ONLINE SUPPLEMENT

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 ^{o}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 CO_2 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 ^{o}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 $\pm$ 4%. Apparent LPS values were calculated as (measured LPS) / ((% recovery) X 0.01).

Analysis of aortic neutal 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 ^{o}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 ^{o}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 [8]. SMC were used for experiments at passage 3, and plated at 25,000 cells/cm^2 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) [10, 11]. 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

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. P*ost 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

1. Breslow JL. Mouse models of atherosclerosis. *Science.* 1996;272:685-688.
2. Nakashima Y, Plump AS, Raines EW, Breslow JL, Ross R. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arterioscler Thromb.* 1994;14:133-140.
3. Piedrahita JA, Zhang SH, Hagaman JR, Oliver PM, Maeda N. Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. *Proc Natl Acad Sci U S A.* 1992;89:4471-4475.
4. Spiller S, Dreher S, Meng G, Grabiec A, Thomas W, Hartung T, Pfeffer K, Hochrein H, Brade H, Bessler W, Wagner H, Kirschning CJ. Cellular recognition of trimyristoylated peptide or enterobacterial lipopolysaccharide via both TLR2 and TLR4. *J Biol Chem.* 2007;282:13190-13198.
5. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. Cutting edge: toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product. *J Immunol.* 1999;162:3749-3752.
6. Mullick AE, Tobias PS, Curtiss LK. Modulation of atherosclerosis in mice by Toll-like receptor 2. *J Clin Invest.* 2005;115:3149-3156.
7. Liu X, Ukai T, Yumoto H, Davey M, Goswami S, Gibson FC, 3rd, Genco CA. Toll-like receptor 2 plays a critical role in the progression of atherosclerosis that is independent of dietary lipids. *Atherosclerosis.* 2008;196:146-154.
8. Yang X, Coriolan D, Murthy V, Schultz K, Golenbock DT, Beasley D. Proinflammatory phenotype of vascular smooth muscle cells: Role of efficient Toll-like receptor 4 signaling. *Am J Physiol (Heart and Circ Physiol).* 2005;289:H1069-1076.
9. Koopman R, Schaart G, Hesselink MK. Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. *Histochem Cell Biol.* 2001;116:63-68.
10. Rong JX, Shapiro M, Trogan E, Fisher EA. Transdifferentiation of mouse aortic smooth muscle cells to a macrophage-like state after cholesterol loading. *Proc Natl Acad Sci U S A.* 2003;100:13531-13536.
11. Rong JX, Kusunoki J, Oelkers P, Sturley SL, Fisher EA. Acyl-coenzymeA (CoA):cholesterol acyltransferase inhibition in rat and human aortic smooth muscle cells is nontoxic and retards foam cell formation. *Arterioscler Thromb Vasc Biol.* 2005;25:122-127.

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

Sex(age)	Female(20)	Male (18)	Male (18)	Male(36)
Diet	Chow	Chow	Western	Chow
BL6		83 ± 3		
ApoE KO	381 ± 20	417 ± 25 *	1261 ± 61	980± 63
TLR2 DKO	340 ± 15	443 ± 16 *	1349 ± 76	939± 58
TLR4 DKO	403 ± 21	462 ± 36 *	1357 ± 105	1050±142

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

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

Sex(age)	Female (20)	Male (36)
ApoE KO	43 ± 5	69± 6
TLR2 DKO	63 ± 7	62 ± 4
TLR4 DKO	43 ± 4	57 + 8

All mice were chow fed.

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

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

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)

Sex(age)	Female (20)	Male (18)	Male (36)
Diet	Chow	Western	Chow
ApoE KO	654±33	761±32	828±44
TLR2 DKO	630±21	802±50	804±57
TLR4 DKO	621±36	729±34	779±45

Values are mean±SE.

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

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

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.