Deficient p27 phosphorylation at serine 10 increases macrophage foam cell formation and aggravates atherosclerosis through a proliferation-independent mechanism

Fuster et al.

SHORT TITLE: p27 phosphorylation at serine10 in atherosclerosis

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ABSTRACT

OBJECTIVE: Genetic ablation of the growth suppressor $p27^{Kip1}$ (p27) in the mouse aggravates atherosclerosis coinciding with enhanced arterial cell proliferation. However, it is unknown whether molecular mechanisms that limit p27's protective function contribute to atherosclerosis development, and if p27 exerts proliferation-independent activities in the arterial wall. This study aims to provide insight into both questions by investigating the role in atherosclerosis of p27 phosphorylation at serine 10 (p27-phospho-S10), a major post-translational modification of this protein.

METHODS AND RESULTS: Immunoblotting studies reveal a marked reduction in p27-phospho-S10 in atherosclerotic arteries from apolipoprotein E-null mice, and expression of the non-phosphorylatable mutant p27S10A, either global or restricted to bone marrow, accelerates atherosclerosis. p27S10A expression does not affect cell proliferation in early and advanced atheroma, but activates RhoA/ROCK signalling and promotes macrophage foam cell formation in a ROCK-dependent manner. Supporting the clinical relevance of these findings, human atherosclerotic coronary arteries exhibit a prominent reduction in p27-phospho-S10 and increased ERM phosphorylation, a marker of RhoA/ROCK activation.

CONCLUSIONS: Scarce phosphorylation of p27 at S10 is a hallmark of human and mouse atherosclerosis and promotes disease progression in mice. This proatherogenic effect is mediated by a proliferation-independent mechanism that involves augmented foam cell formation owing to increased RhoA/ROCK activity. These findings unveil a new atheroprotective action of p27, and identify p27-phospho-S10 as an attractive target for the treatment of atherosclerosis

KEY WORDS: atherosclerosis; macrophage foam cells; p27; RhoA; ROCK

Atherosclerosis and associated ischemic events are the leading cause of morbidity and mortality in industrialized countries. Atherosclerotic plaque formation is a multifactorial chronic inflammatory process characterized by the interaction between oxidized low-density lipoproteins (oxLDL), endothelial cells, macrophages, T-cells and vascular smooth muscle cells (VSMCs). Atherosclerosis is initially driven by the deposition in the arterial wall of oxLDL that provoke a chronic inflammatory response. Macrophages in the atherosclerotic plaque avidly internalize oxLDL and become foam cells that critically contribute to plaque development by secreting a plethora of inflammatory mediators. [1-3]

Human and animal studies have demonstrated that excessive cell proliferation in the arterial wall is a hallmark of atherosclerosis.[4, 5] Mammalian cell proliferation is controlled by a large number of proteins that regulate the cell cycle. Major positive regulators of cell proliferation are holoenzymes composed of a catalytic cyclin-dependent protein kinase (CDK) and a regulatory cyclin. Cell cycle progression is negatively regulated by CDK inhibitory proteins of the Cip/Kip (CDK interacting protein/kinase inhibitory protein: $p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$) and Ink4 (inhibitor of CDK4: $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$, $p19^{Ink4d}$) families.[6] Mitogen-induced downregulation of $p27^{Kip1}$ (p27) is critical for the activation of specific CDK-cyclin complexes and the subsequent transcriptional activation of genes required for the G1/S-phase transition and the initiation of DNA replication.[7] In most cell types, the activity of p27 is regulated by post-translational modifications that affect its stability and/or subcellular localization.[7, 8] Phosphorylation of p27 at serine 10 (p27-phospho-S10) is a major mechanism of p27 regulation in different scenarios,[9-15] and it has been estimated to be the most abundant post-translational modification of p27 in cultured cells, accounting for 70-75 % of its phosphate incorporation.[9] This phosphorylation event contributes importantly to p27 stabilization in the

G0 phase of the cell cycle,[9, 11, 13-15] and promotes p27 exit from the nucleus in certain settings.[10, 12, 15, 16]

p27 is a major negative regulator of cell proliferation in various pathophysiological settings, including cancer[17] and vasculoproliferative disease (eg, atherosclerosis and restenosis).[4, 18, 19] We have previously demonstrated that both global and hematopoietic cell-restricted inactivation of p27 accelerate diet-induced atherosclerosis in apolipoprotein E-deficient mice (apoE-/-), coinciding with increased cell proliferation within the atheroma.[20, 21] The atheroprotective action of p27 in this animal model has been corroborated by others.[22] However, it remains unknown whether molecular mechanisms that limit p27's protective function indeed occur during human or experimental atherosclerosis. Moreover, although compelling *in vitro* evidence has emerged suggesting that p27 has cell-cycle-independent activities,[23] their pathophysiological relevance, and in particular their possible atheroprotective role, remains unexplored. In the present study, we provide insight into these questions by demonstrating that p27-phospho-S10 is markedly reduced in human and mouse atherosclerosis, and that expression of the non-phosphorylatable p27S10A mutant aggravates disease progression through a cell-cycle-independent mechanism that involves increased RhoA/ROCK signalling and augmented foam cell formation.

METHODS

NOTE: see additional Methods in the online supplement

Mice. Care of animals was in accordance with institutional guidelines and regulations. p27S10A knock-in mice[14] were backcrossed for more than eight generations in a C57BL/6J background and then interbred with apoE-/- mice (C57BL/6J, Charles River) to generate apoE-/-p27S10A mice and apoE-/- littermates. After weaning, mice were maintained on a low-fat standard diet (2.8% fat; Panlab, Barcelona, Spain). For diet-induced atherosclerotic studies, two-month-old mice were placed on atherogenic diet (10.8% total fat, 0.75% cholesterol, S4892-E010, Ssniff, Germany) for the indicated periods of time.

Quantification of atherosclerosis burden. Mice were sacrificed and the aorta was removed after *in situ* perfusion with PBS followed by 4% paraformaldehyde/PBS. Fixation was continued overnight at 4ºC. An operator who was blinded to genotype quantified the extent of atherosclerosis by computer-assisted morphometric analysis (SigmaScan Pro 5) of both whole-mounted aorta stained with Oil Red-O (O0625, Sigma, 0.2% Oil Red-O in 80% MeOH) and in hematoxylin/eosin-stained cross-sections of aortic tissue as previously described.[24, 25]

RhoA activity assay. RhoA activity in peritoneal macrophages obtained from 5-month-old mice fed standard chow (pool of three mice) was measured as the amount of RhoA-GTP using the absorbance-based RhoA G-LISA™ Activation Assay according to manufacturer's instructions (BK-124, Cytoskeleton).

Analysis of modified LDL uptake and macrophage foam cell formation. For modified acetylated LDL (acLDL) uptake studies, peritoneal macrophages from female fat-fed apoE-/- and apoE-/-p27S10A mice were incubated for 3 hours with AlexaFluor488-labelled acLDL (1

μg/mL, Invitrogen) in serum-free media. Cells were then collected and acLDL uptake was quantified by flow cytometry as the relative median fluorescence intensity. For *in vivo* oxLDL uptake by arterial macrophages, fat-fed mice received an intravenous injection of 20 μg diI-labelled oxLDL (Biomedical Technologies). One day post-administration, mice were sacrificed and the aorta was digested as described.[26] Cells were then collected and stained with an Alexa647-conjugated antibody against the macrophage-specific antigen F4/80 (Serotec). oxLDL uptake was quantified by flow cytometry as the relative median fluorescence intensity in F4/80-positive cells from aortic cell suspensions. For *in vivo* foam cell counting, freshly-isolated resident peritoneal macrophages were plated on coverslips for 60 min and extensively washed to remove non-adherent cells. After fixation with 4% paraformaldehyde, cells were stained with Oil-Red-O and counterstained with hematoxylin.

Statistical analysis. Data are presented as mean±SEM. In experiments with two groups, statistical significance was evaluated using a 2-tail, unpaired Student's t-test. Otherwise, a two-way ANOVA with Bonferroni's post-hoc test was performed (GraphPad Prism software). Differences were considered statistically significant at $p<0.05$.

RESULTS

Reduced level of p27-phospho-S10 is associated with atherosclerosis and aggravates disease progression in apoE-/- mice. To gain insight into the potential role in atherosclerosis of p27 phosphorylation at S10, we first performed Western blot analysis in murine atherosclerotic arteries using a phospho-specific antibody. We found that p27-phospho-S10 is markedly down-regulated in the atherosclerotic aortic arch of fat-fed apoE-/- mice (which exhibited prominent atherosclerotic lesions) compared with non-atherosclerotic tissue from age-matched controls fed standard chow **(Fig.1A)**. To assess if impaired p27-phospho-S10 is causally linked to atherogenesis, we interbred apoE-/- mice with p27S10A knock-in mice, which have both p27 alleles replaced by a version carrying a Ser→Ala mutation at position 10 that blocks phosphorylation at this residue.[14] Both under standard chow and high-fat diet, circulating lipids levels **(Fig.1B, Supplemental Fig.IA),** body weight **(Supplemental Fig.IB,C)** and blood cell populations **(Supplemental Fig.ID)** were similar in apoE-/- and apoE-/-p27S10A mice. However, Oil Red-O staining revealed increased atheroma size in the aortic arch and thoracic aorta of fat-fed male apoE-/-p27S10A compared with apoE-/- mice **(Fig.1C)**. Likewise, we observed augmented atherosclerosis in cross-sections from the aortic sinus and three different regions of the ascending aorta separated by approximately 30 μm (**Fig.1D**). Expression of p27S10A also increased atherosclerosis burden in fat-fed female apoE-/- mice (**Supplemental Fig.II**) and in mice of both genders fed standard chow (**Supplemental Fig.III**). Overall, the relative content of macrophages and VSMC in the atherosclerotic plaque was comparable in both genotypes, with the exception of a significant but very modest decrease in VSMC content in the ascending aorta of apoE-/-p27S10A mice (**Fig.2**). Similarly, no significant differences were observed in the collagen content of atheromata (**Supplemental**

Fig.IV). Collectively, these findings demonstrate that expression of the non-phosphorylatable p27S10A mutant hastens both native and diet-induced atherosclerosis development in male and female hypercholesterolemic apoE-/- mice.

Proatherogenic effect of p27S10A expression is cell proliferation-independent. We have previously shown that genetic inactivation of p27 in apoE-null mice increases cell proliferation within atherosclerotic plaques and aggravates disease progression.[20, 21] To assess whether p27S10A expression similarly affects neointimal cell proliferation, we performed immunofluorescence assays in the aortic sinus of fat-fed mice to detect expression of the proliferation marker Ki67 together with different cell-type specific antigens (SMA for VSMCs; CD3 for T-cells; Mac3 for macrophages). We noted similar percentage of neointimal Ki67-positive VSMCs (**Fig.3A**), T-cells (**Fig.3B**) and macrophages (**Fig.3C**) in advanced plaques of both genotypes. Likewise, no significant differences were observed in the percentage of Ki67-immunoreactive Mac3-positive cells in early fatty-streaks consisting mostly of macrophages (**Fig.3D**). Moreover, cultured bone marrow-derived macrophages (BMDM) of both genotypes exhibited similar cell-cycle kinetics (**Supplemental Fig.VA)** in spite of lower p27 levels in apoE-/-p27S10A macrophages at all time-points analyzed **(Supplemental Fig.VB).** These findings suggest that expression of the non-phosphorylatable p27S10A mutant promotes atherosclerosis by a proliferation-independent mechanism.

Expression of p27S10A in bone marrow-derived cells accelerates atherosclerosis in apoE-/- mice. Given that macrophages are the most abundant cell type in atheromas of apoE-/- mice (confer Fig.2) and play key roles in atherosclerosis,[1-3] we performed BM transplants to assess whether expression of the p27S10A mutant restricted to BM-derived cells affects the

development of atherosclerosis. Lethally-irradiated female apoE-/- mice were transplanted with male apoE-/- or apoE-/-p27S10A BM and then fed a high-fat diet for 8 weeks. Transplant efficiency in the BM was similar in both groups (apoE-/-BM: 88±12%; apoE-/-p27S10A-BM: 83±10%). Remarkably, atheroma size in the aortic arch and the thoracic aorta was increased in apoE-/- mice transplanted with apoE-/-p27S10A BM compared with apoE-/- BM **(Fig.4)**. These results demonstrate that abrogating p27-phospho-S10 specifically in hematopoietic cells is sufficient to aggravate atherosclerosis, and suggest that this post-translational modification plays an important role in the regulation of the atheroprotective actions that p27 exerts in macrophages, the most abundant BM-derived cell in the atherosclerotic plaque.

Expression of p27S10A in macrophages reduces p27 total levels but increases its nuclear localization. Previous studies have reported that p27S10A expression leads to decreased p27 protein levels by reducing its stability in quiescent fibroblasts and lymphocytes,[14, 15] and may also alter p27 subcellular localization by blocking its nuclear export.[10, 12, 15, 16] Consistent with these findings, we found a ~two-fold reduction in total p27 protein levels (**Fig.5A**) and increased relative nuclear accumulation of p27 in apoE-/-p27S10A macrophages (**Fig.5B**).

Expression of p27S10A increases RhoA/ROCK signalling. The results presented thus far suggest that expression of p27S10A aggravates atherosclerosis via a cell proliferation-independent mechanism that operates in macrophages. Previous studies have shown that p27 can interact in the cytoplasm with the small GTPase RhoA, blocking its activation by RhoGEFs and thereby restricting signalling through the RhoA/ROCK pathway,[27] a major contributor to cardiovascular disease.[28, 29] To address whether p27S10A expression affects RhoA/ROCK signalling, we first analyzed the degree of phosphorylation of the ezrin/radixin/moesin proteins

(ERM), a reliable marker of the activity of this pathway.[30] Consistent with a previous study,[30] phospho-ERM (pERM) was abundant in neointimal macrophages but absent in medial VSMCs (**Fig.6A**). Importantly, we found increased pERM in the atherosclerotic aortic arch of fat-fed apoE-/-p27S10A compared to apoE-/- mice (**Fig.6B**). Likewise, peritoneal macrophages obtained from fat-fed apoE-/-p27S10A mice exhibited increased pERM, as well as higher levels of GTP-bound-RhoA and cofilin phosphorylation, two additional markers of RhoA/ROCK activation (**Fig.6C).**

Expression of p27S10A augments modified lipoprotein uptake and foam cell formation in a ROCK-dependent manner. We next investigated the internalization of modified lipoproteins by macrophages, an essential pro-atherogenic process that is facilitated by RhoA/ROCK signalling.[31] To this end, we analyzed *in vitro* the uptake of fluorescently-labelled acLDL by resident peritoneal macrophages. We found increased uptake of acLDL in apoE-/-p27S10A macrophages, and this was blunted by pharmacological inhibition of ROCK with either Y-27632 or hydroxyfasudil (**Fig.7A**). We also observed increased ^{3}H-cholesterol accumulation in acLDL-loaded p27S10A macrophages (**Fig.7B**) without significant effects on cholesterol efflux (**Fig. 7C**). Supporting the *in vivo* relevance of these findings, we found increased uptake of diI-labelled-oxLDL by macrophages within the atheroma of apoE-/-p27S10A mice, which almost reached statistical significance (p=0.08) (**Fig.7D**), and a higher percentage of foam cells in peritoneal macrophages of fat-fed apoE-/-p27S10A mice (**Fig.7E**). The latter occurred without changes in the expression of either the prototypical scavenger receptors CD36 and SRA or other membrane proteins that may mediate lipoprotein uptake, such as VLDLR, TLR2, TLR4 or LOX1 (**Supplemental Fig.VI**), but coincided with increased bead phagocytosis by apoE-/-p27S10A macrophages, which was blunted by pharmacological inhibition of ROCK (**Fig.7F**).

Human atherosclerotic arteries exhibit reduced p27-phospho-S10 and increased ERM phosphorylation. To address the clinical relevance of our findings, we examined the degree of phosphorylation of p27 and ERM in human coronary arteries (**Fig.8**). These studies revealed abundant p27-phospho-S10 in non-atherosclerotic vessels, but undetectable levels in atherosclerotic specimens, which correlated with increased pERM in atherosclerotic arteries. These findings support the notion that p27-phospho-S10 regulates the activity of the RhoA/ROCK signalling pathway in human arteries.

DISCUSSION

Animal studies have demonstrated an important atheroprotective role of the tumour suppressor p27, which has been attributed to its function as a negative regulator of cell proliferation.[20, 21] Consistent with this idea, human studies revealed frequent colocalization of p27 and TGF-β receptors in atherosclerotic coronary arteries[32] and abundant expression of p27 in non-proliferating cells within both normal and atherosclerotic arteries.[33] However, the possibility that p27 exerts proliferation-independent activities in the arterial wall has not been analyzed to date, in spite of an increasing body of evidence suggesting that p27 modulates the activity of various signalling proteins other than CDKs and cyclins. It also remains largely unexplored whether changes in the expression or function of p27 occur during human or experimental atherosclerosis and are causally linked to disease progression. In this study, we address these questions by combining cell culture experiments and studies with atherosclerosis-prone apoE-/- mice and human specimens. To the best of our knowledge, we demonstrate for the first time the impairment of a post-translational modification of p27 during atherosclerosis

that might be causally linked to disease progression by limiting a proliferation-independent atheroprotective function of p27 in macrophages.

In most cell types, the activity of p27 is regulated by post-translational modifications, predominantly phosphorylation at different residues.[7, 8] We previously found that atherosclerosis development is not affected in apoE-/- mice unable to phosphorylate p27 at threonine 187,[24] a post-translational modification of p27 that controls its stability and function in several tissues and cell types.[7, 8] In this study, we assessed the role in atherosclerosis of p27 phosphorylation at S10, which appears to be the most abundant post-translational modification of p27 and modulates its stability in different scenarios.[9-15] Using a phospho-specific antibody which exhibits high specificity in Western blot (confer Fig.5A), we found a marked down-regulation of p27-phospho-S10 in the atherosclerotic aortic arch of fat-fed apoE-/- mice versus non-atherosclerotic arteries of controls fed with standard diet. Supporting a cause-effect relationship between impaired p27-phospho-S10 and atherosclerosis, we found that expression of the non-phosphorylatable p27S10A mutant accelerates atherosclerosis in different vascular beds in apoE-/- mice of both genders fed either standard chow or high-fat diet. Our BM transplantation studies demonstrate that expression of the p27S10A mutant restricted to hematopoietic cells is sufficient to accelerate atherosclerosis development in apoE-/- mice, suggesting that lack of p27-phospho-S10 critically affects the function of macrophages in the vascular wall. Supporting this notion, we found reduced total p27 protein levels and increased nuclear p27 localization in cultured macrophages expressing p27S10A. These results are consistent with previous studies showing that expression of this non-phosphorylatable mutant reduces total p27 protein levels in fibroblasts and lymphocytes,[14, 15] and affects p27's subcellular localization in some cell types by restraining its exit from the nucleus to the cytoplasm.[10, 12, 15, 16]

We have previously shown that genetic disruption of p27 aggravates atherosclerosis in apoE-/- mice coinciding with increased VSMC and macrophage proliferation in the vessel wall.[20, 21] Moreover, mice lacking p27 exhibit increased body size and organomegaly which have been attributed to increased cell proliferation.[34-36] Therefore, we hypothesized that expression of p27S10A promotes atherogenesis by increasing neointimal cell proliferation. However, our studies revealed comparable amounts of proliferating VSMCs, T-cells and macrophages in advanced atherosclerotic lesions of fat-fed apoE-/- and apoE-/-p27S10A mice. Similarly, we found no differences in the proliferation of macrophages in early fatty streaks, and similar kinetics of cell-cycle progression in cultured macrophages obtained from apoE-/- and apoE-/-p27S10A mice. Moreover, unlike p27-null mice,[34-36] apoE-/-p27S10A mice have normal body weight. These results are consistent with previous studies showing that p27S10A expression does not affect body size and cell-cycle progression of lymphocytes and fibroblasts in spite of lowering p27 levels,[14, 15] suggesting that impaired p27-phospho-S10 does not affect cell proliferation. It is plausible that higher nuclear localization of p27 in cells expressing p27S10A compensates for the overall lower level of p27, thus allowing normal cell cycle progression. Supporting this notion, Besson and coworkers reported that the p27S10A mutant exhibits increased interaction with CDK/cyclin complexes due to its nuclear accumulation.[15] Taken together, our previous results in apoE-/- mice lacking p27[20, 21] and the findings in apoE-/-p27S10A presented herein strongly support that p27 exerts both cell cycle-dependent and independent atheroprotective functions.

Having discarded abnormal cell proliferation as the mechanism underlying the aggravation of atherosclerosis in apoE-/-p27S10A mice, we examined whether p27S10A expression promotes atherogenesis by limiting a yet unidentified atheroprotective activity of p27 independent of its growth suppressive function. Indeed, p27 can regulate the activity of a number of regulatory proteins other than CDKs and cyclins.[27, 37, 38] We focused our attention on

the small GTPase RhoA, which is inhibited through interaction with p27.[27] RhoA regulates a plethora of cellular processes via its downstream kinases ROCK1/2,[39] and strong evidence exists that activation of this pathway contributes to neointimal thickening in the setting of atherosclerosis, vessel ligation and balloon angioplasty in different murine models.[28, 29, 31, 40-44]. Our mechanistic studies provide the first evidence supporting a pathophysiologically relevant link between p27 and RhoA. We have shown that p27S10A expression augments RhoA/ROCK signalling in both atherosclerotic plaques of apoE-/- mice and in cultured macrophages. Moreover, apoE-/-p27S10A macrophages exhibit increased uptake of modified LDL and phagocytic activity, and these responses are blunted by pharmacological inhibition of ROCK1/2. These findings are consistent with previous studies showing that ROCK1 inactivation reduces foam cell formation,[31] and that ROCK2 promotes phagocytosis.[45] We also find that the effect of p27S10A expression on lipoprotein uptake correlates with augmented accumulation of cholesterol and is not mediated neither by reduced cholesterol efflux nor by changes in the expression of scavenger receptors (including SRA, CD36, LOX-1, VLDL-R, TLR2 and TLR4). Therefore, considering that the RhoA/ROCK pathway is involved in actin cytoskeletal remodeling during the engulfment of foreign material,[46] it is tempting to speculate that p27S10A macrophages exhibit RhoA-mediated cytoskeletal alterations that favour lipoprotein endocytosis/phagocytosis leading to increased foam cell formation.

Supporting the clinical relevance of our findings, we found a marked reduction in p27-phospho-S10 level in atherosclerotic human coronary arteries. Human atherosclerosis was also accompanied by increased ERM phosphorylation in the arterial wall, suggesting augmented RhoA/ROCK signalling. On the basis of the results presented herein, we propose that scarce phosphorylation of p27 at S10 in the atherosclerotic plaque contributes to disease progression in a proliferation-independent manner, at least in part owing to reduced p27 levels in macrophages, which lead to increased foam cell formation through RhoA/ROCK activation.

When taken together with our previous studies with p27-null mice,[20, 21] these findings indicate that p27 exerts both cell cycle-dependent and independent atheroprotective functions that could be potentiated by overexpressing p27 and preventing the loss of p27-phospho-S10, respectively. However, given that p27 overexpression may be expected to indiscriminately block cell proliferation and thus compromise plaque stability (e.g., by decreasing the thickness of VSMC-containing fibrous caps), the development of drug-based therapies preventing the loss of p27-phospho-S10 merits further investigation.

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DISCLOSURE

None declared

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FIGURE LEGENDS

Fig.1: Reduced p27 phosphorylation at Ser10 is associated with murine atherosclerosis and accelerates disease progression in hypercholesterolemic mice. (A) Western Blot analysis of a pool of six aortic arches obtained from male apoE-/- mice fed either control chow (tissue essentially free of atheroma) or a high-fat atherogenic diet for 12 weeks (tissue with prominent atherosclerotic lesions). The p27-phosho-S10/p27 ratio was normalized to actin as loading control and the average result of three different blots is shown (p27-pS10: p27 phosphorylated in serine 10). (**B, C, D**) Male apoE-/- and apoE-/-p27S10A mice were fed a high-fat diet for 12 weeks. (B) Plasma lipid levels before and after fat-feeding. (C) Atheroma size in the aortic arch and the thoracic aorta quantified by en-face Oil Red-O staining. Representative images are shown. (D) Atheroma size quantified in histological sections from the aortic sinus and from three different regions of the ascending aorta (I, II, III). Representative images of hematoxylin/eosin-stained sections are shown below (atherosclerotic plaques delineated by discontinuous lines).

Fig.2. Effects of p27S10A expression in atherosclerotic plaque composition. Male mice were fed a high-fat diet for 12 weeks and the neointimal content of macrophages and VSMCs was quantified in the aortic sinus and the ascending aorta by immunostaining of Mac3 and SMA, respectively. Representative images are shown below (atherosclerotic plaques delineated by discontinuous lines).

Fig.3: p27S10A expression does not affect cell proliferation in the atheroma. Double immunofluorescent staining to detect Ki67 and different cell-type-specific antigens within atherosclerotic plaques in the ascending aorta of male apoE-/- and apoE-/-p27S10A mice fed atherogenic diet for 12 weeks (A-C) or 4 weeks (D). Arrows point to double-positive cells. TOPRO3 stains nuclei. The graphs show the percentage of Ki67/SMA double-positive cells within the SMA-positive population (A), Ki67/CD3 double-positive cells within the CD3-positive population (B), and Ki67/Mac3 double-positive cells within the Mac3-positive population (C, D). ns: non-significant ($p>0.05$).

Fig.4: Transplant of bone marrow expressing the p27S10A mutant accelerates atherogenesis in hypercholestherolemic apoE-/- mice. Quantification of atheroma size by en-face Oil Red-O staining in the aortic arch and the thoracic aorta of irradiated apoE-/- female mice transplanted with male apoE-/- or apoE-/-p27S10A BM. Representative images are shown.

Fig.5: Effects of p27S10A expression on p27 protein levels and subcellular localization (**A)** Representative Western Blot analysis of BM-derived and resident peritoneal macrophages from apoE-/- and apoE-/-p27S10A mice. The relative level of p27 was determined as the p27/tubulin or p27/actin ratio and the average result of two different experiments is shown (p27-pS10: p27 phosphorylated in serine 10). (**B)** Representative Western blot analysis of cytoplasmic and nuclear fractions from apoE-/- and apoE-/-p27S10A BMDM. The relative level of p27 was determined as the ratio p27/GAPDH (cytoplasmic extracts) or p27/Lamin (nuclear extracts). The graph shows the relative nuclear/cytoplasmic p27 distribution (n=2 experiments).

Fig.6: p27S10A expression increases RhoA/ROCK signalling in atheromata and cultured macrophages. (**A**) Immunofluorescence staining to detect simultaneously Mac3, SMA and phosho-ERM (marker of RhoA/ROCK signalling) in atherosclerotic aorta of fat-fed apoE-/- mice. (**B**) RhoA/ROCK activity in atherosclerotic aorta of fat-fed apoE-/- and apoE-/-p27S10A mice measured as the degree of ERM phosphorylation. The average quantification of two different experiments is shown. (**C**) RhoA/ROCK activity in apoE-/- and apoE-/-p27S10A peritoneal macrophages measured as the amount of RhoA bound to GTP (top), phospho-cofilin (middle) and phospho-ERM (bottom).

Fig.7: p27S10A expression promotes macrophage foam cell formation through increased RhoA/ROCK signalling. (**A**) Flow cytometry quantification of fluorescently-labelled acLDL uptake by cultured peritoneal macrophages in the absence or presence of ROCK1/2 inhibitors (Y-27632, hydroxyfasudil). (**B**) ^{3}H-cholestherol accumulation in acLDL-loaded cultured peritoneal macrophages. (**C**) ^{3}H-cholesterol cholesterol efflux in peritoneal macrophages treated with 100 μg/ml HDL for 24 h (n=4, see details in Supplemental Methods). (**D**) Mice were injected with fluorescently-labelled oxLDL to quantify by flow cytometry *in vivo* uptake by arterial macrophages. (**E**) Percentage of peritoneal macrophages exhibiting cytoplasmic lipid droplets after Oil Red-O staining. Representative images are shown. (**F**) Flow cytometry quantification of fluorescent bead phagocytosis by peritoneal macrophages in the absence or presence of Y-27632.

Fig.8: Human atherosclerosis features deficient p27 phosphorylation at S10 and increased ERM phosphorylation. A total of 7 non-atherosclerotic and 10 atherosclerotic coronary artery samples were processed from different patients. Representative Western blots are shown. p27-pS10: p27 phosphorylated at serine 10.

FIGURE 1

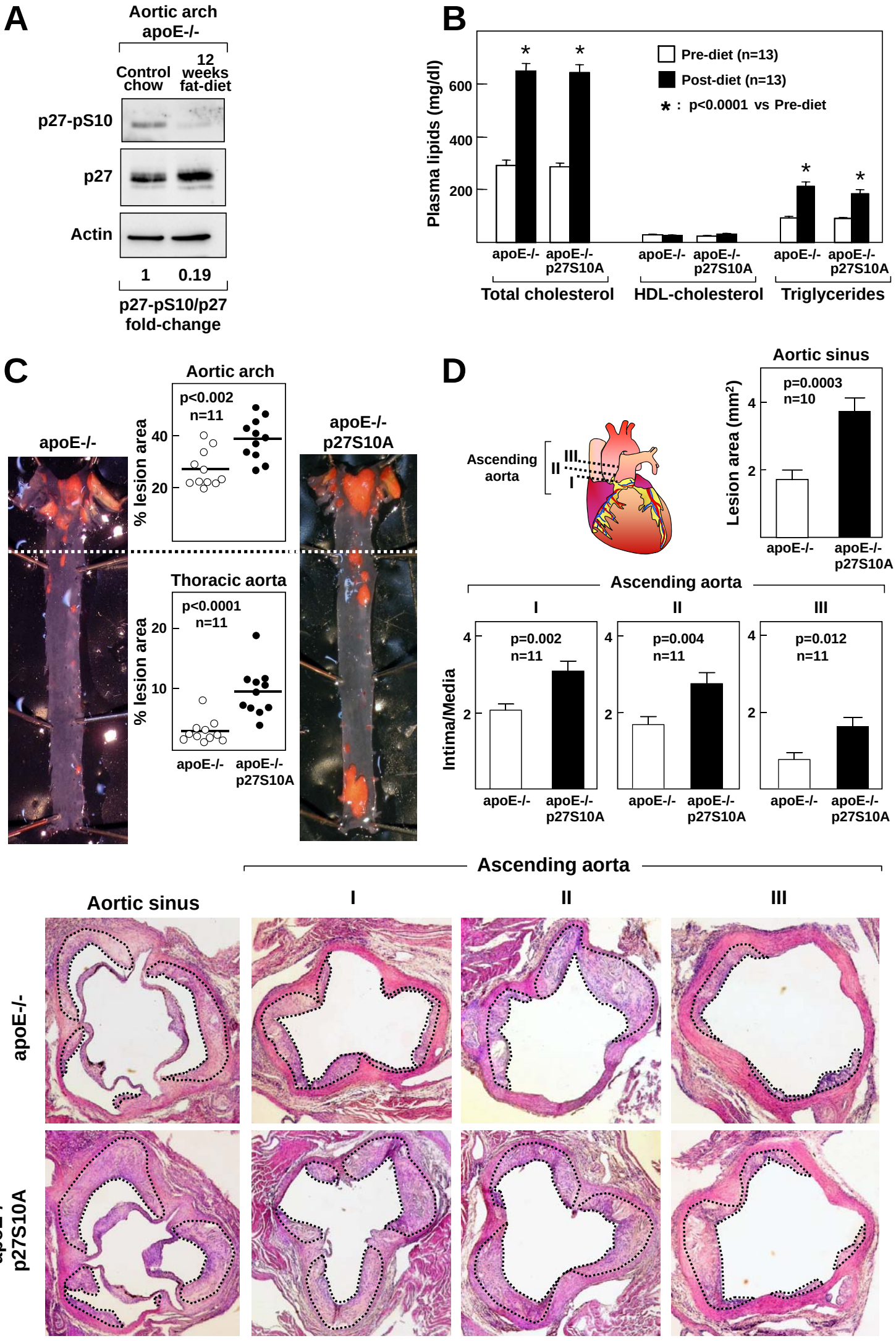

A
Aortic arch
apoE-/-
Control chow
12 weeks fat-diet
p27-pS10
p27
Actin
1
0.19
p27-pS10/p27 fold-change
B
Plasma lipids (mg/dl)
600
400
200
Pre-diet (n=13)
Post-diet (n=13)
* : p<0.0001 vs Pre-diet
apoE-/-
apoE-/- p27S10A
Total cholesterol
HDL-cholesterol
Triglycerides
C
Aortic arch
apoE-/-
apoE-/- p27S10A
% lesion area
p<0.002
n=11
Thoracic aorta
p<0.0001
n=11
D
Ascending aorta
III
II
I
Aortic sinus
Lesion area (mm²)
p=0.0003
n=10
Intima/Media
p=0.002
n=11
p=0.004
n=11
p=0.012
n=11
Aortic sinus
Ascending aorta
apoE-/-
apoE-/- p27S10A

FIGURE 2

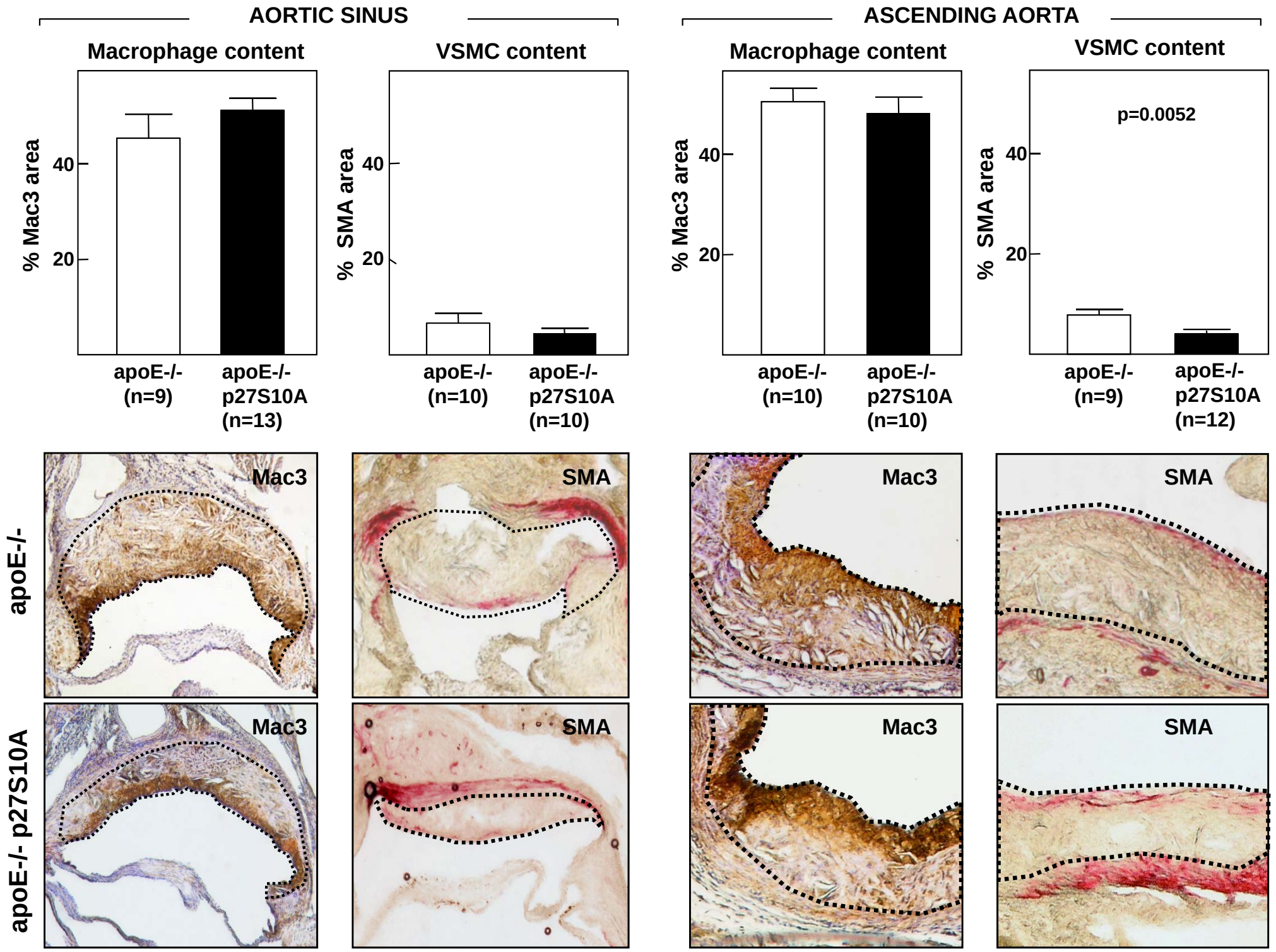

AORTIC SINUS
ASCENDING AORTA
Macrophage content
VSMC content
Macrophage content
VSMC content
% Mac3 area
% SMA area
% Mac3 area
% SMA area
40
20
40
20
40
20
40
20
p=0.0052
apoE-/- (n=9)
apoE-/- p27S10A (n=13)
apoE-/- (n=10)
apoE-/- p27S10A (n=10)
apoE-/- (n=10)
apoE-/- p27S10A (n=10)
apoE-/- (n=9)
apoE-/- p27S10A (n=12)
apoE-/-
apoE-/- p27S10A
Mac3
SMA
Mac3
SMA
Mac3
SMA
Mac3
SMA

FIGURE 3

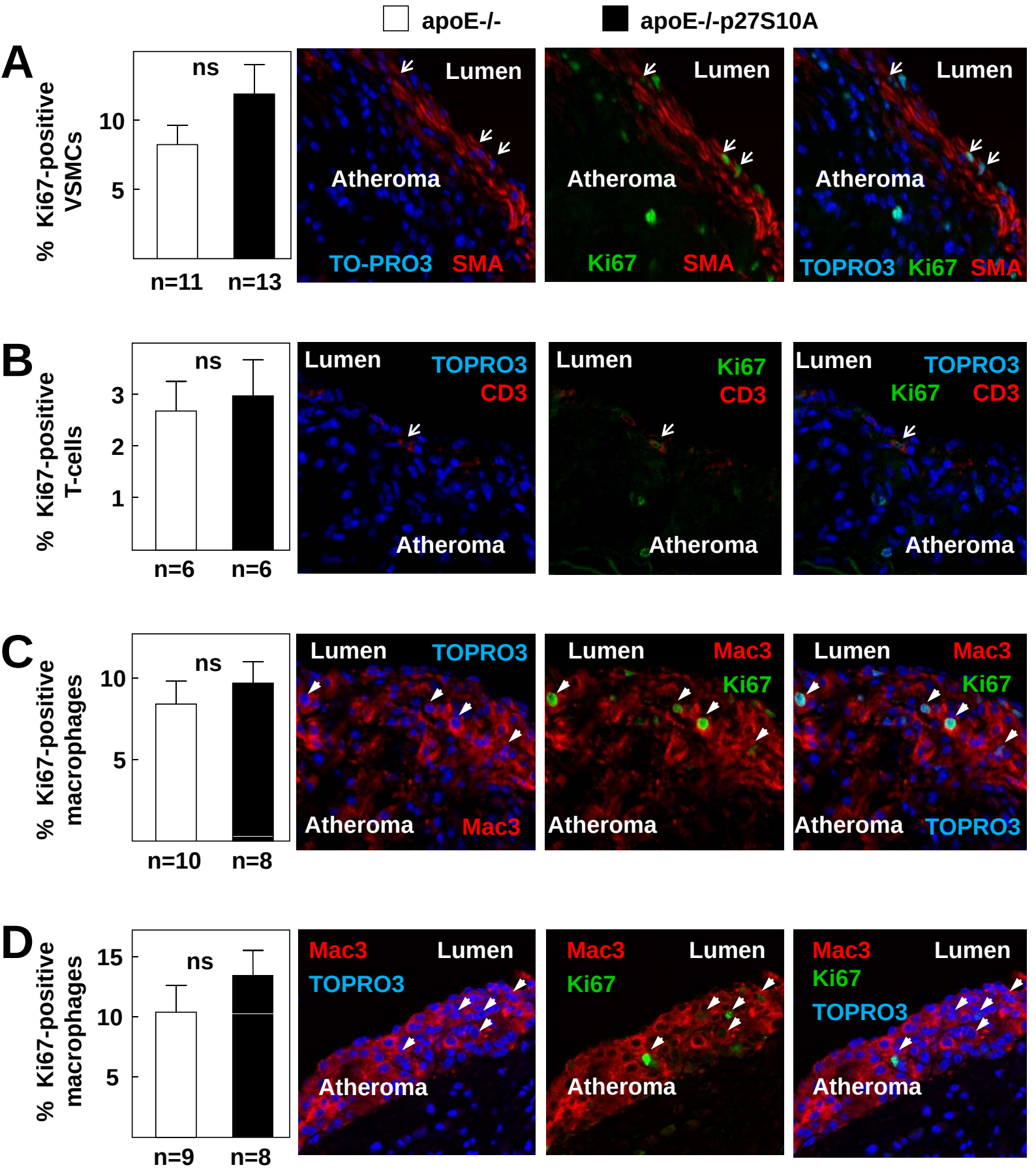

apoE-/-
apoE-/-p27S10A
A
% Ki67-positive VSMCs
ns
10
5
n=11
n=13
Lumen
Atheroma
TO-PRO3
SMA
Ki67
TOPRO3
B
% Ki67-positive T-cells
3
2
1
n=6
CD3
C
% Ki67-positive macrophages
n=10
n=8
Mac3
D
15
n=9

FIGURE 4

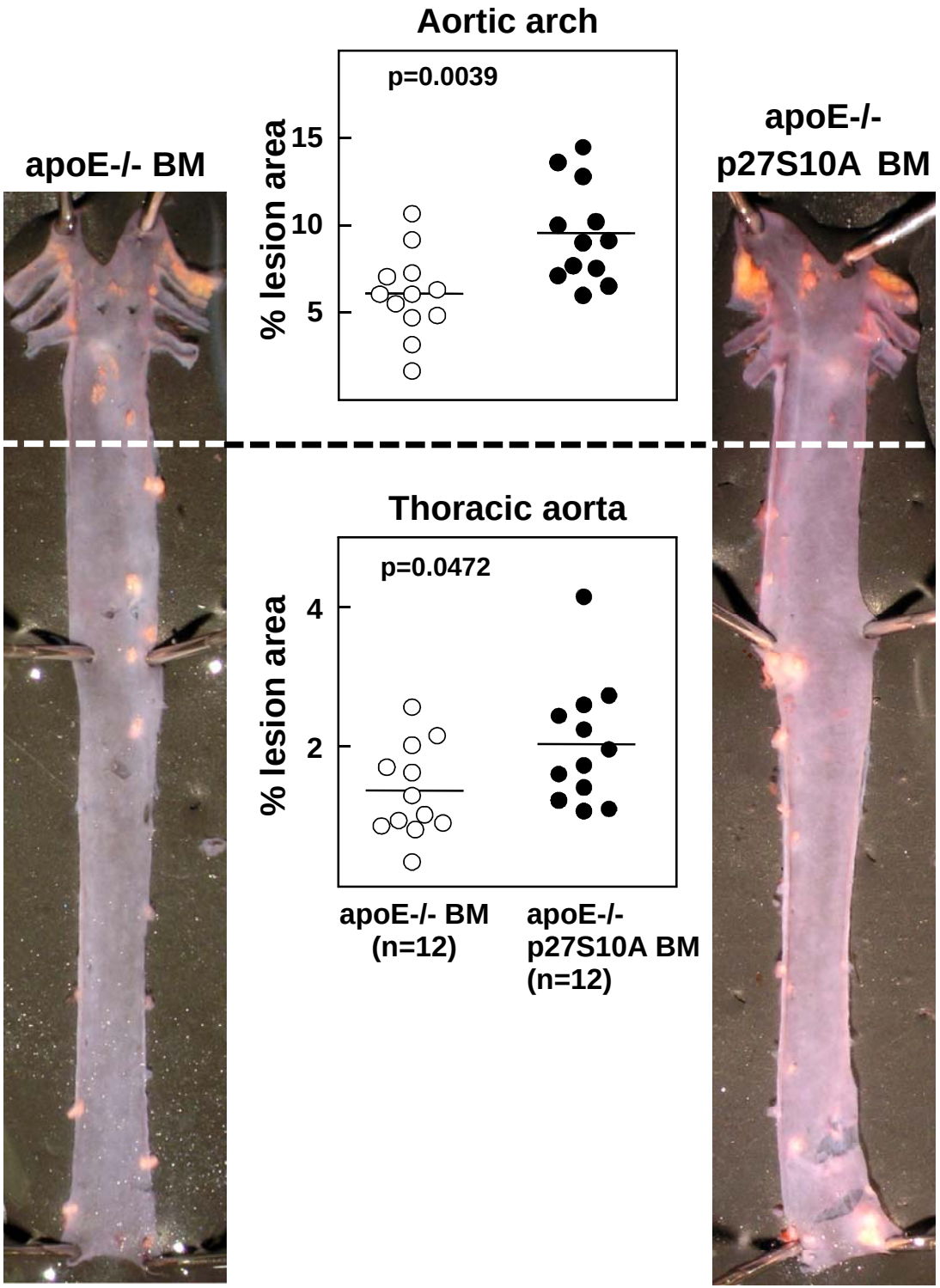
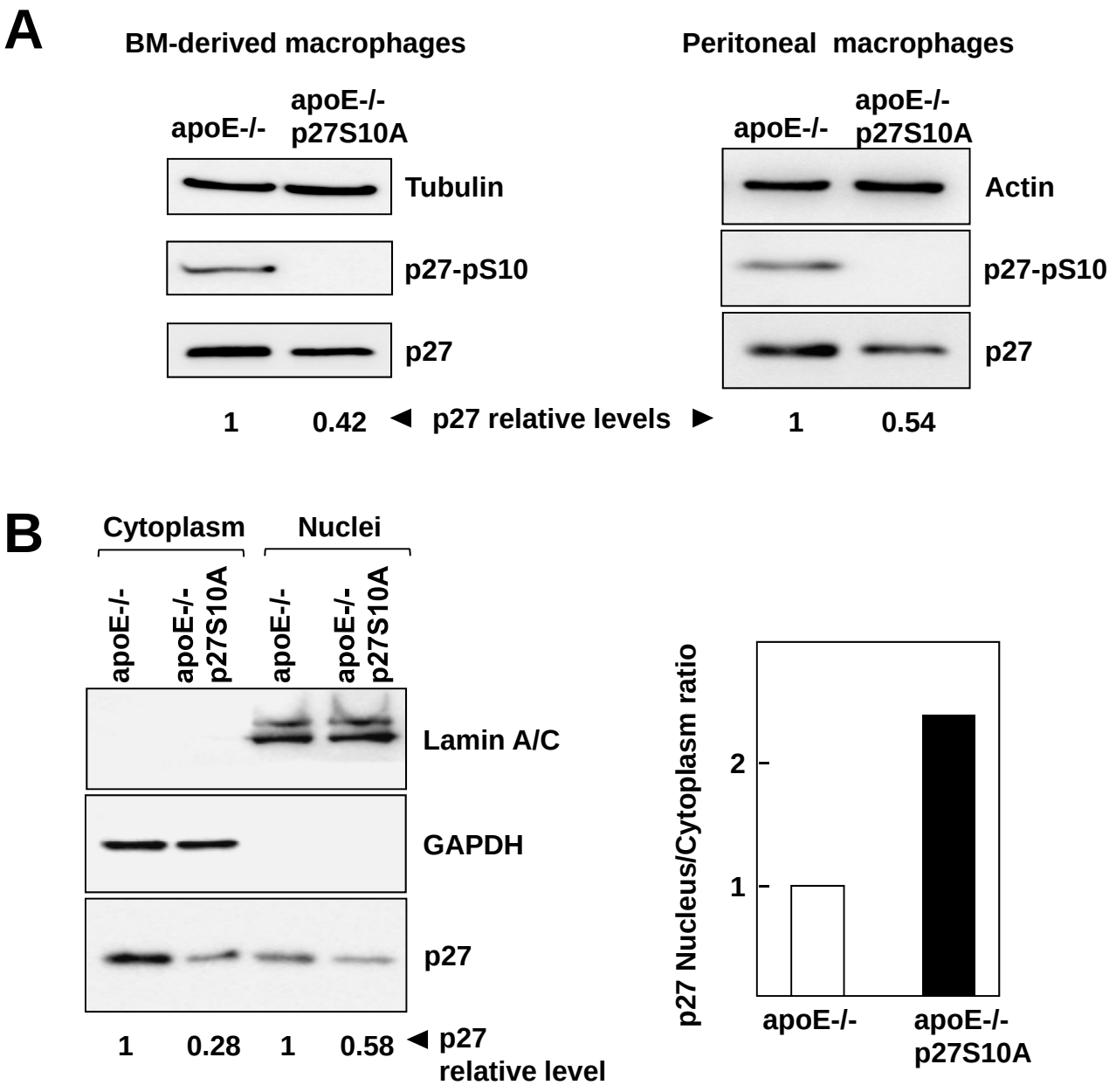
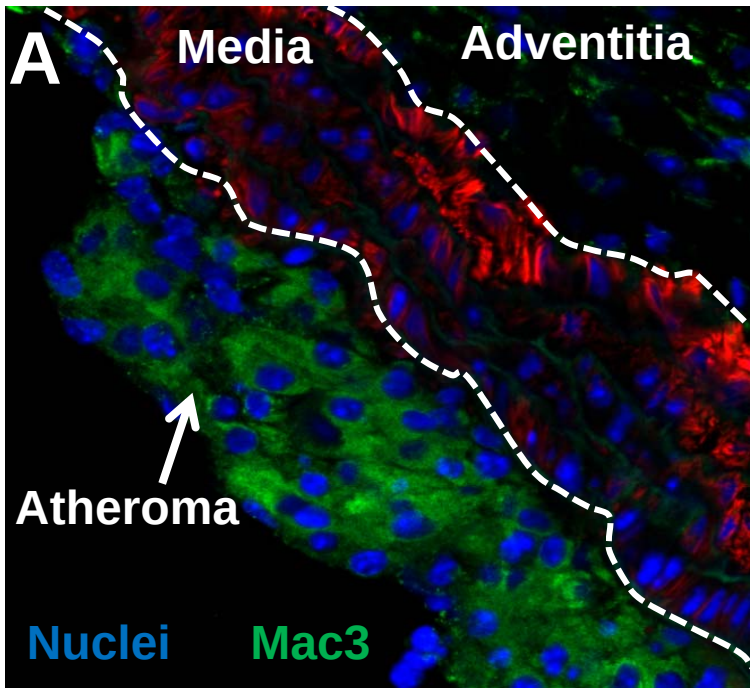
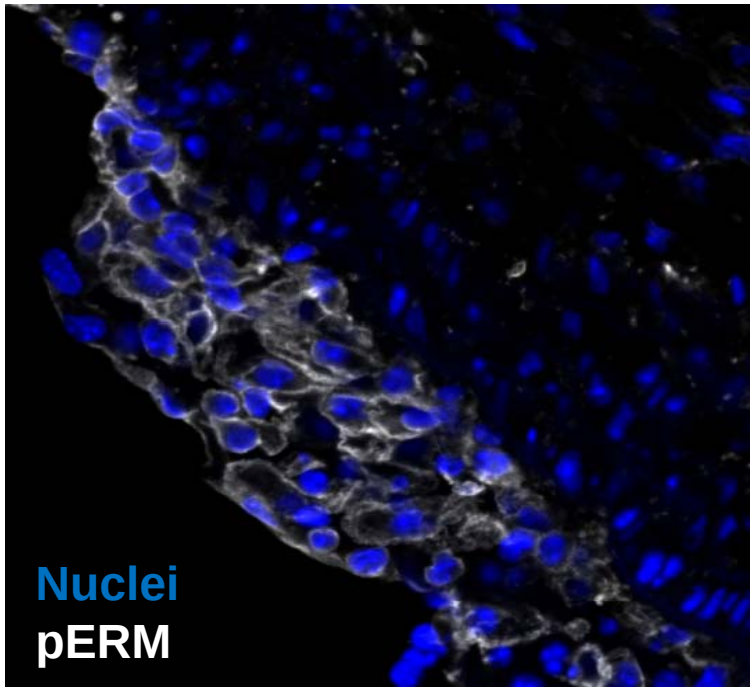
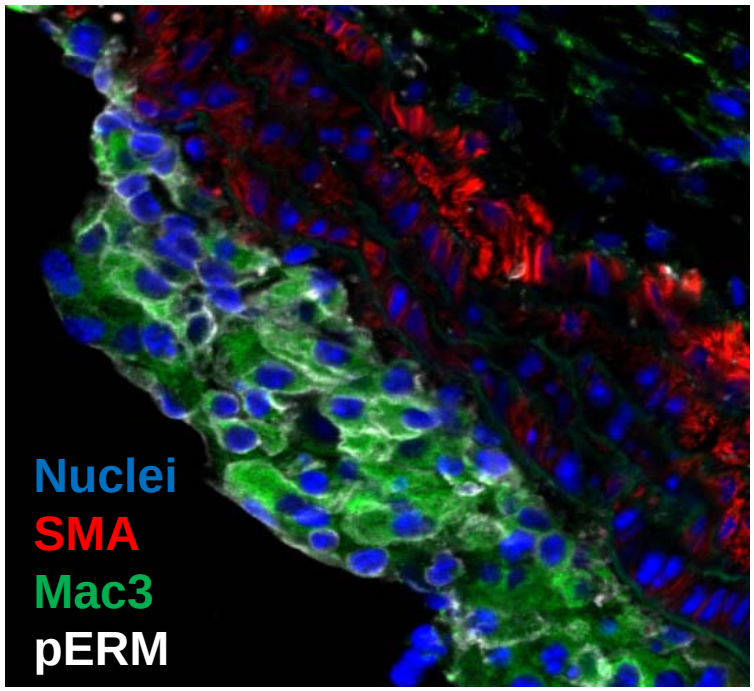

Aortic arch
p=0.0039
% lesion area
15
10
5
apoE-/- BM
apoE-/-
p27S10A BM
Thoracic aorta
p=0.0472
% lesion area
4
2
apoE-/- BM
(n=12)
apoE-/-
p27S10A BM
(n=12)

FIGURE 5

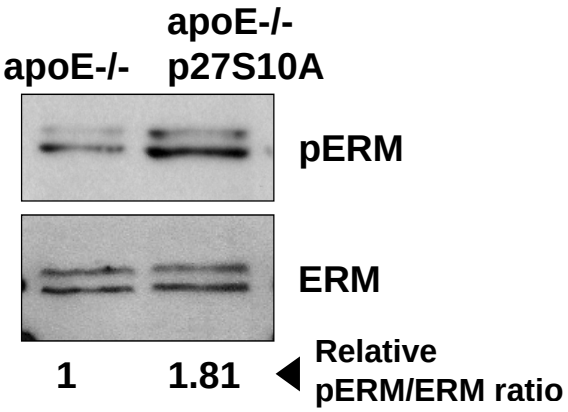

A
BM-derived macrophages
Peritoneal macrophages
apoE-/-
apoE-/- p27S10A
Tubulin
p27-pS10
p27
Actin
1
0.42
p27 relative levels
1
0.54
B
Cytoplasm
Nuclei
apoE-/-
apoE-/- p27S10A
Lamin A/C
GAPDH
p27
1
0.28
1
0.58
p27 relative level
p27 Nucleus/Cytoplasm ratio
2
1
apoE-/-
apoE-/- p27S10A



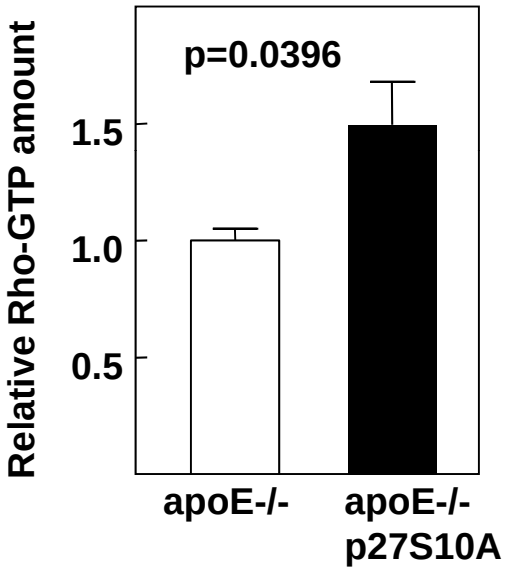


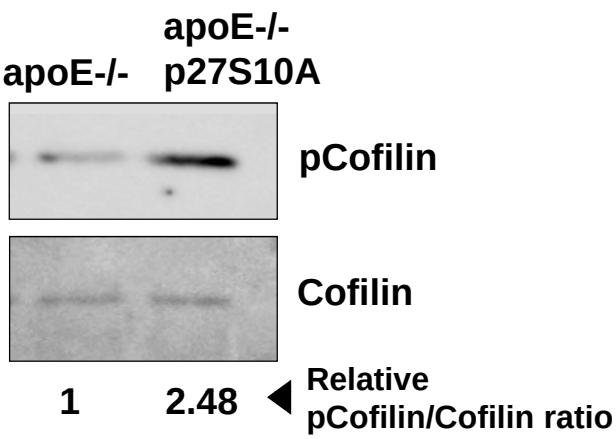


B Atherosclerotic aortic arch



C Peritoneal macrophages





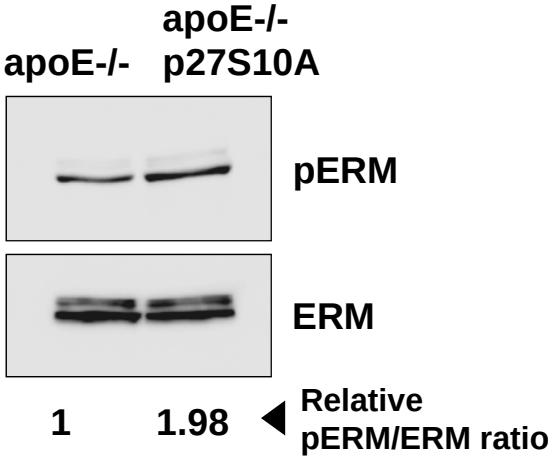


FIGURE 7

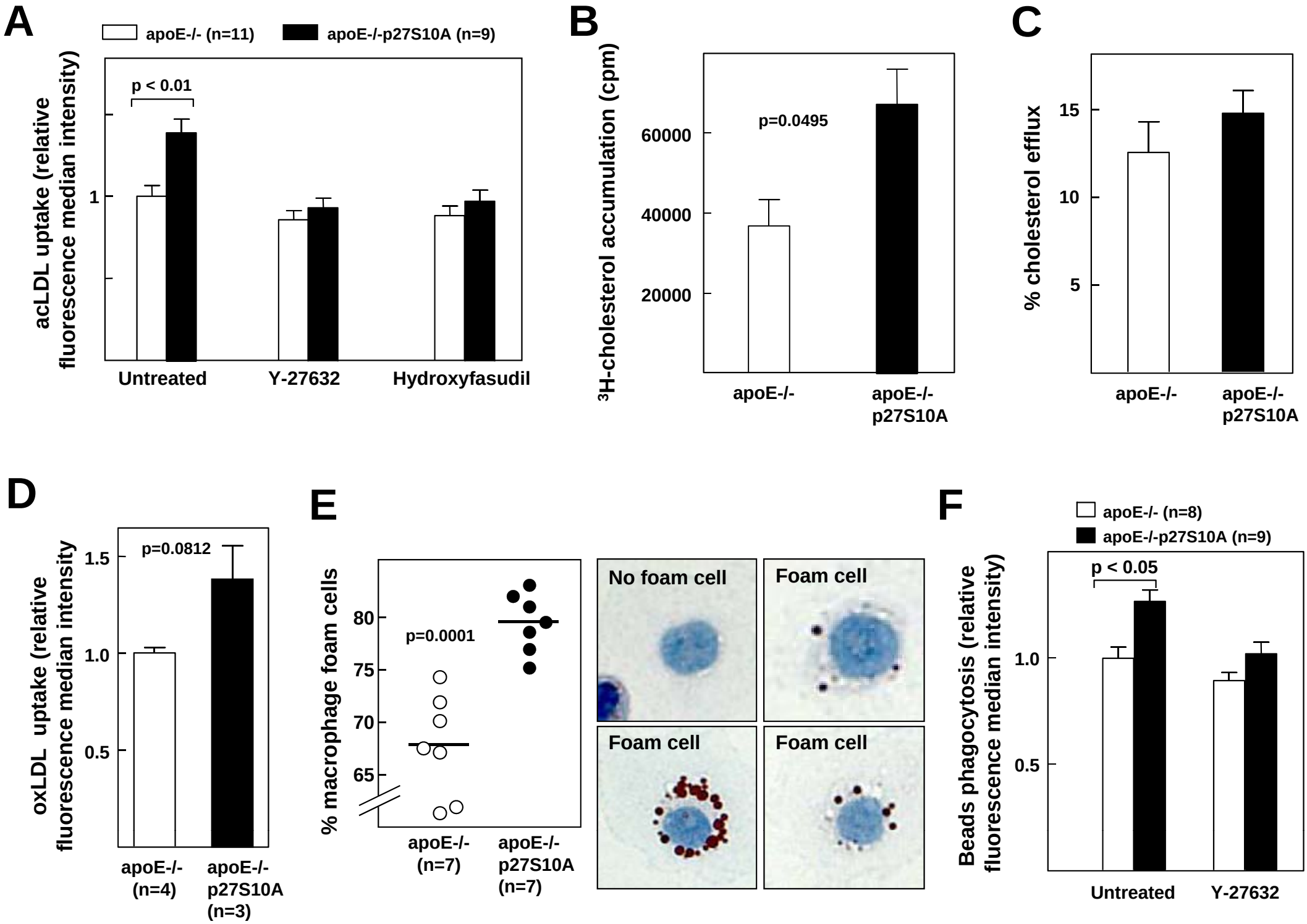
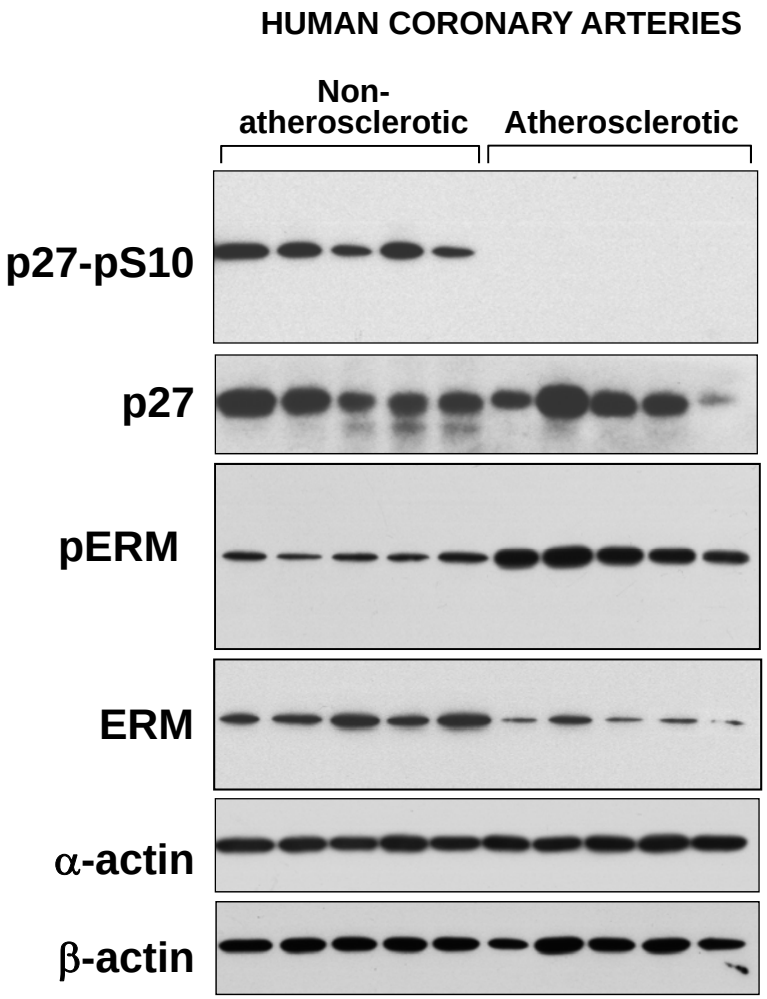

A
apoE-/- (n=11)
apoE-/-p27S10A (n=9)
acLDL uptake (relative fluorescence median intensity)
p < 0.01
1
Untreated
Y-27632
Hydroxyfasudil
B
^{3}H-cholesterol accumulation (cpm)
p=0.0495
60000
40000
20000
apoE-/-
apoE-/- p27S10A
C
% cholesterol efflux
15
10
5
apoE-/-
apoE-/- p27S10A
D
oxLDL uptake (relative fluorescence median intensity)
p=0.0812
1.5
1.0
0.5
apoE-/- (n=4)
apoE-/- p27S10A (n=3)
E
% macrophage foam cells
p=0.0001
80
75
70
65
apoE-/- (n=7)
apoE-/- p27S10A (n=7)
No foam cell
Foam cell
Foam cell
Foam cell
F
apoE-/- (n=8)
apoE-/-p27S10A (n=9)
Beads phagocytosis (relative fluorescence median intensity)
p < 0.05
1.0
0.5
Untreated
Y-27632

FIGURE 8


HUMAN CORONARY ARTERIES
Non-atherosclerotic
Atherosclerotic
p27-pS10
p27
pERM
ERM
α-actin
β-actin