Apolipoprotein A-I Modulates Atherosclerosis Through Lymphatic Vessel-Dependent Mechanisms in Mice

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Background—Subcutaneously injected lipid-free apoA-I (apolipoprotein A-I) reduces accumulation of lipid and immune cells within the aortic root of hypercholesterolemic mice without increasing high-density lipoprotein–cholesterol concentrations. Lymphatic vessels are now recognized as prerequisite players in the modulation of cholesterol removal from the artery wall in experimental conditions of plaque regression, and particular attention has been brought to the role of the collecting lymphatic vessels in early atherosclerosis-related lymphatic dysfunction. In the present study, we address whether and how preservation of collecting lymphatic function contributes to the protective effect of apoA-I.

Methods and Results—Atherosclerotic Ldlr<sup>−/−</sup> mice treated with low-dose lipid-free apoA-I showed enhanced lymphatic transport and abrogated collecting lymphatic vessel permeability in atherosclerotic Ldlr<sup>−/−</sup> mice when compared with albumin-control mice. Treatment of human lymphatic endothelial cells with apoA-I increased the adhesion of human platelets on lymphatic endothelial cells, in a bridge-like manner, a mechanism that could strengthen endothelial cell–cell junctions and limit atherosclerosis-associated collecting lymphatic vessel dysfunction. Experiments performed with blood platelets isolated from apoA-I-treated Ldlr<sup>−/−</sup> mice revealed that apoA-I decreased ex vivo platelet aggregation. This suggests that in vivo apoA-I treatment limits platelet thrombotic potential in blood while maintaining the platelet activity needed to sustain adequate lymphatic function.

Conclusions—Altogether, we bring forward a new pleiotropic role for apoA-I in lymphatic function and unveil new potential therapeutic targets for the prevention and treatment of atherosclerosis. (J Am Heart Assoc. 2017;6:e006892. DOI: 10.1161/JAHA.117.006892.)

Key Words: apolipoprotein A-I • atherosclerosis • collecting lymphatic vessels • platelets

The lymphatic system is an open, unidirectional system playing multiple roles in immunity, chylomicron transport, and clearance of wastes from the periphery. It is characterized by a network of vessels that carry a clear fluid called lymph. Sequentially, ultrafiltrates from peripheral tissues are absorbed by initial lymphatics (also called lymphatic capillaries), a highly permeable and specialized compartment constituted by discontinuous “button-like” junctions between endothelial cells. These initial lymphatics are characterized by the absence of smooth muscle cells and the presence of lymphatic vessel endothelial hyaluronan receptor (LYVE-1) at the surface of lymphatic endothelial cells (LECs). Following its absorption, lymph moves on to converge into larger vessels called collecting lymphatic vessels that are partially covered with smooth muscle cells, and characterized by the expression of podoplanin. Collecting vessels are composed of contractile lymphangions that propel lymph in a unidirectional manner, with the help of intraluminal bi-leaflet valves, as well as smooth muscle cells. Eventually, lymph passes through the lymphovenous junction and reaches the blood circulation via the subclavian vein. Junctional organization of LECs in initial lymphatics and collecting vessels is crucial to vessel integrity. The transcriptional factor FOXC2 (Forkhead box protein C2) has been identified to play a key role in cell–cell junctions and lymphatic valve integrity. Depletion of FOXC2 has led to major lymphatic dysfunction and lethality. However, many
Clinical Perspective

What Is New?

- We bring forward a new pleiotropic role for apolipoprotein A-I in preserving and restoring lymphatic function by direct and indirect mechanisms that include platelet activity.

What Are the Clinical Implications?

- Our work unveils new potential therapeutic targets for the prevention and treatment of atherosclerosis.

Whereas the adventitial lymphatic vessels are now accepted as important modulators of cholesterol transport between the atherosclerotic lesion and the bloodstream, the apoA-I (apolipoprotein A-I) has been identified as a key regulator of cellular cholesterol efflux via the ABCA1 receptor present at the cell surface. ApoA-I is the main protein constituent of plasma high-density lipoprotein (HDL) and participates in its highly heterogeneous properties.

Whereas subcutaneous injections of low-dose lipid-free apoA-I treatment do not significantly increase plasma HDL-cholesterol concentrations, lipid-free apoA-I reduces excess cellular cholesterol and reverses the autoimmune-like phenotype that develops in high cholesterol diet–fed Ldlr\(^{-/-}\) apoA-I\(^{-/-}\) double knockout mice. Recently, the mechanistic basis explaining this protective effect of small and consistent amounts of apoA-I in reducing lipid and immune cell accumulation within the aortic root has emerged. HDL and apoA-I have been shown to modify atherogenic and antigen presentation properties by interfering with the cell membrane lipid raft. In the same vein, it has been demonstrated that apoA-I acts in hypercholesterolemic mice by systemically reducing excess cellular cholesterol accumulation in membrane lipid rafts. The latter are tightly regulated microdomains contained in cell membranes and they form a platform responsible for organizing the signaling of receptors and proteins of various cell types. In a recent publication, in vitro treatment with apoA-I, on a tumor necrosis factor-\(\alpha\) background, has been found to cause lymphangiogenesis. ApoA-I treatment has been demonstrated to increase podoplanin mRNA level, which could potentially help the CLEC-2/podoplanin interaction with platelets.

In the present study, we hypothesize that the protective effect of apoA-I is mediated at least in part by preserving collecting lymphatic vessel function by mechanisms that include modulating platelet adhesion on LECs. Our findings could bring forth a new pleiotropic role for apoA-I in lymphatic function and unveil new potential therapeutic targets for the prevention and treatment of atherosclerosis.

Methods

Experimental Setup

Ldlr\(^{-/-}\) mice on a C57BL/6 background were purchased from Jackson Laboratories. Animals were housed in a pathogen-free environment under 12-hour light–dark cycles with free access to water. The experimental design of the in vivo section of our study is illustrated in Figure 1A. Female Ldlr\(^{-/-}\) mice at 6 weeks of age were fed a high-fat diet (HFD—adjusted calories diet, 0.2% total cholesterol and 42% from fat, Harlan 88137) for 8 weeks, after which they were...
separated into 3 age-matched groups for a period of 6 more weeks. To assess the direct effect of apoA-I on lymphatic function, 1 group was injected intradermally with lipid-free apoA-I (200 µg/25 g of body weight) 3 times a week for 6 weeks while still under a HFD. The control group received concentration-matched BSA alone. A third group was switched to standard chow diet, in order to lower plasma cholesterol and promote lesion regression. At the end of the sixth week, lymphatic function assays were performed and blood and lymph were collected under anesthetic conditions. Following this, mice were euthanized by cardiac puncture following ketamine anesthesia (100 mg/mL ketamine administered at 0.10 mL/10 g of body weight), or by carbon dioxide (CO₂), and were perfused with 15 mL PBS. Finally,

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**Figure 1.** Study design and assessment of circulating total cholesterol and lipid deposition in \( Ldlr^{-/-} \) mice. A, Pictogram of the experimental design used. At 6 weeks of age, female \( Ldlr^{-/-} \) mice began a high-fat diet (HFD, hatched bar). After 8 weeks, mice were divided into 3 groups. Two groups (groups #1 and #3) remained on the HFD during the treatment phase of the study and were divided as follows: group #1—intradermal (i.d.) injections of 200 µg of BSA, 3 times/wk (black bar); group #3—intradermal injections of 200 µg of lipid-free apolipoprotein A-I (apoA-I), 3 times/wk (white bar). Group #2 was switched on a chow diet instead of receiving injections (light gray bar). After 20 wks of age, the mice were evaluated and lymphatic function assessed. B and C, Neutral lipid- (Oil Red O; ORO) area was quantified in the 3 groups of mice (en face) using ImageJ software. Experiments were performed with 7 to 11 mice per experimental group. Total cholesterol was assessed in (D) plasma and (E) lymph of BSA-, diet switch-, and apoA-I-treated \( Ldlr^{-/-} \) mice. Experiments were performed with 4 mice per experimental group. **\( P \leq 0.01 \) and ***\( P \leq 0.001 \), as determined by 1-way ANOVA with Tukey’s post-hoc test.
organs were collected. All experiments were performed in accordance with the Canadian Council on Animal Care guidelines and approved by the Montreal Heart Institute Animal Care Committee.

ApoA-I Preparation
ApoA-I was purified from human plasma by sequential ultracentrifugation as previously described. The apoA-I was lyophilized to dryness, dissolved in 6 mol/L guanidine hydrochloride, and then refolded by dialyzing exhaustively against 10 mmol/L ammonium bicarbonate, pH 7.4. Mass spectrometry and 12% SDS-PAGE were used to ensure that there were no contaminating proteins and that apoA-I methionines were not oxidized to the sulfoxide form, a common contaminant in apoA-I preparations. Protein concentration was determined using the Lowry assay.

ApoA-I Kinetics
To ensure that the lipid-free apoA-I injected was absorbed by the initial lymphatics present in the back skin dermis and that apoA-I kinetics in lymph reflect that of blood, we used wild type mice on a C57BL/6 background purchased from the Jackson Laboratories (Figure S1). Wild type mice were anesthetized with isoflurane (4% for induction, 2–3% for maintenance). For lymph collection, the animal was anesthetized and positioned on its right side. A cannula was inserted into the thoracic lymph duct above the cisterna chyli between the transverse lumbar artery and the diaphragm as previously described. Lymph was collected continuously for 45 minutes with a tube attached to a syringe coated with EDTA 0.1 mol/L. Blood was collected by cardiac puncture with a syringe coated with EDTA 0.1 mol/L followed by euthanasia of the animal. Collected lymph was centrifuged at 1200g for 10 minutes and blood was centrifuged at 2400g for 10 minutes. To avoid thawing-related damage on lipoprotein conformation, sucrose (5%) was added before samples were stored at −80°C for further batch analysis. Human ApoA-I was measured by ELISA tests.

Lymphatic Functional Assessment
Lymphatic function was assessed by 3 complementary methods. First, the propensity of dendritic cells to migrate through the lymphatic vessels from the periphery to draining lymph nodes (LNs) was measured as described previously. The animals were euthanized 18 hours 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. Of importance, FITC was applied to a different location than the apoA-I injection site, as our interest was to assess the global lymphatic function. The corresponding skin-draining LNs were recovered and enzymatically digested in collagenase D for 25 minutes 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 Biosciences, 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 x # of cells/LN).

Second, 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 following Evans Blue intradermal injection in the footpad, popliteal collecting lymphatic vessels were visualized using a Stereo Discovery V8 (Zeiss). Both the effusion of Evans Blue around the vessel, as well as the area the leakage covers were analyzed using ImageJ software.

Third, lymphatic function was assessed by quantifying the dermal clearance of dextran by the initial lymphatics, as described previously. Briefly, a total of 1 μL fluorescent (Cy5) dextran (70 kDa) at a concentration of 2 mg/mL in sterile PBS was injected intradermally in the ear pinnae of anesthetized mice. Because of its large size, the tracer is specifically uptaken by blind-ended lymphatic capillaries, avoiding absorption by blood capillaries. Fluorescence decay was observed through the skin using a fluorescence stereomicroscope and images of the skin were acquired every minute for 30 minutes. The rate of clearance was determined by calculating the area under the curve of fluorescence intensity at each time point, and normalized to the initial value. The normalized rate of fluorescence decay was then calculated from the slope of area under the curve versus time, which is considered proportional to the actual rate of dextran-Cy5 clearance.

Mouse Platelet Isolation
Mouse blood was withdrawn in 1-mL syringe containing 50 μL of diluted heparin (dilute stock heparin 1:10 to obtain 1000 U/mL). Blood from 2 to 3 mice was pooled to obtain sufficient platelets. Blood was then diluted in Tyrode’s buffer (1/2) containing 0.2 μg/mL of prostaglandin E1 (Sigma) and centrifuged at 164g for 8 minutes. An additional centrifugation was made with diluted red blood cells in Tyrode’s buffer containing 0.2 μg/mL of prostaglandin E1 to obtain more platelets. Prostaglandin E1 0.2 μg/mL was added to the pooled platelet-rich plasma and centrifuged at 1000g for

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5 minutes. The pellet was resuspended in modified Tyrode’s buffer at a concentration of 250×10⁶ platelets/mL.

Mouse Platelet Aggregation
Isolated platelets were submitted to a constant shear rate (1000 rpm) at 37°C in a 4-channel optical aggregometer (Chronolog Corp.). A volume of 250 μL of the washed platelet preparation was used per channel. Platelet aggregation was then induced with high concentration of thrombin (0.5–1 U/mL) in the first channel or podoplanin (5 μg/mL) in the second channel, and the percent aggregation was recorded when platelets reached a plateau.

Immunoblotting of Mouse Platelets
Following aggregations, platelets were lysed by adding 62.5 μL of 4X SDS in 250 μL of washed platelets, and heated at 95°C for 5 minutes. Samples were stored at −20°C until further analysis. Proteins from the three mouse groups’ platelets were separated by 10% SDS-PAGE followed by transfer to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk for 1 hour at room temperature. Antibodies against CLEC-2 (R&D Systems, AF1718) and pAkt (Cell Signalling, 9275) were incubated with the membranes overnight at 4°C, and a horseradish peroxidase–conjugated secondary antibody (Abcam, AB6741) was used for detection using the Western Lightning Ultra chemiluminescence kit (PerkinElmer).

Organ Harvesting
LNs, ears, dermal back skin sections, aortas, hearts, and popliteal collecting lymphatic vessels were harvested and either freshly processed for flow cytometry analysis and/or Western blots, or fixed in 4% paraformaldehyde and 10% formalin for future analysis.

Immunohistochemistry and Immunofluorescence of the Skin Dermis
The back skin of the animals was shaved and harvested, fixed in 10% formalin, and embedded in paraffin. Eight-micrometer (μm)-thick back-skin sections were stained with hematoxylin and eosin. Pictures were taken with an Olympus B45 microscope and visualized using ImagePro Plus 7.0 software. Another batch of 8-μm-thick back-skin sections was incubated with anti-CD206 (Abcam, ab64693), -CD68 (Biologend, 137001), and -LYVE-1 (Abcam, ab14917) antibodies. Secondary antibodies anti-Alexa-fluo 555 (Abcam, ab150074), -Cy3, and -Cy5 (Jackson ImmunoResearch, 712-165-150 and 711-606-152, respectively), as well as 4’,6-diamidino-2-phenylindole (DAPI) were then added and images were acquired with an LSM 710 Confocal Microscope (Zeiss) equipped with a ×63/1.4 oil mic objective.

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 vessel integrity, whole-mount immunofluorescence analysis following incubation with anti–smooth muscle actin already coupled to FITC (Sigma) and -FOXC2 (R&D Systems, AF6989) antibodies was performed on isolated popliteal lymphatics of mice. Secondary antibody donkey anti-sheep 555 (Jackson ImmunoResearch, 713-165-147) and DAPI were then added. Images were acquired with an LSM 710 Confocal Microscope (Zeiss) equipped with a ×63/1.4 oil mic objective. All image handling was performed using ImageJ software.

Atherosclerotic Lesion and Initial Lymphatic Density Quantification
The heart and aorta were removed and fixed in 4% paraformaldehyde for 2 hours. The heart was transferred into PBS containing 30% sucrose (wt/vol) overnight at 4°C before being immersed in optimal cutting temperature compound and stored at −80°C. Eight-micrometer-thick cryosections of the aortic sinus were prepared. Cross-sections of the aortic sinus were stained with anti-LYVE-1 (Abcam, ab14917) and anti-CD68 (Biologend, 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 described previously.42 Ear dermis was stained for lymphatic capillaries (anti-LYVE-1, Abcam) at 4°C, and then sections were incubated with Alexa Fluor 647-conjugated donkey anti-rabbit antibody and Cy3 donkey anti-rat (Jackson ImmunoResearch, 711-606-152 and 712-165-150, respectively). All imaging was performed on a Fluoview FV10i (Olympus). Vessel counts were performed by 1 observer. The relative quantification of the number of initial lymphatics (LYVE-1⁺ vessels), their diameter, and the total surface area they occupy was determined by computer-assisted morphometric analysis. 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).
Human Platelet Isolation

All experiments performed with human specimens were approved by our institutional review committee and every subject gave informed consent. Human blood from healthy donors was collected with a syringe containing Anticoagulant Citrate Dextrose Solution and centrifuged at 200 g for 15 minutes. Prostaglandin E1 (1 μg/mL) was added to the platelet-rich plasma and centrifuged at 1000 g for 10 minutes. Platelets were washed with Hanks balanced salt solution citrate buffer and the suspension was centrifuged at 800 g for 10 minutes. Lastly, platelets were resuspended in Hanks balanced salt solution for a total concentration of 250×10^6 platelets/mL.

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 1 μ-Slide VI.4 tissue culture treated flow chamber (Ibidi) or in a glass-bottom culture dishes chamber (Mattek) for experiments that did not require a steady perfusion flow rate. In both of these cases, HMVEC-dLyAd at 80% confluence were treated with apoA-I (0.6 mg/mL) for 24 hours.

Platelet Perfusion Under Physiological Lymph Flow

Following treatment, for underflow, isolated human washed platelets were perfused over the HMVEC-dLyAd at a wall shear rate of 50/s at 37°C for 8 minutes. PBS was then added to remove the nonadhered platelets. For static treatments, the HMVEC-dLy-Ad were treated with washed platelets for 1 hour. Cells were then fixed with paraformaldehyde 2% and immunofluorescence analysis was performed following incubation with anti-CD61 (Clone: VI-PL2, Biolegend).

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 minutes. Proteins were separated by electrophoresis on a 12% SDS-PAGE, then transferred on nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBST, 0.1% Tween 20) for 1 hour at room temperature, then incubated with an anti-podoplanin (OriGene, DP3512S), an anti-vascular endothelial growth factor receptor (VEGFR)-3 (Abcam, AB27278), 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 hour at room temperature. Western Lightning Ultra chemiluminescence kit (PerkinElmer) was used for detection. Each sample was normalized with its respective beta-actin expression.

Platelet-Activating Factor Acetylhydrolase Activity

Plasma and lymph from the 3 mouse groups was used to assess total platelet-activating factor acetylhydrolase (PAF-AH) activity. We used the Cayman’s platelet-activating factor (PAF) Acetylhydrolase Assay Kit (Cayman Chemical, 760901) and followed manufacturer instructions.

Statistical Analyses

Data are presented as mean and SEM. Statistical differences were assessed using a 2-tailed parametric Student t test, ANOVA or nonparametric tests, with P<0.05 reported as statistically significant, using Prism software version 6.0 c (GraphPad) or SPSS version 23.0 (SPSS Inc., Chicago, IL, USA).

Study Approval

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. All experiments performed with human specimens were approved by our institutional review committee and every subject gave written informed consent before inclusion in the study.

Results

ApoA-I Treatment Reduces Aortic Lipid Without Significantly Affecting Total Cholesterol Level in Ldlr^-/- Mice

As our goal is to assess the effect of apoA-I on the lymphatic vasculature per se, we performed intradermal injections to allow a more direct drug uptake by the dermal initial lymphatic vessels (experimental design illustrated in Figure 1A). After subtracting the background value, we first measured the time course appearance of human apoA-I in plasma and lymph following intradermal injection of lipid-free apoA-I in HFD-fed Ldlr^-/- mice, and confirmed that the injected apoA-I reach lymph efficiently. Approximately 1.5%
and 3% of the injected human apoA-I appears and peaks by 3 and 8 hours postinjection, in lymph and blood, respectively (Figure S1).

We next tested the effect of this route of injection on the extent of atherosclerosis in our 3 groups of mice. En face aorta ORO staining is decreased following apoA-I treatment; Figure 1B shows representative images of the atherosclerotic lesions present in the thoracic aorta, as assessed after staining with ORO. The results of ORO quantification are expressed as percent of total surface (Figure 1C). \( \text{Ldlr}^{-/-} \) mice that have been switched from a HFD to a chow diet have significantly less plasma cholesterol than control- (BSA-) or apoA-I-treated mice on continuous HFD (Figure 1D). ApoA-I does not significantly decrease plasma (Figure 1D) or lymph (Figure 1E) cholesterol compared with control. Contrarily to mice that had been switched to a chow diet, apoA-I treatment, when injected intradermally, did not, however, significantly reduce lesion size or ORO area, nor CD68+ cells in the aortic sinus (Figure S2A through S2C, respectively) of \( \text{Ldlr}^{-/-} \) mice fed for 8 weeks on a HFD. Figure S2D and S2E show representative images of the aortic root stained with ORO and CD68 antibody, respectively.

**ApoA-I Restores Systemic Cellular Lymphatic Transport in \( \text{Ldlr}^{-/-} \) Mice**

To test whether apoA-I might exert its beneficial effects through its interaction with the lymphatic system, we first measured the transport of dendritic cells from the peripheral tissue (skin) to the corresponding draining LN through the lymphatic system in our different groups.\(^4^0\) We show that apoA-I treatment rescues lymphatic function in atherosclerotic HFD-fed \( \text{Ldlr}^{-/-} \) mice, with respect to the number of dendritic cells that have migrated from the skin to the corresponding draining LN (Figure 2A). In diet switch mice, this effect was not seen, despite a decrease in plasma cholesterol as previously illustrated (Figure 1D). In addition to improving dendritic cell transport from peripheral tissues, this low-dose apoA-I treatment was associated with accumulation of fewer immune cells in the skin dermis of \( \text{Ldlr}^{-/-} \) mice. CD206+ (Figure 2B) and CD68+ (Figure 2C) macrophage levels were decreased in apoA-I-treated mice. Whereas resident CD206+ macrophages are significantly less abundant in the diet switch group, CD68+ macrophages density was not significantly affected by this decrease in plasma cholesterol.

**ApoA-I Does Not Alter Obesity-Related Parameters**

Obesity causes chronic systemic and local inflammation,\(^4^3\) and impairs lymphatic function.\(^4^4\) We therefore sought to investigate whether apoA-I could have acted on lymphatic transport by first reducing obesity-related parameters. Our results revealed that subcutaneous adipose tissue (Figure 3A and 3B), visceral fat (Figure 3C), or total body weight variation (Figure 3D) in \( \text{Ldlr}^{-/-} \) mice are not priori affected following apoA-I treatment in our experimental design.

**ApoA-I Enhances the Prevalence of Initial Lymphatic Vessel Number Without Affecting Their Function**

We have recently demonstrated that lymphatic dysfunction, at least in an early stage of atherosclerotic disease, is mainly

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**Figure 2.** Effect of apoA-I on systemic cellular lymphatic transport in \( \text{Ldlr}^{-/-} \) mice. Using a contact sensitization assay, the assessment of dendritic cell migration through the lymphatic was assessed by flow cytometry. Skin-draining lymph nodes were harvested after 18 hours and the number of dendritic cells that migrated from the skin to the lymph node was determined. We quantified the (A) number of CD45+CD11c+FITC+ cells in BSA-, diet switch-, and apoA-I-treated \( \text{Ldlr}^{-/-} \) mice. Experiments were performed using 5 to 8 mice per experimental group (mean±SEM). Analysis was performed using a BD LSRII flow cytometer. The percentage of (B) CD206+DAPI+ and (C) CD68+DAPI+ macrophages were quantified in 10-μm-thick skin sections from the 3 groups of mice. Experiments were performed with 7 to 11 mice per experimental group and were quantified using ImageJ software. *\( P<0.05 \) and **\( P<0.01 \), as determined by 1-way ANOVA with Tukey’s post-hoc test. apoA-I indicates apolipoprotein A-I; DAPI, 4’,6-diamidino-2-phenylindole.
caused by collecting lymphatic vessel impairment. In our current experimental design, as the atherosclerotic plaque is already advanced, we do not exclude the fact that the defect in lymphatic transport could now reside in both the initial and the collecting lymphatic vessels. We therefore explored lymphatic vessel density in the skin dermis (back skin and ear dermis, Figure 4A and 4B, respectively) and in the adventitia of the aortic sinus (Figure 4C). Reversing the diet to a chow diet does not increase lymphangiogenesis (Figure 4D through 4F). Although no changes are observed in the back skin, apoA-I treatment is reflected by a small albeit significant increase of initial lymphatic vessels in the aortic sinus and in the ear dermis of Ldlr/−/− mice. However, an increase in branching points or initial lymphatic hyperplasia could not be observed concomitantly (Figure 4G and 4H, respectively). To test whether this slight increase in LYVE-1+ vessels was sufficient to improve the uptake of interstitial molecules, we quantified the dermal clearance of a large molecule (dextran Cy5) by the initial lymphatics over a time course of 30 minutes and observed no changes in the dye uptake (Figure 4I).

**Figure 3.** Obesity-related parameters in Ldlr/−/− mice. A, Hematoxylin and eosin staining was performed on 8-µm-thick paraffin skin sections in BSA-, diet switch-, and apoA-I-treated Ldlr/−/− mice. B, Quantification of adipose tissue thickness in back skin is illustrated. Experiments were performed with 5 to 7 replicates per experimental group (mean±SEM). Pictures were taken with an Olympus B45 microscope and analyzed using ImagePro Plus 7.0 software. All image handling was performed using ImageJ software. C, Visceral fat was excised and weighed. Visceral fat percentage was calculated based on the visceral fat and the mouse total body weight. D, Weight gain variation percentage was calculated using the variation between the final and initial weight of the mouse. Experiments were performed with 10 to 12 replicates per experimental group (mean±SEM). Scale bar=500 µm. apoA-I indicates apolipoprotein A-I.
ApoA-I Reduces the Atherosclerosis-Associated Increased Collecting Lymphatic Vessel Permeability

Whereas we observe an enhanced number of initial lymphatic vessels in the apoA-I-treated group, our results suggest that the interstitial fluid uptake does not seem to be improved overall. Thus, we hypothesize that it is only once reaching the collecting lymphatic vessel that lymph flow is compromised and that apoA-I would mediate its beneficial effect solely at that level during atherosclerosis progression. Therefore, we next wanted...

Figure 4. Assessment of initial lymphatic vessels morphology and function in Ldlr−/− mice. Initial lymphatic vessels (Lyve-1+) were imaged by immunofluorescence and their number was quantified in (A and D) the back skin, (B and E) ear dermis, and (C and F) aortic sinus of BSA-, diet switch-, and apoA-I-treated Ldlr−/− mice. In back skins and aortic sinuses, only distinguishably round Lyve-1+ vessels were included, as indicated by the yellow arrows. Data are expressed as the percentage of BSA (control group) in each set of tissues. Experiments were performed with 7 to 11 replicates per experimental group (mean±SEM). Quantification of the (G) branching and (H) diameter of Lyve-1+ vessels in the ear dermis. I, Lymphatic molecular transport was assessed by Cy5-labeled Dextran (70 kDa) injection in the ear dermis of BSA-, diet switch-, and apoA-I-treated Ldlr−/− mice. Experiments were performed with 5 to 11 replicates per experimental group (mean±SEM). Pictures were taken using Fluoview FV10i (Olympus). All image handling was performed using ImageJ software. *P≤0.05 as determined by 1-way ANOVA with Tukey’s post-hoc test. Scale bars=100 μm (B) and 50 μm (A and C). apoA-I indicates apolipoprotein A-I; AUC, area under the curve; DAPI, 4′,6-diamidino-2-phenylindole.

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to investigate whether apoA-I adequately restores collecting lymphatic vessel function. We first determined the effect of apoA-I on collecting lymphatic vessel permeability by injecting Evans Blue in the footpad skin of the mouse. Images of the lower limb were taken to visualize the popliteal lymphatic vessels. Evans Blue leakage surrounding the vessel was quantified (Figure 5A). Our results show that leakage is nearly nonexistent in mice treated with apoA-I, indicative of proper lymphatic vessel integrity (Figure 5B and 5C). In the diet switch group, the area of leakage was also decreased, whereas the perpendicular leakage distance was as impaired as in the BSA group.

Several factors could account for the loss of lymphatic vessel permeability during atherosclerosis. Specific deletion of the Forkhead transcription factor FOXC2 on LECs is associated with a loose and disorganized extracellular matrix, which is indicative of increased endothelial permeability.6 To test whether apoA-I could regulate lymphatic permeability through FOXC2 expression, popliteal lymphatic vessels were harvested, and FOXC2 expression was assessed by immunofluorescence. Our images reveal that FOXC2 expression is similar in Ldlr−/− mice that underwent apoA-I treatment and control mice (Figure S3). However, feeding mice with chow diet appears to increase FOXC2 expression, suggesting that normalizing circulating cholesterol could enhance cell–cell junction stability and endothelial integrity. Our results indicate that apoA-I most likely uses another mechanism to improve lymphatic vessel permeability.

**ApoA-I Enhances Human Platelet Adhesion to Lymphatic Endothelial Cells in Culture**

Among the potential players involved in regulating vessel function and integrity, platelets are a target of interest in lymphatic physiology. Platelets are essential in proper lymphatic function: the interaction between CLEC-2 on platelets and podoplanin on LECs is crucial for the formation of the lymphovenous junction and for the lymphatic vessel integrity per se, during development and throughout life.5 As platelets are gatekeepers for lymphatic endothelial cells, we thus sought to investigate the potential role of platelets in repairing the loss of lymphatic vessel permeability during atherosclerosis. First, we isolated platelets from human blood and assessed platelet adherence to primary HMVEC-dLyAd pretreated for 24 hours with either apoA-I or BSA (control). Our results show that, regardless of the treatment, platelets adhere to the lymphatic endothelium and exert a bridging role in maintaining lymphatic vessel integrity.

![Figure 5](image-url)

**Figure 5.** Assessment of collecting lymphatic vessel permeability in Ldlr−/− mice. A, Lymphatic vascular integrity was assessed following Evans Blue (EB) dye intradermal injection in the footpad of BSA-, diet switch-, and apoA-I-treated Ldlr−/− mice. After 30 minutes, lymphatic vessels were visualized using a Stereo Discovery V8. Pictures were taken by Canon Rebel XSi. BSA and diet switch mice display EB dye leakage around the vessels (green arrows). Histograms on top of each vessel illustrate the propagation of EB dye leakage. Both the (B) leakage distance and (C) its area were assessed and calculated using ImageJ software. Experiments were performed with 4 replicates per experimental group (mean±SEM). * P<0.01 and ** P<0.05, as determined by 1-way ANOVA with Tukey’s post-hoc test. apoA-I indicates apolipoprotein A-I.
Figure 6. Effect of lipid-free apoA-I treatment on platelet adhesion to lymphatic endothelial cells. A through C, Human platelets were isolated and incubated with a confluent monolayer of primary HMVEC-dLyAd for 1 hour at 37°C. LECs and platelets were identified by immunofluorescence using DAPI and anti-CD61 antibodies, respectively. A, Representative images indicating that, under static conditions, apoA-I-treated LECs display a “bridge effect” mediated by platelets pseudopodia, thus assembling LECs together. B, Representative images and (C) quantification of the number of adhered platelets interacting with HMVEC in BSA- (upper panel) and apoA-I- (lower panel) treated LECs. Results are the averages of 5 independent experiments. *P<0.05, as determined by 1-tailed t test. D and E, Human platelets were isolated and perfused over primary HMVEC-dLyAd seeded at maximum confluence in tissue culture treated flow chambers, at a wall shear rate of 50/s at 37°C for 8 minutes. D, LECs and platelets were identified using DAPI and anti-CD61 antibodies, respectively. E, The % augmentation in the number of adhered platelets following treatment is indicated above the bars. P=0.043, using a Wilcoxon signed rank test. Scale bars=10 μm (A and B) and 100 μm (E). apoA-I indicates apolipoprotein A-I; DAPI, 4',6-diamidino-2-phenylindole; HMVEC-dLyAd, human dermal lymphatic microvascular endothelial cells-adult; LECs, lymphatic endothelial cells.
effect through which as little as 1 platelet assembles together several LECs (Figure 6A, left panel). Furthermore, an assembling effect was observed, in which several platelets will aggregate together in an attempt to bring together several LECs (Figure 6A, right panel). Using the same experimental setting, quantification of pseudopodia-shaped platelet adhesion to the lymphatic endothelium allowed us to observe a significant increase in the number of strongly adhered platelets\(^4\) per LECs when the latter were pretreated with apoA-I (Figure 6B and 6C).

As podoplanin is more potent to bind to platelets under flow\(^4\), we exposed apoA-I- or BSA-pretreated LECs to a constant venous shear for 8 minutes using 6-channel \(\mu\)-slides, as depicted in Figure S4. Platelets isolated from 5 different healthy volunteers were perfused over HMVEC-dLyAd at a wall shear rate of 50/s at 37\(^\circ\)C for 8 minutes. Figure 6D illustrates that, under a physiological flow, the prevalence of CD61\(^+\) platelet adherence per LEC (DAPI\(^+\) cells) was increased when HMVEC-dLyAd were first treated with apoA-I. Platelet adhesion was improved in each individual donor, with an increase ranging from 3% to 74% in all our healthy volunteers, and an average increase in adherence of 36.2\(\pm\)11.8\% (mean\(\pm\)SEM) was noted (Figure 6E). This large span between values is to be expected as human physiology is highly variable, but nonetheless, in all cases, we see an increase in platelet adherence to the lymphatic endothelium following apoA-I treatment \((P=0.043\) as determined by Wilcoxon signed rank test).

To test whether an increase in podoplanin expression on LECs could account for the beneficial effect of apoA-I, we performed immunoblotting of human LECs pretreated with apoA-I. Our results revealed no changes in podoplanin expression (Figure 7A). We therefore turned to another potential actor in lymphatic function regulation, namely, the vascular growth factor receptor 3 (VEGFR-3). The detection of VEGFR-3 on HMVEC-dLyAd by Western blots shows that VEGFR-3 expression is increased when LECs are exposed to apoA-I (Figure 7B).

**ApoA-I Reduces Ex Vivo Platelet Aggregation**

Activated platelets have long been known for their ability to promote thrombus formation and coagulation.\(^47\) Increased platelet activation in blood is associated with the lethal outcomes of atherosclerosis.\(^48\) Our results suggest that pretreating LEC with apoA-I before platelet infusion enhances platelet arrest at a physiological-like lymph flow on LECs in vitro, a phenomenon that would most likely be beneficial in preserving the lymphatic system integrity.\(^5\) Therefore, we next evaluated whether apoA-I treatment in \(Ldlr\(^{-/-}\) mice would solely enhance the platelet activity needed to maintain proper lymphatic function, while limiting

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**Figure 7.** Effect of lipid-free apoA-I treatment on podoplanin and VEGFR3 expression. Primary HMVEC-dLyAd were seeded at maximum confluence and incubated for 20 hours with BSA or apoA-I. Cells were then analyzed by Western blotting with the use of (A) podoplanin and (B) VEGFR3. Podoplanin protein was observed at 37 kDa and VEGFR3 was observed at 170 kDa. Experiments were performed with 6 replicates per experimental group. *\(P<0.05\), as determined by 1-tailed \(t\) test. apoA-I indicates apolipoprotein A-I; HMVEC-dLyAd, human dermal lymphatic microvascular endothelial cells-adult; VEGFR, vascular endothelial growth factor receptor.
the platelet activity that leads to blood thrombosis in circulation. We measured the effect of in vivo apoA-I treatment on platelet aggregation induced by platelet agonists ex vivo.

Close to 2 decades ago, Li et al introduced the concept that apoA-I Milano inhibits arterial thrombus formation through inhibition of thrombin activity,39 and recent data have shown an inhibitory effect of isolated human apoA-I on human platelet activation and murine arterial/venous thrombosis.50 Recently, it has been shown that, in vivo, apoA-I deficiency abrogates flow restriction–induced thrombosis in a mouse model of deep vein thrombosis, and intravenous human apoA-I infusion in wild-type mice decreases thrombi prevalence from 55% in vehicle-infused mice to 0%.51 Figure 8A shows representative aggregation curves, obtained with a Chrono-log Optical aggregometer. Platelet aggregation was performed using either high concentrations of the classic agonist thrombin (0.5–1 U/mL, blue curves) or the CLEC-2 specific agonist podoplanin (5 μg/mL, black curves) on platelets isolated from BSA+, diet switch+, or apoA-I-treated Ldlr+/− mice. The histogram depicted in Figure 8B and 8C represents the mean data of percent platelet aggregation with thrombin and podoplanin, monitored under a constant shear (1000 rpm) at 37°C. ApoA-I-treated Ldlr+/− mice displayed lower aggregation amplitude than that isolated from the BSA-treated or diet switch group once it reached a plateau after addition of thrombin (Figure 8B). At this arterial shear rate, there was a statistically insignificant trend of decreased aggregation of platelets isolated from apoA-I-treated mice with podoplanin (Figure 8C). At least in plasma, Figure 8C also revealed that podoplanin-induced platelet aggregation is decreased in apoA-I–treated mice compared with BSA-control mice, as calculated by a 2-tailed t test.

To test whether apoA-I infusion could suppress flow restriction–induced aggregation caused by enhanced baseline levels of CLEC-2 on platelets, we determined by immunoblotting the level of CLEC-2 expression on resting isolated platelets. Neither the level of CLEC-2 (Figure 8D) nor the phosphorylation of its downstream effector Akt (Figure 8E) were statistically affected by apoA-I or diet reversal. PAF is involved in platelet aggregation and is produced by a variety of cells, including platelets. Its production is controlled by the activity of PAF acetylhydrolases. Thus, because HDL and HDL–associated PAF acetylhydrolase (PAF-AH) has been shown to restore normal dendritic cell migration and priming,52 we sought to determine whether an increase in PAF-AH activity could have accounted for the beneficial effect on platelet activity, and consequently, on lymphatic vessel integrity. Figure S5 rather shows that PAF-AH activity, either in plasma or in lymph, is unchanged among the 3 different groups of Ldlr+/− mice, suggesting that apoA-I does not mediate its beneficial effect on lymphatic function via a PAF-AH-related mechanism.

Discussion

Atherosclerosis is one of the principal causes of mortality worldwide, instigating most cardiovascular diseases (coronary artery disease). In recent years, a lot of emphasis has been placed on increasing levels of blood HDL in clinical studies. However, from a global perspective, increasing HDL-cholesterol levels did not demonstrate any clinical benefits, nor improve macrophage reverse cholesterol transport or decrease coronary artery disease,53–55 leading scientists to redefine our understanding of the cholesterol excretion pathway. ApoA-I, the main protein constituent of plasma HDL, quickly became a target of interest. It has been shown to be a key player in macrophage reverse cholesterol transport regulation through its capacity to package large amounts of cholesterol following its interaction with ABCA1 on cell membranes.29,56 Interestingly, subcutaneously injected lipid-free apoA-I has been reported to reduce accumulation of lipid and immune cells within the aortic root of hypercholesterolemic mice without increasing HDL-cholesterol concentrations.33,57 Whereas apoA-I has been extensively studied in the past decade, the mechanisms by which it mediates its atheroprotective effect are still unclear. In the present study, we addressed whether and how preservation of collecting lymphatic function contributes to the protective effect of apoA-I.

For several decades, the lymphatic system had been suspected as being a potential player in lipoprotein transport during atherosclerosis.21,22,58 Several years ago, Martel et al quantitatively demonstrated, for the first time, that lymphatic vessels are important components involved in macrophage reverse cholesterol transport.26 Therefore, the lymphatic system is now recognized as the missing link between the atherosclerotic plaque and the blood circulation. Improving lymphatic function to either prevent or abrogate atherosclerosis would be a potentially attractive therapeutic target. We therefore herein sought to connect the beneficial effects of apoA-I in atherosclerosis to lymphatic function, and we proposed that apoA-I might reduce the lymphatic dysfunction observed during atherosclerosis.26 Our findings reveal that a continuous low-dose intradermal injection of diet-fed Ldlr+/− mice with lipid-free apoA-I reverses atherosclerosis-associated collecting lymphatic vessel dysfunction, without significantly affecting plasma or lymph total cholesterol concentrations. The direct effect of apoA-I on LECs combined with its role in platelet activity highlight the versatility of this apolipoprotein in the modulation of lymphatic function. Altogether, our work suggests that preservation of collecting lymphatic function contributes to the protective effect of apoA-I.

The method of injection chosen in our experimental design comes from the fact that lymphatic vessels are present and abundant in the skin dermis.59 Therefore, to directly assess
Figure 8. Effect of lipid-free apoA-I treatment on platelet activity. A, Representative aggregation curves, obtained with a Chrono-log Optical aggregometer. Platelet aggregation was performed using either thrombin (blue curve) or podoplanin (black curve) on platelets isolated from BSA-, diet switch-, and apoA-I-treated Ldlr<sup>−/−</sup>/C0/C0 mice. Histogram represents the mean data of percent platelet aggregation with (B) thrombin (0.5–1 U/mL) and (C) podoplanin (5 µg/mL) monitored under a constant shear (1000 rpm) at 37°C. *P<0.05 and #P<0.06, as determined by Kruskal–Wallis analysis with Dunn’s multiple comparison post-hoc test. Extracts from resting platelets were analyzed by Western blotting with the use of (D) CLEC-2 and (E) Akt phosphorylation (pAkt). CLEC-2 protein was observed at 28 kDa and pAkt protein was observed at 60 kDa. Experiments were performed with 4 replicates per experimental group, each consisting of 2 mice per replicate. apoA-I indicates apolipoprotein A-I; CLEC2, C-type lectin-like receptor 2.
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the effect of the drug on the lymphatic vasculature per se, we performed intradermal injections, rather than subcutaneous injections as performed in previous work. Following the injection, apoA-I is first retrieved in lymph before appearing in the blood circulation, thus acting upon the lymphatic vasculature as soon as it gets within the mouse. We observe longitudinal plaque regression in the thoracic aorta of Ldlr<sup>−/−</sup> mice following lipid-free apoA-I treatment, independently of cholesterol accumulation in lymph or plasma. As reported by Wilhelm et al, this lack of effect in plasma cholesterol could be explained by the potent effect of apoA-I on immune cell cholesterol balance instead of affecting whole body cholesterol balance. ApoA-I has the ability to relieve excess cholesterol in lipid rafts/microdomains and thus to affect numerous types of signal transduction pathways that rely on these microdomains. The same rationale could be applied to lymph: the positive effects of apoA-I on lymphatic and platelet function suggest that despite high plasma cholesterol, cells present in lymph have the ability to efflux their excess cholesterol via lipid-free apoA-I to the liver. Therefore, it is very likely that LEC and platelet signaling pathways depend on the amount of cholesterol in their microdomains, consequently modulating platelet aggregation and adhesion to the lymphatic endothelium. As we hypothesize that apoA-I plays a positive role in preserving lymphatic transport, the expected repercussion would be observable, primarily, at the level of the collecting lymphatic vessel, the entity that is first defective during atherosclerosis-associated lymphatic dysfunction. By improving lymphatic vessel integrity, we believe that apoA-I would improve lymph transport per se, thus promoting the movement of lymph content toward the blood circulation. Sequentially, cholesterol would be taken up by the initial lymphatics, and efficiently pumped out of the collecting lymphatic vessels to subsequently allow the exit of cholesterol from the lymphatic vessel, to eventually reach the liver to complete the reverse cholesterol transport loop, rather than being stagnant in lymph.

Subcutaneous injections of apoA-I<sup>33</sup> had more potent effects than intradermal injections to reduce the inflammatory cell content in the aortic sinus. This is most likely because of a more direct access to the blood vasculature. However, we show that interstitial spaces near the skin-draining lymphatics had less macrophage accumulation in the apoA-I-injected mice compared with the diet switch- or the BSA-injected control group. Following contact sensitization on the back-skin dermis, we also show that dendritic cells are more potent to migrate to the corresponding draining lymph nodes when lipid-free apoA-I is injected, compared with the 2 control groups. Our results suggest that apoA-I, but not diet switch, can regulate immune cell content and improve lymphatic function before influencing obesity-related parameters such as subcutaneous fat accumulation.

Recent studies in animals provide evidence that affecting lymphatic drainage by promoting the growth of lymphatic vessels can modulate inflammation. Lympathic vessel hyperplasia has been associated with hypercholesterolemia, but we show here that apoA-I does not reduce vessel diameters. In addition, we report that the transport of larger molecules is not seemingly different between the groups, reflecting that the capacity of the peripheral ultrafiltrate to be uptaken is not modified, despite the increased number of lymphatic vessels observed solely in the ear dermis. Altogether, these observations point out that more numerous initial lymphatic vessels do not necessarily reflect better functioning lymphatic vessels in our atherosclerotic mouse model. We have recently reported that lymphatic dysfunction associated with the early stage of atherosclerosis appears to be first and foremost linked to a defect in the collecting lymphatic vessel per se. Aging and chronic HFD have also both been associated with a defect in the collecting lymphatic vessels as well, stressing the importance of targeting this portion of the vessel in chronic inflammatory diseases. As the defect is first found in the collecting vessels, lymphangiogenesis by itself might not be sufficient to reduce plaque formation. Therefore, we next investigated collecting lymphatic vessel permeability in our models. We first showed that, following apoA-I treatment, Ldlr<sup>−/−</sup> mice have restored collecting lymphatic vessel integrity as shown by a decrease in Evans Blue leakage around the vessel, both with respect to the area and the perpendicular distance of leakage within the surrounding tissue. This is potentially reflected in previous studies where HDL was shown to increase endothelial barrier integrity, implicating sphingosine 1-phosphate as a mediator. In our case, we suspected that apoA-I acted in a similar way on the lymphatic vessels. Surprisingly, although FOXC2 absence/reduction is well known to be implicated in lymphatic valve failure, thereby potentially causing lymphedema and more importantly a dysfunction in the lymphatic collecting vessels, our results did not report a direct effect of apoA-I on FOXC2. The switch to a chow diet from HFD, however, significantly restored FOXC2 levels by immunofluorescence imaging, an avenue that deserves further attention. So far, variations in FOXC2 may have a minor role in body weight control and seem to be involved in the regulation of basal glucose turnover and plasma triglyceride levels, particularly in women. For now, we believe that modulating FOXC2 might not be sufficient to rescue atherosclerosis-related lymphatic dysfunction and that apoA-I uses another mechanism to improve lymphatic vessel permeability.

Whereas an excess of platelet activation in blood circulation is often deleterious and is associated with clinically devastating outcomes in atherosclerosis, platelets are also known to support the semipermeable function of the blood vessel endothelium. In lymphatic physiology, they play a...
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ApoA-I enhances lymphatic function by direct and indirect mechanisms. This work contends that lipid-free apoA-I mediates beneficial effects through the upregulation of the VEGFR-3 pathway. In addition, apoA-I might exert a protective effect on the lymphatic endothelium, as by limiting platelet aggregation, it would clear the way for platelet adhesion on LECs. Although there is evidence of a morphological role for platelets in maintaining LECs integrity, this indirect mechanism could also subsequently involve the activation of the signaling pathway to contribute to proper lymphatic function. Altogether, these studies bring forward a new pleiotropic role for apoA-I in lymphatic function and unveil new potential therapeutic targets for the prevention and treatment of atherosclerosis.
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Disclosures

None.

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Supplemental Material
**Figure S1. ApoA-I kinetics in wild type mice.**

Human lipid-free apoA-I was injected intradermally in the back skin of wild-type mice. Lymph and plasma were collected at different time points following injection. Human apoA-I levels were differentially detected in both lymph and plasma at each time point by ELISA and the background was subtracted. Experiments were performed with 4 mice per experimental group.
Figure S2. Assessment of atherosclerosis in Ldlr<sup>-/-</sup> mice. A, D, Total lesion, B, D, neutral lipid- (Oil Red O) and C, E, macrophage (CD68<sup>+</sup>)-positive areas were quantified in the three groups of mice in 8 µm-thick aortic sinus sections using ImageJ software. Experiments were performed with 7-11 mice per experimental group. *p≤0.05, **p≤0.01 and ***p≤0.001, as determined by one-way ANOVA with Tukey’s post-hoc test. Scale bars = 500 µm.
Figure S3. Effect of apoA-I treatment on FOXC2 expression on lymphatic endothelial cells. Representative images of FOXC2 and smooth muscle cells (smooth muscle actin, SMA) expression in collecting lymphatic vessels of BSA, diet switch and apoA-I treated Ldlr⁻/⁻ mice. Popliteal collecting lymphatic vessels were harvested and processed for immunofluorescence detection. Longitudinally imaged single plan vessels were acquired with an LSM 710 Confocal Microscope (Zeiss) equipped with a 63X/1.4 oil DIC objective. Experiments were performed with 4 replicates per experimental group. Scale bar = 50 µm.
Figure S4. Analysis of platelet adherence on lymphatic endothelial cells under a physiological lymph flow. Human platelets were isolated and perfused over primary HMVEC-dLyAd seeded at 80% confluence in IbiTreated flow chambers at a wall shear rate of 50 s-1 at 37 °C for 8 minutes. Orange arrows indicate the direction of flow.
Figure S5. PAF-AH activity in plasma and lymph. Total PAF-AH activity was assessed in A, plasma and B, lymph of BSA, diet switch and apoA-I treated Ldlr⁻/⁻ mice. Experiments were performed with 3-4 mice per experimental group.