

# Overexpression of Angiopoietin-Like Protein 4 Protects Against Atherosclerosis Development

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**Objective**—Macrophage foam cells play a crucial role in several pathologies including multiple sclerosis, glomerulosclerosis, and atherosclerosis. Angiopoietin-like protein 4 (Angptl4) was previously shown to inhibit chyle-induced foam cell formation in mesenteric lymph nodes. Here we characterized the regulation of Angptl4 expression in macrophages and examined the impact of Angptl4 on atherosclerosis development.

**Approach and Results**—Macrophage activation elicited by pathogen-recognition receptor agonists decreased Angptl4 expression, whereas lipid loading by intralipid and oxidized low-density lipoprotein increased Angptl4 expression. Consistent with an antilipotoxic role of Angptl4, recombinant Angptl4 significantly decreased uptake of oxidized low-density lipoprotein by macrophages, via lipolysis-dependent and -independent mechanisms. Angptl4 protein was detectable in human atherosclerotic lesions and localized to macrophages. Transgenic overexpression of Angptl4 in atherosclerosis-prone apolipoprotein E\*3-Leiden mice did not significantly alter plasma cholesterol and triglyceride levels. Nevertheless, Angptl4 overexpression reduced lesion area by 34% ( $P<0.05$ ). In addition, Angptl4 overexpression decreased macrophage content (−41%;  $P<0.05$ ) and numbers of monocytes adhering to the endothelium wall (−37%;  $P<0.01$ ). Finally, plasma Angptl4 was independently and negatively associated with carotid artery sclerosis measured by 3-T MRI in subjects with metabolic syndrome and low-grade systemic inflammation.

**Conclusions**—Angptl4 suppresses foam cell formation to reduce atherosclerosis development. Stimulation of Angptl4 in macrophages by oxidized low-density lipoprotein may protect against lipid overload. (*Arterioscler Thromb Vasc Biol*. 2013;33:1529-1537.)

**Key Words:** atherosclerosis ■ inflammation ■ lipoprotein lipase ■ lipoproteins ■ macrophages

Macrophages are an important component of the innate immune system. Via phagocytosis of foreign pathogens, macrophages play a critical role in the body's first line of defense. In addition, macrophages are involved in removal of cellular debris and clearance of postapoptotic cells. The ability of macrophages to secrete various cytokines allows them to communicate with other immune cells and orchestrate the inflammatory response.

Besides cellular debris and foreign particles, macrophages can, under certain conditions, also engulf lipid particles via the expression of various scavenger receptors, thereby becoming foam cells. Foam cell formation has been studied primarily in the context of atherosclerosis characterized by accumulation of foam cells in the atherosclerotic plaque.<sup>1,2</sup> However, foam cells also participate in other pathologies including

multiple sclerosis,<sup>3</sup> obesity,<sup>4</sup> nonalcoholic steatohepatitis,<sup>5</sup> and glomerulosclerosis.<sup>6,7</sup>

Recently, we described the accumulation of macrophage foam cells in mesenteric lymph nodes of mice fed a highly saturated fat diet.<sup>8</sup> In particular, foam cell formation specifically occurred in mice lacking angiopoietin-like protein 4 (Angptl4), an endogenous inhibitor of the triglyceride-hydrolyzing enzyme lipoprotein lipase (LPL) that catalyzes uptake of circulating lipids into tissues.<sup>9</sup> Angptl4 irreversibly inhibits LPL activity by converting active LPL dimers into inactive monomers.<sup>10,11</sup> Consequently, overexpression of Angptl4 leads to hypertriglyceridemia and reduced fatty acid uptake in tissues, whereas Angptl4 deletion causes lowering of circulating triglyceride levels.<sup>12-15</sup>

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Angptl4 is produced by various tissues including adipose tissue, liver, skeletal muscle, and intestine.<sup>16</sup> In addition, Angptl4 as well as LPL are expressed at high levels in macrophages.<sup>8</sup> Other than a role of lipids and lipid-activated transcription factors peroxisome proliferator activated receptor (PPAR)  $\beta/\delta$  and PPAR $\gamma$ ,<sup>8</sup> currently little is known about specific stimuli impacting Angptl4 expression in macrophages. Taking into account the importance of foam cells in atherosclerosis,<sup>2</sup> and given the facilitative role of LPL in macrophage foam cell formation,<sup>17,18</sup> we hypothesized that changes in Angptl4 expression may influence atherosclerosis development. Accordingly, the present study was aimed at better characterizing the regulation of Angptl4 expression in macrophages and examining the potential impact of Angptl4 on atherosclerosis development in a well-established model for human-like lipoprotein metabolism.<sup>19</sup>

**Materials and Methods**

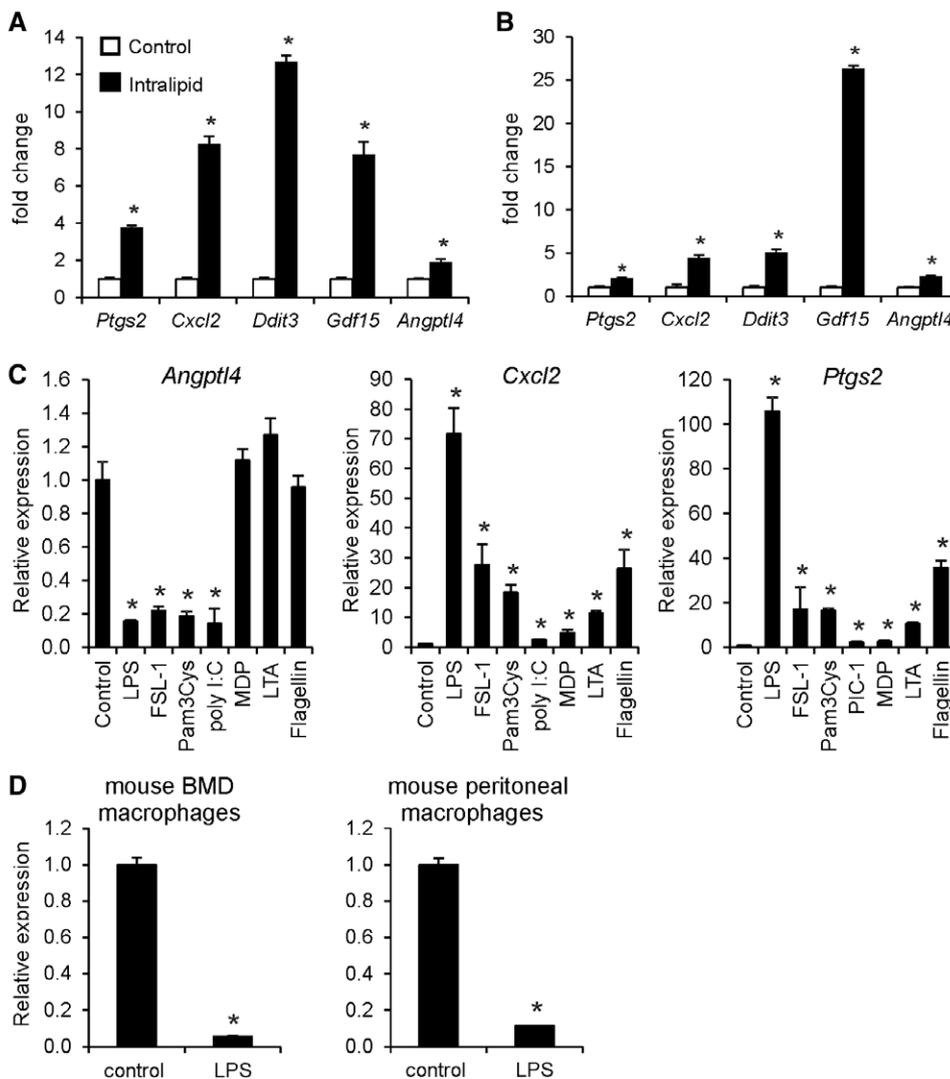
Materials and Methods are available in the online-only Supplement.

**Results**

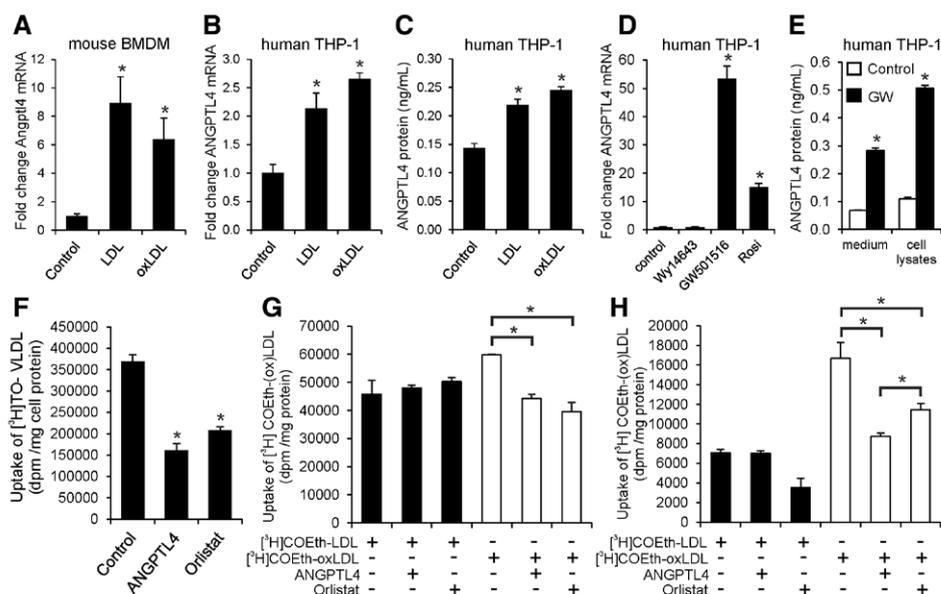
**Macrophage Angptl4 Is Regulated by Lipid Emulsion and Toll-Like Receptors 3 and 4 Agonists**

First, we characterized the regulation of Angptl4 expression in cultured macrophages. Previous studies have shown that Angptl4 expression is highly upregulated by chylomicrons and fatty acids in peritoneal macrophages.<sup>8</sup> In line with these data we observed that incubation of mouse RAW 264.7 macrophages with a triglyceride emulsion, which causes foam cell formation and macrophage activation (Figure I in the online-only Data Supplement), increases Angptl4 mRNA expression as well as expression of inflammatory markers (ie, *Ptgs2*, *Cxcl2*) and endoplasmic reticulum stress-marker *Ddit3* (Figure 1A). Similar results were obtained in mouse bone marrow-derived macrophages (BMDMs; Figure I in the online-only Data Supplement; Figure 1B).

To investigate whether activation of macrophages independent of foam cell formation may also alter Angptl4 expression, macrophages were treated with various pattern-recognition



**Figure 1.** Regulation of Angptl4 in macrophages. Expression of various genes in RAW 264.7 mouse macrophages (A), or bone marrow-derived macrophages (BMDMs; B) treated with 2 mmol/L intralipid for 6 hours. C, Changes in mRNA expression of selected genes in mouse RAW 264.7 macrophages on treatment with Toll-like receptor agonists for 4 hours. D, Effect of lipopolysaccharide (LPS; 1  $\mu$ g/mL) on Angptl4 mRNA expression in mouse BMD macrophages and mouse peritoneal macrophages after 24-hour treatment. Error bars represent SEM. Differences between experimental treatment and control were evaluated by Student *t* test (\**P* value <0.05). FSL indicates Pam2CGDPKHPKSF; LTA, lipoteichoic acid; and MDP, muramyl dipeptide.



**Figure 2.** Angptl4 reduces uptake of oxidized low-density lipoprotein (oxLDL) by macrophages. Changes in Angptl4 mRNA (**A** and **B**) or protein secretion (**C**) in mouse bone marrow–derived macrophages (BMDMs; **A**) and human THP-1 (**B** and **C**) macrophages after 24-hour incubation with LDL or oxLDL (25  $\mu\text{g}/\text{mL}$ ). **D**, ANGPTL4 mRNA expression in THP-1 macrophages treated with agonists for PPAR $\alpha$  (Wy14643, 1  $\mu\text{mol}/\text{L}$ ), PPAR $\delta$  (GW501516, 0.5  $\mu\text{mol}/\text{L}$ ), and PPAR $\gamma$  (rosiglitazone, 0.5  $\mu\text{mol}/\text{L}$ ) for 6 hours. **E**, ANGPTL4 protein levels in medium and lysate from THP-1 macrophages treated with GW501516 (0.5  $\mu\text{mol}/\text{L}$ ) or vehicle for 6 hours. **F**, Quantification of the uptake of  $^3\text{H}$ -activity by human THP-1 macrophages after 24-hours incubation with [ $^3\text{H}$ ]TO-labeled very-low-density lipoprotein (VLDL; 30  $\mu\text{g}$  protein/mL), in the presence or absence of human recombinant ANGPTL4 (1.5  $\mu\text{g}/\text{mL}$ ) or orlistat (30  $\mu\text{mol}/\text{L}$ ). **G**, Quantification of 6-hours uptake of [ $^3\text{H}$ ]COEth-labeled LDL or oxLDL (10  $\mu\text{g}$  protein/mL) by THP-1 macrophages preincubated with unlabeled VLDL (30  $\mu\text{g}$  protein/mL) and recombinant ANGPTL4 (1.5  $\mu\text{g}/\text{mL}$ ) or orlistat (30  $\mu\text{mol}/\text{L}$ ) for 24 hours. **H**, Quantification of 6-hours uptake of [ $^3\text{H}$ ]COEth-labeled LDL or oxLDL (10  $\mu\text{g}$  protein/mL) in presence or absence of recombinant ANGPTL4 (1.5  $\mu\text{g}/\text{mL}$ ) or orlistat (30  $\mu\text{mol}/\text{L}$ ) in THP-1 macrophages that had been preincubated with nonradioactive VLDL (150  $\mu\text{g}/\text{mL}$ ) for 2 hours. Error bars represent SEM. Differences between experimental treatment and control were evaluated by Student *t* test (\**P* value <0.05).

receptor agonists. In contrast to the lipid emulsion, several pattern-recognition receptor agonists, including lipopolysaccharide (LPS; Toll-like receptor 4 [TLR4] agonist), Pam2CGDPKHPKSF-1 (TLR2/6 agonist), and polyI:C (TLR3 agonist), markedly reduced Angptl4 mRNA levels in mouse RAW 264.7 macrophages while inducing inflammatory markers Ptg2 and Cxcl2 (Figure 1C). Downregulation of Angptl4 mRNA by various pattern-recognition receptor agonists in BMDMs could be partially relieved by an inhibitor of the nuclear factor- $\kappa\text{B}$  pathway, which effectively blunted induction of inflammatory markers (Figure II in the online-only Data Supplement). However, the major portion of the effect of pattern-recognition receptor agonists on Angptl4 seemed to be independent of nuclear factor- $\kappa\text{B}$  activation. Downregulation of Angptl4 by LPS was confirmed in mouse BMDMs and peritoneal macrophages (Figure 1D). Taken together, these data show that expression of Angptl4 in macrophages is differentially regulated by lipid emulsions and LPS.

### Angptl4 Decreases the Uptake of Oxidized Low-Density Lipoprotein in Macrophages

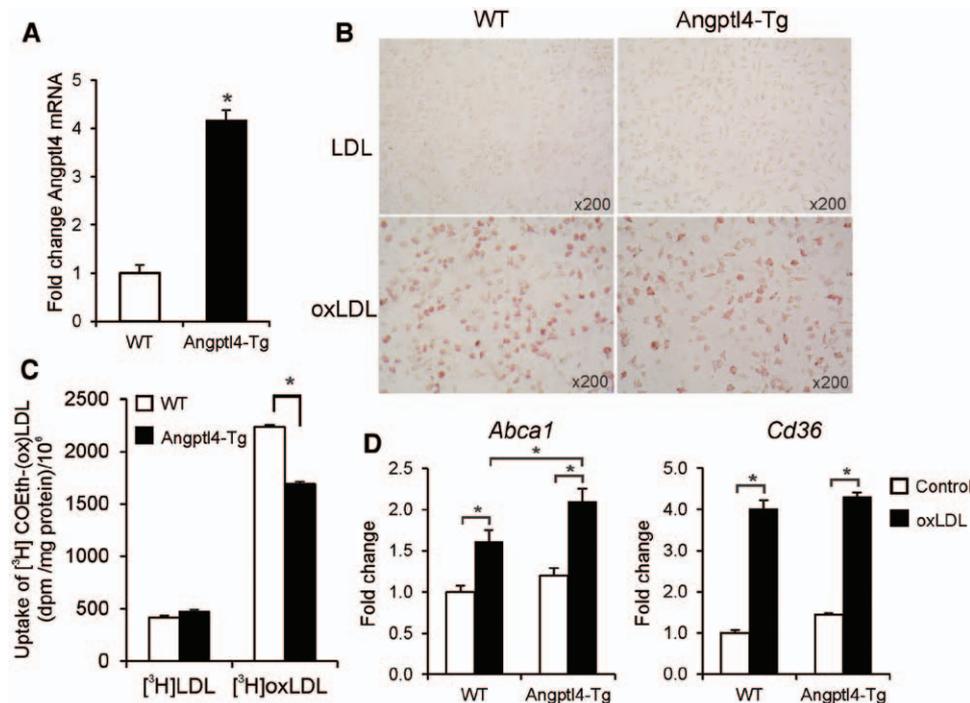
Oxidized low-density lipoprotein (oxLDL) is often used as substrate to induce macrophage foam cell formation and mimic the events in the atherosclerotic plaque. Treatment of mouse BMDMs (Figure 2A) and human THP-1 macrophages (Figure 2B) with oxLDL significantly increased expression of Angptl4 mRNA. Induction of ANGPTL4 mRNA in THP-1 cells was paralleled by a marked increase in ANGPTL4

protein secretion (Figure 2C). Similar results were obtained for native LDL.

The PPAR transcription factors were previously shown to at least partially mediate the effects of oxLDL on gene expression in macrophages.<sup>20–22</sup> Synthetic agonists for PPAR $\gamma$  (rosiglitazone) and especially PPAR $\delta$  (GW501516) markedly induced ANGPTL4 mRNA levels in THP-1 macrophages (Figure 2D). Induction of ANGPTL4 by GW501516 was paralleled by a significant increase in ANGPTL4 protein in cell lysate and medium (Figure 2E). Accordingly, PPAR $\delta$  and possibly PPAR $\gamma$  may be suspected to mediate the effect of oxLDL on ANGPTL4 gene transcription.

By inhibiting LPL activity, Angptl4 was previously shown to reduce macrophage uptake of triglycerides-derived fatty acids and impair macrophage activation,<sup>8</sup> which may indirectly lead to decreased uptake of oxLDL. Using THP-1 macrophages, we confirmed that human recombinant Angptl4 as well as the LPL inhibitor orlistat markedly decreased the uptake of triglycerides from glycerol tri[ $^3\text{H}$ ]oleate-labeled very-low-density lipoprotein (VLDL; Figure 2F). We subsequently preincubated THP-1 cells with unlabeled VLDL in the presence or absence of recombinant Angptl4 for 24 hours followed by a wash and treatment of the cells with [ $^3\text{H}$ ]cholesteryl oleoyl ether (COEth)-labeled oxLDL for 6 hours. Consistent with our expectation, Angptl4 and orlistat significantly decreased the uptake of oxLDL (Figure 2G).

To examine whether Angptl4 may directly inhibit oxLDL uptake, we first pretreated THP-1 macrophages with VLDL in



**Figure 3.** Angptl4 overexpression reduces uptake of oxidized low-density lipoprotein (oxLDL) by macrophages. **A**, Angptl4 mRNA expression in bone marrow–derived macrophages (BMDMs) derived from wild-type (WT) and Angptl4Tg mice. **B**, Oil Red O staining on fixed BMDMs from WT and Angptl4Tg mice treated with 25  $\mu$ g/mL oxLDL or LDL for 24 hours. **C**, Quantification of <sup>3</sup>H-activity in BMDM cell lysates, after 48-hours incubation with 15  $\mu$ g protein/mL of [<sup>3</sup>H]COEth-labeled oxLDL or [<sup>3</sup>H]COEth-labeled LDL. Values represent dpm per mg protein. **D**, mRNA expression of *Abca1* and *Cd36* in BMDM from wild-type (WT) and Angptl4Tg mice treated with 25  $\mu$ g/mL oxLDL for 24 hours. Error bars represent SEM. Differences between control and Angptl4Tg mice were evaluated by Student *t* test (\**P* value <0.05).

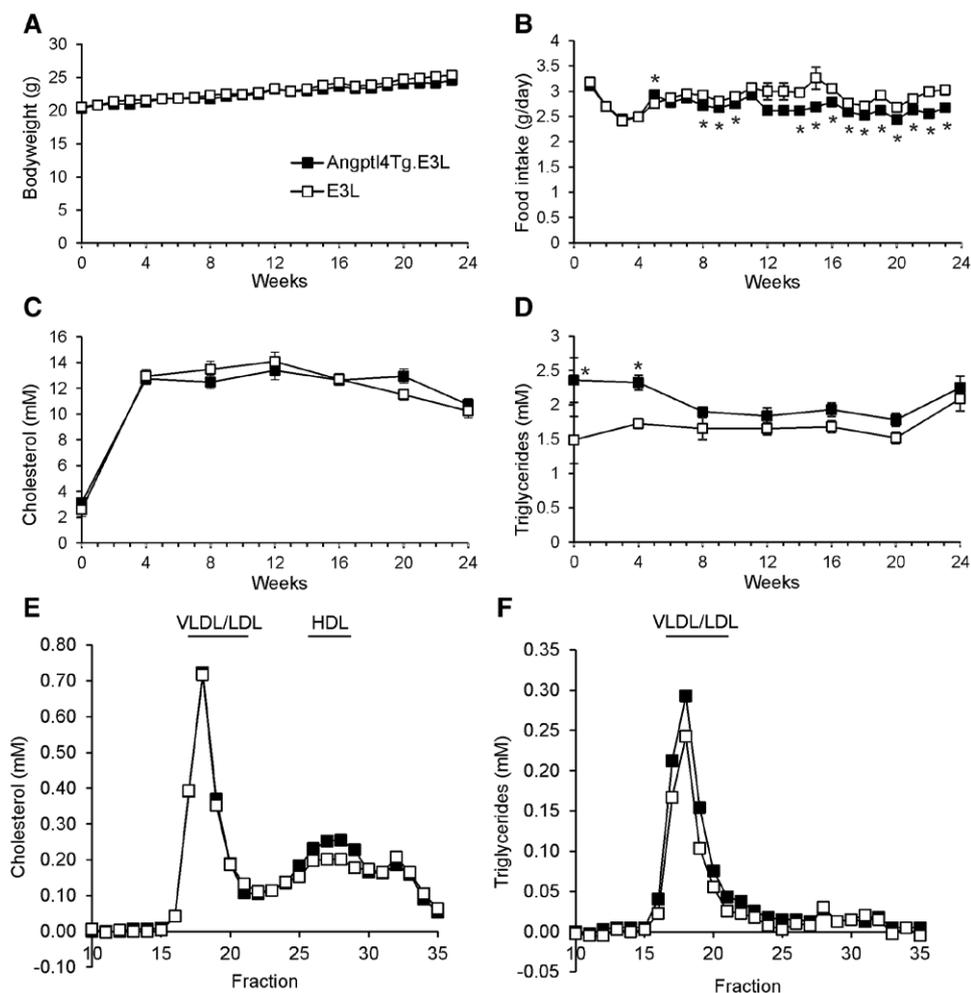
the absence of Angptl4 to provoke a proinflammatory phenotype that may facilitate uptake of oxLDL.<sup>23–25</sup> After 2 hours, VLDL was washed away and cells were treated with [<sup>3</sup>H]COEth-labeled oxLDL or [<sup>3</sup>H]COEth-labeled LDL in the presence or absence of human recombinant Angptl4 or orlistat for 6 hours. Recombinant Angptl4 significantly reduced uptake of oxLDL by 50%, compared with 30% for orlistat. Recombinant Angptl4 had no effect on uptake of native LDL (Figure 2H). Because orlistat specifically inhibits the lipolytic activity of LPL, these data suggest that Angptl4 reduced oxLDL uptake via a combination of lipolysis-dependent and independent mechanisms.

Subsequently, we assessed the impact of Angptl4 overexpression on oxidized LDL uptake and consequent formation of foam cells, using BMDMs from wild-type (WT) and Angptl4Tg mice. Angptl4Tg mice moderately overexpress Angptl4 in a variety of tissues including macrophages (Figure 3A). In contrast to native LDL, oxLDL efficiently promoted foam cell formation in BMDMs of both WT and Angptl4Tg mice (Figure 3B). To quantitatively assess macrophage uptake of oxLDL, we incubated BMDMs from WT and Angptl4Tg mice with [<sup>3</sup>H]COEth-labeled oxLDL or [<sup>3</sup>H]COEth-labeled LDL for 48 hours. Remarkably, uptake of oxLDL was significantly lower in Angptl4Tg compared with that in WT macrophages, whereas uptake of LDL was unaltered (Figure 3C). Consistent with previous data, oxLDL significantly induced expression of its receptor Cd36 and the cellular cholesterol exporter *Abca1* in BMDMs (Figure 3D).<sup>21,26</sup> Interestingly, *Abca1* expression was significantly higher in Angptl4Tg compared with WT macrophages, whereas no difference was observed for Cd36. Expression of *Abcg1* and *Msr1* (scavenger receptor class A member 1) were also not different between WT and Angptl4Tg BMDMs (Figure III in the online-only Data Supplement). Overall, these data indicate that endogenous and exogenous Angptl4 suppress oxLDL uptake in macrophages.

### Impact of Angptl4 Overexpression in Apolipoprotein E\*3-Leiden Mice on Plasma Cholesterol and Triglycerides

To study the potential role of Angptl4 in atherosclerosis, we first ascertained the presence of ANGPTL4 protein in human atherosclerotic plaques. Mouse plaques could not be studied because of lack of suitable antibody. Staining of serial sections from human carotid tissue with antibodies against CD68 and ANGPTL4 revealed colocalization of ANGPTL4 with CD68, suggesting ANGPTL4 is present in macrophages (Figure IV in the online-only Data Supplement).

To explore the role of Angptl4 in atherosclerosis development, we crossbred Angptl4Tg mice with apolipoprotein E (ApoE)\*3-Leiden (E3L) mice. E3L mice represent a unique human-like model for atherosclerosis characterized by plasma cholesterol levels that are proportional to dietary cholesterol content and development of diet-induced atherosclerosis in the presence of the endogenous LDL receptor and ApoE.<sup>27</sup> Both Angptl4Tg.E3L mice and control E3L mice were fed a Western-type diet containing 0.4% cholesterol for 24 weeks. Weight gain was equal between the 2 groups (Figure 4A). In contrast, Angptl4Tg.E3L mice ate slightly less than E3L mice (Figure 4B). After 4 weeks of being fed the Western-type diet, all animals were hypercholesterolemic and plasma cholesterol levels remained high until the end of the study. Importantly, plasma cholesterol levels were not significantly different between the 2 groups (Figure 4C). Whereas plasma triglycerides were increased in Angptl4Tg.E3L mice up to week 4, triglycerides subsequently dropped to levels that were not significantly different from the E3L group (Figure 4D). Elevated plasma cholesterol levels in Angptl4Tg.E3L and E3L mice could be attributed to elevated VLDL/LDL levels, as determined by fast liquid protein chromatography (Figure 4E and 4F). Quantitative polymerase chain reaction analysis



**Figure 4.** Metabolic parameters in Angptl4Tg.E3L and E3L mice. **A**, Body weight of Angptl4Tg.E3L and E3L mice during 24 weeks on a Western diet containing 0.4% cholesterol. **B**, Food intake expressed as grams per day. Plasma cholesterol (**C**) and triglycerides (**D**) levels were measured at the indicated time points. FPLC lipoprotein profiling of pooled plasma collected at 24 weeks. Fractions were assessed for cholesterol (**E**) and triglyceride (**F**) levels. Error bars represent SEM. Differences between E3L and Angptl4Tg.E3L mice were evaluated by Student *t* test (*P* value <0.05). Numbers of animals per group were 14 to 16. E3L indicates E\*3-Leiden; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and VLDL, very-low-density lipoprotein.

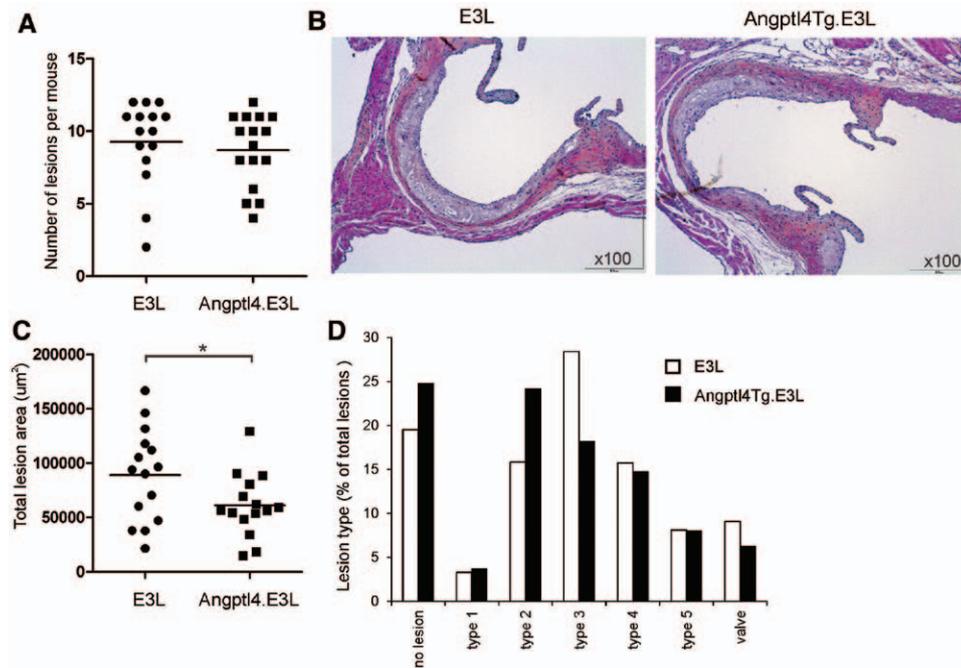
verified that after 24 weeks of feeding Angptl4 mRNA expression was significantly elevated in liver, white adipose tissue, and aorta of Angptl4Tg.E3L mice compared with E3L mice (Figure VA and VB in the online-only Data Supplement).

### Angptl4Tg.E3L Mice Develop Smaller Lesions Than E3L Mice

After 24 weeks of Western-type diet, we investigated atherosclerosis development in the aortic root by measuring the lesion area and lesion types based on cellular composition of the plaques (see Materials and Methods). Whereas Angptl4Tg.E3L mice developed on average an equal number of plaques per cross section compared with E3L mice (Figure 5A), average lesion size expressed as total lesion area was reduced by 34% in Angptl4Tg.E3L mice ( $P < 0.05$ ; Figure 5B and 5C). We next classified lesion types and determined the distribution of lesions according to severity in the 2 groups. Angptl4Tg.E3L mice showed a tendency toward development of less severe lesions (no lesion, type 1 and type 2) compared with E3L mice

(Figure 5D), although the difference did not reach statistical significance.

We next evaluated the effect of Angptl4 overexpression on monocyte recruitment and lesion composition with respect to macrophage content, collagen content, and smooth vascular muscle cell content. Angptl4 overexpression decreased the number of monocytes adhering to the vessel wall compared with E3L mice by 37% ( $P < 0.05$ ; Figure 6A). The decrease in adhering monocytes was accompanied by a 41% decrease in macrophage content in the intima of Angptl4Tg.E3L mice ( $P < 0.05$ ; Figure 6B). The collagen area tended to be reduced ( $-27\%$ ;  $P = 0.07$ ) in Angptl4Tg.E3L mice compared with E3L mice (Figure 6C), and vascular smooth muscle cell area was not different between the 2 groups (Figure 6D). Thus, overexpression of Angptl4 in E3L mice reduces lesion size and leads to a less inflammatory lesion phenotype characterized by decreased monocyte recruitment and macrophage accumulation. Interestingly, no major differences in expression of relevant genes were observed between aortas of E3L and Angptl4Tg.E3L mice, including adhesion molecule



**Figure 5.** Angptl4Tg.E3L mice develop smaller lesions than E3L mice. **A**, Number of lesions per mouse in Angptl4Tg.E3L and E3L mice. Only positive lesions were included in the calculations. **B**, Representative images of mouse atherosclerotic lesions obtained by hematoxylin-phloxin-saffron staining. **C**, Angptl4 overexpression decreased average lesion size. The total lesion area per mouse was calculated as the average of 12 sections (50-µm intervals) per mouse. **D**, Distribution of lesions among different levels of severity expressed as percentage of total lesions. Error bars represent SEM. Differences between E3L and Angptl4Tg.E3L mice were evaluated by Mann-Whitney test (\**P* value <0.05). Numbers of animals per group were 14 to 16. E3L indicates E<sup>3</sup>-Leiden.

Vcam1, metalloproteinase Mmp2, macrophage marker Cd68, chemokine Ccl2 (monocyte chemoattractant protein 1) and its receptor Ccr2, proapoptotic Bax, and antiapoptotic Bcl2 (Figure VB in the online-only Data Supplement).

Consistent with an inhibitory effect of Angptl4 on foam cell formation in E3L mice, unelicited monocytes/macrophages isolated from the peritoneal cavity of Angptl4Tg.E3L mice after 24 weeks of Western-type diet showed virtually no Oil Red O droplets, in contrast to extensive Oil Red O droplets in monocytes/macrophages isolated from E3L mice (Figure 6E).

In the early stages of atherogenesis, monocytes/macrophages are recruited to the vessel wall in response to chemokines such as monocyte chemoattractant protein 1 (Ccl2) produced by the inflamed endothelium.<sup>28</sup> To further investigate the effect of Angptl4 on the chemotactic recruitment of macrophages, we performed an *in vitro* macrophage migration assay. Strikingly, BMDMs from Angptl4Tg mice were unable to migrate toward the chemotactic signal monocyte chemoattractant protein 1 compared with WT macrophages (Figure 6F). These results suggest a suppressive effect of Angptl4 on macrophage migration and chemotaxis. Gene-expression analysis of unstimulated and oxLDL-stimulated BMDMs from WT and Angptl4Tg mice showed that oxLDL mildly induced Ccl2 and markedly suppressed Ccr2 and Il1b expression (Figure VI in the online-only Data Supplement), consistent with a previous study.<sup>29</sup> No major differences in gene expression were observed between WT and Angptl4Tg macrophages. A trend toward reduced expression of Tnf was observed in Angptl4Tg macrophages.

### Angptl4 and MRI-Derived Measurements of Atherosclerosis

Finally, to evaluate the potential role of Angptl4 in atherosclerosis development in humans we determined whether plasma ANGPTL4 levels are associated with carotid atherosclerosis as measured by MRI. Clinical characteristics and MRI results

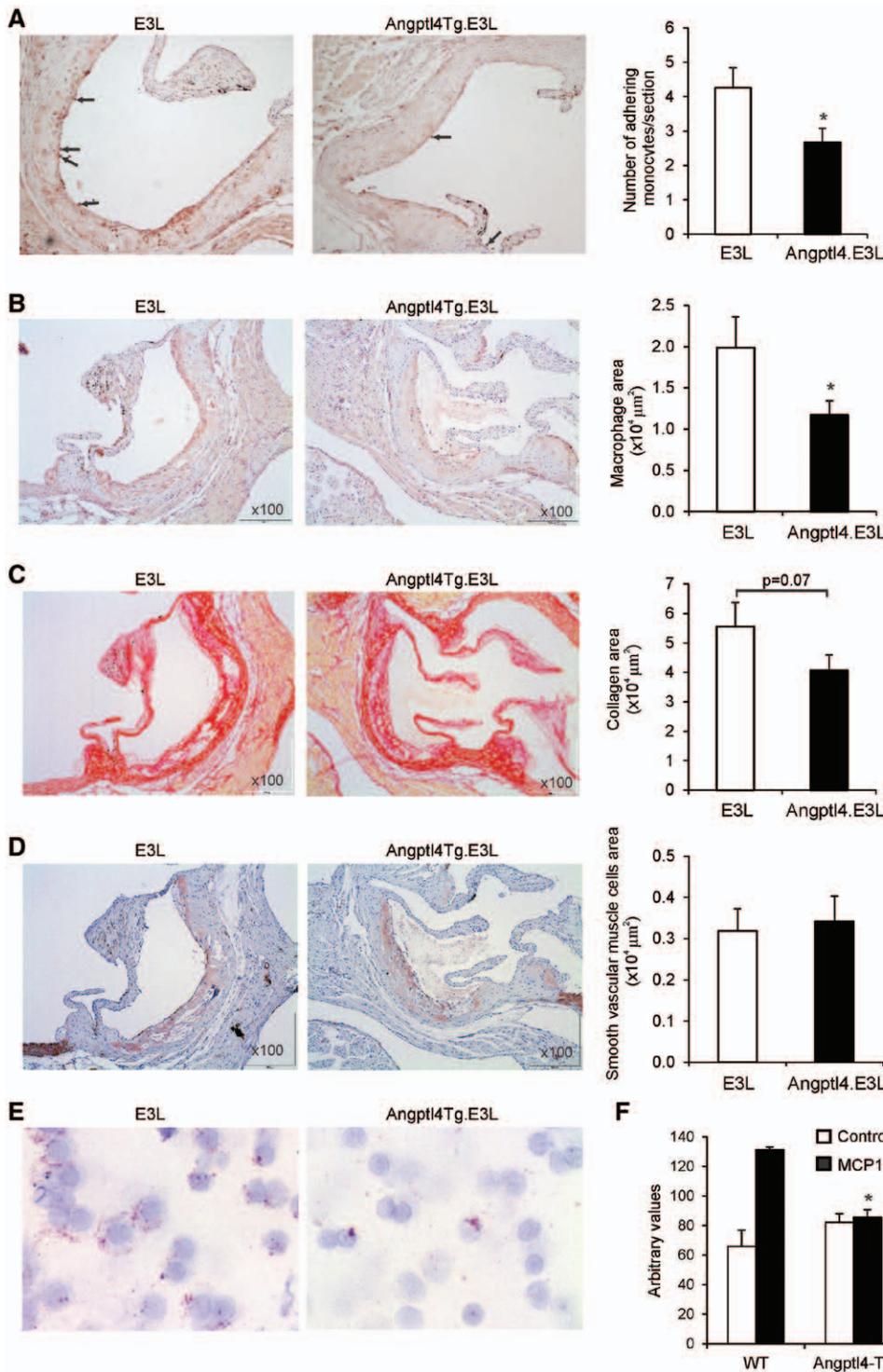
are given in Table II in the online-only Data Supplement. The median plasma ANGPTL4 level was 6.75 ng/mL and ranged from 3.30 to 13.40 ng/mL. Median (inter quartile range) common maximal vessel-wall thickness was 1.50 mm and median (inter quartile range) common vessel-wall area was 1.68 cm<sup>2</sup>.

Remarkably, plasma ANGPTL4 showed a significant negative correlation with an early marker of the degree of focal plaque formation measured by common maximal vessel-wall thickness ( $r=-0.242$ ,  $P=0.041$ ; Figure VII in the online-only Data Supplement). Multiple regression analysis with common maximal vessel-wall thickness as dependent variable showed that the association with ANGPTL4 was still significant ( $\beta -0.226$ ;  $P=0.045$ ) after correcting for age, smoking, blood pressure, waist circumference, glucose, high-density lipoprotein cholesterol, triglycerides, and hsCRP. The model explained 26% of variance of the common maximal vessel-wall thickness. No relation was observed between plasma ANGPTL4 and common vessel-wall area ( $r=-0.126$ ;  $P=0.292$ ).

### Discussion

The data presented show that Angptl4 reduces foam cell formation and decreases atherosclerosis in atherosclerosis-prone E3L mice. This effect was not caused by reduction of plasma cholesterol and triglycerides, because levels were similar between the 2 groups. Importantly, Angptl4Tg.E3L mice exhibited a less proinflammatory phenotype, with decreased accumulation of monocytes/macrophages in the atherosclerotic plaque, suggesting an anti-inflammatory role of Angptl4 in atherosclerosis development. Finally, we found that plasma Angptl4 is negatively associated with carotid artery sclerosis measured by 3-T MRI in subjects with the metabolic syndrome and low-grade systemic inflammation.

The impact of Angptl4 on atherosclerosis has been previously investigated.<sup>30</sup> In that study Angptl4<sup>-/-</sup> mice on an ApoE<sup>-/-</sup> background developed less atherosclerotic lesions on a chow diet compared with control mice. It should be realized



**Figure 6.** Angptl4Tg.E3L mice show decreased monocyte recruitment and altered plaque composition compared with E3L mice. Representative images (left) and quantification (right) of numbers of monocytes adhering to the endothelial wall per section (arrows; **A**), macrophage area (brown staining; **B**), collagen area (red staining; **C**), and vascular smooth muscle cell area (brown staining) in the atherosclerotic plaque (**D**). **E**, Representative images of Oil Red O staining of peritoneal unelicited monocytes/macrophages from E3L and Angptl4Tg.E3L mice after 24 weeks being fed a Western-type diet. **F**, Quantification of DNA-binding fluorescent dye (CyQuant GR Dye) from bone marrow-derived macrophages migrated toward the chemotactic signal monocyte chemoattractant protein 1 (MCP-1) or control (PBS). Error bars represent SEM. Differences between E3L and Angptl4Tg.E3L mice were evaluated by Student *t* test (\**P* value <0.05). Numbers of animals per group were 14 to 16. E3L indicates E<sup>3</sup>-Leiden.

that ApoE<sup>-/-</sup> mice are characterized by a severe deficiency in clearance of VLDL remnants, develop severe atherosclerosis from birth, and have impaired innate immunity. In contrast, E3L mice only develop hyperlipoproteinemia after being fed a diet rich in fat and cholesterol,<sup>19</sup> which we believe better mimics the lifestyle-dependent development of atherosclerosis in humans. Angptl4<sup>-/-</sup> mice on ApoE<sup>-/-</sup> background exhibited a decrease in circulating LDL-cholesterol and triglyceride levels, which very likely accounted for the improvement in atherosclerosis in that model. In our study on ad libitum fed

mice, plasma VLDL/LDL-cholesterol, total cholesterol, and plasma triglycerides were minimally affected in Angptl4Tg.E3L mice compared with E3L mice after several weeks of feeding the atherogenic diet. The minor elevation of levels of plasma triglycerides and cholesterol in Angptl4Tg.E3L mice is consistent with the relative minor effect of Angptl4 overexpression on plasma triglyceride and cholesterol levels in fed mice, as opposed to fasted mice.<sup>10,12</sup> Interestingly, the reduction in hypertriglyceridemia in Angptl4Tg.E3L mice on starting the Western-type diet coincided with the

development of hypercholesterolemia. Recently, it was shown that triglyceride-rich lipoproteins may interfere with the ability of Angptl4 to inhibit LPL,<sup>31</sup> a property that may also extend to LDL. Accordingly, the hypercholesterolemia in the ApoE3Leiden transgenic model may interfere with the effect of Angptl4 on circulating triglycerides levels. In contrast, such a mechanism may be expected to have minimal effect on macrophage Angptl4 action in the atherosclerotic plaque.

Because the slight increase in VLDL levels would be expected to increase rather than decrease atherosclerosis, Angptl4 overexpression apparently reduces atherosclerosis via a mechanism independent of its effect on plasma lipid levels. Previously, we have shown that Angptl4 dramatically reduced foam cell formation in peritoneal macrophages incubated with triglyceride-rich lipoproteins.<sup>8</sup> The present study indicates Angptl4 also inhibits oxLDL uptake by macrophages, presumably via a direct and an indirect mechanism: (1) by directly inhibiting oxLDL uptake by macrophages; and (2) by inhibiting lipid loading from triglyceride-rich lipoproteins and associated activation of macrophages, leading to downregulation of subsequent oxLDL uptake.

Previously, it was found that externally added and endogenously produced LPL enhance binding and uptake of oxLDL in macrophages.<sup>32</sup> Accordingly, it is plausible that Angptl4 inhibits oxLDL uptake via its effect on LPL. Supporting a stimulatory effect of macrophage LPL on atherosclerosis in vivo, macrophage-specific overexpression of LPL stimulated the formation of atherosclerotic lesions and accumulation of macrophage-derived foam cells, which occurred in the absence of any changes in circulating lipoproteins,<sup>17,18,33,34</sup> whereas the opposite was observed in macrophage-specific LPL knockout mice.<sup>35,36</sup> Transgenic mice expressing catalytically active or inactive LPL were used to show that the noncatalytic bridging function of LPL is sufficient for its proatherogenic effect.<sup>37</sup> Whereas Angptl4 was shown to potently inhibit macrophage LPL catalytic activity,<sup>8</sup> it is unclear whether Angptl4 inhibits the bridging function of LPL. In the present study we found that Angptl4 more effectively reduced uptake of oxLDL compared with orlistat, which suggests that Angptl4 reduces oxLDL uptake via a combination of lipolysis-dependent and -independent mechanisms. Expression of oxLDL receptors Cd36 and Msr1 was not different between Angptl4Tg and WT macrophages. Interestingly, expression of Abca1 was higher in Angptl4Tg and WT macrophages, indicating a potential effect of Angptl4 on cholesterol efflux. However, additional studies are required to better delineate the mechanisms of Angptl4 action in macrophages.

Besides lowering oxLDL uptake, Angptl4 may reduce atherosclerosis by reducing chemotaxis. Specifically, we found that Angptl4 overexpression led to decreased accumulation of monocytes/macrophages in the atherosclerotic plaque. Additionally, Angptl4Tg macrophages exhibited a decreased chemotactic response in an in vitro migration assay. No major differences in expression of chemotactic and inflammatory genes were observed between WT and Angptl4Tg macrophages at baseline or on stimulation with oxLDL. In addition, no in vivo effect of Angptl4 overexpression could be observed on expression of chemotaxis and adhesion-related genes in

aortas. Angptl4 thus might influence chemotaxis independently of any changes in gene expression.

Evidence has been provided that oxLDL may trigger inflammatory signaling in mouse macrophages via a mechanism involving TLR4.<sup>38,39</sup> Because TLR4 activation by LPS reduced Angptl4 expression in mouse macrophages, it is unlikely that the stimulatory effect of oxLDL and lipid emulsion on Angptl4 expression is mediated by TLR4. Instead, induction of Angptl4 by oxLDL may occur via PPARs, which are potent activators of Angptl4 expression in macrophages and are activated by oxidized lipoproteins or their component oxidized lipids.<sup>8,20,21</sup> We found that activation of especially PPAR $\delta$  led to a dramatic induction of ANGPTL4 protein and mRNA in THP-1 macrophages. At the functional level, induction of Angptl4 in intimal macrophages by oxidized lipoproteins may be a protective mechanism to reduce foam cell formation and mitigate anti-inflammatory responses.

Expression of Angptl4 in mouse RAW 264.7 macrophages was markedly reduced by TLR2/6, TLR3 and TLR4 agonists, as well as by the NOD2 agonist muramyl dipeptide. These results are consistent with a recent study showing suppression of Angptl4 mRNA by LPS, zymosan, polyI:C, and imiquimod. How TLR activation leads to downregulation of Angptl4 expression requires further study.

In conclusion, the present study reveals a protective role of Angptl4 in atherosclerosis development, which is independent of changes in levels of plasma lipoproteins. Furthermore, the study suggests an inhibitory effect of Angptl4 on macrophage oxLDL uptake and chemotaxis. We postulate that stimulation of Angptl4 gene expression in macrophages by oxLDL may be part of a protective feedback mechanism aimed at minimizing lipid overload.

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## Disclosures

None.

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## Significance

Angiopietin-like 4 (Angptl4) is a secreted factor that reduces lipid uptake in cells by inhibiting the enzyme lipoprotein lipase. Macrophages are immune cells that accumulate in atherosclerotic plaques and take up oxidized low-density lipoprotein to become foam cells. This article shows that oxidized low-density lipoprotein stimulates Angptl4 production by macrophages. Angptl4 protein was detectable in human atherosclerotic lesions and localized to macrophages. Overexpression of Angptl4 in an atherosclerosis-prone mouse model suppresses development of atherosclerosis. It is concluded that stimulation of Angptl4 in macrophages by oxidized low-density lipoprotein protects against excess lipid uptake and foam cell formation.