Overexpression of Tissue Inhibitor of Metalloproteinase 3 in Macrophages Reduces Atherosclerosis in Low-Density Lipoprotein Receptor Knockout Mice

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Objective—Tissue inhibitor of metalloproteinase 3 (TIMP3) is a stromal protein that inhibits the activity of proteases and receptors. TIMP3 is downregulated in metabolic and inflammatory disorders, such as type 2 diabetes mellitus and atherosclerosis, particularly in regions enriched with monocyte/macrophage cells. To investigate the role of TIMP3 in atherosclerosis, we generated a new mouse model in which Timp3 was overexpressed in the atherosclerotic plaque via a macrophage-specific promoter (MacT3). We elucidated any potential antiatherosclerotic effects of TIMP3, including regulation of monocyte/macrophage recruitment within atherosclerotic plaques, in MacT3 mice crossbred with low-density lipoprotein receptor knockout (LDLR−/−) mice.

Methods and Results—MacT3/LDLR−/− mice had an improvement of atherosclerosis and metabolic parameters compared with LDLR−/−. En face aorta and aortic root examination of MacT3/LDLR−/− mice revealed smaller atherosclerotic plaques with features of stability, such as increased collagen content and decreased necrotic core formation. Atherosclerotic plaques in MacT3/LDLR−/− mice contained fewer T cells and macrophages. Furthermore, TIMP3 overexpression in macrophages resulted in reduced oxidative stress signals, as evidenced by lower lipid peroxidation, protein carbonylation, and nitration in atheromas.

Conclusion—Our study confirmed that macrophage-specific overexpression of TIMP3 decreases the inflammatory content and the amplitude of atherosclerotic plaques in mice. (Arterioscler Thromb Vasc Biol. 2012;32:74-81.)

Key Words: atherosclerosis • macrophages • metalloproteinases • inflammation • lipotoxicity

In patients with type 2 diabetes mellitus and cardiovascular disease, the expression of tissue inhibitor of metalloproteinase 3 (TIMP3) is reduced in carotid atherosclerotic plaques, particularly in regions enriched with monocyte/macrophage cells.1 TIMP3 is a secreted 24- to 27-kDa stromal protein that belongs to the TIMP family. It binds to the extracellular matrix and inhibits metalloproteinases such as matrix metalloproteinase-2/9/14 and a disintegrin and a metalloprotease domain 17 (ADAM17), whose activities are increased in atherosclerotic plaques.1-3 We previously identified TIMP3 as a modifier factor for insulin resistance and vascular inflammation in mice, and these results were partially explained by the inhibitory effect of TIMP3 on ADAM17.3-6 In particular, analysis of metabolic and vascular tissues from different strains of Insr+/− revealed that those with diabetes were characterized by deficiency for TIMP3, insulin resistance, unrestrained metalloproteinase activity, and increased activation of inflammatory signals.3 This phenotype partially reversed when the Insr+/− mice were crossed with ADAM17 haploinsufficient mice or treated with a metalloprotease inhibitor.3 Moreover, in a mouse model combining insulin resistance with nutrient overload and decreased TIMP3 activity, the loss of TIMP3 was associated with greater infiltration of F4/80 positive cells, increased monocyte chemoattractant protein-1 expression, and enhanced oxidative stress markers in the subendothelial space of aortic roots.4 These studies suggest that a deficit in TIMP3 may contribute to the pathogenesis of vascular disorders via upregulation of inflammatory mediators that recruit monocytes and T cells from the circulation, such as tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1.3,4 Experimental models have recently revealed the pivotal role of ADAM17 in the inflammatory response to endotoxin stimulation. In fact, in mouse monocytes, the absence of TIMP3 provoked massive release of TNF-α and other cytokines, whereas the absence of ADAM17 prevented the inflammatory response.7,8 Therefore,
we generated a new mouse model with targeted overexpression of TIMP3 in monocyte/macrophage lineage cells, which results in overexpression of TIMP3 directly at inflammatory sites. This mouse was crossed with the atherosclerosis-prone low-density lipoprotein receptor–null (LDLR−/−) mouse to test the therapeutic role of TIMP3 in atherosclerosis. We confirmed that increased expression of TIMP3 inside the atherosclerotic plaque slows down the progression of vascular damage associated with atherosclerosis.

Methods

Generation of MacT3 Transgenic Mice

The mouse Timp3 gene was amplified by polymerase chain reaction (PCR) using cDNA obtained from mouse muscle as a template. Timp3 cDNA was cloned into a vector containing a CD68 promoter/enhancer and amplified in Escherichia coli strain DH5α. For transgenic mouse generation, the construct was linearized with endonuclease enzymes (EcoRI/NotI) and microinjected into mouse pronuclei by standard methods. Offspring derived from the injected embryos were genotyped by PCR analysis on DNA isolated from tail biopsies performed with primers that amplified a 300-bp fragment of the transgenic construct. PCR results were confirmed by Southern blotting of EcoRV-digested DNA followed by probing of the membrane with the CD68-Timp3 fragment labeled with [α-32P]dCTP. To evaluate hematopoietic cell lineage distribution, peripheral blood was collected from the tail vein and analyzed on a blood cell analyzer (Simply Cell, BPC BioSed, Rome, Italy) to determine cell counts. MacT3 mice used in this study had been backcrossed into the C57BL/6J background for 5 generations. MacT3/LDLR−/− mice were obtained by breeding MacT3 with LDLR−/− mice (Harlan) to generate MacT3/LDLR−/− mice. To induce atherosclerosis, 6-week-old male mice were fed a Western diet (15% [w/w] fat and 0.25% [w/w] cholesterol, Research Diets) for 16 (aortic root analysis) or 24 (aorta en face analysis) weeks as previously described.

Isolation of Bone Marrow–Derived Macrophages

For bone marrow–derived macrophages (BMDMs) preparation, femurs and tibia were removed from euthanized mice, and bone marrow cells were resuspended in 10 mL of macrophage medium (Isoculture modified Dulbecco’s medium containing 20% FCS, 50 ng/mL macrophage colony-stimulating factor, 2 mmol/L L-glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin) to stimulate proliferation and differentiation of the marrow progenitors into macrophages. After 5 days, the resulting BMDMs were replated and used for experiments within 2 days. In some experiments, lipopolysaccharide (200 ng/mL, Sigma-Aldrich, St. Louis, MO) was added 24 hours before the cells were harvested.

Gene Expression Analysis

Total RNA was isolated from BMDMs, adipose tissues, spleens, livers, and aortas with Trizol reagent (Invitrogen Corp, Eugene, OR). A total of 2 µg of RNA was reverse transcribed into cDNA using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed, and the relative gene copy number was calculated as 2^ΔCt as previously described.

Western Blot and Reverse Zymography

Western blots for TIMP3 on extracellular matrix extracts and reverse zymography were performed as previously described. A representative image of 3 to 4 mice per group is shown.

Metalloprotease Activity and TNF-α Levels

Metalloprotease activity was determined as previously described. TNF-α levels were measured in cell culture medium using a commercial ELISA kit (R&D Systems, Minneapolis, MN).

Lipoprotein Profiles

To obtain lipoprotein profiles, plasma samples were analyzed by size-exclusion high-performance liquid chromatography on a Supersose column (Superox 6PC 3.2/30, Amersham Pharmacia Biotech, Uppsala, Sweden) to separate very-low-density, low-density, and high-density lipoproteins. Total cholesterol levels in the column effluents were continuously measured via in-line mixture with a commercially available enzymatic colorimetric cholesterol detection reagent (Cholesterol RTU, Biomerieux, Marcy l’Étoile, France) followed by downstream spectrophotometric detection of the reaction products at 500 nm absorbance. The first peak of cholesterol eluted from the column was attributed to very-low-density lipoprotein, the second to low-density lipoprotein, and the third to high-density lipoprotein. The area under each peak was calculated using the software provided with the fast protein liquid chromatograph (Chromelone, Dionex, Sunnyvale, CA). To calculate the cholesterol concentration for each lipoprotein fraction, the ratio of the corresponding peak area to total peak area was multiplied by the total cholesterol concentration measured in the sample.

Histology and Quantification of Arterial Lesions

Mice were anesthetized with isoflurane (IsoVet, Schering-Plough, Whitehouse Station, NJ) and bled via cardiac puncture. The heart with the proximal aorta was fixed in 4% paraformaldehyde and embedded in paraffin or OCT blocks for Oil Red O staining. Atherosclerotic lesions were assessed using the method of Paigen et al as previously described.

Migration Analysis of Bone Marrow–Derived Monocytes

Donor mice were euthanized, and their femurs and tibias were removed aseptically. Femur and tibia marrow cavities were flushed with RPMI medium containing fetal bovine serum and HEPES, pH 7.4, using a 25-gauge needle. Single-cell suspensions were prepared by repeat pipetting, and the cell preparations were passed through a 70-µm nylon mesh to remove particulate matter. Cells were centrifuged, washed twice in RPMI, and counted. For adoptive transfer experiments, 10⁶ bone marrow cells were depleted of Ly6Glow cells using anti-Ly6G magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Depletion columns (LD) were used according to the MACS separation system protocol provided by the manufacturer (Miltenyi Biotec). Next, Ly6Glow cells were incubated with anti-CD11b magnetic beads and Ly6Glow CD11b+ cells were obtained using the MACS cell sorting system protocol. Cell purity was confirmed by 2-color flow cytometry analysis of the different cell populations. Antibodies against CD11b (BD Biosciences Pharmingen, San Diego, CA) and Ly6G (Miltenyi Biotec) were used. mRNA was extracted from Ly6Glow and Ly6Ghigh cells, and Timp3 expression was analyzed by real-time PCR. Ly6Glow CD11b+ cells were labeled with 5-(and-6)-carboxyfluorescein diacetate (CFDA-SE, Molecular Probes, Carlsbad, CA), and 5×10⁵ cells were resuspended in 200 µL of 0.9% NaCl and then injected retroorbitally into mice that had received a high-cholesterol diet for 16 weeks. Sixty hours after the injection, mice were euthanized by asphyxiation with carbon dioxide. The base of the heart and the ascending aorta were isolated then gently perfused with 1 mL of heparinized saline via the left ventricle. The aortic root was cut 2 mm distal from the heart, fixed in 4% paraformaldehyde and embedded in paraffin or OCT blocks for Oil Red O staining. Atherosclerotic lesions were assessed using the methods of Paigen et al as previously described.

For the en face analysis, the aorta was cut 2 mm distal from the heart, fixed in 4% paraformaldehyde-PBS, opened longitudinally, and pinned flat on a siliconized plate. The en face preparation was then stained with Sudan IV and stored in paraffin. The atherosclerotic lesion area and the total area of the aorta were measured on the dissecting microscope using computer-assisted image analysis software (Optimas 6.5, Biosoft, Cambridge, MA). Immunostaining was performed using antibodies against F4/80, CD3, N-scarboxy methyl lysine, nitrotyrosine, Ki-67, and active caspase-3 (Abcam Inc, Cambridge, MA) following the manufacturer’s instructions.
the valves) were cut, stained with anti-Ly-6G- eFluor 625NC antibody (eBioscience, San Diego, CA), and analyzed by fluorescence microscopy (Olympus BX51 equipped with Olympus C-3030 digital camera, Tokyo, Japan). The number of fluorescent leukocytes attached to the intimal surface or to atheromatous plaques was counted in each cross-section of the aortic root. Data were averaged per mouse, and these numbers were used to calculate the mean ± SEM for each group.

For in vitro experiments, $1 \times 10^5$ bEnd.3 cells were cultured on 1.5% gelatin or 4 g/cm² fibronectin-coated Transwell filters for 3 days to allow the formation of confluent monolayers. Following overnight treatment with 20 ng/mL recombinant TNF-α and 200 U/mL mouse interferon-γ (R&D Systems), the inserts were placed into new plates containing 50 ng/mL recombinant mouse monocyte chemoattractant protein-5 (R&D Systems), and $10^5$ CFDA-SE labeled CD11b+ cells were added to endothelial monolayers. After 2 hours at 37°C, the inserts were removed, and the cells that had transmigrated to the bottom of the plates were counted using an inverted microscope.

**Statistical Analysis**

Results of the experimental studies are mean ± SD. Statistical analyses were performed using 1-way ANOVA or the unpaired Student $t$ test as indicated. Values of $P < 0.05$ were considered to be statistically significant.

**Results**

To study the protective role of TIMP3 in myeloid cells during the development of atherosclerosis, we generated transgenic...
mice (MacT3) overexpressing TIMP3 under the control of the monocyte/macrophage lineage-specific promoter CD68 (Figure 1A, B). This strategy allowed us to increase TIMP3 expression directly in atherosclerotic plaques where monocytes/macrophages are gradually recruited during disease progression. We found increased expression of TIMP3 at the mRNA level in MacT3 splenocytes and BMDMs compared with the wild-type (WT) mice (Figure 1C). Hematogram results were similar among the littermates of WT and MacT3 mice (Figure 1D).

To test their physiological function, MacT3 BMDMs and WT BMDMs were stimulated in vitro with lipopolysaccharide.

MacT3 BMDMs showed increased TIMP3 release in the extracellular matrix and enhanced TIMP3 activity compared with WT BMDMs, as assessed by Western blot and reverse zymography, respectively (Figure 1E). Although the basal levels of metalloproteinase activity and TNF-α secretion were not significantly different between WT and MacT3 BMDMs (data not shown), lipopolysaccharide stimulation significantly reduced MacT3 BMDMs responses compared with WT BMDMs (Figure 1F and 1G, respectively). These results demonstrate that MacT3 BMDMs display increased levels of a functional TIMP3 protein when the promoter CD68 is activated.

Next, we investigated whether TIMP3 could reduce the development of atherosclerotic lesions in a mouse model combining genetic predisposition (LDLR<sup>−/−</sup>) and environmental stress (atherogenic Western diet). To study the infiltration of monocytes into the aortic roots<sup>10</sup> and the extension of atherosclerosis into the aorta surface,<sup>11</sup> MacT3 mice were crossed with LDLR<sup>−/−</sup> mice and fed a Western diet. Hematogram results did not differ between LDLR<sup>−/−</sup> and MacT3/LDLR<sup>−/−</sup> mice (Supplemental Figure I, available online at http://atvb.ahajournals.org). After 16 weeks of a Western diet, an analysis of the cholesterol content in circulating lipoproteins revealed that MacT3/LDLR<sup>−/−</sup> mice had a mild but not significant reduction in total cholesterol and low-density lipoprotein cholesterol compared with LDLR<sup>−/−</sup> mice (Figure 2A). MacT3/LDLR<sup>−/−</sup> mice showed a slight but significant reduction in weight and random fed glucose and insulin levels (Figure 2B). Analysis of the expression of inflammatory genes and cytokines in white adipose tissue and liver revealed a decreased inflammatory profile in MacT3/LDLR<sup>−/−</sup> mice compared with LDLR<sup>−/−</sup> mice (Figure 2C and 2D). Because inflammation is a cause of insulin resistance, these results could in part explain the improvement of random fed glucose and insulin levels in MacT3/LDLR<sup>−/−</sup> mice.

Aortas from MacT3/LDLR<sup>−/−</sup> mice showed a significant reduction of atherosclerotic plaques compared with the LDLR<sup>−/−</sup> mice as demonstrated by en face staining with Sudan IV (Figure 3A). Oil Red O and Weigert–Van Gieson staining of MacT3/LDLR<sup>−/−</sup> aortic roots clearly confirmed a significant reduction of lesion areas in atherosclerotic plaques compared with LDLR<sup>−/−</sup> mice. A significant decrease of the necrotic lipid core, as well as an increase of collagen content in plaques from MacT3/LDLR<sup>−/−</sup> mice, was also observed (Figure 3B and 3C).

We then determined whether the rescue effects observed in MacT3/LDLR<sup>−/−</sup> mice were the result of a diminished recruitment of proinflammatory cells into the aorta. TIMP3 overexpression in macrophages rescued the aortic wall from the accumulation of proinflammatory cells as demonstrated by a reduction of CD3<sup>+</sup> and F4/80<sup>+</sup> infiltrated cells (Figure 4A). Upregulation of TIMP3 in MacT3/LDLR<sup>−/−</sup> aortas was coupled with a significant reduction of inflammatory cytokines, such as monocyte chemoattractant protein-1, interleukin-1α, and interleukin-6 (Figure 4B), compared with LDLR<sup>−/−</sup> aortas. In addition, signs of oxidative stress, such as nitrotyrosine and N-g-carboxymethyl-lysine, were reduced in MacT3/LDLR<sup>−/−</sup> mice compared with control mice (Figure 4C).

To evaluate whether the overexpression of TIMP3 changed the cellular composition inside atherosclerotic plaques in vivo, we analyzed markers of cell proliferation and apoptosis. The number of proliferating (Ki67 positive) or apoptotic (caspase-3 positive) endogenous cells in plaque areas did not change, demonstrating that TIMP3 upregulation does not influence atherosclerotic plaque turnover in vivo (Supplemental Figure II). Therefore, we investigated whether the rescue effects observed in MacT3/LDLR<sup>−/−</sup> mice were partially explained by an impaired migration of MacT3 myeloid cells. We initially performed an in vitro transmigration assay using mouse bEnd.3 endothelial cell monolayers preactivated with proinflammatory cytokines. There was a 2-fold impairment of migration in MacT3 bone marrow–derived CD11b<sup>+</sup> cells compared with WT CD11b<sup>+</sup> cells, independent of whether gelatin or fibronectin was used for coating (Figure 5A). However, freshly isolated CD11b<sup>+</sup> cells, derived from mouse bone marrow, consist of a heterogeneous...
population of monocytes/macrophages and granulocytes. Therefore, to investigate the role of monocytes, we obtained a pure population of CD11b<sup>+</sup>/Ly6G<sup>−</sup> cells depleted of neutrophils (CD11b<sup>+</sup>/Ly6G<sup>−</sup> cells) (Figure 5B). mRNA analysis confirmed that TIMP3 expression levels were significantly higher in CD11b<sup>+</sup>/Ly6G<sup>−</sup> cells than in Ly6G<sup>−</sup> cells (Figure 5C). To investigate in vivo whether the monocyte fraction was the primary mediator of the effects observed in MacT3 mice, we intravenously injected hypercholesterolemic recipient LDLR<sup>−/−</sup> mice with fluorescently labeled bone marrow–derived CD11b<sup>+</sup>/Ly6G<sup>−</sup> cells from donor LDLR<sup>−/−</sup> or MacT3/LDLR<sup>−/−</sup> mice. Recipient mice showed no apparent adverse effects, and we isolated aortas 60 hours after injection. Histological sections of the aortic sinuses were analyzed by light and fluorescent microscopy to count the number of labeled monocytes that were adherent or within the atherosclerotic plaques. When injected into recipient LDLR<sup>−/−</sup> mice, a lower number of fluorescently labeled MacT3/LDLR<sup>−/−</sup> bone marrow–derived CD11b<sup>+</sup>/Ly6G<sup>−</sup> cells infiltrated into the atherosclerotic plaque compared with fluorescently labeled LDLR<sup>−/−</sup> bone marrow–derived CD11b<sup>+</sup>/Ly6G<sup>−</sup> cells (Figure 5D).

Discussion

In this study, we analyzed the effects of upregulation of TIMP3 on atherosclerotic plaques in vivo using a transgenic approach. We observed that at the macroscopic level, TIMP3 was able to diminish the lipid deposits in the aorta after 24 weeks of a Western diet. Because lipoprotein levels were not modified by overexpression of TIMP3 in myeloid cells, we hypothesized that increased TIMP3 expression within the atherosclerotic plaques could result in a less severe subendothelial inflammatory infiltrate and diminished macrophage foam cell formation. Reduced TIMP3 expression was associated with reduced accumulation of foam cells in a rabbit model of atherosclerosis, but no functional evidence for a direct role of TIMP3 was provided. More recently, reduced TIMP3 expression was found in a new inflammatory subpopulation of macrophages in the liver. In our study, increased TIMP3 expression was associated with reduced accumulation of foam cells in a rabbit model of atherosclerosis, but no functional evidence for a direct role of TIMP3 was provided. More recently, reduced TIMP3 expression was found in a new inflammatory subpopulation of macrophages in the liver.
effect of TIMP3 on metalloproteinase proteolytic activity. Therefore, we provide functional evidence that overexpression of TIMP3 during atherosclerosis progression stabilizes atherosclerotic plaques in the hypercholesterolemic LDLR⁻/⁻ mouse model. In a recent study, overexpression of adenovirus encoding TIMP2 but not TIMP1 resulted in regression of atherosclerotic plaques. Given the multiple actions exerted by TIMP3 and TIMP2, additional studies are necessary to understand whether the results from this study are due to inhibition of specific proinflammatory proteases or other targets.

When TIMP3 was overexpressed in myeloid cells, the vascular wall was less inflamed and contained a lower number of macrophages, which could inhibit their differentiation into foam cells and their contributing to plaque progression. Our adoptive transfer model suggests that these results may be due to regulation of monocyte recruitment into the atherosclerotic plaque. However, bone marrow–derived CD11b⁺ cells also include a small fraction of other cell types, such as granulocytes; therefore, despite that they express CD68 at a very low level, we cannot exclude that these cells actively contribute to the observed results.

TIMP3 also affects cell migration, apoptosis, proliferation, and inflammation. TIMP3 stimulates hematopoietic stem cell proliferation by causing quiescent hematopoietic stem cells to enter the cell cycle, and TIMP3 overexpression results in decreased frequency of B and T lymphocytes and increased frequency of myeloid cells in the blood and bone marrow. In our study, MacT3 and MacT3/LDLR⁻/⁻ hematogram analyses did not reveal

Figure 4. MacT3/low-density lipoprotein receptor knockout (LDLR⁻/⁻) mice accumulate significantly lower numbers of macrophages in atherosclerotic lesions compared with LDLR⁻/⁻. A, Representative sections of aorta, after 16 weeks of a Western diet, analyzed by immunohistochemistry for the presence of inflammatory cells using CD3 or F4/80 antibodies. Quantitative analysis of CD3⁺ T cells and F4/80⁺ cells (n=5 per group, *P<0.05, Student t test, data are mean±SD). B, mRNA expression. Results are expressed relative to LDLR⁻/⁻ mice and normalized to expression of 18S rRNA (n=5 per group, *P<0.05, Student t test, data are mean±SD). C, Representative sections of aorta immunostained with nitrotyrosine or carboxy methyl lysine antibodies (magnification ×250) (n=5 per group, *P<0.05, Student t test, data are mean±SD).
significant differences in the basal state or during the Western diet, possibly because TIMP3 overexpression was restricted to CD68-positive cells and limited to sites of vascular inflammation.

Our in vivo analyses did not reveal any effects of TIMP3 overexpression on proliferation and apoptosis markers in the atherosclerotic plaque as a whole, although we cannot exclude the possibility that TIMP3 regulates these processes in specific cell types during the dynamics of atherosclerotic plaque growth.

We observed a reduction of atherosclerotic lesion development with different techniques at 16 (aortic root analysis) and 24 (en face aorta and in vivo injection of CD11b-H11001/Ly6G-H11002 cells) weeks after beginning a Western diet. Although our data suggest that TIMP3 overexpression slows the atherosclerotic process at least up until 24 weeks after starting on a Western diet, the reduction in lesion development might not persist at a later time point.

In conclusion, our results reveal that TIMP3 overexpression in monocytes/macrophages reduces the progression of atherosclerosis during a long-term treatment in a genetic model. Whether this effect was only due only TIMP3 expressed by monocytes/macrophages or may result from the interaction of increased TIMP3 with other cell populations must be clarified in future studies.

**Acknowledgments**

We thank Dr Peter Murray (St. Jude Children’s Research Hospital, Memphis, TN) for the CD68 promoter, the Telethon Core Facility for Conditional Mutagenesis, S. Raffaele (Milano) at the Istituto di Ricerca e Cura a Carattere Scientifico for generating transgenic mice, Dr Marie-France Champy at the Mouse Clinical Institute (Illkirch, France) for the lipoprotein assay, and Dr Manlio Vinciguerra (European Mouse Biology Laboratory, Monterotondo, Italy) for the bEnd.3 mouse endothelial line.

**Sources of Funding**

This work was funded by Telethon GGP08065, Fondazione Roma 2008, Juvenile Diabetes Research Foundation Regular Research Grant 1-2007-665, and Framework Programme 7-Health-241913-FLORINASH, Ministry of Health RF 2007 (all to M. Federici). We thank BioMed Proofreading for manuscript editing.

**Disclosures**

None.
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Arterioscler Thromb Vasc Biol. 2012;32:74-81; originally published online October 20, 2011; doi: 10.1161/ATVBAHA.111.238402

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplement Material

**Supplementary Figure I.** Hematogram analyses demonstrating normal blood cell lineage distributions, including lymphocytes (Lym), monocytes (Mono) and granulocytes (Gran) in MacT3/LDLR\(^{-/-}\) and LDLR\(^{-/-}\) mice fed a Western Diet for 16 weeks (n=3 per group and expressed as mean ± SD).

**Supplementary Figure II.** Representative sections of aortas from LDLR\(^{-/-}\) and MacT3/LDLR\(^{-/-}\) mice after 16 weeks of Western Diet, analyzed by immunohistochemistry for the presence of apoptosis using Anti Active Caspase-3 antibody and proliferation using Anti Ki-67 antibody. Quantitative analysis of positive cells is reported (n = 5 per group, data are mean ± SD).
Supplemental Figure I
Supplemental Figure II

[Image of Supplemental Figure II showing histological sections and bar charts comparing LDLR⁻/⁻ and MacT3/LDLR⁻/⁻ genotypes for active Casp-3 and Mφ47 positive cells/mm² of plaque area.]