Lymphatic Vessel Insufficiency in Hypercholesterolemic Mice Alters Lipoprotein Levels and Promotes Atherogenesis

Taina Vuorio, Harri Nurmi, Karen Moulton, Jere Kurkipuro, Marius R. Robciuc, Miina Öhman, Suvi E. Heinonen, Haritha Samaranayake, Tommi Heikura, Kari Alitalo, Seppo Ylä-Herttuala

Objective—Lymphatic vessels collect extravasated fluid and proteins from tissues to blood circulation as well as play an essential role in lipid metabolism by taking up intestinal chylomicrons. Previous studies have shown that impairment of lymphatic vessel function causes lymphedema and fat accumulation, but clear connections between arterial pathologies and lymphatic vessels have not been described.

Approach and Results—Two transgenic mouse strains with lymphatic insufficiency (soluble vascular endothelial growth factor 3 [sVEGFR3] and Chy) were crossed with atherosclerotic mice deficient of low-density lipoprotein receptor and apolipoprotein B48 (LDLR−/−/ApoB100/100) to study the effects of insufficient lymphatic vessel transport on lipoprotein metabolism and atherosclerosis. Both sVEGFR3×LDLR−/−/ApoB100/100 mice and Chy×LDLR−/−/ApoB100/100 mice had higher plasma cholesterol levels compared with LDLR−/−/ApoB100/100 control mice during both normal chow diet (16.3 and 13.7 versus 8.2 mmol/L, respectively) and Western-type high-fat diet (eg, after 2 weeks of fat diet, 45.9 and 42.6 versus 30.2 mmol/L, respectively). Cholesterol and triglyceride levels in very-low-density lipoprotein and low-density lipoprotein fractions were increased. Atherosclerotic lesions in young and intermediate cohorts of sVEGFR3×LDLR−/−/ApoB100/100 mice progressed faster than in control mice (eg, intermediate cohort mice at 6 weeks, 18.3% versus 7.7% of the whole aorta, respectively). In addition, lesions in sVEGFR3×LDLR−/−/ApoB100/100 mice and Chy×LDLR−/−/ApoB100/100 mice had much less lymphatic vessels than lesions in control mice (0.33% and 1.07% versus 7.45% of podoplanin-positive vessels, respectively).

Conclusions—We show a novel finding linking impaired lymphatic vessels to lipoprotein metabolism, increased plasma cholesterol levels, and enhanced atherogenesis. (Arterioscler Thromb Vasc Biol. 2014;34:1162-1170.)

Key Words: atherosclerosis ■ lipoproteins ■ lymphatic vessels

Cardiovascular complications caused by atherosclerosis, such as coronary artery disease and stroke, are major health problems in the Western world. Several factors contribute to atherogenesis, but its main causes are elevated levels of low-density lipoprotein (LDL) and low levels of high-density lipoprotein resulting from genetic factors and excessive consumption of dietary fat. The primary characteristics of atherosclerotic lesions are intimal accumulation of cholesterol-rich macrophages and proliferation of smooth muscle cells.1-3 In addition, some advanced lesions stimulate proliferation of vasa vasorum that arise from the adventitia and invade into the vessel wall. The increased vascularity around atheromas is hypothesized to provide oxygen and nutrients and conduits for recruitment of inflammatory cells to the expanding vessel wall. However, they can also lead to hemorrhages that make lesions more prone to rupture.4-6

Lymphatic vessels are responsible for collecting and transporting protein-rich extravasated fluid from tissues to blood circulation and are required for chylomicron absorption from the intestinal microvillus. In addition, they participate in inflammatory reactions by transporting antigens and inflammatory cells to lymph nodes and sites of inflammation.7,8 Vascular endothelial growth factor receptor 3 (VEGFR3) is a receptor for vascular endothelial growth factor (VEGF)-C and VEGF-D and a key mediator of lymphangiogenesis and maintenance of lymphatic endothelium.9,10 VEGFR3 is required for normal vascular development,11 but its postnatal expression is mainly restricted to lymphatic vessels.12

We have here studied effects of high-fat feeding on serum lipids and atheroma formation in mouse models of lymphatic dysfunction. Soluble VEGFR3 (sVEGFR3) mice have impaired lymphatic vessels in the skin and some more distant...
organs because they secrete, under the control of the basal keratinocyte K14 promoter, a chimeric fusion protein that consists of the ligand-binding portion of VEGFR3 extracellular domain and the fragment crystallizable domain of immunoglobulin γ-chain.13 Chy mice have impaired lymphangiogenesis attributable to an inactivating point mutation in the VEGFR3 gene.14 Crossing the mice with atherogenic mice deficient of low-density lipoprotein receptor and apolipoprotein B48 (LDLR−/−/ApoB100/100)15 showed that sVEGFR3×LDLR−/−/ApoB100/100 mice and Chy×LDLR−/−/ApoB100/100 mice have increased cholesterol levels leading to accelerated atherogenesis especially in young and intermediate mouse cohorts. Our results suggest that lymphatic vessels have an important role in maintaining proper lipoprotein metabolism and vascular homeostasis.

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

Results
Effects of Western Diet on Blood Lipid Levels in Mice With Dysfunctional Lymphatic Vessels
Weight gain and food consumption were similar between the Chy mice and their wild-type littermates during the 14-week feeding period on a Western diet (data not shown). Dual-energy x-ray absorptiometry analysis demonstrated a modest but significant reduction of fat mass in Chy mice compared with wild-type littermates (11.6±1.1 versus 15.1±0.8 g; P<0.03). Serum cholesterol levels were significantly higher in Chy mice, both on chow and Western diet (Figure 1A). However, no significant differences in the triglyceride levels were observed between the mice of the 2 genotypes (Figure 1B). The sVEGFR3 mice and their wild-type littermates had similar body weight and food consumption after 14 weeks on Western diet. No significant changes in total cholesterol or triglycerides (Figure 1C and 1D) levels were observed between the genotypes although a small trend toward higher cholesterol values was apparent in the sVEGFR3 animals.

Characterization of sVEGFR3×LDLR−/−/ApoB100/100 Mice and Chy×LDLR−/−/ApoB100/100 Mice
sVEGFR3×LDLR−/−/ApoB100/100 mice were viable and fertile, and there was no difference in weight gain compared with controls. sVEGFR3 protein was present in blood plasma in detectable amounts (Figure 2A). Chy×LDLR−/−/ApoB100/100 mice had a normal lifespan and weight but remarkably smaller litter sizes (3.8 pups/litter) and a lower frequency of the mutant allele (17% of born pups). In addition, ≈10% of the Chy×LDLR−/−/ApoB100/100 pups developed severe ascites in the abdomen and needed to be euthanized at weaning.

Lymphatic vessels were absent in the skin of sVEGFR3×LDLR−/−/ApoB100/100 mice and Chy×LDLR−/−/ApoB100/100 mice. In particular, sVEGFR3×LDLR−/−/ApoB100/100 mice had swollen feet and tails (Figure 2B and 2C) compared with LDLR−/−/ApoB100/100 mice (Figure 2D and 2E), which indicated accumulation of fluid and fat in the tissues. Lymphatic function was investigated after Evans Blue dye injections into footpads. In sVEGFR3×LDLR−/−/ApoB100/100 mice, transport of Evans Blue from feet to collecting lymphatic vessels was inhibited (Figure 2F) and no blue dye was visible in the thoracic duct 30 minutes after the injection. Transport of Evans Blue dye was normal in LDLR−/−/ApoB100/100 mice (Figure 2G). The old cohort of sVEGFR3×LDLR−/−/ApoB100/100 mice on Western diet had accumulation of inflammatory cells in the livers (Figure 2H), such cells were not present in the livers of Chy×LDLR−/−/ApoB100/100 mice or LDLR−/−/ApoB100/100 control mice (Figure 2I). These inflammatory cells were mostly CD3-positive T cells that accumulated in large quantities around the central veins (Figure 2I). In addition, some sVEGFR3×LDLR−/−/ApoB100/100 mice had swelling in their jaws, with histological evidence of fat tissue and inflammatory giant cells in their neck area (Figure 2K).
Analysis of Metabolic Factors in sVEGFR3×LDLR−/−/ApoB100/100 Mice and Chy×LDLR−/−/ApoB100/100 Mice

Cholesterol and triglyceride levels in blood plasma were measured to determine the effect of impaired lymphatic function on blood lipids. Cholesterol levels (Table) were significantly elevated in sVEGFR3×LDLR−/−/ApoB100/100 mice (16.3±1.2 mmol/L) and Chy×LDLR−/−/ApoB100/100 mice (13.7±0.8 mmol/L) on chow diet compared with LDLR−/−/ApoB100/100 control mice (8.2±0.4 mmol/L). Significantly higher cholesterol levels were maintained in sVEGFR3×LDLR−/−/ApoB100/100 mice and in Chy×LDLR−/−/ApoB100/100 mice after starting the Western diet (Table). Triglyceride levels were also significantly higher in sVEGFR3×LDLR−/−/ApoB100/100 mice before Western diet and Chy×LDLR−/−/ApoB100/100 mice (13.7±0.8 mmol/L) on chow diet compared with LDLR−/−/ApoB100/100 control mice (8.2±0.4 mmol/L). Significantly higher cholesterol levels were maintained in sVEGFR3×LDLR−/−/ApoB100/100 mice and in Chy×LDLR−/−/ApoB100/100 mice after starting the Western diet (Table). Triglyceride levels were also significantly higher in sVEGFR3×LDLR−/−/ApoB100/100 mice before Western diet.

Table. Cholesterol and Triglyceride Levels of sVEGFR3×LDLR−/−/ApoB100/100 Mice, Chy×LDLR−/−/ApoB100/100 Mice, and LDLR−/−/ApoB100/100 Control Mice on Chow (Week 0) and Western Diet for 2, 4, 6, and 12 Weeks

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<td>Cholesterol, mmol/L</td>
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<tr>
<td>sVEGFR3×LDLR/ApoB</td>
<td>16.3±1.2 (19)*</td>
<td>45.9±3.0 (20)*</td>
<td>58.8±5.2 (11)*</td>
<td>50.8±4.4 (16)†</td>
<td>52.7±8.7 (7)</td>
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<td>Chy×LDLR/ApoB</td>
<td>13.7±0.8 (5)‡</td>
<td>42.6±0.7 (5)‡</td>
<td>46.3±8.1 (4)</td>
<td>53.5±10.5 (3)</td>
<td>53.5±3.1 (2)</td>
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<td>LDLR/ApoB</td>
<td>8.2±0.4 (21)</td>
<td>30.2±1.2 (21)</td>
<td>31.8±4.8 (14)</td>
<td>36.9±3.0 (20)</td>
<td>46.0±3.0 (9)</td>
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<td>Triglycerides, mmol/L</td>
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<tr>
<td>sVEGFR3×LDLR/ApoB</td>
<td>1.9±0.1 (20)§</td>
<td>2.6±0.3 (21)</td>
<td>4.3±0.8 (12)</td>
<td>3.8±0.8 (16)</td>
<td>4.2±1.9 (7)</td>
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<tr>
<td>Chy×LDLR/ApoB</td>
<td>1.6±0.1 (5)</td>
<td>3.4±0.5 (5)</td>
<td>2.3±0.7 (4)</td>
<td>4.3±1.9 (3)</td>
<td>3.1±0.6 (2)</td>
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<tr>
<td>LDLR/ApoB</td>
<td>1.4±0.1 (21)</td>
<td>2.9±0.3 (21)</td>
<td>2.5±0.4 (10)</td>
<td>3.3±0.4 (19)</td>
<td>2.5±0.4 (9)</td>
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Values are mean±SEM. No. of animals is marked in parenthesis. Values from sVEGFR3×LDLR−/−/ApoB100/100 mice and Chy×LDLR−/−/ApoB100/100 mice are compared with values from LDLR−/−/ApoB100/100 control mice at the same time point. Values are combined from all age cohorts and both sexes. Conversion factors from mmol/L to mg/dL for cholesterol and triglycerides are 38.67 and 88.57, respectively. ApoB indicates apolipoprotein B; LDLR, low-density lipoprotein receptor; and sVEGFR3 indicates soluble vascular endothelial growth factor 3.

*P<0.0001.
†sVEGFR3×LDLR−/−/ApoB100/100 mice, P<0.05.
‡Chy×LDLR−/−/ApoB100/100 mice, P<0.05.
§P<0.001.
compared with controls (Table). To assess the distribution of cholesterol and triglycerides in different lipoprotein fractions, lipoprotein profiles were determined by fast protein liquid chromatography analysis of plasma samples from sVEGFR3×LDLR−/−/ApoB100/100 mice and LDLR−/−/ApoB100/100 control mice after 6 weeks on Western diet. sVEGFR3×LDLR−/−/ApoB100/100 mice had higher levels of cholesterol (Figure 3A) and triglycerides (Figure 3B) in very-low-density lipoprotein and LDL fractions when compared with control mice. Both groups had low levels of cholesterol and triglycerides in high-density lipoprotein fraction.

To further analyze lipid metabolism in sVEGFR3×LDLR−/−/ApoB100/100 mice, intestinal triglyceride absorption was measured with radioactive triolein either with or without the lipoprotein lipase inhibitor Triton WR1339. After lipid bolus, radioactivity in plasma did not differ between sVEGFR3×LDLR−/−/ApoB100/100 mice and LDLR−/−/ApoB100/100 control mice (Figure IA in the online-only Data Supplement). To test whether the lipoprotein uptake in the liver is altered, LDL turnover was analyzed. sVEGFR3×LDLR−/−/ApoB100/100 mice tended to have a slower clearance rate of 125I-LDL than controls (75.1% versus 64.5% of 125I-LDL remaining in the blood 12 hours after the injection, respectively). However, the difference was not statistically significant. The half-life of 125I-LDL was around 24 hours in both groups (Figure IB in the online-only Data Supplement). To identify differences in high-density lipoprotein functions, in vivo reverse cholesterol transport (RCT) rates were compared between these 2 strains after injection of macrophages labeled with 3H-cholesterol in the peritoneum. In vivo RCT was similar in both groups (Figure IC in the online-only Data Supplement). In addition, the effect of impaired lymphatic vessels on glucose metabolism was analyzed with glucose tolerance tests. Both sVEGFR3×LDLR−/−/ApoB100/100 mice and Chy×LDLR−/−/ApoB100/100 mice had similar fasting glucose levels compared with controls, and plasma glucose levels returned to basal levels 60 minutes after the glucose injection in all groups (Figure ID in the online-only Data Supplement).

Progression of Atherosclerosis in sVEGFR3×LDLR−/−/ApoB100/100 Mice and Chy×LDLR−/−/ApoB100/100 Mice

Atherosclerotic lesions were measured from young, intermediate, and old mouse cohorts fed Western diet for 3 to 12 weeks as the percent area of lesions in the entire aorta and by the mean cross-sectional area of intima at the aortic root. sVEGFR3×LDLR−/−/ApoB100/100 mice had increased areas of atherosclerotic lesions in en face opened aortas compared with controls and reached statistical significance in the intermediate cohort on Western diet for 6 weeks (Figure 4A–4C). Intimal lesion areas in the aortic roots were also generally larger in sVEGFR3×LDLR−/−/ApoB100/100 mice and reached statistical significance compared with controls in the young and intermediate cohorts on Western diet for 12 and 2 weeks, respectively (Figure 4D–4F). Atherosclerotic lesion size was also increased in Chy×LDLR−/−/ApoB100/100 mice. Lesion development was compensated with outward remodeling in sVEGFR3×LDLR−/−/ApoB100/100 mice because the area within the internal elastic lamina was increased at the latest time point in young cohort and at all time points in intermediate cohort of sVEGFR3×LDLR−/−/ApoB100/100 mice compared with controls (Figure 4G–4I). In the old cohort, area within internal elastic lamina was similar between groups. Differences in the lesion sizes can be easily visualized in the en face opened aortas (Figure 4J and 4K) and hematoxylin–eosin stained cross sections (Figure 4L–4N, respectively).
The impact of aging and Western diet on atherosclerotic lesion composition was examined on modified Movat stains of aortic cross sections of intermediate cohort of sVEGFR3×LDLR−/−/ApoB100/100 mice and LDLR−/−/ApoB100/100 control mice after 2, 6, and 12 weeks of Western diet. Lesions from sVEGFR3×LDLR−/−/ApoB100/100 mice contained cholesterol crystals as early as 2 weeks on Western diet, whereas lesions developed more slowly in control LDLR−/−/ApoB100/100 mice and consisted mainly of foam cells ≤6 weeks on Western diet (Figure 5A–5D). After 12 weeks of Western diet, many lesions in LDLR−/−/ApoB100/100 mice contained atheromatous cores, whereas lesions in sVEGFR3×LDLR−/−/ApoB100/100 mice still had some cellularity (Figure 5E and 5F). Results were also confirmed with immunohistochemical staining for macrophages (Figure 5G and 5H). Intermediate cohorts of sVEGFR3×LDLR−/−/ApoB100/100 mice and LDLR−/−/ApoB100/100 seemed to have similar quantities of lipids in the lesions (Figure 5I and 5J). Lesions of young cohort of Chy×LDLR−/−/ApoB100/100 mice after 12 weeks on Western diet showed cholesterol crystals and some foam cells (Figure 5K and 5L). Plaque neovascularization and lymphatic vessels associated with atherosclerotic lesions in the descending aortas were visualized by confocal microscopy after intravenous injection of glucan synthase-like 1 lectin in vivo, followed by in situ staining with antibodies reactive for podoplanin, a marker on lymphatics. CD31 was expressed on both blood vessels and lymphatics, but only blood perfused blood vessels stained positive for glucan synthase-like 1. Lymphatic vessels stained positive for podoplanin and CD31 but excluded hematogenously delivered glucan synthase-like 1 lectin (Figure 6). Atherosclerotic regions in the descending aortas from all genotype groups had similar densities of plaque-associated neovascularization (sVEGFR3×LDLR−/−/ApoB100/100 median, 11.2% area of glucan synthase-like 1 lectin+vessels per high power field; range, 4.36–21.34; Chy×LDLR−/−/ApoB100/100 median, 9.18%; range, 4.03–17.2; and LDLR−/−/ApoB100/100 controls median, 8.20%; range, 2.45–17.5; Kruskal–Wallis, P=0.5491; Dunn post-test comparison between groups, P>0.05; Figure 6D). Despite their similar or greater extents of atherosclerosis, lymphatic vessels were less abundant in the adventitia around atheromas.
of sVEGFR3×LDLR−/−/ApoB100/100 mice (median, 0.33% area podoplanin+vessels per high power field; range, 0.09–1.33; Kruskal–Wallis, \( P = 0.0014 \); Dunn post-test comparison, \( P < 0.05 \)) and Chy×LDLR−/−/ApoB100/100 mice (median, 1.07%; range, 0.04–4.36; \( P < 0.05 \)) compared with controls (median, 7.45%; range, 0.12–19.59; Figure 6D).

**Discussion**

This study showed that 2 different mouse strains with impaired lymphatic vessel function develop increased cholesterol levels and atherosclerosis when bred into an atherosclerotic background. Indeed, lymphatic vessels are not only required for lipid absorption from the intestine, but may have systemic effects on circulating levels of lipoproteins, inflammatory reactions in peripheral tissues, and atherosclerosis.1,5

In normal physiological situations, chylomicrons are taken up from intestinal enterocytes into lacteals, small lymphatic vessels in the mesentery, and transported to blood circulation via the thoracic duct.16 In addition, lymphatic vessels are involved in endogenous lipoprotein metabolism as extravasated lipoproteins are taken back to blood through lymphatic vessels.17 Peripheral lymph contains all classes of lipoproteins, but concentrations of LDL and very-low-density lipoprotein are significantly lower than in plasma, suggesting that these lipoproteins are cleared before returning to blood.18–20 If the function of lymphatic vessels is impaired either in experimental animals13,14,21 or in humans with hereditary conditions or surgical procedures,22,23 adipose tissue is expanded and tissues develop lymphedema. Emerging evidence now indicates that lymphatic vessels have roles in lipid transport, RCT, and in modulating adipose tissue, but knowledge of their functions in hypercholesterolemia and atherosclerosis is limited.24 Both sVEGFR3×LDLR−/−/ApoB100/100 mice13 and Chy×LDLR−/−/ApoB100/100 mice,14 which have impaired lymphatic function especially in their skin and some developmental lymphatic defects in other parts of the body, developed higher plasma cholesterol on a Western-type high-fat diet compared with controls in the nonatherosclerotic background and atherosclerosis-prone LDLR−/−/ApoB100/100 background. It should be noted that the lipoprotein profiles of the mice used in this study and in other studies by our group25 differ from originally published lipoprotein profiles of LDLR−/−/ApoB100/100 mice.15 Mice used in this study have clearly a hypercholesterolemic phenotype and higher levels of cholesterol especially in very-low-density lipoprotein–sized particles and lower levels of cholesterol in LDL-sized particles than in the original publication.15 This difference could be attributable to the natural transgenic drift of the mouse strains, use of the different background strain, or differences in the lipoprotein analysis methods. However, these differences do not explain the main findings of this study because adequate controls have been used throughout the experiments.

Lipid absorption, LDL turnover, and in vivo RCT after intraperitoneal injection of labeled macrophages were not
different between sVEGFR3×LDLR−/−/ApoB100/100 mice and LDLR−/−/ApoB100/100 control mice. Thus, sVEGFR3×LDLR−/−/ApoB100/100 mice seemed to have no changes in lipoprotein absorption from the intestine or uptake by the liver when compared with LDLR−/−/ApoB100/100 control mice. Other studies have shown that RCT is reduced when cholesterol-labeled macrophages were injected in the skin of Chy mice or into distal extremities or peripheral tissues where lymphatics were interrupted. It is possible that the RCT rates from intraperitoneal macrophages into plasma were not significantly altered in sVEGFR3×LDLR−/−/ApoB100/100 mice compared with controls because peritoneal lymphatics were not sufficiently impaired by sVEGFR3. In addition, because hyperlipidemia itself has shown to alter lymphatic vessels in hypercholesterolemic apolipoprotein E–deficient mice, dyslipidemic conditions may have affected lymphatic function also in control mice in this study and obtunded RCT differences. On the contrary, VEGF-A and VEGF-B can alter genes involved in lipoprotein metabolism and lipid uptake. Such effects have not been reported for VEGF-C, but if present, then sVEGFR3 might have altered cholesterol and lipoprotein metabolism directly. Therefore, future studies, such as analyzing the lipoprotein production from the liver and evaluating the expression of genes related to lipid metabolism, are needed to clarify the mechanisms how the deficient lymphatic vessels affect hypercholesterolemia and atherosclerosis.

Because lymphatic vessels actively participate in immune cell trafficking and adaptive immunity, attenuated function of lymphatic vessels might alter inflammatory reactions and have secondary effects on atherosclerosis and lipid metabolism. Older sVEGFR3×LDLR−/−/ApoB100/100 mice developed massive infiltrations of inflammatory cells in their livers and giant cells in their neck area, whereas livers in old Chy×LDLR−/−/ApoB100/100 mice resembled livers of control mice. Accumulation of fat in the liver may further activate proinflammatory cytokines (eg, tumor necrosis factor-α) and reactive oxygen species that augment vascular inflammation.

Recent studies have shown that although Chy mice have normal immune cell transport from the skin to lymph nodes, K14-VEGFR3-immunoglobulin mice have impaired dendritic cell trafficking and B-cell function in the skin and altered systemic T-cell ratios, and 1-year-old K14-VEGFR3-immunoglobulin mice have many signs of autoimmunity. Depletion of regulatory T cells alone can increase plasma cholesterol levels and induce an atherogenic lipoprotein profile. These results indicate that abnormal lymphatic function could have systemic effects on inflammatory cell homing and lipoprotein metabolism that promote atherosclerosis.

Although systemic changes in hypercholesterolemia or immune functions may account for the increased atherosclerosis in mice with deficient lymphatic vessels, impaired lymphatic functions in aortic plaques could have exerted a local effect on plaque growth. An important step in atherosclerotic progression is angiogenesis and growth of vasa vasorum. Intravital microscopy studies of atherosclerotic mice have demonstrated that plaque-associated vessels function as more efficient conduits for inflammatory cell recruitment compared with the arterial endothelium overlying atheromas. Angiogenesis is
driven by several factors (e.g., VEGF) produced as a response to hypoxia and inflammatory stimuli. In addition to arterial and venous microvessels, lymphatic vessels develop in the adventitia of large arteries. 

Macrophages in human atherosclerotic lesions express lymphangiogenic factors VEGF-C and VEGF-D and their receptor VEGFR3, and lymphatic growth occurs in the human arteries with calcified and fibrous atherosclerotic plaques. In this study, the density of plaque-associated neovascularization imaged en face from the adventitial surface by confocal microscopy remained at similar levels in all mouse strains, but lymphatic vessels were less abundant in vascularized atherosclerotic plaques of LDLR−/−/ApoB100/100 mice. 

Mice rarely contain CD31+ blood vessels or vasa vasorum in the aorta, whereas the experimental design of aorta RCT studies used LDLR−/−/ApoB100/100 mice or Chy×LDLR−/−/ApoB100/100 mice compared with control LDLR−/−/ApoB100/100 mice. 

The study by Martel et al observed absorptive lymphatics in transverse sections of the aortic sinus and descending aorta and noted the lymphatics weaved in and out of adjacent adipose tissue and were not absent in Chy mice. The Chy mice however were not evaluated in the atherosclerotic background. 

Methods of study and vascular immune responses that are involved during both progression and regression phases of atherosclerosis. In conclusion, we show a novel association among impaired lymphatic vessels, lipoprotein metabolism, and increased atherogenesis. These results support the emerging view that lymphatic vessels play an important role in regulating lipoprotein metabolism and vascular biology and may provide new options for the treatment of lipid-related diseases. 

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Disclosures 

None. 

References 

Increasing evidence suggests that lymphatic vessels play an important role in the lipoprotein metabolism. Here, we show that deficiency of lymphatic vessels affects cholesterol and triglyceride levels and accelerates the development of atherosclerosis in mice. Further understanding of lymphatic functions in lipoprotein metabolism may reveal new avenues for the treatment of vascular diseases.
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Supplemental Figure I. Lipid and glucose metabolism in the young cohorts of sVEGFR3 x LDLR-/-/ApoB<sup>100/100</sup> mice and LDLR-/-/ApoB<sup>100/100</sup> mice on Western diet. Both genders were used in the analysis. A) Lipid absorption after Triton WR1339 injection and lipid bolus. Radioactivity in plasma is presented as the percentage of activity in lipid stock. B) Turnover of <sup>125</sup>I-LDL. Radioactivity remaining in the blood is presented as the percentage of radioactivity 3 min after the injection of <sup>125</sup>I-LDL. Data is presented as a two-phase decay curve. C) In vivo reverse cholesterol transport. Radioactivity in plasma is presented as the percentage of radioactivity in macrophage suspension. D) Glucose tolerance test. Data points are presented as mean ± SEM.
MATERIALS AND METHODS

**Mouse model**
sVEGFR3 and Chy in mice were crossbred with LDLR−/−/ApoB100/100 mice (originally stock number 003000, The Jackson Laboratory, Bar Harbor, ME) in C57Bl/6 background. sVEGFR3 x LDLR−/−/ApoB100/100 mice were genotyped with polymerase chain reaction (PCR) for sVEGFR3 using primers 5′-GAA AGC CCA AAA CAC TCC AAA CAA TG-3′ and 5′-TCC TTG TCT CCG GCT GCT G-3′ and Chy x LDLR−/−/ApoB100/100 mice for mutation in VEGFR3 with primers 5′-AGG CCA AAG TCG CAG AA-3′ and 5′-GAA GAC CTT GTA TGC TAC-3′. Both strains were genotyped for LDLR with primers 5′-CCA TAT GCA TCC CCA GTC TT-3′, 5′-GCG ATG ACA CTC ACT GC-3′ and 5′-AAT CCA TCT TGT TCA ATG GCC GAT C-3′. Protein expression of sVEGFR3 was verified by western blotting of plasma samples with anti-human Fc antibody (α-hFc, Sigma-Aldrich, St.Louis, MO) using donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Dallas, TX) as a secondary antibody. Female and male sVEGFR3 x LDLR−/−/ApoB100/100 mice (n = 33), Chy x LDLR−/−/ApoB100/100 mice (n = 7) and LDLR−/−/ApoB100/100 control mice (n = 40) in the young, intermediate and old cohorts were first fed rodent chow for 3-4 months, 7-8 months or 11-12 months, respectively, and then the high-fat Western type diet (TD.88137 Harlan Teklad, Indianapolis, IN; 42% of energy from fat, 0.2 % cholesterol) for 2, 6 and 12 weeks before euthanasia (n = 2 - 7 in each group). Subgroup of 6 wk old male sVEGFR3 (n = 4) mice, Chy (n = 5) mice and their wild type littermates (n = 5 and n = 10, respectively) in NMRI background were kept in separate cages and fed Western-type, high fat diet (D12451, Research Diets, New Brunswick, NJ; 45% calories from fat) for 14 weeks. Food consumption was monitored weekly and body composition was analyzed using the DEXA scanner (LunarPiximus2, General Healthcare, Waukesha, WI). The general health, appearance and weight of the mice were recorded every two weeks. All animal experiments were approved by National Experimental Animal Board of Finland.

**Functionality of lymphatic vessels**
Lymphatic vessel function was determined by injecting Evans Blue (30 µl, 30 mg/ml in 0.7 % sodium chloride (NaCl)) into footpads of anaesthetized (ketamine 75 mg/kg - xylasins 10 mg/kg) mice (n = 4 in sVEGFR3 x LDLR−/−/ApoB100/100 and n = 4 LDLR−/−/ApoB100/100 mice). Mice were euthanized after 30 min and transport of the dye to thoracic duct was evaluated.

**Clinical chemistry**
Serum samples from sVEGFR3 and Chy mice were collected after 6 h fasting from saphenous vein or heart puncture. Total serum cholesterol and triglyceride levels were measured utilizing specific colorimetric assays (Cobas, Roche Diagnostics Limited, West Sussex, Great Britain). Blood samples were collected from the tail vein of sVEGFR3 x LDLR−/−/ApoB100/100 mice and Chy x LDLR−/−/ApoB100/100 mice, into lithium heparin tubes (BD Microtainer, 365971, BD, Franklin Lakes, NJ) after 4 h fasting. Plasma was separated and analyzed for cholesterol (Konelab™/ T Series, Cholesterol, 981812, Thermo Scientific, Waltham, MA) and triglycerides (Konelab™/ T Series, Triglycerides, 981301, Thermo Scientific) by Movet Oy, Kuopio, Finland. Blood glucose levels were measured after 4 h fasting (Ascencia Elite XL Glucose meters and test strips, Bayer, Leverkusen, Germany) (n = 7 in sVEGFR3 x LDLR−/−/ApoB100/100 mice and n = 7 LDLR−/−/ApoB100/100 mice). For the analysis of lipoprotein profiles, pooled plasma from sVEGFR3 x LDLR−/−/ApoB100/100 mice and LDLR−/−/ApoB100/100 on Western diet was fractionated by fast-performance liquid chromatography (FPLC) as previously described. To test the glucose tolerance, sVEGFR3 x LDLR−/−/ApoB100/100 mice (n = 7) and LDLR−/−/ApoB100/100 mice (n = 7) were given a bolus of glucose in NaCl (1.5 mg/g) i.p. and glucose levels in blood were measured 30, 60, 90 and 120 min after the injection.
**Intestinal lipid absorption**

To measure intestinal lipid absorption, 4 hour fasted young sVEGFR3 x LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice (n = 4) and LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice (n = 7) on Western diet received an intragastric load of [\(^3\)H] triolein (5 µCi) (Perkin Elmer, Waltham, MA) in 200 µl olive oil. In addition, second set of mice sVEGFR3 x LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice (n = 2) and LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice (n = 3) were treated with Triton WR1339 before gavage (500 mg/kg, 10 % solution in NaCl) to inhibit plasma LPL function. Blood samples were taken before gavage and 1, 2 and 3 hours after gavage. Plasma samples were prepared for liquid scintillation counting and radioactivity was measured with liquid scintillation counter (Microbeta plus 1450, Wallac Oy, Turku, Finland) according to manufacturer’s protocol.

**LDL turnover studies**

Human LDL was iodinated with \(^{125}\)Iodine as previously described. \(^3\)\(^{125}\)I-LDL was diluted with NaCl to give a specific activity of 110 cpm/ng. sVEGFR3 x LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice (n = 6) and LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice (n = 6) on Western diet were sedated with ketamine (37.5 mg/kg) - xylatins (5 mg/kg) s.c. and injected with 100 µl of \(^{125}\)I-LDL (10 µg) via the tail vein. Blood samples were collected 3 min and 0.5, 1, 6, 12, 24 and 36 h after the injections. Radioactivity was measured using a gamma counter (Gammamaster 1277, Wallac Oy, Turku, Finland) and calculated as the percentage of \(^{125}\)I-LDL activity remaining in the blood 3 min after the injection. A decay curve was fitted using a two-phase decay.

**In vivo RCT**

Bone marrow derived macrophages were harvested from LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice by flushing femurs and tibiae with PBS and cells were plated in RPMI medium containing 20 ng/ml murine colony stimulating factor (M-CSF, Miltenyi Biotec, Bergisch Gladbach, Germany). After 8 days of culturing, cells were loaded with 50 µg/ml of acetylated LDL (aclLDL) and 5 µCi/ml \(^{3}\)H-cholesterol (Perkin Elmer, Waltham, MA). After incubation of 48 hours, cells were harvested and sVEGFR3 x LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice (n = 3) and LDLR\(^{-/-}\)/ApoB\(^{100/100}\) mice (n = 3) on Western diet received i.p. injection of 1,3 x 10\(^5\) cells in 400 µl 1 x PBS. Blood was collected at 4, 24 and 48 hours. Mice were sacrificed and livers were collected and radioactivity was measured with liquid scintillation counter according to manufacturer’s protocol.

**Histology**

Mice were euthanized with carbon dioxide (CO\(_2\)) and perfused with phosphate-buffered saline (PBS) through the left ventricle. Aortas were dissected from aortic arch to bifurcation and fixed in 4 % paraformaldehyde (PFA)-PBS overnight. Aortas were opened longitudinally and attached to a black surface. Aortas were stained with 0.5 % Sudan IV dye (198102, Sigma-Aldrich) and photographed for en face evaluation. For Hematoxylin-Eosin, modified Movat’s pentachrome and immunohistochemical stainings tissue samples were collected in 4 % paraformaldehyde (PFA)-PBS for overnight fixation, processed to paraffin and cut as 4 or 7 µm sections. For Oil-red-O stainings, tissue samples were snap frozen in liquid nitrogen and cut as 10 µm frozen sections. Cross-sectional lesion areas were quantified from aortic roots stained with Hematoxylin-Eosin. Percentage of the lesion area from intima was calculated from a tissue section of aortic sinus level, defined by the presence of three valve cusps. Vascular outward remodeling was determined by measuring the area within internal elastic lamina as previously.

Immunohistochemical stainings were performed with antibodies recognizing T-cells (CD3, ab166699, 1:200, Abcam, Cambridge, UK), macrophages (mMQ, AIA31240, 1:6500, Accurate Chemical & Scientific Corp., Westbury, NY), endothelium (CD31, 553370, 1:50, BD Pharmingen, Franklin Lakes, NJ) and lymphatic vessels (LYVE-1, 103-PA50AG, 1:1000, Reliatech, Oakland, CA). To improve the specificity of the stainings, antigen retrieval with citrate buffer boiling was used for CD3 and LYVE-1 antibodies. For all stainings, biotinylated IgG secondary antibodies (Vector Laboratories, Burlingame, CA) were used followed by

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avidin-biotin-HRP step (Vector Laboratories) and DAB (Invitrogen, Carlsbad, CA) as a chromogen. Controls for immunohistochemical staining included stainings with class and species matched irrelevant primary antibodies and incubations where primary antibodies were omitted. En face –analysis, cross-sectional lesion areas and immunohistological stainings were analyzed with analySIS software (Olympus Soft Imaging Systems GmBH, Münster, Germany) connected to Olympus AX70 microscope (Olympus Optical, Tokyo, Japan).4

**Confocal microscopy of blood perfused neovascularization and lymphatic vessels associated with atheromas**

Subgroups of mice in old cohorts of sVEGFR3 x LDLR−/−/ApoB100/100 mice, Chy x LDLR−/−/ApoB100/100 mice and LDLR−/−/ApoB100/100 control mice were anesthetized with ketamine (75 mg/kg) - xylazine (10 mg/kg) and then injected with rhodamine *Griffonia Simplicifolia* lectin (GSL-R, 200 µg, Vector Laboratories) and CD31 antibody (553370, 80 µg, BD Pharmingen) via the tail vein. After 10 min, rabbit anti-rat IgG-FITC (FI-4001, 50 µg, Vector Laboratories) was injected i.v. Mice were euthanized after 5 min and perfused with 1 % bovine serum albumin (BSA)-PBS and then 4 % PFA-PBS through the left ventricle. Aortas were fixed in 4 % PFA-PBS for 30 min, stored in PBS, trimmed of periaortic fat and opened longitudinally. Aortic segments were blocked with 5 % BSA-PBS and incubated overnight with antibodies recognizing mouse CD31 (1 µg/ml) and podoplanin (1 µg/ml; Syrian hamster anti-mouse, Biolegend, San Diego, CA), secondary reagents (anti-rat IgG-FITC and biotinylated goat anti-syrian hamster IgG (Biolegend) and finally streptavidin Alexa 633. Aortic segments were mounted en face in Dapi containing Vectamount with adventitia adjacent to the coverslip. Descending aortas were initially viewed at low magnification to identify atheromas that were associated with neovascularization containing intravenous GSL-I lectin. Dapi-labeled nuclei of intimal plaque cells distinguished the plaque borders from uninvolved aorta. Regions of interest (ROI) were imaged with a laser-scanning confocal microscope LSM510 META NLO (Carl Zeiss Inc, Oberkochen, Germany) using a water immersion 40x lens, 1.2NA C Apochromat and analyzed using ZEN2009 software. Z stacks were collected at 1 µm spacing intervals starting from the outer adventitia layer beneath any residual traces of periaortic fat and extending through an average 24 µm depth towards the intima. The Z-stack images were rendered into composite Z-projections using ImageJ 1.45s software (NIH, Bethesda, MD). Plaque neovascularization was positive for GSL-I and the pan-endothelial marker CD31. Lymphatic channels stained positive for C31 and podoplanin but were negative for intravenously injected GSL-I lectin. The densities of plaque blood and lymphatic vessels in 10 plaques from 3 sVEGFR3 x LDLR−/−/ApoB100/100 aortas, 9 plaques from 2 Chy x LDLR−/−/ApoB100/100 aortas and 16 plaques from 3 LDLR−/−/ApoB100/100 aortas were quantified using Image J software and expressed as the percent areas of GSL-I lectin+ or podoplanin+ vessels per high power field, respectively.

**Statistical analysis**

Statistical analysis was performed with GraphPad Prism version 5.04 (GraphPad Software, La Jolla, CA). Data is presented as mean ± SEM. Two-tailed unpaired t-test, one-way ANOVA followed by Bonferroni correction or Kruskal-Wallis test with Dunn’s correction were used. P < 0.05 was considered significant.
REFERENCES


