Supplementary Information:

Extended Materials and Methods.

Summary of contents: This file has the extended Materials and Methods as well as their references for the manuscript entitled: "Obesity but not High-Fat Diet impairs lymphatic function."

Extended Materials and Methods

Animals and Diets

All experimental protocols were reviewed and approved by the IACUC committee at Memorial Sloan Kettering Cancer Center. The institution operates under the Animal Welfare Act (AWA) and Health Research Extension Act of 1985. Male C57BL/6J, BALB/cJ, and lean (Ln) allele of myostatin ($MSTN^{ln}$) mice based on the same genetic background (C57BL/6J-$MSTN^{ln}$) were purchased from Jackson Laboratories (Bar Harbor, Maine) and maintained in a pathogen-free, temperature- and light-controlled environment. Previous studies have demonstrated that C57B6/6J mice fed a high-fat diet (HFD; 60% kcal from fat) develop moderate obesity as compared to animals fed a normal chow or low-fat diet after a period of 10-17 weeks.(1) In contrast, BALB/cJ and MSTN null mice are resistant to HFD-induced obesity even after prolonged exposure to this diet.(2, 3) As a result, the use of these mouse strains enabled us to analyze the effect of HFD with or without concomitant obesity on lymphatic function.

Male C57BL/6J, BALB/cJ, and $MSTN^{ln}$ mice were randomized to a HFD (Purina Test Diet 60% kcal from fat; W.F Fisher & Son, Inc., NJ) or NCD at 6 weeks of age, and maintained on those diets *ad libitum* for 10–12 weeks. Age-matched control male animals on the same genetic backgrounds were maintained on a normal chow diet (13% kcal from fat; Purina PicoLab Rodent Diet 20) for the same period of time. Animals were excluded from the study and sacrificed if wound infection or skin ulceration was observed. When performing experiments that required anesthesia, mice were anesthetized using isofluorane. Respiratory rate and tail pinching were used to monitor the depth of anesthesia. At the conclusion of the experiment, animals were weighed using a digital scale (Sartorius, Bradford, MA) and euthanized by carbon dioxide asphyxiation as recommended by the American Veterinary Medical Association, following the ethical principles under which this journal operates.

Metabolic Analysis

Metabolic analysis was performed following a 12-hour fasting blood draw from the retro-orbital sinus. Fasting serum glucose, cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) levels were analyzed using standard assays (ALX laboratories, New York, NY). For oral glucose tolerance testing, mice were fasted for 12 hours and baseline glucose measurements were obtained from the tail following distal amputation using a handheld glucometer (True2go glucometer; Nipro Diagnostics, Inc). Glucose (45% D-(+)-Glucose; Sigma Aldrich, St Louis, MO) was administered via oral gavage at a dose of 1.5 g/kg of body weight. Glucose measurements were taken at 30 min intervals for 2 hours after glucose administration.

Analysis of Lymphatic Function

Lymphoscintigraphy was used to analyze lymph node uptake following peripheral injection of a technetium labeled colloid as previously reported.(4) Briefly, 50µl of technetium-99m (^{99m}Tc) labeled sulfur colloid was injected in the distal hindlimb and subsequent inguinal lymph node uptake was assessed using an X-SPECT camera (Gamma Medica, Northridge, CA) for 90 minutes. Region-of-interest analysis was performed to derive decay-adjusted activity using ASIPro software (CTI Molecular Imaging, Knoxville, TN), enabling us to calculate rate and peak nodal uptake. All experiments were performed in a minimum of 5 animals per group.

We used a modification of the methods outlined by Lim *et al.* in order to analyze migration of DCs from the periphery to draining lymph node basins in experimental and control animals.(5) Briefly, the spleens of CD45.1 isotype C57B6/6J or BALB/cJ mice (Jackson Laboratories) were harvested and digested with collagenase D (Sigma-Aldrich, St Louis, MO) at 37°C for 15 minutes with gentle agitation. The DC population was enriched using a magnetic microbead-based positive selection kit for $CD11c^+$ cells (Miltenyi Biotech, Gladbach, Germany) according to the manufacturer's recommendations. The isolated DCs were resuspended in sterile PBS and injected into the right hindlimb (10^6 cells per injection) of experimental or control animals, and 24 hours later the right popliteal and inguinal lymph nodes were harvested and analyzed

to determine the number of migrating DCs ($CD45.1^+/CD11c^+/MHC\text{-}II^{high}$) using flow cytometry (Fortessa II; BD Biosciences, San Jose, CA) and Flowjo software (Tree Star, Ashland, OR). Each experiment was repeated in 5 animals per group.

We used a previously described method of ferritin injection to visualize functional lymphatic drainage.(6) Ferritin (Sigma-Aldrich) was injected in the distal hindlimb 60 minutes prior to sacrifice and draining lymph nodes were harvested and fixed. Because the iron III component of ferritin is transported selectively by lymphatics, staining tissues with 5% potassium ferrocyanide enables identification of functional lymphatic vessels in the lymph node.

Histology

Trachea and ears were harvested from all groups and fixed with 4% paraformaldehyde (PFA, Affymetrix, Cleveland, OH) then embedded in O.C.T. compound (Scigen Scientific, Gardena, CA), and sectioned at 10 microns. Whole ears were harvested, and cross sections cut longitudinally so as to analyze the sagittal plane cross sections of the entire ear. Back skin of all 6 groups was harvested utilizing 5mm punch biopsy kits (Fray Products Corp, Buffalo, NY). 5mm thick sections of front-limbs were harvested, fixed in 4% PFA, decalcified in Sodium EDTA 0.5M (Chem Cruz, Dallas, TX), and backskin and frontlimb specimens were then placed in 70% alcohol and subsequently paraffin-embedded; 5μm sections were cut for analysis. In another set of experiments, ears were harvested for whole mounts, depilated with Nair® (Church and Dwight Co., Princeton, NJ), washed and fixed in 4% PFA at 4°C. The cartilage was then excised and the ear was split to expose the subcutaneous tissues.

Immunohistochemical staining was performed to localize the expression of CD3 (Dako, Glostrup, Denmark), CD11b (BD Bioscience, San Jose, CA), LYVE-1, CCL21 (R&D Systems, Minneapolis, MN), p-AKT (Cell-signaling Techonology, Danvers, MA), VEGFR-3 (BioLegend, San Diego, CA), PROX1 (AngioBio, San Diego, CA), and DAPI (Sigma-Aldrich). All secondary antibodies were obtained from Vector Laboratories (Burlingame, CA) and eBioscience (San Diego, CA). Only $LYVE\text{-}1^+$ vessels (capillary lymphatics) close to the dermis and epidermis were analyzed for perilymphatic inflammation. Skin cross

sections were stained with hematoxillin (Dako) and eosin (Thermo Fisher Scientific, Waltham, MA), analyzed using brightfield microscopy, and scanned using Mirax slide scanner (Zeiss, Munich, Germany). Cell counts were performed on high-powered sections using Pannoramic Viewer (3D HISTECH, Budapest, Hungary) from a minimum of 4-6 animals per group and 4-5HPF/animal by two blinded reviewers. Ear whole mounts were imaged using Leica MZFLIII SP5 upright confocal microscope (Leica Biosystems, Buffalo Grove, IL) and images processed using Imaris software (version 7.2.3, Bitplane, Concord, MA).

BODIPY Lipid Stain

In order to identify lipid droplets within LECs a double stain using LYVE-1 and boron-dipyrromethene (BODIPY) was used in ear whole mounts. BODIPY is a nonpolar structure that can be utilized to stain for neutral lipids and nonpolar lipids. Sections were blocked in 20% donkey serum, and then incubated in LYVE-1 monoclonal antibodies, followed by visualization with secondary fluorophore antibodies (donkey anti-goat Alexa Fluor 594, Invitrogen). The tissues were then incubated with BODIPY (BODIPY 493/503, Life technologies, Grand Island, NY) diluted to 1ug/mL in PBS and DAPI. Imaging was performed using a Leica MZFLIII SP5 upright confocal microscope and Imaris software.

Lymphatic Endothelial Cell Isolation and PCR

Dermal LECs were isolated from the abdominal skin of C57BL/6J, BALB/cJ, and MSTN^{lu} mice using a modification of previously reported methods.(7) Briefly, a 2x2 cm piece of the abdominal skin was excised, diced into small pieces, and digested in a 0.4% Collagenase IV digestion buffer (Collagenase IV; MP Biomedicals, Solon, OH) for 45 minutes. The digested tissue was then filtered through a 100μm nylon cell strainer, resuspended, and filtered through a 70μm nylon cell strainer. From these single cell suspensions, flow cytometric cell sorting was then performed using fluorophore-conjugated flow cytometry optimized monoclonal antibodies (eBioscience) to identify LECs (CD45 negative, CD31 positive, podoplanin positive).

RNA extraction was performed on sorted LECs using TRIZOL (Invitrogen, Life Technologies, Carlsbad, CA) according the manufacturer's recommendations. RNA quality and quantity was assessed using

an Agilent bio analyzer (Agilent Technologies, Inc; Santa Clara, CA). The isolated RNA was converted to cDNA utilizing a TaqMan reverse transcriptase kit (Roche, Branchburg, NJ) and relative gene expression between groups was performed using delta-delta CT PCR analysis and normalizing gene expression using GAPDH RNA amplification as previously described.(8) Relative expression was calculated using the formula: 2[-(Ct gene of interest – Ct endogenous control) sample A – (Ct gene of interest – Ct endogenous control) sample B)]. All samples were performed in triplicate. The primers used for the PCR targets of interest were for Prospero homeobox protein 1 (*Prox1,*), vascular endothelial factor receptor 3 (*VEGFR-3*), chemokine ligand 21 (*CCL21*), endothelial intracellular adhesion molecule 1 (*ICAM-1*), and Bcl2 associated X protein (*Bax*, all from Applied Biosystems, Life Technologies, Carlsbad, CA).

Protein Quantification and ELISA

Skin and subcutaneous tissues from C57B/6J mice fed a NCD or HFD were harvested for protein analysis, flash frozen, crushed and extracted with tissue extraction protein reagent (ThermoFisher Scientific, Waltham, MA) mixed with phosphatase and protease inhibitor (Sigma-Aldrich, St. Louis, MO). 20–30 mg of protein from samples (n= 6-8 animals/group) was analyzed by ELISA to quantify CCL21, IL-1β, TNF-α (Sigma-Aldrich) or VEGF-C (United States Biological, Salem, MA) according to the manufacturer's protocol.

In order to quantify serum FFA levels, blood was collected via retro-orbital blood draw into serum separator tubes (BD Microtainer, Franklin Lakes, NJ) and spun at 13,000RPM for 10 minutes. The supernatant was collected for serum analysis. Protein concentrations were measured via Bradford protein quantification. Serum FFA analysis (n=6-8 animals per group) was performed by ELISA according to the manufacturer's protocol (Abcam, Cambridge, MA). All experiments were run in duplicate.

Cell Culture and Immunocytochemistry and Western blot Analysis

Human dermal LECs and adipose derived mesenchymal stem cells (ASCs) were obtained from PromoCell (Heidelberg, Germany). LECs are tested by the supplier for cell morphology and cell-type specific markers as well as growth performance and viral (HIV-1, HIV-2, HBV, HCV, HTLV-1, HTLV-2) or

microbial contamination (fungi, bacteria and mycoplasma). LECs were culture expanded *in vitro* using ECGM-MV2 media containing 5%FCS, epidermal growth factor (EGF) (5ng/mL), hydrocortisone (0.2 μg/ml), basic fibroblast growth factor (bFGF) (10 ng/ml), insulin-like growth factor (IGF) (20 ng/ml), VEGF 165 (0.5 ng/ml), ascorbic acid (1 μg/ml), and penicillin-streptomycin (50 U/ml) (Invitrogen). ASCs were culture expanded *in vitro* using MesenPRO reduced serum (2%) Basal Medium (Life Techonologies, Grand Island, NY) containing 2 mM L-glutamine and 5 μg/ml gentamicin and MesenPRO RS growth supplement. Cells were passaged every 48 hours and morphology confirmed with immunofluorescent staining for PROX1 and VEGFR-3. Experiments were performed using early-passage cells (<10). Plates were imaged using an inverted microscope (Carl Zeiss, Oberkochen, Germany).

In order to obtain a stearic acid stock solution for our experiments, stearic acid (Sigma Aldrich, St. Louis, MO) was conjugated with bovine serum albumin (Roche Diagnostics, Mannheim, Germany) at a 4:1 molar ratio before treatment. Stearic acid was then dissolved in 95% ethanol at 60°C and mixed with prewarmed BSA (10%) to yield a stock concentration of 5.4 mM. Primary antibodies used for chamber slide immunofluorescent staining included PROX1, Ki67 (both from Abcam), p-AKT (Cell Signaling Technology) and VEGFR-3 (EMD Millipore, Billerica, MA). All secondary antibodies were obtained from Vector Laboratories and eBioscience. Chamber slides were scanned using the Mirax slide scanner and analyzed using Pannoramic Viewer (3D HISTECH, Budapest, Hungary). PROX1, Ki67 and p-AKT signal intensity per cell was measured using MetaMorph analysis (Molecular Devices, Sunnyvale, CA).

Western blot analysis was performed with total cellular protein harvested from LECs using our previously published methods.(9) Briefly, LECs were treated with 10μM stearic acid and/or 3nM PTENi, (SF1670, Sigma Aldrich)(10) 100nM insulin (Human recombinant insulin, Sigma-Aldrich)(11-13) or 100ng/mL VEGF-C (Human recombinant VEGF-C, Sigma-Aldrich)(14, 15) for 12hrs and protein was isolated from cell lysate using the NP40 lysis buffer (Invitrogen, Camarillo, CA) in addition to protease and phosphatase inhibitors (Roche Diagnostics) and quantified by using the Bradford method. Western blotting was performed as previously described for VEGFR-3 (ABCAM, Cambridge, MA), p-AKT and p-eNOS (all

from Cell-signaling Techonology, Danvers, MA). Equal loading was confirmed by using GAPDH (EMD Millipore). For relative expression analysis, NIH Image J software was used to determine band intensity. Immunoreactivity was determined by using the Enhanced Chemiluminescence (ECL) chemiluminescence detection system (Amersham, Arlington Heights, IL). All experiments were performed in triplicate.

Cellular Apoptosis Assays

LEC apoptosis in response to increasing levels of stearic acid and/or 0.3nM PTENi were quantified using caspase-3 assay (R and D systems, Minneapolis, MN) and Annexin V-FITC Apoptosis Detection Kit (eBioscience). Cells were first lysed to collect their intracellular contents.

Caspase-3 is an intracellular cysteine protease that exists as a proenzyme, which becomes activated during the cascade of events associated with apoptosis.(16) The presence of caspase-3 in cells of different lineages suggests that caspase-3 is a key enzyme required for the execution of apoptosis. Caspase-3 Fluorometric Assay was carried as per the manufacturer's recommendations. Briefly, LECs and ASCs were plated at a seeding density of $9x10^6$ cells/ml and treated with growth media containing 0.1,1,10,100μM stearic acid (SA) in media, SA and 0.3nM PTENi or media alone (control). After 12hrs of treatment, cells were harvested, washed and lysed. The cell lysate was incubated on ice for 10min and then centrifuged at 10,000 x g for 1min. 50μL of reaction buffer and DTT, as well as 5μL of caspase-3 fluorogenic substrate, were added per 10^6 cells, and each reaction was incubated at 37°C 1hr, and then read on a Spectra Max M4 Microplate reader (Molecular devices, Sunnyvale, CA) using filters that allow light excitation at a wavelength of 400nm and can collect emitted light at a wavelength of 505nm.

Annexin V assay was undertaken according to the manufacturer's recommendations. Briefly, LECs were plated, exposed to SA and PTENi, harvested, lysed as previously described, and then stained with Annexin V-FITC/propidium iodide (PI). Data acquisition was performed using flow cytometry (LSRII; BD Biosciences, San Jose, CA) and analysis was performed using FlowJo software (Tree Star, Ashland, OR). Viable cells were negative for both Annexin V and PI while cells in late stage apoptosis were positive for both Annexin V and PI.

Cellular Viability Assay

A WST-8 cytotoxicity cell assay kit (Sigma Aldrich, St Louis, MO) was used according to the manufacturer's instructions as a method to measure LEC viability. Briefly, cells were seeded in a 96-well plate at a density of 10^4–10^5 cells/well and incubated 24hrs at 37 °C, 5% CO2. Wells were then treated with 0.1, 1, 10, 25, 50, 100μM of stearic acid and equal concentrations with 0.3 nM PTENi. In other experiments with media, media plus 25μM of stearic acid and media plus 25μM of stearic acid with 100nM insulin, 100ng/mL VEGF-C or both. The plate was incubated 12hrs after which 10μL CCK-8 solution was added to each well. Plate was incubated for 2hrs and absorbance was measured at 450nm using the microplate reader.

Statistical Analysis and Justification of Mouse Numbers

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. For multiple groups, an analysis of variance with post-hoc tests (Tukey Kramer) was used to compare differences overall and between individual groups. Data are expressed as mean ± standard deviation unless otherwise noted, with $p<0.05$ considered significant. Data points greater or smaller than two standard deviations from the mean were considered statistical outliers and excluded from the analysis. With the aid of our statistician we performed a detailed statistical analysis to determine the number of animals that will be required for our experiments based on our preliminary and previous studies. We have previously found that our in vivo analyses have a standard deviation of 20-30% depending on the experiment that is performed and our analysis. All experiments were performed using a minimum of 5-8 animals per group. All histological quantifications were performed by two reviewers blinded to the individual treatment group.

Materials and Methods References:

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