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Early rescue of lymphatic function limits atherosclerosis progression in *Ldlr*$^{-/-}$ mice

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HIGHLIGHTS

Early treatment with VEGF-C 152s:

- Prevents lymphatic dysfunction and maintains the contractile capacity of the collecting lymphatic vessels throughout the whole atherosclerotic process;
- Limits plaque buildup, then stabilizes plaque progression in *Ldlr*$^{-/-}$ mice.

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ABSTRACT

Background and aims: Our previous data showed that lymphatic function impairment occurs before the onset of atherosclerosis in mice and is precociously associated with a defect in the propelling capacity of the collecting lymphatic vessels. Concomitantly, we found that lymphatic transport can be restored in mice by systemic injections of a mutant form of VEGF-C (VEGF-C 152s), a growth factor known to increase mesenteric collecting lymphatic vessel pumping through a VEGFR-3-dependent mechanism in rats. In the present study, we aimed to determine whether and how early modulation of collecting lymphatic vessel function could restrain atherosclerosis onset and limit its progression.

Methods: Before the administration of a pro-atherosclerotic regimen, *Ldlr*$^{-/-}$ mice at 6 weeks of age were injected intraperitoneally with VEGF-C 152s or PBS every other day for 4 weeks, fed on high fat diet (HFD) for an additional 8 weeks to promote plaque progression, and switched back on chow diet for 4 more weeks to stabilize the lesion.

Results: Early treatment with VEGF-C first improved lymphatic molecular transport in 6-week-old *Ldlr*$^{-/-}$ mice and subsequently limited plaque formation and macrophage accumulation, while improving inflammatory cell migration through the lymphatics in HFD-fed mice. The contraction frequency of the collecting lymphatic vessels was significantly increased following treatment throughout the whole atherosclerotic process and resulted in enhanced plaque stabilization. This early and maintained rescue of the lymphatic dysfunction was associated with an upregulation of VEGFR3 and FOXC2 expression on lymphatic endothelial cells.

Conclusions: These results suggest that early treatments that specifically target the lymphatic contraction capacity prior to lesion formation might be a novel therapeutic approach for the prevention and treatment of atherosclerosis.

1. Introduction

Atherosclerosis, characterized by the over accumulation of cholesterol and immune cells within the arterial wall, is the principal cause of mortality worldwide and is at the origin of most cardiovascular diseases [1]. The lymphatic system is now increasingly emerging as a potential contributor to cardiovascular disease. Although it has been well known for many years that lymphatic vessels are present within the adventitia of blood vessels [2], it is only recently that Martel et al. introduced a new integrated model of reverse cholesterol transport (RCT), in which they clearly showed that without a functional lymphatic network, cholesterol cannot be properly conducted out of the artery wall [3]. As

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lymphatic vessels are responsible for the transport of immune cells, their functional properties have become of great interest in many pathological settings. In contrast to the blood vascular network, the lymphatic vasculature is an open, unidirectional and low-pressure vascular system [4]. The lymphatic vessels (LVs) are composed of two different entities, bearing distinct but complementary roles. Firstly, initial LVs, also called lymphatic capillaries, help absorb plasma ultrafiltrates from the periphery, they are highly permeable and they specifically express lymphatic vessel endothelial hyaluronan receptor (LYVE-1) at the surface [5]. Secondly, collecting LVs are contractile entities that propel lymph in a unidirectional manner with the help of intraluminal bi-leaflet valves. They are covered sparsely with smooth muscle cells (SMC) [5].

Different transcription factors regulate lymphatic development, some of the most crucial ones being vascular endothelial growth factors (VEGFs). The vascular endothelial growth factor receptor-3 (VEGFR-3) serves as a receptor for LV-specific VEGFs, such as VEGF-C, which is important for normal development of lymphatic vessels and is critical in the process of lymphangiogenesis [6]. In addition to this crucial role, VEGF-C was also shown to stimulate lymphatic pumping [7]. VEGF-C 156s is a point mutant that only binds to and activates signaling through VEGFR-3, which unlike wild type VEGF-C, is unable to bind VEGFR-2, a well-known receptor that contributes to angiogenesis [8]. Supporting data from the literature suggests that this mutant form of VEGF- C increases lymphatic contraction frequency, dilation, and pump flow through its action on VEGFR-3[7, 9]. Interestingly, VEGF-C also reversed hypercholesterolemia-associated lymphatic dysfunction in *apoE*$^{-/-}$ mice [3]. VEGF-C 152S is analog to the human VEGF-C 156S mutant and also a selective VEGFR-3 agonist. Of interest, the rat VEGF-C cDNA encodes a pre-pro-protein of 416 amino acids residues making it almost identical to the mouse VEGF-C protein. When treating young *Ldlr*$^{-/-}$*; hApoB100*$^{+/+}$ mice systemically with VEGF-C 152s, this rescued the lymphatic impairment apparent before atherosclerosis onset, in a cholesterol-independent manner [10]. In another inflammatory model, VEGF-C-dependent stimulation of lymphatic function ameliorated experimental inflammatory bowel disease [11]. To date, no study addressed whether VEGF-C-dependent stimulation of lymphatic function before the onset of atherosclerosis had an impact on subsequent atherosclerotic plaque buildup and characteristics of lesion stability.

This present study suggests that early treatment with VEGF-C 152s instigates and maintains rescue of the lymphatic dysfunction throughout the whole atherosclerotic process, restraining atherosclerotic plaque size and CD68^{+} macrophages content, and stabilizing plaque progression.

2. Materials and methods

2.1. Sex of mice

This study was conducted on *Ldlr*$^{-/-}$ female mice on a C57BL/6 background that were purchased from Jackson Laboratories. As the most widely reported sex effect on atherosclerosis is that female mice have larger aortic root lesion areas than male mice [12], we deemed fit to start our present study on this one gender with exacerbated plaques.

2.2. Experimental model

One of the most commonly used atherosclerotic models is the LDL receptor–deficient mouse [13]. The normal diet–fed *Ldlr*$^{-/-}$ mice only develop small atherosclerotic lesions, even at an advanced age (beyond 6 months) and therefore, this mouse strain is fed a Western diet (HFD—adjusted calories diet, 0.2% total cholesterol and 42% from fat, Harlan 88137) to accelerate atherosclerosis. To our knowledge, no systematic pathological analysis of lesion development and lymphatic function has been reported in normal diet–fed *Ldlr*$^{-/-}$ mice that are not yet baring an atherosclerotic plaque, but prone to develop atherosclerosis under a pro-atherogenic regimen.

2.3. Experimental design

Animals were housed in a pathogen-free environment under 12-h light–dark cycles with free access to water. The experimental design of our study is illustrated in Supplementary Fig. 1. At 6 weeks of age, mice were injected intraperitoneally with VEGF-C 152s or control (phosphate buffer saline, PBS), 50 ng/25 g of body weight. Following, for 8 weeks, mice were fed a high-fat diet (HFD - adjusted calories diet, 0.2% total cholesterol and 42% from fat, Harlan 88137) to induce plaque progression. Contrarily to a 10–12-week diet that will lead to a well-established lesion, we aimed to assess an early-stage evaluation of the plaque, which is the main reason why our time lapse is shorter. Afterwards, mice were switched back to a chow diet for a remainder of 4 weeks to stop plaque growth. At the end of each milestone, 4 weeks of VEGF-C 152s treatment, 8 more weeks of HFD and 4 more weeks of chow diet, lymphatic function assays were performed, and blood and lymph were collected under anesthetic conditions. Following this, mice were euthanized by cardiac puncture while on isoflurane administration (4% for induction, 2–3% for maintenance), or by carbon dioxide (CO_2), and were perfused with 15 mL PBS. Finally, organs were collected and stored until batch analysis. Lymph nodes (LNs), ears, dermal back skin sections, aortas, hearts, and popliteal collecting lymphatic vessels were harvested and either freshly processed for flow cytometry analysis or fixed in 4% paraformaldehyde, 10% formalin or radioimmunoprecipitation assay buffer (RIPA buffer) for future analysis. All animal studies were performed in accordance with the Canadian Council on Animal Care guidelines and approved by the Montreal Heart Institute Animal Care Committee.

2.4. Atherosclerotic lesion assessment

The heart and aorta were removed and fixed in 4% paraformaldehyde. The heart was transferred into PBS containing 30% sucrose (wt/vol) overnight at 4 °C before being immersed in optimal cutting temperature compound (OCT) and stored at −80 °C. Eight-micrometer-thick cryosections of the aortic sinus were prepared. Aortas were cleaned by removing the surrounding fat and were then split along their outer curvature. Neutral lipid assessment in atherosclerotic lesions in the aortic sinus and *en face* aorta was performed by Oil-red-O (ORO) staining (Sigma, O-0625).

2.5. Total circulating cholesterol, lipoprotein profile, triglycerides and apolipoprotein B levels

Lymph was collected as previously described [14]. Blood was collected on ethylenediamine tetraacetic acid (EDTA) by cardiac puncture and plasma was obtained following centrifugation at 2400*g* for 10 min, after which sucrose was added before samples were stored at −80 °C. Mouse lipoproteins were separated from plasma by size exclusion chromatography [fast protein LC (FPLC)] using a Superose 6 column on a FPLC system with a Model 500 pump from Waters (Milford, MA). In short, a 100 µl aliquot of mouse plasma pooled equally from five (5) different mice was injected into a 1.0-ml sample loop and separated with a buffer (0.15 M NaCl, 0.01 M Na_2HPO_4, and 0.1 mM EDTA) at a flow rate of 0.5 ml/min. Forty-three fractions of 0.3 ml each were collected with the lipoproteins being contained within. Batch analysis was performed to measure circulating total cholesterol (Wako) in lymph, plasma and plasma lipoprotein fractions according to the manufacturer's protocol. Due to technical limitations for lymph volume, triglyceride (#10010303, Cayman Chemicals) and apolipoprotein B (apoB, ab230932, Abcam) circulating levels were measured solely in plasma according to manufacturer's protocols.

2.6. Initial lymphatic density quantification and immune cell accumulation

Cross-sections of the aortic sinus were stained with anti-LYVE-1 (Abcam, ab14917) and anti-CD68 (Biolegend, 137001) antibodies, and then incubated with the appropriate secondary antibodies. As macrophages can also be positive for LYVE-1, adventitial initial lymphatics were identified as LYVE-1$^+$CD68$^-$ cells forming vessel-like shapes. Whole-mount immunohistochemical analysis of the ear dermis to visualize lymphatic vessels was performed as previously described. Ear dermis was stained for lymphatic capillaries (anti-LYVE-1) at 4 °C, and then sections were incubated with Alexa Fluor 647-conjugated donkey anti-rabbit antibody (Abcam, ab150075) and Cy3 donkey anti-rat (Jackson ImmunoResearch, 712-165-150). All imaging was performed on a Fluoview FV10i (Olympus). The relative quantification of the number of initial lymphatics (LYVE-1$^+$ vessels) and the total surface area they occupy was determined by computer-assisted morphometric analysis.

2.7. Atherosclerotic lesion calcium and collagen content

Histological visualization of calcium deposits (ab150687) and collagenous connective tissue fibers (ab150686) in 8 µm aortic sinus frozen sections was performed as per manufacturers' instructions. Briefly, for calcium staining, frozen sections were treated with a silver nitrate solution and the silver is deposited by replacing the calcium reduced by the strong light. Calcium in mass deposits will show as black, whereas dispersed deposits will be visualized as metallic silver.

2.8. Lymphatic functional assessment

Lymphatic function was analyzed by four complementary methods.

2.8.1. Cellular transport

The propensity of dendritic cells to migrate through the lymphatic vessels from the periphery to draining LNs was measured as described previously [15]. The animals were euthanized 18 h after the application of a solution that contains fluorescein isothiocyanate (FITC), dibutyl phthalate, and acetone solution, in order to instigate an immune response and thus dendritic cell trafficking. The corresponding skin-draining LNs were recovered and enzymatically digested in collagenase D for 25 min at 37 °C. Cells were then passed through a 70-µm cell strainer, washed, counted, and stained for analysis by flow cytometry (BD Biosciences LSR II). Conjugated antibodies CD11b PerCp-Cy5.5 (BioLegend, CA101227-BL), CD11c PeCy7 (Tonbo Biosciences, 60–0114), MHCII-VioletFluor 450 (Tonbo Biosciences, 75–5321), and CD45-APC (Tonbo Bio-sciences, 20–0451) were used. The number of dendritic cells that uptake FITC and traveled to the corresponding skin-draining LN was then counted based on the total LN cellularity (% of FITC$^+$ cells/# of cells/LN).

2.8.2. Lymphatic vessel permeability

Lymphatic vessel permeability was assessed using Evans Blue dye for tracing the path of lymph through popliteal lymphatic vessels. Mice were anesthetized with isoflurane and skin was carefully removed from the legs. Following Evans Blue intradermal injection in the footpad, popliteal collecting lymphatic vessels were visualized using a Stereo Discovery V8 (Zeiss). The effusion of Evans Blue around the vessel marks the area the leakage covers and was analyzed using ImageJ software.

2.8.3. Lymphatic vessel contractility

Lymphatic vessel contraction capacity was assessed following injection of ovalbumin, coupled to Alexa Fluor 488 (Fisher, catalog #O34781) in the dermis of the footpad of the mouse to enable proper visualization of collecting lymphatic vessels [9]. Briefly, the mouse was placed on his abdomen on a heat pad at 40 °C and an incision was made all around his upper hind limb. Following, skin was removed ensuring no blood vessels were severed while pulling the skin away. Warm saline was administered to keep the limb tissue hydrated at all times. 10 µL of Alexa Fluor 488-coupled ovalbumin (2 mg/mL) were injected in the footpad, following which the foot was moved three times for the vessels to appear on the screen. Once adequate perfusion of tracer was confirmed, the vessel was observed for 5 min to ensure that the lymphatic contractility was active and that no obstructions had occurred or tearing of vessels. Timing was cautiously kept equal for all subjects. The *in vivo* contraction imaging was performed for a duration of 10 min with photos being taken at intervals of 1 s with an Axiozoom V.16 microscope. It has been proposed that alterations in lymphatic smooth muscle activity significantly impact lymphatic function. Numerous endogenous vasoactive agents are known to constrict lymph vessels. We used 10 µL of phenylephrine (1.0 µM, Sigma-Aldrich), which acts upon alpha-adrenoceptors on lymphatic smooth muscle [16], and injected in the footpad of the mouse. Thereafter, the foot was moved three times after which it was left to rest for 5 min. Acquisition was performed for 10 min and photos taken at intervals of 1 s, as mentioned above.

2.8.4. Lymphatic molecular transport

Following the aforementioned analysis of lymphatic vessel contractility, blood was collected, and presence of fluorescent ovalbumin was quantified using a fluorescence plate reader to assess the efficiency of lymphatic vessels to propel lymph into the bloodstream.

2.8.5. Lymphatic contraction frequency and amplitude analysis

Videos of the collecting lymphatic vessel contraction capacity were analyzed using ImageJ. We traced a line of equivalent length at 3 different regions of interest (ROI) along the vessels. The raw integrated density (RawIntDen) was assessed for each and a graph was computed. Appropriate peaks and valleys were manually identified and counted by two different observers. The frequency and amplitude of each ROI were assessed and an average per mouse was computed [17,18].

2.9. Immunofluorescence of the collecting lymphatic vessel

Popliteal collecting lymphatic vessels were identified following Evans Blue dye intradermal injection, as described above and harvested. For analysis of lymphatic vessel integrity, whole-mount immunofluorescence analysis following incubation with anti -smooth muscle actin already coupled to FITC (Sigma), -FOXC2 (R&D Systems, AF6989), -phosphorylated eNOS, -VE-cadherin, -podoplanin and -VEGFR3 antibodies was performed on isolated popliteal lymphatics of mice. Secondary antibodies donkey anti-rat 488 (Jackson ImmunoResearch, 712-546-153), donkey anti-sheep 555 (Jackson ImmunoResearch, 713-165- 147), donkey anti-hamster 555 (Abcam, ab150106), donkey anti-rabbit 647 (Abcam, ab150075) and DAPI were then added. Images were acquired with an LSM 710 Confocal Microscope (Zeiss) equipped with a 63/1.4 oil dic objective. All image handling was performed using ImageJ software.

2.10. Cytokine analysis

A BD Cytometric Bead Array (CBA) Mouse Inflammatory Cytokines Kit was performed in plasma to measure the circulating levels of IL-10, MCP-1 and TNF-α proteins. No results were obtained in lymph as the quantity is inferior to the detection threshold of the kit.

2.11. Cell culture

Primary human dermal lymphatic microvascular endothelial cells-adult (HMVEC-dLyAd) were cultured according to the manufacturer's protocol (Lonza) in EBM-2 medium containing the EGM-2 MV SingleQuots. Cells were seeded in culture dishes at 80% confluence and were treated with VEGF-C 152s (50 ng/mL) or concentration matched

PBS for 24 h.

2.12. Immunoblotting of human lymphatic endothelial cells

Proteins were extracted using radioimmunoprecipitation assay buffer and the protein concentration was established using the Bradford Protein Assay Kit (Bio Basic). Protein samples were diluted in 4X Laemmli buffer, then heated at 95 °C for 5 min. Proteins were separated by electrophoresis on a 12% SDS-PAGE, then transferred on polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non fat dry milk in Tris-buffered saline (TBST, 0.1% Tween 20) for 1 h at room temperature, then incubated with an anti-phosphorylated-eNOS (Cell Signaling, #9275), an anti-vascular endothelial growth factor receptor (VEGFR)-3 (Abcam, AB27278), and anti-phosphorylated-AKT (Cell Signaling, #9570) or an anti-beta-actin (Abcam, AB8227) overnight at 4 °C. The membranes were washed with TBST and incubated with horseradish peroxidase–conjugated secondary antibodies (Abcam, AB6721 and AB6721) for 1 h at room temperature. Clarity Max Western ECL Blotting Substrates (BioRad) was used for detection. Each sample was normalized with its respective beta-actin expression.

2.13. Statistics

Data are expressed as the mean ± SEM. Statistical significance was evaluated by unpaired *t*-test or, for multiple comparisons, one-way ANOVA using appropriate corrections when data was not normally distributed, or for unequal variances. All calculations were done with GraphPad Prism v6c software (GraphPad Software, La Jolla, CA, USA), and p values < 0.05 were considered statistically significant.

3. Results

3.1. Early treatment with VEGF-C 152s before the onset of atherosclerosis first boosted, then sustained lymphatic transport in *Ldlr*$^{-/-}$ female mice, while modulating the inflammatory response throughout development of the disease

Our previous studies show that lymphatic function is impaired even before the development of atherosclerosis in 3-month-old *Ldlr*$^{-/-}$; *apoB100*$^{+/+}$ mice that, unlike the *Ldlr*$^{-/-}$ mice, are prone to develop atherosclerosis on a regular chow diet [19]. This dysfunction is presumably associated with a defect in the collecting lymphatic vessels, first in a non-specific cholesterol- but LDLR-dependent manner, as chow-fed *Ldlr*$^{-/-}$ mice also had a significant defect in dendritic cell migration through the lymphatics, without having as much total circulating cholesterol in plasma [10]. Therefore, we subsequently aimed to test whether specifically targeting lymphatic function in *Ldlr*$^{-/-}$ mice before the onset of the risk factors, such as an atherosclerotic regimen, could restrain the development of the atherosclerotic plaque and limit its progression. To test this, we first verified whether lymphatic function can be efficiently improved early on in the disease process. We measured both molecular and cellular lymphatic transport before plaque onset in 3-month-old *Ldlr*$^{-/-}$ mice that experienced a VEGF-C 152*s* treatment while fed on a chow diet. Following intradermal injection of ovalbumin 488 in the footpad of mice to assess the capacity of large molecules to travel within the lymphatics up to the blood circulation, a higher fluorescence was observed in the plasma of mice treated with VEGF-C 152s before the administration of the pro-atherogenic regimen (Fig. 1A). Whereas chemoattractant protein monocyte chemoattractant protein 1 (MCP-1) was significantly down-regulated (Fig. 1B) immediately following VEGF-C treatment, no differences were yet observed with respect to cellular transport by the lymphatic vessels (Fig. 1C). Hypercholesterolemia has been reported as a risk factor for lymphatic dysfunction [20], and VEGF-C proved to be effective in restoring lymphatic function in hypercholesterolemic mice [21]. We thus next sought to confirm that VEGF-C 152s, even if applied before the atherosclerosis regimen, would be as effective to improve lymphatic function during hypercholesterolemia. Therefore, *Ldlr*$^{-/-}$ mice that were pre-treated with VEGF-C 152s (or control) were fed a HFD for an additional 8 weeks to induce atherosclerotic lesion formation. We observed that the effect of VEGF-C treatment on dendritic cell migration through the lymphatics actually appeared to kick in when mice were switched to a HFD (Fig. 1C), as it almost doubled the amount of cells that travelled up to the lymph nodes. Therefore, we show that VEGF-C 152s palliates to the hypercholesterolemic status to improve lymphatic function when atherosclerotic plaque build-up should be at its apogee, at least in our protocol. This effect is reflective of the observation made when a chow diet is given to PBS-treated mice to reverse the hypercholesterolemic phenotype. Interestingly, in hypercholesterolemic plasma, associated proinflammatory cytokine tumor necrosis factor alpha (TNF-α) was significantly lower in VEGF-C treated-mice than control (Fig. 1D), thus preventing systemic inflammation, while MCP-1 levels were comparable between groups (Fig. 1B). While during the early stages of disease development IL-10, an anti-inflammatory cytokine, was not different between groups, early treatment with VEGF-C 152s significantly increased circulating levels of IL-10 in *Ldlr*$^{-/-}$ mice following a switch to a chow diet (Fig. 1E).

3.2. Early treatment with VEGF-C 152s restrains buildup of atherosclerotic plaque

As our results presage that early treatment with VEGF-C 152s would prepare the lymphatic vessels for subsequent harmful situations by enhancing its clearance capacity, we then sought to investigate whether this pre-treatment with VEGF-C 152s would eventually protect mice from subsequent plaque accumulation in the presence of excess circulating cholesterol. The atherosclerotic lesion size and composition were examined in the aortic sinus and *en face* aorta using Oil Red O (ORO) staining. A decrease in ORO^{+} staining of 30% in the aortic sinus (Fig. 2A) and 40% in *en face* aorta (Supplementary Fig. 2A) was observed following a HFD in mice pretreated with the VEGF-C 152s treatment. No effect on ORO^{+} staining is observed in the aortic sinus, nor in *en face* aorta lesion composition in mice that were switched back to a chow diet. The results of neutral lipid quantification are expressed as percent of control based on percentages of ORO^{+} total surface.

To test whether this reduction in plaque is due to an enhanced reduction in plasma cholesterol, we have measured total cholesterol in lymph, plasma and plasma lipoprotein fractions. Albeit total circulating cholesterol levels were not affected by VEGF-C 152s treatment before the onset of the atherosclerotic regimen, a significant reduction was observed thereafter in both lymph and plasma compared to control when mice were given a HFD (Fig. 2B). Furthermore, when mice where switched back to a regular chow diet, VEGF-C 152s and PBS equally reduced circulating plasma cholesterol levels, while lymph cholesterol remained at the same low level in the VEGF-C-treated group, whereas it drastically reversed the hypercholesterolemic phenotype in the PBS-treated group (Fig. 2C).

Lacteals are lymphatic vessels that reside in the small intestines and are responsible for dietary lipid absorption [22]. Without a HFD, *Ldlr*$^{-/-}$ mice are not hypercholesterolemic [23], and it is not surprising that, despite a positive effect on lymphatic function as described above, systemic injections of VEGF-C 152s had no effect on the lipoprotein profile (Fig. 2D) at this point. When the pro-atherogenic regimen was applied, however, VEGF-C 152s improved the absorptive capacity of the lacteals, as reflected by the presence of plasma chylomicron (CM) remnants. This observation was paralleled with lower levels of plasma VLDL and LDL/IDL as compared to the PBS control group, whereas TG (Supplementary Fig. 3A) or total apoB (Supplementary Fig. 3B) levels were undistinguishable between the PBS- and the VEGF-C- treated groups. Once the mice were switched back to a chow diet, TG concentrations went down in both groups, while total apoB levels remained

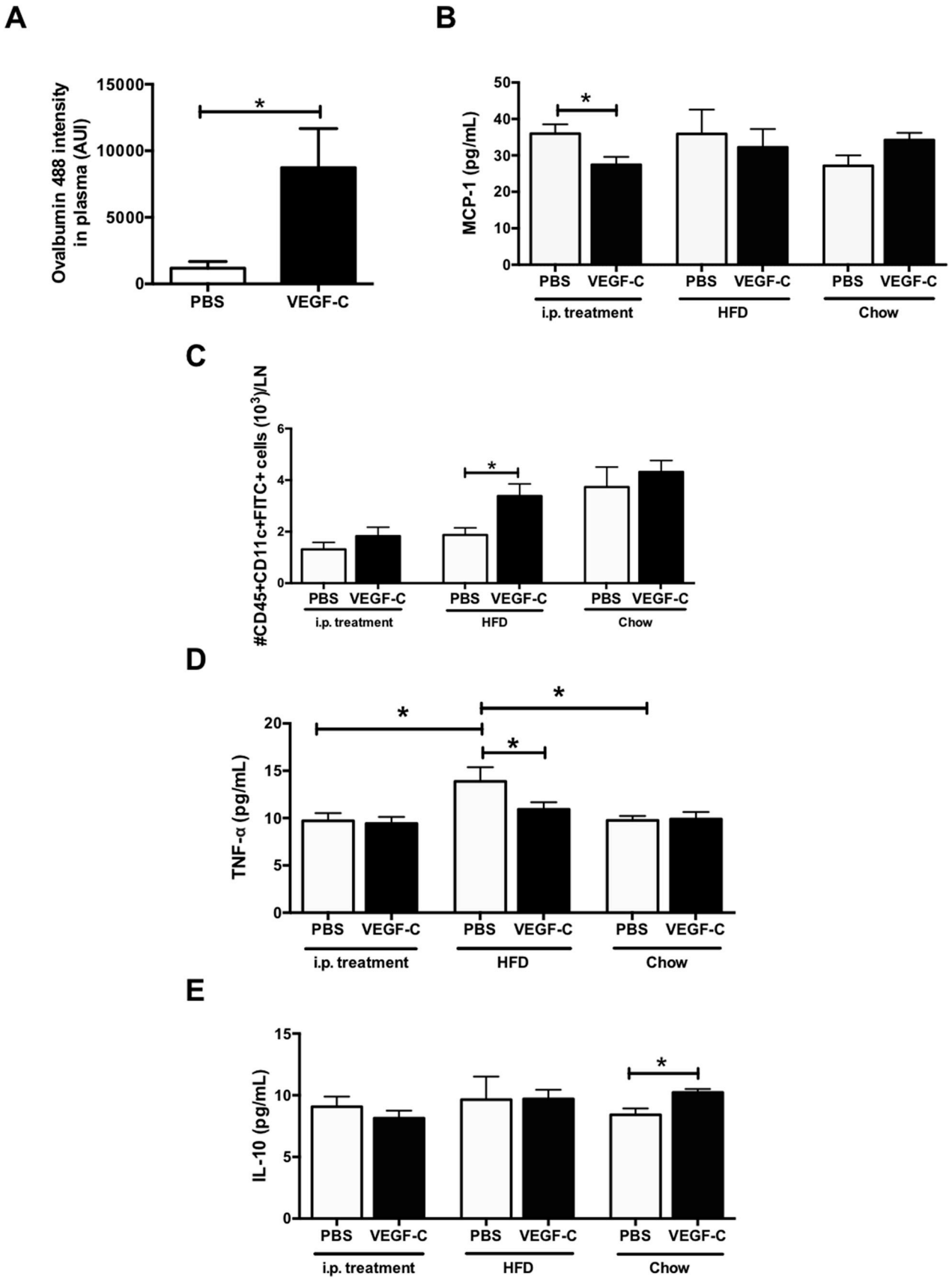

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Fig. 1. Early treatment with VEGF-C 152s before the onset of atherosclerosis first boosted, then sustained, lymphatic transport, and modulated inflammatory cytokines in *Ldlr*$^{-/-}$ female mice.
Immediately following VEGF-C 152s treatment, (A) molecular transport from the dermis of the mouse footpad to the plasma was assessed using Alexa Fluor 488-coupled ovalbumin. Dye intensity in plasma was measured and reported in arbitrary units of intensity (AUI). Mann-Whitney *t*-test. (B, D, E) Quantification of monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor alpha (TNF-α) and interleukin-10 (IL-10) was assessed in mouse plasma by a Cytometric Bead Assay (CBA) kit. (C) Skin-draining lymph nodes were harvested after 18 h and the number of dendritic cells ($CD45^+CD11c^+FITC^+$) that migrated from the skin to the lymph node was reported. ANOVA with Bonferroni *post hoc* test. Experiments were performed with 4–5 mice per experimental group. $^*p < 0.05$.

high. VLDL and IDL/LDL greatly diminished in the control group, whereas the reduction in the VEGF-C-treated group was more modest. Throughout the whole experimental design, VEGF-C 152s did not significantly modulate HDL levels.

3.3. Early treatment with VEGF-C 152s allows the stabilization of the atherosclerotic lesion following cessation of high fat diet

The atherosclerotic plaque is characterized by accumulation of lipids and immune cells, such as macrophages, which produce proinflammatory mediators in the artery wall, and the formation by vascular SMC of a fibrous cap composed mostly of collagen [24]. Furthermore, the majority of coronary thrombi are caused by plaque rupture [25]. Inflammation modulates atherogenesis and plaque destabilization. Thus, inflammatory cytokines may attenuate interstitial collagen synthesis, increase matrix degradation, and promote apoptosis in several atheroma-associated cell types, and all these cellular events may enhance plaque vulnerability [26]. Particularly of interest are the mechanisms leading to plaque instability which includes the proteolysis of collagen by metalloproteinases released by activated macrophages, and apoptosis of intimal SMC which impedes collagen synthesis [27,28]. We assessed $CD68^+$ macrophages within the atherosclerotic lesion (Fig. 3A). As expected, the reversal of the hypercholesterolemic phenotype with the chow diet significantly decreased the accumulation of macrophages, notwithstanding of the pre-treatment applied. While on HFD, however, the sole impact of VEGF-C was as important as the one mediated by the diet reversal in the PBS-treated group. The chow diet further reduced macrophage content by half in the VEGF-C treated group. The macrophage area/smooth muscle cell area ratio was significantly lower in the VEGF-C treated-animals when compared to control mice (Fig. 3B), notwithstanding of the diet. Although plaque calcification was not significantly different between groups or dietary conditions (Supplementary Fig. 2B), we observe a marked increase in collagen content during atherosclerosis progression in VEGF-C pretreated *Ldlr*$^{-/-}$ mice, suggesting a less pronounced proinflammatory phenotype of the lesions (Fig. 3C).

3.4. VEGF-C 152s treatment modulates atherosclerosis development and progression independent of lymphatic collecting vessel permeability

Next, we sought to investigate the mechanisms by which atherosclerosis-related lymphatic function is rescued by VEGF-C 152s throughout the disease process. Lymphatic vessels are known to absorb interstitial fluid from the periphery through the initial lymphatics, and since VEGF-C is a well-known factor of lymphangiogenesis, we sought to characterize lymphatic vessel growth in the aortic sinus, as well as the ear dermis. As reported by others [11], VEGF-C has a positive effect on lymphangiogenesis in hypercholesterolemic mice, as reflected by the quantification of the LYVE-1-positive vessels both in skin dermis and the aortic sinus of atherosclerotic mice. Whereas both the number of vessels per se (Supplementary Fig. 4A) and the number of branching (Supplementary Fig. 4B) were higher in the VEGF-C treated group immediately following the treatment, the number of LYVE-1-positive vessels was not elevated at this early stage in the aortic sinus (Supplementary Fig. 4C). Twelve weeks following the interruption of the treatment, when mice were no longer on the pro-atherogenic regimen, VEGF-C 152s seems to lose its lymphangiogenic potential. The effect of increased density in lymphatic capillaries could not account for the sustained lymphatic function improvement observed at this stage.

We have previously reported that collecting lymphatic vessels are impaired in mice with an already well-established plaque and therefore, transport is less efficient as there is significant leakage surrounding the vessels [29]. To assess whether the improved lymphatic transport we observed following VEGF-C 152s treatment throughout the atherosclerotic process was mediated through mechanisms that first affect lymphatic vessel permeability, we injected Evans Blue in the footpad of the mouse and quantified any blue dye that may have leaked surrounding the vessel. Our results revealed that VEGF-C 152s had no significant effect on the leakage (Supplementary Fig. 5A). Concomitantly, VE-cadherin expression throughout the atherosclerotic process was also unchanged (Supplementary Fig. 5B).

3.5. Early treatment with VEGF-C 152s increases collecting lymphatic vessel capacity to contract throughout the entire atherosclerotic process

As the results stated above suggest that VEGF-C 152s does not act through permeability-related mechanisms to maintain proper integrity of the lymphatic vasculature from the initiation to the progression of the atherosclerotic process, we turned our attention to its contraction inducing capabilities. The VEGF-C/VEGFR3 axis has previously been shown to modulate the contraction capacity of rat mesenteric lacteals, so it is with great interest that we observed *in vivo* the effect of baseline, as well as stimulated contractions in our different groups. At rest, approximately 1/3 of lymph transport in the human lower extremities results from compression by skeletal muscle contractions and 2/3 to active pumping of the collecting vessel network [30]. We wanted to evaluate the effects of VEGF-C 152s on lymphatic endothelial cells and following injection with phenylephrine, we sought to assess the response to adrenergic stimulation of the smooth muscle cells surrounding the vessels. Previous studies support the existence of alpha-adrenoceptors on lymphatic smooth muscle [16,31]. It has been concluded that conditions characterized by increased sympathetic outflow may augment lymphatic function through alpha 1- but not alpha 2-adrenoceptors [32]. Phenylephrine (PE, 0.1–1.0 μM) was shown to produce dose-dependent increases in frequency and decreases in diameter [16]. In our experimental design, lymphatic contraction frequency was assessed under basal conditions and significantly more contractions were observed in mice pretreated with VEGF-C 152s throughout atherosclerosis onset and progression (Fig. 4A). The amplitude of contraction remained unchanged (Fig. 4B). Following PE addition, the same trend as baseline was observed with the exception of the period of time that mice were fed on an HFD (Fig. 4C). This time, the amplitude of the contraction seems to have been favored (Fig. 4D). A possible mechanism that seemed plausible for the modulation of the contraction capacity is nitric oxide (NO) produced by the endothelial NO synthase (eNOS). In fact, eNOS affects the function of the whole lymphatic system and is regulated via the collecting lymphatics [33]. VEGF receptor-2 and -3 stimulation in lymphatic endothelial cells activates downstream effectors such as Akt and increases the phosphorylation of eNOS [34]. We show that eNOS expression in the collecting lymphatic vessels of our different groups of mice (Supplementary Figs. 6A and B) is not modulated in mice that undergo early treatment with VEGF-C 152s. *In vitro*, lymphatic endothelial cells treated with VEGF-C 152s had no significant changes in the phosphorylation of Akt

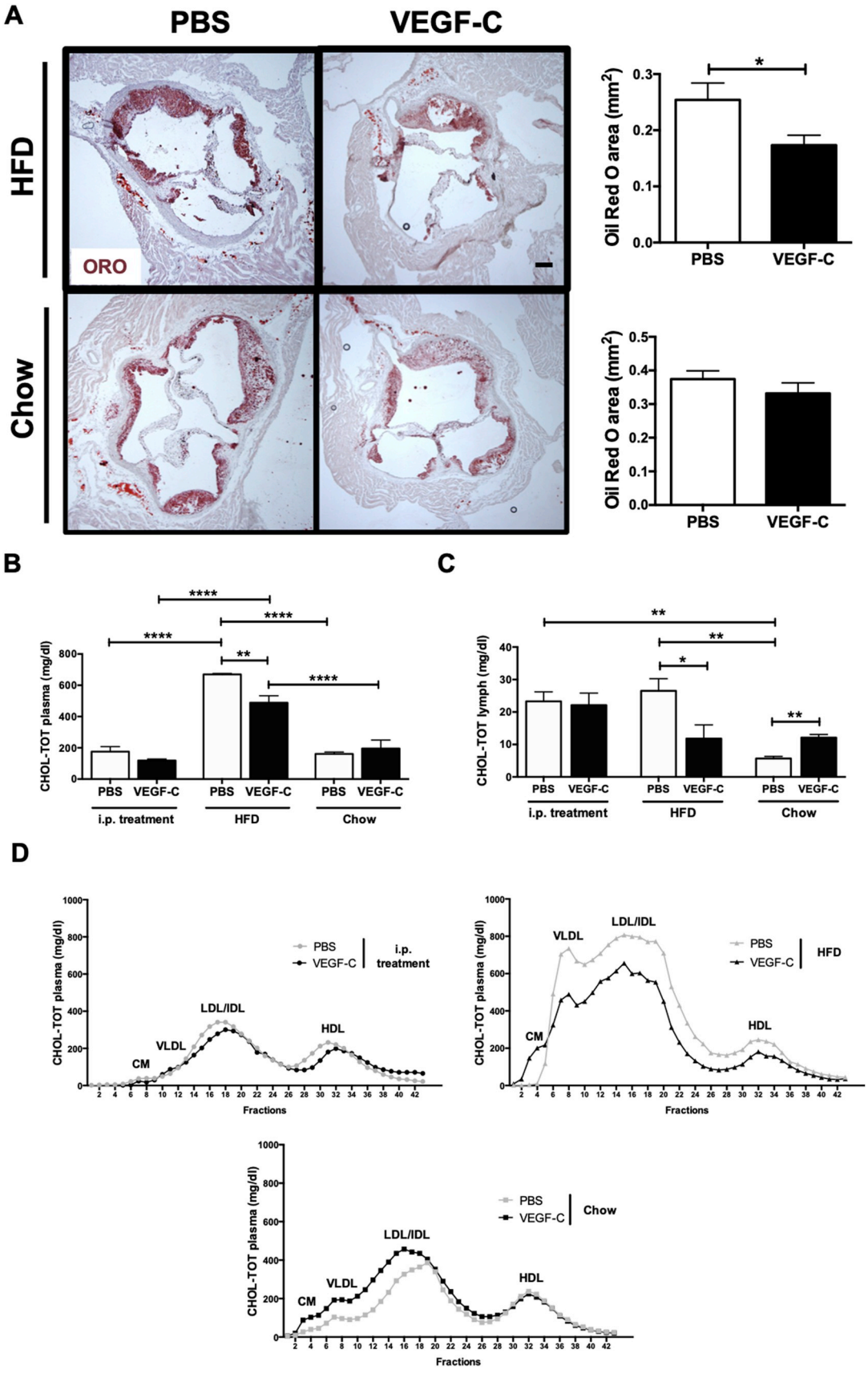

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Fig. 2. Early treatment with VEGF-C 152s enhances total cholesterol mobilization through lymphatic vessels and restrains buildup of atherosclerotic plaque.
Oil Red O (ORO) percentage was quantified in the two groups of mice, (A) in 8 μm-thick aortic sinus sections, parametric *t*-test with Welch correction. Experiments were performed with 8–10 mice per experimental group. Total cholesterol was measured in (B) plasma and (C) lymph of $Ldlr^{-/-}$ mice treated with VEGF-C 152s or PBS, at all timepoints. ANOVA with Bonferroni *post hoc* test. (D) Plasma from mice at all time points was pooled and subject to gel filtration by FPLC. The concentration of lipoproteins in each fraction (total of 43) was measured. Experiments were performed with 5 mice per experimental group. Scale bar = 100 μm. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{****}p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Supplementary Fig. 6C) or eNOS (Supplementary Fig. 6D).

3.6. Pre-treatment with VEGF-C 152s instigates and maintains high levels of VEGFR3 expression on lymphatic endothelial cells

The VEGF-C/VEGFR3 signaling pathway contributes to resolving chronic skin inflammation by activating lymphatic vessel function [35]. Of particular interest, VEGF-C stimulates the lymphatic pump by a VEGF receptor-3-dependent mechanism [7]. We wanted to see if VEGFR3 is modulated in an atherosclerotic setting in mice pre-treated with VEGF-C 152s. We observed that VEGFR3 expression at the surface of collecting lymphatic vessels is higher compared to the control group before atherosclerosis onset and maintained all throughout the atherosclerotic process (Fig. 5A). Control mice had a significantly lower VEGFR3 expression at the surface, but this significantly caught up with treated-mice levels once the atherosclerotic disease progressed and a lesion was formed, an effect maintained even after mice were switched to a normal diet. Podoplanin is also expressed on lymphatic endothelial cells and facilitates blood/lymphatic vessel separation [36]. No changes in podoplanin expression were observed at any time point (Supplementary Fig. 7A).

3.7. FOXC2 might contribute to the maintenance of collecting lymphatic vessel phenotype following onset of atherosclerosis

Previous studies indicate that flow is required to initiate valve formation, to direct the vascular remodeling that converts a primary mesenteric plexus into a hierarchical drainage system, and to control the extent of SMC coverage [37]. FOXC2-deficient animals exhibit defects in both valve formation and SMC recruitment in lymphatic collecting vessels [38]. As valves play a crucial role in proper lymphangion contraction [39], we sought to investigate if VEGF-C 152s treatment might affect FOXC2 expression on the collecting lymphatic vessels. We have shown in a recent study that FOXC2 is downregulated while mice are on a high fat diet [29], and have reproduced this finding here. The results we report herein suggest that VEGF-C 152s prevents against this decrease mediated by a HFD (Fig. 5B). This may showcase a potential mechanism of valve rescue and overall collecting lymphatic vessel integrity that allows for a good contraction capacity despite the onset of atherosclerosis. As FOXC2 is known to recruit SMC on the collecting vessels, which play a thorough role in the muscle stimulated contraction of the vessel, we also looked at SMC coverage of collecting lymphatic vessels but saw no differences in our treated mice compared to control (Supplementary Fig. 7B). This indicates that FOXC2 may not be a primary mechanism of action in this case and is responsible at the very least in maintaining proper lymphatic remodeling, and we assume proper valve function as well, in mice treated with VEGF-C 152s.

4. Discussion

As atherosclerosis develops from an early stage, depending on numerous genetic and environmental risk factors, prevention would be the best treatment of all. Therapies that could prevent or at least diminish atherosclerotic plaque buildup should be the ultimate goal. Systemic inflammation is a critical part of the atherosclerotic process, and studies of VEGF-C in inflammatory bowel disease (IBD), a chronic inflammatory disease, proved to be useful in better understanding atherosclerosis. Crohn's disease, for example, is associated with an aberrant mucosal immune response and, furthermore, D'Alessio et al. demonstrated that adenoviral induction of VEGF-C expression provides marked protection against the development of acute and chronic colitis in two different animal models [11]. These authors believe that VEGF-C offers protection mediated by decreasing the production of M1 macrophages (pro-inflammatory), thus allowing the alternative activation of repair macrophages, M2 (anti-inflammatory). This all leads to a decrease in pathological inflammation, once more suggesting that treatment with VEGF-C may serve to delay the development and/or progression of atherosclerosis.

Lymphatic vessels are now recognized as prerequisite players in the modulation of cholesterol removal from the artery wall [3]. Previous studies have demonstrated a rescue in lymphatic dysfunction associated with the early stages of atherosclerosis development [3,10,29,40]. In this study we aimed to investigate if by administering lymphatic vessel specific treatment before the onset of atherosclerosis, we would be able to modulate the initiation of atherosclerosis and its progression. VEGF-C, an important growth factor for the lymphatic vasculature, has been under thorough investigation with respect to his abilities to induce the formation of new lymphatic vessels. However, despite VEGF-C being often associated with lymphangiogenesis, we herein evaluated its effect on the contractile capacity of the collecting lymphatic vessels, the main subunits dysfunctional in the early stages of atherosclerosis development [10]. Specific mutants of VEGF-C that solely bind VEGFR3 have proven efficient in the past, both by rescuing lymphedema through mechanisms of lymphangiogenesis, but most importantly, by increasing the pumping capacity of the collecting lymphatic vessels in the rat mesentery with a different isoform than the VEGF-C we used [7].

Pre-treatment with VEGF-C 152s before mice were fed a pro-atherosclerotic regimen led to promising results in the quest of new treatments that may one day prevent altogether any signs of cardiovascular disease [10,41]. Following treatment administration, before the development of the atherosclerotic lesion, the molecular transport capacity was already higher, an effect that may be mirroring an increase in cholesterol transport by the lymphatic vessels. Enhanced expression of MCP-1 was demonstrated in a variety of pathologic conditions associated with inflammation and mononuclear cell accumulation. Extensive experimental evidence suggests that MCP-1 is highly expressed in atherosclerotic plaques and mediates macrophage recruitment in the atheromatous lesion [42]. In our study, immediately following VEGF-C 152s treatment, MCP-1 is significantly downregulated, as a possible pre-emptive move to prevent macrophage recruitment, which might be at least partially responsible for a limited macrophage accumulation, once mice are fed on an HFD to promote atherosclerotic lesion formation, as compared to control. This prevention is important, as newly differentiated macrophages fail to penetrate significantly deeper than the limited depth they reside on initial entry, regardless of plaque progression, or regression [43].

In this present study, cholesterol levels are similar between groups before the administration of the pro-atherogenic regimen, notwithstanding of the pre-treatment applied. However, once these mice are switched to a HFD they have drastically increased total circulating cholesterol levels in plasma, and in the control mice the concentration is significantly higher than VEGF-C 152s treated mice. Contrarily, in previous studies [10], $Ldlr^{-/-}/hapoB100^{+/+}$, a different strain of mice that do not require a HFD for plaque to be induced, had increased

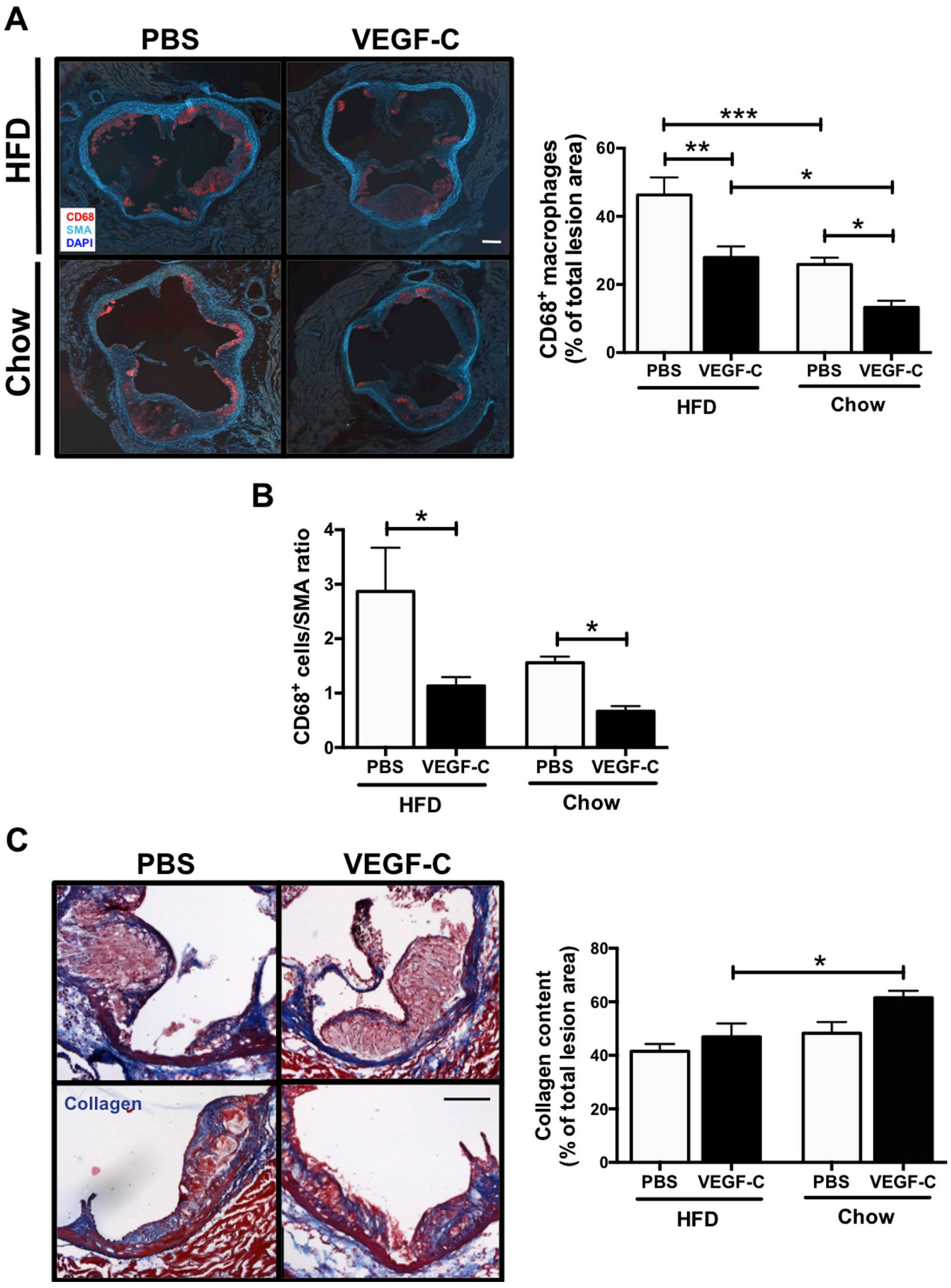

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Fig. 3. Early treatment with VEGF-C 152s modulates atherosclerotic plaque development in HFD- and chow-fed *Ldlr*$^{-/-}$ female mice.
The percentage of (A) CD68^{+}DAPI^{+} macrophages and (B) CD68^{+} cells/SMA area was quantified in 8-μm-thick aortic sinus sections using ImageJ software in HFD- and chow-fed *Ldlr*$^{-/-}$ female mice. (C) The percentage of collagen content using Masson Trichrome staining (blue fibres) was quantified using ImagePro software. Experiments were performed with 7–10 mice per experimental group and 3 to 6 different sinuses per mouse were averaged for the final result. Images were quantified using ImageJ software. Scale bar = 100 μm *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$; DAPI, 4′,6-diamidino-2-phenylindole. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cholesterol levels following early treatment with VEGF-C 152s despite restauration of the lymphatic function. The *Ldlr*$^{-/-}$*/hapoB100*$^{+/+}$ mice genetically overexpress human apoB100, which is synthesized by the liver and secreted within VLDLs that are metabolized in plasma to form LDL [44]. Of notice is that chylomicrons produced by the intestine contain apoB48, whereas VLDLs made by the liver contain apoB100. Our values do not differentiate between these two entities, as the total apoB concentrations were measured. As observed, systemic injections of VEGF-C 152s had no effect on the lipoprotein profile immediately following the VEGF-C 152s treatment. When the pro-atherogenic regimen was applied, however, pre-treatment with VEGF-C 152s improved the absorptive capacity of the lacteals, as reflected by the presence of plasma chylomicron remnants. This observation was paralleled with lower levels of plasma VLDL and LDL/IDL as compared to the PBS control group, whereas TG or total apoB levels were undistinguishable between the PBS- and the VEGF-C- treated groups. Once the mice were switched back to a chow diet, TG concentrations went down in both groups, while total apoB levels remained high. VLDL and IDL/LDL greatly diminished in the control group, whereas the reduction in the VEGF-C-treated group was more modest. This is probably due to the fact that chylomicron remnants are left over due to the increase of lacteal function and supposed absorption. The chylomicron remnants are taken up by the liver and processed to VLDL/IDL/LDL [45]. Throughout the whole experimental design, VEGF-C 152s did not significantly modulate HDL levels. Interestingly, cholesterol was increased in lymph compared to control, meaning that instead of being deposited into the plaque like it does in control, VEGF-C 152s treated mice have proper and constant cholesterol mobilization out of peripheral tissues. This supports the results of significantly higher collecting lymphatic vessel contractions when mice undergo atherosclerosis development and are fed on an HFD or chow diet.

As soon as atherosclerosis development progressed, cellular transport was rescued in comparison to control. In parallel to lower circulating cholesterol levels, in both lymph and plasma, mice fed on an HFD also showed significant signs of restrained accumulation of atherosclerotic plaques, as well as decreased immune cell accumulation. Interestingly, once the HFD was removed and replaced by a chow diet to diminish plaque burden, and assess if the lesion was further stabilized, we observed that mice having been pre-treated with VEGF-C 152s had plaques that contained a higher level of collagen, decreased CD68^{+} cells accumulation and a lower macrophage/smooth muscle cell ratio. Further supporting these data was an augmented concentration of circulating IL-10, a known atheroprotective cytokine that reduces immune cell accumulation and maintains collagen levels [46]. One possible mechanism for these beneficial effects could be the modulation of systemic circulatory levels of this atheroprotective cytokine. IL-10 plays critical roles in both atherosclerotic lesion formation and stability. Atherosclerotic lesions of IL-10–deficient mice showed increased immune cell accumulation and decreased collagen content [47].

The integrity of lymphatic vessels in inflammatory settings has quickly emerged in recent years as a crucial factor in the severity of the atherosclerotic process. Previous studies have shown that the lymphatic dysfunction, which we attributed to occur at the level of the collecting lymphatic vessels, could be rescued in mice with already established atherosclerosis. Although VEGF-C 152s does promote lymphangiogenesis, as observed in both the aortic sinus and systemically, we do not attribute any significant role to these supplementary vessels in combatting atherosclerosis. We cannot eliminate the fact that the initial lymphatic vessels may compensate in cases of increased interstitial pressure [48,49], augmented cell accumulation in the periphery [50] and local changes in matrix stiffness that regulate cellular functions such as adhesion and invasiveness [51]. VEGF-C mediated enhancement of lymphatic drainage and reduced intestinal inflammation in experimental chronic colitis [52]. Furthermore, human studies have shown increased levels of VEGF-C and LYVE-1 in postmortem atheroma samples compared with healthy controls which indicated that cytokine secretion activates the lymphatic system and this later causes increased lymphangiogenesis towards the inflamed atheroma plaque combined with increased lymphocyte/macrophage trafficking in atherosclerosis [53]. However, an increased absorption does not lead to automatic proper propulsion of the lymph through the collecting lymphatic vessels. Indeed, studies [10,29,54,55] support the fact that more vessels do not necessarily lead to more functioning vessels overall.

Although treatments such as apolipoprotein A-I (apoA-I) rescue the lymphatic permeability associated with atherosclerosis by modulating platelet [29] or platelet extracellular vesicles adhesion to the lymphatic endothelium, unlike apoA-I treatment, early treatment with VEGF-C 152s had no significant effects on lymphatic collecting vessel permeability. This effect was further supported by analyses of lymphatic endothelial cell junctions where VE-cadherin levels did not differ between our different groups.

The VEGF-C/VEGFR3 axis was shown to promote mesenteric lymphatic vessel contractions in the rat under *ex vivo* conditions. Our results were observed *in vivo* and for the first time, we were able to follow changes in lymphatic vessel contraction frequency and amplitude even before the onset and progression of atherosclerosis. The intrinsic lymphatic vessel contraction frequency was significantly higher than control under all conditions. To assess the effects on muscle tonus, phenylephrine was injected in the footpad dermis of the mice. Lymphatic vessels possess no basal adrenergic tone [56]. Phenylephrine (1–1.0 μM) has already been shown to produce dose-dependent increases in frequency and decreases in diameter in the rat [16]. In the present study, results show that with the exception of the period of time where mice were fed on a HFD, the frequency of contraction was significantly higher than control in mice that had undergone early treatment with VEGF-C 152s. This suggests that PE could not overcome the decrease in contractions that is well known to occur once mice are fed a chronic HFD [57]. Lymphatic endothelial cells serve as sensors of local lymph flow and transduce signals to lymphatic smooth muscle [58]. As smooth muscle cell coverage did not differ between conditions in our two different groups, we can exclude any alterations with respect to lymphatic vessel muscle coverage following VEGF-C 152s treatment. Furthermore, at the only time point (HFD) that we did not observe any differences in contraction pumping after addition of phenylephrine, other changes systemically due to the VEGF-C 152s treatment still counteracted the negative effects of the disease observed in control. Indeed, the intrinsic capacity of the lymphatic vessels resisted the negative effects of a HFD through systemic downregulation of TNF-α. The pro-inflammatory cytokine TNF-α inhibits lymphatic pumping via activation of the NF-κB-iNOS signaling pathway [59].

Although we did see some changes at the molecular level of different genes expression in lymphatic vessels, we stipulate that VEGF-C 152s seems to be important in a multi-faceted sequential way. From the very beginning of the atherosclerotic process, VEGF-C 152s increases VEGFR3 expression and maintains it throughout the atherosclerotic process, improves lymphatic contraction capacity, and reduces

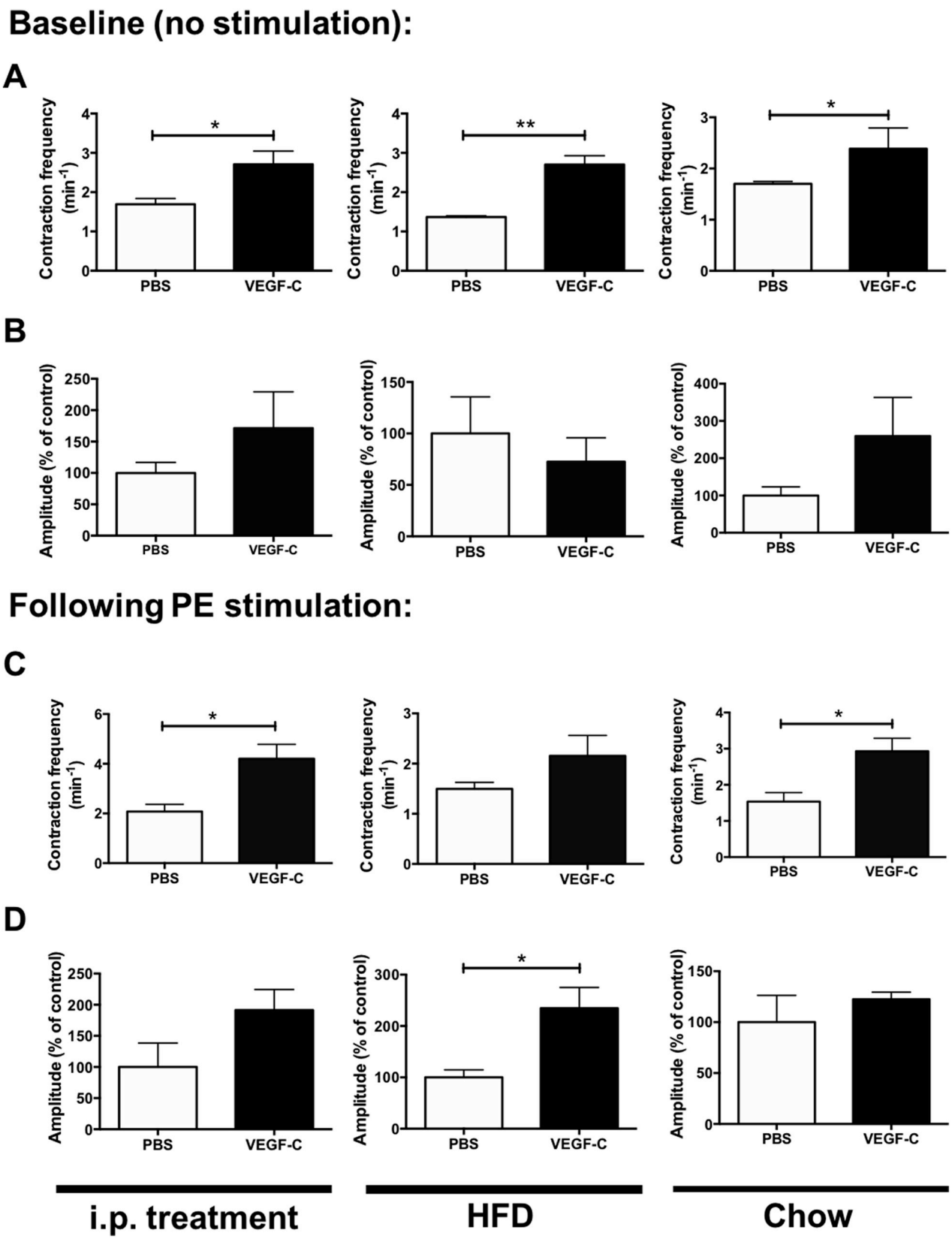

Fig. 4. Early treatment with VEGF-C 152s increases collecting lymphatic vessel capacity to contract throughout the whole atherosclerotic process.
Popliteal lymphatic vessels exhibit consistent pumping activity *in vivo*. Representative lymphatic contraction frequency curves at (A) baseline and (C) following phenylephrine (PE) stimulation show reduced contraction frequency in control compared to VEGF-C 152s treated *Ldlr*$^{-/-}$ mice. Lymphatic (B) baseline and (D) following PE analyses of amplitude, respectively. Experiments were performed using 5 mice per experimental group and three different regions of interest were analyzed at random. Parametric *t*-test with Welch correction. *$p < 0.05$ and **$p < 0.01$.

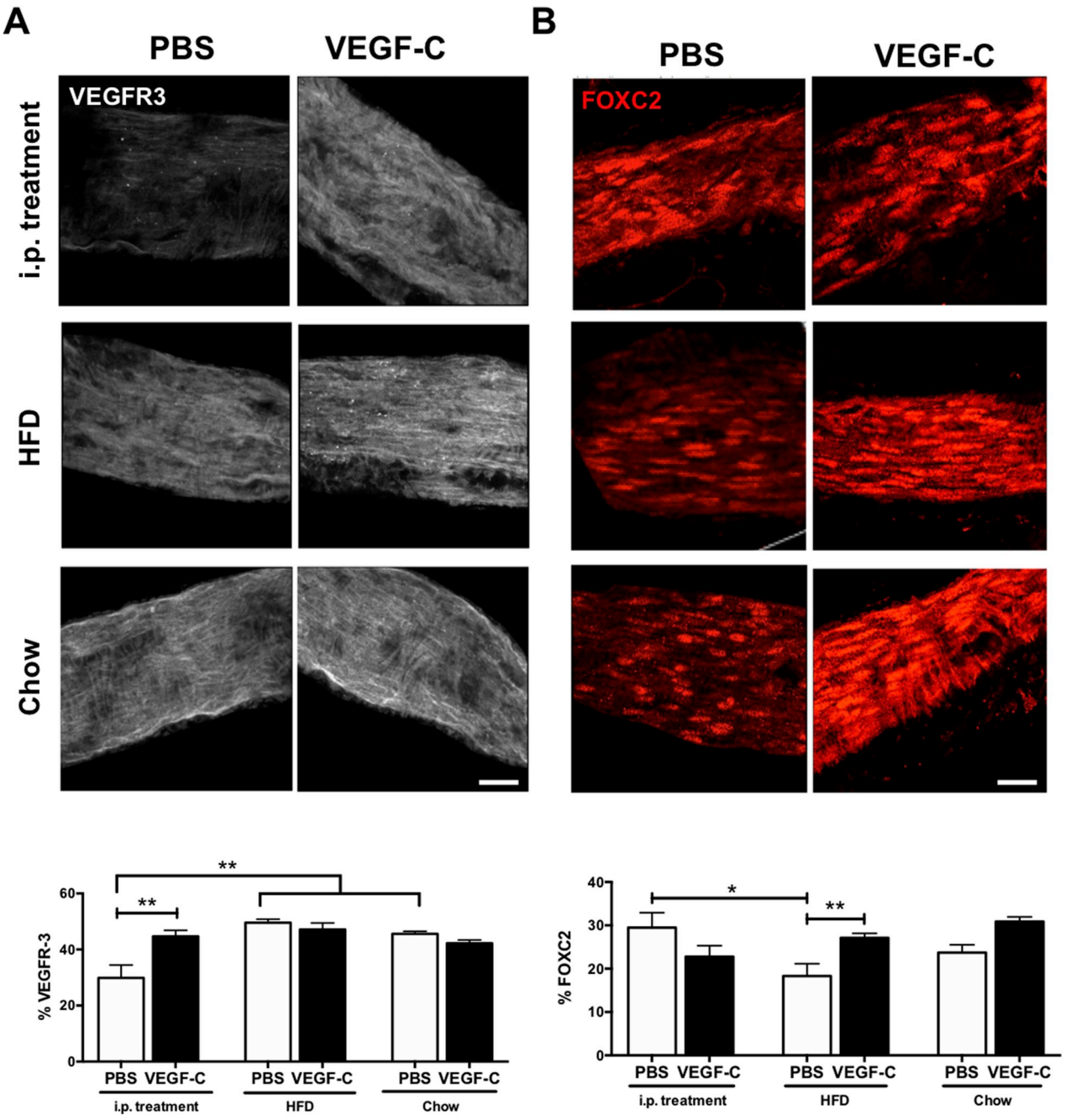

Fig. 5. Modulation of the expression of different lymphatic endothelial cell markers maintains collecting lymphatic vessel characteristics at different stages of the atherosclerotic process.

Popliteal collecting lymphatic vessels were harvested and processed for immunofluorescence detection. Representative images of (A) VEGFR-3 and (B) FOXC2 expression and quantification on collecting lymphatic vessels of PBS and VEGF-C 152s treated $Ldlr^{-/-}$ mice throughout the entire atherosclerotic process are illustrated. Longitudinally imaged vessels were acquired with an LSM 710 Confocal Microscope (Zeiss) equipped with a 63X/1.4 oil dic objective. Experiments were performed with 5 mice per experimental group and three different regions of interest were analyzed at random. ANOVA with Bonferroni *post hoc* test. Scale bars = 10 μm. $*p < 0.05$ and $**p < 0.01$.

inflammatory cell accumulation. We propose that this is the instigating mechanism by which VEGF-C 152s modulates inflammation systemically and within plaque, both leading to a smaller and more stable plaque. Subsequently, VEGF-C 152s administration prevented down-regulation of FOXC2 whose absence/reduction is well known to be implicated in lymphatic valve failure [38], thereby contributing to the protection of the associated lymphatic dysfunction while mice were switched to a HFD.

4.1. Conclusion

Altogether, our results highlight the multiple mechanisms underlying the proper role of lymphatic vessels throughout the process of atherosclerosis. These findings show that atherosclerosis can be modulated if early treatments that specifically target the lymphatic vasculature are administered. In this study, early treatments with VEGF-152s restrained the accumulation of plaque and contributed to its stabilization thereafter. The responsible mechanisms interact at

different stages of the disease and in different ways, all aiming to preserve the lymphatic network and allow for proper reverse cholesterol transport from plaque. To our knowledge, we are the first to correlate *in vivo*, in a timely matter, that a rescue in lymphatic vessel contractions leads to beneficial effects with respect to plaque accumulation, its properties, and overall systemic inflammation that leads to many deleterious effects. Further investigations into different kinds of treatments targeting the lymphatic vessels, particularly at the level of the collecting vessels, crucially deserve further attention and might actually lead to not only an efficient atherosclerosis treatment, but above all, prevent the risk of developing an atherosclerotic lesion.

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

AM and CM: Study conception and design; data acquisition, analysis and interpretation; manuscript writing and critical revision. AS: data acquisition support.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.01.031.

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