Macrophage-specific overexpression of group IIa $sPLA_2$ increases atherosclerosis and enhances collagen deposition

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Abstract **Atherosclerosis is a chronic inflammatory disease of the vessel wall characterized by the accumulation of lipid-laden macrophages and fibrotic material. The initiation of the disease is accompanied by the accumulation of modified lipoproteins in the vessel wall. Group IIa secretory phospholipase A_2 ($sPLA_2$ IIa) is a key candidate player in the enzymatic modification of low density lipoproteins. To study the role of $sPLA_2$ IIa in macrophages during atherogenesis, transgenic mice were generated using the human $sPLA_2$ IIa gene and the CD11b promoter. Bone marrow transplantation to LDL receptor-deficient mice was performed to study $sPLA_2$ IIa in atherosclerosis. After 10 weeks of high-fat diet, mice overexpressing $sPLA_2$ IIa in macrophages showed 2.3-fold larger lesions compared with control mice. Pathological examination revealed that $sPLA_2$ IIa-expressing mice had increased collagen in their lesions, independent of lesion size. However, smooth muscle cells or fibroblasts in the lesions were not affected. Other parameters studied, including T-cells and cell turnover, were not significantly affected by overexpression of $sPLA_2$ IIa in macrophages. These data clearly show that macrophage $sPLA_2$ IIa is a proatherogenic factor and suggest that the enzyme regulates collagen production in the plaque and thus fibrotic cap development.**—Ghesquiere, S. A. I., M. J. J. Gijbels, M. Anthonsen, P. J. J. van Gorp, I. van der Made, B. Johansen, M. H. Hofker, and M. P. J. de Winther. **Macrophage-specific overexpression of group IIa $sPLA_2$ increases atherosclerosis and enhances collagen deposition.** ***J. Lipid Res.*** **2005.** 46: **201–210.**

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Atherosclerosis is a progressive disease of the large arteries. The accumulation of LDLs in the intima of the vessel wall is a crucial factor in driving atherogenesis in both the early onset and subsequent progression to an advanced plaque (1). It is widely accepted that oxidized and enzymatically modified LDLs are proatherogenic (2). Modified LDLs attract monocytes, which subsequently differentiate into macrophages. The macrophages transform into foam cells as they take up the modified LDL particles. In addition, inflammatory mediators are released from the modified lipids, which contribute to an inflammatory response in the vessel wall (3, 4).

Several enzymes are known to modify LDL particles. Group IIa secretory phospholipase A_2 ($sPLA_2$ IIa) has been detected in atherosclerotic lesions in CD68-positive macrophages (5–7). $sPLA_2$ IIa can hydrolyze LDL-associated phospholipids, resulting in the formation of the so-called "bioactive" lipids, such as lysophospholipids and arachidonic acid. $sPLA_2$ IIa is a member of a large family of related enzymes that are able to catalyze the hydrolysis of phospholipids at the *sn*-2 ester bond, a process that results in the generation of free fatty acids and lysophospholipids (8). $sPLA_2$ IIa is widely expressed in the human body (9) and is considered to be important in the amplification of inflammation in many disease processes, including atherosclerosis (6, 7, 10, 11). Furthermore, $sPLA_2$ IIa-modified LDLs also show an enhanced affinity for proteoglycans (10, 12, 13) and glycosaminoglycan binding (14).

Recent experiments with phospholipase group V, which is a potent modifier of LDL, showed reduction in LDL particle size and subsequent aggregation (15). Smaller LDL particles have an increased preference for binding to proteoglycans (13). Although $sPLA_2$ IIa exhibits low enzymatic activity on intact LDL particles, binding to extracellular matrix components such as decorin, versican, and biglycan enhances the hydrolytic activity dramatically (11, 13, 16). The interaction between LDL and proteoglycans changes the LDL properties, enabling human $sPLA_2$ IIa, which is normally weakly active on LDL, to induce LDL aggregation and fusion, further increasing binding to the proteoglycan molecules (17, 18) and making it more proatherogenic.

Grass et al. (19) generated a transgenic mouse model to study the role of $sPLA_2$ IIa in inflammation. In this model, the human $sPLA_2$ IIa gene was used with its own promoter

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and regulatory elements, leading to a natural pattern of overexpression. Interestingly, these mice developed atherosclerotic lesions even on a normal chow diet (20). However, LDL levels in these mice were increased, combined with decreased HDL levels and a reduction in paraoxonase activity. All of the systemic changes observed in this overexpression model are known risk factors contributing to increased atherosclerosis, which makes this mouse model not very well suited to study the precise role of $sPLA_2$ IIa in the vessel wall on lesion formation.

More recently, Webb et al. (21) used an elegant approach to avoid systemic effects by transplanting transgenic bone marrow from the above mentioned model to lethally irradiated LDL receptor knockout ($LDLR^{-/-}$) mice. After a period of a mild fatty diet, the mice showed a 73% increase in lesion size at the aortic root area. It was concluded that $sPLA_2$ IIa can contribute to atherosclerotic lesion development independent of the systemic lipoprotein metabolism. However, how $sPLA_2$ IIa affects the pathology of lesion development was not addressed in detail.

We have previously shown that $sPLA_2$ IIa is not detectable in monocytes or differentiated, unstimulated macrophages, but its expression is induced upon minimally modified LDL stimulation of the latter (5). To acquire a better understanding of the role of $sPLA_2$ IIa in lesion development and the effects on lesion composition, we created a macrophage $sPLA_2$ IIa overexpression mouse model. In this mouse model, selective expression was obtained by using the myeloid-specific CD11b promoter fused to the human genomic $sPLA_2$ IIa gene lacking the endogenous promoter. The CD11b promoter has been shown to drive transgene expression in granulocytes, monocytes, and macrophages (22). Bone marrow from these mice was transplanted to $LDLR^{-/-}$ mice to study atherogenesis. Macrophage $sPLA_2$ was shown to strongly increase atherosclerosis and increase collagenous cap formation. Other inflammatory parameters, such as T-cells, granulocytes, and cell turnover, were not affected.

MATERIALS AND METHODS

Mouse models

A 3.8 kb fragment, containing the genomic $sPLA_2$ IIa gene, was isolated from vector pBG34:3.8 with *Hin*dIII and blunted. This fragment corresponds to the *Eag*I/*Not*I fragment described by Kramer et al. (23). The blunted *Hin*dIII fragment was ligated into a pB202 vector containing a 1.7 kb human CD11b promoter (22) (a generous gift from Dr. D. Tenen, Beth Israel Hospital, Boston, MA), which was cut with *Bam*HI and *Not*I and also blunted. Correct orientation was confirmed with digestion and sequencing from the CD11b promoter. To generate transgenic mice, the pB202:$sPLA_2$ construct was partial digested with *Hin*dIII and *Sac*I restriction enzymes. The complete 5.6 kb fragment (CD11b + $sPLA_2$ IIa) was isolated with electrophoresis on an agarose gel, followed by electroelution. Oocyte injection to generate transgenic mice was performed according to standard procedures (24). Two transgenic mouse lines were obtained (lines 1 and 2). Before their use in experiments, the transgenic mice were tested for the presence of endogenous $sPLA_2$ IIa with Southern blot analysis using a ^{32}P-labeled probe.

Because of a mutation, C57Bl/6 mice are natural knockouts for mouse $sPLA_2$ IIa (25). The transgenic $sPLA_2$ IIa bone marrow donors were backcrossed five times with C57Bl/6 mice. $LDLR^{-/-}$ mice have been described (26). They were backcrossed four times to C57Bl/6J mice and originally obtained from the Jackson Laboratory (Bar Harbor, ME). All animal experiments were approved by the Committee for Animal Welfare of Maastricht University.

Northern blotting

To study the expression of the transgene, total RNA was isolated from various tissues and from thioglycollate-elicited and bone marrow-derived macrophages, which were isolated as described before (27). The RNA Instapure System (Eurogentec S.A., Seraing, Belgium) was used to isolate RNA from the samples. When appropriate, the tissues were first frozen and mechanically pulverized. The RNA samples were separated by electrophoresis through a denaturing agarose gel (1%, w/v) containing 7.5% formaldehyde and then transferred to Hybond N according to the manufacturer's recommendations. Hybridization was performed on the blots with ^{32}P-labeled human $sPLA_2$ IIa DNA probe at 54°C in hybridization mixture (50% formamide, 1% SDS, 10% dextran sulfate, 5× SSC, 1× Denhardt's solution, 0.2 M Na_2PO_4, and 50 mg/ml sonicated salmon sperm DNA).

Phospholipase activity

Levels of $sPLA_2$ protein in cell lysates of peritoneal macrophages were assessed by measuring the hydrolysis of FFAs from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (Sigma), as described in detail before (28). Phospholipase activity in blood of the transplanted mice was measured in pooled plasma samples collected at week 8 after the start of the diet, using a photometric assay (number 765001; Cayman Chemical Co., Ann Arbor, MI).

In vitro foam cell formation

Bone marrow macrophages were cultured as described before (27). LDL was isolated according to standard procedures and modified as described before (27). Cells were incubated for 24 h in culture medium with or without either 25 μg/ml native LDL or 25 μg/ml Cu^{2+}-oxidized LDL. Cholesteryl esters were determined as described before (29).

Bone marrow transplantation

Thirty-four 9 week old female littermate $LDLR^{-/-}$ mice were put in filter-top cages. The mice received acidified water supplemented with neomycin (100 mg/l) and polymyxin B sulfate (60,000 U/l) during the week before transplantation. One day before the actual transplantation, the mice were irradiated with a lethal dose of 10 Gy röntgen. The bone marrow of five female wild-type mice and five female transgenic $sPLA_2$ IIa littermates was collected and pooled. Bone marrow cells were derived by flushing the femur and tibia of the mice. For transplantation, 16 mice received wild-type bone marrow (wt-tp) and 18 mice received transgenic bone marrow ($sPLA_2$-tp). Bone marrow cells (10^7) were injected in the tail vein of each mouse.

Blood samples and analysis

Four weeks after transplantation, the mice were put on a high-fat diet containing 16% fat, 0.15% cholesterol, and no cholate (Hope Farms, Woerden, The Netherlands) for 10 weeks. At two time points (4 and 8 weeks of diet), blood was collected from the mice after overnight fasting. Plasma cholesterol was determined with an enzymatic essay kit (catalog number 40; Sigma-Aldrich, Zwijndrecht, The Netherlands). The blood lipid profile was analyzed with pooled plasma collected after 8 weeks of diet. The samples were separated over a Superose 6PC 3.2/30 column in an AKTABasic chromatography system (Amersham Biosciences,

Roosendaal, The Netherlands). In the collected fractions (50), the cholesterol levels were determined by adding Roche Cholesterol CHOL_PAP reagent (catalog number 1489232; Roche Diagnostics, Mannheim, Germany) and measured with a plate reader (Bio-Rad Laboratories, Veenendaal, The Netherlands). Paraoxonase activity was measured in the blood of 10 randomly selected mice (5 wt-tp and 5 $sPLA_2$-tp). The activity was determined as the rate of conversion of paraoxon into *p*-nitrophenol and diethylphosphate by hydrolysis (30, 31). Serum of each mouse was incubated with 5 mM diethyl *p*-nitrophenyl phosphate (Sigma-Aldrich Chemie Gmbh, Steinberg, Germany). The reaction was kept at 37°C, and the extinction rate was recorded at 450 nm with a microplate reader (Bio-Rad Laboratories).

Atherosclerosis assessment and lesion analysis

After 10 weeks of high-fat diet, the mice were killed. Atherosclerosis was analyzed as described before (27). Briefly, the animals were dissected, and hearts and aortic arches were removed and bisected perpendicularly to the heart axis, just below the atrial tips. The tissue was frozen in Tissue-Tec (Shandon, Veldhoven, The Netherlands) with the base facing downward. Cryosectioning was performed from the atrioventricular area. Sections (7 μm) were made with an interval of 42 μm and collected on slides. The aortic lesion area was analyzed using serial sections with 42 μm intervals, beginning from the onset of the aortic valves until the valves had disappeared. The collected sections were stained with toluidine blue and digitally photographed and quantified using digital image software (Adobe Photoshop 6.0).

Pathology

All pathology measurements were done without knowledge of the genotype. Lesion sections from the aortic root were fixed in acetone and incubated with antibodies against various cell types and cell markers. The following antibodies were used: FA11 (macrophages), NIMP (granulocytes), 1A4 (smooth muscle cells), ERTR7 (fibroblasts), KT3 (T-cells), Ki-67 (cell proliferation), and $sPLA_2$ IIa (a generous gift from Dr. Timo Nevalainen, Turku University, Turku, Finland). Terminal deocynucleotidyl transferase end labeling (TUNEL) assay (Roche) was used to detect apoptosis. Sirius red staining was performed to visualize collagen. The stained sections were all photographed in the same conditions with a digital microscope camera (Nikon DXM1200). To quantify the percentage of collagen within the lesions, the lesion area was selected manually and subtraction filters (red channel minus the green channel) were applied to select the stained pixels (Adobe Photoshop 6.0). The same filter preferences were used on all sections, and quantification was assessed blindly (i.e., without knowledge of the genotype).

To quantify necrosis, necrotic areas were measured using toluidine-stained sections as described before (27). Cellular density was determined by counting nuclei and measuring area. All data are expressed relative to the lesion area size. For statistical analysis, one observation was the average value per mouse.

Statistical analysis

All data were analyzed using Graphpad Prism 4.01. Groups were compared using Welch's corrected two-tailed, nonpaired *t*-tests, and data are expressed as means ± SEM. Data were considered statistically significant at $P < 0.05$.

RESULTS

Generation and phenotyping of the $sPLA_2$ IIa transgenic mice

To assess the local, vascular role of $sPLA_2$ IIa in atherosclerosis in the absence of systemic effects of $sPLA_2$ IIa, a macrophage overexpression mouse model was created. The human genomic $sPLA_2$ IIa gene was cloned behind the CD11b promoter (22) (**Fig. 1A**). Two transgenic mouse lines were successfully generated. The mRNA expression pattern was examined by Northern blot analysis. The mice of line 1 showed expression (Fig. 1B) in liver, spleen, lung, and isolated primary macrophages. A similar expression pattern was observed in line 2, but expression was lower (data not shown). Therefore, all of the experiments described here were performed with line 1. Both bone marrow-derived macrophages (data not shown) and thioglycollate-elicited macrophages (Fig. 1B) exhibited high expression of $sPLA_2$ IIa. To test if the observed mRNA expression patterns translated into effective production and presence of the $sPLA_2$ IIa protein, immunohistochemistry using a rabbit anti-human $sPLA_2$ IIa polyclonal antibody was performed on different organs. The antibody used is known not to show cross-reactivity with endogenous mouse $sPLA_2$ IIa and is specific for the $sPLA_2$ IIa (32), confirmed by the lack of signal in wild-type tissues. Immunostaining (Fig. 1C) showed the presence of $sPLA_2$ IIa in macrophages in different tissues. An abundance of positively stained cells was observed in the spleen of the transgenic mice. Lung showed a moderate level of positive cells, whereas liver had only a few positive cells. In all organs tested, wild-type mice remained negative for $sPLA_2$ IIa. Staining with an antibody directed against CD11b (Mac1) confirmed the myeloid-specific expression of the $sPLA_2$ IIa transgene (data not shown). Finally, phospholipase activity was measured in cell lysates from thioglycollate-elicited macrophages. In cells from transgenic mice, 12–18 ng of $sPLA_2$ per milligram of protein was measured, whereas wild-type cells remained below the detection level. In vitro foam cell formation (i.e., cholesteryl ester accumulation) of wild-type or $sPLA_2$ transgenic bone marrow macrophages was not different after overnight incubation without LDL (0.47 ± 0.2 and 0.62 ± 0.1 μg/mg protein, respectively), with 25 μg/ml native LDL (0.88 ± 0.1 and 0.83 ± 0.2 μg/mg protein), or with 25 μg/ml oxidized LDL (5.2 ± 0.2 and 5.1 ± 0.2 μg/mg protein).

Bone marrow transplantation, lipid analysis, and phospholipase activity

To test the effect of $sPLA_2$ IIa on atherogenesis, bone marrow of wild-type mice and transgenic littermates was transplanted to lethally irradiated atherosclerosis-susceptible $LDLR^{-/-}$ mice. The resulting mice were all $LDLR^{-/-}$ with either $sPLA_2$ IIa transgenic hematopoietic cells ($sPLA_2$-tp) or wild-type hematopoietic cells (wt-tp). Both groups were allowed to recover for 4 weeks before high-fat feeding for 10 weeks.

Plasma cholesterol levels in the blood of the mice were examined after 4 and 8 weeks of the diet (**Fig. 2A**). After 4 weeks, the cholesterol levels in the $sPLA_2$-tp mice were slightly higher than in the wt-tp mice (26.8 ± 1.1 and 22.1 ± 1.0 mM, respectively; $P < 0.01$). After 8 weeks, cholesterol levels were further increased but were no longer significantly different between groups (37.2 ± 2.7 and 30.8 ± 2.7 mM for $sPLA_2$-tp and wt-tp, respectively; $P = 0.10$).

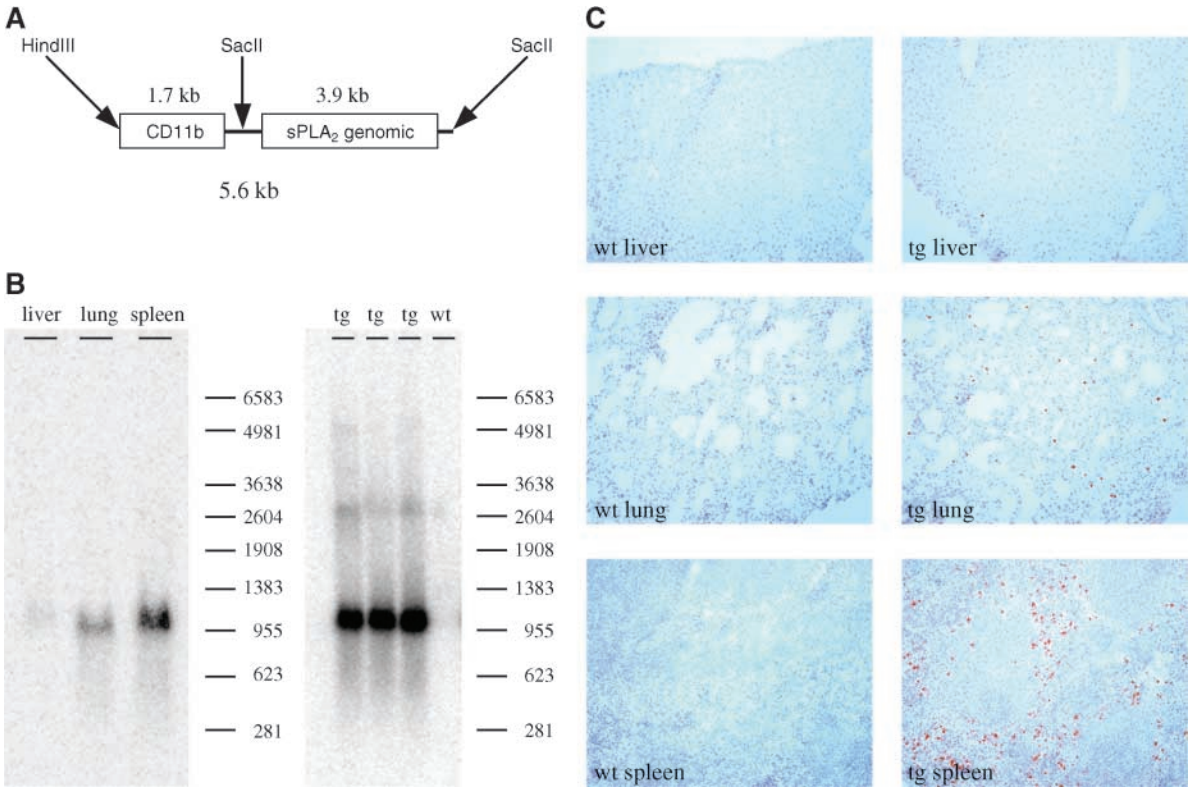

Fig. 1. Generation and characterization of CD11b-group IIa secretory phospholipase A_2 ($sPLA_2$ IIa) mice. A: Schematic representation of the CD11b-$sPLA_2$ IIa construct. The 5.6 kb fragment was used for oocyte injection. B: Northern blot analysis of liver, lung, and spleen (left blot) and thioglycollate-elicited macrophages (right blot) from line 2 mice hybridized with a human $sPLA_2$ IIa probe. tg, $sPLA_2$ IIa transgenic; wt, wild type. C: Immunostaining of tissues with rabbit anti-human $sPLA_2$ IIa serum. Liver, lung, and spleen from wild-type and transgenic mice are shown.

Paraoxonase activity in the blood of the mice after 8 weeks on the diet did not differ between the wild-type and the transgenic animals (1.37 ± 0.11 and 1.39 ± 0.26 IU/ml, respectively). To examine the blood lipid levels in more detail, lipid profiles were generated using plasma samples from week 8 (Fig. 2B). No major differences in VLDL, LDL, and HDL fractions were observed between the wt-tp and $sPLA_2$-tp mice.

Phospholipase activity of the pooled plasma samples was measured at 8 weeks, and no significant difference was detected (22.4 ± 0.2 and 22.3 ± 0.7 μmol/min/ml for wt-tp and $sPLA_2$-tp, respectively).

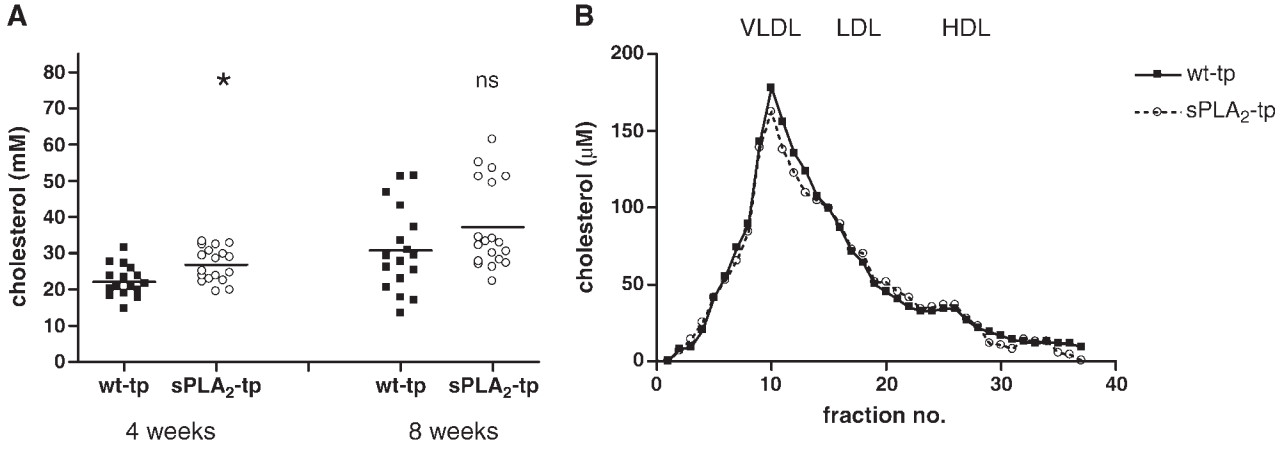

Fig. 2. Cholesterol levels and lipoprotein profiles of $sPLA_2$ IIa and wild-type transplanted LDL receptor knockout ($LDLR^{-/-}$) mice. A: Cholesterol levels in wild-type (wt-tp; closed squares) and $sPLA_2$ IIa ($sPLA_2$-tp; open circles) transplanted $LDLR^{-/-}$ mice after 4 and 8 weeks of high-fat diet. B: Lipoprotein cholesterol profiles of wild-type (closed squares) and $sPLA_2$ IIa (open circles) transplanted $LDLR^{-/-}$ mice after 8 weeks of high-fat diet. The values represented in the graph show the cholesterol content in each fraction.

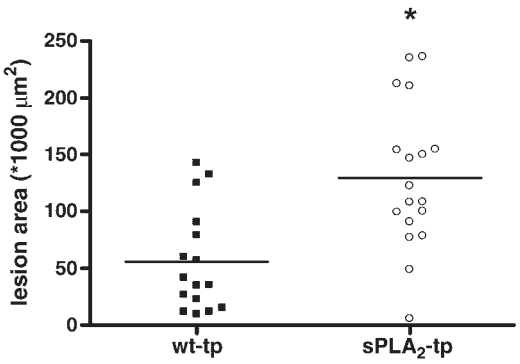

Fig. 3. Atherosclerotic lesion size in $sPLA_2$ IIa and wild-type transplanted $LDLR^{-/-}$ mice. Lesion area measurements of wild-type and $sPLA_2$ IIa transplanted $LDLR^{-/-}$ mice. Symbols represent individual mice. * $P < 0.001$.

Effect of macrophage-specific $sPLA_2$ IIa overexpression on atherosclerotic lesion size

Bone marrow-transplanted mice were killed after 10 weeks of high-fat diet. Analysis of atherosclerotic lesions was done at the aortic root, near the heart valves. Quantification of the lesion area revealed a 2.3-fold increase in lesion size (**Fig. 3**) in $sPLA_2$-tp animals compared with wt-tp. The $sPLA_2$-tp mice had an average lesion size of 130.0 ± 15.1 (×1,000 μm^2; n = 18), whereas the wt-tp mice had an area of 56.0 ± 11.3 (×1,000 μm^2; n = 16) ($P < 0.001$). Regression analysis did not show a clear correlation between lesion area and cholesterol levels at either 4 weeks of diet ($R^2 = 0.11$, $P = 0.25$ and $R^2 = 0.04$, $P = 0.51$ for wt-tp and $sPLA_2$-tp, respectively) or 8 weeks of diet ($R^2 = 0.019$, $P = 0.60$ and $R^2 = 0.16$, $P = 0.12$ for wt-tp and $sPLA_2$-tp, respectively).

In addition to the quantitative approach to measuring the lesion area, the plaques were also studied in more detail for their composition. Representative lesions stained with toluidine blue are shown in **Fig. 4A**, **B**. The major constituents of the lesions were macrosialin-positive macrophages (Fig. 4C, D); some collagenous cap formation was also observed using Sirius red staining (Fig. 4E, F). The presence of $sPLA_2$ IIa in the lesions was confirmed by immunostaining (**Fig. 5**). The $sPLA_2$-tp mice showed clear staining in the lesions, whereas the wt-tp mice remained negative. Interestingly, lesions from $sPLA_2$-tp mice were positive in their macrophage areas, but the majority of the $sPLA_2$ IIa staining was in the medium. This may indicate the accumulation of macrophage-derived $sPLA_2$ in the medium.

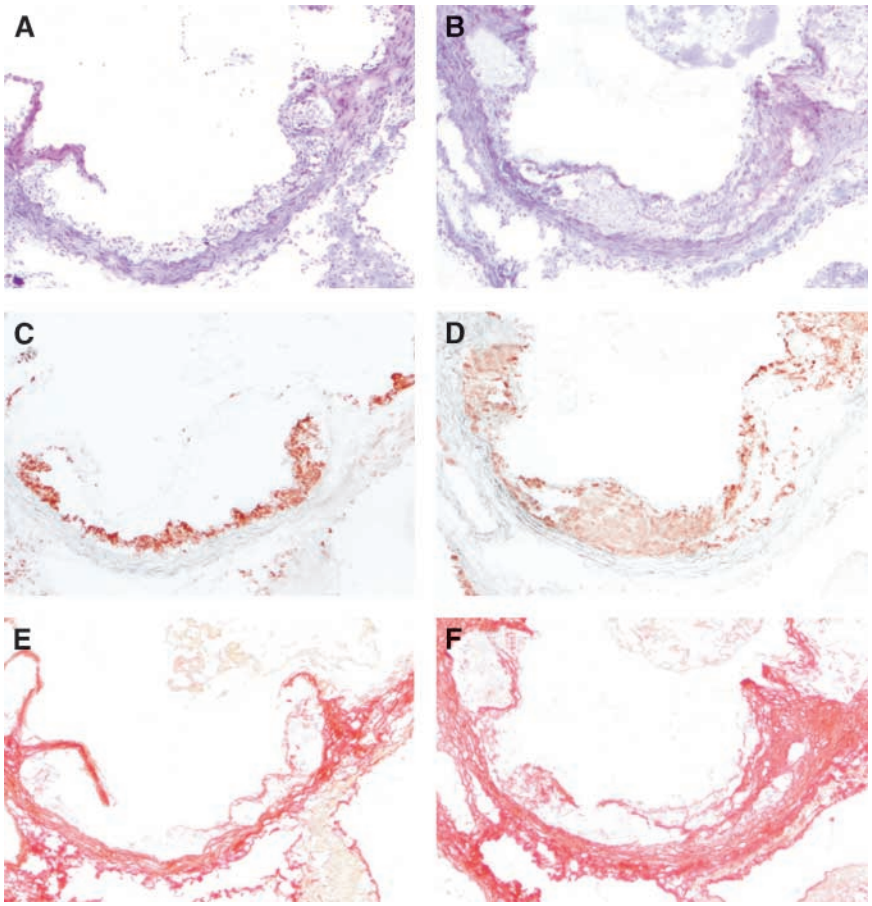

Fig. 4. Atherosclerotic lesions in $sPLA_2$ IIa and wild-type transplanted $LDLR^{-/-}$ mice. Representative lesions from wild-type (A, C, E) and $sPLA_2$ IIa (B, D, F) transplanted $LDLR^{-/-}$ mice stained with toluidine blue (A, B) for general cellular composition, macrosialin (C, D) for macrophages, and Sirius red (E, F) for collagen.

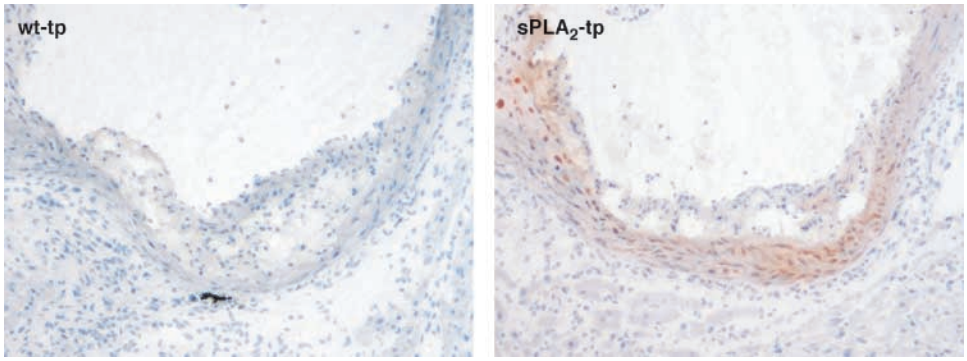

Fig. 5. $sPLA_2$ IIa immunostaining. Immunostaining for human $sPLA_2$ IIa of lesions from wild-type and $sPLA_2$ IIa transplanted $LDLR^{-/-}$ mice.

In addition, the lesions were classified according to three different types as described before (27): *1*) early lesions, with fatty streaks consisting of only foam cells; *2*) moderate lesions, or foam cell lesions with a fibrotic cap; *3*) severe lesions, with increased fibrosis and media involvement. Interestingly, although the lesions in the $sPLA_2$-tp mice were 2.3-fold larger, no significant shift toward a more severe phenotype of the lesions was observed (early lesions, 19.5 and 15.7; moderate lesions, 65.9 and 74.5; advanced lesions, 14.6 and 9.8 for wt-tp and $sPLA_2$-tp, respectively; all as relative percentages of the total number of lesions). This demonstrates that the lesions in $sPLA_2$-tp mice are much larger but not more severe compared with those in control mice.

Plaque phenotype

To characterize atherosclerosis in more detail, different cellular parameters were determined. All were expressed relative to the size of the lesions.

Two factors important for plaque stability (i.e., necrosis and collagen content) were analyzed. Necrotic area did not differ between the two groups (**Fig. 6A**). Also in both groups, no clear difference in correlation between lesion size and necrosis could be found (Fig. 6B). Interestingly,

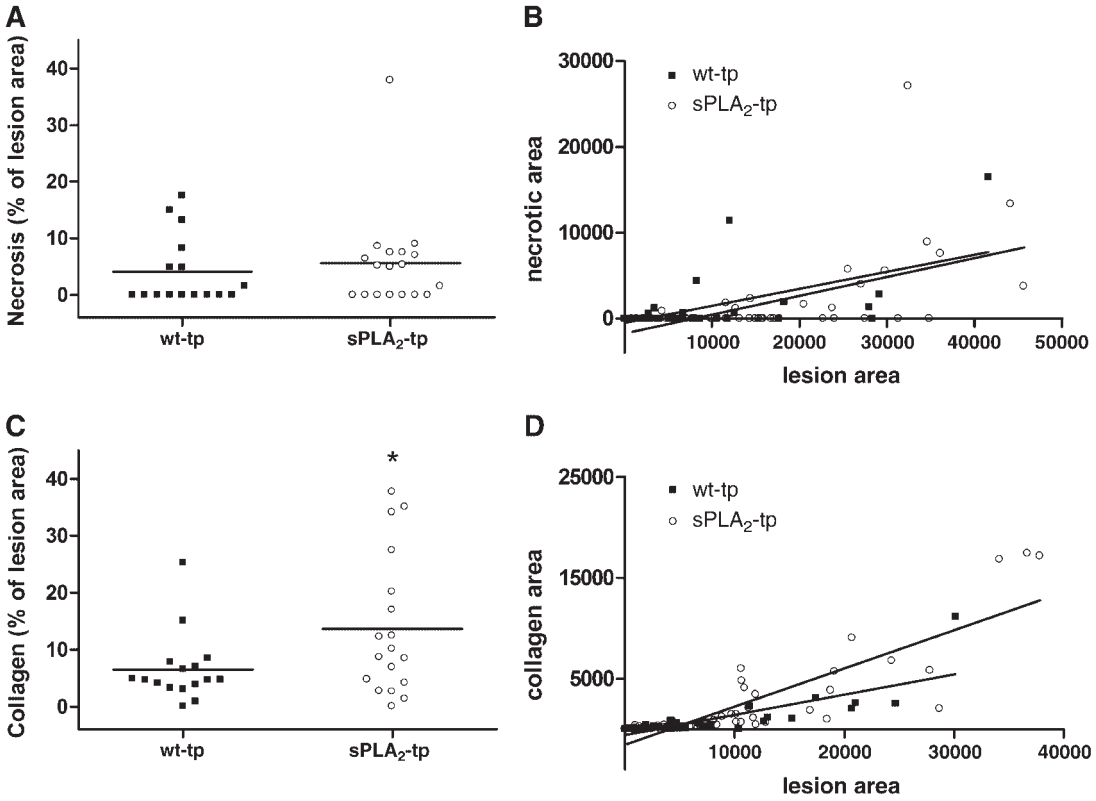

Fig. 6. Necrosis and collagen in lesions from $sPLA_2$ IIa and wild-type transplanted $LDLR^{-/-}$ mice. Necrosis (A, B) and collagen (C, D) were quantified in the lesions of wild-type and $sPLA_2$ IIa transplanted $LDLR^{-/-}$ mice as described in Materials and Methods. Both are expressed as relative percentages of lesion area per animal (A, C) and as individual areas correlated to the lesion size (B, D). In A and C, each symbol represents one animal, and in B and D, each symbol represents one lesion. * $P < 0.05$.

collagen, as measured by Sirius red staining of the lesions, was 2-fold increased in $sPLA_2$-tp lesions (6.5 ± 1.5% and 13.6 ± 2.9% for wt-tp and $sPLA_2$-tp, respectively; $P < 0.05$), even though the values were corrected for lesion size (Fig. 6C). Regression analysis confirmed this difference, showing correlation between lesion size and collagen content ($R^2 = 0.62$, $P < 0.0001$ and $R^2 = 0.73$, $P < 0.0001$ for wt-tp and $sPLA_2$-tp, respectively) with a 2-fold steeper slope for $sPLA_2$-tp mice (0.20 ± 0.02 and 0.38 ± 0.03 for wt-tp and $sPLA_2$-tp, respectively; $P < 0.001$) (Fig. 6D). These data show that overexpression of $sPLA_2$ in macrophages does not affect necrosis but results in atherosclerotic lesions with more collagen content.

Other cellular parameters were also studied. Smooth muscle cell (SMC) content was analyzed because increased collagen might be a reflection of increased SMCs in the lesions. However, quantification of these cells after SMC α-actin staining did not reveal any differences between $sPLA_2$-tp and controls (**Fig. 7A**). Moreover, fibroblasts (ERTR7-positive cells) also did not differ between groups (4.6 ± 1.1% and 5.7 ± 1.6% for wt-tp and $sPLA_2$-tp, respectively). Next, T-cells were quantified. Although they appeared to be reduced (Fig. 7B), the difference did not reach statistical significance ($P = 0.10$). For cell turnover, proliferation (i.e., Ki-67 positivity) and apoptosis (TUNEL staining) were examined. Proliferation appeared to be reduced in $sPLA_2$-tp mice, but the difference was only borderline significant ($P = 0.06$; Fig. 7C). Finally, apoptosis was not changed in the $sPLA_2$-tp group compared with wt-tp mice (Fig. 7D).

Because changes in cell turnover (i.e., apoptosis, necrosis, and proliferation) and SMC or collagen content could not completely account for the 2.3-fold increase in total lesion size observed, macrophage density was determined. The increase in lesion size could be the result of either increased cell size or just increased cell number. Counting of the number of macrophages per selected area showed no differences in cell density between wt-tp and $sPLA_2$-tp (574.5 ± 42.8 and 548.2 ± 24.3 cells/μm^2, respectively), indicating that the size of the macrophages was not changed. Therefore, the increased lesion size is attributable to the increased number of macrophages and not to their increased size.

DISCUSSION

In this study, we examined the effects of macrophage $sPLA_2$ IIa on atherogenesis. We show that macrophage-specific overexpression strongly increases atherosclerotic plaque size without the normally associated lesion progression to a more severe phenotype. In addition, collagen deposition in the lesions was also increased even when corrected for lesion size. Remarkably, other cellular parameters were not affected by $sPLA_2$ IIa.

We could demonstrate that $sPLA_2$ IIa is present at the site of the atherosclerotic lesions of the mice transplanted with $sPLA_2$ bone marrow. Only minimal systemic effects on cholesterol levels were observed, with no change in circulating $sPLA_2$ or paraoxonase activity. Hence, we have generated an animal model to investigate the direct lesional role of $sPLA_2$ IIa in plaque development and pro-

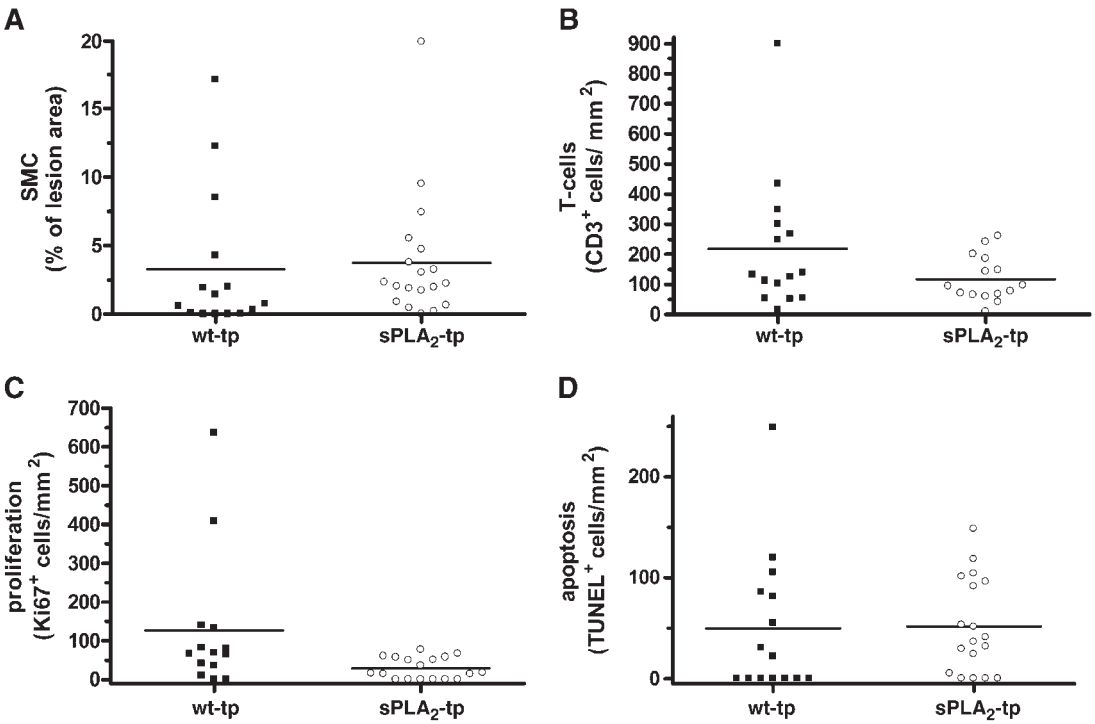

Fig. 7. Cellular characterization of lesions from $sPLA_2$ IIa and wild-type transplanted $LDLR^{-/-}$ mice. Smooth muscle cells (SMC) (A), T-cells (B), proliferation (C), and apoptosis/terminal deoxynucleotidyl transferase end labeling (TUNEL) (D) were quantified in lesions of wild-type and $sPLA_2$ IIa transplanted $LDLR^{-/-}$ mice and expressed relative to lesion size. Each symbol represents one animal.

gression. Quantification of the atherosclerotic lesions revealed a 2.3-fold increase in lesion size attributable to increased macrophage $sPLA_2$ IIa expression. Similar results, albeit less pronounced, have been reported by Webb et al. (21). Closer examination of the lesions in our experimental animals revealed interesting changes in plaque morphology in response to increased levels of $sPLA_2$. Both collagen content and increased macrophage numbers contributed to lesion enlargement in the $sPLA_2$-tp mice. Apoptosis and necrosis were not altered, and cell proliferation did not increase, indicating that the increased cell number in $sPLA_2$-tp mice must be attributed to increased monocyte infiltration in the vessel wall. It was recently shown that one of the products of $sPLA_2$ activity, lysophosphatidylcholine, can be chemotactic for monocytes (33), so this may account, at least in part, for the observed increased cell number and increased atherosclerosis. No other signs of increased inflammation could be measured. No changes were observed in T-cells, inflammatory cells that are often associated with atherosclerotic plaques. Also, granulocyte count (data not shown) did not reveal any differences.

Despite the cell-specific expression of the transgene, the $sPLA_2$ IIa produced in macrophages and monocytes proved to be sufficient to increase the cholesterol levels in mice after 4 weeks of diet. However, at later times, this difference was no longer significant. No significant correlation between lesion area and cholesterol levels at either 4 or 8 weeks of high-fat diet was found. Additionally, no clear change in the blood lipoprotein profile or in paraoxonase activity was detected. Paraoxonase levels have been previously described to be reduced in $sPLA_2$ transgenic animals, and paraoxonase can act as a protective factor against lipoprotein modification (20). From these data, we conclude that total systemic effects of $sPLA_2$ IIa were rather limited in our experimental setup, and it is unlikely that they contributed significantly to the phenotype. This is in contrast to the drastic lipid changes observed by Ivandic et al. (20). They demonstrated that general overexpression results in decreased levels of HDL-cholesterol and increased levels of LDL/VLDL on a chow diet, and even more prominent changes were observed on a high-fat diet. The high expression levels of $sPLA_2$ IIa in the liver of this mouse model (34) could contribute to these changes in lipid parameters.

Immunohistochemistry for $sPLA_2$ IIa revealed the presence of the enzyme in the media of the aorta near atherosclerotic lesions in the sPLA2-tp mice. Nevalainen, Laine, and Grass (34) observed similar accumulation of $sPLA_2$ IIa protein in media without the presence of mRNA for $sPLA_2$ IIa. A mechanism for this specific localization is offered by Sartipy et al. (35), who showed binding of secreted $sPLA_2$ IIa to such proteoglycan molecules as decorin and versican. We observed medial $sPLA_2$ IIa only near the lesions, suggesting the accumulation of diffused $sPLA_2$ IIa from within the lesion. $sPLA_2$ IIa has been shown to bind to a whole range of extracellular matrix components, such as glypican, a glycosylphosphatidylinositol (GPI)-anchored proteoglycan (36), decorin (36), and biglycan (12, 14, 16, 35). Kovanen and Pentikainen (37) demonstrated an increased affinity of modified LDL to collagen as a result of decorin linkage. Moreover, Flood et al. (12) also demonstrated increased affinity for proteoglycans of $sPLA_2$ IIa-modified LDL. Interaction with the extracellular matrix components may result in rearrangement of the phospholipids in the LDL particles, which may increase susceptibility to $sPLA_2$ IIa modifications, in accordance with results shown by Sartipy et al. (11). These mechanisms in turn contribute to prolonged LDL retention and subsequent modifications (38) in the extracellular matrix, resulting in increased atherogenicity of the lipoproteins (39). Besides direct binding to the matrix, $sPLA_2$ IIa-modified LDL has been shown previously to aggregate more and fuse in the presence of proteoglycans (17, 40, 41), which may also increase atherogenesis. The absence of clear inflammatory effects in the lesions of our $sPLA_2$-tp mice, despite the increased lesion size, may indicate that LDL retention in the vessel wall is an important factor in $sPLA_2$ IIa-amplified atherosclerosis.

One of the main features of the lesions in the $sPLA_2$ IIa mice was increased collagen content. The combination of more collagen (i.e., cap thickening) and the absence of increased necrosis or apoptosis suggests a more stable plaque development attributable to the presence of $sPLA_2$. It has not been described before that $sPLA_2$ IIa affects collagen production or fibrosis in atherogenesis. However, using $sPLA_2$ IIa transgenic mice in a model of acute pancreatitis, Mayer et al. (42) also observed increased fibrosis in the pancreas of transgenic animals, whereas fibrosis was absent in the wild-type mice. SMC and fibroblast contents were not changed in $sPLA_2$-tp mice, which may indicate that the difference in collagen in the lesions cannot be attributed to an increase in specific cell types producing collagen. The exact source of the collagen remains unclear, but it should be noted that collagen VIII can be produced by macrophages in atherosclerotic lesions (43). More collagen in atherosclerotic lesions can be the result of either increased overall production in the lesion or decreased degradation. Degradation may be affected by reduction in matrix metalloproteases (MMPs), which degrade collagen, or increases in tissue inhibitors of MMPs. However, no data are available on the effect of $sPLA_2$ IIa on these kinds of enzymes; future experiments should focus on this issue. In the lesions of our $sPLA_2$ IIa-transplanted mice, we found an increase in macrophages and foam cells but no clear signs of increased inflammation (i.e., changes in T-cells or granulocytes). Fibrosis or scar formation is often considered to be a final anti-inflammatory phase (44) and reflects the resolution of inflammation. Interestingly, using an air pouch model, Gilroy et al. (45) recently described a new role for phospholipases in inflammation. $sPLA_2$ IIa and group V were shown to be important in the resolution phase of inflammation, whereas these enzymes did not contribute to the initiation phase of inflammation. Both were capable of upregulating platelet-activating factor and lipoxin A4, resulting in group IV cytosolic PLA_2-mediated release of proresolving eicosanoids (45–47). Moreover, they showed an increase in the number of

inflammatory cells in the resolving phase in C57Bl/6 mice, a natural knockout for $sPLA_2$ IIa compared with BALB/c animals, a difference not observed in the initiating phase (45). These results indicate a role for $sPLA_2$ IIa in the termination of inflammatory responses. Our observed increased collagen in atherosclerotic plaques may be in agreement with such an anti-inflammatory role. Combining our model with activation through other proinflammatory stimuli may shed light on specific effects in the initiation and resolution of inflammation.

In conclusion, we have generated a novel mouse model to study the function of $sPLA_2$ IIa in macrophages and atherogenesis. We show that $sPLA_2$ IIa in macrophages is a proatherogenic factor. Our findings also suggest an LDL accumulation- and retention-based mechanism as a major cause of the effects of $sPLA_2$ IIa. Moreover, we found that overexpression of $sPLA_2$ IIa also affects collagen deposition in the plaque, which indicates a novel function of $sPLA_2$ IIa in the vessel wall. The exact mechanisms involved in $sPLA_2$ IIa-induced collagen deposition remain speculative, and more detailed mechanistic studies are needed. Future studies may also focus on longer lasting atherosclerosis experiments to investigate how more advanced atherosclerosis is affected by $sPLA_2$ IIa and how plaque stability develops in the presence of increased levels of this enzyme.

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