(^{38-40}\) Thus, low levels of LPS may be sufficient to prime the inflammatory response in athero-prone regions of the vasculature. LPS-driven TLR4 signaling has also been postulated to promote human arterial disease.\(^{41}\) For example, epidemiological evidence indicates that low-grade endotoxemia (≥50 pg/mL) is a strong risk factor for progression of early carotid artery atherosclerosis in humans.\(^{42}\) Also, human macrophages, endothelial cells, and SMC are
exquisitely sensitive to LPS, as concentrations as low as 30 to 100 pg/mL stimulate VCAM-1 expression and MCP-1 release, suggesting that circulating LPS levels in healthy people may be capable of stimulating atherogenic inflammation.

An alternative hypothesis for how atherogenic TLR4 activation occurs in ApoE-deficient mice is that nonmicrobial, endogenous TLR4 agonist ligands contribute to a “sterile inflammatory response.” This idea is supported by findings that advanced lesions in older, Western diet–fed ApoE KO mice are similar whether the mice are gnotobiotic and raised in a “sterile” environment free of bacteria and known pathogens or are raised in the presence of ambient pathogens. One class of candidate hypercholesterolemia-associated TLR4 ligands includes molecules associated with minimally or moderately oxidized forms of low-density lipoprotein, which stimulate TLR4-dependent fluid phase low-density lipoprotein uptake, a potential mechanism of foam cell formation, and expression of multiple chemokines.

Additional putative endogenous TLR4 ligands include intracellular proteins released by stressed or dying cells, such as Mrp8. We found that Mrp8 mRNA was expressed in the LCAA of ApoE KO mice, suggesting that Mrp8 may contribute to TLR4 activation in LCAA lesions. Mrp8 mRNA levels were also increased in SMC incubated in cholesterol-enriched media, suggesting the possibility that TLR4-dependent expression of proatherogenic genes in cholesterol-enriched SMC or macrophages may potentially involve enhanced expression of Mrp8, which promotes TLR4 signaling.

Our in vivo immunofluorescent findings revealed an intimate relationship between SMC and developing lesions, supporting a possible role of SMC in modulating foam cell accumulation. First, we found that SMC are prevalent in the intima of the lesion-susceptible lesser curvature but not the lesion-resistant greater curvature of the aortic arch at the early lesion stage. MCP-1 released by such intimal SMC may promote monocyte recruitment, a possibility supported by our findings that cholesterol-enriched SMC express MCP-1 and that the lesion-prone LCAA expresses MCP-1 mRNA in a TLR4-dependent manner. Second, we discovered that bands of SMC surround and permeate developing clusters of lipid-laden foam cells, suggesting that paracrine actions of SMC-derived cytokines or chemokines may also influence foam cell accumulation in nascent lesions. Furthermore, the spindle-shaped SMC that invested developing lesions were intimately associated with SMA-negative spindle-shaped cells that contained abundant neutral lipid, although they themselves appeared to contain none. The origin of these novel spindle-shaped lipid-laden cells remains to be determined, but their close anatomic association with SMC suggests that they might be SMC derived. This hypothesis is supported by the findings that ACAT1 gene expression and intracellular cholesterol ester accumulation were increased in a TLR4-dependent manner in SMC incubated in cholesterol-enriched media, whereas SMA expression is simultaneously diminished. Also supporting this idea, some of the lipid-laden cells present in atherosclerotic lesions of human and primates appear to be of SMC origin.

These findings suggest that TLR4 promotes early foam cell accumulation and expression of IL-1α and other proinflammatory mediators at lesion-prone aortic sites in ApoE KO mice. We found that intimal SMC are situated within nascent lesions in a manner suggesting that they could influence foam cell accumulation. SMC that are intimately associated with nascent lesions may release proinflammatory chemokines in a cholesterol-enriched environment, accumulate intracellular cholesterol ester, or convert to a macrophage-like phenotype via mechanisms involving TLR4 signaling.

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Disclosures

None.

References


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METHODS

**Animals and diet.** We used mice having targeted deletion of the ApoE gene. Because ApoE is a surface component of lipoprotein particles that is necessary for their receptor-mediated uptake \(^1\), ApoE knockout mice have impaired lipoprotein clearance and develop hypercholesterolemia and spontaneous lesions throughout the arterial tree even on a normal chow diet \(^2\). Mice were weaned at 4 weeks of age, received standard chow diet containing 5% fat (Harlan Teklad 2018), and were studied between 12 and 36 weeks of age. One group of mice received Western diet for 3 weeks (TD.88137; containing 42% of calories from fat and 0.15% cholesterol by weight; Harlan Teklad). Mice were housed in a 12-hr light dark cycle (lights on 0600-1800 h), at a temperature of 22 ± 1 °C, in autoclaved cages, and received autoclaved bedding and water, and irradiated chow. The studies were approved by the Institutional Animal Care and Use Committee of Tufts Medical Center.

**Generation of ApoE and TLR4 or TLR2 double knockout mice.** ApoE KO \(^3\) and TLR2 KO \(^4\) mice were obtained from Jackson Laboratories. TLR4 KO mice \(^5\) were kindly provided by Douglas Golenbock (Univ. of Mass. Medical School). All 3 strains of mice had been backcrossed 10-11 times on the C57BL/6 background, to ensure that any differences in atherosclerotic lesion formation were due to targeted deletions rather than to insufficient backcrossing of donor mice. ApoE/TLR double-knockout (DKO) mice were obtained by breeding ApoE KO male mice to TLR2- or TLR4-KO female mice, and then intercrossing the heterozygous littermate mice to obtain the DKO genotype, as determined by PCR analysis of ear-punch DNA. Mouse colonies were maintained by independent breeding of homozygous wild-type ApoE KO, TLR2 DKO, and TLR4 DKO
mice. No significant differences were observed in the overall appearance, health or behaviors of the different strains of mice. However, TLR4 DKO mice did not breed well, and many litters were lost during the first several days after birth. The only overt effect of TLR deficiency observed was on body weight, which was significantly increased by TLR2 deficiency in short term Western-diet fed ApoE KO mice, (Online Table V), but was not affected by TLR4 deficiency. TLR2 deficiency likewise increases body weight in LDLR KO mice fed high-fat diet 6, but not in chow-fed ApoE KO mice 7, as seen here.

**Serum lipid, LPS, and cytokine analysis.** Mice were euthanized by CO2 inhalation, and blood was withdrawn from the left ventricle for analysis of serum lipid levels. The levels of total cholesterol and total triglyceride in serum were assayed using enzymatic methods and commercial kits and standards (Wako Chemical and Sigma Chemical, respectively). Serum IL-1α, MCP-1, and soluble VCAM-1 levels were determined by ELISA (R&D Systems).

Serum endotoxin levels were determined by Limulus amebocyte lysate (LAL) assay (Endochrome; Charles River Endosafe). For LAL analysis, blood was obtained aseptically with a sterile needle and syringe by cardiac puncture through the thoracic muscle layer, after peeling back the skin. Serum was diluted 1:10 in endotoxin-free water and heated to 75 °C for 15 min to inactivate any serum proteins including lipoproteins that may interfere with the analysis by either inhibiting or enhancing the LAL reaction. A separate aliquot of each sample was also spiked with a known amount of endotoxin (37.5 pg/ml) to determine recovery, which was 57 ± 4%. Apparent LPS values were calculated as (measured LPS) / ((% recovery) X 0.01).

**Analysis of aortic neutral lipid accumulation.** Mice were perfused with phosphate-buffered saline (PBS), followed by 10% buffered formalin via the left ventricle, and the
descending and ascending aorta and aortic arch were removed and post-fixed in 10% buffered formalin. Neutral lipid was visualized by staining with Oil Red O solution (0.5% ORO, 1% dextrin in 60% isopropanol). Aortic arch segments were flattened on glycerol gelatin-coated glass slides with glass coverslips. Digital images were obtained using a dissecting microscope and digital camera, and analyzed using the ImageJ software package (NIH).

**RNA isolation and quantitative PCR.** Mice were perfused with 5 ml RNAlater (Ambion), and the entire aortic arch, extending from the heart to 1 mm past the bifurcation of the left subclavian artery was removed, cleared of adventitial tissue, homogenized in buffer, and RNA isolated using RNeasy columns (Qiagen), as directed by the manufacturer. RNA (50–100 ng) was reverse-transcribed and cDNA analyzed by real-time PCR using Taqman primers and probes as we described earlier ⁸, and adapted here for small samples of aortic tissue.

**En face immunostaining analysis.** Mice were perfused with PBS followed by 2% paraformaldehyde, and aortic arch segments were harvested and post-fixed for 30 min at 4 °C. The aortic arch was immersed in cold PBS for removal of adventitial fat, and the lumen then exposed by longitudinal incision open along the greater curvature. To detect intracellular antigens, cell membranes within the tissue were permeabilized by immersion for 10 min in 0.2% Triton X-100/PBS, containing 0.1 M glycine to quench autofluorescence. Tissues were incubated for 1 h with anti-CD16/CD32 Fc-block (BD Biosciences, 1:100) and 10 μg/ml non-immune mouse IgG (Sigma) to block non-specific antibody binding. Primary antibodies were added directly to this blocking solution, and the tissues incubated overnight at 4 °C. Tissues were then washed with PBS, mounted on microscope slides using SlowFade Gold (Molecular Probes) and coverslipped. Neutral lipid was stained 1 h with BODIPY (Molecular Probes), or Oil Red O, as described ⁹. In
some cases, nuclei were counterstained with Topro3 for 15 min (0.2 µg/ml; Molecular Probes).

**Mouse aortic SMC culture.** SMC were isolated from aortas of male mice of various strains, including wild-type (C57BL/6J), TLR2 KO, and TLR4 KO mice as we described previously. SMC were used for experiments at passage 3, and plated at 25,000 cells/cm² the day before the study began.

**Mouse aortic SMC foam cell formation.** SMC plated in 24-well plates were incubated with serum-free media (DMEM) supplemented with 0.25% bovine serum albumin and either 0, 10, or 20 µg/ml water-soluble cholesterol complex (cholesterol:methyl β cyclodextrin complex (Chol:MβCD; 1:6 molar ratio) containing ~1 µg cholesterol per 20 µg complex (Sigma Chemical)). The cyclodextrin-cholesterol reagent was not a significant source of endotoxin (<0.04 EU/ml or 4 pg/ml LPS in the incubation media, as determined by *Limulus* amebocyte lysate assay). Cells were incubated 68 h, fixed in 2% paraformaldehyde for 15 min, washed with PBS, and lipids were extracted in ethanol (200 µl) for 30 min at 4 °C. Aliquots of the extracts were transferred to black-walled 96-well assay plates, dried under nitrogen, and reconstituted in reaction buffer for enzymatic determination of total and free cholesterol using Amplex Red Cholesterol Assay Kit (Molecular Probes). Esterified cholesterol was calculated as total cholesterol minus free cholesterol, determined with and without cholesterol esterase treatment respectively, and normalized to cellular protein content, determined using BCA protein assay reagent (Pierce).

**Mouse aortic SMC gene expression and MCP-1 release.** Mouse aortic SMC were serum-deprived in a low-FCS medium (DMEM supplemented with 0.25% FCS) for 24 h and then incubated in media supplemented with 0.2% BSA with or without added
C chol:MβCD (20 μg/ml). After 24 h, RNA was extracted for analysis of gene expression by real-time RT-PCR as described above. To analyze MCP-1 release, cell supernatants were collected after 48 h from SMC plated in 24-well plates and incubated similarly. MCP-1 levels were analyzed by ELISA (BD Biosciences).

**Statistical Analysis.** Prism Graphpad software was used to determine significant differences by one-way analysis of variance, with the exception of serum IL-1α levels, which were analyzed by non-parametric Mann-Whitney U test due to non-Gaussian distribution of the data. *Post hoc* tests were then performed as indicated, using Dunnett’s method to correct for multiple comparisons to ApoE KO values. Values of *P* < 0.05 were considered significant.
REFERENCES

Table I. Effect of diet but not genotype on serum cholesterol (mg/dl)

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<td>Chow</td>
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<tr>
<td>BL6</td>
<td></td>
<td>83 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoE KO</td>
<td>381 ± 20</td>
<td>417 ± 25 *</td>
<td>1261 ± 61</td>
<td>980 ± 63</td>
</tr>
<tr>
<td>TLR2 DKO</td>
<td>340 ± 15</td>
<td>443 ± 16 *</td>
<td>1349 ± 76</td>
<td>939 ± 58</td>
</tr>
<tr>
<td>TLR4 DKO</td>
<td>403 ± 21</td>
<td>462 ± 36 *</td>
<td>1357 ± 105</td>
<td>1050 ± 142</td>
</tr>
</tbody>
</table>

Age is indicated in weeks. Values are mean ± SE. *P = 0.001 vs. BL6.

Table II. Genotype does not affect serum triglyceride (mg/dl)

<table>
<thead>
<tr>
<th>Sex(age)</th>
<th>Female (20)</th>
<th>Male (36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE KO</td>
<td>43 ± 5</td>
<td>69 ± 6</td>
</tr>
<tr>
<td>TLR2 DKO</td>
<td>63 ± 7</td>
<td>62 ± 4</td>
</tr>
<tr>
<td>TLR4 DKO</td>
<td>43 ± 4</td>
<td>57 ± 8</td>
</tr>
</tbody>
</table>

All mice were chow fed.

Table III. Effect of genotype on serum LPS levels (pg/ml) and recovery

<table>
<thead>
<tr>
<th></th>
<th>LPS (pg/ml)</th>
<th>Recovery(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL6</td>
<td>99±25 (5)</td>
<td>56±6</td>
</tr>
<tr>
<td>ApoE KO</td>
<td>103±15 (7)</td>
<td>57±4</td>
</tr>
<tr>
<td>TLR2 DKO</td>
<td>52±7 (6) *</td>
<td>105±16 *</td>
</tr>
<tr>
<td>TLR4 DKO</td>
<td>91±10 (3) *</td>
<td>58±10%</td>
</tr>
</tbody>
</table>

Chow-fed female mice aged 12 wk. Values are mean±SE (n). *P = 0.01 vs. ApoE KO.

Table IV. Genotype does not affect serum sVCAM-1 (ng/ml)

<table>
<thead>
<tr>
<th>Sex(age)</th>
<th>Female (20)</th>
<th>Male (18)</th>
<th>Male (36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td>Chow</td>
<td>Western</td>
<td>Chow</td>
</tr>
<tr>
<td>ApoE KO</td>
<td>654±33</td>
<td>761±32</td>
<td>828±44</td>
</tr>
<tr>
<td>TLR2 DKO</td>
<td>630±21</td>
<td>802±50</td>
<td>804±57</td>
</tr>
<tr>
<td>TLR4 DKO</td>
<td>621±36</td>
<td>729±34</td>
<td>779±45</td>
</tr>
</tbody>
</table>

Values are mean±SE.

Table V. Effect of genotype on body weight (g)

<table>
<thead>
<tr>
<th>Sex(age)</th>
<th>Female (20)</th>
<th>Male (18)</th>
<th>Male (18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td>Chow</td>
<td>Chow</td>
<td>Western</td>
</tr>
<tr>
<td>BL6</td>
<td></td>
<td>26.1±0.4</td>
<td></td>
</tr>
<tr>
<td>ApoE KO</td>
<td>21.5±0.5</td>
<td>30.9±0.5</td>
<td>28.3±1.2</td>
</tr>
<tr>
<td>TLR2 DKO</td>
<td>23.4±0.6</td>
<td>32.2±0.8</td>
<td>33.5±1.0 *</td>
</tr>
<tr>
<td>TLR4 DKO</td>
<td>22.4±0.4</td>
<td>29.2±1.3</td>
<td>30.4±0.8</td>
</tr>
</tbody>
</table>

Values are mean±SE. *P = 0.01 vs. ApoE KO.
**Online Figure Legends**

**Figures I and II.** Smooth muscle cells (SMC) infiltrate nascent lipid-rich lesions in ApoE KO mice. Sequential images in the z direction, proceeding from the luminal to medial aspect of raised lesions within the lesser curvature of the aortic arch of 25 week-old chow-fed mice. 

I: Neutral lipid was visualized with Oil Red O (red) and SMC were identified by anti-smooth muscle α-actin (SMA) antibody conjugated with FITC (green). Note label colors are opposite to those in the study shown in II. 

II: Neutral lipid was visualized with BODIPY (green) and mouse anti-SMA was detected with Cy5-conjugated anti-mouse IgG (red). The lesion is composed of a cluster of lipid-laden foam cells. Spindle-shaped SMA+ cells surround and infiltrate the foam cell clusters. Also note spindle-shaped cells adjacent to SMA+ cells contain neutral lipid.