

SUPPLEMENTAL MATERIAL

Deficient p27 Phosphorylation at Serine 10 Increases Macrophage Foam Cell Formation and Aggravates Atherosclerosis Through a Proliferation-Independent Mechanism

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METHODS

Metabolic measurements. Plasma lipid levels in mice fasted overnight were measured using enzymatic procedures (WAKO). HDL-cholesterol (HDL-C) was determined after precipitation of the apolipoprotein B-containing lipoproteins with dextran-sulphate/MgCl₂ (SIGMA) as previously described.¹

Immunohistochemical analysis of atherosclerotic plaque composition. Plaque composition was examined by immunohistochemical techniques performed by a researcher blinded to genotype. Vascular smooth muscle cells (VSMCs) were identified with mouse anti-smooth muscle α -actin (SMA) monoclonal alkaline phosphatase-conjugated antibody (1/20 dilution, clone 1A4) and Fast Red substrate (both from Sigma). Macrophages were detected with a rat anti-Mac3 monoclonal antibody (1/200 dilution, clone M3/84, sc-19991, Santa Cruz Biotechnology), followed by biotin-conjugated goat anti-rat secondary antibody (1/300 dilution, sc-2041, Santa Cruz Biotechnology), streptavidin-HRP (Ref. TS-060-HR, LabVision Corporation) and DAB substrate (BUF021A, AbD SEROTEC). Specimens were counterstained with hematoxylin. Collagen content was determined by Masson's trichrome staining.

Assessment of cell proliferation in the atherosclerotic plaque. Identification of proliferating macrophages, VSMCs and T-cells within the atherosclerotic lesions was achieved by double immunofluorescence assays to detect expression of the proliferation marker Ki67 together with the cell-type-specific antigens Mac3 (macrophages) CD3 (T-cells) and SMA (VSMCs). After deparaffinization, antigen retrieval and blocking of non-specific interactions (5% horse serum in PBS, 45 minutes), histological sections were incubated overnight at 4°C with anti-Ki67 antibody (prediluted, Clone SP6; Vitro) together with anti-Mac3 (1/500 dilution, clone M3/84, sc-19991, Santa Cruz Biotechnology), anti-SMA (1/75 dilution, Cy3-conjugated, C6198, SIGMA) or anti-CD3 (1/75 dilution, A0452, DAKO). Mac3 and CD3 were visualized with Alexa488-conjugated secondary antibodies (A11006, A-11034, Invitrogen) and Ki67 with an Alexa555-conjugated anti-rabbit IgG antibody (A21429, Invitrogen). Cell nuclei were stained with TOPRO-3 (T3605, Invitrogen). Slides were mounted with Slow-Fade Gold Antifade reagent (S36936, Invitrogen) and images were acquired on a Leica TCS/SP2 confocal microscope with a 40X oil immersion objective. Settings were adjusted to produce the optimum signal-to-noise ratio. Moreover, the sequential mode was used for image acquisition in order to avoid any interference from overlapping fluorescence. Quantification was done using the Metamorph software (Molecular Devices).

Blood cells counting. Circulating blood cells were counted and identified using an Abacus junior automated cell counter (Diatron).

Western blot analysis. Protein extracts from cultured macrophages and mouse aortic tissue were obtained using an ice-cold lysis buffer containing 50 mM Tris·Cl, pH 7.2, 1% (w/v) Triton X-100, 0.1% (w/v) SDS, 500 mM NaCl and 10 mM MgCl₂, supplemented with phosphatase and protease inhibitors (Roche). Protein extracts from human aortic samples were obtained using an ice-cold lysis buffer containing 50mM Tris-HCl pH 7.5, 1% (w/v) Triton X-100, 150mM NaCl and 1mM DTT, supplemented with phosphatase and protease inhibitors. Polyacrilamide gel-electrophoresis and Western blot analysis were done as reported¹ and detection of proteins was performed with the following antibodies: anti-p27 (610242), from BD Transduction Laboratories; anti-actin (A2066), from SIGMA; anti-tubulin

(sc-8035) and anti-cofilin (sc12549), from Santa Cruz Biotechnologies; anti-phospho-cofilin (Ab3831), from Chemicon-Millipore; anti- α -actin (M0851), from Dako; anti-ERM (3142) and anti-phospho-ERM (3149), from Cell Signalling; and anti- β -actin (ab8227) and anti-p27-phospho-S10 (ab62364), from Abcam. HRP-conjugated secondary antibodies were from Santa Cruz Biotechnologies. Immunocomplexes were detected with the ECL Plus detection kit (Amersham Biosciences).

Macrophage culture. Bone marrow-derived macrophages (BMDMs) were obtained from suspensions of femoral BM and differentiated for 7 days in the presence of Dulbecco's Modified Eagle Medium supplemented with antibiotics, 10% fetal bovine serum and 10% L929-cell conditioned medium as a source of macrophage colony-stimulating factor (MCSF). Peritoneal macrophages were obtained from the peritoneal cavity of mice, plated in standard cell culture dishes for 2 h and then extensively washed to eliminate non-attached cells.

Macrophage cell cycle analysis. For in vitro cell-cycle analysis, BMDM were synchronized in G0/G1 by 36 h of MCSF deprivation (DMEM, 10% FBS, 0.5% L929-cell-conditioned medium) and then stimulated for different times with complete medium (DMEM, 10% FBS, 10% L929-cell-conditioned medium). Macrophages were trypsinized and collected by centrifugation for 5 min at 300g. After fixation in 80% ethanol for 1 h at -20°C, cells were incubated for at least 30 min with 50 μ g/mL propidium iodide (P4170, SIGMA) containing 0.25 mg/mL RNase A (R4642, SIGMA). Labelled cells were analyzed in a FACSCanto flow cytometer (Becton Dickinson) and DNA histograms were fitted into cell cycle distributions using the ModFit 3.0 software (Verity Software House).

BM transplantation. BM transplantation was carried out essentially as we have previously described.² Briefly, female apoE^{-/-} mice were irradiated with 2 doses of 4 Gy and transplanted with BM cells (2×10^6) obtained from five pooled femurs of male apoE^{-/-} or apoE^{-/-}-p27S10A mice. After 4 weeks on standard diet, transplanted mice were placed on atherogenic diet for 2 months. Transplant efficiency was assessed as the relative proportion of donor cells in the BM of recipient mice by real-time quantitative DNA amplification of Y-chromosome sequences essentially as previously described.³

Phospho-ERM immunofluorescence. After deparaffinization, antigen retrieval and blocking of non-specific interactions (1 % BSA in PBS, 45 minutes), aortic cross-sections were incubated overnight at 4°C with anti-phospho-ERM antibody (1/500, clone 41A3, 3149, Cell Signalling) together with anti-Mac3 (1/500 dilution, clone M3/84, sc-19991, Santa Cruz Biotechnology) and anti-SMA (1/75 dilution, Cy3-conjugated, C6198, SIGMA). Phospho-ERM and Mac3 were visualized with an Alexa635-conjugated anti-rabbit-IgG antibody (A31577, Invitrogen), and an Alexa488-conjugated anti-rat IgG antibody (A11006, Invitrogen), respectively. Cell nuclei were stained with DAPI (D3571, Invitrogen). Slides were processed and images were acquired as described above (see *Assessment of cell proliferation in the atherosclerotic plaque*).

Gene expression analysis by quantitative real-time PCR (qPCR). RNA from peritoneal macrophages was obtained using TRIzol Reagent (Invitrogen). After verification of purity and concentration by the $A_{260/280}$ ratio, RNA (0.5-1 μ g) was retro-transcribed and amplified with Superscript III First Strand Synthesis Supermix and Platinum Quantitative PCR Supermix-UDG with Rox dye (both from Invitrogen). The following primers (Forward: Fw;

Reverse: Rv) designed with the Primer Express software (Applied Biosystems) were used (mouse sequences):

Cd36:

Fw 5'-TCGGAAGTGTGGGCTCATTG-3'
Rv 5'-CCTCGGGGTCCTGAGTTATATTTTC-3'

Sra:

Fw 5'-CATGAACGAGAGGATGCTGACT-3'
Rv 5'-GGAAGGGATGCTGTCATTGAA-3'

Lox-1:

Fw: 5'-TGCGAATGACGAGCCTGAT-3'
Rv: 5'-AGAAAGCAAATGCAGACCTTTAGG-3'

Vldlr:

Fw: 5'-GAAGGAATGCCATATCAACGAAT-3'
Rv: 5'-AGGTCTTTGCAGATATGGGAACA-3'

Tlr2:

Fw: 5'-CCCTGTGCCACCATTTCC-3'
Rv: 5'-GCCACGCCACATCATTC-3'

Tlr4:

Fw: 5'-CCTGACACCAGGAAGCTTGAA-3'
Rv: 5'-TCTGATCCATGCATTGGTAGGT-3'

Cyclophilin:

Fw 5'-TGGAGAGCACCAAGACAGACA-3'
Rv 5'-TGCCGGAGTCGACAATGAT-3'

Reactions were run on a thermal Cycler 7500 Fast System and results were analyzed with the software provided by the manufacturer (Applied Biosystems). mRNA levels in apoE^{-/-}p27S10A mice were expressed relative to those in apoE^{-/-} mice. Gene expression in both genotypes was normalized to the expression of the endogenous cyclophilin control.

Cholesterol accumulation and efflux assays. For cholesterol accumulation assays, peritoneal macrophages were plated at a density of 5×10^5 cells/well in 24-well plates. After 24 h, triplicate wells were cultured in DMEM with 10% human lipoprotein deficient serum, in the presence of 2 μ Ci/ml of ³H-cholesterol (Perkin Elmer) and 50 μ g/ml of acLDL (Biomedical Technologies). ³H-cholesterol accumulation in macrophages was measured by scintillation counting after extraction of cellular lipids with hexane/isopropanol (2:1). For cholesterol efflux assays, peritoneal macrophages were subjected to ³H-cholesterol loading as described above for 36 h. Next, cells were extensively washed in PBS, and then incubated in DMEM plus either 100 μ g/ml HDL (Biomedical Technologies) or 0.1 % BSA for 24 h. Radioactivity was measured by scintillation counting in the medium and in cellular lipids

after extraction with hexane/isopropanol (2:1). HDL-induced cholesterol efflux was calculated using the following equation:

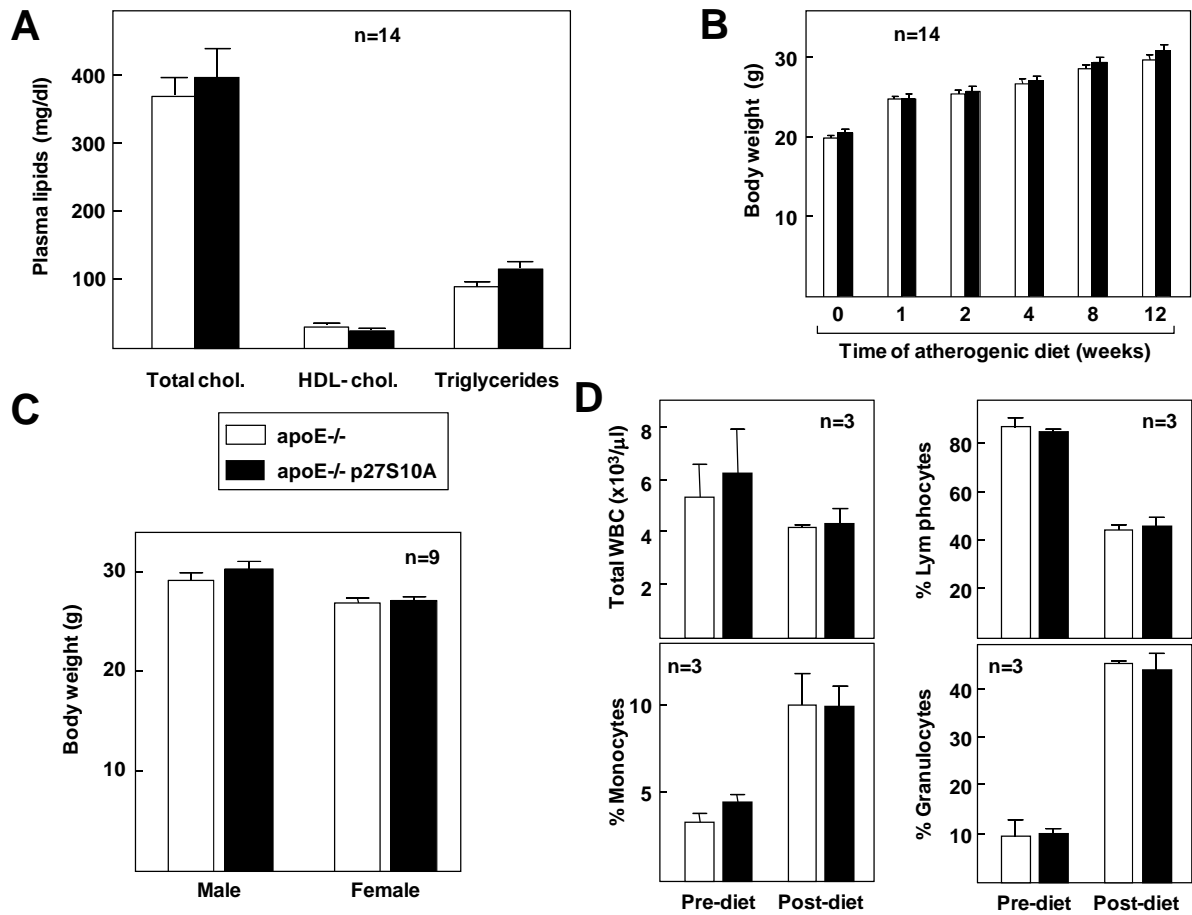
$$\% \text{ efflux} = \left(\frac{\text{cpm in medium}}{\text{cpm in medium} + \text{cpm in cells}} \cdot 100 \right)^{\text{HDL}} - \left(\frac{\text{cpm in medium}}{\text{cpm in medium} + \text{cpm in cells}} \cdot 100 \right)^{\text{BSA}}$$

Beads phagocytosis assays. Peritoneal macrophages were plated at a density of 1×10^6 cells/well in 6-well plates and incubated for 90 minutes in the presence of $1 \mu\text{m}$ carboxylate-coated fluorescent beads (Sigma). After extensive washes in PBS, macrophages were collected by trypsinization and the internalization of beads was examined by flow cytometry.

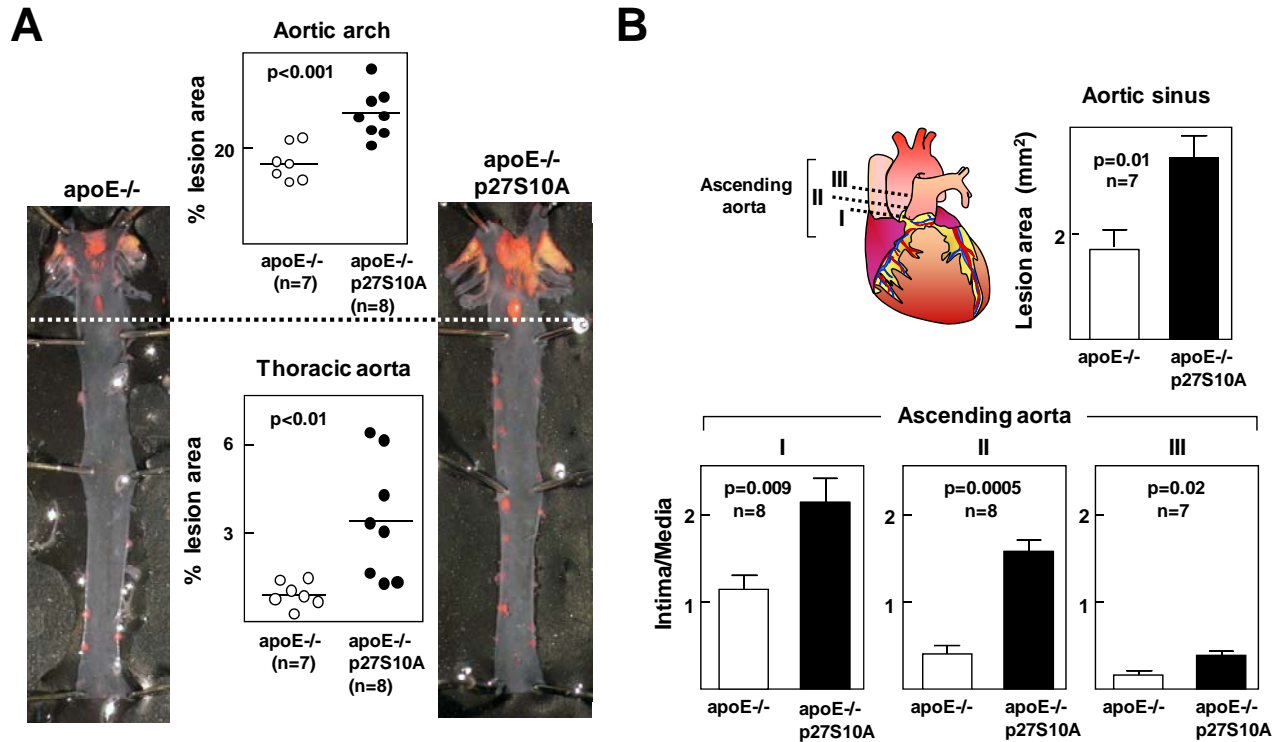
Human artery sampling and preservation. Human coronary artery samples were collected from freshly excised hearts removed during transplant operations at the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain). The study was approved by the local ethics committee of and was conducted according to the Declaration of Helsinki (revised in 2000). Atherosclerotic samples were obtained from coronary artery disease patients aged 41-63 years, while non-atherosclerotic samples were from patients aged 14-48 years who had healthy coronary arteries but with serious dysfunctions affecting myocardium or great vessels that require heart transplantation (i.e. dilated cardiomyopathy, great vessel transposition). Immediately after surgical excision, the common trunk of the left coronary artery was dissected, cut in rings of approximately 0.5 cm, immersed in cell-maintenance media, and cleaned of connective tissue and fat under low magnification with a zoom stereo microscope. This examination permitted the classification of tissue as either atherosclerotic, assessed by the presence of evident atherosclerotic lesions, or non-atherosclerotic, as deduced from absence of fibro-fatty tissue or visible plaques. Specimens were frozen in liquid nitrogen and stored at -80°C until protein extraction.

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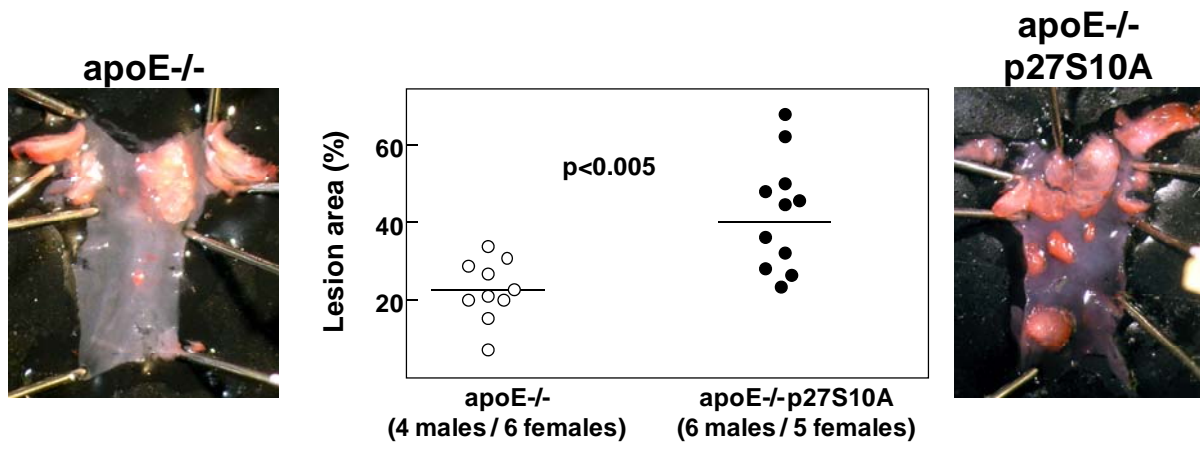
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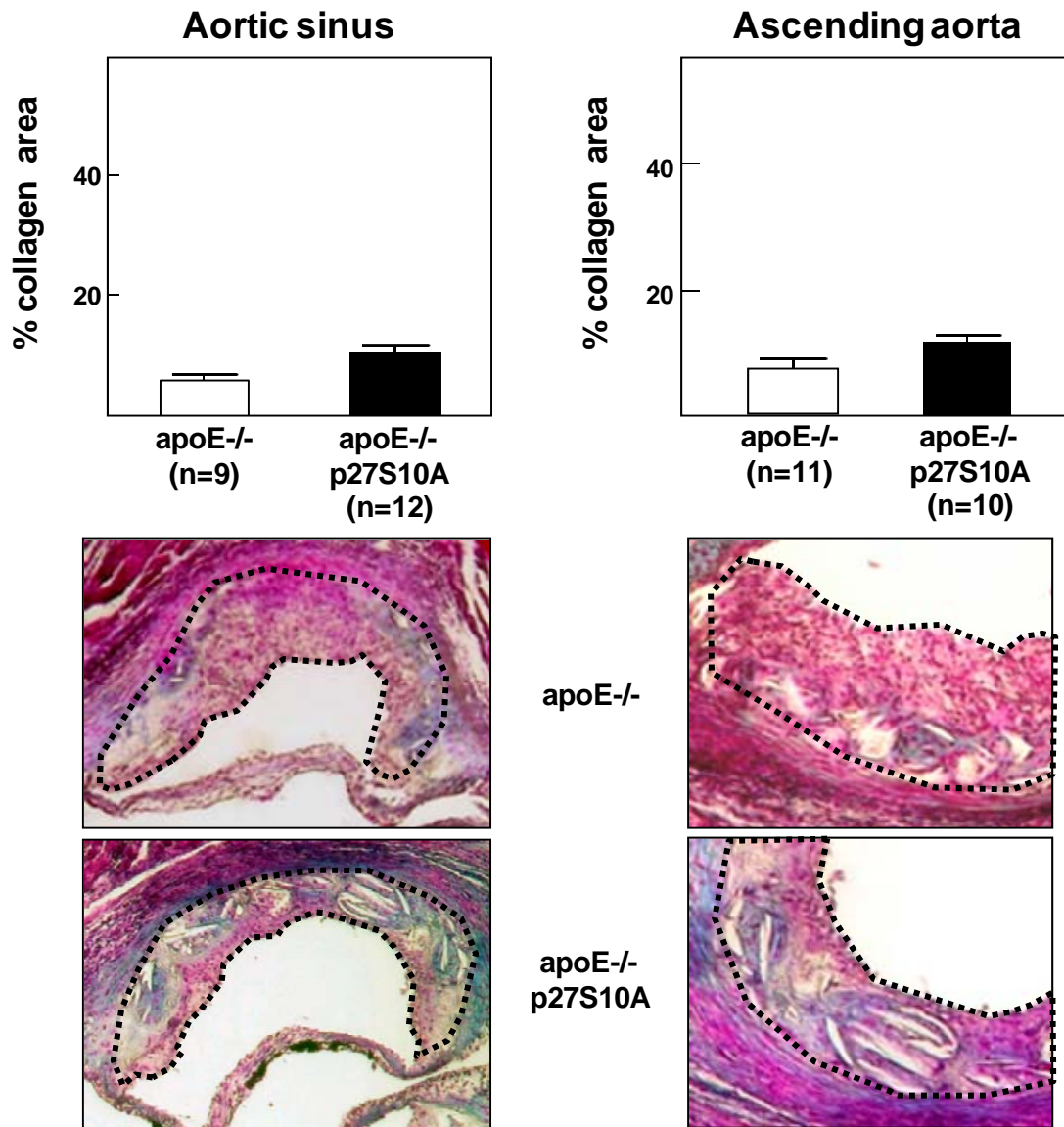
Supplemental Figure I. Effects of p27Ser10Ala mutant expression on plasma lipid levels, body weight, and circulating blood cell counts. Mice were fed standard chow (A, C) or high-fat diet for 12 weeks (B, D). Total chol.: Total cholesterol; HDL-chol.: HDL-cholesterol.



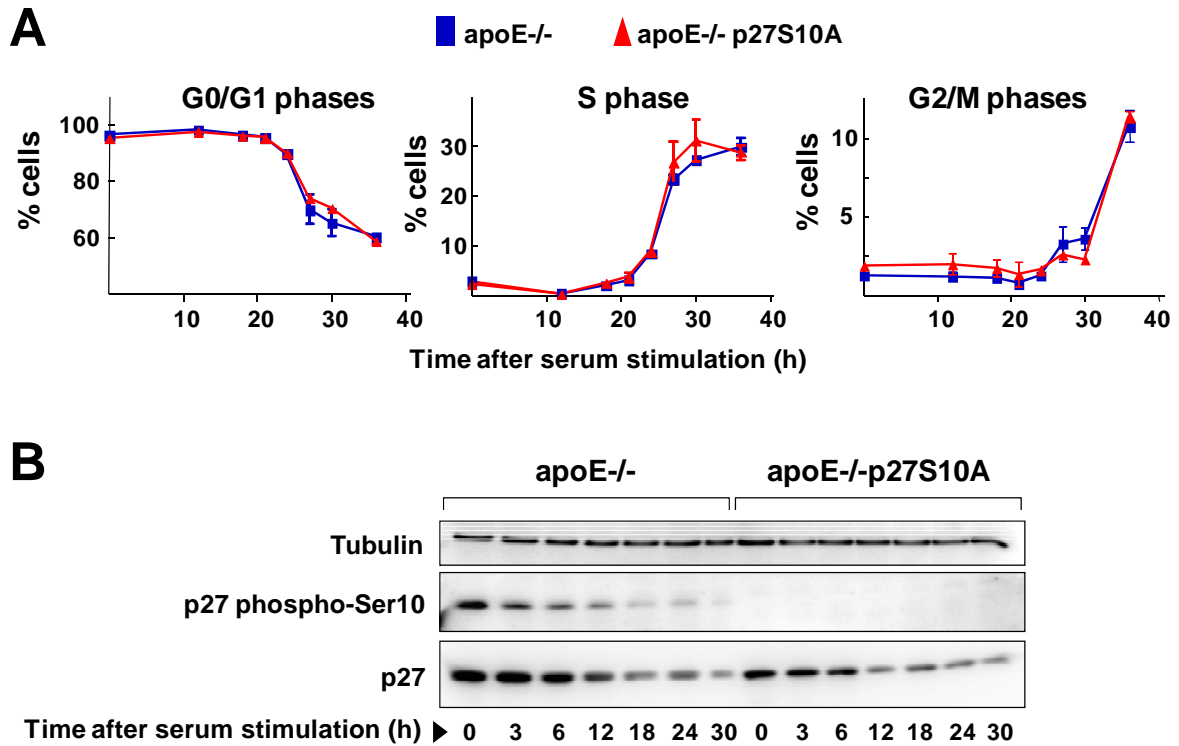
Supplemental Figure II. Expression of p27Ser10Ala mutant hastens atherosclerosis in female apoE^{-/-} mice. Atherosclerosis burden was quantified in female mice fed a high-fat diet for 8 weeks. **(A)** Atheroma size in the aortic arch and the thoracic aorta quantified by en-face Oil Red-O staining. Representative images are shown. **(B)**. Atheroma size quantified in histological sections from the aortic sinus and from three different regions of the ascending aorta separated by approximately 30 μ m (Ascending Aorta I, II and III, starting at the end of the aortic valve). Representative images of hematoxylin/eosin-stained sections are shown below. Atherosclerotic plaques are delineated by discontinuous lines.



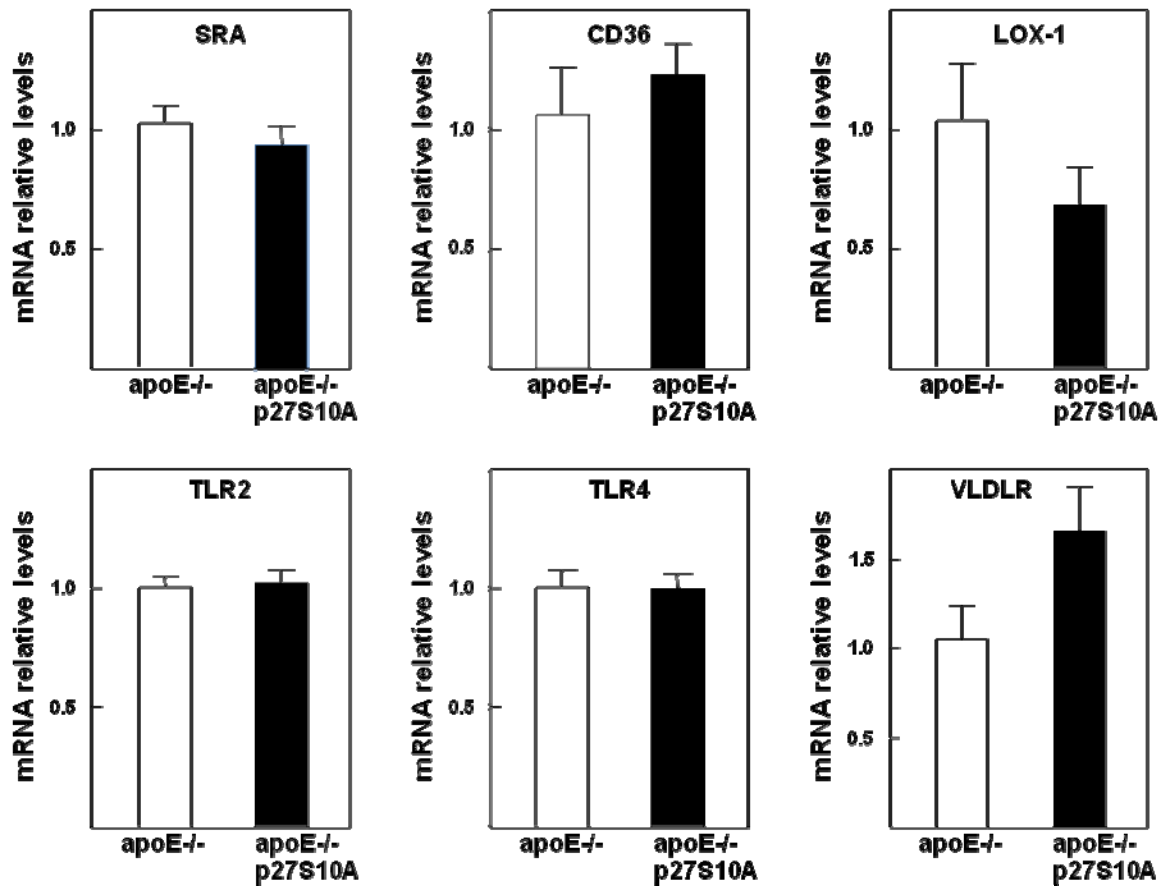
Supplemental Figure III. Expression of p27Ser10Ala mutant accelerates native atherosclerosis in apoE^{-/-} mice fed standard chow. Atheroma size in the aortic arch of 9-month-old mice was quantified by en-face Oil Red-O staining. Representative images are shown and the number of mice analyzed is indicated.



Supplemental Figure IV. Effects of p27Ser10Ala mutant expression on collagen content in atheromata. Collagen content was determined by Masson's trichrome staining of histological sections from apoE-/- and apoE-/-p27Ser10Ala mice fed a high-fat diet for 12 weeks. No statistically-significant differences were observed.



Supplemental Figure V: Expression of p27Ser10Ala mutant in macrophages reduces p27 protein levels but does not affect cell-cycle progression. Bone-marrow-derived macrophages were synchronized in G0 by 36 h of MCSF deprivation and then stimulated for the indicated times with 10% L929-cell conditioned medium as a source of MSCF. **(A)** Cell cycle kinetics were analyzed by propidium iodide staining and flow cytometry. **(B)** p27 levels were analyzed by Western blot.



Supplemental Figure VI: Expression of p27Ser10Ala mutant in macrophages does not affect the expression of receptors involved in the uptake of modified LDL. Transcript levels in peritoneal macrophages were analyzed by quantitative real-time PCR (n=4). No statistically-significant differences were observed. SRA: Scavenger receptor A; LOX-1: lectin-like oxidized low-density lipoprotein receptor-1; TLR2/4: Toll-like receptor 2/4; VLDLR: very low-density lipoprotein receptor.