Chapter 10

The epigenetic factor PCAF regulates vascular inflammation and is essential for accelerated atherosclerosis development

M.M. Ewing[1,2,3], J.C. Karper[2,3], A.J.N.M. Bastiaansen[2,3], H.A.B. Peters[2,3], M.R. de Vries[2,3], P.J. van den Elsen[4,5], K. Ozato[6], T. Maurice[7,8], C. Gongora[7,9], J.W. Jukema[1,3], P.H.A. Quax[2,3]

1 Dept. of Cardiology, Leiden University Medical Center (LUMC), Leiden, The Netherlands
2 Dept. of Surgery, LUMC, Leiden, The Netherlands
3 Einthoven Laboratory for Experimental Vascular Medicine, LUMC, Leiden, The Netherlands
4 Dept. of Immunohematology and Blood Transfusion, LUMC, Leiden, The Netherlands
5 Dept. of Pathology, VU University Medical Center, Amsterdam, the Netherlands
6 Laboratory of Molecular Growth Regulation, National Institute of Child Health and Human Development, National Institutes of Health , Bethesda, Maryland
7 Université de Montpellier 2, 34095 Montpellier, France
8 INSERM U 710, 34095, Montpellier, France
9 Institut de Recherche en Cancérologie de Montpellier INSERM U 896, 34298, Montpellier, France

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Abstract

Objective Genetic P300/CBP-associated factor (PCAF) variation affects restenosis-risk in patients. PCAF has lysine acetylase activity and promotes inflammation, which drives post-interventional vascular remodeling and accelerated atherosclerosis development. We studied the contributing role of PCAF in post-interventional vascular remodeling.

Methods and Results PCAF contribution to inflammation and vascular remodeling was assessed in macrophages in vitro and in a mouse model for vascular remodeling and accelerated atherosclerosis. PCAF regulates MHC class-II, but not MHC class-I expression in macrophages through CIITA, inducing a pro-inflammatory reaction. PCAF$^{-/-}$ mice are used to show that PCAF deficiency is associated with 73.2% (p=0.001) reduction of intimal hyperplasia, intima/media ratio and luminal stenosis by preventing smooth muscle cell (SMC) accumulation. This was confirmed using the potent natural PCAF inhibitor garcinol in vivo which reduced arterial leukocyte and macrophage adherence and infiltration following injury and accelerated atherosclerosis development by 71.9% (p=0.004) in operated hypercholesterolemic ApoE3*Leiden mice. This occurred due to downregulation of MCP-1 and TNFα expression by garcinol, similarly to PCAF siRNA, as demonstrated on cultured splenocytes, SMCs and macrophages and in vivo.

Conclusions These results identify a vital role for the epigenetic factor PCAF in the regulation of local inflammation after arterial injury, responsible for vascular remodeling and accelerated atherosclerosis development.

Introduction

Percutaneous coronary intervention (PCI) remains the main choice of revascularization therapy for coronary artery disease. However, restenosis is a common complication and inflammation plays a pivotal role in its development[1-3]. Endothelial injury and underlying plaque exposure during PCI, together with sub-endothelial LDL cholesterol retention evoke thrombocyte adhesion and activation, promoting leukocyte attachment and extravasation[2, 4]. Macrophages and T-cells play a central role and their pro-atherogenic cytokine expression is responsible for further chemotaxis. T-cells secrete interferon (IFN)γ, which enhances class II transactivator (CIITA) and MHC class II-molecule expression in macrophages. Although it can both stimulate SMC proliferation and lead to SMC apoptosis in vitro[5], it has been shown to function as an important pro-atherogenic cytokine in (accelerated) atherosclerosis development[6].
Gene-environmental interactions that stimulate nuclear factor kappa-beta (NFκB) expression are regulated by epigenetic factors that strongly modulate gene expression patterns without DNA sequence modification, mainly by regulating histone acetylation and de-acetylation[7]. Inflammatory gene expression is the result of the counterbalancing and reversible actions of lysine acetyltransferases (KATs) and lysine deacetylases (KDACs), which together determine chromatin structure modification and accessibility to transcription factors[8, 9]. P300/CBP associated factor (PCAF/KAT2B) is a transcriptional co-activator with intrinsic KAT-activity and is involved in lysine acetylation of histones at the site of NFκB-regulated genes[9, 10]. PCAF binds the cyclooxygenase (COX)-2 promoter region following cellular exposure to inflammatory mediators and thereby regulates the general inflammatory response through prostaglandin H2 formation[11]. PCAF also enhances the p65-mediated increase in tumor necrosis factor (TNF)α promoter activity and both TNFα and COX-2 regulate the inflammatory response that lead to atherosclerosis[12, 13].
Few natural inhibitors of PCAF have been described, of which only the natural inhibitor garcinol, derived from the Garcinia Indica fruit rind, has been shown to be extremely potent[14]. It inactivates PCAF activity within 3min[15] and has strong apoptosis-inducing effect on leukemia cell lines[16] and prostate and pancreatic cancer cells[17] through NFκB downregulation. These properties make garcinol an extremely potent inhibitor of PCAF-regulated inflammation in vascular remodeling and accelerated atherosclerosis development.
Previously, association between the -2481C variant allele of the PCAF gene and reduced vascular mortality was shown in three independent large prospective studies[18-20], identifying PCAF as possible diagnostic marker for CHD mortality and restenosis21. Increased intravascular PCAF mRNA levels after injury suggested PCAF involvement in inflammatory-mediated remodeling, although the nature of this elevation remained unexplored[21].
In the present study, PCAF knock-out mice[22, 23] were used to investigate the PCAF contribution to the inflammatory response following vascular injury in a reactive stenosis mouse model[24, 25]. Mice lacking PCAF are developmentally normal without a distinct phenotype, although they have been shown to be resistant to amyloid toxicity[22] and display an altered memory capacity in response to stress[22]. Furthermore, PCAF inhibition with siRNA and garcinol is shown to functionally affect the cellular inflammation reaction after stimulation, resulting in reduced inflammatory cell re-

cruitment to the site of vascular injury and eventually attenuated accelerated atherosclerosis development in vivo. This highlights inflammation-regulation by PCAF as a contributing factor in post-interventional vascular remodeling.

Materials and methods

PCAF$^{-/-}$ and control macrophages were used to study expression of MHC-I, II and CIITA, the master regulator of class II MHC transcription, at RNA and protein level using quantitative real-time PCR and flow cytometry in resting conditions and following INFγ stimulation. We performed multiple in vivo studies in which control, PCAF$^{-/-}$ and garcinol-treated hypercholesterolemic ApoE3*Leiden mice were subjected to femoral artery cuff placement to induce vascular remodeling. This evokes rapid leukocyte recruitment and infiltration (3d) and inflammation-dependent concentric intimal lesions (21d) that consists predominately of SMCs and connective tissue. In these vascular segments, inflammatory cell adhesion, infiltration, intimal thickening and lesion composition were assessed using histology, morphometry and immunohistochemistry (IHC). Cultured splenocytes, SMCs and macrophages were used to study the effects of PCAF inhibition by siRNA and garcinol on the expression of MCP-1 and TNFα by ELISA. A detailed description of materials and methods can be found in the supplemental material section.

Results

PCAF is essential for CTIIA and MHCII, but not MHCI, expression in macrophages

We analyzed the expression of MHC-I and II at the RNA and protein levels and the expression of the master regulator of class II MHC transcription, CIITA, at the RNA level. First we confirmed by quantitative real-time PCR (QPCR) analysis that PCAF is not expressed in PCAF$^{-/-}$ mice (fig 1A) and we observed that PCAF expression is not induced by INFγ.

We compared IFNγ-induced MHC-II expression in WT and PCAF$^{-/-}$ macrophages. Constitutive MHC-II and IFNγ expression was inhibited in PCAF$^{-/-}$ macrophages, compared to the WT macrophages at the mRNA level measured by QPCR (fig 1B) and at the protein level at the cell surface, both constitutively and following IFNγ induction, measured by flow cytometry (fig 1C). The mRNA level of CIITA was assessed by QPCR and we observed that WT and PCAF$^{-/-}$ macrophages displayed the same level (fig 1D), indicating that the levels of IFNγ-induced CIITA transcription are not modulated in theses macrophages, but its activity is impaired due to the lack of PCAF. As CIITA also contributes to MHC-I gene transcription, the cell surface expression of MHC-I in macrophages incubated or untreated with IFNγ was measured in WT and PCAF$^{-/-}$ cells (fig 1E). We observed that the MHC-I expression was not altered in PCAF$^{-/-}$ macrophages.

Intimal thickening is significantly reduced in PCAF$^{-/-}$ mice

Quantitative analysis of cuffed femoral artery segments of C57Bl/6 control and PCAF$^{-/-}$ mice stained with Weigert's elastin revealed, despite similar plasma total choleste

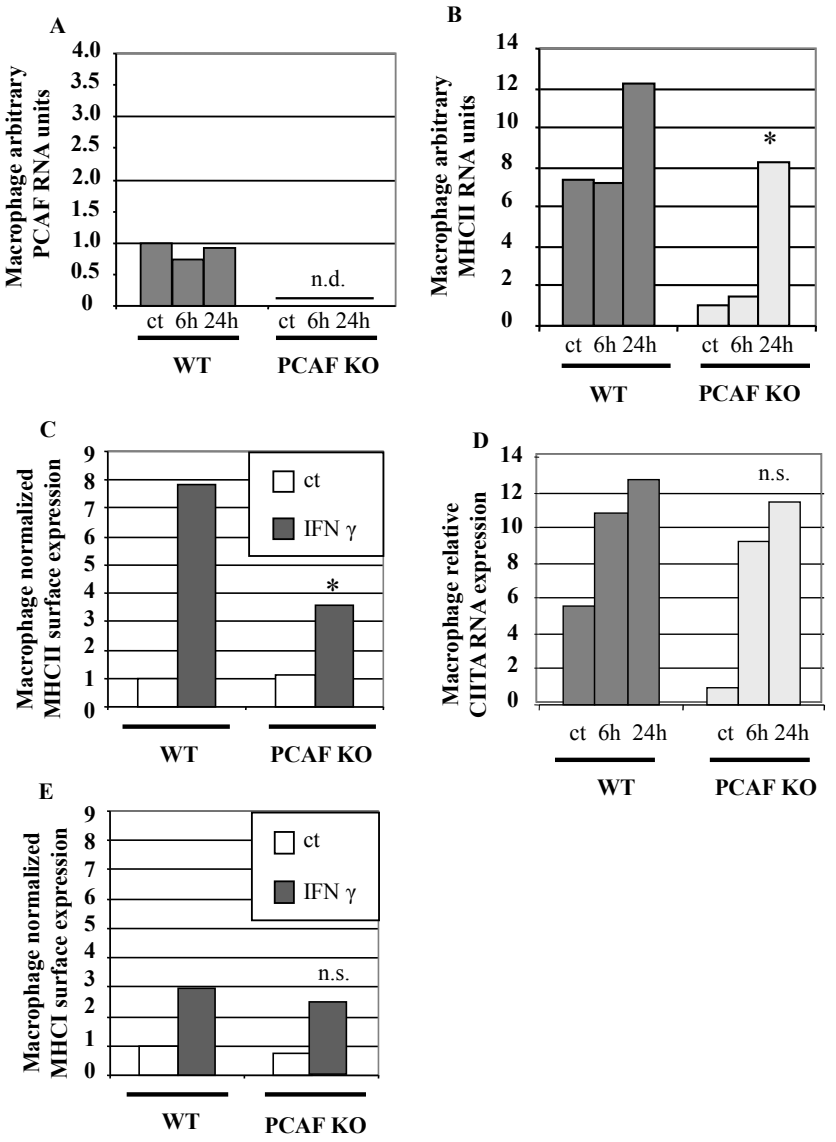

Figure 1. PCAF is necessary for MHC-II expression in macrophages. The loss of PCAF inhibits IFNγ-induced MHC-II expression in peritoneal macrophages at RNA and protein level. A, B and D: Analysis of mRNA levels by semi-quantitative Q-PCR of PCAF, MHCII and CIITA respectively. Constitutive and IFNγ-induced levels of transcripts were analyzed at times 0, 6 and 24h following IFNγ exposure respectively. C and E: Analysis of protein expression at the cell surface by flow cytometry of MHCI and MHCII respectively. Endogenous and IFNγ-treated cells during 24h were analyzed and displayed against untreated cells (ct: consecutively, n.d.: non-detectable, n.s.: not-significant). Results are representative of three separate experiments (mean±SEM, n=3, * $p<0.05$).

rol concentrations (supplemental table I), 73.2% reduced intimal thickening in PCAF$^{-/-}$ mice when compared to controls (WT: 9777±1608 μm^2, PCAF$^{-/-}$: 2623±368 μm^2, n=10, p=0.001, fig 2A). Since the media area was similar, this was accompanied by a reduced intima/media ratio by 73.7% (WT: 0.94±0.18, PCAF$^{-/-}$: 0.25±0.03, n=10, p=0.001, fig 2B) and reduced luminal stenosis by 60.1% after 21d (WT: 51.5±4.6%,

PCAF$^{-/-}$: 20.5±2.5%, n=10, p=0.001, fig 2C). IHC was performed to assess lesion composition (fig 2D) and it was observed that PCAF$^{-/-}$ mice displayed a significantly smaller area of α-SMC actin expressing cells (positively-stained area per cross section in μm^2) by 61.8% (p=0.028) within the intimal layer (fig 2E).

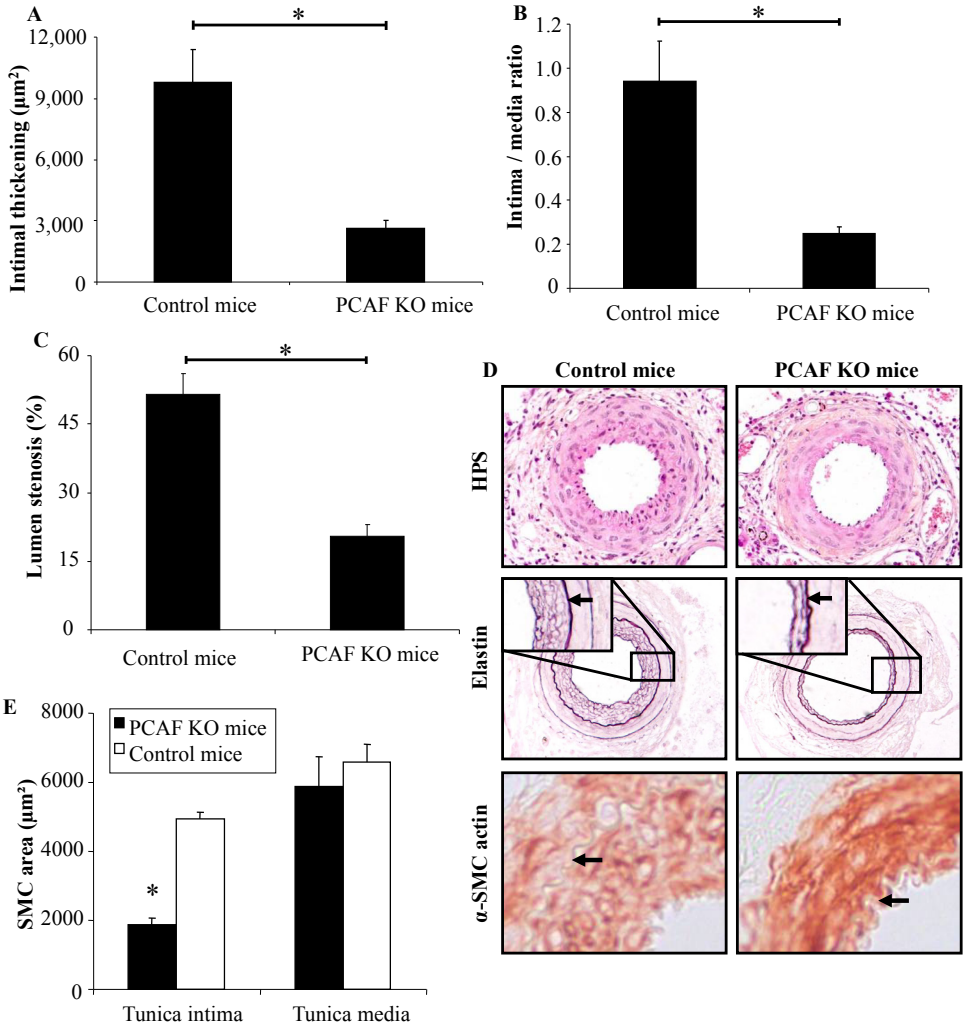

Figure 2. Reduced vascular remodeling in PCAF$^{-/-}$ mice. A: intimal thickening (μm^2), (B) intima / media ratio and (C) luminal stenosis (%) in wildtype and PCAF$^{-/-}$ mice, 21d after femoral arterial cuff placement (mean±SEM, n=10, * p<0.05). D: representative cross-sections of cuffed arterial segments, stained with HPS (200x), Weigert's elastin 200x (arrows in inserts (400x) indicate internal elastic lamina) and for SMC α-actin (200x). E: quantification of SMC area (μm^2) in the intimal and medial layers of wildtype and PCAF$^{-/-}$ mice (mean±SEM, n=10, * p<0.05).

PCAF regulates the functional leukocyte and SMC inflammatory response

Vascular injury leads to inflammatory cytokine expression by recruited leukocytes and resident VSMCs which drives subsequent vascular remodeling. The anti-inflammatory potential of pharmacological PCAF inhibition through garcinol, the most po-

tent natural PCAF inhibitor described, was first tested in vitro. Analysis of garcinol-treated wildtype and PCAF$^{-/-}$ cultured splenocytes showed reduced expression of TNFα by 25.7% (p=0.037) and 42.3% (p=0.030) respectively (fig 3A) and of MCP-1 by 41.3% (p=0.057) and 71.4% (p=0.029) respectively (fig 3B) after overnight lipopolysaccharide (LPS) stimulation. Next, splenocytes were subjected to siRNA-mediated knock-down of PCAF, incorporation assessed with siRNA luciferase (fig 3C) and control siRNA as controls and were again exposed to LPS. When compared to vehicle-treated splenocytes (which responded similarly to control siRNA-exposed cells, p=0.240), knockdown of PCAF led to reduced TNFα expression by 41.2% (p=0.029) as did PCAF$^{-/-}$ splenocytes by 55.5% (p=0.004). No differences in TNFα expression were measured between PCAF deficient splenocytes and wildtype splenocytes following PCAF knockdown (fig 3D).

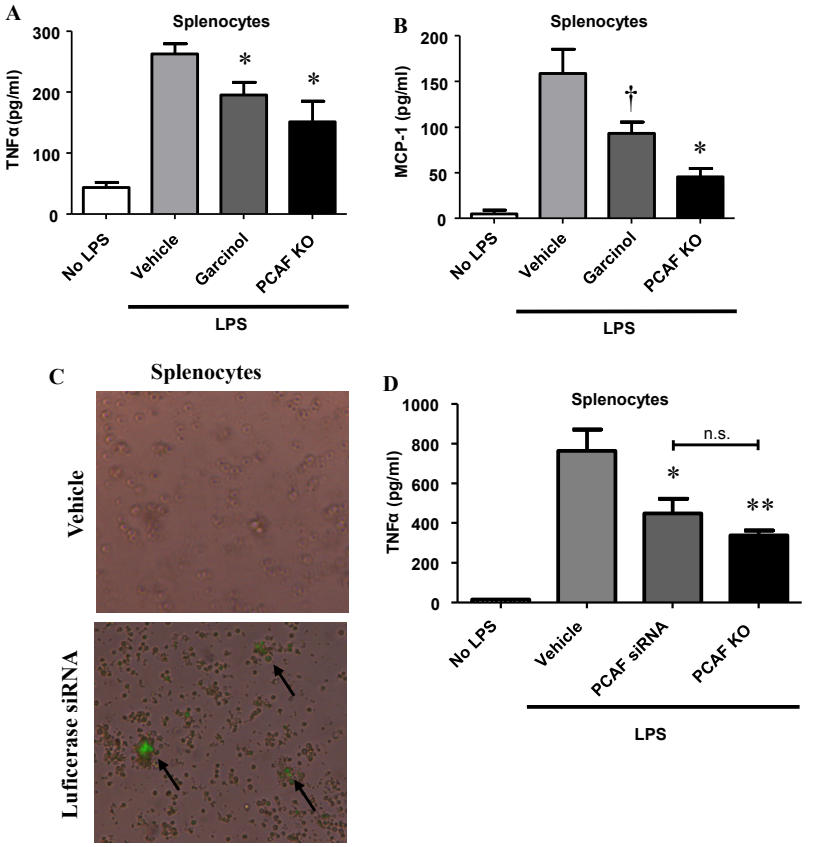

Figure 3. PCAF inhibition prevents inflammatory cytokine expression in vitro. Compared to overnight LPS-stimulated (100 ng/ml) wildtype splenocytes, garcinol-incubated (15 μM) and PCAF$^{-/-}$ splenocytes display a reduced expression of TNFα (pg/ml) (A) and MCP-1 (pg/ml) (B). Luciferase siRNA controls display effective splenocyte tranfection after 24h (C). TNFα expression (pg/ml) is also reduced after siRNA-mediated PCAF knockdown in wildtype splenocytes following overnight LPS-stimulation (300 ng/ml) as effectively as in PCAF$^{-/-}$ splenocytes (D). Results are representative of three separate experiments (mean±SEM, n=3, * p<0.05, † 0.05<p<0.10, n.s. not significant, n.d. not detectable).

PCAF was shown to regulate macrophage MHCII expression through CTIIA. Functional macrophage dampening was investigated and it was found that TNFα expression after overnight LPS stimulation was profoundly reduced by simultaneous garcinol incubation (fig 3E) ($p=0.0*10^{-6}$) in vitro. Finally, MCP-1 expression by LPS-stimulated SMCs was reduced by 24.5% (p=0.043, fig 3F) in the presence of garcinol (15 μM), although this could not be demonstrated at the lowest garcinol concentration (5.0 μM). Next, analysis to which extent PCAF contributes to MCP-1 expression by VSMCs was performed in a dose-response experiment using (0.1-10.0 ng/ml) LPS. It is shown that VSMC-secreted level of MCP-1 (pg/ml) is reduced by garcinol by 19.2-26.0% at LPS concentrations of 0.1, 1.0 and 10 ng/ml respectively (fig 3G, p=0.049, p=0.021 and p=0.043 respectively), measured by ELISA.

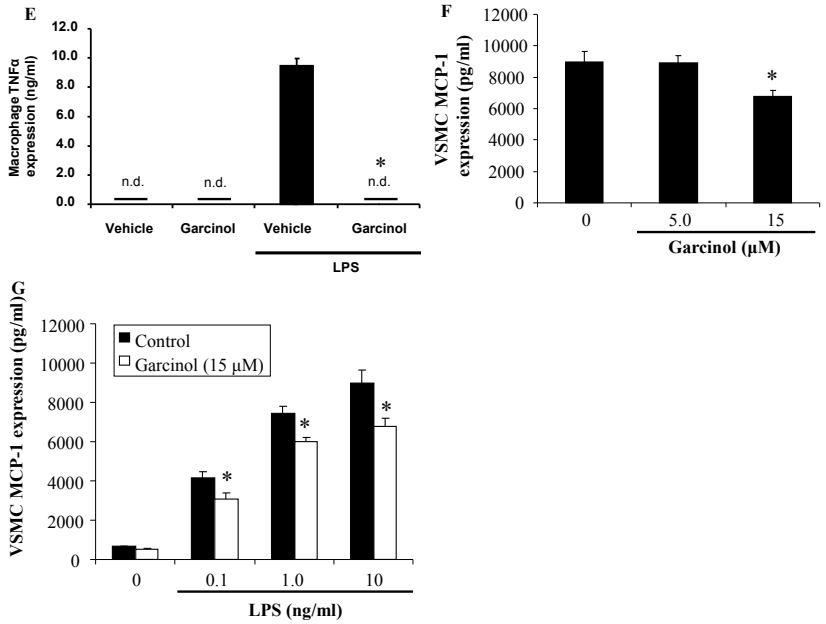

Figure 3. PCAF inhibition prevents inflammatory cytokine expression in vitro. Macrophage TNFα (ng/ml) expression following overnight inflammatory mediator (LPS) stimulation is significantly reduced by garcinol (15 μM) to non-detectable levels (E). Garcinol (15 μM) downregulates MCP-1 (pg/ml) expression by cultured vascular SMCs (F) and reduces vascular SMC MCP-1 (pg/ml) expression following a dose-dependent increase of LPS (0.1-10 ng/ml) (G). Results are representative of three separate experiments (mean±SEM, n=3, * p<0.05, † 0.05<p<0.10, n.s. not significant, n.d. not detectable).

PCAF inhibition prevents injury-induced leukocyte recruitment in vivo

PCAF inhibition to prevent post-interventional remodeling and accelerated atherosclerosis development was investigated by garcinol in the femoral artery cuff model in hypercholesterolemic ApoE3*Leiden mice. Viability of circulating leukocytes in the presence of garcinol (0-250 μM) was investigated using the redox indicator Alamar blue. Fluorescence intensity (FI) remained constant at ~5700 AU (615 nm) at garcinol concentrations 0-20 μM, indicating complete cellular viability compared to positive controls (100%) (p=0.448, fig 4A), with cytotoxicity at concentrations ≥30 μM (FI: ~900 AU).

Independently of elevated total plasma cholesterol concentrations (supplementary

table I), garcinol in pluronic gel (10 µl of 41.5 mmol/L lubricated around operated arterial segment) reduced the percentage of endothelial leukocyte adhesion out of all cells within the internal elastic lamina by 62.1% (p=0.028) and of macrophages by 54.6% (p=0.010) after 3d compared to vehicle only. Medial leukocyte infiltration was reduced by garcinol by 61.7% (p=0.005) and macrophages by 84.5% (p=0.004) (fig 4C, D). Garcinol reduced the percentage of cells in the tunica intima (predominately endothelial cells) that expressed MCP-1 by 65.0% (p=0.003) and of cells in the tunica media by 57.1% (p=0.010) (fig 4E), suggesting that garcinol reduced leukocyte adherence and infiltration by affecting chemo-attracting factors.

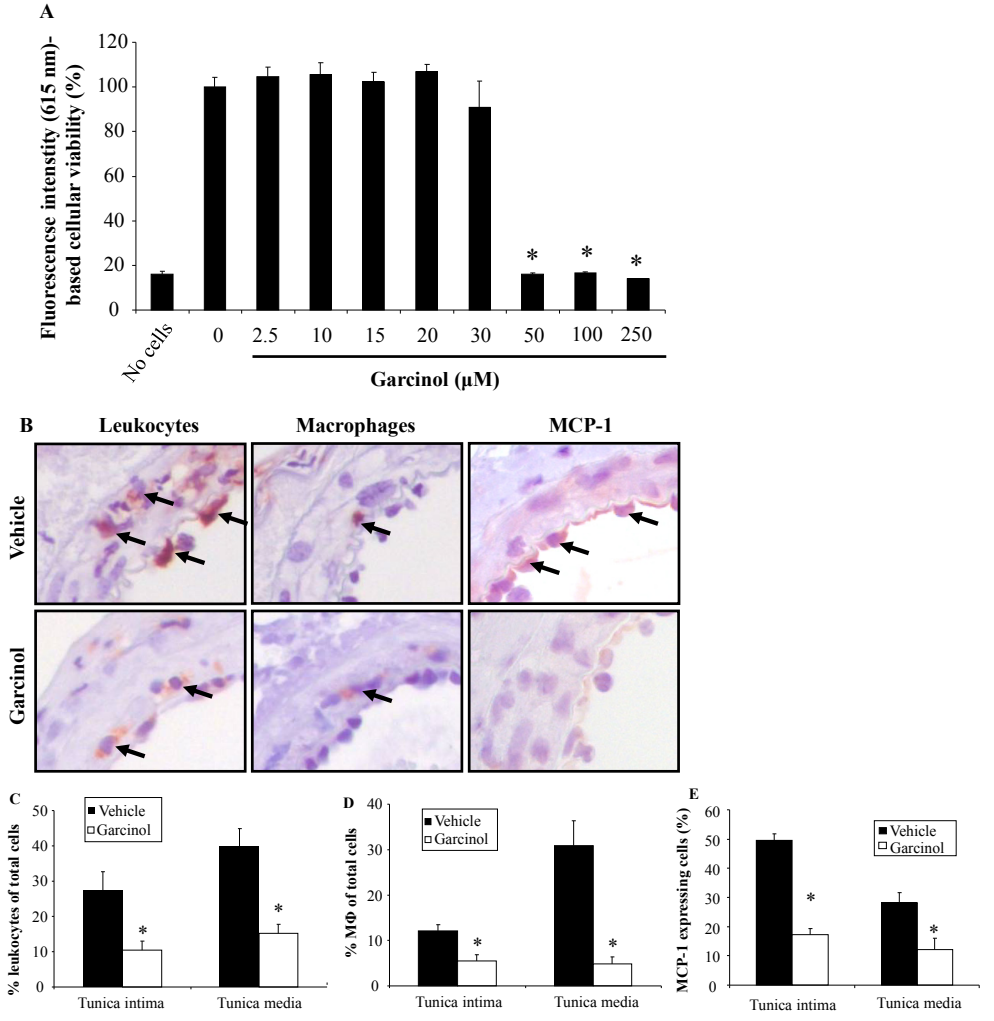

Figure 4. Garcinol-mediated PCAF inhibition reduces the leukocyte inflammatory response. A: ApoE3*Leiden-derived whole blood viability assay after overnight 0-250 µM garcinol incubation using Alamar blue conversion (emission 615 nm), expressed as percentage fluorescence intensity (of positive controls) (mean±SEM, n=10, * p<0.05). B: representative cross-sections of cuffed arterial segments of ApoE3*Leiden mice, 3d after surgery (CD45+ leukocytes, macrophages and MCP-1 expressing cells, 400x, arrows indicate positive staining). Quantification of (endothelial)-adhered or medial infiltrated leukocytes (C), macrophages (D) and MCP-1 expressing cells (E), expressed as the percentage of all cells

adhering to the endothelium or in the media. The entire length of the cuffed femoral artery segment was used for analysis (mean±SEM, n=10, * p<0.05).

PCAF inhibition prevents accelerated atherosclerosis development

IHC showed that PCAF expression is absent in non-injured hypercholesterolemic arteries (fig 5A). Negative staining was confirmed in PCAF$^{-/-}$ arteries (fig 5B). PCAF presence was increased 3d after surgery in ApoE3*Leiden mice (fig 5C). Staining was observed in both endothelial-adhering leukocytes and in the SMC-rich tunica media. Garcinol reduced PCAF staining (fig 5C) after 3d.

Early anti-inflammatory effects of PCAF-inhibition were substantiated by garcinol application during femoral artery cuff placement. Despite similarly elevated total plasma cholesterol concentrations (supplementary table I), garcinol (10 µl of 41.5 mmol/L concentration lubricated around operated arterial segment) significantly reduced intimal thickening by 71.9% (p=0.004) in ApoE3*Leiden mice when compared to vehicle only (fig 5D, G). Since medial surface was similar, this was accompanied by a reduced intima / media ratio by 72.5% (p=0.002) (fig 5E) and reduced luminal stenosis by 63.3% (p=0.001) after 14d (fig 5F).

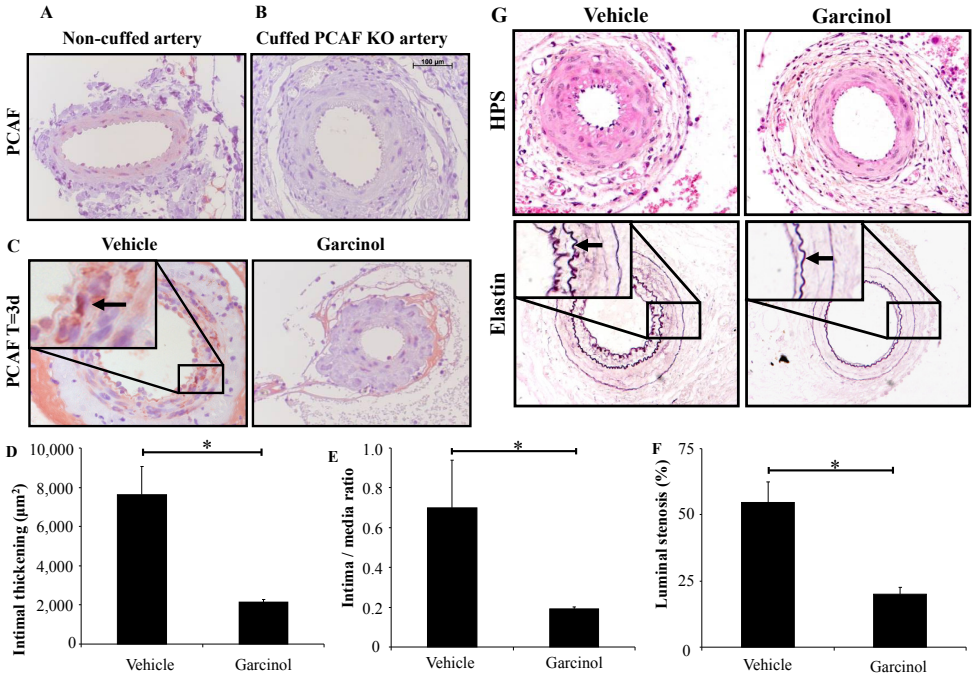

Figure 5. PCAF inhibition prevents vascular remodeling. Representative cross-sections of (un)cuffed-femoral arterial segments, stained for the presence of PCAF (200x). A: non-cuffed artery from ApoE3*Leiden mouse. B: cuffed femoral artery of PCAF$^{-/-}$ mouse (negative control). C: PCAF expression in cuffed femoral arteries of ApoE3*Leiden mice ± garcinol treatment, 3d and 14d after surgery. Arrow in insert indicates positive medial staining. Garcinol-treatment in ApoE3*Leiden mice reduced intimal thickening (µm^2) (D), intima / media ratio (E) and luminal stenosis (%) (F), 14d after femoral arterial cuff placement (mean±SEM, n=10, * p<0.05). G: representative cross-sections of cuffed femoral arteries (HPS and Weigert's elastin staining, 200x, arrow in inserts (400x) indicate internal elastic lamina).

PCAF inhibition affects vessel wall composition

IHC was performed to assess lesion composition in vehicle and garcinol-treated animals (fig 6A), revealing a reduced inflammatory phenotype following garcinol treatment. This led to reduced relative macrophage presence (in %) by 75.7% in the intimal layer (p=0.023) and by 74.7% in the medial layer (p=0.004) (fig 6B). In garcinol-treated arteries, the relative α SMC actin+ area (%) was 14.6% (p=0.042) higher in the tunica media, although there were no relative differences measured in the tunica intima (fig 6C). However, absolute α SMC actin intimal thickening was prevented by garcinol, indicated by 40.0% (p=0.042) reduced SMC surface area (μm2) (fig 6D). Cellular proliferation in the arterial wall was assessed using BrdU staining, injected once daily for 3d after surgery. Control arteries displayed more elaborate BrdU staining, suggesting PCAF inhibition could affect injury-induced cellular proliferation in vivo (fig 6A). Together, these data identify clear effects of PCAF inhibition on vascular inflammatory phenotype.

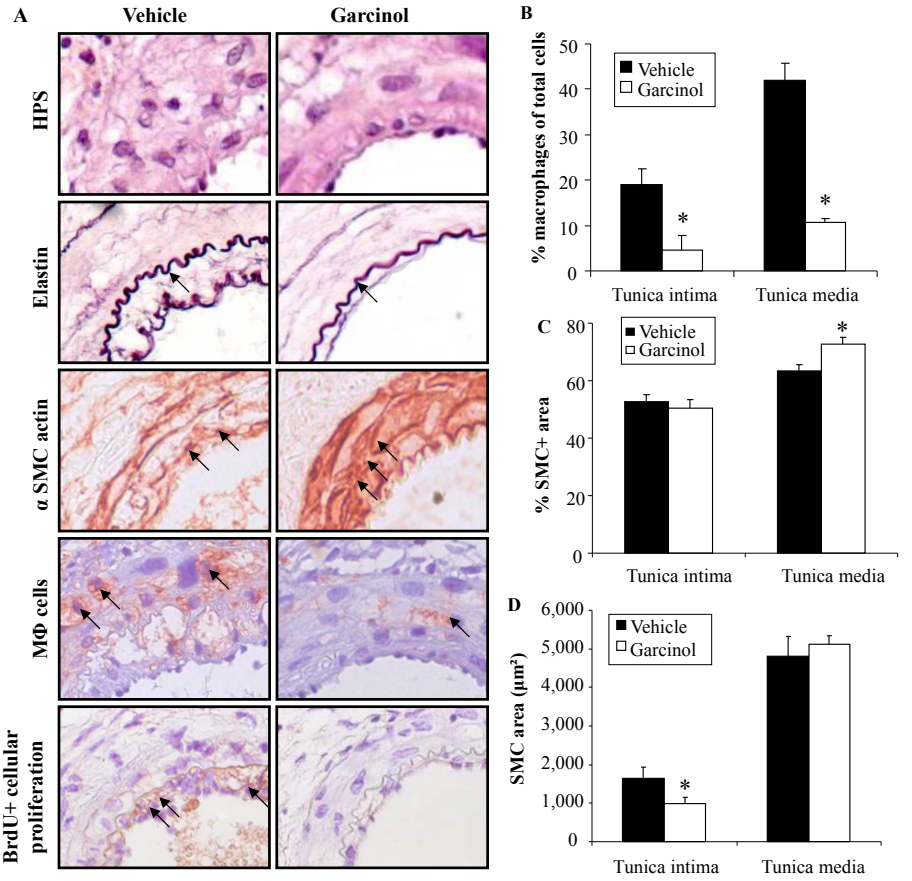

Figure 6. PCAF inhibition affects accelerated atherosclerotic lesion morphology. A: cross-sections of cuffed arterial segments of ApoE3*Leiden mice, 14d after surgery, treated with vehicle only or garcinol-containing pluronic gel, 200x. Arteries are stained with HPS, Weigert's elastin (arrows indicate internal elastic lamina) and for SMC α-actin, macrophages and BrdU-incorporation (arrows indicate positive staining). Quantification of macrophages (B) and relative SMC α-actin+ surface area (C) in the intimal and medial layers, expressed as the positively-stained area as percentage of the total area. D: quantification of α-actin SMC area (μm^2) in the tunica intima and media (mean±SEM, n=10, * p<0.05).

Discussion

Here we demonstrate that compared to controls, post-interventional vascular remodeling is reduced in PCAF$^{-/-}$ mice, as is post-interventional accelerated atherosclerosis development in hypercholesterolemic mice treated with a PCAF inhibitor. Furthermore, we demonstrate that this reduced remodeling is due to an attenuated inflammatory response, shown by reduced MCP-1 and TNFα-expression in vivo and in cultured cells. It was shown that PCAF regulates MHC class II, but not I, expression in macrophages through CIITA and becomes upregulated in the early period following vascular injury. To our knowledge, this is the first paper to show that PCAF has a contributing role in vascular remodeling by regulating the inflammatory response.
PCAF$^{-/-}$ mice were used to demonstrate clear effects of PCAF deficiency on inflammatory-regulated vascular remodeling. Animals developed significantly smaller SMC-rich neointimal lesions with reduced luminal stenosis. The only described natural potent PCAF inhibitor garcinol, was used next to siRNA-mediated PCAF knockdown and PCAF$^{-/-}$ cells to demonstrate its essential contribution in the production of pro-inflammatory cytokines (MCP-1 and TNFα) by various cell types including splenocytes, macrophages and vascular SMCs. This contribution was confirmed in vivo where garcinol prevented post-interventional leukocyte recruitment to the site of arterial injury and accelerated atherosclerosis development in operated hypercholesterolemic mice. These results demonstrate an important role for the epigenetic factor PCAF in the post-interventional arterial inflammatory response[26].
The role of PCAF was further investigated in macrophages, which play a vital role in the progression of accelerated atherosclerotic lesions. MHC class II, but not class I, expression was found to be inhibited in PCAF$^{-/-}$ macrophages at both mRNA and protein level, whilst CIITA mRNA was normalized. This indicates that IFNγ-induced CIITA transcription is not affected by PCAF deficiency, but its activity is impaired due to the lack of PCAF[27, 28], suggesting PCAF-requirement for adequate adaptive immune responses in this leukocyte subset. Although PCAF is important for MHC class II expression, it cannot be fully excluded that other inflammatory genes are also affected by PCAF inhibition (e.g. COX-2), albeit not MHC class I.
Previously, mouse PCAF mRNA levels were demonstrated to be reactively increased within 48h after vascular injury, after which levels were reduced below resting values[21]. Using IHC, we show abundant PCAF protein expression 72h after surgery in endothelial-adhering cells (e.g. macrophages) and in the SMC-rich tunica media. Since vascular injury in known to elicit inflammation and provoke SMC proliferation, regulation of inflammation by PCAF could strongly contribute to vascular remodeling. Indeed, short-term PCAF inhibition early after surgery led to reduced PCAF expression after 14 days, indicative of a reduced inflammatory phenotype of arterial wall. Consistent with PCAF expression by SMC, SMC-rich intimal lesions which occurred in wildtype mice were significantly less present in PCAF$^{-/-}$ mice (fig 2E), indicating that PCAF-regulated inflammation contributes strongly to lesion formation in these animals.
An ex vivo LPS stimulation experiment in splenocytes, macrophages and vascular SMCs from either wildtype or PCAF$^{-/-}$ mice was performed to test if PCAF was involved in inflammatory cytokine expression by these various cell types. Indeed, TNFα expression by splenocytes and macrophages was severely compromised in

PCAF deficient animals and similarly in wildtype cells subjected to siRNA-mediated PCAF knockdown or garcinol-treatment, indicating a vital role for PCAF in inflammatory gene transcription. Since cytokine levels were differently affected in different cell types such as VSMCs and macrophages, this might differences in PCAF expression concentrations in different cell types or altered histone acetylation status between cells.

Garcinol was used successfully in preclinical cancer studies in rats, where oral garcinol application reduced the severity of tongue carcinoma by inhibiting tumor cell proliferation[29, 30]. Anti-proliferative drugs, originally applied as cytostatic anti-cancer agents such as sirolimus and paclitaxel, have been extensively used against restenosis in drug-eluting stents[31, 32]. However, they affect cellular proliferation non-specifically, whereas PCAF inhibition could be a potent promising anti-restenotic therapy. It is speculated that the protective function of garcinol is primarily through PCAF inactivation in macrophages, affecting their MHCII expression. However, garcinol also downregulates KDAC-induced tissue-type plasminogen activator in endothelial cells[33]. Unlike the quiescent state in an uninjured artery, the fibrinolytic system becomes activated after vascular trauma with strong t-PA expression in SMC, endothelial cells and platelets[34] and can increase SMC migration. Inhibition of plasmin activity at the cell surface inhibits intimal thickening in our mouse model for reactive stenosis[35] and it can therefore not be ruled out that garcinol affects intimal thickening by influencing plasmin activity, provided this is KDAC-induced.

It is also conceivable that PCAF itself influences post-interventional vascular remodeling through indirect effects on macrophages. High mobility group 1 protein (HMGB1) is a chromatin component that can be secreted by activated monocytes and macrophages and functions as a late mediator of inflammation. These cells can tilt the acetylation / deacetylation balance in favor of acetylation upon activation and PCAF is known to acetylate HMGB1[8], which occurs extensively upon activation with LPS[36]. LPS also provokes significant intimal thickening, which could indicate that PCAF may not directly affect inflammatory gene transcription, but indirectly through LPS-induced HMGB1 acetylation in stimulated monocytes and macrophages. Moreover, HMGB1 may act as an endogenous ligand for TLR4 and TLR4 signaling is also a key regulatory process in vascular remodeling[37]. A significantly impaired inflammatory response by LPS-stimulated PCAF$^{-/-}$ whole blood ex vivo is supportive of this possibility.

In conclusion, using PCAF$^{-/-}$ mice, evidence is provided that PCAF contributes to post-interventional vascular remodeling. Further, local delivery of the PCAF-inhibitor garcinol inhibited reactive stenosis and accelerated atherosclerosis development in hypercholesterolemic ApoE*3-Leiden transgenic mice. This could be explained by an as yet unrecognized direct or indirect effect of PCAF on inflammation and SMC proliferation, leading to reduced post-interventional remodeling. These results might therefore shed light on the possible contribution of PCAF as an important epigenetic factor in vascular remodeling and accelerated atherosclerosis development in stented human coronary lesions and identify it as a possible new clinical target against vascular remodeling after PCI.

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Supplemental material

Materials and Methods

Mice

All experiments were approved by the Institutional Committee for Animal Welfare at the LUMC. The generation of PCAF knockout (KO) mice has been described previously[1]. Male C57Bl/6 PCAF KO mice[2] and wildtype (WT) C57Bl/6 controls were used, as were transgenic male ApoE*3-Leiden mice (both bred in our own laboratory), backcrossed for more than 20 generations on a C57BL/6 background. ApoE3*Leiden (at the start of a dietary run-in period) and WT and PCAF KO mice aged 10-12 weeks, were used for femoral artery cuff experiments.

Peritoneal macrophage culture

Peritoneal macrophages (PM) were elicited by intraperitoneal (IP) injection with thioglycollate broth. 2 ml of thioglycollate was administered IP to 4-6 week old control or PCAF KO mice and PM were harvested 3d later under anesthesia, as described in the section below. Thioglycollate elicited PM were plated at $5*10^6$ cells/dish in 100 mm tissue culture dish in RPMI 5% fetal calf serum (FCS) overnight. The PM were then washed with RPMI 5% FCS and were either treated or untreated with IFNγ (100 U/ml) for 24 or 48 hours.

Peritoneal macrophage RNA isolation and real time (RT) PCR

Real time PCR analysis was done with an ABI PRISM 7700 sequence detection system (Applied Biosystems). Real-time PCR was used in analysis of RNA samples from macrophages-like cells isolated from PCAF WT and KO mice. RT-PCR reactions were done with a kit from Perkin Elmer (SYBR Green PCR master kit). Primer sets for CIITA, MHC-II and HPRT cDNAs were (forward and reverse) 5'-TGCAGGCGACCAGGAGAGACA–3' and 5'–GAAGCTGGGCACCTCAAAGAT–3'; 5'–TATGTGGACTTGGATAAGAAG–3' and 5'–ACAAAGCAGATAAGGGTGTTG–3'; 5'–GGGAGGCCATCACATTGTG –3' and 5'–TCCAGCAGGTCAGCAAAGAAC–3', respectively. Real time PCR values were determined by reference to a standard curve generated by real time PCR amplification of serial dilution of cDNA using CIITA, MHC-II and HPRT primers. Values obtained for levels of CIITA and MHC-II mRNAs were normalized to the levels of HPRT mRNA expression as determined by real time PCR. Data are representative of at least three separate experiments on different RNA.

Flow cytometry analysis

Macrophages-like cells were plated at a concentration of $0.5\text{-}1*10^6$/ml and were either treated or untreated by IFNγ for 48h. Cells were removed from the plates, washed in PBS and resuspended in medium. For surface marker analysis, cells were first blocked with anti-mouse FcγR antibody (CD16/CD32 2.4G2, BD Pharmingen) followed by incubation with specific antibodies or isotype control for 30 min at 4ºC. FITC-conjugated antibodies against H-2Kb (AF6-88.5), PE-conjugated anti-mouse I-A/I-E (M5/114.15.21), were purchased from BD Pharmingen. PE-conjugated anti-

F4/80 (Ly-71) antibody was obtained from Caltag. Cells were collected on FACS Caliber and analyzed using Cell Quest software (Becton Dickinson).

Diet

PCAF KO and WT mice received chow diet. Transgenic male ApoE*3-Leiden mice were fed a Western-type diet containing 1% cholesterol and 0.05% cholate to induce hypercholesterolemia (AB Diets). The diet was given three weeks prior to surgery and was continued throughout the experiment. All animals received food and water ad libitum during the entire experiment.

Femoral artery cuff mouse model

To investigate the role of PCAF in restenosis development, mice underwent a non-constrictive cuff placement around the femoral artery after three weeks of diet to induce vascular inflammation and remodeling. Mice were anesthetized before surgery with a combination of intraperitoneally (IP) injected Midazolam (5mg/kg, Roche), Medetomidine (0.5mg/kg, Orion) and Fentanyl (0.05mg/kg, Janssen). The right femoral artery was isolated and sheathed with a rigid non-constrictive polyethylene cuff (Portex, 0.40mm inner diameter, 0.80mm outer diameter and an approximate length of 2.0mm). 3d, 14d or 21d after cuff placement, mice were anesthetized as before and euthanized.

The thorax was opened and pressure-perfusion (100mm Hg) with 3.7% formaldehyde in water (w/v) was performed for 5 minutes by cardiac puncture in the left ventricle. After perfusion, the cuffed femoral artery was harvested, fixed overnight in 3.7% formaldehyde in water (w/v) and paraffin-embedded. Serial perpendicular cross-sections (5µm thick) were taken from the entire length of the artery for analysis.

In vivo garcinol treatment

During non-constrictive cuff placement, ApoE*3Leiden mice were treated with 10 µl pluronic gel (40%, maintained at 0°C, Sigma Aldrich) ± 25 mg/ml garcinol (Enzo Life Sciences), which was lubricated around the isolated femoral artery and was allowed to harden out and settle around the artery, which occurred within 20 seconds after application.

Biochemical analysis

Total plasma cholesterol concentration (Roche Diagnostics, kit 1489437) was measured enzymatically. Inflammatory cytokine concentration of monocyte chemotactic protein (MCP)-1 and TNFα were determined using ELISA kits (2665KI and 558534, both Becton Dickinson), according to the manufacturer's instructions.

Immunohistochemistry (IHC)

To detect the presence of PCAF, inflammatory cells, vessel wall characteristics and effects of garcinol therapy, IHC was performed on paraffin-embedded sections of cuffs harvested after 3 and 14 days (ApoE3*Leiden) or 21 days respectively (PCAF KO and WT mice). All samples were stained with hematoxylin-phloxine-saffron (HPS) and Weigert's elastin staining was used to visualize elastic laminae. Presence of PCAF was assessed using an anti-PCAF antibody (1:500, Abcam). Inflammatory

cell presence in the vascular wall was visualized using antibodies against leukocytes (anti-CD45 antibodies 1:200, Pharmingen) and macrophages (MAC3, 1:200, Pharmingen). To assess vessel wall morphology, smooth muscle cells (SMC) were stained using anti-smooth muscle α-actin (1:800, Dako). Since garcinol can alter global gene expression and can inhibit proliferation, incorporation of 5-bromo-2'-deoxyuridine (BrdU) into DNA as a marker of DNA synthesis was used to determine the rate of cell proliferation in cuffed vessel segments. Mice (n=5 per group) were injected IP with 25 mg/kg (in 100 µl) BrdU (Sigma Aldrich) three times, once daily starting at time of surgery or 72, 48 and 24h prior to sacrifice. Sections were incubated with a rat anti-BrdU antibody (1:200, Abcam).

Immunohistochemical analysis

All quantification in this study was performed on six equally spaced (150µm distance) serial stained perpendicular cross-sections throughout the entire length of the vessel and was performed by (at least) two blinded observers. The number of leukocytes, macrophages and proliferating cells were counted and expressed as a percentage of the total number of cells (stained with hematoxylin). Vessel wall morphology after 14d is expressed as the percentage of total medial and intimal area stained positive for SMC α-actin or MAC3. Using image analysis software, (Qwin, Leica), total cross-sectional medial area (between both elastic laminae), neointimal area (between internal elastic lamina and lumen) and luminal area was measured. These values were used to calculate the intima / media ratio and percentage luminal stenosis.

Cell-cultures

Murine aortas were harvested from C57Bl/6 PCAF KO mice and WT controls. The aortas were cut longitudinally to expose the luminal side. The endothelial cells were removed by gently scraping. The aortas were cut in small pieces and placed on gelatin-coated culture dishes. The explants were cultured in DMEM (PAA laboratories) containing 20% FCS heat-inactivated (Lonza), 1% penicillin/streptomycin (Invitrogen) and 1% NEAA (PAA laboratories). Cells were cultured and used for experiments at passages 2 to 4.

Splenocytes were isolated from PCAF$^{-/-}$ and control WT mice and were transfected during 24 hours with 100 nmol/L control short-interfering (si) RNA or directed towards murine KAT2B (PCAF) 1, KAT2B 5, KAT2B 6 and KAT2B 7 (all from Qiagen) using Lipofectamine 2000 (Invitrogen).

Bone-barrow derived cells were isolated from PCAF$^{-/-}$ and control mice and subjected to murine macrophage colony-stimulating factor (M-CSF) (20 ng/µl; Miltenyi Biotec) to stimulate differentiation into macrophages. To evaluate the effects of inflammation on inflammatory cytokine expression, confluent layers of human SMC were trypsinized and seeded out in DMEM supplemented with 8% FCS and 1% penicillin/streptomycin and cultured for 24h. SMCs, splenocytes and macrophages were stimulated by exposure to 8% FCS in the presence and absence of 10-100 ng/ml lipopolysaccharide (LPS) from Escherichia coli K-235 L2018 (Sigma Aldrich) alone or together with a serial dilution of garcinol (0, 5 or 15 µM). The cells were incubated overnight at 37°C in 5% CO2 atmosphere. After 24 hr incubation the supernatants were collected and analyzed by ELISA. Data are one representative of three independent experiments.

Cell viability assay

Since garcinol can affect cellular viability in high concentrations, garcinol-induced apoptosis in ex vivo whole blood was assessed in heparinized venous whole blood drawn from WT mice. Blood was diluted 1:25 in RPMI together with (0, 2.5, 10, 15, 20, 30, 50, 100 or 250 μM) garcinol for 24h at 37°C in 5% CO2 atmosphere. After 24h incubation, the medium of all cells including garcinol treated cells was refreshed with RPMI supplemented with 8% FCS and 10% (vol/vol) Alamar blue (Invitrogen). The optical density of each well was measured in a Millipore CytoFluor 2300 plate-reading Fluor meter with excitation at 560 nm and emission at 615 nm when medium in untreated samples turned pink (±4h). Cell viability (%) was calculated compared with positive (untreated) control cells.

Statistical analysis

All data are presented as mean±standard error of the mean (SEM), unless otherwise indicated. Overall comparisons between data from groups were performed using the Kruskal-Wallis test. If a significant difference was found, groups were compared using a Mann-Whitney sum test. All statistical analyses were performed with SPSS 14.0 software for Windows. P-values less than 0.05 were regarded as statistically significant and are indicated with an asterisk (*).

Supplemental figures

	Body Weight (g)	Total plasma cholesterol (mmol/L)
Control mice	24.9 ± 0.3	2.5 ± 0.2
PCAF$^{-/-}$ mice	27.0 ± 1.3	2.4 ± 0.2
Leukocyte adhesion (3d)		
ApoE3*Leiden + vehicle	27.0 ± 0.7	10.9 ± 0.9
ApoE3*Leiden + garcinol	26.9 ± 0.9	11.4 ± 1.2
Vascular remodeling (14d)		
ApoE3*Leiden + vehicle	30.5 ± 0.5	13.3 ± 0.9
ApoE3*Leiden + garcinol	30.8 ± 1.0	12.4 ± 1.1

Table I. Mean body weights (gram) and total plasma cholesterol (mmol/L) at surgery. Values are shown as mean±SEM (n=10/group). No significant differences were observed between groups.

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