Apolipoprotein A-I Limits the Negative Effect of Tumor Necrosis Factor on Lymphangiogenesis

Radjesh Bisoendial, Fatiha Tabet, Paul P. Tak, Francine Petrides, Luisa F. Cuesta Torres, Liming Hou, Adam Cook, Philip Barter, Wolfgang Weninger, Kerry Anne Rye

Objective—Lymphatic endothelial dysfunction underlies the pathogenesis of many chronic inflammatory disorders. The proinflammatory cytokine tumor necrosis factor (TNF) is known for its role in disrupting the function of the lymphatic vasculature. This study investigates the ability of apo AI, the principal apolipoprotein of high-density lipoproteins, to preserve the normal function of lymphatic endothelial cells treated with TNF.

Approach and Results—TNF decreased the ability of lymphatic endothelial cells to form tube-like structures. Preincubation of lymphatic endothelial cells with apo AI attenuated the TNF-mediated inhibition of tube formation in a concentration-dependent manner. In addition, apo AI reversed the TNF-mediated suppression of lymphatic endothelial cell migration and lymphatic outgrowth in thoracic duct rings. Apo AI also abrogated the negative effect of TNF on lymphatic neovascularization in an ATP-binding cassette transporter A1-dependent manner. At the molecular level, this involved downregulation of TNF receptor-1 and the conservation of prospero-related homeobox gene-1 expression, a master regulator of lymphangiogenesis. Apo AI also re-established the normal phenotype of the lymphatic network in the diaphragms of human TNF transgenic mice.

Conclusions—Apo AI restores the neovascularization capacity of the lymphatic system during TNF-mediated inflammation. This study provides a proof-of-concept that high-density lipoprotein–based therapeutic strategies may attenuate chronic inflammation via its action on lymphatic vasculature. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.115.305777.)

Key Words: apolipoprotein A-I ■ inflammation ■ lymphangiogenesis ■ lymphatic system ■ receptors, tumor necrosis factor

The lymphatic system comprises a highly organized network of vessels that is critical for immune cell trafficking, lipid transport, and tissue fluid homeostasis. Rather than being passive conduits, lymphatic vessels directly control local and distal inflammation.1,2 Impairment of lymphatic endothelial cell (LEC) function exacerbates inflammation and is believed to contribute to the pathogenesis of chronic inflammatory disorders, such as rheumatoid arthritis, Crohn disease, and psoriasis.3–6 Tumor necrosis factor (TNF), a key pleiotropic cytokine, is a major driver of inflammation in these pathologies. It has strong negative effects on the lymphatic vasculature in experimental arthritis and in patients with rheumatoid arthritis.4,6 Strategies targeted at reversing dysfunction of the lymphatic system in these pathological conditions hold promise for ameliorating or even promoting resolution of inflammatory states.4,6

High-density lipoproteins (HDLs) and their principal apo AI have multiple cardioprotective functions.7 The best studied cardioprotective function of HDLs is related to their role in reverse cholesterol transport, the process that mediates the removal of excess cholesterol from peripheral tissues and its subsequent transport to the liver for recycling or excretion.7 The initial steps in reverse cholesterol transport are dependent on the ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1. ABCA1 effluxes cellular cholesterol to lipid-free apo AI and discoidal, nascent HDL, whereas ABCG1 mediates efflux of cholesterol and oxysterols to spherical HDLs. HDLs and apo AI also have...
Apo AI Inhibits the Negative Effect of TNF on LEC Migration

The scratch assay was used to determine whether TNF affected the migratory capacity of LECs and, if so, whether this was modulated by apo AI (Figure 2A and 2B). When LECs were incubated with TNF, the number of cells that migrated across the scratch was 79.7±5.6% lower than that of control cells incubated in the absence of TNF (P<0.05). When the cells were preincubated with apo AI at a final concentration of 1.2 mg/mL, the number of cells that migrated across the scratch increased to 146.9±28.8% of what was observed for the control cells (P<0.01 versus TNF alone). To exclude the possibility that the increased migration of the LECs that were preincubated with apo AI was a reflection of LEC proliferation, incorporation of bromodeoxyuridine into the cells was quantified. These results established that neither TNF alone nor incubation with apo AI and TNF affected bromodeoxyuridine incorporation into LECs (Figure 2C). Collectively, these data demonstrate that preincubation with apo AI reverses the inhibitory effect of TNF on the migratory capacity of LECs.

Materials and Methods

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

Results

Apo AI Reverses the TNF-Mediated Inhibition of LEC Tube Formation

The ability of LECs (characterized in Figure I in the online-only Data Supplement) to form tube-like structures in the presence of TNF was assessed by counting the number of branch points (Figure 1A). When LECs were incubated with TNF alone, the number of branch points was 87.1% lower than that in the control cells (P<0.0001). Preincubation of the LECs with apo AI before incubation with TNF inhibited the TNF-mediated reduction in tube formation in a concentration-dependent manner (P<0.01). When the cells were preincubated with apo AI at final concentrations of 0.1, 0.3, 0.6, and 1.2 mg/mL, the number of branch points progressively increased to 19.6±1.4%, 37.3±1.4%, 57.1±3.1%, and 97.1±2.2% of the control, respectively (P<0.0001 for TNF alone versus apo AI at 0.3–1.2 mg/mL). When the LECs were incubated with apo AI at a final concentration of 1.2 mg/mL and then incubated for an additional 6 hours in the absence of TNF, the number of branch points was comparable with that of the control cells (Figure 1B). Neither TNF alone nor incubation with apo AI and TNF affected cell viability (Figure 1C). Collectively, these results indicate that apo AI inhibits the adverse effects of TNF on the tube-forming capacity of LECs in a dose-dependent manner but does not directly promote tube formation.

Lymphatic Protective Properties of Apo AI Are Dependent on ABCA1

To determine whether the protective effects of apo AI were dependent on ABCA1, TD rings were incubated for 1 hour with ABCA1-specific antibodies or an IgG isotype control antibody. The TD rings were then incubated for 24 hours with apo AI and subsequently treated with TNF for 14 days. The use of blocking antibodies against ABCA1 has been shown previously to specifically abolish the anti-inflammatory effects of apo AI. The protective effects of apo AI on lymphatic outgrowth in the presence of TNF were no longer apparent in the TD rings that were incubated with the anti-ABCA1 antibody. Lymphatic outgrowth was unaffected by incubation with the IgG isotype control (P<0.05; Figure 3C). These results indicate that apo AI abrogates the TNF-mediated suppression of the sprouting capacity of TD rings in an ABCA1-dependent manner.
Apo AI Maintains Prox-1 Expression in LECs in the Presence of TNF

Prox-1 is a key determinant of inflammation-driven lymphangiogenesis. The loss of the transcriptional activity of Prox-1 has previously been associated with suppression of LEC tube formation and lymphatic sprouting of TD rings, underscoring its fundamental role for determining the lymphatic phenotype. This was confirmed as shown in Figure 4A, which shows that Prox-1 mRNA levels are decreased 2.3-fold when LECs are incubated with TNF (P<0.05 compared with control). However, when LECs were preincubated with apo AI before stimulation with TNF, Prox-1 mRNA levels did not decrease (P<0.05 compared with TNF alone). These results were confirmed by confocal microscopy (Figure 4B).

To validate these findings, we looked at 2 other LEC-specific genes: lymphatic vessel endothelial receptor-1 (Lyve-1) and podoplanin. Lyve-1 mRNA levels were reduced 34.1-fold when LECs were incubated with TNF alone (Figure 4A; P<0.001 versus control). This inhibitory effect was partly reversed in LECs that were preincubated with apo AI, with Lyve-1 mRNA levels being restored to 45.2±14.8% of that in control cells (P<0.05; Figure 4A). Podoplanin mRNA levels were increased by 1.9-fold and 1.5-fold in LECs that were preincubated with apo AI compared with control cells (P<0.01) and LECs incubated with TNF alone (P<0.05), respectively (Figure 4A). Apo AI, by contrast, did not affect the transcription of other genes involved in lymphangiogenesis, such as Forkhead box protein C2, GATA-binding protein 2, neuropilin-2, vascular endothelial growth factor receptor 3, and notch ligand δ–like ligand 4 (Figure IIA in the online-only Data Supplement). As crosstalk has been reported between the vascular endothelial growth factor family and the Notch pathway in the regulation of lymphangiogenesis, expression of vascular endothelial growth factor receptor 3 and notch ligand δ–like ligand 4 was assessed by Western blotting (Figure IIB and IIC in the online-only Data Supplement). There was no difference in notch ligand δ–like ligand 4 or vascular endothelial growth factor receptor 3 protein levels between LECs that were preincubated with apo AI and TNF and those that were incubated with TNF alone.

Apo AI Attenuates the Effects of TNF and Decreases TNF-R1 mRNA Levels in LECs

To determine whether apo AI conserves Prox-1 expression and LEC function in the presence of TNF directly or by limiting TNF bioactivity, LECs were incubated for 24 hours with increasing amounts of apo AI before quantifying Prox-1 mRNA levels. These results established that apo AI does not directly affect the expression of Prox-1 (Figure III in the online-only Data Supplement). We then wanted to find whether the ability of apo AI to limit the bioactivity of TNF reflected reduced the expression of the endothelial receptor TNF-R1.16,17 TNF-R1 mRNA levels in LECs were not...
affected by incubation with TNF alone (Figure 5A). However, when the LECs were preincubated with apo AI before activation with TNF, TNF-R1 mRNA levels were 2.3-fold lower than those in control cells (P<0.01) and 1.8-fold lower than those in cells that were incubated with TNF alone (P<0.05; Figure 5A). This result was confirmed by Western blotting. Compared with cells that were incubated with TNF alone, TNF-R1 protein expression was decreased 1.9-fold in LECs that had been preincubated with apo AI (P<0.05; Figure 5B and 5C). As whole-cell lysates were used for these experiments, this result reflects the membrane pool of TNF-R1 that dictates cell sensitivity to TNF.

Preincubation with apo AI also decreased mRNA levels of TNF-R–associated factor 2, an adaptor protein that is downstream of TNF-R1, by 2.9-fold and 2.2-fold relative to control cells (P<0.01) and LECs that were incubated with TNF alone (P<0.05), respectively (Figure 5A). Apo AI, by contrast, did not affect the transcription of other genes, such as receptor-interacting protein-1, an adaptor kinase that is crucial to TNF-R1 signaling, or the nuclear factor-κB inhibitor, A20 (Figure 5A). Compared with cells that were incubated with TNF alone, p38 MAPK phosphorylation was decreased 1.9-fold in LECs that were preincubated with apo AI and then incubated with TNF (P<0.05; Figure 5D and 5E). Taken together, these results are consistent with apo AI inhibiting the detrimental effects of TNF on LECs by decreasing TNF-R1 expression and signaling.

Additional experiments were carried out to ascertain whether preincubation with apo AI lowers TNF-R1 expression and signaling before stimulation with TNF. These results indicated that 24 hours of preincubation with apo AI in the absence of TNF did not affect TNF-R1 mRNA and protein expression (Fig. IV A–IVC in the online-only Data Supplement). Similarly, TNF-α–associated factor 2, A20, and receptor-interacting protein-1 mRNA levels were unaltered by incubation of LECs with apo AI alone (Figure IVA in the online-only Data Supplement). These results indicate that the apo AI–mediated reduction in TNF-R1 expression and activity is apparent only in LECs that have been stimulated with TNF.

Preincubation With Apo AI Increases TNF-α–Converting Enzyme Expression

A potential explanation for the reduced TNF-R1 expression involves the shedding of TNF-R1 from the cell surface through proteolytic cleavage by TNF-α–converting enzyme (TACE). This sheddase regulates cellular responsiveness to TNF by releasing soluble TNF-R1 from the cell surface, which inactivates TNF. The possibility that TACE-mediated cleavage was responsible for the reduced TNF-R1 expression was addressed by using flow cytometry...
to quantify TACE expression in LECs. After preincubation with apo AI, the number of TACE-expressing LECs was increased by 1.4-fold relative to LECs that were incubated with TNF alone ($P<$0.05; Figure V in the online-only Data Supplement). Given the close correlation between the surface protein expression of TACE and its cleaving activity,18 this result is consistent with increased TACE activity enhancing the shedding of TNF-R1 and thus reducing cell surface TNF-R1 levels.

Apo AI Restores a Normal Lymphatic Phenotype in hTNFtg Mouse Diaphragms
Apo AI or phosphate-buffered saline was administered intraperitoneally 3× a week for 2 weeks into 6-week-old hTNFtg mice. Compared with littermate controls, the density of the lymphatic system in the diaphragms of the hTNFtg mice that received phosphate-buffered saline decreased ($P<$0.001), with fewer branch points ($P<$0.001) and blind-ended sacs ($P<$0.05; Figure 6A and 6B). The lymphatics in the hTNFtg mice were also smaller in diameter ($P<$0.05) than those of their littermate controls (Figure 6C). By contrast, a normal lymphatic phenotype, with morphological features similar to those of the control animals, was observed when the hTNFtg mice were treated with apo AI (Figure 6A–6C).

Discussion
This study uncovers a novel function of apo AI: the restoration of the neovascularization capacity of lymphatic endothelium in the presence of TNF, the cytokine that is regarded as the major driver of lymphatic dysfunction in chronic inflammatory disorders. The results show that the protective effect of apo AI is ABCA1 dependent, is associated with reduced TNF-R1 signaling, and the conservation of Prox-1 expression in LECs. These results were further corroborated in hTNFtg mice,3,6,19 where the phenotype of the lymphatic vasculature of the diaphragm was normalized by apo AI treatment. This suggests that apo AI may regulate protective molecular pathways in lymphatic endothelium, and therefore, it is a potential therapeutic target with the capacity to improve lymphatic function and promote resolution of acute and chronic inflammatory states.

Inflammatory lymphangiogenesis is a dynamic process, featuring accelerated neoforation of lymphatic vessels to compensate for the increased amounts of extravasated tissue fluid and immune cell trafficking. Perturbation of this tightly regulated process has been reported to exacerbate inflammation in chronic inflammatory disorders, including rheumatoid arthritis, experimental arthritis, psoriasis, and Crohn disease.5,20–22 TNF is involved in the pathogenesis of all of these disorders.

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Figure 5. Apo AI attenuates the effects of tumor necrosis factor (TNF) and reduces TNF receptor-1 (TNF-R1) expression and signaling. 

A. Lymphatic endothelial cells (LECs) were incubated without or with apo AI (final concentration, 1.2 mg/mL) for 24 hours and then incubated for 3 days with TNF (final concentration, 10 ng/mL). mRNA levels of TNF-R1, receptor-interacting protein-1 (RIP), TNF-R-associated factor 2 (TRAF2), and A20 were quantified by quantitative polymerase chain reaction. 

B. Western blot of TNF-R1 protein expression. 

C. Quantification of TNF-R1 protein expression relative to control. 

D. Western blot of p38 MAPK and p-p38 MAPK protein expression. 

E. Quantification of p38 MAPK and p-p38 MAPK protein expression relative to control. Values represent the mean±SEM of at least 3 separate experiments. *$P<$0.05 vs TNF alone; **$P<$0.01 vs control. Samples were loaded undiluted (1×) and after 3× dilution. Results for the undiluted samples were used for quantification.
Apo AI treatment restores a normal lymphatic phenotype in (human tumor necrosis factor transgenic) hTNFtg mouse diaphragms. Six-week-old hTNFtg mice were injected intraperitoneally 3× a week for 2 weeks (6 injections in total) with 1.2 mg/kg of apo AI or phosphate-buffered saline (PBS). The central tendon side of the diaphragm was used for whole-mount immunofluorescence. For morphological analysis, lymphatic vessels were detected with an antibody. The diaphragms were mounted in DAPI-containing mounting media and imaged using confocal microscopy. A. Representative images of the diaphragm lymphatic system. B and C. Systematic quantification of the lymphatics. Values represent the mean±SEM of at least 3 separate experiments. *P<0.05 vs control; ***P<0.001 vs control; **P<0.01 vs PBS; ***P<0.001 vs PBS.

The current findings showing that TNF inhibits key LEC functions, including tube formation, migratory capacity, and lymphatic sprouting, confirm previous reports that demonstrate the overt antilymphatic effects of TNF. TNF has been reported to impair capillary tube formation and barrier function in mouse and human LECs.19 Intradiscal administration of TNF in mice also results in an acute and systemic deterioration of lymphatic propulsion.24 In addition, hTNFtg mice, a model of chronic inflammatory arthritis, exhibit decreased lymph flow from the joints compared with wild-type littermates.6 Finally, TNF blockade in patients with rheumatoid arthritis and in experimental arthritis has been shown to promote lymphatic vessel formation and the resolution of chronic synovial inflammation.

The current report provides the first evidence that apo AI protects LECs from the negative effects of TNF on lymphangiogenesis in several in vitro models and in hTNFtg mice. The results show that preincubation with apo AI restores the tube-forming capacity of LECs in the presence of TNF in a dose-dependent manner, as well as the migratory and sprouting capacity of LECs and TD rings, respectively.

Rather than having a direct effect, apo AI most likely overcame the negative effects of TNF by activating signaling pathways in the LECs. This possibility was strengthened by showing that preincubation of LECs with apo AI conserved the expression of Prox-1 in the presence ofTNF. Prox-1 function is essential for inflammatory lymphangiogenesis1 and is regulated by hypoxia-responsive genes that promote cell survival and proliferation, including hypoxia-inducible factor-α and hypoxia-inducible factor-2α as well as the interleukin-3, interleukin-7, and the JAK2/STAT3 pathway. Moreover, the Kaposi sarcoma herpes virus depends on the JAK2/STAT3 pathway for Prox-1–mediated lymphatic reprogramming of ECs.29 On the other hand, the loss of Prox-1 activity results in defective lymphangiogenesis.13,14

In a murine contact hypersensitivity model, downregulation of Prox-1 is inversely correlated with local TNF and interferon-γ production.29 Furthermore, mice heterozygous for a mutation that inactivates Prox-1 exhibit features of leaky lymphatic vessels and are prone to develop obesity and inflammation.30 The present report is consistent with these findings and further show that apo AI prevents the TNF-mediated downregulation of Prox-1 expression in LECs in response to TNF.

Apo AI may have conserved Prox-1 expression by 2 potential mechanisms: (1) limiting TNF-R1 signaling and (2) activation of the JAK2/STAT3 signaling pathway. In doing so, apo AI sustained lymphatic neovascularization in the presence of TNF. The present results are consistent with preincubation of LECs with apo AI before TNF treatment reducing total TNF-R1 expression, including the receptor molecules at the cell surface, which confer cellular sensitivity to TNF, and in the Golgi, which is responsible for replenishing cell surface TNF-R1 levels.26 Several lines of evidence were consistent with reduced TNF-R1 signaling in LECs that were preincubated with apo AI. These include reduced TNF-R–associated factor 2 mRNA levels, reduced phosphorylation of p38 MAPK, and partial recovery of Lyve-1 expression.23 The partial recovery of LWYE-1 expression in LECs preincubated with apo AI is most likely because of reduced sensitivity of the cells to TNF, which reversibly downregulates the membrane expression of LWYE-1. This is also in line with the reduced TRAF2 mRNA levels (Figure 5A) and lower phosphorylation of p38 MAPK (Figure 5D and SE). Given the inverse correlation between Prox-1 expression in inflamed lymphatic endothelium and local TNF production, it is also conceivable that decreased TNF-R1 signaling as a consequence of apo AI pretreatment may have resulted in conservation of Prox-1 expression.

It is also possible that the reduced TNF-R1 expression in LECs preincubated with apo AI is a reflection of enhanced activation of TACE29 and increased shedding of TNF-R1 from the cell surface.23 The integrity of lipid rafts and caveolae, where TACE and TNF-R1 are sequestered in the cell membrane, is critical for efficient TNF-R1 shedding.22 Disruption of these domains either by incubation with methyl-β-cyclodextrin or by silencing of caveolin-1 has been shown to attenuate TNF-R1–membrane shedding.25 Through interaction with caveolin-1, apo AI may stabilize lipid rafts and promote TACE-mediated cleavage of TNF-R1.25,36 The observation that apo AI reduces TNF-R1 signaling only after stimulating LECs with TNF favors the hypothesis that apo AI enhances the TNF-induced increase in TACE activity without affecting TNF-R1 expression directly. The decreased TNF-R1 mRNA levels in LECs preincubated with apo AI (Figure 5A) suggest that reduced protein synthesis may also have contributed to the reduction in TNF-R1 cell surface protein levels.
Another mechanism by which apo AI may have conserved Prox-1 expression is via the JAK2/STAT3 axis. Previous studies in macrophages have shown that apo AI can limit responsiveness to lipopolysaccharide via the ABCA1/JAK2/STAT3 pathway.37 Further investigation of the contribution of ABCA1, and the downstream JAK2/STAT3 signaling pathway, to the protective effects of apo AI was hindered by the low ABCA1 protein levels in cultured LECs. As apo AI did not affect other signaling pathways that control lymphangiogenesis, such as vascular endothelial growth factor receptor 3 and notch ligand δ-like ligand 4 (Figure II in the online-only Data Supplement), it is possible that alternative mechanisms, including the fibroblast growth factor 2–Lyve1 axis, might be involved.38 Furthermore, as apo AI alone did not increase Prox-1 expression or stimulate tube formation, it follows that this apolipoprotein is unable to directly maintain Prox-1 expression or mediate tubulogenesis in the presence of TNF.

In conclusion, this study identifies apo AI as a critical regulator of lymphatic vascular homeostasis during inflammation and provides the first proof-of-concept evidence that HDL-based therapeutic strategies may increase lymphatic neovascularization and function in chronic inflammatory disorders and reverse chronic inflammation. The results also indicate that lymphatic vascular dysfunction can be reversed by apo AI in an inflammatory setting. When taken together, these findings represent a powerful stimulus for ongoing efforts targeting HDL in chronic inflammatory disorders in addition to the current therapeutic arsenal of anti-inflammatory drugs.

Acknowledgments
We thank G. Kollia (Biomedical Sciences Research Center, Alexander Fleming, Varka, Greece) who offered the Tg197 mice and G. Schett (University of Erlangen-Nuremberg, Erlangen, Germany) for providing them.

Sources of Funding
This work was supported by the Dutch Arthritis Association (NR 09/11/103) postdoctoral fellowship and a Ruitinga van Swieten grant to R. Biosoendial and the National Health and Medical Research Council of Australia (grant 1037905).

Disclosures
P.P. Tak is an employee at GlaxoSmithKline (Stevenage, United Kingdom). The other authors report no conflicts.

References
Chronic inflammatory disorders, such as rheumatoid arthritis, are associated with increased morbidity and mortality and have a major socioeconomic burden. We wanted to find whether apo AI, the main apolipoprotein in high-density lipoproteins, can resolve the lymphatic dysfunction that accompanies chronic inflammatory disorders. The results show that apo AI prevents the proinflammatory cytokine, tumor necrosis factor, from reducing lymphatic endothelial growth and the neovascularization capacity of lymphatic endothelial cells. At the molecular level, this involves reduced tumor necrosis factor receptor-1 expression and the maintenance of the expression of prospero-related homeobox gene-1, a key transcriptional regulator of inflammation-driven lymphangiogenesis. Therefore, this study provides a proof-of-concept that high-density lipoprotein–based therapeutic strategies may attenuate the chronic inflammatory state by acting on the lymphatic vasculature. The results also indicate that high-density lipoprotein–raising agents currently under consideration as a therapeutic option for treating cardiovascular disease may have broader applications in chronic inflammatory disorders.
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Arterioscler Thromb Vasc Biol, published online September 10, 2015;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2015/09/10/ATVBAHA.115.305777

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Materials and Methods

Apolipoprotein A-I limits the negative impact of tumor necrosis factor on lymphangiogenesis

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Running title: Lymphatic protective functions of apoA-I
Reagents
Human basic fibroblast growth factor, vascular endothelial growth factor C, and tumor necrosis factor (TNF) were from R&D Systems (Minneapolis, MN; Catalog numbers 233FB, 2179VC, and 210-1A-005). Polyclonal rabbit antibodies against ATP binding cassette transporter A1, and isotype control were from Novus Biologicals (Littleton, CO; Catalog numbers NB400-105 and NB810-56910). The APC BrdU flow kit was from BD Biosciences (San Jose, CA; Catalog number 557892).

Preparation of lipid-free apolipoprotein (apo) A-I
High density lipoproteins were isolated from pooled samples of normal human plasma (Healthscope Pathology, Adelaide, South Australia) by sequential ultracentrifugation (1.063<d<1.21 g/mL) and delipidated by standard techniques. ApoA-I was isolated by chromatography using a Q Sepharose Fast Flow column attached to an Äkta-FPLC system (GE Healthcare; Chalfont St Giles, Bucks, UK). Purified apoA-I was lyophilized, reconstituted in 3 mM/L guanidine hydrochloride/10 mM/L Tris/0.01% (w/v) EDTA (pH 8.2) and dialyzed against endotoxin-free PBS before use.

Cell culture
Primary human lymphatic endothelial cells (LECs) (Lonza, Waverly, VIC, Australia; Catalog number CC-2810) were cultured for up to 7 passages in endothelial growth medium-2-MV (EGM-2-MV), containing human epidermal growth factor, fibroblast growth factor, insulin-like growth factor, vascular endothelial growth factor, hydrocortisone, ascorbic acid, gentamycin/amphotericin B and 5% (v/v) fetal bovine serum; Lonza; Catalog number CC-3202) at 37°C in a 5% CO₂ incubator. The dependency of the cell culture conditions on vascular endothelial growth factor (VEGF)-C signaling was underlined by our observations that LEC tube formation and lymphatic sprouting of thoracic duct rings increased 1.5-fold and 1.8-fold, respectively, when VEGF-C was included in the incubations (data not shown). Exogenous VEGF-C was not added to the medium in the assays below unless indicated otherwise.

For characterization by immunofluorescence, LECs were cultured on coverslips coated with fibronectin (Roche, Dee Why, NSW, Australia; Catalog number 10838039001) until they were fully confluent. The LECs were then fixed, blocked, and incubated at 4 °C with primary antibodies including mouse anti-human lymphatic vessel endothelial receptor-1 (Abcam, Cambridge, UK; clone 4G1, Catalog number Ab33477), mouse anti-human intercellular adhesion molecule-1 (Abcam; Clone MEM-111, Catalog number Ab2213), goat anti-human prospero homeobox gene-1 (R&D systems; Catalog number AF2727), and mouse anti-human cluster of differentiation 31 (Biolegend, San Diego, CA; clone WM59, Catalog number 303102). After overnight incubation, the cells were washed and incubated for 2 h at room temperature with secondary antibodies conjugated to Alexa Fluor 488 alone or in combination with Alexa Fluor 555 (Life Technologies, Melbourne, VIC, Australia; Catalog numbers A11001 and A21432). The LECs were then washed, mounted in 4',6-diamidino-2-phenylindole-containing media (Vector Laboratories, Burlingame, CA; Catalog number VEHE1200), and imaged using a Leica SP5 confocal system. Images were analyzed using Volocity (PerkinElmer, Melbourne, VIC, Australia).

Tube formation assay
LECs were cultured for 24 h without or with apoA-I (final concentration 0.1-1.2 mg/mL). Matrigel (30 µl, BD Biosciences; Catalog number 356237)³ was added to 24-well plates and distributed evenly then incubated for 30 min at 37 °C. Care was taken to avoid bubble formation. The media was removed from the LECs, which were then rinsed with PBS, gently detached, and added to the 24-well Matrigel-coated plates (3x10⁴ cells/well; total volume 500 µl). The cells were then incubated with or without TNF (final concentration 10 ng/mL) for 6 h. The assay was completed within 6.5 h. Images were acquired in less than 30 min using an Olympus IX71 inverted microscope. The number of vessel branchpoints was determined for
Scratch wound assay
LEC$s (5 \times 10^5$/well) were plated in 24-well plates pre-coated with fibronectin (Roche) and incubated for 24 h without or with apoA-I (final concentration 1.2 mg/mL) prior to scratching the centre of the monolayer with a 20 μL pipette tip$^4$. Under basal conditions, EGM-2-MV culture medium did not promote LEC migration in the scratch assay. However, addition of exogenous FGF (50 ng/mL) and VEGF-C (100 ng/mL) to the medium increased the number of cells that migrated across the scratch by 3.4-fold (data not shown). These experiments were thus carried out with media supplemented with exogenous fibroblast growth factor (50 ng/mL) and VEGF-C (100 ng/mL) as described previously$^4$. After scratching, the media was removed and the LECs were incubated for 16 h without or with TNF (final concentration 10 ng/mL). For each well, three representative regions were chosen for imaging using an Olympus IX71 inverted microscope. The number of migrated cells was calculated using Fiji.

Quantitative Real-Time PCR (qPCR)
Total RNA was extracted from LECs using an RNeasy minikit (Qiagen, Chadstone Centre, VIC, Australia). cDNA was synthesized using iScript Reverse Transcription Reagents (Bio-Rad, Gladesville, NSW, Australia; Catalog number 170-8840). Target gene amplification was performed using iQ SYBR Green Super mix (BioRad; Catalog number 170-8880) and run in sets of three replicates using a CFX96 real-time PCR detection system (Bio-Rad). The average threshold cycle ($C_T$) was determined from triplicate reactions. Relative mRNA quantification was calculated by the $2^{\Delta \Delta C_T}$ method. The results were invariant irrespective of whether they were normalized to β-actin, GAPDH, or β2 microglobulin. Results normalized to β-actin are shown throughout. Primers for the genes that were evaluated are listed in Table 1.

Thoracic duct ring assay
Mouse thoracic ducts were harvested from 2-4 month old C57BL/6 mice, as previously described$^3$, cut into 1 mm rings, and implanted into a three dimensional culture system comprising collagen gel type I (Cultrex-Trevigen, Gaithersburg, MD; Catalog number 3443-100-01). The rings were pre-incubated for 24 h without or with apoA-I (final concentration 1.2 mg/mL). The media was removed and replaced by EGM-2-MV without or with TNF (final concentration 10 ng/mL) then incubated for 14 days under hypoxic (5% O$_2$) conditions$^5$. The media was changed every 3 days. At the end of the incubation the rings were imaged using an Olympus IX71 inverted microscope. The outgrowth area of the sprouted lymphatic vessels was calculated by subtracting the thoracic duct ring area from the area of capillary outgrowth using the area-calculating tool from Fiji. In some experiments, the rings were pre-incubated for 1 h with an anti-ATP binding cassette transporter A1 antibody (1:100) or isotype control (1:100), washed with PBS, then treated with apoA-I for 24 h prior to exposure to TNF. The experiments were performed with approval of the Sydney Local Health Network Animal Ethics Committee (Protocol number 2011/009A).

Quantification of prospero homeobox gene-1 expression
Prospero homeobox gene-1 protein expression was quantified by seeding LECs (5 \times 10^5$/well) onto fibronectin-coated coverslips, followed by incubation for 24 h without or with apoA-I (final concentration 1.2 mg/mL). The media was replaced by EGM-2-MV containing TNF (10 ng/mL) and changed daily over 3 days. The cells were then fixed, permeabilized and blocked prior to overnight incubation at 4 °C with a polyclonal goat anti-human prospero homeobox gene-1 antibody and a monoclonal mouse anti-human cluster of differentiation 31 antibody. Secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 555 were used for detection. Samples were acquired using a Leica SP2 confocal system and analyzed with Volocity software (PerkinElmer; Melbourne, VIC, Australia).
Proliferation assay
LECs (5x10⁴ cells/well) were plated into collagen type I-coated 24 well plates, and incubated for 24 h in EGM-2-MV medium without or with apoA-I (final concentration 1.2 mg/mL). The apoA-I was removed, and the cells were incubated for a further 16 h with EGM-2-MV without or with TNF (final concentration 10 ng/mL). BrdU (final concentration 10 µM/L) was added for the last 5 h. The cells were then fixed, permeabilized, re-fixed and incubated for 1 h at 37 °C with DNAse (100 µL of a 300 µg/mL solution) according to the manufacturer's instructions (BD Biosciences). After staining with an allophycocyanin-anti-BrdU monoclonal antibody for 20 min at room temperature, BrdU uptake was measured using a LSR Fortessa flow cytometer (BD Biosciences).

LEC viability assay
Confluent LECs were incubated for 24 h in T25 flasks in EGM-2-MV medium without or with apoA-I (final concentration 1.2 mg/mL). The media was replaced by EGM-2-MV containing TNF (10 ng/mL) and changed daily over 3 days. The LECs were added to the cell suspension (1 mL), which was mixed and incubated for 30 min on ice. Cell viability was measured with a violet laser (excitation at 405 nm; emission 525 nm) using an LSR Fortessa flow cytometer.

Western blot analysis
LECs were incubated for 24 h without or with apoA-I (final concentration 1.2 mg/mL), then incubated for 3 days with TNF (final concentration 10 ng/mL). Whole cell extracts were prepared by direct lysis in Laemmli buffer. After supplementation with benzonase nuclease (Millipore, North Ryde, NSW, Australia; Catalog number 70746), the cell extracts were heated at 95 °C for 5 min, electrophoresed on a 4-12% NuPAGE Bis-Tris gel (Life Technologies), and transferred to a nitrocellulose membrane. Non-specific binding was blocked by incubation with TBS containing 0.1% (v/v) Tween-20, and 5% (w/v) non-fat dry milk or 5% (w/v) BSA (Sigma, Castle Hill, NSW, Australia; Catalog number A7906). The membranes were incubated overnight at 4 °C with primary antibodies in TBS-Tween and 5% (w/v) non-fat dry milk or 5% (w/v) BSA. The primary antibodies were mouse anti-human VEGFR3 (Abcam; clone 44CT92.4.4, Catalog number Ab72240), rabbit-anti human Dll4 (Cell Signaling, Danvers, MA; Catalog number 2589), rabbit anti-human phospho-p38 MAPK (Cell Signaling; Catalog number 9211), rabbit anti-human p38 MAPK (Cell Signaling; Catalog number 9212), mouse anti-human TNF-R1 (R&D systems; clone 16803, Catalog number MA8225), and mouse anti-β-actin (Sigma; clone AC-74, Catalog number A2228). Goat anti-rabbit IgG HRP (Santa Cruz, Dallas, TX; Catalog number sc-2004) or goat anti-mouse IgG HRP (Santa Cruz; Catalog number sc-2005) was used as a secondary antibody. The membranes were developed with Luminata Forte Western HRP substrate (Millipore; Catalog number WBLUF0100) and imaged using a Chemidoc MP Imaging System (Bio-Rad). The chemiluminescent signal was quantified using Fiji. For some experiments, 3x dilutions of the samples were used as an additional loading controls. Signal intensities of the undiluted cell lysates (1x dilution) were used for quantification.

In vivo studies involving the lymphatic vasculature in the diaphragm
Human TNF transgenic mice (Tg197) were kindly offered by Professor G. Kollias and provided by Professor G. Schett for the in vivo lymphangiogenesis study. These mice spontaneously develop a chronic inflammatory polyarthritis at 4-6 weeks of age. Heterozygous human TNF transgenic mice were injected intraperitoneally with apoA-I (1.2 mg/kg) or PBS three times a week between 6 and 8 weeks of age (6 injections in total). After harvesting the diaphragms, the central tendon side was used for whole mount immunofluorescence. For morphological analysis, diaphragms were incubated for 3 days with a rat-anti mouse lymphatic vessel endothelial receptor-1 antibody (R&D systems;
clone 223322, Catalog number MAB2125). A goat anti-rat antibody conjugated to Alexa Fluor 488 (Life Technologies; Catalog number A11006) was used for detection. The diaphragms were mounted in 4',6-diamidino-2-phenylindole-containing mounting media. Images were acquired using a Leica SP5 confocal system. Systematic quantification of the lymphatics in the diaphragm (density, branch points, loops, blind-ended sacs and diameter) was performed using the Lymphatic Vessel Analysis Protocol plug-in for Fiji®.

**Quantification of cell surface tumor necrosis factor-α-converting enzyme**
LECs (5×10⁴ cells/well) were plated into collagen type I-coated 24 well plates, and incubated for 24 h in EGM-2-MV medium without or with apoA-I (final concentration 1.2 mg/mL). The apoA-I was removed, and the cells were incubated with EGM-2-MV without or with TNF (final concentration 10 ng/mL). The LECs were lifted in Accutase (Sigma), blocked, and incubated for 1 h on ice with a 1:100 dilution of goat anti-human tumor necrosis factor-α-converting enzyme (R&D systems; Catalog number AF9301). The cells were washed and incubated for 30 min on ice with an Alexa 647-conjugated donkey-anti-goat polyclonal antibody (Life Technologies; A21447). Fluorescence was measured using an LSR Fortessa flow cytometer.

**Statistics**
Data are presented as the mean±SEM (unless indicated otherwise) of at least three independent experiments. Differences between two groups were compared using the unpaired t-test (two-sided). Multi-group comparisons were performed by ANOVA with the Tukey-Kramer post hoc test using GraphPad Prism Version 6 (San Diego, CA, US). A value of P<0.05 was considered statistically significant.
<table>
<thead>
<tr>
<th>Table 1. Sequence of primers used for qPCR</th>
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<tr>
<td><strong>Prox-1</strong></td>
</tr>
<tr>
<td>Forward 5’-GCTCCAATATGCTGAAGACC,</td>
</tr>
<tr>
<td>Revere 5’-ATCGTTGATGGCTTGACGTG</td>
</tr>
<tr>
<td><strong>ABCA1</strong></td>
</tr>
<tr>
<td>Forward 5’-GAGCGGGGTCAGCAAAGCAA</td>
</tr>
<tr>
<td>Revere 5’-GGAGGACACATAGGACTTCTG</td>
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<tr>
<td><strong>ß-actin</strong></td>
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<tr>
<td>Forward 5’-CGGGAAAATCGTGCGTGACAT</td>
</tr>
<tr>
<td>Revere 5’-AAGGAAGGCTGGAAGAGTGC</td>
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<tr>
<td><strong>DLL4</strong></td>
</tr>
<tr>
<td>Forward 5’-ACAACTTGTCCGGACTTCCAG</td>
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<td>Revere 5’-CAGCTCCTCTTCTCGGTTTG</td>
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<td><strong>FoxC2</strong></td>
</tr>
<tr>
<td>Forward 5’-GCCCAGCAGCAAACTTTCC</td>
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<td>Revere 5’-CCCGAGGGGTCGAGTTCTCA</td>
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<td><strong>GATA2</strong></td>
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<td>Forward 5’-GCTGCACAATGTGAACAGGC</td>
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<td>Revere 5’-TCTCCTGCATGCACTTTGAC</td>
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<td><strong>Lyve1</strong></td>
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<td>Forward 5’-GCAAGGACCAAGTGAACAGCCTTG</td>
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<td>Revere 5’-CTGGAATGCGACGAGTTAGTCCAAAGTA</td>
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<td><strong>NRP2</strong></td>
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<tr>
<td>Forward 5’-AGCACTAATGGAGAGGACTG</td>
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<tr>
<td>Revere 5’-CCGTTTAGGCTGTAGGAGAC</td>
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<td><strong>Podoplanin</strong></td>
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<tr>
<td>Forward 5’-AGT GTCAACATGTGAACAGGCATT</td>
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<td>Revere 5’-TGA CAAAACCATTTTCTCAACTGT</td>
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<td><strong>RIP1</strong></td>
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<tr>
<td>Forward 5’-AGTCCTGGTTTGCTCCTTCCC</td>
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<tr>
<td>Revere 5’-GCGTCTCCTTTCTCCTCTCTCTG</td>
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<tr>
<td><strong>TNFAIP3 (A20)</strong></td>
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<tr>
<td>Forward 5’-CGGAAATTCTATGGCTGAACAGTCTTCC,</td>
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<tr>
<td>Revere 5’-ACGCGTCGACCTAGCCATACTCTGCCTTA</td>
</tr>
<tr>
<td><strong>TNF-R1</strong></td>
</tr>
<tr>
<td>Forward 5’-CTGCCAGGAGAAACAGAACC</td>
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<td><strong>TRAF2</strong></td>
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<td>Revere 5’-GCATAAGATGGACTTGCCG</td>
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<td><strong>VEGFR3</strong></td>
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<td>Forward 5’-CTTGTCGAAGATGCTGGCAGG</td>
</tr>
<tr>
<td>Revere 5’-GGAGAAAGTCTCCCGAGCAG</td>
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</table>
References

1. Rye KA, Garrety KH, Barter PJ. Preparation and characterization of spheroidal, reconstituted high-density lipoproteins with apolipoprotein A-I only or with apolipoprotein A-I and A-II. *Biochim Biophys Acta.* 1993;1167:316-325.


Supplemental Material

Apolipoprotein A-I limits the negative impact of tumor necrosis factor on lymphangiogenesis

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Running title: Lymphatic protective functions of apoA-I
Supplemental Fig I: Characterization of LECs by immunofluorescence
Confluent LEC monolayers were incubated overnight at 4 °C with mouse anti-human Lyve-1, mouse anti-human ICAM-1, goat anti-human Prox-1 and mouse anti-human CD31, then incubated for 2 h at room temperature with secondary antibodies conjugated to Alexa Fluor 488 alone or in combination with Alexa Fluor 555. The LECs were washed, mounted in DAPI-containing media and imaged by confocal microscopy.
Supplemental Fig II: ApoA-I does not alter proteins that regulate lymphatic function

Panel A: LECs were pre-incubated for 24 h without or with apoA-I (final concentration 1.2 mg/mL), then incubated for 3 days with TNF (10 ng/mL). mRNA levels of FoxC2, GATA2, NRP2, VEGFR3, and the notch ligand DLL4 were quantified by qPCR. Panel B: Western blot of DLL4 and VEGFR3 protein expression. Samples were loaded onto a NuPAGE Bis-Tris gel undiluted (1x) and after 3x dilution. Panel C: Quantification of undiluted DLL4 and VEGFR3 protein expression from Panel B.
Supplemental Fig III: ApoA-I does not increase Prox-1 expression directly
LEC s were incubated for 24 h without or with apoA-I (final concentration 0.3-1.2 mg/mL). Prox-1 mRNA levels were quantified by qPCR.
Supplemental Fig IV: Pre-incubation with apoA-I does not affect TNF-R1 expression
LECs were incubated for 24 h without or with apoA-I (final concentration 1.2 mg/mL).
Panel A: TNF-R1, A20 and RIP mRNA levels were quantified by qPCR. Panel B: Western blot of TNF-R1 protein expression. Panel C: Quantification of TNF-R1 protein expression from Panel B.
TNF receptor-associated factor 2 (TRAF2); receptor-interacting protein (RIP)
Supplemental Fig V: ApoA-I increases tumor necrosis factor-α-converting enzyme expression

LECs were incubated for 24 h in EGM-2-MV medium without or with apoA-I (final concentration 1.2 mg/mL), then stimulated with TNF (10 ng/mL) for 3 days. The cells were stained with Alexa 647 and analyzed for TACE expression by flow cytometry. Values represent the mean±SEM of three separate experiments.

TACE: tumor necrosis factor-α-converting enzyme

*P<0.05 vs control or TNF alone.