Promyelocytic leukemia protein promotes the phenotypic switch of smooth muscle cells in atherosclerotic plaques of human coronary arteries

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Promyelocytic leukemia protein (PML) is a constitutive component of PML nuclear bodies (PML-NBs), which function as stress-regulated SUMOylation factories. Since PML can also act as a regulator of the inflammatory and fibroproliferative responses characteristic of atherosclerosis, we investigated whether PML is implicated in this disease. Immunoblotting, ELISA and immunohistochemistry showed a stronger expression of PML in segments of human atherosclerotic coronary arteries and sections compared with non-atherosclerotic ones. In particular, PML was concentrated in PML-NBs from α-smooth muscle actin (α-SMA)-immunoreactive cells in plaque areas. To identify possible functional consequences of PML-accumulation in this cell type, differentiated human coronary artery smooth muscle cells (dHCASMCs) were transfected with a vector containing the intact PML-gene. These PML-transfected dHCASMCs showed higher levels of small ubiquitin-like modifier (SUMO)-1-dependent SUMOylated proteins, but lower levels of markers for smooth muscle cell (SMC) differentiation and revealed more proliferation and migration activities than dHCASMCs transfected with the vector lacking a specific gene insert or with the vector containing a mutated PML-gene coding for a PML-form without SUMOylation activity. When dHCASMCs were incubated with different cytokines, higher PML-levels were observed only after interferon γ (IFN-γ) stimulation, while the expression of differentiation markers was lower. However, these phenotypic changes were not observed in dHCASMCs treated with small interfering RNA (siRNA) suppressing PML-expression prior to IFN-γ stimulation. Taken together, our results imply that PML is a previously unknown functional factor in the molecular cascades associated with the pathogenesis of atherosclerosis and is positioned in vascular SMCs (VSMCs) between upstream IFN-γ activation and downstream SUMOylation.

Introduction

Atherosclerotic heart disease is one of the most frequent causes of death in humans worldwide [1]. Despite the ever-growing understanding of the molecular processes underlying atherosclerosis, the molecular factors that initiate blood vessel degeneration, i.e. the formation of plaques and their sudden breakage are still widely unknown. This lack of knowledge leads to limited treatment options and slows down the development of new drugs and therapies.

Morphological changes in atherosclerotic blood vessels are caused by inflammatory and fibroproliferative processes that facilitate the proliferation and migration of vascular smooth muscle cells (VSMCs),
endothelial cells (ECs) and white blood cells (macrophages/monocytes), which, in addition to the deposition of subendothelial lipids, contribute to typical atherosclerotic lesions known as plaques [2,3]. During the formation of atherosclerotic plaques, the VSMCs from the arterial media undergo an extensive, not yet fully understood differentiation process towards a proliferative phenotype and migrate from the media into the intima [3]. Especially inflammatory processes appear to play an important role in the development of advanced atherosclerotic lesions which are associated with the secretion of pro-inflammatory cytokines such as tumor necrosis factor \( \alpha \) (TNF-\( \alpha \)), transforming growth factor \( \beta \) (TGF-\( \beta \)), interferon \( \gamma \) (IFN-\( \gamma \)) and interleukins, preferably released by activated T cells and macrophages [4,5].

Interestingly, the expression of these cytokines is also related to the intracellular expression and signaling of the promyelocytic leukemia protein (PML) [6]. PML was originally identified in acute promyelocytic leukemia and was therefore primarily considered to act as a tumor suppressor [7]. Subsequently, PML was shown to play important roles in stress response, cell cycle regulation, apoptosis, senescence, transcriptional and post-transcriptional regulation, DNA repair, inflammatory responses and intermediary metabolism [8,9]. Remarkably, many of these cellular functions are involved in or altered during plaque formation in atherosclerotic vessels. PML expression itself is also modulated under various stress conditions such as inflammation [9,10]. In addition, a microarray study identified PML target genes that play a role in atherogenesis [11].

Together with a wide variety of over 100 functionally diverse proteins, PML forms dynamic aggregates inside the nucleus known as ‘PML nuclear bodies’ (PML-NBs), which are potent modifiers of proteins and their functions [8,12]. Almost all PML-associated proteins are modified by small ubiquitin-like modifier (SUMO), and SUMOylation of PML itself is essential for the integrity of PML-NBs [12]. SUMOylation is a post-translational modification which is characterized by reversible covalent binding of SUMO to target proteins and is involved in the regulation of protein–protein interactions, subcellular nuclear localization, protein–DNA interactions and enzymatic activity [13].

Several molecular key findings and clinical studies indicate that SUMOylation of components of PML-NBs also play an essential role in cardiac development, function and cardiovascular diseases, in particular atherosclerosis [14–16]. Examples for SUMOylated proteins within PML-NBs, which are relevant for the pathophysiology of atherosclerosis, are extracellular signal-regulated kinase 5 (Erk5) [17], p53 [17], protein inhibitor of activated STAT-1 (PIAS1) [15] and sentrin-specific protease 1 (SENP1) [18].

Based on its role in promoting inflammation and its established biochemical impact on SUMOylation, we hypothesized that PML plays a hitherto unknown function in the pathogenesis of atherosclerosis. To address this issue, we investigated whether PML is expressed in human coronary arteries, especially in association with atherosclerotic plaques, and assessed in a cell culture system if and how PML affects atherogenesis.

### Materials and methods

#### Human artery specimens

Coronary arteries were obtained from 16 patients receiving heart transplantation at the German Heart Institute Berlin. The patients with failing hearts had previously suffered from either dilated cardiomyopathy or ischemic heart disease (Table 1). Samples were examined by means of conventional microscopy and were classified into atherosclerotic vessels with plaques or non-atherosclerotic vessels. Specimens of human coronary arteries from explanted hearts were analyzed under the written informed consent of the patients to their HTx.

Aorta tissue samples were provided by the tissue bank of the University Medical Center Mainz in accordance with the regulations of the tissue biobank and the approval of the ethics committee of the University Medical Center Mainz.

| Comorbidities                        | Sex |
|--------------------------------------|-----|
| Ischemic heart disease               | M = 7 |
| (all were found to be atherosclerotic)| F = 0 |
| Dilated cardiomyopathy               | M = 7 |
| (all were found to be non-atherosclerotic) | F = 2 |
Cell culture

Human coronary artery smooth muscle cells (HCASMCs; Cell Applications, San Diego, CA, U.S.A.; Cat. No: 350-05a) were cultured in smooth muscle cell growth medium (Cell Applications, San Diego, CA, U.S.A.; Cat. No: 311-500). Cells were maintained under standard conditions (37°C in a humidified atmosphere of 5% CO₂). To induce differentiation, cells were grown to 70–80% confluence and then transferred to smooth muscle cell differentiation medium (Cell Applications, San Diego, CA, U.S.A.; Cat. No: 311D-250) for 5 days. HCASMCs from passage 3–6 were used for the experiments.

Transfection with PML expression vector

Transfection of differentiated human coronary artery smooth muscle cells (dHCASMCs) with vectors containing PML-IV inserts was performed as previously described [19]. Briefly, for transient transfection of dHCASMCs with full-length PML-IV, the human expression plasmids pEGFP-C1-PML-IV [20], Flag-PMLIV/pRK5 [21] and PML-3/pSG5 [22] were used in combination with the TurboFect reagent (Thermo Fisher Scientific, Waltham, MA, U.S.A.; Cat. No: R0533) according to the manufacturer’s instructions. The transfection rates were controlled by monitoring GFP expression with a Zeiss Axioskop 40 microscope and a Zeiss object lens Neofluar 16/0.4 (Carl Zeiss, Oberkochen, Germany), and/or expression efficiency was tested by RT-qPCR and immunoblot analysis. pEGFP-C1-PML-IV was a kind gift from Peter Hemmerich (Jena, Germany), Flag-PMLIV/pRK5 was a gift from Xiaolu Yang (Addgene plasmid #59742; http://n2t.net/addgene:59742; RRID: Addgene 59742) and PML-3/pSG5 was a gift from Pier Pandolfi (Addgene plasmid # 50939; http://n2t.net/addgene:50939; RRID: Addgene_50939). The term ‘control cells’ usually refers to cells transfected with a corresponding empty vector lacking a specific gene insert.

Transfection with small interfering RNA

Transfection of dHCASMCs was performed using a mixture of four unrelated small interfering RNA (siRNA) species (25 nM final) against the PML nucleotide sequence (Dharmacon, Lafayette, CO, U.S.A.; Cat. No: LQ-006547-00-0010). A non-gene-specific ‘scrambled’ siRNA was used as a negative control. HCASMCs were transfected using the transfection reagent Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, U.S.A.; Cat. No: 11668027) according to the manufacturer’s protocol. Knockdown of the target mRNA was monitored 24 h after transfection of siRNAs by RT-qPCR and immunoblotting. dHCASMCs were transfected with 25 nM PML-specific siRNAs or scrambled siRNA, respectively, for 4 h to be then incubated with IFN-γ in a final concentration of 100 ng/ml for additional 24 h.

Cell stimulation experiments

For stimulation experiments, dHCASMCs were treated with final concentrations of IFN-γ 100 ng/ml (Peprotech, Hamburg, Germany; Cat. No: 300-02), TGF-β 5 ng/ml (Peprotech, Hamburg, Germany; Cat. No: 100-21) or TNF-α 100 ng/ml (Peprotech, Hamburg, Germany; Cat. No: 300-01A). Cytokine stock solutions were dissolved in phosphate buffered saline (PBS, pH 7.4) containing 1% bovine serum albumin (BSA) (AppliChem, Darmstadt, Germany; Cat. No: 1391). PBS, pH 7.4 containing 1% BSA served as a negative control.

Immunohistochemistry with chromogenic substrates

Hematoxylin–Eosin (HE) and Elastic Van Gieson (EvG) staining were performed on cross-sections (4 μm-thick) of formalin-fixed and paraffin-embedded vessel tissue. For immunohistochemistry, human coronary artery sections were incubated with the chromogenic substrates 3,3′-diaminobenzidine (DAB), Fast Red and Vector Blue. For the human aortic sections, the EnVision™ G2 Doublestain System (Agilent Technologies, Santa Clara, CA, U.S.A.; Cat. No: 5361) was used for the simultaneous detection of two antigens according to the manufacturer’s instructions. Antibodies used: anti-PML (clone E-11, dilution 1:100, Santa Cruz Biotechnology, Dallas, TX, U.S.A.; Cat. No: sc-377390), anti-CD31 (clone JC70A, ready to use, Agilent Technologies, Santa Clara, CA, U.S.A.; Cat. No: M0823), anti-smooth muscle actin (clone 1A4, ready to use, Agilent Technologies, Santa Clara, CA, U.S.A.; Cat. No: IR61161-2) and anti-CD68 (clone PG-M1, ready to use, Agilent Technologies, Santa Clara, CA, U.S.A.; Cat. No: IR61361-2). Isotype-matched, non-binding primary IgG antibodies of the same species in the same concentration as the specific primary antibodies served as negative controls.

The number of PML-positive cells per area of atherosclerotic and non-atherosclerotic arteries was counted using the HALO image analysis software (Indica Labs, Albuquerque, NM, U.S.A.).
**Immunocytochemistry**

Cellular localization of PML, SUMO-1 and α-smooth muscle actin (α-SMA) was assessed on cross-sections of human coronary arteries or fixed dHCASMCs by immunocytochemistry evaluated by confocal laser scanning microscopy, as previously described [23]. In brief, after blockage with 10% milk powder (AppliChem, Darmstadt, Germany; Cat. No: A0830) in PBS, sections were incubated with a primary antibody 1:100 (anti-PML H-238 and anti-PML E-11) showing identical expression patterns as determined in preliminary experiments; anti-SUMO-1 D-11; Santa Cruz, Dallas, TX, U.S.A.; Cat. No: sc-5308; anti-CD31 clone D8V9E, Cell Signaling Technology, Danvers, MA, U.S.A.; Cat. No: 77699S; anti-CD68 clone PG-M1, Agilent Technologies, Santa Clara, CA, U.S.A.; Cat. No: IR61361-2) in blocking solution overnight at 4°C, afterwards washed with PBS and then a secondary antibody (Alexa Fluor, Thermo Fisher Scientific, Waltham, MA, U.S.A.) was added for 30 min at room temperature. Isotype-matched, non-binding primary IgG antibodies of the same species in the same concentration as the specific primary antibodies served as negative controls. Nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; 1:10000, Merck, Darmstadt, Germany; Cat. No: 124653).

Cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and were permeabilized by PBS containing 0.5% Triton X-100. After blocking in 10% FCS in PBS, cells were incubated overnight at 4°C with an anti-PML (E-11, Santa Cruz, Dallas, TX, U.S.A.; Cat. No: sc-377930), an anti-SUMO-1 (D-11, Santa Cruz, Dallas, TX, U.S.A.; Cat. No: sc-5308), or an α-SMA (Santa Cruz, Dallas, TX, U.S.A.; Cat. No: sc-130617) antibody in blocking buffer. Alexa Fluor-conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA, U.S.A.) were diluted in blocking buffer containing 5 μmol/l Draq5 (Thermo Fisher Scientific, Waltham, MA, U.S.A.; Cat. No: 65-0880-92). Cells were incubated with the corresponding secondary antibody mix for 1 h at room temperature, washed three times with PBS, and covered with fluorescence mounting medium (Agilent Technologies, Santa Clara, CA, U.S.A.; Cat. No: S3023).

Sections and cells were assessed with a confocal laser microscope (Leica DMI 6000, Wetzlar, Germany) equipped with 20× and 63× oil immersion lens, a single photon argon laser, a solid-state laser and a helium–neon laser. At higher magnification, the microscopic setting was adjusted to the fluorescence signal. Digital images were processed using the Leica LAS AF Lite software.

**Immunoblotting**

Cells or tissues were homogenized in RIPA buffer (Santa Cruz, Dallas, TX, U.S.A.; Cat. No: sc45001) containing protease inhibitors (Sigma–Aldrich, St. Louis, MO, U.S.A.; Cat. No: S8820) and 20 mM N-ethylmaleimide (Sigma–Aldrich, St. Louis, MO, U.S.A.; Cat. No: E3876) for 15 min at 4°C and lysates were collected after centrifugation. The protein concentration was determined using BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.; Cat. No: 23225). Total protein extracts (20 μg) from artery lysates of at least six of each classified artery-type or cell lysates of dHCASMCs were resolved by SDS/PAGE gel electrophoresis and transferred to nitrocellulose membranes (GE Healthcare, Chicago, IL, U.S.A.; Cat. No: 10600002) for immunoblotting. Target protein was detected using specific primary antibodies. Primary antibodies were diluted as follows: anti-PML (1:1000, Novus Biologicals, Littleton, CO, U.S.A.; Cat. No: NB100-59787), anti-SUMO-1 (1:500, Elabscience, Wuhan, Hubei, China; Cat. No: E-AB-32998), anti-α-SMA (1:500, Santa Cruz, Dallas, TX, U.S.A.; Cat. No: sc-130617), anti-calponin 1 (1:1000, Cell Signaling Technology, Danvers, MA, U.S.A.; Cat. No: 17819) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:10000, Proteintech, Rosemont, IL, U.S.A.; Cat. No: 60004). Bound antibodies were detected by a peroxidase-conjugated secondary antibody (Santa Cruz, Dallas, TX, U.S.A.), and the signals were visualized using a chemiluminescence kit (Bio–Rad, Hercules, CA, U.S.A.; Cat. No: 1705061). Densitometric analysis was performed using ImageJ.

**ELISA analysis**

PML protein concentrations were quantified in tissue lysates using a commercial human PML-ELISA kit (Cloud Corporation, Houston, TX, U.S.A.; Cat. No: SEC221Hu) following the manufacturer’s guidelines. TNF-α, IFN-γ and TGF-β levels in cell culture supernatants were measured using commercial human ELISA kits (Thermo Fisher Scientific, Waltham, MA, U.S.A.; Cat. No: 88-7316-88/88-7346-88/88-8350-22) according to the manufacturer’s guidelines.

**RNA isolation, reverse transcription and real time PCR**

Isolation of total RNA, reverse transcription and realtime-PCR (RT-qPCR) using the primers listed in Table 2 were performed, as previously reported [24]. RNA was extracted from dHCASMCs using the GeneMATRIX Universal RNA Purification kit from EURx (Gdansk, Poland) according to the manufacturer’s instructions. mRNA of 1 μg total
RNA was transcribed into complimentary DNA (cDNA) using a reverse transcriptase kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.; Cat. No: K1612).

Semi-quantitative realtime PCR analysis was carried out using the QuantStudio 5 system (Applied Biosystems, Foster City, CA, U.S.A.). Amounts of specific cDNAs were analyzed using the GoTaq qPCR Master Mix (Promega Corporation, Madison, WI, U.S.A.; Cat. No: A6001). Primer sequences, product sizes and annealing temperatures are listed in Table 2. In each experiment, melting curve analysis was performed to verify that a single transcript was produced. RT-qPCR relative mRNA levels were calculated using the comparative $C_T$ ($2^{-\Delta\Delta C_T}$) method, with GAPDH as a reference. Non-RT and non-template controls were run for all reactions.

### Scratch wound assay

Equal numbers of HCASMCs were grown to 70–80% confluence in small Petri dishes (Ø 4 cm) and then transferred to smooth muscle cell differentiation medium for 5 days. The monolayer was scratched with a pipette tip along a ruler 48 h after transfection. After 24 and 48 h of incubation at 37°C in a humidified atmosphere (5% CO₂), images of the scratch were taken with a digital camera (Kappa, DX4-285fW, Turin, Italy). The size of the cell-free (open) area was calculated using analysis algorithm-based T-scratch software [25].

### Proliferation assay

Two proliferation assays were performed. For both proliferation assays, HCASMCs were seeded at the same density on 96-well plates. The MTS dye (CellTiter96 proliferation assay, Promega Corporation, Madison, WI, U.S.A.; Cat. No: G3582) was added to the culture medium of dHCASMCs (48 h after transfection of full-length PML-IV or the corresponding control vector) and incubated for 2 h at 37°C. The extinction was measured in triplicates by microplate reading (Tecan Sunrise, Männedorf, Switzerland) at 490 nm. Alternatively, the proliferation rate was measured using a BrdU ELISA kit (Abcam, Cambridge, U.K.; Cat. No: ab126556) according to the manufacturer’s instructions.

### Statistical analysis

Data were analyzed with SigmaPlot 13.0 software and presented as mean ± SD ($n\geq 3$). All datasets were tested by Shapiro–Wilk for their normality of distribution prior to statistical analysis and the Brown–Forsythe test was used to test the equality of variance. Comparisons between two groups were performed by Student’s $t$ test (two-tailed unpaired), between more than two groups by one-way ANOVA followed by Dunnett’s test or by two-way ANOVA followed by Bonferroni’s post hoc test. In case of non-normally distributed datasets or data without equal variance the Welch’s $t$ test or the Mann–Whitney $U$-test were performed. The differences were considered significant if $P<0.05$ in all statistical tests.
Results

PML protein levels in atherosclerotic human coronary arteries

Using a specific ELISA, PML was quantitatively assessed in coronary arteries isolated from sixteen human hearts—seven vessels with plaques from patients with atherosclerosis and nine vessels without plaques from patients showing no signs of atherosclerosis when undergoing heart transplantation (Table 1); 2.8-fold more \( (P < 0.001) \) PML was found in lysates of the atherosclerotic arteries than in those of the non-atherosclerotic arteries (Figure 1A). Six of the atherosclerotic arteries were divided in two segments of which both were subjected to the PML-specific ELISA: 3.1-fold more \( (P < 0.01) \) PML was measured in the plaque-containing segments than the non-plaque segments of these arteries (Figure 1B). Lysates of the coronary arteries that had been subjected to the ELISA were subsequently assessed by immunoblotting followed by densitometric analysis showing a 2.2-fold higher \( (P < 0.001) \) level of PML protein in the six atherosclerotic coronary arteries than the six non-atherosclerotic ones (Figure 1C). Furthermore, 3.3-fold more \( (P < 0.001) \) PML protein was demonstrated in plaque segments than non-plaque segments of six coronary arteries isolated from the hearts of patients with atherosclerosis (Figure 1D).

Immunohistochemical demonstration of cellular and subcellular PML expression in human arteries

To determine and compare the cellular PML localization in coronary arteries isolated from the transplanted hearts of the two patient groups, immunohistochemistry was conducted (Figure 2; Supplementary Figure S1). In non-atherosclerotic arteries, moderate PML-immunoreactivity was confined to most cells present in the intima and the adventitia as well as weak PML-immunoreactivity in many cells of the media (Supplementary Figure S1A2,A3,A5,A6). In atherosclerotic arteries, PML-immunoreactivity in the non-plaque regions corresponded to that observed in the non-atherosclerotic arteries (Supplementary Figure S1B2,B3,B5,B6). Most striking, however, was the PML-immunoreactivity in the thickened intima-media area of the plaque, with most of the cells there being strongly immunoreactive and showing the highest signal-density at the vulnerable shoulder region of the plaque cap (Supplementary Figure S1B5).

Quantification in the cross-sections revealed a 1.7-fold higher \( (P < 0.05) \) number of PML-immunoreactive cells in the intima-media area of atherosclerotic arteries than in non-atherosclerotic arteries (Supplementary Figure S1D).

Double immunohistochemistry with antibodies against PML and markers specific for the three cell types most frequently present in plaques (ECs, smooth muscle cells (SMCs) and macrophages) was performed to determine the cell type-specific distribution of PML in atherosclerotic coronary arteries in more detail (Figure 2). In the non-plaque area, ECs of the intima as well as many SMCs of the media were clearly immunoreactive (Figure 2C,E,G). In the plaque area, \( \alpha \)-SMA-positive cells, which were the majority of the cells found in the plaque area (Supplementary Figure S1B2) and macrophages were strongly PML-positive (Figure 2B,D,F). If the signal density of PML-immunoreactivity was compared between the atherosclerotic and the non-atherosclerotic regions, \( \alpha \)-SMA-immunoreactive cells showed the greatest differences in PML-signal intensities: \( \alpha \)-SMA-immunoreactive cells of the intact media contained less PML-immunoreactivity than the \( \alpha \)-SMA-immunoreactive cells present in the thickened intima-media of the plaque area (Figure 2).

Higher microscopic magnification revealed most of the PML-immunoreactivity in the three cell types to be consistently localized in intranuclear aggregates likely representing PML-NBs (Figure 2H-K). Strikingly, in the \( \alpha \)-SMA-immunoreactive cells in the thickened intima-media of the plaque area, more and larger PML-NBs were visible than in the \( \alpha \)-SMA-immunoreactive cells of the media (Figure 2J,K).

Taken together, the immunohistochemical analyses revealed that 1. PML is expressed in the three major cell types present in atherosclerotic coronary arteries and 2. significantly higher PML levels were detected in \( \alpha \)-SMA-positive cells of the plaque than in \( \alpha \)-SMA-positive cells of the media.

To examine the cell type-specific distribution pattern of PML in another type of atherosclerotic vessel besides the coronary artery, a section of a human aorta was subjected to immunohistochemistry. All three layers of the blood vessel contained cells with strong PML-immunoreactivity in the nuclei, such as ECs (data not shown), SMCs of the media (Supplementary Figure S2C) and macrophages (Supplementary Figure S2D). Thus, the cellular expression pattern of PML in the atherosclerotic aorta corresponds to that in atherosclerotic coronary arteries.

PML promotes the phenotypic switch of coronary artery SMCs

To investigate whether PML plays a role in the phenotypic switch in which SMCs of the media transform from a contractile (or differentiated) phenotype to a synthetic (or dedifferentiated) phenotype, a cell culture model with primary VSMCs isolated from human coronary arteries (HCASMCs) was implemented. Therefore, the primary HCASMCs
Figure 1. Higher levels of PML in atherosclerotic than non-atherosclerotic vascular segments of human coronary arteries

Total lysates of human non-atherosclerotic and atherosclerotic coronary arteries (A,C) as well as total lysates of non-plaque and plaque segments from human atherosclerotic coronary arteries (B,D) were analyzed for PML by quantitative ELISA (A,B) and immunoblotting (C,D). (A) Quantification of the PML concentration in total lysates of non-atherosclerotic (n=9) and atherosclerotic (n=7) coronary arteries by ELISA. ***P<0.001 using Student’s t test. (B) Quantification of the PML concentration in total lysates of non-plaque (n=6) and plaque (n=6) segments from atherosclerotic coronary arteries by ELISA. **P<0.01 using Student’s t test. (C) Representative immunoblots (left panel) of three non-atherosclerotic and three atherosclerotic arteries together with the quantitative data of the densitometric analysis (right panel) including all non-atherosclerotic (n=6) and atherosclerotic arteries (n=6) analyzed. ***P<0.001 using Student’s t test. (D) Representative immunoblots (left panel) of two non-plaque and two plaque segments of two atherosclerotic coronary arteries together with the quantitative data of the densitometric analysis (right panel) including all plaque (n=6) and non-plaque (n=6) segments analyzed. ***P<0.001 using Student’s t test. All graphs reported as mean ± SD.
Figure 2. Nuclear localization of PML in different cell types present in atherosclerotic human coronary arteries

Cross-sections of formalin-fixed and paraffin-embedded human atherosclerotic coronary arteries (n=5) were subjected to immunohistochemistry using chromogens as substrate (A–G) or fluorescent dyes (H–K) combined with histological staining. (A–G) Nuclei were stained with Hematoxylin (blue). Immunoreactivity for cell-specific markers was displayed with Fast Red (α-SMA specific for smooth muscle cells; CD68 = macroasialin = scavenger receptor D specific for cells of the monocyte lineage), Vector Blue (CD31 = PECAM-1 specific for ECs) or DAB (PML). (A) Overview of a representative atherosclerotic plaque in a human coronary artery with boxes marking the vessel areas, which are displayed at higher magnification in B–G. (B) Co-immunoreactivity for CD68 (red) and PML (brown) demonstrated that macrophages appeared preferentially in the shoulder area of the plaque containing PML in most cases. (C) Co-immunoreactivity for CD31 (blue) and PML (brown) demonstrated PML expression in ECs. (D,F) Co-immunoreactivity for α-SMA (red) and PML (brown; D) or immunoreactivity for PML alone (brown; F) demonstrated strong PML expression in most α-SMA-positive cells within the atherosclerotic plaque of the coronary artery. (E,G) Co-immunoreactivity for α-SMA (red) and PML (brown; E) or immunoreactivity for PML alone (brown; G) demonstrated weak PML expression in nuclei of SMCs in the media of the coronary artery. Sites of co-localized immunoreactivity in cell nuclei are marked by black arrows. Dashed lines in F, G indicate the presumptive surface of the PML-labeled cells. H–K Nuclei were stained with Draq5 (blue). (H) Co-immunoreactivity for CD31 (cyan) and PML (red) demonstrated PML expression in ECs. (I) Co-immunoreactivity for CD68 (yellow) and PML (red) demonstrated PML expression in most macrophages. (J,K) Co-immunoreactivity for α-SMA (green) and PML (red) demonstrated strong PML expression in most α-SMA-positive cells within the atherosclerotic plaque (J) of the coronary artery and only weak PML expression in nuclei of SMCs in the media (K) of the coronary artery. Abbreviations: Lu, lumen; Me, media; Plq, plaque; SMC, smooth muscle cell.
(pHCASMCs) were cultivated for 5 days in differentiation medium to obtain populations of differentiated HCASMCs (dHCASMCs), as shown in Supplementary Figure S3A. Compared with the pHCASMCs, dHCASMCs actually expressed higher concentrations of marker genes specific for cell differentiation such as smooth muscle 22 α (SM22α; ∼480%, P < 0.01), α-SMA (∼650%, P < 0.01) and smooth muscle myosin heavy chain (SM-MyHC; ∼340%, P < 0.01) at the mRNA level as well as α-SMA (∼1050%, P < 0.001) and calponin (∼1300%, P < 0.01) at the protein level (Supplementary Figure S3B,C).

Next, dHCASMCs were transfected with the expression plasmid pEGFP-C1 containing either the complete PML-IV gene or lacking a specific gene insert as control (Figure 3, Supplementary Figure S4). The PML-overexpressing dHCASMCs did not show the SMC-characteristic spindle-like, elongated shape and were not arranged in parallel as the control-transfected dHCASMCs. They furthermore exhibited less pronounced α-SMA immunoreactivity compared with the control cells (Figure 3B). Accordingly, lower levels of α-SMA (∼50%, P < 0.01; Figure 3D) and the contractile protein calponin 1 (∼60%, P < 0.05; Figure 3E) were found in the cells overexpressing PML versus control-transfected dHCASMCs. RT-qPCR showed that mRNA levels of markers for SMC differentiation (α-SMA, SM22α, CNN1, SM-MyHC) were lower (∼10, ∼15 ∼20, ∼30%; P < 0.05) and the connexin 43 (Cx43) mRNA levels were higher (∼420%, P < 0.01) in the PML-overexpressing dHCASMCs than the control-transfected cells (Figure 3C). Furthermore, in dHCASMCs transfected with the PML gene, proliferation was increased to ∼150% (Figure 3F, P < 0.01). In contrast, knockdown of PML with specific siRNAs resulted in lower proliferation of dHCASMCs (Figure 3F; ∼25%, P < 0.05). In addition, scratch closure (i.e. migration) was increased to ∼300% 48 h after scratching (Figure 3G, P < 0.01). In summary, these experiments revealed that characteristic markers for differentiation are lower and markers for proliferation and migration are higher in dHCASMCs transfected with the PML-vector than in those transfected with the empty vector.

PML increases the SUMO-1-dependent SUMOylation of proteins in cultured coronary artery SMCs

In the next step, dHCASMCs overexpressing the PML-IV gene were subjected to co-immunocytochemistry with PML and SUMO-1 antibodies. A co-localization of PML and SUMO-1 was observed within many PML-NBs of these cells (Figure 4A). Interestingly, overexpression of PML by transfection with the PML-IV gene did not change the SUMO-1 mRNA levels (∼11% increase, P ≥ 0.05, Figure 4B). On the other hand, immunoblotting with anti-SUMO-1 antibodies revealed more SUMO-1-dependent protein SUMOylation in PML-IV-transfected dHCASMCs and less SUMO-1-dependent protein SUMOylation in PML-siRNA transfected dHCASMCs compared with control-transfected dHCASMCs (Figure 4C). Together, these findings show that SUMO-1-dependent protein SUMOylation in dHCASMCs is related to PML expression.

To investigate whether dedifferentiation, proliferation and migration of dHCASMCs depend on the SUMOylation state and SUMOylation activity of PML, dHCASMCs were transfected with either a vector without a specific gene insert or a mutated PML-IV gene that is translated into a PML-IV protein with three altered lysines within the SUMOylation consensus motif (K65, K160, K490) and thus lacking SUMOylation activity. The qualitative and quantitative SUMO-1-dependent patterns of SUMOylated proteins were similar in the dHCASMC populations subjected to transfection with the vector containing the gene for the mutated SUMOylation-defective PML-IV form or with the vector lacking a specific gene insert (Supplementary Figure S5A). Furthermore, neither the expression levels of the differentiation marker α-SMA protein (Figure 4D) nor the proliferation nor the migration activity of the cells (Figure 4E, Supplementary Figure S5B) differed (P ≥ 0.05) between the two dHCASMCs transfections. These results imply that the PML protein, increased by overexpression, must undergo SUMOylation to induce dedifferentiation, proliferation and migration of dHCASMCs.

SUMO-1 is highly expressed and co-localizes with PML in atherosclerotic human arteries

As for PML, SUMO-1 levels in atherosclerotic arteries exceeded SUMO-1 levels in non-atherosclerotic arteries by ∼5-fold (P < 0.001) in immunoblots (Figure 5A,B).

In non-atherosclerotic arteries, SUMO-1 immunoreactivity was mainly located at the luminal side of the internal elastic lamina (IEL), i.e. in the intima (Figure 5C, upper row). In the case of an intima-media thickening, a high signal-density of SUMO-1 immunoreactivity was found in the plaque region of atherosclerotic arteries, especially at the plaque cap (Figure 5C, lower row).
Figure 3. PML induced a phenotypic switch of dHCASMCs

HCASMCs were cultured in SMC differentiation medium for 5 days and then transfected with a plasmid encoding the PML-IV gene (PML↑) or a corresponding plasmid without PML insert (ctrl). (A) Representative images from five independent experiments showing dHCASMCs. Upper row: cells incubated with anti-PML antibody (red) and counterstained with DRAQ5 (blue) for demonstration of nuclei showing nuclear agglomerates of PML, i.e., PML-NBs; lower row: cells subjected to double immunocytochemistry with anti-PML antibody (red) and anti-α-SMA antibody (green) showing also staining of PML in the cytoplasm. (B) PML or control-transfected dHCASMCs were subjected to co-immunocytochemistry with anti-PML and anti-α-SMA antibodies. More immunoreactivity for PML and less immunoreactivity for α-SMA accompanied by a loss of α-SMA-defined structural arrangement were present in PML-transfected dHCASMCs compared to control-transfected dHCASMCs; n=4. (C) RT-qPCR for quantification of the mRNA levels of SMC differentiation markers in PML or control-transfected dHCASMCs. Relative expression values. n=4, *P<0.05, **P<0.01 using Welch's t test and Mann–Whitney U test. (D) Immunoblotting for determination of α-SMA (D) and calponin (E) protein levels in total lysates of PML or control-transfected dHCASMCs (upper panel: representative immunoblots; lower panel: densitometric data of all immunoblots analyzed). (D) n=5, **P<0.01 using Student’s t test. (E) n=4, *P<0.05 using Student’s t test. (F) Proliferation activity of PML, PML↑, PML siRNA, or ctrl-transfected dHCASMCs was assessed by a colorimetric MTS proliferation assay. (G) Migration activity of PML or control-transfected dHCASMCs was assessed in a scratch assay. Left panel: Representative microscopic images of scratch areas from four independent experiments with ctrl-transfected and PML-transfected cells (PML↑) dHCASMCs immunoreactive for α-SMA. Right panel: Quantification of the open wound area analyzed 24 and 48 h after scratching a confluent layer of PML-transfected cells versus ctrl-transfected dHCASMCs. n=3, **P<0.01 using Student’s t test. All graphs reported as mean ± SD.
Figure 4. Co-localization and interaction of PML with SUMO-1 in cultured dHCASMCs

HCASMCs were cultured in SMC differentiation medium for 5 days and then transfected with either a plasmid containing an intact PML-IV insert (PML↑), a plasmid containing a PML-IV mutant gene insert leading to a PML protein form with three substituted amino acids within the SUMOylation active site (PMLmut) or a plasmid without PML gene insert (ctrl). (A) PML-transfected dHCASMCs subjected to co-immunocytochemistry with anti-PML and anti-SUMO-1 antibodies demonstrating the co-localization (yellow) of PML and SUMO-1 in cell nuclei; n=4. (B) RT-qPCR for the quantification of the mRNA levels of SUMO-1 in transfected dHCASMCs. Relative expression values; n=4; statistical significance was tested using Student’s t test. (C) Immunoblotting for the determination of SUMO-1 and PML protein levels in total lysates of PML-IV and PML siRNA transfected dHCASMCs; n=3. (D) Immunoblotting for the determination of α-SMA protein levels in total lysates of transfected dHCASMCs (left panel: representative immunoblots; right panel: densitometric data of all immunoblots); n=4, **P<0.01 using one-way ANOVA. (E) Proliferation activity of transfected dHCASMCs was quantified by a BrdU assay. n=3, *P<0.05 using one-way ANOVA. All graphs reported mean ± SD.
In addition, immunofluorescence double labeling demonstrated that PML and SUMO-1 are co-localized in a variety of cell types that are present in atherosclerotic and non-atherosclerotic arteries as well as in adventitial capillaries (Figure 5D).

**IFN-γ increases expression of PML and SUMO-1 in dHCASMCs**

Since PML expression is induced by different cytokines in a variety of cells, the effect of three cytokines on dHCASMCs phenotype was evaluated. For this purpose, dHCASMCs were exposed to TNF-α, TGF-β or IFN-γ for 24 h to be subsequently analyzed by immunocytochemistry, RT-PCR and immunoblotting. Immunocytochemistry revealed higher signal-density for nuclear PML-NBs and more co-localization of SUMO-1 after incubation especially with IFN-γ compared with untreated control cells (Figure 6A). Counting of the PML-NB numbers in the dHCASMCs exposed to the various cytokines showed higher numbers (P<0.05) of PML-NBs in the cells incubated with TNF-α (~25%) and IFN-γ (~40%) while stimulation with TGF-β (~10%, P≥0.05) did not cause any significant changes (Figure 6B). Immunoblotting (~280%, Figure 6D) and RT-PCR analyses (~320%, Figure 6C) likewise revealed a significant increase in PML after IFN-γ incubation, which was accompanied by suppression of α-SMA transcription (~20%, P<0.05, Figure 6E,F), while TGF-β stimulation resulted in higher (220%; P<0.05) α-SMA mRNA levels (Figure 6E). The down-regulation of α-SMA mRNA levels observed after IFN-γ incubation was abolished if PML mRNA expression was knocked-down with specific siRNAs (~40%, P<0.01, Figure 6F). A similar effect could also be observed for the mRNA level of SM-MyHC (~45%, P<0.01, Figure 6G). Remarkably, PML expression in dHCASMCs did not have significant effects (P≥0.05) on mRNA and protein secretion levels of TNF-α, IFN-γ or TGF-β (Supplementary Figure S6).
Figure 6. The IFN-γ-mediated de-differentiation of cultured dHCASMCs was dependent on PML

HCASMCs were cultured in SMC differentiation medium for 5 days and then incubated with TGF-β (5 ng/ml), TNF-α (100 ng/ml), IFN-γ (100 ng/ml) or under control conditions (vehicle alone, ctrl) for 24 h prior harvesting. (A) Immunocytochemistry of dHCASMCs with anti-PML and anti-SUMO-1 antibodies. Incubation of dHCASMCs with IFN-γ resulted in higher numbers of PML-NBs as well as higher rates of co-localization of PML and SUMO-1 than in dHCASMCs incubated with TNF-α, TGF-β or without cytokine supplementation. Representative images of n=3 independent experiments. (B) Determination of the numbers of PML-NBs per nucleus in cytokine-stimulated dHCASMCs. n=3 independent experiments each with 60 cell nuclei evaluated, ***P<0.001 using one-way ANOVA. (C) RT-qPCR to quantify mRNA levels of PML in dHCASMCs that were stimulated by cytokines as indicated. Relative expression values. n=4, *P<0.05 using one-way ANOVA. (D) Immunoblotting for determination of the PML protein levels in total lysates of dHCASMCs incubated with cytokines as indicated (upper panel; representative immunoblots; lower panel: densitometric data of all immunoblots). n=3, *P<0.05 using one-way ANOVA. (E) RT-qPCR analysis of α-SMA dHCASMCs following cytokine stimulation. Relative expression values. n=4, *P<0.05 using one-way ANOVA. (F,G) RT-qPCR to quantify mRNA levels of α-SMA (F) and SM-MyHC (G) in dHCASMCs that were stimulated with IFN-γ after transfection with scrambled siRNA (IFN-γ), scrambled siRNA (without cytokine = control), or siRNA against PML (siPML), or stimulated with IFN-γ and transfected with siRNA against PML (siPML+IFN-γ). Relative expression values. n=3, **P<0.01, ***P<0.001 by Bonferroni’s post hoc test after two-way ANOVA. All graphs reported mean ± SD.
Discussion

The aim of the present study was to investigate whether the PML protein is related to atherosclerosis by affecting plaque formation. Major observations were: (1) significantly higher levels of PML in atherosclerotic plaques than in non-atherosclerotic segments of human coronary arteries and (2) an enrichment of PML in nuclei of SMCs particularly at the vulnerable shoulder region of atherosclerotic plaques in coronary arteries. (3) A phenotype change (i.e. dedifferentiation) of dHCASMCs through higher levels of PML, which was (4) SUMOylation-dependent. (5) The phenotypic shift of dHCASMCs was adjustable by IFN-γ in a PML-dependent fashion.

Immunoblotting, ELISA and immunohistochemistry consistently demonstrated higher levels of PML in atherosclerotic plaques of human coronary arteries than in non-atherosclerotic segments of the same vessels or non-atherosclerotic human coronary arteries. These results are in accordance with those previously reported which found significantly higher levels of PML in inflamed than non-diseased tissues of humans [10].

Alternative mRNA splicing leads to the generation of seven PML isoforms (PML I–VII) with slightly different molecular weights, functions and intracellular localization patterns [26]. In addition to the complexity of PML expression, PML is post-translationally modified (e.g. SUMOylation [27]). In our immunoblots with anti-PML antibodies, several protein bands at ~100 and 70 kDa were immunoreactive. The sizes of these bands correspond to the molecular weights of the PML-isoforms I/II/III/IV/V [26]. Since the band pattern appeared similar in all immunoblots, it is unlikely that PML expression in coronary arteries is shifted during atherosclerosis in favor of a specific PML variant.

Because PML is expressed in many tissues and cells of humans [10,12], it was not surprising that in the non-atherosclerotic and atherosclerotic coronary arteries examined, PML-immunoreactivity was present in all three layers of the vessel wall. In all cell types identified (ECs, SMCs and macrophages) PML was consistently aggregated mainly in subnuclear domains known as PML-NBs [8]. When compared with the signal in the normal media from both non-atherosclerotic and atherosclerotic arteries, the PML-immunoreactivity appeared strongest in the thickened intima-media region, particularly at the vulnerable shoulder region in atherosclerotic arteries. Additional immunocytochemistry with α-SMA antibodies revealed the normal intima to be replaced by plaques with invading VSMCs. At this point, it should at least be pointed out that various cells in atherosclerotic plaques, including macrophages and adventitial cells, express SMC markers, while some VSMCs lose this ability when they appear in plaques [28,29]. Therefore, in the present study, we should correctly speak of smooth muscle-like cells (SMLCs) within the thickened intima-media region.

An increase in PML levels was especially observed in SMLCs of the thickened intima-media. These microscopic observations suggest that the higher PML concentrations, measured in plaque-containing specimens of atherosclerotic arteries using immunoblot and ELISA techniques, are primarily due to increased numbers of invaded VSMCs along with higher PML levels in these cells.

Like in human coronary arteries, the cells in the atherosclerotic plaque of a human aorta also showed more PML-immunoreactivity than the cells in the non-plaque areas of the vessel wall. Therefore, higher levels of PML appear to be a common plaque-associated feature of atherosclerotic human arteries.

VSMCs can change their function and undergo phenotypic changes, thereby adapt to or even promote vascular remodeling or vessel injury; including changes present in atherosclerotic blood vessels [30,31]. During the formation of atherosclerotic lesions, VSMCs switch from a ‘contractile’ or ‘differentiated’ state to a ‘synthetic’ or ‘dedifferentiated’ phenotype which goes along with reduced expression of SMC differentiation markers, increased capacity for cell proliferation, migration and secretion of various extracellular matrix proteins and cytokines [30,31]. However, the molecular mechanisms underlying this phenotypic switch are not fully understood.

Due to the observation of stronger PML-immunoreactivity in α-SMA-positive cells of the plaque area than in those of the non-diseased media, we hypothesized that PML is involved in this phenotypic transformation of VSMCs. To investigate this issue, pHCASMCs were cultivated to obtain dHCASMCs. These cells were then transfected with a PML isoform IV-specific vector, which is the most intensively characterized PML-isoform [8] and involved in the regulation of many cellular processes such as apoptosis, senescence, DNA damage response and viral protein sequestration [32,33].

The PML-IV transfected dHCASMCs contained lower mRNA levels of α-SMA, CNN1, SM22α and SM-MyHC which are marker genes of the contractile smooth muscle phenotype of VSMCs and a higher expression of Cx43, which is a characteristic of the synthetic smooth muscle phenotype [34]. Increased proliferation and migration activity of dHCASMCs overexpressing PML supported the concept that PML plays a mechanistic role in atherosclerosis-associated phenotypic switching of VSMCs.

Although PML transfection of dHCASMCs increased and knockdown of PML by siRNA decreased the SUMO-1-dependent SUMOylation pattern in dHCASMCs, SUMO-1 mRNA expression levels were not influenced.
We hypothesize that the transfection-induced up-regulation of PML expression resulted in more PML-NB aggregates into which soluble SUMO-1 was recruited. As a result, higher SUMOylation rates were achieved in dHCASMCs even without induction of SUMO-1 mRNA expression. In addition, since PML itself exhibits SUMO E3 ligase activity [35] which causes covalent SUMO-binding to protein substrates, the higher availability of PML after transfection should lead to more SUMOylation activity even without changes in SUMO-1 mRNA expression levels. Accordingly, we interpret the results of the experiments with PML siRNA, in which the PML availability revealed to be down-regulated, as a reduction in SUMOylation platform activity.

If dHCASMCs were transfected with a gene coding for a SUMOylation defective PML-IV mutant that lost its ability to recruit specific interacting proteins despite the formation of morphological normal PML-NBs [12,36], neither α-SMA protein levels nor cellular proliferation and migration were changed. These results indicate that SUMO-1-dependent SUMOylation of PML is a prerequisite for the PML-induced phenotypic switch of VSMCs. The SUMOylation site of PML could thus be a new target to prevent the progress of atherosclerosis.

The expression profile of marker genes for the contractile phenotype is regulated in VSMCs by a network of transcription factors and cofactors. Interestingly, some of these transcription factors such as serum response factor (SRF), Kruppel-like factor 4 (KLF4), myocardin and protein Elk-1 (ELK1) containing ETS domains are known to be modified by SUMO-1 [37–40]. In addition, some of these proteins have been shown to colocalize with PML-NBs, such as SRF whose activity is inactivated by its SUMOylation [37]. Another factor that is important for the regulation of differentiation is PIAS1, which plays a role in the control of SMC-selective gene expression, when it interacts with PML-NBs to act as SUMO E3 ligase within the SUMOylation cascade [41,42]. The disruption of the SUMO E3 ligase activity of PIAS1 abolishes its ability to activate the α-SMA promoter [43].

PML-NBs and SUMO-1 co-localized not only in cultivated VSMCs but also in human coronary arteries in situ. This observation underlines a possible role of PML-NBs in controlling post-translational SUMOylation of proteins during the formation of atherosclerotic plaques.

Since PML expression is induced by different cytokines [6,44] and cytokines influence the growth of SMCs [45], dHCASMCs were exposed to TNF-α, TGF-β and IFN-γ. Stimulation with IFN-γ increased the expression of PML as well as number and size of PML-NBs, which was accompanied with enhanced co-localization of SUMO-1 to PML-NBs.

The pro-inflammatory cytokine IFN-γ is secreted by activated T-cells and therefore present at high levels in atherosclerotic lesions. A higher IFN-γ availability could cause the up-regulation of PML in VSMCs and thus induce or at least promote their phenotype change. Interestingly, it has been previously shown that IFN-γ suppresses the expression of α-SMA, at least in arterial SMCs and myofibroblasts [46,47], with the signaling pathway being unidentified so far. As our study demonstrated, the mRNA transcription level of α-SMA in dHCASMCs was reduced after stimulation with IFN-γ. On the contrary, as already shown in the literature before, treatment of SMCs with TGF-β elevated their level of α-SMA [48,49]. To elucidate whether the known IFN-γ-mediated suppression of α-SMA was due to the effect of IFN-γ on PML, PML was knocked-down by the use of specific siRNAs prior to stimulating the cells with IFN-γ. This showed that the effect of IFN-γ on α-SMA was mediated by PML, at least in the model tested. A similar influence of PML was also demonstrated on the IFN-γ-mediated suppression of SM-MYHC. Thus, our molecular studies suggest that PML is critically involved in IFN-γ-stimulated pathways of VSMCs dedifferentiation, as it occurs during atherogenesis.

Taken together, the findings of the current study support the hypothesis that PML promotes atherogenesis by promotion of the phenotypic switch of VSMCs from the ‘contractile’ to the ‘synthetic’ state, which included dedifferentiation as well as cellular migration and proliferation. PML thereby served as a mediator of IFN-γ-induced upstream effects. Co-localization of PML-NBs with SUMO-1, as known from other tissues, supported the speculation that SUMOylation is required as downstream signal in this PML-dependent pathway. Remarkably, neither TNF-α nor IFN-γ nor TGF-β were influenced by PML in dHCASMCs suggesting that these cytokines are only acting as regulators but not as downstream mediators of PML expression.

In the present study, we combined the in vivo analysis of PML expression in atherosclerotic coronary artery samples of patients with the phenotypic characterization of dHCASMC batches that were transfected with genes coding differently modified PML variants. To validate these two sets of findings, it would be consequent to carry out studies on transgenic animals. In fact, a knockout-mouse strain has been developed that lacks total PML expression in all tissues and cells, which did not result in striking phenotypic changes of the animals [50]. However, since we have investigated the special issue of whether PML-IV is involved in the phenotypic switch of SMC-derived cells in atherosclerotic plaques, it would not be sufficient to use total PML knockout-mice for the validation of the key observations of the present study. Instead, it would be necessary to work experimentally with conditional PML knockout-mice, PML-sensitive atherosclerosis-prone mice (e.g. PML/ApoE double knockout-mice) or
PML-isform-specific knockout-mice, which, to the best of our knowledge, have not yet been generated. And even if such genetically engineered mice were available, they would not necessarily be useful tools to adequately mimic the hemodynamic conditions in human physiology (e.g. mice do not develop plaque ruptures or coronary lesions in their vasculature [51–53]). We therefore decided not to extend the study to the analysis of transgenic mice.

The present study is focused on the biochemical analysis of PML-transfected dHCASMCs, which are originally derived from VSMCs and represent an experimental model for the cell type most commonly found in atherosclerotic plaques. However, significant levels of other cell types, such as ECs or macrophages, also occur in these plaques. PML in macrophages selectively activates the NLRP3 inflammasome [54], while PML in ECs influences angiogenesis and cell migration [55,56]. Inflammation, angiogenesis and cell migration are cellular processes associated with atherosclerosis [3,4]. We therefore speculate that the pathogenesis of atherosclerosis is exacerbated if PML is also up-regulated in these cells being present in atherosclerotic plaques besides VSMCs.

Some biological and methodological constraints may limit the significance of our findings: (1) although the effects described here were statistically significant already with coronary artery specimens of 16 patients, a higher number of samples/subjects would further increase the validity of the quantitative data. (2) The coronary artery specimens analyzed in the present study were derived from patients suffering from complex advanced stages of atherosclerosis. Thus, the plaques may have grown over decades. It is therefore difficult to make a valid statement about the role of PML and PML-NBs in the etiology and course/progression of atherosclerosis. (3) Furthermore, PML-NBs are very complex and highly dynamic subnuclear structures and, thus, may differ in composition/function in plaque cells at different stages of atherosclerosis. Future studies should therefore focus on a more detailed analysis of such changes in the PML interactome during the time course of atherogenesis.

Our study clearly demonstrated that PML is a hitherto unknown actor in the development of atherosclerosis, which may be important for a better understanding of the progression of this disease and the development of additional treatment options.

Clinical perspectives

- Since a growing body of evidence suggests that PML is a key regulator of inflammatory responses, it has been investigated whether PML plays a role in atherogenesis.

- In human atherosclerotic coronary arteries, expression of PML was significantly higher, especially in α-SMA-immunoreactive cells of plaques where it was co-localized with SUMO-1. In cultured dHCASMCs PML increased global SUMOylation pattern as downstream event and triggered a phenotypic switch by promoting dedifferentiation as well as proliferation and migration of the cells.

- Identification of the PML-dependent SUMOylation pathway as a so far unrecognized modulator system for the phenotypic switch of VSMCs in atherosclerotic plaques that may facilitate the development of new tools for the biochemical characterization and histological diagnosis of atherosclerosis. In addition, our results may offer new therapeutic options to treat atherosclerosis using specific drugs, e.g. already existing SUMOylation inhibitors.

Data Availability

All data generated or analysed during the present study are included in this published article and its supplementary files.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Weronika Karle: Formal analysis, Validation, Investigation, Writing—original draft. Samuel Becker: Investigation. Philipp Stenzel: Resources, Investigation. Christoph Knosalla: Resources. Günter Siegel: Conceptualization, Data curation,
Writing—original draft. **Oliver Baum**: Conceptualization, Writing—original draft. **Andreas Zakrzewicz**: Conceptualization, Supervision, Validation. **Janine Berkhof**: Conceptualization, Data curation, Supervision, Investigation, Writing—original draft.

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**Abbreviations**

α-SMA, α-smooth muscle actin; BSA, bovine serum albumin; CNN1, gene encoding the protein calponin; Cx43, connexin 43; dHACSMC, differentiated human coronary artery smooth muscle cell; EC, endothelial cell; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HACSMC, human coronary artery smooth muscle cell; IFN-γ, interferon γ; PBS, phosphate buffered saline; PML, promyelocytic leukemia protein; PML-NB, promyelocytic leukemia protein nuclear body; siRNA, small interfering RNA; SM-MyHC, smooth muscle myosin heavy chain; SM22α, smooth muscle 22α; SMC, smooth muscle cell; SMLC, smooth muscle-like cell; SUMO, small ubiquitin-like modifier; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α; VSMC, vascular smooth muscle cell.

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