Lysine Acetyltransferase PCAF Is a Key Regulator of Arteriogenesis


Objective—Therapeutic arteriogenesis, that is, expansive remodeling of preexisting collaterals, using single-action factor therapies has not been as successful as anticipated. Modulation of factors that act as a master switch for relevant gene programs may prove more effective. Transcriptional coactivator p300-CBP–associated factor (PCAF) has histone acetylating activity and promotes transcription of multiple inflammatory genes. Because arteriogenesis is an inflammation-driven process, we hypothesized that PCAF acts as multifactorial regulator of arteriogenesis.

Approach and Results—After induction of hindlimb ischemia, blood flow recovery was impaired in both PCAF−/− mice and healthy wild-type mice treated with the pharmacological PCAF inhibitor Garcinol, demonstrating an important role for PCAF in arteriogenesis. PCAF deficiency reduced the in vitro inflammatory response in leukocytes and vascular cells involved in arteriogenesis. In vivo gene expression profiling revealed that PCAF deficiency results in differential expression of 3505 genes during arteriogenesis and, more specifically, in impaired induction of multiple proinflammatory genes. Additionally, recruitment from the bone marrow of inflammatory cells, in particular proinflammatory Ly6C^hi monocytes, was severely impaired in PCAF−/− mice.

Conclusions—These findings indicate that PCAF acts as master switch in the inflammatory processes required for effective arteriogenesis. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: inflammation • monocytes • p300-CBP–associated factor

Peripheral arterial occlusive disease is a leading cause of morbidity and mortality. Blood flow to ischemic tissues in the affected limb can be restored by distinct processes, namely vasculogenesis, angiogenesis, and arteriogenesis, of which arteriogenesis, the remodeling of preexisting collateral arterioles into larger arteries, has the greatest impact.

Effective arteriogenesis requires coordination of multiple events. Arteriogenesis is triggered by an increase in fluid shear stress across preexisting collaterals cross-connecting adjacent arterial trees, which is caused by a pressure gradient created by occlusion or atherosclerotic stenosis of one of the trees. This leads to activation of the endothelial cells and adjacent vascular smooth muscle cells (VSMCs) of the collateral wall. Induction of adhesion molecules, cytokines, and chemokines then follows as the first step of an inflammatory cascade essential for arteriogenesis. Recruitment of leukocytes from blood and bone marrow follows, in particular monocytes, and CD4+, CD8+, and regulatory T cells and natural killer cells. These cells infiltrate into the perivascular space around collaterals and release additional paracrine signaling molecules and growth factors. Subsequent degradation and reorganization of the extracellular matrix by released matrix metalloproteases (MMPs), including MMP2 and MMP9, create space required for expansive remodeling of the preexisting collaterals. Proliferation of collateral endothelial cells, VSMCs, and fibroblasts is stimulated, resulting in an increased anatomic lumen diameter. All of the steps described above underline the crucial role of inflammation in effective arteriogenesis.

Although stimulation of collateral remodeling is considered as a promising therapeutic alternative to surgical interventions, clinical trials aimed at modulating individual growth factors or cytokines have thus far not been as successful as anticipated. Now, we know that the coordinated inflammatory and immune modulatory processes driving collateral
growth are multifactorial and too complex to be modulated by therapeutics that target a single gene or pathway. In contrast, modulation of a factor that acts as a master switch for multiple relevant gene programs may be a more effective strategy to augment arteriogenesis.

A protein with such master switch potential is p300-CBP–associated factor (PCAFl), a transcriptional coactivator with intrinsic histone acetyltransferase activity. PCAFl acetylates histones H3 and H4, but there is also increasing evidence that PCAFl modulates nonhistone proteins,13–16 including hypoxia-inducible factor 1α and Notch.18 Furthermore, the histone acetylating activity of PCAFl is essential for nuclear factor κB (NF-κB)–mediated gene transcription19 and facilitates inflammatory gene regulation.20 Because arteriogenesis is an inflammatory-like process, we hypothesized that PCAFl acts as master switch that stimulates multiple inflammatory processes important for collateral remodeling.

Recently, it was shown in a large patient population study (>3000 individuals)31 that a variation in the promoter region of PCAFl is associated with coronary heart disease–related mortality.22 In support of this observation, we recently demonstrated a role for PCAFl in vascular remodeling in a mouse model for reactive stenosis. However, whether PCAFl participates in arteriogenesis has not yet been investigated.

In the present study, we investigated the contribution of PCAFl to postschismic neovascularization in a hindlimb ischemia (HLI) model,23 using PCAFl-deficient (PCAF−/−) mice. When studying arteriogenesis in a knockout model, it is possible that the gene deletion may affect vascular development in the embryo, including collateralogenesis, thus affecting the number of collaterals available for remodeling after an occlusive event in the adult. To investigate whether observed effects were caused by differences in arteriogenesis, in the native collateral circulation or a combination of both, we examined the preexisting collateral density as well as the effect of administration of the PCAFl inhibitor Garcinol to wild-type (WT) mice after induction of HLI. We also studied gene expression and leukocyte recruitment in PCAFl−/− and WT mice after induction of HLI to examine potential mechanisms by which PCAFl regulates arteriogenesis.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

PCAF Contributes to Collateral Remodeling

PCAF−/− mice showed impaired blood flow recovery after HLI (Figure 1A and 1B). Postoperative blood flow was decreased to ≈6% of blood flow in the contralateral limb in both groups, with a trend toward reduced blood flow in PCAFl−/− mice compared with WT mice (Figure 1C; P=0.07). Thereafter, blood flow recovery in PCAFl−/− mice was reduced and did not recover completely before termination at 28 days. Moreover, PCAFl−/− mice showed significantly more necrotic toe nails than WT mice (PCAF−/− 2.9±0.6 versus WT 0.45±0.2; P<0.001; Figure 1D). No autoamputation of hindlimb digits was observed in either group. The reduced blood flow recovery in PCAFl−/− mice was confirmed by quantification of smooth muscle α-actin positive (αSMA+) vessels in the adductor muscle group, 28 days after HLI (Figure 1E). Both the number of αSMA+ vessels (Figure 1F) and the diameter of αSMA+ vessels (Figure 1G and 1H) in PCAFl−/− mice were significantly reduced, resulting in a reduced blood flow. The mean lumen area per αSMA+ vessel (PCAF−/− 139±15 versus WT 297±26 μm2; P<0.001) and total lumen area per section (PCAF−/− 447±46 versus WT 1253±117 μm2; P<0.001) were severely reduced in PCAFl−/− mice (Figure 1G and 1H). Thus, PCAFl deficiency leads to reduced arteriogenesis after induction of HLI.

To assess whether the reduction in blood flow recovery in PCAFl−/− mice was caused by reduced collateral remodeling or by fewer preexisting collaterals, we performed 2 additional experiments. First, we inhibited PCAFl by pharmacological intervention with Garcinol to rule out any effects on number of preexisting collaterals in the PCAFl−/− mice. In WT mice, local PCAFl inhibition by Garcinol resulted in reduced blood flow restoration compared with the empty pluronic gel control group (Figure 2A and 2B). Second, the preexisting vascular bed of PCAFl−/− and WT mice was assessed in the pial circulation using an arterial vascular casting (Figure 2C). Pial collateral density in PCAFl−/− mice was reduced by 11% compared with WT mice, reflecting a moderate but significant contribution of PCAFl in determining the abundance of the native collateral circulation (Figure 2D and 2E; P=0.02). This was in agreement with the trend toward decreased blood flow perfusion in PCAFl−/− mice directly after HLI.

PCAF Is Required for In Vitro Inflammatory Response

We investigated the role of PCAFl in the inflammatory response of multiple cell types, given the above evidence for decreased collateral remodeling and the known involvement of these cells in arteriogenesis. Analysis of circulating cells in a whole blood LPS stimulation assay showed dose-dependent increase of tumor necrosis factor α (TNFα) in blood from WT mice, which was significantly reduced in blood from PCAFl−/− mice (Figure 3A). Next, the splenic cell reservoir was subjected to LPS stimulation and pharmacological PCAFl inhibition with Garcinol. LPS (300 ng/mL)-stimulated membrane cofactor protein (MCP)-1 levels of splenocytes from both PCAFl−/− mice (63±32 pg/mL) and WT splenocytes treated with 20 μmol/L Garcinol (195±35 pg/mL) were both significantly reduced in comparison with WT splenocytes (372±13 pg/mL; P=0.005 and P=0.04, respectively; Figure 3B). Also the inflammatory phenotype of PCAFl−/− VSMCs was assessed. Similar to the splenocyte stimulation, MCP-1 levels were markedly reduced after LPS (0.1 ng/mL) stimulation of PCAFl−/− VSMCs (689±49 pg/mL) and WT splenocytes treated with 20 μmol/L Garcinol (195±35 pg/mL) were both significantly reduced in comparison with WT splenocytes (372±13 pg/mL; P<0.001 and P=0.049, respectively; Figure 3C). In addition, upregulation of MCP-1 mRNA was significantly reduced by 53% in PCAFl−/− VSMCs (Figure 3D; P=0.01). To exclude nonspecific effects of Garcinol, these experiments were repeated in WT VSMCs treated with siRNAs against PCAFl mRNA instead of Garcinol. Transfection with siRNAs targeting PCAFl mRNA
efficiently decreased PCAF mRNA expression by 61% and, like Garcinol, inhibited MCP-1 production (Figure IA–IC in the online-only Data Supplement).

PCAF Modulates Postischemic Gene Regulation

PCAF staining showed enhanced expression in cells of large developing collaterals in the adductor muscle group compared with surrounding skeletal muscle (Figure 4A). To study differential gene expression after HLI between PCAF−/− and WT mice, total RNA isolated from the adductor muscle group was used in a whole-genome expression analysis using Illumina Beadchips. Statistical analysis by SAM on t1/t0avg ratios identified 1963 genes with a significant lower ratio and 1542 genes with a higher ratio in PCAF−/− relative to WT mice (q<5%), indicating that PCAF exhibits a large effect on gene transcription after HLI (Figure 4B).

Table I in the online-only Data Supplement shows the top 50 genes with impaired upregulation in PCAF−/− mice compared with WT mice, including MMP9, critical in matrix degradation for collateral artery expansion. Because PCAF has been shown to regulate inflammatory gene transcription, we selected inflammatory genes that were significantly regulated (Table II in the online-only Data Supplement; Figure II in the online-only Data Supplement). Among the inflammatory genes showing a more pronounced induction in WT mice compared with PCAF−/− mice were genes encoding cytokines CXCL12, CCL9, and TNFα, chemokine receptor CXCR1, transcription factor IRF7, TNF receptor–associated factors TRAF2 and TRAF3, TNF receptor–associated protein TRAP1 and members of the TNF receptor superfamily TNFRSF19 and TNFRSF11a (also known as RANK). The total of inflammatory genes with greater induction in PCAF−/− mice was much smaller than the number of genes more strongly induced in WT, and included inhibitors of the NF-κB pathway like NFKBIA and NKRAS1. Aberrant regulation of several relevant regulated factors (MMP9, TNFα, CCL9, CXCL12, and IRF7) was confirmed using real-time quantitative polymerase chain reaction (Figure 4C–4G).

PCAF Deficiency Alters Leukocyte Recruitment

We quantified leukocyte subtypes that are involved in arteriogenesis, including T cells (helper CD4+, cytotoxic CD8+, and regulatory T cells) and natural killer cells, and subtypes, which have not been previously implicated in arteriogenesis, including B cells and dendritic cells. Blood samples from before (t0) and 1 day after (t1) HLI were analyzed by FACS.
PCAF deficiency had effects on most of the leukocyte subtypes examined. After HLI, circulatory T cells were significantly decreased in PCAF−/− mice compared with WT mice. This difference was caused mainly by a reduction in CD4+ T cells, especially by the fraction of activated CD4+ T cells, defined by the loss of CD62L (L-selectin), and regulatory T cells (CD4+CD25+FoxP3+ T cells). The number of circulatory CD8+ T cells did not differ between WT and PCAF−/− mice. Also counts of other leukocyte subtypes, including B cells and natural killer cells were decreased by PCAF deficiency.

To investigate the migratory behavior of the leukocyte subtypes, the spleen, bone marrow, and lymph nodes were harvested from both mouse strains before (t0) and 1 day after (t1) HLI. Compared with WT mice, we observed reduced numbers of dendritic cells in the draining inguinal lymph nodes of PCAF−/− mice after HLI. Accordingly, the fraction of dendritic cells expressing the costimulatory molecule CD86+ was smaller in the draining lymph nodes of PCAF−/− mice. Furthermore, nearly all tested leukocyte subtypes were increased in the bone marrow of PCAF−/− mice compared with WT mice, including CD4+ and CD8+ T cells, natural killer cells, and dendritic cells, suggesting that these subpopulations are retained in the bone marrow of PCAF−/− mice during recovery after HLI (Figure IIIA–IIIII in the online-only Data Supplement).

Because monocytes play a key role in arteriogenesis and are among the first leukocytes recruited to remodeling collaterals, we evaluated different monocyte populations in blood, spleen, and bone marrow. After HLI, the absolute number of circulating monocytes in WT mice was equal to baseline numbers, but monocytes in PCAF−/− mice significantly decreased compared with baseline (PCAF−/− 0.13±0.05 versus WT 0.37±0.02×10⁶/mL; P=0.002; Figure 5A). In WT mice, the monocyte population increased in the spleen and decreased in the bone marrow after HLI. In contrast, bone marrow monocytes in PCAF−/− mice increased compared with baseline and were significantly higher after HLI compared with WT mice (Figure 5B and 5C). The differences in monocyte numbers were caused mainly by the specific subtype of proinflammatory Ly6Chi monocytes (Figure 5D–5F). The activation state of total and Ly6C+ monocytes did not differ between the 2 strains, measured by mean fluorescent intensity of the adhesion molecule CD11b (Figure 5G and 5H).

Finally, we assessed the number of monocytes/macrophages in the adductor muscle group by fluorescent staining with antibodies against MOMA-2 and αSMA (Figure 6A). Although PCAF−/− mice showed a significant increase in MOMA-2–positive cells 24 hours after HLI, the increase in WT mice was significantly higher (PCAF−/− 3.2±0.35 versus WT 6.0±0.43/section; P=0.001; Figure 6B). Differences were most evident in the perivascular space of remodeling collaterals (PCAF−/− 1.4±0.16 versus WT 3.5±0.76/section; P=0.01; Figure 6C).

**Discussion**

We demonstrate that blood flow recovery after induction of HLI is strongly impaired in PCAF−/− mice, in association with reduced expansive remodeling of collaterals. Furthermore, local PCAF inhibition by Garcinol in WT mice also reduces recovery, indicating that PCAF is directly required for normal arteriogenesis. PCAF gene deficiency results in a repressed in vitro inflammatory response in many cell types known to be involved in arteriogenesis. One day after induction of HLI,
3505 genes are differentially regulated in the adductor muscle group of PCAF−/− mice compared with WT mice. Additionally, recruitment of different proarteriogenic leukocyte subtypes in PCAF−/− mice, in particular inflammatory monocytes, is significantly impaired at this time. Therefore, our data demonstrate that PCAF plays a key role in postischemic arteriogenesis. Compared with WT mice, PCAF−/− mice showed an impaired blood flow recovery after HLI. Our findings suggest that 2 deficiencies caused by a lack of PCAF are involved. First, the expansive remodeling of αSMA+ arterioles at the center of the adductor muscle group, of which most are collaterals, was reduced by 53% in PCAF−/− mice compared with WT mice. Correspondingly, local application of PCAF inhibitor Garcinol in healthy WT mice also resulted in impaired blood flow recovery compared with control animals. Hence, PCAF has a major impact on arteriogenesis. Second, we observed that the density of native preexisting collaterals in the pial circulation of PCAF−/− mice was reduced by 11%. Even changes of this magnitude have significant effects on collateral-dependent perfusion of tissue downstream from an arterial obstruction.24–26 Previous studies have shown that genetic-dependent variation in collateral number in the cerebral pial circulation is shared, at least qualitatively, by similar differences in collateral density in other tissues.24–26 Accordingly, we also observed a trend toward a decrease in blood flow directly after induction of HLI in PCAF−/− mice. In mice, the density of the native collaterals in tissues varies widely among strains from differences in genetic background.24–26 Hence, besides collateral remodeling, genetic PCAF deficiency also contributes to reduced formation of the collateral circulation, which occurs during embryonic development.

In a clinical setting, the outcome after an ischemic event varies among individuals, and differences in abundance of the native collateral circulation have also been reported in patients.27,28 Moreover, a previous study found that the −2481G allele in the promoter region of the PCAF gene associates with an increased risk of mortality in patients with coronary heart disease,22 which further supports our findings that PCAF deficiency impairs collateral function.

For PCAF to serve as a master switch in collateral remodeling, it needs to impact multiple critical phases in the process, namely activation of the endothelium and vessel wall, leukocyte recruitment, matrix degradation, and arteriolar expansion. We examined gene expression in the adductor muscle group containing remodeling collaterals in the initial phase after HLI. More than 3500 genes were differentially regulated between PCAF−/− and WT mice. This suggests that PCAF impacts expression of a large number of genes activated in this setting. More specifically, PCAF−/− mice showed impaired induction of multiple proarteriogenic and proinflammatory genes, including MMP9 and chemokines CXCL12 (SDF1) and CCL9.

MMP9 is critical in degradation and remodeling of the extracellular matrix allowing cell migration and outward expansion of the collaterals and, thus, effective arteriogenesis.29 CXCL12...
is elevated in ischemic skeletal muscle of patients with critical limb ischemia and acts as chemoattractant for CXCR4+ cells, including leukocytes and progenitor cells. CXCL12-mediated recruitment of bone marrow–derived cells to ischemic tissues results in enhanced neovascularization. Also CCL9, which is a strong chemoattractant for bone marrow–derived cells, is upregulated after muscle injury.

In addition, PCAF −/− mice showed impaired induction of multiple factors related to the proinflammatory TNFα pathway. TNFα−/− mice have reduced collateral artery perfusion, and anti-TNFα therapy attenuates arteriogenesis. Thus, reduced TNFα expression in PCAF−/− mice likely contributes to the impaired arteriogenesis in these mice. Our data suggest that PCAF regulates many factors that have previously been described, thus, playing an important role in both inflammation and arteriogenesis.

It should be noted that RNA was isolated from the adductor muscle group as a whole and not from the embedded collateral arteries alone, as was described previously. In that report, a whole-genome microarray analysis was performed on collaterals microdissected from the gracilis muscle 24 hours after HLI. Here, we found exceedingly more differentially expressed genes, then the 404 genes that were found upregulated in gracilis collaterals of WT mice. Using the entire adductor muscle group for microarray analysis, not only the collaterals but also infiltrating leukocytes and surrounding nonvascular tissues were included in these analyses.

As discussed in the introduction, an inflammatory-like process plays a role in all stages of arteriogenesis. To investigate the impact of PCAF on the inflammatory response of the different cell types involved in arteriogenesis, we studied circulating cells in whole blood, splenic leukocytes and VSMCs in vitro. PCAF is critical for the regulation of transcription factor NF-κB, which consists of a p65 and p50 subunit bound to inhibitory proteins in the cytoplasm. On stimulation NF-κB is translocated to the nucleus and activates the expression of multiple genes, including TNFα and MCP-1. PCAF binds to the NF-κB p65 subunit and activates NF-κB–related inflammatory gene expression.
clearly demonstrate that PCAF deficiency results in decreased production of proinflammatory cytokines by multiple cell types after stimulation with LPS. LPS-stimulated whole blood from PCAF−/− mice produced less TNFα than blood from WT mice, indicating a reduced inflammatory phenotype of circulating cells. Also PCAF−/− cells isolated from the spleen, one of the major leukocyte reservoirs, showed a reduced inflammatory response compared with splenocytes from WT mice. PCAF−/− VSMCs produced less MCP-1 than WT VSMCs in response to LPS, which would favor reduced monocyte recruitment and, therefore, reduced VSMC proliferation, which is essential for collateral remodeling. We obtained similar results using either Garcinol or PCAF-specific siRNA knockdown in WT VSMCs, thus excluding effects of any preexisting differences in PCAF-deficient cells. Our data correspond with a report that TNFα-induced NF-κB activity increases in human airway smooth muscle cells overexpressing PCAF and provide strong evidence for a wide effect of PCAF on inflammatory gene transcription.

The p65 subunit of NF-κB recruits coactivator PCAF and activates NF-κB–mediated gene transcription. In contrast, the NF-κB p50 subunit lacks the transcriptional activation domain and inhibits gene transcription. Mice deficient of the NF-κB p50 subunit showed enhanced blood flow recovery after HLI as the result of increased monocyte recruitment to the perivascular space of collaterals. Whereas arteriogenesis and monocyte recruitment are enhanced by NF-κB activation in NF-κB p50−/− mice, reduced regulation of the NF-κB p65 subunit in PCAF−/− mice could likely explain the impaired arteriogenesis by inhibition of monocyte recruitment. In WT mice, the monocyte population increased in the spleen and decreased in the bone marrow after HLI. This is in line with earlier reports that monocytes are mobilized from the bone marrow after HLI. Activation of monocytes and Ly6Chi monocytes was described to originate from a specific proinflammatory subtype, which is characterized by high expression of Ly6C. These Ly6Chi monocytes are recruited in the early stage of collateral remodeling, and our data confirm that they are mobilized from the bone marrow in WT mice. In contrast, recruitment of monocytes proved to be severely impaired in PCAF−/− mice. PCAF−/− mice showed reduced numbers of circulating monocytes after HLI, particularly reduced numbers of Ly6C+ monocytes. Whereas monocytes migrated away from the bone marrow in WT mice, PCAF−/− mice showed an increase in bone marrow monocytes, suggesting a defect in monocyte mobilization. Concomitantly, 24 hours after HLI fewer monocytes were recruited to the collaterals in PCAF−/− mice.
monocytes stimulate arteriogenesis by secretion of growth factors and degradation of extracellular matrix at the site of collateral remodeling. Therefore, the lack of monocyte accumulation along collaterals likely contributes to the impaired arteriogenesis in PCAF−/− mice.

Besides monocytes, PCAF also affected numerous other leukocyte subtypes. In PCAF−/− mice, we demonstrated decreased numbers of circulating leukocytes involved in arteriogenesis, like T cells46 (predominantly activated CD4+ T cells),7,11 natural killer cells,11 and regulatory T cells,9,10 and also in those cells that have not previously been implicated in arteriogenesis, including B cells. Furthermore, fewer dendritic cells were found in draining inguinal lymph nodes compared with WT mice, where ing B cells. Furthermore, fewer dendritic cells were found in draining inguinal lymph nodes compared with WT mice, where ing B cells. Furthermore, fewer dendritic cells were found in draining inguinal lymph nodes compared with WT mice, where ing B cells.

In conclusion, PCAF−/− mice demonstrated impaired collateral remodeling after HLI, together with a reduction in the number of native preexisting collaterals present before arterial obstruction. PCAF deficiency resulted in altered expression of a large number of genes, including those in immune and inflammatory pathways, and an attenuated inflammatory response in multiple cell types involved in arteriogenesis. These findings indicate that PCAF is a key regulator in post-ischemic blood flow recovery by impacting the inflammatory processes required for robust arteriogenesis.

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Disclosures
None.

References


**Significance**

Although the induction of arteriogenesis is a promising therapeutic alternative to surgical interventions for patients with severe peripheral arterial disease, clinical trials aimed at modulating individual growth factors or cytokines have thus far not been as successful as anticipated. Master switches for multiple relevant gene programs will likely be a more effective strategy to stimulate arteriogenesis. The transcriptional coactivator p300-CBP–associated factor (PCAF) acts as such a master switch because it induces multiple inflammatory processes crucial to arteriogenesis. Here, we show that PCAF is an essential factor in arteriogenesis in a model for peripheral arterial disease. Our findings clearly demonstrate that PCAF plays a key role in the formation of preexisting collateral arteries and postischemic arteriogenesis. PCAF alters the expression of >3500 genes and impacts inflammatory processes required for effective arteriogenesis, signifying PCAF as an unanticipated target gene that yields new mechanistic insight in arteriogenesis.
Lysine Acetyltransferase PCAF Is a Key Regulator of Arteriogenesis

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**Material and Methods**

**Animals**

Experiments were approved by the committee on animal welfare of the Leiden University Medical Center (Leiden, The Netherlands). Male C57BL/6 mice were purchased from Charles River (France). The generation of PCAF−/− mice (C57BL/6 background) has previously been described and the animals were kindly provided by Dr. C. Gongora (Montpellier, France). All animals received regular chow diet and water ad libitum.

**Induction of hind limb ischemia**

Mice were anesthetized by intraperitoneal (i.p.) injection of midazolam (8 mg/kg, Roche Diagnostics), medetomidine (0.4 mg/kg, Orion) and fentanyl (0.08 mg/kg, Janssen Pharmaceutica). Unilateral HLI was induced by electrocoagulation of the left femoral artery proximal to the superficial epigastric arteries alone, or combined with electrocoagulation of the distal femoral artery proximal to the bifurcation of the popliteal and saphenous artery. After surgery, anesthesia was antagonized with flumazenil (0.7 mg/kg, Fresenius Kabi), atipamezole (3.3 mg/kg, Orion) and buprenorphine (0.2 mg/kg, MSD Animal Health). For pharmacological PCAF inhibition in WT mice, 20 µl 40% pluronic gel (Sigma-Aldrich) with or without 25 mg/ml Garcinol (Santa Cruz Biotechnology) was applied topically to the adductor muscle before skin closure.

**Laser Doppler perfusion imaging**

Hind limb perfusion was measured with laser Doppler perfusion imaging (LDPI) (Moor Instruments) after intraperitoneal injection of midazolam (8 mg/kg) and medetomidine (0.4 mg/kg). The regions of interest analyzed consisted of the foot distal to the base of the first digit. Perfusion was expressed as the ratio of ligated to non-ligated foot. After measurement, anesthesia was antagonized with flumazenil (0.7 mg/kg) and atipamezole (3.3 mg/kg).

**Pre-existing collateral density**

Pre-existing collateral density in the pial circulation of the dorsal cerebral cortex predicts collateral density in skeletal muscle and other vascular beds. However, unlike in other tissues where arterial trees are arranged three-dimensionally and difficult to image with fidelity, all cerebral collaterals are contained within the pia and can thus be directly identified and counted. Methods for measurement of collateral density between the anterior cerebral artery (ACA), middle cerebral artery (MCA), and posterior cerebral artery (PCA) were described elsewhere. Briefly, animals were heparinized systemically and anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) prior to vascular casting. Maximal dilation was accomplished by cannulation of the thoracic aorta and infusion of sodium-nitroprusside (30 μg/ml) and papaverine (40 μg/ml) in PBS at 100 mmHg for 3 minutes. Yellow Microfil™ (Flow Tech Inc.) with viscosity adjusted to prevent capillary and venous filling was infused under a stereomicroscope after craniotomy. The dorsal cerebral circulation was fixed with topical application of 4% paraformaldehyde to prevent any reduction in vessel dimensions after Microfil injection.
Brains were incubated in Evans Blue (2 μg/ml) for several days to improve contrast for visualization of the vasculature. Digital images were obtained at 13X (Leica) of the dorsal brain surface and processed with ImageJ software (NIH). Collateral density was calculated by determining the total number of pial collaterals between the ACA-MCA, ACA-PCA and MCA-PCA and dividing by the dorsal surface area of the cerebral hemispheres. Areas that sustained damage, were incompletely filled, or were otherwise uncountable were excluded from analysis.

**Immunostaining and analysis**

Mice were sacrificed and the adductor muscle group medial to the femur was excised en bloc. Tissues were snap frozen in liquid nitrogen or fixed in 3.7% formaldehyde. Serial 5-μm-thick paraffin-embedded sections were used for histological analysis of collateral artery number and size. Vessels at the midpoint of the adductor muscle group, stained using anti-smooth muscle α-actin (αSMA) (DAKO), are likely composed of collaterals but may also include arterioles of the opposing tree. Randomly photographed images through the central part of the adductor muscle group were used to quantify the number and lumen diameter of αSMA* vessels using ImageJ software (total of 9 images of 3 sections per mouse). To correct for non-perpendicularly cut sections, the circular lumen area of αSMA* vessels was calculated from the lumen diameter measured at the narrowest point.

Frozen 5-μm-thick sections were fixed in ice-cold acetone and stained with anti-PCAF (Abcam) and Cy3 conjugated anti-αSMA (Sigma-Aldrich). PCAF was visualized using Alexa 488 conjugated secondary antibody (Invitrogen). Nuclei were stained using Vectashield with DAPI (Vector Laboratories). Fluorescent images were taken on a LSM700 microscope (Carl Zeiss) and contrast-stretched using Zen 2009 software (Carl Zeiss). Collaterals were detected with Cy3 conjugated anti-αSMA, and perivascular monocytes with anti-MOMA-2 (Millipore), visualized using Alexa 488 conjugated secondary antibody (Invitrogen). Monocytes were quantified from at least six consecutive sections per mouse and expressed as the number of MOMA-2 positive cells in the perivascular space of αSMA* vessels.

**In vitro immune response**

*Whole blood*

Blood was collected from the tail vein of PCAF<sup>−/−</sup> and WT mice and diluted 1:25 with RPMI 1640 (Invitrogen) supplemented with non-essential amino acids (PAA Laboratories) and glutamax (Invitrogen). Blood was incubated overnight at 37ºC, 5% CO₂, in the presence of lipopolysaccharide (LPS) (0-500 ng/ml) from Escherichia coli K-235 (Sigma-Aldrich). Cell-free supernatant was collected and TNFα level was measured by ELISA (BD Biosciences).

*Splenocytes*

Spleens were isolated from PCAF<sup>−/−</sup> and WT mice, minced through a 40μm-cell strainer (Biosciences) and, after erythrolysis with ammonium chloride solution, single cell suspensions were resuspended in DMEM (PAA Laboratories) supplemented with 10% heat-inactivated FCS (Lonza). Splenocytes (1x10<sup>6</sup>) from PCAF<sup>−/−</sup> and WT mice were plated and incubated for 24 hours with LPS (300 ng/ml) or control. Splenocytes of WT
mice were also incubated with Garcinol (20 μM) in combination with LPS (300 ng/ml) or control. MCP-1 level in the cell-free supernatant was measured by ELISA (BD Biosciences).  

**Vascular smooth muscle cells**

VSMCs were isolated from abdominal aortas from PCAF-/- and WT mice. For stimulation assays, cells (passage 2-4) were plated (5x10^3) and incubated for 24 hours with LPS (0.1 and 1 ng/ml) or control. VSMCs of WT mice were also incubated with Garcinol (15 μM) in combination with LPS (0.1 and 1 ng/ml) or control. MCP-1 level in the cell-free supernatant was measured by ELISA (BD Biosciences). RNA was isolated from LPS stimulated (1 ng/ml) WT and PCAF-/- VSMCs (1x10^5) using RNeasy minikits (Qiagen).

**Whole-genome expression**

The adductor muscle group of PCAF-/- and WT mice was harvested before (t0) and 1 day after (t1) induction of HLI, and total RNA was extracted using RNeasy fibrous tissue minikit (Qiagen). RNA integrity was checked by NanoDrop 1000 Spectrophotometer (NanoDrop Technologies) and 2100 Bioanalyzer (Agilent Technologies). For whole-genome expression profiling, amplified biotinylated RNA was generated using the Illumina TotalPrep RNA Amplification Kit. For array analysis, MouseWG-6 v2.0 Expression Beadchips (Illumina), which contain more than 45,200 transcripts, were used. Expression levels were Log2-transformed and after quantile normalization, transcripts showing background intensity, both at baseline and after induction of HLI, were removed from the analysis. Gene expression levels at t1 were expressed relative to average baseline levels generating t1/t0avg ratios for all 15,555 regulated genes, and compared between both mouse strains. To define the effect of PCAF on inflammatory gene transcription, gene descriptions, as provided by Illumina, containing any of these criteria (interleukin, chemokine, interferon, TGF, TNF, NF-κB) were selected and ratios were tested for significance.

**Real-time quantitative PCR**

RNA was reverse transcribed using High Capacity RNA-to-cDNA kit (Applied Biosystems). Quantitative PCR was performed on the ABI 7500 Fast system, using commercially available TaqMan gene expression assays for HPRT1, MCP-1, MMP9, TNFα, CCL9, IRF7, CXCL12 and CXCR4 (Applied Biosystems).

**Flow cytometry**

Blood, spleen, bone marrow and non-draining lymph nodes were harvested before (t0) and 1 day after (t1) induction of HLI. Draining lymph nodes were dissected from the inguinal region. Total circulating leukocytes were measured using the KX-21N Hematology Analyzer (Sysmex). Tissues were minced through a 40μm-cell strainer (BD Biosciences) to obtain single cell suspensions which were resuspended in IMDM (Lonza) with 2% FCS. For dendritic cell-specific cell surface staining, the spleen and lymph nodes were first perfused with collagenase (1mg/ml) and DNase (0.02mg/ml) for 10 minutes and minced. Erythrocytes were lysed and samples for intracellular staining were permeabilized. Fluorochrome-conjugated monoclonal antibodies specific for CD3, CD4, CD8, CD11c, CD11b, CD19, CD25, CD86, CD115, FoxP3, Ly6C, Ly6G, B220,
DX5 were used. Cells were measured on a LSRII flow cytometer (BD Biosciences) and data was analyzed using FlowJo software (Tree Star, Inc.).

Statistical analysis

All results are presented as mean ± standard error of the mean (SEM) or as scatter plot. Comparisons between groups were performed using Student’s T-test. All statistical analyses were performed using SPSS 17.0 software. P-values < 0.05 were considered statistically significant and are indicated with *; p-values < 0.01 and < 0.001 are indicated by ** and *** respectively. Statistical Analysis of Microarray data (SAM) was used for the analysis on t1/t0avg ratios in the whole-genome expression array. A false discovery rate (expressed as q-values) of less than 5% was considered significant.

References


Supplemental data for

Bastiaansen et al, “The lysine acetyltransferase PCAF is a key regulator of arteriogenesis”

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Supplemental Figure I. PCAF knockdown by siRNA in vascular smooth muscle cells. (A) Vascular smooth muscle cells (VMSCs) were transfected with non-target fluorescent siGlow (Dharmacon) to test transfection efficiency, using Lipofectamine 2000 according to the manufacturer's instructions. VMSCs were visualized on a Leica fluorescence microscope. (B) VMSCs were plated and transfected with control short-interfering RNA (siRNA) or a combination of 4 siRNAs directed towards PCAF for 4 hours. Untransfected VMSCs were used as control. VMSCs were incubated with LPS (1 ng/ml) for 24 hours. To confirm PCAF knockdown, PCAF mRNA was measured by real-time quantitative PCR. Levels were normalized against the expression of HPRT1. PCAF specific siRNA reduced PCAF expression with 61% in comparison to scrambled siRNA. (C) Cell-free supernatant of LPS stimulated VMSCs was collected for MCP-1 quantification, measured by ELISA. Transfection with PCAF specific siRNA inhibited MCP-1 production of VMSCs in comparison to scrambled siRNA. All samples were performed in triplicates. *P < .05, **P < .01, scrambled siRNA versus PCAF siRNA.
Supplemental Figure II. Differential inflammatory gene expression in PCAF-/- and WT mice. Heatmap of differentially expressed inflammatory genes in adductor muscle group of PCAF-/- and WT mice, 1 day after HLI. Gene definitions containing any of these criteria (interleukin, chemokine, interferon, TGF, TNF, NFKB) were selected. Included are genes that were significantly different between PCAF-/- and WT mice (q-value < 5). Data are presented as the fold change in expression between day 1 and average preoperative baseline levels, generating t1/t0avg ratios. Red indicates increased and green indicates reduced expression relative to average baseline levels.
Supplemental Figure III. Leukocyte subtypes in PCAF-/- and WT mice after HLI. Flow cytometry analysis of lymphocytes in blood (nx103/mL), spleen and bone marrow (% of total cells). In succession, values are presented for (A) CD4+ T helper cells, (B) CD8+ cytotoxic T cells, (C) activated CD4+ T helper cells, (D) activated CD8+ cytotoxic T cells, (E) regulatory T cells (Treg), (F) Natural Killer (NK) cells and (G) B lymphocytes. (H) Dendritic cells (DCs) in spleen, bone marrow, non-draining and draining (inguinal) lymph nodes (LN). (I) Activated DCs in spleen, bone marrow, non-draining and draining (inguinal) lymph nodes.
Supplemental Table I. List of differentially expressed genes in de adductor muscle group of PCAF-/- and WT mice. Data are presented as the log fold change in expression between day 1 after HLI and average preoperative baseline levels, generating t1/t0avg ratios. Listed are the top 50 genes which showed an impaired upregulation in PCAF-/- mice compared to WT mice. Q-values less than 5% were considered significant. FC = fold change.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene Name</th>
<th>PCAF-/- logFC</th>
<th>WT logFC</th>
<th>q-value</th>
<th>PCAF-/- vs WT FC</th>
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<tbody>
<tr>
<td>Arg1</td>
<td>arginase 1, liver</td>
<td>1.55</td>
<td>3.11</td>
<td>4.39</td>
<td>0.34</td>
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<td>Pdk4</td>
<td>pyruvate dehydrogenase kinase, isoenzyme 4</td>
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<td>3.00</td>
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<td>Ptpn6</td>
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<td>0.86</td>
<td>0.50</td>
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<td>Lst1</td>
<td></td>
<td>0.37</td>
<td>1.36</td>
<td>0.86</td>
<td>0.50</td>
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<td>Arxa2</td>
<td>annexin A2 (Arxa2), mRNA.</td>
<td>0.39</td>
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<td>0.51</td>
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<td>Sytnb1a</td>
<td>synaptojanin (or cysteine) peptidase inhibitor, clade B, member 1a</td>
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<td>1.23</td>
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<td>1.01</td>
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<td>1.60</td>
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<td>Angpt4</td>
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<td>Ctf</td>
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<td>Cdt1</td>
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<td>Kcnab2</td>
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<td>1.66</td>
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<td>Sirpa</td>
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<td>0.60</td>
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<td>Alox5ap</td>
<td>arachidonate 5-lipoxygenase activating protein</td>
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<td>Arhgdib</td>
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<td>1.23</td>
<td>0.61</td>
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<td>Emilin2</td>
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<td>0.10</td>
<td>0.82</td>
<td>0.86</td>
<td>0.61</td>
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<td>Lrcr33</td>
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<td>Ly6e</td>
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<td>0.66</td>
<td>0.33</td>
<td>0.64</td>
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<td>Hdac4</td>
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<td>0.02</td>
<td>0.66</td>
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<tr>
<td>Dok2</td>
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<td>1.14</td>
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<td>0.64</td>
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<tr>
<td>Fcgr4</td>
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<td>2.26</td>
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<tr>
<td>Alox5ap</td>
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<tr>
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<td>0.45</td>
<td>0.65</td>
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<tr>
<td>Gnb2</td>
<td>guanine nucleotide binding protein (G protein), beta 2</td>
<td>0.07</td>
<td>0.68</td>
<td>0.00</td>
<td>0.66</td>
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<td>Gnaa</td>
<td>guanine nucleotide binding protein (G protein), alpha polypeptide</td>
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<td>1.17</td>
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<td>Gm1</td>
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<td>Fxyd5</td>
<td>FXYD domain-containing ion transport regulator 5</td>
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<td>Cdh15</td>
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<td>0.69</td>
<td>0.33</td>
<td>0.66</td>
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<td>Rabgef1</td>
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<td>0.82</td>
<td>0.45</td>
<td>0.67</td>
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<td>0.62</td>
<td>0.98</td>
<td>0.67</td>
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<tr>
<td>Arb2</td>
<td>arrestin, beta 2</td>
<td>0.04</td>
<td>0.62</td>
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<td>0.67</td>
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<td>Emp3</td>
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<td>0.67</td>
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<td>0.68</td>
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<td>St100a11</td>
<td>S100 calcium binding protein A11</td>
<td>0.54</td>
<td>1.11</td>
<td>4.39</td>
<td>0.68</td>
</tr>
</tbody>
</table>
### Symbol | Gene Name | PCAF-/- WT | logFC | logFC | q-value
--- | --- | --- | --- | --- | ---
Ccl9 | chemokine (C-C motif) ligand 9 | | 1.91 | 2.81 | 3.60
Tgfb1 | transforming growth factor, beta induced | | 0.36 | 0.99 | 3.00
Tnfrsf19 | tumor necrosis factor receptor superfamily, member 19 | | -0.64 | -0.04 | 1.66
C1qtnf9 | C1q and tumor necrosis factor related protein 9 | | -0.95 | -0.50 | 3.00
LOC100041504 | PREDICTED: similar to beta chemokine Exodus-2 | | -0.16 | 0.27 | 3.60
Cxc12 | chemokine (C-X-C motif) ligand 12, transcript variant 3 | | -0.58 | -0.16 | 3.60
C1qtnf2 | C1q and tumor necrosis factor related protein 2 | | -0.57 | -0.16 | 0.00
Traf2 | Tnf receptor-associated factor 2 | | -0.28 | 0.12 | 3.60
Iftm3 | interferon induced transmembrane protein 3 | | 1.00 | 1.39 | 2.42
Tgfb3 | transforming growth factor, beta 3 | | -0.40 | -0.02 | 4.39
Il8ra | interleukin 8 receptor, alpha | | 0.07 | 0.43 | 0.98
Ifngr2 | interferon gamma receptor 2 | | -0.48 | -0.15 | 1.94
Il3ra | interleukin 3 receptor, alpha chain | | -0.02 | 0.30 | 0.89
C1qtnf5 | C1q and tumor necrosis factor related protein 5, transcript variant 1 | | -0.11 | 0.19 | 0.33
Traf3 | Tnf receptor-associated factor 3, transcript variant 1 | | -0.26 | 0.04 | 4.39
Tnfap8l2 | tumor necrosis factor, alpha-induced protein 8-like 2 | | 0.07 | 0.36 | 1.94
Ifi3 | interferon-induced protein with tetratricopeptide repeats 3 | | -0.23 | 0.06 | 4.39
Irf7 | interferon regulatory factor 7 | | 0.00 | 0.24 | 1.94
Fadd | Fas (TNFRSF6)-associated via death domain | | -0.04 | 0.19 | 4.39
Tnfrsf11a | tumor necrosis factor receptor superfamily, member 11a | | 0.14 | 0.37 | 2.42
Prkra | protein kinase, interferon inducible double stranded RNA dependent activator | | -0.14 | 0.08 | 4.39
Il17c | interleukin 17 receptor C | | 0.01 | 0.23 | 0.98
Trap1 | TNF receptor-associated protein 1 | | -0.10 | 0.11 | 1.66
Tnf | tumor necrosis factor | | -0.08 | 0.11 | 1.94
Il18 | interleukin 18 | | -0.12 | 0.04 | 4.39

### Symbol | Gene Name | PCAF-/- WT | logFC | logFC | q-value
--- | --- | --- | --- | --- | ---
Irai1 | interleukin-1 receptor-associated kinase 1 | | 0.18 | 0.00 | 1.66
Ifi3 | interleukin enhancer binding factor 3, transcript variant 3 | | 0.19 | 0.00 | 0.98
Tgfb2 | transforming growth factor, beta receptor II, transcript variant 1 | | 0.47 | 0.24 | 3.60
LOC100048583 | PREDICTED: similar to interferon-inducible protein 203, transcript variant 1 | | 0.16 | -0.08 | 0.33
Ccr11 | chemokine (C-C motif) receptor-like 1 | | -0.15 | -0.42 | 3.00
LOC545396 | PREDICTED: similar to TGF beta-inducible nuclear protein 1 | | -0.06 | -0.33 | 3.00
Nfkbia | nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha | | 0.36 | 0.06 | 3.60
Irf2bp2 | PREDICTED: interferon regulatory factor 2 binding protein 2 | | 0.09 | -0.22 | 3.00
Nkirs1 | NFKB inhibitor interacting Ras-like protein 1 | | 0.15 | -0.17 | 0.00
Ccl9 | chemokine (C-C motif) ligand 19 | | 1.02 | 0.68 | 0.40
Il15r4 | interleukin 15 receptor, alpha chain, transcript variant 2 | | 0.20 | -0.15 | 2.42
Il8ra | interleukin 6 receptor, alpha | | 0.69 | 0.21 | 1.66
Il4i1 | interleukin 4 induced 1 | | -0.39 | -1.10 | 0.00
LOC100044430 | PREDICTED: similar to Interferon activated gene 205 | | 1.27 | 0.50 | 1.23
Cxc1 | chemokine (C-X-C motif) ligand 1 | | 2.93 | 1.45 | 1.66

Supplemental Table II. List of significantly differentially expressed inflammatory genes in de adductor muscle group of PCAF-/- and WT mice. Data are presented as the log fold change in expression between day 1 after HLI and average preoperative baseline levels, generating t1/t0avg ratios. Gene definitions containing any of these criteria (interleukin, chemokine, interferon, TGF, TNF, NFKB) were selected. Q-values less than 5% were considered significant. FC = fold change.