Supporting Information

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SI Materials and Methods

Stimulation with Cytokines and Toll-Like Receptor Ligands. Smooth muscle cells (SMC) were cultured in 50-cm^2 tissue culture dishes and grown until near confluence. Atheroma-derived smooth muscle cells (AthSMC) and aortic smooth muscle cells (AoSMC) were serum starved for 24 h and then cultured in Dulbecco's modified Eagle's medium (DMEM) either alone or in the presence of 10 ng/mL IL1a, 100 ng/mL Pam3Cys, 100 ng/mL FSL-1 (Pam2CGDPKHPKSF), 25 μg/mL Poly(I:C), 100 ng/mL lipopolysaccharide, 1 μg/mL R837 (Imiquimod) and 1 μg/mL PolyU (all purchased from InvivoGen). Supernatants were collected 24 h after stimulation and frozen at −80 °C for batch analysis via ELISA. In the experiments performed on the mixed human atheroma cell culture, cells were cultured immediately after isolation at 10^6 cells per milliliter in Roswell Park Memorial Institute (RPMI) medium containing 5% FBS (Biosera) in the presence or absence of 25 μg/mL Poly(I:C), 1 μM Cytosine-phosphate-Guanine (CpG) ODN2006, and 1 μM CpG ODN2006 control (Invivogen) for 24 and 48 h. The concentrations used were selected on the basis of dose–response experiments as the dose with maximal effect in absence of cell death monitored via 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium (MTT).

ELISA Analysis of Human Cytokine Levels. Cytokine production in supernatants of AthSMC and AoSMC were measured by ELISA using IL-6, IL-8, and CCL2/MCP-1 (Pharmingen). IFNα was detected via a high sensitivity ELISA kit from R&D Systems. Each condition was tested in triplicate and each triplicate was analyzed separately. Concomitantly, viability was monitored with the use of MTT (Sigma).

Gene Expression Profiling and Quantitative PCR in Human SMC. SMC were plated in 9.6-cm^2 dishes and grown until near confluence. SMC were serum starved for 24 h before stimulation with 25 μg/mL Poly (I:C) for 5 h. Total cellular RNA was extracted from SMC using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. To remove any residual genomic DNA, RNA samples were treated with DNase (TurboDNase, Ambion) according to the manufacturer's instructions. RNA was reverse-transcribed to cDNA using M-MLV Reverse Transcriptase (Promega). Quantitative PCR (Q-PCR) analysis of 84 atherosclerosis-related genes was performed using Atherosclerosis RT2 Profiler PCR Arrays (SA Bioscience) as per the manufacturer's protocol. The complete list of the genes analyzed is available at http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-038A.html. RT2 Profiler PCR arrays were run on an ABI 7900HT machine (Applied Biosystems). Duplicate arrays were run per condition for unstimulated and Poly (I:C)-stimulated AoSMC and AthSMC. Data analysis was performed using the manufacturer's integrated web-based software package for the PCR Array System using cycle threshold (Ct)-based fold-change calculations.

Alternatively, AoSMc and AthSMC were stimulated with 25 μg/mL Poly(I:C) or IFNα at 10 ng/mL or IFNγ at 10 ng/mL. Total RNA was extracted using RNeasy Mini Kit as above, and Toll-like receptor 3 (TLR3) gene expression was quantified via Q-PCR with TaqMan Gene Expression Assays (Hs01551078_m1*; Applied Biosystems).

Detection of TLR3 Expression on SMC via Flow Cytometry. SMC were grown until near confluence in 50-cm^2 tissue culture dishes and serum-starved for 24 h. Cells were then scraped in cold PBS and washed in FACS buffer (PBS in 1% FBS, 0.09% NaN_3). Cells were either left untouched or permeabilized with BD Perm/Wash buffer (BD Biosciences). Cells were subsequently stained with FITC-conjugated anti-TLR3 antibody or isotype control (Abcam) and analyzed by FACScan (Becton Dickinson) and Flow-Jo Software (TreeStar).

RT-PCR of Murine Tissues. Total RNA was isolated from murine tissues using the Qiagen RNeasy kit according to the manufacturer's instructions. Total RNA was treated with DNase I and reverse-transcribed to cDNA using M-MLV Reverse Transcriptase RNase H-, Point Mutant (Promega) and oligo(dT) primer. RT-PCR was performed using the following TaqMan (Applied Biosystems) gene expression assays: CCL5 (Mm01302427_m1), VCAM1 (Mm01320970_m1), CCL2 (Mm00441242_m1), TLR3 (Mm01207403_m1), IL-10 (Mm01288386_m1), PD-L1 (Mm00452054_m1*), PD-L2 (Mm00451734_m1*), and IFNβ (Mm00439552_s1*) and TaqMan universal PCR Master Mix (Applied Biosystems) on a 7900HT Fast Real-Time PCR System (Applied BioSystems). PCR amplification was carried out for 40 cycles. Samples were normalized to β-actin. The 2-ΔΔCt method was used to analyze the relative changes in gene expression.

Morphometric Measurement of Neointima Formation in Perivascular Collar Injury. Twenty days following collar placement, mice were euthanized, terminal blood was collected via cardiac puncture, and the vasculature perfused with 0.9% saline. Injured carotids were dissected out and frozen at −80 °C in optimal cutting temperature (OCT) compound (ThermoScientific). Sham-operated contralateral arteries were used as controls. Mice were fed regular chow throughout the duration of the experiment.

For the injured carotid artery, serial 5-μm cryosections were taken of the carotid tissue distal to the collar. Five sections were collected on each slide, and 15–25 slides were collected per arterial segment. The first five alternate slides were stained with Accustain elastic stain kit (Sigma) according to manufacturer's instructions. Measurement of lesion and vessel areas was performed on one section per stained slide using ProgRes CapturePro image analysis software (version 2.5.2.0; Jenoptik). The area between the internal and external elastic arteries was taken as the medial area, and the intimal area was calculated by subtracting the lumen area from the internal elastic lamina area. The intimal medial ratio (IMR) was then calculated by dividing the intimal area by the medial area. The IMR measurements were then averaged for each mouse.

Analysis of Elastic Lamina Breaks in Perivascular Collar Injury. Elastin-stained slides were also used to assess the integrity of the elastic laminae. The portion of the carotid artery distal to the collar (the same as used for neointima assessment) was divided into five segments, and representative sections from each segment were evaluated for the presence of interruptions in the elastic lamina. The width of any observed break was measured by drawing a line between the start and end of a break in the external elastic lamina using ProgRes CapturePro image analysis software (version 2.5.2.0; Jenoptik). The width of any elastic lamina break was calculated as the mean width of break across all sections examined for each mouse. Absolute values for size of elastic lamina break were obtained by calibrating the software using an image of a micrometer slide taken at the same magnification.

Morphometric Measurement of Aortic Root Atherosclerotic Lesion Development. Mice were weaned at 4 wk of age and fed a standard chow diet for the duration of the experiment. At either 15 or

30 wk of age, ApoE$^{-/-}$ and ApoE$^{-/-}$TLR3$^{-/-}$ mice were euthanized with a barbiturate overdose, and terminal blood was collected from the right ventricle. Hearts were perfused in situ with saline via a cannula inserted into the left ventricle (outflow via an incision in the right atria) and then frozen in OCT.

Five-micrometer cryosections of the aortic root were taken for the entire region of the valve leaflets, and every 20th section (100 μm) was stained with Oil Red O and counterstained with hematoxylin. Aortic root sections were coded and analyzed blind. Images were captured under identical microscope, camera, and light conditions. Quantification was performed by drawing around the atherosclerotic lesions and the aortic wall using Clemex Vision Lite version 5.0. Absolute values for the cross-sectional area were obtained by calibrating the software using an image of a micrometer slide taken at the same magnification. The individual lesion areas per aortic root section were averaged to obtain the mean lesion area per mouse. The lesion area fraction was calculated by dividing the mean lesion area by the mean area of the aortic wall and expressed as a percentage.

Immunohistochemistry. Immunohistochemistry was performed on 5-μm cryosections of aortic root sections using standard avidin biotinylated enzyme complex (ABC) methods. In brief, sections were fixed in ice-cold acetone before incubation with 10% normal rabbit or goat serum for 1 h. Following a wash in PBS, endogenous avidin and biotin were blocked using the Vector avidin/biotin blocking kit (Vector Labs) according to manufacturer's instructions. Sections were then incubated with a primary antibody against CD68 for macrophages (clone FA-11; AbD Serotec) for 45 min at room temperature followed by relevant biotinylated secondary antibodies. Following blocking of endogenous peroxidase activity with 0.3% hydrogen peroxide, sections were incubated with avidin and biotinylated horseradish peroxidase macromolecular complexes using the Vectastain Elite ABC kit (Vector Labs) according to manufacturer's instructions. Bound peroxidase was detected using 3,3′-diaminobenzidine and nuclei were counterstained with hematoxylin. Staining using an appropriate isotype-matched control was performed on a consecutive section as a control.

Masson Trichrome Staining. Masson trichrome staining was performed using standard staining protocols. In brief, slides were incubated for 1 h in 5% chromic acid before a 4-min incubation in Celestine blue. After 4 min in Harris hematoxylin, slides were then dipped briefly in acid alcohol before a 5-min incubation in ponceau red/acid fuchsin solution. Following 30 s in 1% phosphomolybdic acid and a 3-min incubation in 1% fast green solution, slides were dehydrated and coverslipped.

Quantification of Immunohistochemical and Masson Trichrome Staining. Aortic root lesion area staining positive for CD68 (brown staining) or collagen (green staining) was quantified using Clemex Vision Lite version 5.0. Images were captured under identical microscope, camera and light conditions, coded and analyzed blind. Using the image analysis software, positive staining was detected and lesion area was measured. Absolute values were obtained by calibrating the software using an image of a micrometer slide taken at the same magnification. Lesion area fraction staining positive for CD68 or collagen was calculated by dividing the positive area by the lesion area and expressing it as a percentage.

Serum Cholesterol Quantification. Total serum cholesterol levels in ApoE$^{-/-}$ and ApoE$^{-/-}$TLR3$^{-/-}$ mice were determined using a Cholesterol/Cholesteryl Ester Quantitation Kit (BioVision) according to manufacturer's instructions.

Statistical Analysis. Data were analyzed with STATA (Version 10, StataCorp LP) or GraphPad Prism (version 5.02) as appropriate. All data are expressed as mean ± SEM unless otherwise stated. Rank ANCOVA was used to assess the effect of treatment of cells in culture to take into account the effect of baseline cytokine production. For data that passed a normality test, Student's *t* tests or one-way analysis of variance with Dunnett's multiple comparison test were used as appropriate. Where data did not pass a normality test, Mann–Whitney *U* tests were performed. Chi-square tests were also performed as appropriate. An α-level of 0.05 was considered as statistically significant. All tests used were two-tailed.

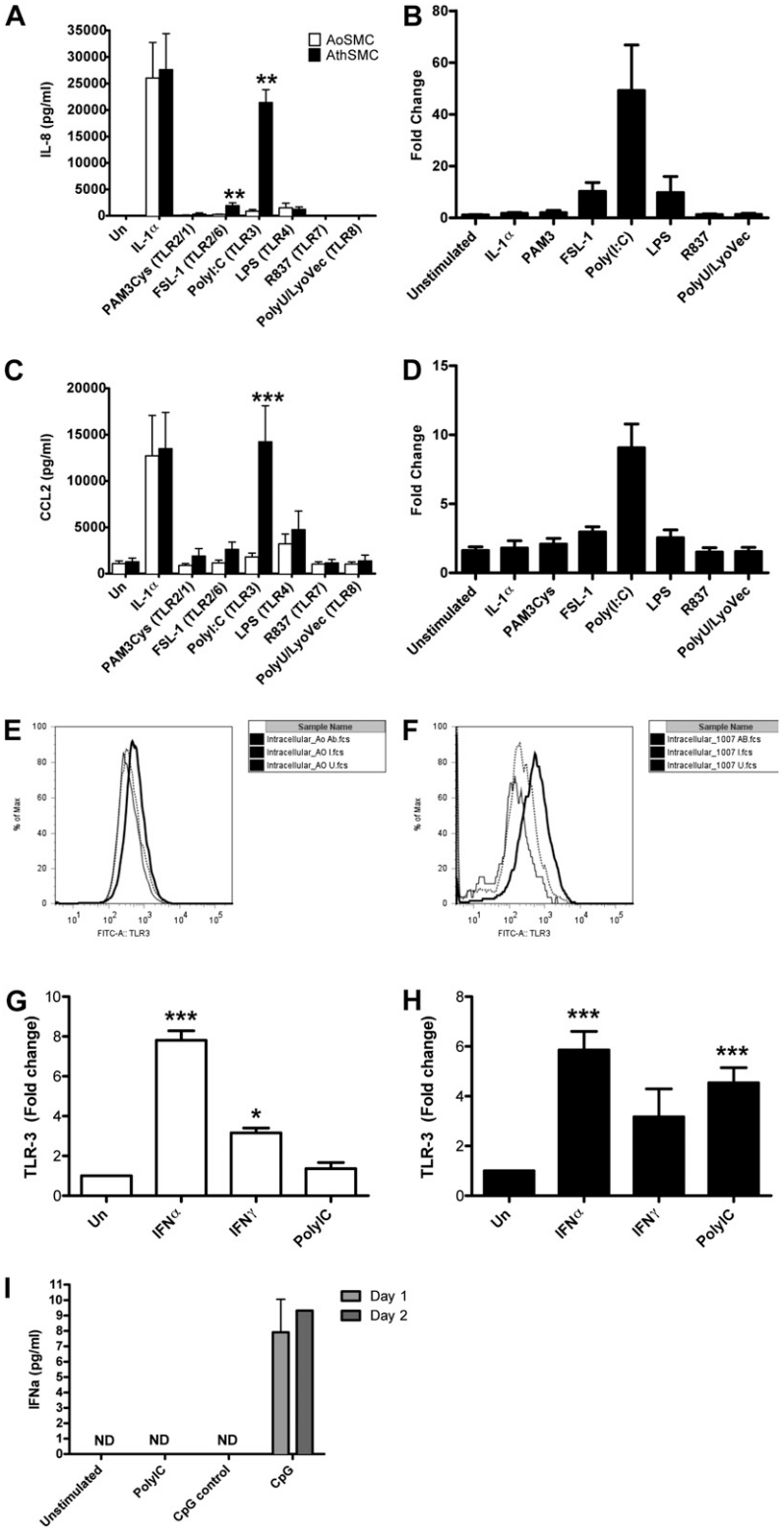

Fig. S1. (*A–D*) AthSMC exhibit increased cytokine responses to TLR3 stimulation. (*A* and *C*) Concentration of IL-8 (*A*) and CCL2/MCP-1 (*C*) in the supernatants of SMC stimulated with various TLR agonists as shown for 24 h. Bars show mean ± SEM ($n = 6$ donors per group) AthSMC displayed enhanced expression of IL-8 and CCL2/MCP-1 when stimulated with Poly(I:C) and increased IL8 also when stimulated with FSL-1 when compared with AoSMC (**$P < 0.01$, ***$P < 0.001$; rank ANCOVA). (*B* and *D*) The same data as in *A* and *C* are shown here as fold change in IL-8 (*B*) and CCL2/MCP-1 (*D*) production between AthSMC and AoSMC following TLR agonist stimulation. Bars show mean ± SEM ($n = 6$ donors per group). (*E–I*) AthSMC express increased intracellular TLR3 compared with AoSMC. (*E* and *F*) Analysis of intracellular TLR3 expression by flow cytometry showed that TLR3 expression was higher on AthSMC (*F*) compared with AoSMC (*E*). One representative staining of three separate experiments is shown here. TLR staining was present only after permeabilization, but not on the cell surface. (*G* and *H*) TLR3 expression was augmented by stimulation with 10 ng/mL IFNs and 25 μg/mL Poly(I:C). AoSMC (*G*) or AthSMC (*H*) were stimulated with the indicated

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agonists for 5 h before total cellular RNA was extracted and quantitative PCR was performed. Data are shown as mean ± SEM (n = 3 donors per group; *$P < 0.05$, ***$P < 0.001$ vs. unstimulated; one-way analysis of variance with Dunnett's multiple comparison test). (*I*) Type I interferons are produced in the mixed cell culture population in response to TLR9 stimulation. Carotid endarterectomy specimens were digested with an enzymatic mixture and the cell culture population was placed in culture for 24 h in the presence or absence of 25 μg/mL Poly(I:C) or 1 μM CpG ODN2006 or 1 μM CpG ODN2006 control. IFNα was detected by ELISA. One representative experiment is shown from three that were performed.

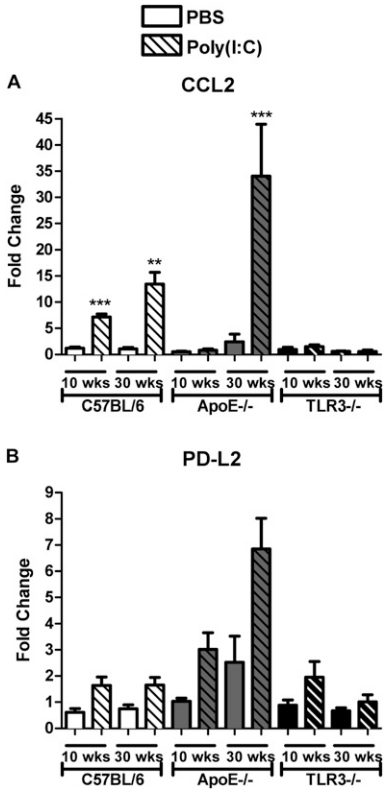

Fig. S2. Induction of pro- and anti-inflammatory factors following Poly(I:C) stimulation in aortas. Ten- and 30-wk-old C57BL/6, ApoE$^{-/-}$, and TLR3$^{-/-}$ mice were stimulated with PBS or 250 μg Poly(I:C) in PBS. Twenty-four hours poststimulation, mice were killed, aortas were harvested, and RNA was extracted. Gene expression of CCL2 (*A*) and PDL2 (*B*) was examined by quantitative RT-PCR. Bars show overall mean ± SEM [n = 3–5 mice per group; **$P < 0.01$, ***$P < 0.001$ PBS vs. Poly(I:C); unpaired Student's t test]. PD-L2: programmed death-ligand 2.

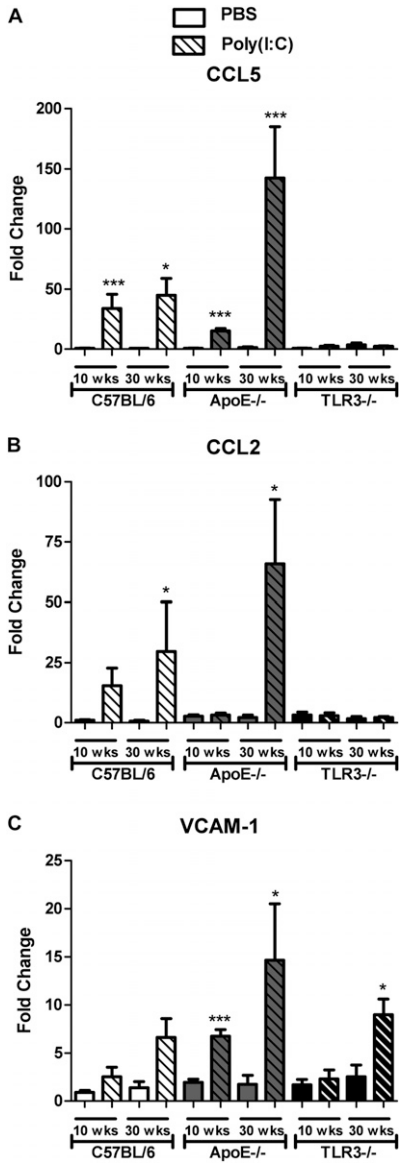

Fig. S3. Carotid gene expression of proinflammatory factors is induced by Poly(I:C) stimulation. Ten- and 30-wk-old C57BL/6, ApoE$^{-/-}$, and TLR3$^{-/-}$ mice were stimulated with PBS or 250 μg Poly(I:C). Twenty-four hours poststimulation, mice were killed, carotid arteries were harvested, and RNA was extracted. Gene expression of CCL5 (*A*), CCL2 (*B*), and VCAM1 (*C*) in the carotid arteries was examined by quantitative RT-PCR. Bars show overall mean ± SEM [n = 4–5 mice per group; *$P < 0.05$, ***$P < 0.001$ PBS vs. Poly(I:C); unpaired Student's t test]. CCL2: chemokine (C-C motif) ligand 2; CCL5: chemokine (C-C motif) ligand 5; VCAM1: vascular cell adhesion molecule 1.

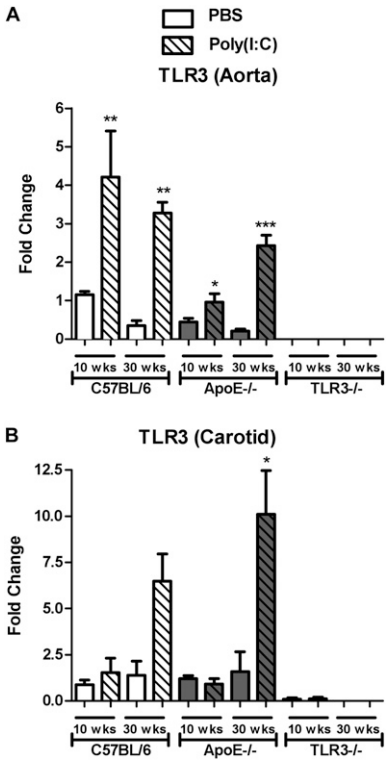

Fig. S4. TLR3 gene expression is induced in murine aortas and carotids following stimulation with Poly(I:C). Ten- and 30-wk-old C57BL/6, ApoE$^{-/-}$, and TLR3$^{-/-}$ mice were stimulated with PBS or 250 μg Poly(I:C). Twenty-four hours poststimulation, mice were killed, aortas and carotid arteries were harvested, and RNA was extracted. TLR3 gene expression was examined in the aorta (*A*) and the carotid arteries (*B*) by quantitative RT-PCR. Bars show overall mean ± SEM [n = 3–5 mice per group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; PBS vs. Poly(I:C); unpaired Student's t test].

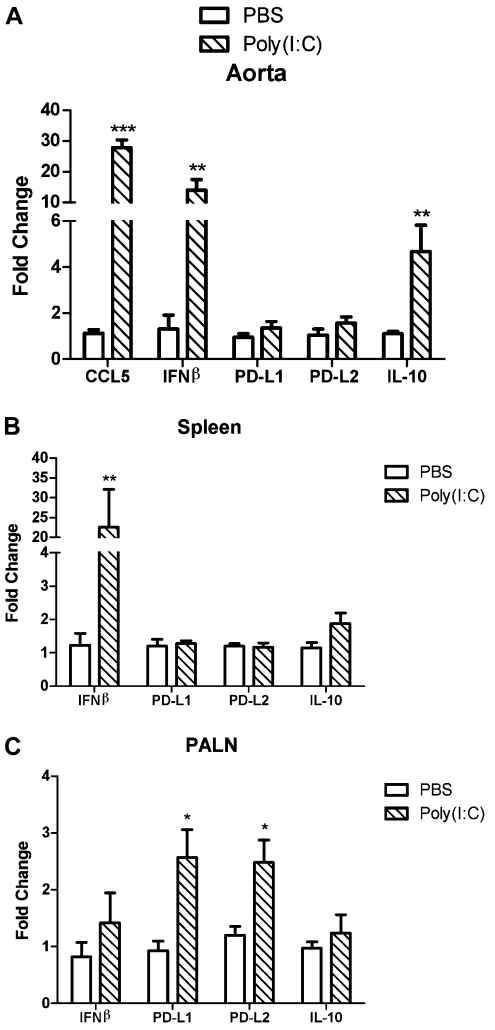

Fig. S5. Gene expression of anti-inflammatory factors is increased in aortas and lymphoid tissues in C57BL/6 mice treated with Poly(I:C). Aortas, spleens, and para-aortic lymph nodes (PALN) of C57BL/6 mice that underwent carotid artery injury and PBS or Poly(I:C) treatment were collected at sacrifice. RNA was extracted, and gene expression of CCL5, IFNβ, PD-L1, PD-L2, and IL-10 in the aorta (*A*), spleen (*B*), and PALN (*C*) was examined by RT-PCR. Bars show overall mean ± SEM [$n = 4$ mice per group; $^{*}P < 0.05$, $^{**}P < 0.01$ PBS vs. Poly(I:C); unpaired Student's t test]. CCL5: chemokine (C-C motif) ligand 5; IL-10: interleukin-10; PD-L1: programmed death-ligand 1; PD-L2: programmed death-ligand 2.

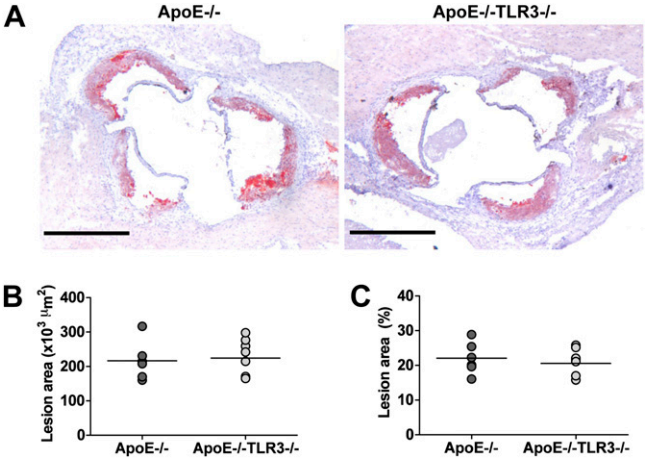

Fig. S6. TLR3 deficiency does not affect late atherosclerotic lesion development in the aortic root. (*A*) Representative photomicrographs of aortic roots from 30-wk ApoE$^{-/-}$ and ApoE$^{-/-}$TLR3$^{-/-}$ mice stained with Oil Red O and hematoxylin. (Scale bars: 500 μm.) (*B*) Cross-sectional aortic root lesion area (×10^3 μm^2) in 30-wk ApoE$^{-/-}$ ($n = 6$) and ApoE$^{-/-}$TLR3$^{-/-}$ mice ($n = 8$). (*C*) Cross-sectional aortic root lesion area (%) in 30-wk ApoE$^{-/-}$ and ApoE$^{-/-}$TLR3$^{-/-}$ mice. (*B* and *C*) Each circle represents the mean lesional area per individual mouse. Line represents the mean lesional area per group ($n = 6$–8; $P > 0.05$; unpaired Student's t test).

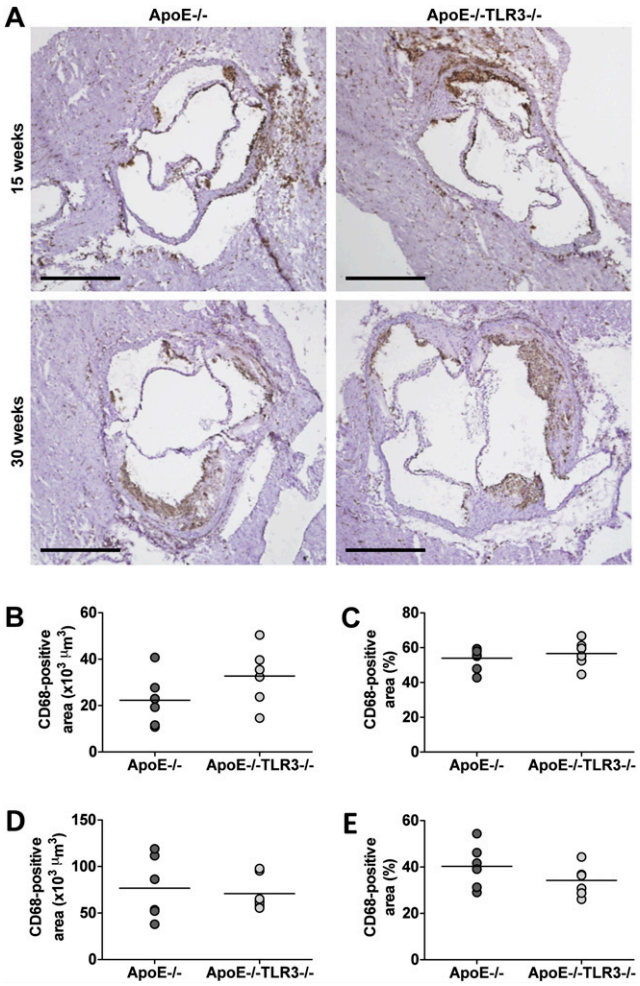

Fig. S7. TLR3 deficiency does not affect lesional macrophage content. (*A*) Representative photomicrographs of aortic roots from 15- and 30-wk ApoE$^{-/-}$ and ApoE$^{-/-}$TLR3$^{-/-}$ mice stained with an antibody against CD68 for macrophages and counterstained with hematoxylin. (Scale bars: 500 μm.) (*B* and *D*) Aortic root lesion area staining positive for CD68 (×10^3 μm^2) in 15-wk (*B*) and 30-wk (*D*) ApoE$^{-/-}$ and ApoE$^{-/-}$TLR3$^{-/-}$ mice. (*C* and *E*) Aortic root lesion area staining positive for CD68 (%) in 15-wk (C) and 30-wk (*E*) ApoE$^{-/-}$ and ApoE$^{-/-}$TLR3$^{-/-}$ mice. (*B*–*E*) Each circle represents the mean area staining positive per individual mouse. Line represents the mean area staining positive per group (n = 6–8; $P > 0.05$; unpaired Student's t test).

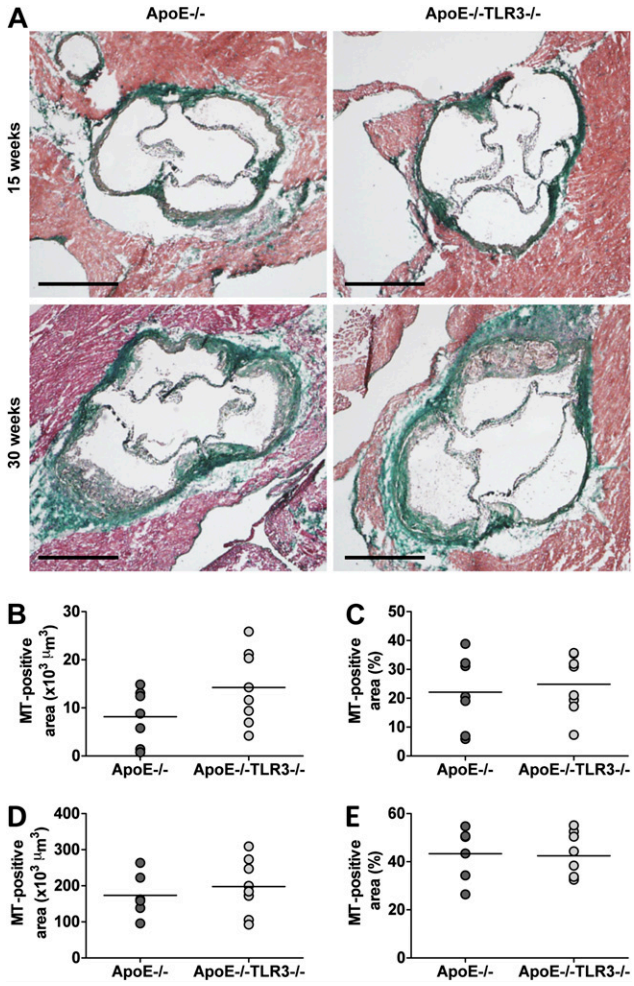

Fig. S8. TLR3 deficiency does not affect lesional collagen content. (*A*) Representative photomicrographs of aortic roots from 15- and 30-wk ApoE$^{-/-}$ and ApoE$^{-/-}$TLR3$^{-/-}$ mice stained with Masson trichrome staining for collagen (green) and muscle (pink). (Scale bars: 500 μm.) (*B* and *D*) Aortic root lesion area staining positive for collagen (×10^3 μm^2) in 15-wk (*B*) and 30-wk (*D*) ApoE$^{-/-}$ and ApoE$^{-/-}$TLR3$^{-/-}$ mice. (*C* and *E*) Aortic root lesion area staining positive for collagen (%) in 15- (*C*) and 30-wk (*E*) ApoE$^{-/-}$ and ApoE$^{-/-}$TLR3$^{-/-}$ mice. (*B*–*E*) Each circle represents the mean area staining positive per individual mouse. Line represents the mean area staining positive per group (n = 6–8; $P > 0.05$; unpaired Student's t test).

Table S1. Baseline characteristics of ApoE$^{-/-}$ and ApoE$^{-/-}$TLR3$^{-/-}$ mice aged 15 and 30 wk

Mouse genotype	Age (wk)	n	Final body weight ± SD (g)	Total cholesterol ± SEM (mg/dL)
ApoE$^{-/-}$	15	7	30.43 ± 2.79	356.0 ± 20.34
ApoE$^{-/-}$TLR3$^{-/-}$	15	8	29.61 ± 1.12	334.4 ± 26.98
ApoE$^{-/-}$	30	6	34.42 ± 2.25	388.6 ± 40.22
ApoE$^{-/-}$TLR3$^{-/-}$	30	8	34.21 ± 3.57	356.7 ± 34.22