Obesity but not High-Fat Diet impairs lymphatic function

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Abstract

Background/Objectives—High-fat diet (HFD)-induced obesity has significant negative effects on lymphatic function, but it remains unclear whether this is a direct effect of HFD or secondary to adipose tissue deposition.

Methods—We compared the effects of HFD on obesity-prone and obesity-resistant mice and analyzed lymphatic function *in vivo* and *in vitro*.

Results—Only obesity-prone mice had impaired lymphatic function, increased perilymphatic inflammation, and accumulation of lipid droplets surrounding their lymphatic endothelial cells (LECs). LECs isolated from obesity-prone mice, in contrast to obesity-resistant animals, had decreased expression of VEGFR-3 and Prox1. Exposure of LECs to a long-chain free fatty acid increased cellular apoptosis and decreased VEGFR-3 expression, while inhibition of intracellular inhibitors of VEGFR-3 signaling pathways increased cellular viability.

Conclusions—Collectively, our studies suggest that HFD-induced obesity decreases lymphatic function by increasing perilymphatic inflammation and altering LEC gene expression. Reversal of diminished VEGFR-3 signaling may rescue this phenotype and improve lymphatic function.

Keywords

Obesity; high-fat diet; lymphatic function; free fatty acid; lymphatic endothelial cell; inflammation

Introduction

The incidence of obesity is rapidly rising in Western countries; in the United States alone, it is estimated that 1 in 3 adults and 1 in 6 children are obese.(1) This is important because obesity is a significant risk factor for a variety of systemic disorders and adds nearly 200

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billion dollars to our annual healthcare budget.(2) Therefore, understanding the mechanisms that regulate obesity-related pathology is an important goal.

Chronic adipose tissue inflammation is thought to contribute to a large number of pathologic responses in obesity, including metabolic syndrome and malignancy. These responses are modulated by T cells and macrophages, leading to increased local/systemic expression of inflammatory cytokines and release of free fatty acids (FFA) with direct toxic effects on surrounding tissues.(3–6) In addition, adipose tissue functions as an endocrine organ that produces substances that regulate function and obesity-related pathology.(5, 7)

Recent studies have shown that obesity has significant negative effects on the lymphatic system. For example, our group and others have shown that HFD-induced obesity markedly decreases interstitial fluid transport, immune cell trafficking, and lymphatic collecting vessel pumping.(8–10) These negative consequences were correlated with increasing body mass suggesting that adipose deposition plays a role in this response. In contrast, others have reported that high fructose diet-induced metabolic syndrome(11) or hypercholesterolemia, (12) decrease collecting vessel pumping and result in lymphatic system abnormalities independent of obesity. Thus, although it is clear that obesity can cause lymphatic dysfunction, the independent effects of dietary changes and weight gain on the lymphatic system remain unknown.

In this study, we sought to determine the independent effects of prolonged HFD intake with or without concomitant obesity on lymphatic function. By comparing the effects of HFD in obesity-prone and obesity-resistant mice, we show that dietary changes alone are insufficient to induce lymphatic dysfunction. In addition, we show that lymphatic dysfunction in obesity-prone mice correlates with perilymphatic accumulation of inflammatory cells and lipids, and alterations in LEC gene expression of lymphatic markers and inflammatory receptors. Finally, using correlative *in vitro* studies, we show that exposure of LECs to stearic acid, a long-chain fatty acid known to be increased in obese tissues,(13) results in decreased VEGFR-3 expression, apoptosis, and growth inhibition. Furthermore, intracellular modulation of VEGFR-3 signaling by inhibiting dephosphorylation of phosphatidylinositol (3,4,5)-trisphosphate-tyrosine-kinase (PIP3), addition of recombinant VEGF-C (direct activation of VEGFR-3), or recombinant insulin (indirect activation of PIP3 pathway) rescues LECs and markedly decreases apoptosis. Taken together, our findings suggest that obesity-induced inflammation, and not HFD, is responsible for the pathologic changes observed in the lymphovascular system and that these changes are due at least in part to direct injury to the LECs via byproducts of inflamed adipose tissues, including long-chain free fatty acids.

Materials and Methods

An extended version of Materials and Methods is available on the International Journal of Obesity website.

Animals, Diets, and Metabolic Analysis

Male C57BL/6J, BALB/cJ, and lean (Ln) allele of myostatin (MSTN^{ln}) mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and maintained on a HFD (Purina Test Diet 60% kcal from fat; W.F Fisher & Son, Inc., NJ) for 10–12 weeks. Age-matched controls on the same genetic backgrounds were maintained on a normal chow diet (NCD, 13% kcal from fat; Purina PicoLab Rodent Diet 20) for the same period of time.

Analysis of Lymphatic Function

Lymphoscintigraphy was used to analyze lymph node uptake following peripheral injection of a technetium labeled colloid (^{99m}Tc) as previously reported.[14] We used a modification of previously reported methods to analyze dendritic cell (DCs) trafficking[15] and ferritin uptake.[16]

Histology

Tissues were analyzed with immunofluorescent staining as previously reported.[14]

LEC Isolation and PCR

Dermal LECs were isolated from the abdominal skin by digestion in 0.4% Collagenase IV digestion buffer (MP Biomedicals, Solon, OH) and flow cytometry to sort for LECs (CD45$^-$, CD31$^+$, podoplanin$^+$).[17] RNA was extracted using TRIZOL (Invitrogen, Life Technologies, Carlsbad, CA) and converted to cDNA with TaqMan reverse transcriptase kit (Roche, Branchburg, NJ). Relative gene expression between groups was analyzed by normalizing gene expression with GAPDH.[18]

ELISA

Protein isolated from skin/subcutaneous tissues and serum was analyzed with ELISA to quantify CCL21, IL-1β, TNF-α (Sigma-Aldrich), VEGF-C (United States Biological) or FFA (Abcam) following the manufacturer's protocol.

Cell Culture, Immunocytochemistry, and Western Blot

Human dermal LECs and adipose derived mesenchymal stem cells (ASCs) were obtained from PromoCell (Heidelberg, Germany) and stained in chamber slides for Prox1, Ki67 (Abcam, Cambridge, MA), p-AKT (Cell Signaling Technology, Danvers, MA), and VEGFR-3 (EMD Millipore, Billerica, MA). Cell signal intensity was quantified using MetaMorph (Molecular Devices, Sunnyvale, CA). Western blot analysis for VEGFR-3 (Abcam), p-AKT, and p-eNOS (Cell-signaling Technology) was performed with total cellular protein harvested from LECs and quantified with NIH Image J.[19]

Cellular Apoptosis and Viability assays

Apoptosis in response to stearic acid (SA; 0.1, 1, 10, 100μM) with/without 0.3nM Phosphatase and tensin homolog inhibitor (PTENi; SF1670, Sigma-Aldrich), [20] was quantified using caspase-3 assay (R and D systems, Minneapolis, MN) and Annexin V-FITC Apoptosis Detection Kit (eBioscience, San Diego, CA). Viability was measured using a WST-8 cytotoxicity cell assay kit (Sigma-Aldrich).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 software (La Jolla, CA) and differences between 2 groups were analyzed using a Student's T test. Data are expressed as mean ± standard deviation with $p < 0.05$ considered significant.

Results

High-fat diet (HFD) decreases lymphatic vascular density in obesity-prone mice

In order to study the effects of obesity and HFD on lymphatic function, obesity-prone C57BL/6J mice were compared to obesity-resistant BALB/cJ[21] and MSTN[ln] mice.[22] As expected, feeding male C57BL/6J mice a HFD for 10 weeks resulted in obesity characterized by marked weight gain (164% increase), 9-fold increase in subcutaneous adipose deposition, mild glucose intolerance, and increased fasting serum insulin levels (Fig. 1A, C and Supplemental Fig. 1A, B). In contrast, BALB/cJ mice fed a HFD gained only a small though significant amount of weight (8%), with modest increases (2.86-fold) in subcutaneous adipose tissue thickness, mildly increased fasting serum insulin levels, and no signs of glucose intolerance. HFD-fed MSTN[ln] mice remained lean throughout the study and displayed no abnormalities in glucose metabolism. All HFD-fed mice, regardless of strain, had modestly increased serum levels of total and HDL cholesterol and increased levels of FFA in serum; HFD-fed C57BL/6J and BALB/cJ mice also displayed increased serum LDL (Supplemental Fig. 1C, D). HFD-induced obesity in C57BL/6J mice correlated with a decrease in lymphatic (LYVE-1^{+}) vessel density in the dermis and subcutaneous tissues as compared with their lean NCD-fed controls (Fig. 1B). In contrast, obesity-resistant HFD-fed BALB/cJ and MSTN[ln] mice demonstrated no significant differences.

Obesity impairs lymphatic transport

The rate of uptake and peak transport of macromolecules by the lymphatic system can be quantified by injecting [99mTc] labeled sulfur colloid in the distal hindlimb and analyzing its uptake by the inguinal lymph node.[14] Consistent with our previous studies, analysis of lymphatic transport in HFD-fed C57BL/6J revealed significant decreases in both total and rate of lymph node [99mTc] uptake (Fig. 2A–C). In contrast, HFD-fed obesity-resistant mice had no significant decrease in peak or rate of [99mTc] uptake as compared with their respective lean controls.

Peripherally injected ferritin is transported by, and remains bound to functional lymphatic vessels enabling histochemical localization using Prussian blue staining.[23] Consistent with our lymphoscintigraphic findings, only obese C57BL/6J animals had significantly decreased lymph node ferritin staining (2-fold) as compared to their NCD-fed controls, whereas HFD-fed BALB/cJ and MSTN[ln] mice did not (Fig. 2D, E).

Functional lymphatic vessels also regulate migration of antigen presenting cells to regional lymph nodes where they interact with other immune cells.[24] Therefore, we analyzed migration of isolated DCs from the distal hindlimb (injection site) to the inguinal lymph node of HFD- and NCD-fed animals over a 24-hour period. Flow cytometry of draining lymph nodes harvested from HFD-fed obese C57BL/6J mice demonstrated a more than 50%

reduction in the number of trafficked DCs as compared to their NCD-fed controls (Fig. 2F, G). In contrast, HFD-fed BALB/cJ and MSTN^{In} mice demonstrated no changes when compared to their NCD-fed controls. Taken together, these findings show that obesity, rather than simply prolonged exposure to HFD, is necessary for impaired lymphatic function in mice.

Obesity results in perilymphatic inflammation

We next analyzed tissues harvested from multiple anatomical locations and co-localized lymphatic vessels (LYVE-1$^+$) with T cells (CD3$^+$) or macrophages (CD11b$^+$) since previous studies have shown that these inflammatory cell types play a key role in the regulation of pathological changes in obesity.(3–6) This approach enabled us to quantify perilymphatic inflammation, which we defined as the number of inflammatory cells located within 50μm of a lymphatic vessel.

Analysis of ear tissues harvested from HFD-fed obese C57BL/6J mice demonstrated significant increases in the number of perilymphatic CD3$^+$ and CD11b$^+$ cells (3.6- and 3-fold increase from controls, respectively; Fig. 3A–B, Supplemental Fig. 2). Similar findings were noted in the trachea and back skin, suggesting that perilymphatic inflammation in obese C57BL/6J mice is a systemic phenomenon (Supplemental Fig. 3, 4). In contrast, HFD-fed BALB/cJ or MSTN^{In} mice failed to show significant changes as compared with their NCD-fed controls, indicating that HFD alone is insufficient for perilymphatic inflammation.

Previous studies have shown that VEGF-C expression is increased in obese individuals and that this growth factor plays a key role in macrophage infiltration and differentiation.(25, 26) Consistent with these studies and our finding of increased macrophage infiltration in obese mice, we noted a marked increase in VEGF-C expression in both serum (1.75-fold) and skin (2-fold) of HFD-fed obese C57BL/6J mice, as compared to their NCD-fed controls (Fig. 3 C, D). In addition, analysis of skin tissues from obese HFD-fed C57BL/6J mice also demonstrated increased expression of inflammatory cytokines including TNF-α (20-fold) and IL-1β (8.32-fold) as compared to their NCD controls (Fig. 3 E, F).

Obesity downregulates expression of lymphatic markers in LECs

We next analyzed gene expression in LECs isolated from the subcutaneous tissues of obesity-prone and obesity-resistant mice fed either a HFD or NCD. PCR analysis of sorted LECs isolated from obese HFD-fed C57BL/6J mice demonstrated a nearly 2-fold decrease in the expression of *Prox1* (a transcription factor necessary for lymphatic differentiation), *VEGFR-3* (a signaling receptor necessary for LEC proliferation and function), and *CCL21* (a chemotactic cytokine that regulates migration of DCs to lymphatic channels), as compared with their NCD-fed controls (Fig. 4A). Immunofluorescent co-localization of lymphatic vessels (LYVE-1$^+$) with CCL21 confirmed our PCR findings demonstrating a marked reduction in CCL21 expression in lymphatic vessels of HFD-fed obese C57BL/6J mice as compared to their NCD-fed controls (Fig. 4B). Furthermore, LECs isolated from obese HFD-fed C57BL/6J mice demonstrated a more than 2-fold increase in the expression of the inflammatory cell receptor *ICAM-1* and the pro-apoptotic gene *Bax*. In contrast, we

noted no significant gene expression changes in cell sorted LECs isolated from HFD-fed BALB/cJ or MSTN[ln] mice as compared with their respective NCD-fed controls.

Previous studies have shown that gradients of CCL21 expression are necessary for migration of antigen presenting cells (e.g. dendritic cells) to lymphatic channels.(27, 28) In light of our observation that obese C57BL/6J mice had decreased DC trafficking and that their LECs had downregulated *CCL21* expression, we next sought to determine if tissue gradients of CCL21 expression are altered in obesity by analyzing protein levels in the skin and subcutaneous tissues. Interestingly, we found that cutaneous tissues harvested from HFD-fed C57BL/6J mice had a more than 2-fold increase in CCL21 expression as compared to NCD-fed controls suggesting that clearance of inflammatory cells by the lymphatic system in obese animals is decreased, at least in part, due to a loss of gradients of CCL21 (i.e. low expression in LEC and high expression in tissues) (Fig. 4C).

To confirm our PCR findings, tissues were immunostained for LYVE-1 with VEGFR-3, Prox1, and p-AKT (a downstream mediator of VEGFR-3 signaling). Consistent with our PCR findings, we noted a marked decrease in VEGFR-3 and Prox1 expression by lymphatic vessels in HFD-fed obese C57BL/6J mice but not in HFD-fed BALB/cJ or MSTN[ln] mice (Fig. 4D, Supplemental Fig. 5). In addition, only the lymphatic vessels in obese C57BL/6J mice had a marked decrease in expression of p-AKT as compared to their lean controls.

Obesity results in perilymphatic lipid accumulation and in vitro exposure of LECs to FFA decreases cellular viability

We subsequently sought to identify potential mechanisms that may regulate LEC injury and gene expression changes in obesity. Previous studies have shown obesity results in macrophage accumulation, which subsequently engulf necrotic adipocytes resulting in the formation of "crown-like structures" (CLS).(29, 30) Adipocyte breakdown by CLS releases toxic by-products such as long-chain FFA, leading to increased expression of inflammatory mediators and worsening subcutaneous tissue inflammation.(31, 32) Indeed, immunohistochemical analysis of skin harvested from obese HFD-fed C57BL/6J mice demonstrated the presence of CLS in the subcutaneous fat (Fig. 5A). Additionally, co-localization of lymphatic vessels (LYVE-1^{+}) and lipids (BODIPY[+]), demonstrated the presence of numerous large lipid droplets in close proximity to LECs of obese HFD-fed C57BL/6J mice (Fig. 5B). In contrast, we found little lipid accumulation in NCD-fed C57BL/6J controls.

We next performed corroborative *in vitro* studies on isolated LECs in order to better understand the effects of lipid by-products and FFA at the cellular level. SA is known to be increased in obesity where it has been shown to have toxic effects on blood endothelial cells. (6, 33) We therefore compared the effects of SA on LECs with those on ASCs, since these cells are also present in adipose tissues. Interestingly, we found that SA, even in micromolar concentrations, was highly toxic to LECs resulting in a dose-dependent increase in cellular apoptosis as reflected by increasing activity of caspase-3 and annexin V (Fig. 5C, D, and Supplemental Fig. 6A). In contrast, ASCs were highly resistant to SA-induced apoptosis or injury. In addition, similar to our PCR findings, we noted that exposing LECs to even low doses of SA resulted in a significant decrease in VEGFR-3 expression (3-fold) as compared

with LECs cultured in control media (Fig. 5E, F). This is important since a major function of VEGFR-3 signaling in LECs is prevention of apoptosis through activation of AKT and subsequent downregulation of apoptotic pathways.(34)

VEGFR-3, similar to the insulin receptor, is a tyrosine kinase receptor (TKR) and activates intracellular signaling via a variety of mechanisms. A major intracellular signaling pathway of TKRs is regulated by phosphorylation of Phosphatidylinositol 4,5-bisphosphate (PIP2) to PIP3, which then activates downstream signaling pathways, including AKT phosphorylation. PTEN is an intracellular protein that dephosphorylates PIP3 to PIP2, thereby functioning as an intracellular inhibitor of VEGFR-3 signaling. To determine if VEGFR-3 downregulation and subsequent decreased PIP3 activation in response to SA exposure plays a causal role in increased apoptosis, we cultured LECs with SA, either with or without media supplementation with PTENi. Analysis of caspase-3 activity and cellular viability in these cells demonstrated a statistically significant protective effect of PTEN inhibition in LECs as compared to cells cultured without PTENi (Fig. 5G, H). Similarly, we found that culturing isolated LECs with SA and either recombinant VEGF-C (direct activation of VEGFR3 downstream pathways) or recombinant insulin (indirect activation of PIP3 pathway via tyrosine kinase activation) restored the viability of LECs cultured with SA (Supplemental Figure 6B).

Our VEGFR-3 immunocytochemistry findings were confirmed with Western blot analysis of protein isolated from LECs cultured in media containing SA, with or without the addition of PTENi, VEGF-C, or insulin (Fig. 6A, B). Consistent with our immunocytochemistry findings, we noted that exposure of LECs to SA resulted in a 2.9-fold decrease in expression of VEGFR-3, 3.7-fold decrease in expression of p-AKT, and a 2.6-fold decrease in the expression of phosphorylated eNOS (p-eNOS, another downstream mediator of VEGFR-3). Addition of PTENi, recombinant VEGF-C, or insulin to LECs cultured in media containing SA normalized VEGFR-3, p-AKT, and p-eNOS expression to levels noted in LECs cultured in control (i.e. no SA) media. Addition of PTENi, recombinant VEGF-C, or insulin alone to LECs also significantly increased expression of these proteins independent of SA, suggesting that intracellular PTEN function acts as a feedback mechanism regulating VEGFR-3 signaling.

Consistent with our cell-sorted PCR on LECs obtained from obese C57BL/6J mice, we noted a marked reduction in the expression of *Prox1* by LECs after exposure to SA (Fig. 6C). This was normalized to control levels with the addition of PTENi, VEGF-C or insulin. We noted similar findings with Ki67 (a marker of cellular proliferation) and p-AKT expression. Taken together, our *in vitro* findings suggest that long-chain FFA are harmful to LECs by decreasing the expression of master lymphatic regulators and that these effects can be mitigated by modulating intracellular pathways downstream of VEGFR-3 through PIP3 pathway and subsequently AKT activation.

Discussion

Previous clinical and laboratory studies have shown that obesity is a negative regulator of lymphatic function.(3–10) However, it remains unclear if the observed effects on lymphatic

function were secondary to weight gain, prolonged exposure to HFD, or toxic metabolic by-products of adipose tissue. In order to address this question, we compared lymphatic function in obesity-prone and obesity-resistant mice after prolonged exposure to HFD. Our results are consistent with previous reports demonstrating that C57BL/6J mice become morbidly obese on HFD feeds,(8, 9) while BALB/cJ and MSTN*ln* mice do not(21, 22) even though serum FFA levels were significantly increased in all HFD-fed mice.(13, 35) This approach is clinically relevant since the incidence and severity of obesity in patients is also highly variable and modulated by genetic factors. Our findings imply that obesity, but not HFD, is necessary for the development of lymphatic dysfunction since obesity-resistant mice maintained essentially normal lymphatic function despite prolonged exposure to HFD feeds. Our study also suggests that obesity-induced tissue changes or alterations in glucose and/or FA metabolism (or a combination of all) are necessary for lymphatic dysfunction and that prolonged exposure to HFD alone does not cause direct injury to the lymphatic system.

Alternate hypotheses could explain the differences we noted in lymphatic function of obesity-prone and obesity-resistant mice after prolonged HFD feeds. For example, it is possible that strain-related differences may contribute to the disparities we observed in lymphatic function. Although our study does not definitely disprove this possibility, we believe that genetic differences in baseline lymphatic function are less important than the effects of obesity since MSTN*ln* mice are derived from a B6 background, and because *both* obesity-resistant mice strains (MSTN*ln* and BALB/cJ) were protected from lymphatic injury despite dissimilar genetic backgrounds. Additionally, although previous studies reported lymphatic dysfunction in ApoE deficient mice with very high serum cholesterol levels (1000–2000mg/mL),(36) it is unlikely that changes in cholesterol levels are responsible for the lymphatic dysfunction we observed in our obese mice since the total cholesterol levels in our study were an order of a magnitude lower (<200mg/mL) than those reported by Angeli *et al*. In addition, total and LDL cholesterol levels were elevated in all HFD-fed mice (i.e. both obesity-prone and obesity-resistant mice). In fact, BALB/cJ mice had the highest serum levels of LDL cholesterol. Nonetheless, despite increases in total and LDL cholesterol in all HFD-fed animals, only the obese C57BL/6J mice displayed decreased lymphatic function indicating that modestly increased levels of cholesterol do not have deleterious effects on the lymphatic system.

A key histological difference between obesity-prone and obesity-resistant mice in our study was perilymphatic inflammation. This finding extends the results of previous studies demonstrating chronic, low-grade perivascular inflammatory responses in obesity.(37, 38) This is important because perivascular inflammation plays a key role in the pathological outcomes of obesity on the blood vascular system by diverse mechanisms including altered expression of adipokines, impaired nitric oxide signaling, and increased production of reactive oxygen species.(7, 39, 40) These effects are compounded by infiltration of macrophages that phagocytose necrotic adipocytes, leading to the release of long-chain FFA(41) that, in turn, have deleterious effects on endothelial cells.(6, 31, 42) Indeed, in our studies we observed the presence of numerous CLS, and close association of large lipid droplets with lymphatic vessels, in the subcutaneous tissues of obese mice. Thus, chronic perilymphatic inflammatory responses in obese animals may act via a variety of mechanisms to inhibit lymphatic function in this setting.

We analyzed gene expression changes in cell sorted LECs from NCD and HFD mice, as well as isolated LECs cultured with SA, a long-chain fatty acid known to be increased in obesity. (41) This analysis demonstrated that obese animals (but not obesity-resistant mice fed a HFD) and LECs cultured with SA had markedly decreased expression of Prox1 and VEGFR-3. Consistent with this effect, we noted that LECs harvested from obese C57BL/6J mice had a marked increase in the expression of the pro-apoptotic gene *Bax* and decreased expression of p-AKT, the downstream mediator of VEGFR-3 activation. These findings are important as previous studies have shown that the transcription factor Prox1 is a master lymphatic regulator necessary for the maintenance of the lymphatic phenotype and VEGFR-3 expression.(43) Similarly, VEGFR-3 signaling plays a crucial role in a variety of physiologic functions in LECs, including proliferation, migration, differentiation, protection from apoptosis, and expression of nitric oxide.(34, 44) To further explore the role of VEGFR-3 and TKR signaling in LEC response to injury, we cultured LECs with SA with or without an inhibitor of PTEN, VEGF-C, or insulin. This analysis demonstrated that direct activation of VEGFR-3 signaling pathways with VEGF-C or indirect activation of tyrosine kinase signaling with a PTEN inhibitor or insulin confers significant protective effects to LECs cultured with SA. Thus, downregulation of Prox1 and VEGFR-3 in obesity may serve as a key cellular mechanism regulating lymphatic dysfunction in this setting and represents a potential target for therapeutic interventions. Our findings also suggest that obesity or exposure to high levels of long-chain fatty acids decrease insulin sensitivity in lymphatic endothelial cells and that subsequent intracellular downregulation of tyrosine kinase activity (e.g. AKT phosphorylation, eNOS, etc.) decreases LEC viability and proliferation.

A large number of studies have shown that inflammation increases lymphangiogenesis.(45, 46) Thus, it may seem contradictory that we found decreased lymphatic vessel density in obese animals in which we also noted marked subcutaneous infiltration of T cells and macrophages. However, numerous other studies have shown that chronic inflammation in some physiologic settings can markedly inhibit lymphangiogenesis(47, 48) and that the initial lymphangiogenic response to acute inflammation subsides over time.(17, 47, 48) Furthermore, these studies have shown that an important mechanism regulating this effect is decreased VEGFR-3 expression. For example, Huggenberger *et al.* showed that chronic inflammation can decrease VEGFR-3 protein and gene expression during oxazolone challenge, despite concomitantly increased expression of pro-inflammatory cytokines TNF-α, IL-1β, and IFN-γ.(49) Others have reported that micro-RNAs regulate inflammation-induced decreased VEGFR-3 and Prox1 expression.(50, 51) Finally, T cell-derived cytokines including IFN-γ, IL-4 and IL-13 have been shown to directly inhibit lymphangiogenesis.(46, 52)

Consistent with previous studies in obese mice and patients,(26) we noted a marked increase in VEGF-C expression in tissues and serum harvested from obese HFD-fed C57BL/6J mice. This effect plays a key role on glucose homeostasis in obesity by regulating macrophage infiltration and differentiation.(25) In addition, the combination of increased VEGF-C in the context of decreased LEC VEGFR-3 expression in obese mice suggest that homeostatic mechanisms contribute to changes in VEGF-C expression. This scenario may be similar to insulin resistance in obesity (i.e. increased insulin/decreased insulin receptor function), suggesting that similar mechanisms regulate lymphatic function, a condition we have termed

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VEGF-C resistance. Our hypothesis is supported by the fact that receptor downregulation/ dysfunction is a common pathologic outcome in obesity, regulating sensitivity to a number of molecules including insulin and leptin.(53, 54) In addition, VEGFR-3, similar to the insulin receptor, is a TKR,(34) and its expression in obesity may therefore be down-regulated by similar mechanisms, including inflammatory cytokines(55) and free fatty acids. (56) Finally, similar to our findings in LECs *in vitro*, previous studies have shown that deletion of PTEN is protective against insulin resistance by increasing intracellular PI3K/AKT signaling.(57)

An important function of the lymphatic system is the transport of antigen presenting cells to regional lymph nodes. In the current study, we found that obesity markedly decreases DC migration to regional lymph nodes and that this response may be related to decreased clearance of interstitial fluid as well as changes in gradients of CCL21 expression.(27, 58) These findings are in-line with previous studies demonstrating decreased CCL21 gradients and increased LEC ICAM-1 expression in response to contact hypersensitivity.(17) Our findings are also consistent with the known roles of interstitial flow and VEGFR-3 expression in regulating CCL21 expression by LECs.(59) Thus, changes in CCL21 expression gradients and increased expression of ICAM by LECs may effectively trap antigen presenting cells in peripheral tissues and prevent trafficking of activating or suppressive DCs to regional lymph nodes. These data suggest a new cellular mechanism that regulates impaired T cell recall responses and may contribute to increased incidence of autoimmunity in obesity.(60)

In summary, we have shown that obesity, rather than prolonged exposure to HFD is necessary for induction of lymphatic dysfunction in mice and that this response correlates with perilymphatic inflammation and down-regulation of LEC VEGFR-3 and Prox1 expression. In addition, we have shown that LECs are highly sensitive to the toxic effects of long-chain fatty acids that are known to be increased in obesity and that direct or indirect intracellular activation of PIP3 confers significant protection to LECs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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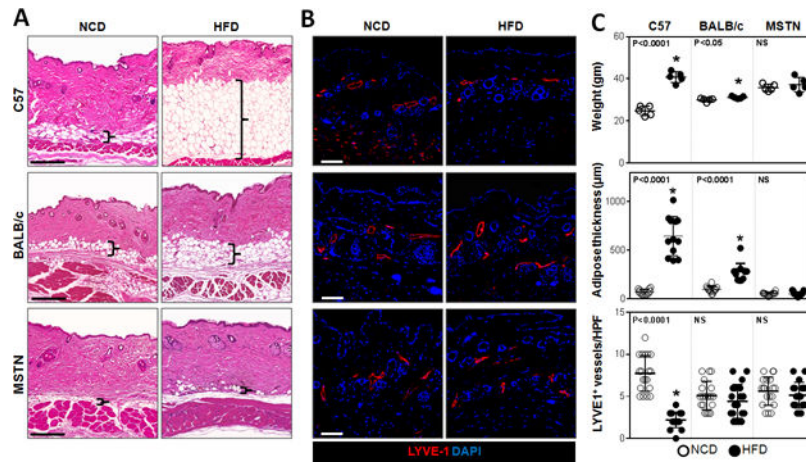
Fig. 1. HFD results in decreased lymphatic vascular density in obesity-prone mice

A. Representative H&E stained sections of back skin from NCD- or HFD-fed C57BL/6J, BALB/cJ, and MSTN[In] mice (Scale bar=200μm; bracket surrounds subcutaneous adipose tissues).

B. Representative immunofluorescent localization of LYVE-1^{+} vessels in upper limb tissues of NCD- or HFD-fed mice in all groups (Scale bar=100μm).

C. *Upper panel:* Body weights of mice on NCD (open circles) and HFD (filled circles) in all groups (n=5/group). C57BL/6J HFD vs. NCD (*p<0.0001). BALB/cJ HFD vs. NCD (*p<0.05), MSTN[In] mice had no significant difference. *Middle panel:* Quantification of subcutaneous soft tissue thickness in NCD- and HFD-fed mice in all groups (n=5–10/group). C57BL/6J HFD vs. NCD (*p<0.0001). MSTN[In] and BALB/cJ mice show no significant difference between NCD and HFD groups. *Lower panel:* Quantification of upper limb LYVE-1^{+} lymphatic vessel density per high powered field (HPF) and quadrant in NCD- and HFD-fed mice in all groups (n=5animals*4HPF/group). C57BL/6J HFD vs. NCD (*p<0.0001). MSTN[In] and BALB/cJ mice show no significant difference between NCD and HFD groups.
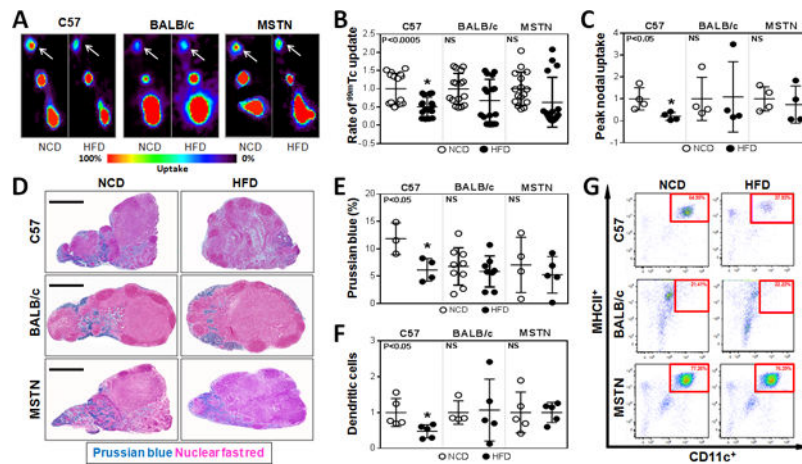
Fig. 2. Obesity impairs lymphatic transport of macromolecules to draining lymph nodes

A. Representative hindlimb ^{99m}Tc heat maps for NCD- and HFD-fed C57BL/6J, BALB/cJ, and MSTN^{ln} mice. White arrows indicate the uptake in the inguinal lymph nodes (n=4/group).

B. Quantification of the rate of ^{99m}Tc uptake in inguinal lymph nodes following hindlimb injection. Data is presented as fold change relative to their NCD controls in each group (n=16/group). C57BL/6J HFD vs. NCD (*$p<0.0005$). MSTN^{ln} and BALB/cJ had no significant difference between NCD and HFD groups.

C. Quantification of peak nodal uptake of ^{99m}Tc in inguinal lymph nodes following hindlimb injection. Data are presented as fold change relative to their NCD controls in each group (n=4/group). C57BL/6J HFD vs. NCD (*$p<0.05$). MSTN^{ln} and BALB/cJ had no significant difference between NCD and HFD groups.

D. Representative Prussian blue stained histological cross sections of axillary lymph nodes harvested from NCD- or HFD-fed mice in all groups. (Scale bar=500µm).

E. Quantification of Prussian blue staining as a percentage of the total lymph node area in NCD- and HFD-fed mice (n=3–9/group). C57BL/6J HFD vs. NCD (*$p<0.05$). MSTN^{ln} and BALB/cJ had no significant difference between NCD and HFD groups.

F. Quantification of migrating CD45.1$^+$ dendritic cells (MHCII$^+$/CD11C$^+$) in inguinal and popliteal lymph nodes of NCD- (open circles) and HFD- (filled circles) fed mice in all groups (n=5/group). Data are presented as fold change from respective NCD-fed controls. C57BL/6J HFD vs. NCD (*$p<0.05$). MSTN^{ln} and BALB/cJ had no significant difference between NCD and HFD groups.

G. Representative dot plot graphs of inguinal/popliteal lymph node flow cytometry analyzing migrating CD45.1$^+$ dendritic cells in all groups. Red square surrounds dendritic cell population.
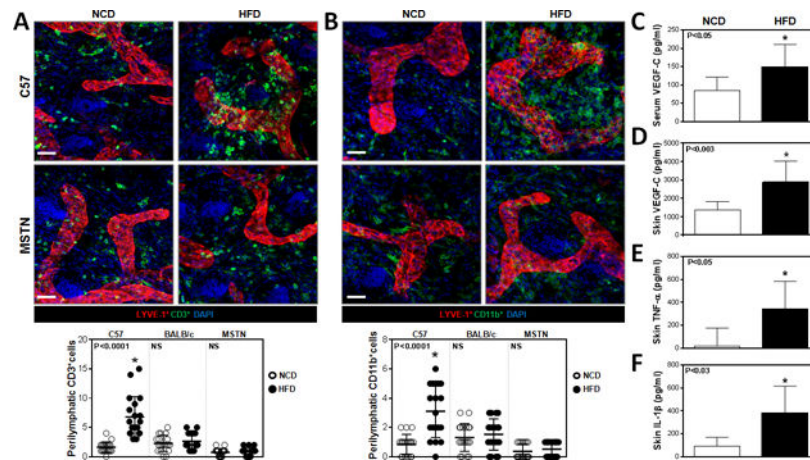
**Fig. 3. Obesity results in local tissue and perilymphatic inflammation**

A. Representative immunofluorescence co-localization (upper panel) of CD3^{+} cells (green) and LYVE-1^{+} vessels (red) in ear skin whole mounts harvested from NCD- and HFD-fed C57BL/6J and MSTN[In] mice. Blue stain is DAPI (Scale bar=30μm). Quantification of perilymphatic CD3^{+} cells in skin ear cross-sections of NCD- (open circles) and HFD- (filled circles) fed mice in all groups (lower panel). C57BL/6J HFD vs. NCD (*p<0.0001, n=4 quadrants *4–5animals/group). MSTN[In] and BALB/cJ mice showed no significant difference between NCD and HFD groups.

B. Representative immunofluorescence co-localization (upper panel) of CD11b^{+} cells (green) and LYVE-1^{+} vessels (red) in ear skin whole mounts harvested from NCD- and HFD-fed C57BL/6J and MSTN[In] mice. Blue stain is DAPI (Scale bar=30μm). Quantification of perilymphatic CD11b^{+} cells in skin ear cross-sections of mice fed NCD (open circles) or HFD (filled circles) in all groups (lower panel). C57BL/6J HFD vs. NCD (*p<0.0001, n=4 quadrants * 5animals/group). MSTN[In] and BALB/cJ mice showed no significant difference between NCD and HFD groups.

C. Quantification of VEGF-C protein levels in serum harvested from lean and obese C57BL/6J mice (n=10/group, p<0.05).

D. Quantification of VEGF-C protein levels in back skin tissues harvested from lean and obese C57BL/6J mice (n=6–10/group, p<0.003).

E. Quantification of TNF-α protein levels in back skin tissues harvested from lean and obese C57BL/6J mice (n=4–6/group, p<0.05).

F. Quantification of IL-1β protein levels in back skin tissues harvested from lean and obese C57BL/6J mice (n=5/group, p<0.03).
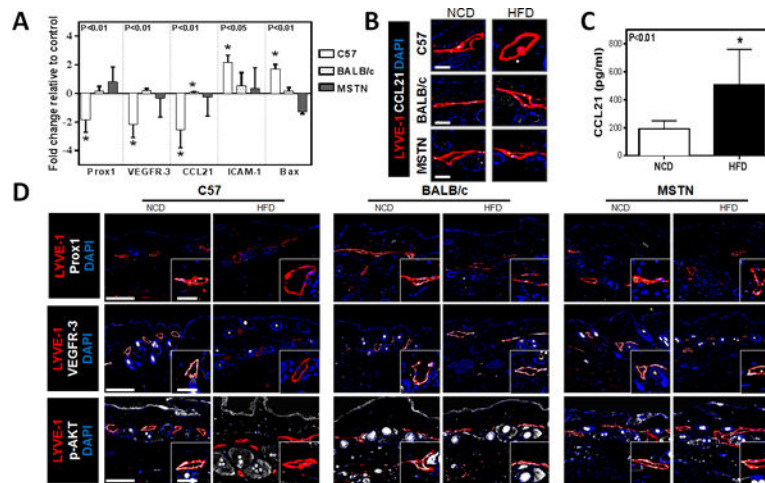
Fig. 4. Obesity downregulates expression of lymphatic markers in LECs

A. Quantification of mRNA expression in LECs sorted from NCD- and HFD-fed mice skin tissues for all groups, demonstrating relative expression of *Prox1, VEGFR-3, CCL21, ICAM-1* and *Bax* (n=4–6/group). Data are presented as fold change relative to the NCD-fed controls in all groups. Note the downregulation of *Prox1, VEGFR-3*, and *CCL21* (*p<0.01 for all) and the upregulation of *ICAM-1* (*p<0.05) and *Bax* (*p<0.01) in obese C57BL/6J when compared to their lean controls.

B. Representative immunofluorescent localization of LYVE-1[+](red) and CCL21[+](white) in NCD- and HFD-fed mice in all groups (DAPI is blue; Scale bar=25μm).

C. Quantification of CCL21 protein levels in hindlimb tissues harvested from lean and obese C57BL/6J mice (n=6–8/group, p<0.01).

D. Representative low and high power (dashed box shown in lower right inset) immunofluorescent localization of LYVE-1^{+} cells (red) and Prox1 (*top*, white), VEGFR-3 (*middle*, white) and p–AKT (*bottom*, white). (DAPI nuclear stain is blue; Scale bars=50μm and 25μm.)
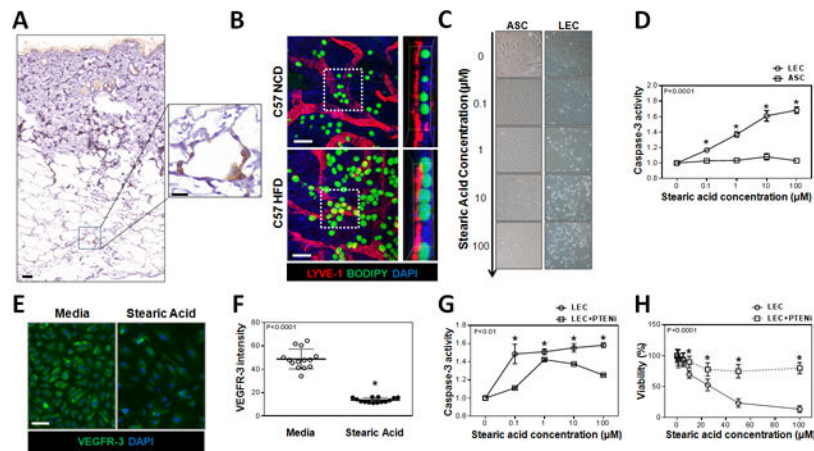
Fig. 5. Obesity results in accumulation of large lipid droplets around lymphatic vessels

A. Representative low (left) and high power (right) immunohistological localization of CD45^{+} cells (brown) surrounding adipocytes (white) in back skin of obese C57BL/6J mice (Scale bars=100μm and 25μm, respectively).

B. Representative immunofluorescent localization (left) and orthoslice (right) from daughter region (white dotted square) of LYVE^{+} cells (red), BODIPY[+] lipids (green) and nuclear DAPI stain (blue), in whole mount ear tissues of NCD- or HFD-fed C57BL/6J mice (Scale bar=20μm).

C. Representative bright field images showing LECs (*top*) and adipose derived mesenchymal stem cells (ASCs; *bottom*) cultured in media containing increasing concentrations of SA for 12hrs.

D. Quantification of caspase-3 activity in LECs (circles) and ASCs (squares) after treatment with SA for 12hrs (n=3–5/group, p<0.0001).

E. Representative immunofluorescent localization of VEGFR-3 expression (green) in LECs cultured with 10μM of SA for 12hrs (DAPI nuclear stain is shown in blue, Scale bar=25μm).

F. Quantification of VEGFR-3 expression intensity in LECs cultured in media (open circle) or media with 10μM SA (closed circle) for 12hrs (n=14/group, p<0.0001).

G. Quantification of caspase-3 activity in LECs cultured with media containing SA (circle) or SA with 0.3nM PTENi (square) for 12hrs (n=3/group, p<0.01).

H. Quantification of LEC viability treated with media containing SA or SA with 0.3nM PTENi for 12hrs (n=12/group, p<0.0001).
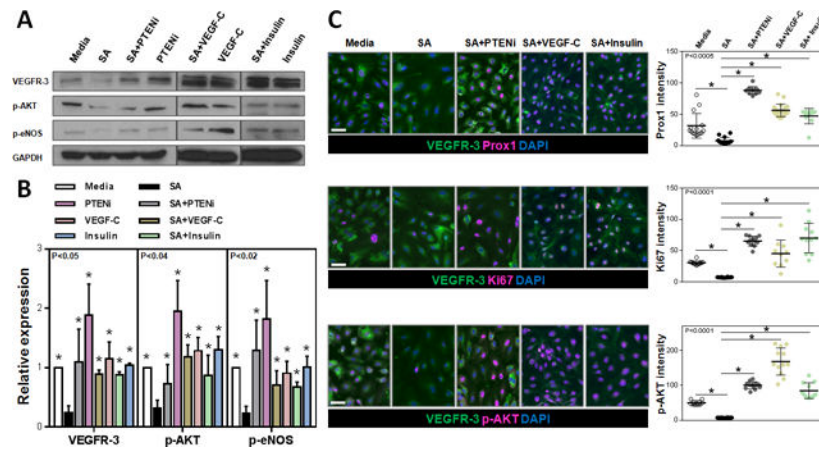
Fig. 6. Exposure of LECs to SA in vitro downregulates expression of VEGFR-3 and its downstream mediators

A, B. Representative Western blot images (**A**) for VEGFR-3, p-AKT, p-eNOS and quantification (**B**) normalized to GAPDH expression of total cellular protein harvested from LECs treated with 1μM SA, 10μM SA+ 0.3nM PTENi, PTENi (0.3nM), SA+ 100ng/mL VEGF-C, VEGF-C (100ng/mL), SA+ 100nM insulin or insulin (100nM) (n=4–5/group, p<0.05 for all antibodies).

C. *Left panels:* Representative immunofluorescent co-localization of VEGFR-3 (green) and Prox1, Ki67, and p-AKT (all shown in pink) in LECs cultured in control media, media containing 10μM SA, media containing 10μM SA with PTENi (0.3nM), 10μM SA with VEGF-C (100ng/mL) or 10μM SA with insulin (100nM) for 12hrs. (Scale bar=25μm). *Right panels:* Quantification of expression of Prox1, Ki67, and p-AKT in groups shown in the left panels (n=10–15/group, p<0.0005).