Lymphatic-specific intracellular modulation of receptor tyrosine kinase signaling improves lymphatic growth and function

Raghu P. Kataru1*, Jung Eun Baik1, Hyeung Ju Park1, Catherine L. Ly1, Jinyeon Shin1, Noa Schwartz2, Theresa T. Lu2,3, Sagario Ortega4, Babak J. Mehrara1

Exogenous administration of lymphangiogenic growth factors is widely used to study changes in lymphatic function in pathophysiology. However, this approach can result in off-target effects, thereby generating conflicting data. To circumvent this issue, we modulated intracellular VEGF-C signaling by conditionally knocking out the lipid phosphatase PTEN using the Vegfr3 promoter to drive the expression of Cre-lox in lymphatic endothelial cells (LECs). PTEN is an intracellular brake that inhibits the downstream effects of the activation of VEGF3 by VEGF-C. Activation of Cre-lox recombination in adult mice resulted in an expanded functional lymphatic network due to LEC proliferation that was independent of lymphangiogenic growth factor production. Furthermore, compared with lymphangiogenesis induced by VEGF-C injection, LECPTEN animals had mature, nonleaky lymphatics with intact cell-cell junctions and reduced local tissue inflammation. Last, compared with wild-type or VEGF-C-injected mice, LECPTEN animals had an improved capacity to resolve inflammatory responses. Our findings indicate that intracellular modulation of lymphangiogenesis is effective in inducing functional lymphatic networks and has no off-target inflammatory effects.

INTRODUCTION

The lymphatic vasculature plays a critical role in numerous physiologic functions, including regulation of immune responses and absorption of interstitial fluid, fats, and cholesterol. As such, abnormal lymphatic function is implicated in various pathological states, including lymphedema, metabolic syndromes, cardiovascular disease, autoimmune responses, impaired tumor surveillance, and chronic inflammatory disorders (1, 2). Given that lymphatic function is highly variable among individuals (3), strategies that improve lymphatic function are clinically important.

Historically, studies seeking to understand the role of the lymphatic system in the regulation of physiological and disease states have relied on loss-of-function strategies using transgenic animals that have impaired lymphatic function. These include Prox1–haplodeficient, vascular endothelial cell receptor 3 (VEGFR3) mutant, K14-VEGFR3–immunoglobulin (IgG), and diphtheria toxin–inducible lymphatic ablative mice (4–8). Other investigations have used a gain-of-function approach by injecting recombinant lymphangiogenic growth factors or using viral expression vectors to promote lymphangiogenesis (9–12). Although these studies are important, the findings can be difficult to interpret because of off-target effects on other cell types. For example, the use of exogenous supraphysiologic doses of vascular endothelial growth factor C (VEGF-C) not only results in lymphangiogenesis but also induces a potent local inflammatory response and vascular leakage (13, 14). This effect is at least partially due to the potent mitogen and chemotactic effects of VEGF-C on macrophages and other inflammatory cells (15, 16). Furthermore, although delivery of VEGF-C (and other lymphangiogenic growth factors) results in robust lymphangiogenesis, these newly formed vessels may be immature, leaky, and nonfunctional (17, 18). Similarly, in K14-VEGFR3-IgG mice, the local levels of VEGF-C are dampened, which potentially affects other cell types that express high-affinity receptors for this growth factor (such as blood, endothelial cells, and macrophages). Given that gain-of-function and loss-of-function strategies can result in conflicting findings that are difficult to interpret (19, 20), developing new methods to induce lymphangiogenesis and improve lymphatic function without off-target effects is important. Such models would have widespread application in multiple pathologic conditions and provide clinically relevant insights into the role of the lymphatics in these settings.

In our current study, we targeted intracellular signaling pathways that regulate lymphatic endothelial cell (LEC) proliferation, differentiation, and migration to improve lymphatic function while avoiding production of growth factors or other changes that may have off-target effects. Our approach was based on VEGFR3 dimerization and phosphorylation induced by the binding of VEGF-C. This phosphorylated receptor dimer is internalized and activates intracellular signaling pathways, including mitogen-activated protein kinase (MAPK) and AKT (also known as protein kinase B; PKB). Phosphorylated and activated AKT is the predominant protein kinase that regulates LEC proliferation, differentiation, and migration. Phosphorylation of AKT depends on the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) by phosphoinositide 3-kinase (PI3K) (21–23). This process is inhibited by phosphatase and tensin homolog (PTEN), which catalyzes the conversion of PIP3 back to PIP2, the inactive form. Thus, PTEN is an intracellular brake that inhibits the downstream effects of VEGF-C/VEGFR3 activation. We hypothesized that deletion of PTEN in LECs would lead to increased phosphorylated AKT, thereby increasing LEC proliferation and differentiation.
Using inducible Cre-lox mice to delete PTEN specifically in LECs, we showed that manipulation of intracellular pathways in LECs was a viable method to regulate lymphangiogenesis and lymphatic function. Our model can be used to study the effects of isolated gain-of-function changes in the lymphatic system without off-target effects of exogenous growth factors in different pathological and physiological settings.

RESULTS
Lymphatic-specific ablation of PTEN induces lymphangiogenesis
Phosphorylation of AKT in the cytoplasm of LECs in capillary lymphatics of the skin was strong under baseline functional conditions (Fig. 1A). Chronically obese mice have dysfunctional lymphatic vessels that are reported to exhibit decreased AKT phosphorylation, indicating the relevance of AKT phosphorylation in lymphatic function (24). Using Cre-lox technology, we generated LEC-specific PTEN knockout mice to regulate AKT phosphorylation in LECs independently of external growth factor stimulation (Fig. 1B). Activation of Cre recombination by tamoxifen injection resulted in downregulation of PTEN mRNA expression and PTEN protein abundance in LECs isolated from ear skin as assessed by flow cytometry (Fig. 1, C and D). PTEN deletion in LECs resulted in significantly increased lymphatic endothelial number, vessel density, diameter, and branching as compared with wild-type (WT) control mice 4 weeks after transgene activation in both peripheral (skin) and LEC number in internal (intestinal wall) tissues (Fig. 1E and fig. S1, A to D). These findings were confirmed by quantification of lymphatic vessel density in the skin and of the percentage of LECs (PDPN+, CD31+, and CD45+) and blood endothelial cells (BECs; PDPN+, CD31+, and CD45+) by flow cytometry in skin and intestine wall (Fig. 1, F and G, and fig. S1, E to H). In both skin and intestinal tissues, we found that deletion of PTEN in LECs resulted in a nearly 1.5-fold increase in the percentage of LECs without significantly changing the number of BECs in these tissues. These findings, together with immunofluorescent analysis of lymphatic vessels in the lymph nodes, diaphragm, pancreas, heart, kidney, and lungs of LECPTEN mice 1 month after Cre activation (figs. S2, A to F, and S3, A to I), suggest that deletion of PTEN in LECs results in expansion of the lymphatic network of these tissues without affecting the blood vasculature.

To determine how PTEN deletion expanded the lymphatic network in the skin, we analyzed lymphatic vessel number using immunofluorescent staining (Fig. 1H) and identified proliferating LECs with flow cytometric analysis using proliferation markers (Fig. 1I) at 1 week (Fig. S3I), 3 weeks, and 5 weeks (fig. S3K) after Cre activation. Histological analysis of skin samples demonstrated that capillary lymphatic vessels in LECPTEN mice began to increase in number by 1 week after Cre activation. This expansion increased by 3 weeks and reached a plateau 4 to 5 weeks after Cre activation. These histological findings correlated with Ki67 expression in LECs in LECPTEN mice 1 week after tamoxifen injection. We noted a nearly threefold increase in the percentage of Ki67+ LECs isolated from LECPTEN mice as compared with controls (Fig. 1I). This difference was more marked at 3 and 4 weeks after Cre activation, resulting in a seven- to ninefold increase in the percentage of proliferating Ki67+ LECs. The proliferation of LECs in LECPTEN mice was also visualized by LYVE-1+ (lymphatic vessel endothelial hyaluronan receptor 1) Ki67+ cells in ear skin tissue 3 weeks after Cre activation (Fig. 1J). In addition, LECPTEN lymph node LECs showed significant proliferation compared to LECs from control mice (Fig. 1K). The increased lymphatic density in LECPTEN mice persisted in skin and intestinal wall as long as 1 year after Cre activation (fig. S4, A to D). We did not observe histologically visible lymphatic tumors or lymphangiosarcomas in major organs (brain, lungs, heart, liver, or kidney) in transgenic mice as late as up to 18 months after tamoxifen injection (fig. S5 A to E).

Lymphangiogenesis in LECPTEN mice is independent of VEGF-C
To investigate the gene expression changes that accompanied PTEN deletion in LECs, we analyzed mRNA expression using reverse transcription polymerase chain reaction (RT-PCR) in LECs sorted from the skin of control and LECPTEN mice 1 month after transgene activation (Fig. 2A). The expression of Prox-1, Vegfr3, Vegfr2, Pdpn (which encodes podoplanin), or Bax was similar in LECPTEN and control mice. We did, however, note a modest though significant increase in Ccl21 expression and a more marked increase in Kit67 expression in LECs isolated from LECPTEN mice. We next analyzed protein abundance by Western blotting sorted LECs to determine how PTEN deletion altered intracellular signaling pathways (Fig. 2B). The phosphorylation but not total abundance of AKT was increased in LECPTEN mice as compared with controls. In contrast, we found no differences in the phosphorylation of extracellular signal–regulated kinase (ERK)1/2, suggesting that the MAPK pathway was not altered in transgenic mice.

To determine whether increased lymphangiogenesis in LECPTEN mice tissues involved increased VEGF-A or VEGF-C production, we analyzed the abundance of these proteins in various tissues using enzyme-linked immunosorbent assay (ELISA; Fig. 2, C and D). Consistent with the hypothesis that lymphangiogenesis was induced without the confounding effects of excess lymphangiogenic growth factors, we found similar levels of both VEGF-A and VEGF-C in transgenic and control mice in all tissues tested. Next, we sought to determine whether lymphatic expansion in LECPTEN mice required VEGFR3 activation by analyzing lymphatic sprouting in thoracic duct explants cultured ex vivo in growth factor–reduced Matrigel (Fig. 2, E and F). We used antibodies that block VEGFR3 ligand binding (ALF4) or receptor dimerization (2E11) or both to understand whether lymphatic growth in LECPTEN mice was independent of VEGFR3 ligand binding and receptor dimerization (25). VEGF-A blocking antibody was used to nullify the effect of VEGF-A on lymphangiogenesis. As expected, lymphatic growth in thoracic ducts from control animals was significantly inhibited upon treatment with ALF4 or a combination of ALF4 and 2E11 antibodies compared to isotype controls. On the contrary, thoracic ducts from LECPTEN animals showed no growth inhibition when treated with either ALF4, 2E11, or both antibodies compared to isotype controls. Thoracic ducts from LECPTEN animals showed significantly higher lymphatic growth in all groups compared to control thoracic ducts as determined by average vessel length. Together, these findings suggest that deletion of PTEN in LECs does not alter expression of lymphangiogenic genes or their receptors in LECs, selectively activated the phosphorylation of AKT, does not alter tissue expression of VEGF-C or VEGF-A, and results in lymphangiogenesis independently of the VEGF-C/VEGFR3 axis.

LECPTEN mice have increased lymphatic function and nonleaky lymphatics
To determine whether the expanded lymphatic network in LECPTEN mice also resulted in increased lymphatic function, we analyzed
lymphatic transport of antigen-presenting cells and cholesterol in control and transgenic mice. Activated dendritic cells (DCs) travel from the skin to regional lymph nodes through afferent lymphatics, and the rate of lymphatic transport of these cells is thought to correlate with lymphatic functional capacity. To analyze lymphatic trafficking of DCs to regional lymph nodes, we painted the ear skin and distal hindlimb of separate sets of control and LECPTEN mice 1 month after transgene activation with fluorescein isothiocyanate (FITC) mixed with a skin-irritating solution of acetone and dibutyl phthalate. Analysis of the percentage of FITC+ DCs (CD11c high) in the cervical (skin draining) and popliteal (hindlimb draining) lymph nodes 10 hours later demonstrated that LECPTEN mice had a nearly threefold increase in DC trafficking as compared with WT mice (Fig. 3, A to C).

Next, we analyzed lymphatic reverse cholesterol transport, an important physiologic function of the lymphatic system (26). Fluorescently labeled cholesterol 2–loaded macrophages were injected in the subcutaneous tissues of the footpad of control and LECPTEN mice 1 month after tamoxifen injection, and the amount of fluorescent cholesterol (expressed as a percentage of the amount injected) was quantified in the footpad, plasma, lymph node, liver, and feces. LECPTEN mice had decreased concentration of labeled cholesterol in the subcutaneous tissues of the footpad 6, 12, 24, 48, and 72 hours after injection (Fig. 3D). This decrease corresponded to increased uptake of fluorescent cholesterol in the plasma (Fig. 3E), lymph node (Fig. 3F), liver (Fig. 3G), and feces (Fig. 3H) of LECPTEN mice, consistent with increased reverse cholesterol transport. Together, these findings suggest that deletion of PTEN in LECs results in significantly increased lymphatic drainage.

To compare lymphangiogenesis in response to recombinant VEGF-C protein injection with that in LECPTEN mice, we analyzed ear skin capillary and collecting lymphatic vessels in control mice injected with phosphate-buffered saline (PBS) or recombinant human VEGF-C (rhVEGF-C) to LECPTEN mice injected with PBS only. Confocal imaging of ear skin revealed strong sprouting lymphangiogenesis and filopodial projections in the skin of mice injected with rhVEGF-C, whereas control mice showed minimal change. These findings suggest that deletion of PTEN in LECs results in significantly increased lymphatic drainage.

Fig. 1. Lymphatic-specific ablation of PTEN induces lymphangiogenesis. (A) Representative confocal images of murine skin lymphatic vessels showing colocalization of LYVE-1 (green) and phosphorylated Akt (p-Akt; red). Scale bar, 20 μm. N = 5 mice. (B) Schematic of how PTEN modulates intracellular receptor tyrosine kinase signaling in LECs. Quantification of PTEN mRNA (C) (N = 5 mice per group from three independent experiments) and PTEN protein (D) (sorted LECs from N = 3 mice per group from three independent experiments). (E) Representative whole-mount microscopic images of ear skin (top) stained for lymphatic (LYVE-1) and blood vessels (CD31) and intestinal wall (bottom) stained for lymphatic vessels (LYVE-1). Scale bars, 500 μm. N = 5 mice per group. (F and G) Flow cytometry quantification of CD45−CD31+PDPN+ LECs from ear skin (F) and intestinal wall (G). N = 5 mice per group from two independent experiments. (H) Representative confocal images of ear skin sections of control mice or LECPTEN mice stained for lymphatic (LYVE-1) and blood capillaries (CD31) at 3 and 5 weeks after tamoxifen injection. N = 5 mice per group. Scale bar, 200 μm. (I) Flow cytometry quantification of proliferating (Ki67+) CD45−CD31+PDPN+ LECs in ear skin at the indicated time points. N = 3 mice per group from two independent experiments. NS, not significant. (J) Representative microscopic images of ear skin lymphatic vessels showing colocalization of LYVE-1 (green) and Ki67 (red). Scale bar, 10 μm. N = 5 mice per group. (K) Quantification of proliferation of sorted mouse primary LECs by MTT assay. N = 4 mice per group from two independent experiments. All quantifications are means ± SD, unpaired Student’s t test or two-way ANOVA with Tukey’s multiple comparison test. C: control; **P < 0.01, ***P < 0.001, and ****P < 0.0001. OD, optical density.
with recombinant VEGF-C and in LECPTEN as compared with WT mice injected with PBS alone (Fig. 4, A and B). Both VEGF-C–injected and LECPTEN mice had expanded lymphatic networks as visualized by Evans blue lymphangiography (Fig. 4C). However, in contrast to mice injected with recombinant VEGF-C, LECPTEN mice had significantly decreased lymphatic vessel leakiness and increased tissue clearance as evidenced by decreased residual Evans blue dye retention in the ear skin 24 hours after dye injection (Fig. 4, D and E). LECPTEN ear skin also had significantly decreased residual dye concentrations as compared with PBS-injected control mice, consistent with improved lymphatic function (Fig. 4, D and E). Lymphatic vessel leakiness was confirmed through confocal imaging of ears injected with FITC lectin, a dye that is taken up and transported exclusively by the lymphatic system. Newly formed capillary and collecting lymphatics in animals injected with recombinant VEGF-C had areas of dye extravasation surrounding the lymphatic vasculature, whereas the dye was maintained in the lumen of the expanded lymphatic networks in LECPTEN mice (Fig. 4, F and G).

Intracellular modulation of lymphangiogenesis does not cause tissue inflammation

VEGF-C is a mitogen and a chemoattractant for macrophages and other inflammatory cells (13, 27, 28). To directly compare local tissue inflammation in mice injected with recombinant VEGF-C with
LECPTEN animals, we analyzed footpad tissue sections from control mice injected with PBS or VEGF-C and LECPTEN mice injected with PBS for the same time period. As expected, control mice injected with PBS had very few CD11b+ inflammatory cells in the skin and subcutaneous tissues of the footpad (Fig. 5A). In contrast, tissues harvested from animals injected with VEGF-C demonstrated a marked increase in the number of CD11b+ cells, consistent with an inflammatory response. In contrast, LECPTEN mice showed attenuated CD11b+ cell infiltration as compared with VEGF-C–injected mice. These findings were corroborated and quantified by flow cytometry analysis of the footpad tissues, which demonstrated a significant increase in the percentage of both CD45+CD11b+ inflammatory cells and CD45+Ly6C+6G8 neutrophils in tissues harvested from mice after VEGF-C injection as compared with control or LECPTEN mice injected with PBS alone (Fig. 5, B and C, and fig. S6A). There were no significant differences between control and LECPTEN mice.

To determine how inflammatory cell infiltration modulated the tissue microenvironment, we harvested footpad tissue and used RT-PCR to analyze the expression of mRNAs encoding inflammatory cytokines (IL12a, IL1β, CCL2, IL10, IL27, and TNFα) and P-selectin, a leukocyte adhesion molecule (fig. S6B). VEGF-C injection significantly increased the expression of mRNAs encoding inflammatory cytokines, monocyte chemoattractant protein (CCL2), and P-selectin as compared with control mice injected with PBS. The expression of IL1β was 5.5-fold higher, whereas IL12a was 2.5-fold higher in VEGF-C–injected compared to PBS-injected control mice (Fig. 5, D and E). In addition, CCL2 was threefold higher in the VEGF-C–injected group compared to PBS-injected control mice (Fig. 5F). P-Sel was significantly elevated in the VEGF-C–injected group compared to the PBS-injected LECPTEN group (Fig. 5G). The mRNAs encoding TNFα, IL27, and IL10 showed a trend toward increased expression in the VEGF-C–injected group, but the difference was not statistically significant compared to the PBS-injected groups (fig. S6B). In contrast, the gene expression profiles of LECPTEN mice injected with PBS were similar to those of control mice injected with PBS. To eliminate any inflammatory response to human VEGC, we compared immune cell and inflammatory responses to human and mouse VEGF-C in separate sets of animals. Compared to PBS-injected controls, footpads injected with either human and mouse VEGF-C showed significantly elevated inflammatory cell infiltration and cytokine gene expression up-regulation that were increased to a similar extent (fig. S6, C to I). Together, these findings show that injection of VEGF-C results in a tissue inflammatory response associated with marked changes in the microenvironment. In contrast, intracellular modulation of the VEGF-C signaling pathway in LECs does not affect tissue inflammation in the microenvironment.

**VEGF-C and IL-1β destabilize VE-cadherin junctions by promoting the phosphorylation of VE-cadherin**

To identify the mechanisms regulating lymphatic vessel leakiness and integrity in VEGF–C–injected mice and LECPTEN mice, we analyzed LEC vascular endothelial cadherin (VE-cadherin) junctional patterns using whole-mount staining of ear skin. Lymphatic vessels have functionally specialized button-like discontinuous VE-cadherin junctions at initial lymphatics and zipper-like continuous junctions on collecting vessels (29). As expected, control mice injected with PBS had a well-organized discontinuous button pattern in capillary lymphatic vessels. LECPTEN mice demonstrated a similar pattern, with well-organized VE-cadherin junctions. In contrast, mice injected with VEGF-C had a disarranged VE-cadherin pattern, with irregular buttons, some transforming and some zippers, at initial lymphatics (Fig. 5H). Similarly, at collecting lymphatics, control and LECPTEN mice injected with PBS showed a well-arranged, continuous zipper pattern of VE-cadherin junctions compared to VEGF–C–injected mice, which displayed aberrant zippers mostly transformed into discontinuous buttons (Fig. 5I). Quantification of confocal image data of the buttons, zippers, and the transforming junctions in VEGF-C–injected mice compared to PBS-injected control and LECPTEN mice also reflected these differences (fig. S7, A and B).
Endothelial cell junctional stability is regulated by the phosphorylation status of VE-cadherin, with the most stable junctions formed by the nonphosphorylated form (30). Therefore, to determine whether VEGF-C injection altered this response directly or by modulating the expression of inflammatory cytokines, we analyzed VE-cadherin phosphorylation by Western blotting in cultured human LECs exposed to supraphysiologic doses of VEGF-C. Considering the mRNAs encoding the inflammatory cytokines interleukin-1β (IL-1β) and IL-12α were significantly increased in vivo after VEGF-C injection, we also tested the effect of IL-1β and IL-12α on VE-cadherin phosphorylation. VEGF-C or IL-1β alone, but not IL-12α, significantly induced VE-cadherin phosphorylation as early as 30 min after treatment compared to untreated LECs (fig. S7, C and D).

LECPTEN mice resolve skin inflammation more quickly than VEGF-C–injected animals

We next sought to understand whether physiologic changes resulting from VEGF-C injection (leaky lymphatics and tissue inflammation) confounded responses to pathologic states. Ultraviolet radiation (UVR) causes skin damage leading to erythema, epidermal thickening, and chronic inflammation. Skin VEGF-C injections mitigate this response by increasing inflammatory cell efflux from the skin and aiding in the resolution of tissue inflammation (31). We compared pathologic changes resulting from UVR exposure in control mice treated with PBS or VEGF-C to LECPTEN mice injected with PBS only (Fig. 6A). After a single dose of UVR light, control animals treated with PBS demonstrated marked tissue inflammation, erythema, skin scaling, and increased epidermal thickness compared to WT ears (Fig. 6, B to F). Pathological changes in these mice peaked on day 7 and slowly improved, although they did not return to baseline values by 14 days after UVR exposure. Mice that were injected with VEGF-C also had skin erythema and increased epidermal thickness. These changes were not statistically different from control mice, although the ear skin of VEGF-C–injected animals subjectively had less erythema. In contrast to control mice injected with PBS or VEGF-C, LECPTEN mice had virtually no skin erythema by day 7, and their ears looked normal, with significantly decreased ear thickness and epidermal thickness compared to those of the other groups (Fig. 6, E and F). These changes correlated with a significant (>2-fold) increase in lymphatic vessel

Fig. 5. Lymphangiogenesis induced by intracellular modulation in LECPTEN mice does not cause tissue inflammation. (A) Representative confocal images of footpad skin sections stained for lymphatic vessels (LYVE-1) and inflammatory cells (CD11b) in WT mice injected with PBS (control), WT mice injected with rhVEGF-C, and LECPTEN mice injected with PBS. Scale bar, 500 μm. (Dotted lines indicate epidermis.) (B and C) Flow cytometry–based quantification of inflammatory cells (B) and neutrophils (C). C: control, VC: VEGF-C injected. N = 6 mice per group from two independent experiments. (D to G) qPCR-based quantification of the expression of genes encoding the cytokines IL-1β (D) and IL-12α (E), the chemokine CCL2 (F), and the adhesion molecule P-selectin (P-Sel) (G). N = 6 mice per group from two independent experiments. (H and I) Representative high-magnification confocal images of lymphatic capillaries stained for VE-cadherin (H) and lymphatic collecting vessels (collectors) stained for VE-cadherin and Prox-1 (I) showing VE-cadherin button, zipper, and transforming junctions in WT mice injected with PBS (control), WT mice injected with rhVEGF-C, and LECPTEN mice injected with PBS. Scale bars, 20 μm. N = 5 mice per group. See fig. S7 (A and B) for quantification of junction types. All quantifications are two-way ANOVA with Tukey’s multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

DISCUSSION

Lymphangiogenesis is directly linked to binding and activation of the ligand VEGF-C to its receptor VEGFR3, leading to LEC proliferation and lymphangiogenesis (21). Because exogenous administration of VEGF-C can have off-target effects in addition to lymphangiogenesis and alter the microenvironment, we sought to induce lymphangiogenesis in vivo while uncoupling VEGFR3 from VEGF-C–mediated activation. We targeted the phosphatase PTEN because it is critical to activating the protein kinase that contributes to proliferation, migration, and cell survival during lymphangiogenesis. Our data showed that inducible, LEC-specific knockout of PTEN in LEC<sup>PTEN</sup> mice resulted in increased lymphatic density in all major organs independently of VEGF-A and VEGF-C. Furthermore, VEGF-C ligand binding and receptor dimerization blockade did not affect the proliferation and growth of new lymphatic vessels in LEC<sup>PTEN</sup> mice, whereas these antibody treatments in control mice resulted in substantially reduced proliferation and growth. These data indicate that we were successful in uncoupling VEGFR3 from its cognate ligands and inducing lymphangiogenesis independently of excessive exogenous growth factors.

Conventional growth factor–induced lymphangiogenesis gain-of-function models have largely local rather than global consequences, depending on the site of administration of exogenous growth factor, or are organ-specific, as in transgenic skin-specific K14 VEGF-C mice (10). The tetracycline-dependent VEGF-C overexpression model results in not only global lymphangiogenesis but also severe lymphangiectasis and related inflammation (32). In contrast to these models, LEC<sup>PTEN</sup> mice have global lymphangiogenesis throughout all major tissues without identifiable off-target effects. Furthermore, increased Ki67 expression in LECs suggests that the increased lymphatic densities observed in these mice are the result of lymphatic proliferation rather than hypertrophy. Because we used a VEGF3Cre driver for lymphatic specificity, this hyperplasia was limited to lymphatic vessels only, and BEC numbers were similar in LEC<sup>PTEN</sup> and control mice at least in skin and intestine tissues. Such results are consistent with the expression of VEGF3R on blood vessels only during embryogenesis, postnatal and pathological conditions, and fenestrated vessels of human kidney and vasa vasorum of aorta (33–35). Macrophages and myeloid-derived cells are reported to express VEGF3R under pathological conditions (36–38). Because we used adult naïve mice in all experiments, the possibility of PTEN knockout on myeloid cells is low and is supported by the similar numbers of CD11b<sup>+</sup> myeloid cells in the skin of adult LEC<sup>PTEN</sup> and control mice. As a result, we specifically chose to induce PTEN knockout only after transgenic mice had reached 6 weeks of age.

PTEN is a tumor suppressor gene whose deletion can cause growth of tumors. In addition, several reports have indicated that endothelial-specific deletion of PTEN may lead to angiosarcomas over time (39). However, we did not observe tumor development in LEC<sup>PTEN</sup> mice, which lived complete life spans. Our results also showed that LEC<sup>PTEN</sup> LECs phenotypically resembled WT LECs and with similar expression of lymphatic-specific genes. Downstream of VEGFR3 signaling, the phosphorylated form of Akt was several-fold higher in LEC<sup>PTEN</sup> LECs, although total Akt abundance did not change, indicating activation of Akt. Total and phosphorylated ERK1/2 were similar between genotypes, suggesting that this signaling pathway was not activated.

VEGF-C is the most potent prolymphangiogenic growth factor and is widely used in preclinical studies as a therapeutic agent to...
induce lymphatic growth and function (40). However, despite its positive effects, there are also important off-target effects, such as inflammation, angiogenesis, macrophage chemotraction, and lymphatic vessel leakage (28, 41–43). Furthermore, VEGF-C binds to VEGFR2 and induces blood vascular permeability and leakage (44). Our results are consistent with these reports. We found that exogenous VEGF-C not only induced sprouting lymphangiogenesis but also resulted in increased neutrophil and macrophage infiltration, elevated inflammatory cytokine concentrations, and leaky lymphatic vessels. In contrast, LECPTENmice demonstrated increased lymphangiogenesis similar to that seen with VEGF-C injection, but without inflammation or lymphatic leakiness. The absence of these effects suggests that VEGF-C–induced lymphatic leakiness is potentially linked to increased vascular permeability through VEGFR2 activation on blood capillaries and indirectly linked to the proinflammatory microenvironment created around the VEGF-C injection site, causing disruption of VE-cadherin junctions on lymphatic vessels. However, it will be interesting to see the extent of inflammatory side effects resulting from continuous, low-level, in situ production of VEGF-C compared to those from injection of concentrated recombinant protein that might overload the local VEGF-3 pool and thus lead to side effects.

The distribution of the endothelial junction protein VE-cadherin plays an important role in lymphatic function. The button pattern on lymphatic capillaries allows for fluid absorption, whereas the zipper pattern on lymphatic collecting vessels prevents fluid leakage (29). The dynamic nature of these junctions and how they remodel during development and inflammation is emerging, but the effect of inflammatory cytokines on VE-cadherin junctions on lymphatic vessels has not been thoroughly studied (45–47). Our data showed a tendency for transformation of buttons into zippers on capillaries and disrupted zippers on lymphatic collecting vessels in the skin of mice injected with VEGF-C, but not in that of LECPTENmice. This is likely a secondary effect of the supraphysiologic VEGF-C levels as well as the resulting increase in inflammatory cytokines such as IL-1β; these cytokines promote phosphorylation of VE-cadherin at Tyr665, which is the unstable form that results in disruption of endothelial junctions (30, 48). Although additional research is required to better understand how excess VEGF-C and inflammation remodels VE-cadherin junctions, this relationship may explain why VEGF-C–induced lymphatic vessels are leaky.

Although supraphysiologic doses of VEGF-C have been associated with increased inflammation, lymphangiogenesis can also aid in the resolution of inflammation (49). Inflammation–induced lymphangiogenesis may have opposing effects on inflammation, depending on the clinical scenario (50). Because of these opposing effects, it has been difficult to separate the true effect of lymphangiogenesis and lymphatic function in inflammation resolution. Because LECPTENmice did not appear to demonstrate inflammation to the same degree as is seen in mice injected with VEGF-C, this transgenic mouse model provides us with a better means to study this phenomenon. In a UVR-induced inflammation model, we found that LECPTENmice evidenced attenuated inflammation and quicker resolution of inflammation compared to both control mice and mice treated with excess VEGF-C. Consistent with a previous report (31), VEGF-C–injected mice demonstrated improved resolution compared to control mice but to a lesser extent compared to LECPTENmice. The less efficient effect of VEGF-C on UVR-induced inflammation resolution compared to LECPTENmice in our study could be due to the use of VEGF-C, whereas the previous report used the VEGF-C 156S variant, which eliminates the effect of VEGF-C on vascular permeability through VEGFR2 (51). In addition, VEGF-C is produced as an inactive precursor protein flanked by N- and C-terminal propeptides, and these propeptides are proteolytically cleaved to form the active-mature forms of VEG-F-C. One caveat of our study is that we used a mature form of VEG-F-C that can activate both VEGFR3 and VEGFR2 and therefore have both lymphatic and vascular effects. However, it would be interesting to use the pro-form of VEG-F-C that provides a differently localized and more sustained stimulus with relatively less off-target effects, especially VEGFR2-related vascular leakage (40, 52, 53).

The lymphatic vasculature plays a critical role in the transport of immune cells, and the efficiency directly depends on lymphatic vessel density (54, 55). Accordingly, we found that lymphatic proliferation in LECPTENmice was associated with enhanced DC migration. Improved lymphatic drainage was corroborated by our data showing that the lymphatic vessels in LECPTENmice were also capable of faster reverse cholesterol transport from the peripheral tissues to the circulation by macrophages, as compared to control mice.

We recognize that the PTEN is a crucial modulator of tumor biology and that knocking out this protein, even selectively in LECs, may be challenging to translate clinically. However, the knowledge we will obtain from these results will identify the mechanisms that regulate lymphatic dysfunction and will enable us to devise more targeted approaches to improve lymphatic function. Another potential issue is that VEGFR3 is expressed by a fraction of activated BECs. However, we did not note any changes in the blood vessels in LECPTENmice. In summary, we have developed a lymphatic gain-of-function mouse model that results in the proliferation of LECs and in the formation of nonleaky, functional lymphatic vessels without notable off-target changes to the surrounding microenvironment. Dysfunctional lymphatic vessels are a major feature of various pathologies, including cancer and metabolic syndromes, but an understanding of their precise role in pathophysiology has only recently begun to be elucidated (56, 57). The model described here is a new tool for studying the lymphatic vasculature in both normal physiology and pathological states.

**MATERIALS AND METHODS**

**Generation of LECPTENmice**

All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Memorial Sloan Kettering Cancer Center (MSKCC). MSKCC adheres to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and operates under the Animal Welfare Act. All mice were maintained in a pathogen-free, temperature- and light-controlled environment and provided with normal chow diet and fresh water ad libitum.

Transgenic mice with inducible lymphangiogenesis were created by crossing Flt4CreERT2 mice (a gift from S. Ortega, Centro Nacional de Investigaciones Oncológicas) (58) with Pten-floxed mice (B6.129S4-Ptenfl/J; the Jackson Laboratory, Bar Harbor, ME). Flt4CreERT2 mice are reported to have high-efficiency recombination specifically in LECs during embryonic and adult stages (58). The expression of both transgenes was confirmed by genotyping (Transnetyx, Cordova, TN), and double-homozygous mice were backcrossed for six to seven generations to ensure consistency. The activation of CreERT2...
These mice were compared to LECPTEN mice injected with vehicle of PBS on days 0, 2, 4, 6, 8, and 11 (see additional details below). rhVEGF-C (R&D Systems; Minneapolis, MN) or an equal volume of PBS was injected into WT mice were randomized to receive intradermal injections of UVR-induced inflammation. A total of nine thoracic duct rings were used per group per time point. All experiments were performed 4 weeks after the first dose of tamoxifen injection unless otherwise specified. When indicated, anesthesia was induced with isoflurane (Henry Schein Animal Health, Dublin, OH). Respiratory rate and tail pinching were used to monitor the depth of anesthesia. At the conclusion of each experiment, the appropriate animals were euthanized by carbon dioxide asphyxiation as recommended by the American Veterinary Medical Association.

**Reverse cholesterol transport**

Reverse cholesterol transport was evaluated following a previous protocol that used 22-NBD-cholesterol, a fluorescent analog of cholesterol (Molecular Probes, Eugene, OR) (26). RAW264.7 macrophages were cultured for 12 hours with 22-NBD cholesterol (5 μg/ml), and 5 × 10⁶ cholesterol-loaded macrophages were subcutaneously injected into the footpad. The footpad, draining lymph node, blood, liver, and feces were then collected at 1, 6, 12, 24, 48, and 72 hours after injection. Samples were homogenized in NBD-cholesterol buffer (80:19:16 isopropanol:hexane:125 mM H₂SO₄), after which fluorescent cholesterol was extracted by centrifugation at 14,800 rpm for 15 min. Fluorescence intensity was measured, and data are presented as a percentage of injected cholesterol.

**Thoracic duct ring assay**

An ex vivo lymphangiogenic assay using mouse thoracic duct ring explants was performed as previously described (59). Briefly, thoracic ducts were isolated from control and LECPTEN mice, cut into 1-mm pieces, implanted into Matrigel (BD Biosciences, San Jose, CA), and cultured in endothelial cell growth basal medium with 10% fetal bovine serum, growth factor supplements, and additional rhVEGF-C (100 ng/ml) for 10 days. Anti-mouse VEGFR-3 ligand binding antibody (10 μg/ml; rat monoclonal; clone: 2E11; catalog no. CMV104, Cell Sciences, MA) or both (5 μg + 5 μg) or nonspecific isotype antibody was added to respective wells at days 0, 3, and 7. VEGF-A blocking antibody was used in all combinations to nullify any effect of VEGF-A on lymphatic growth (150 ng/ml; catalog no. AF-493-NA, R&D Systems). Thoracic ducts were monitored regularly, and phase contrast images were taken at days 0 and 10. The length of thoracic duct ring sprouts was measured using ImageJ software (http://rsb.info.nih.gov/ij; NIH; Bethesda, MD) and represented as bar graphs. A total of nine thoracic duct rings were used per group per time point.

**UV-induced inflammation**

WT mice were randomized to receive intradermal injections of rhVEGF-C (R&D Systems; Minneapolis, MN) or an equal volume of PBS on days 0, 2, 4, 6, 8, and 11 (see additional details below). These mice were compared to LEC PTEN mice injected with vehicle at the same time points. After the first injection, each mouse was placed in an opaque-walled cage, where it could roam freely. UVR light was delivered by four FS40T12/UVB/5 bulbs (Light Sources Inc., Orange, CT) placed on top of the cage for 5 min 2 s to deliver a total dose of 1000 J/m², which was quantified by UVA/B Light Meter 850009 (Sper Scientific, Scottsdale, AZ). Ear inflammation was subsequently quantified by measuring skin thickness with digital calipers on days 3, 7, and 14. Ear samples were also collected, fixed, and processed to assess lymphatic vessels and inflammatory cells as described below.

**Histology and immunohistochemistry**

Histological and immunohistochemical staining were performed using standard protocols. Tissues were fixed in 4% paraformaldehyde (Affymetrix Inc., San Diego, CA) at 4°C, embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek, Torrance, CA) or paraffin, and sectioned at 5 to 10 μm. All tissue sections were rehydrated before staining.

For histological analysis, hematoxylin and eosin (H&E) staining was performed with Mayer’s hematoxylin (Lillie’s Modification; Dako North America, Carpinteria, CA) and eosin Y solution (Thermo Fisher Scientific, Waltham, MA). Sections underwent alcohol-based dehydration and alcohol extraction with xylene, after which they were mounted using VectaMount permanent mounting medium (Vector Laboratories Inc., Burlingame, CA).

For immunohistochemical analysis, nonspecific binding was blocked with a solution of 5% donkey or goat serum (Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature. All tissue sections were incubated overnight at 4°C with the appropriate primary antibodies; goat polyclonal anti–LYVE-1 (1:400; no. 2125-LY) and goat polyclonal anti–VE-cadherin (1:500; no. AF1002) from R&D Systems; rabbit polyclonal anti–LYVE-1 (1:200; no. ab191717), hamster monoclonal anti-podoplanin (PDPN; 1:500; no. ab11936), and rabbit anti-mouse Ki67 (1:200; no. ab16667) from Abcam (Cambridge, MA); mouse monoclonal Cy3-conjugated anti–smooth muscle actin (1:1000; no. C6198) from Sigma-Aldrich; and rat monoclonal anti-CD31 (1:200; no. 553370) and rat monoclonal anti-CD11b (1:300; no. 557395) from BD Biosciences (Franklin Lakes, NJ). Sections or whole-mount tissue preparations were washed with PBS with Triton X-100 (Sigma-Aldrich) and incubated with the appropriate fluorescent-conjugated secondary antibody conjugates (Alexa Fluor 488, 594, or 647; Life Technologies, Carlsbad, CA) for 5 hours followed by 4′6-diamidino-2-phenylindole (DAPI; no. D4571, Molecular Probes/Invitrogen, Eugene, OR) for 10 min before mounting with Mowiol (Sigma-Aldrich) or paraffin. Tissues were sectioned at 5 to 10 μm. All tissue sections were rehydrated before staining.

All sections were scanned using a Mirax slide scanner (Zeiss, Munich, Germany), and whole mounts were imaged using a SP-5 upright confocal microscope (Leica Microsystems, Wetzlar, Germany). Image analysis was performed with Pannoramic Viewer (3D Histech, Budapest, Hungary) and ImageJ software.

**Flow cytometry**

Single-cell suspensions of tissues were prepared by mechanical dissociation and incubated with digestion buffer containing collagenase D, deoxyribonuclease I, and Dispase II (Roche Diagnostics, Indianapolis, IN). Erythrocytes were lysed with red blood cells lysis buffer (eBioscience, San Diego, CA). Samples were stained with different combinations of the following fluorophore-conjugated mouse monoclonal antibodies: anti-PDPN (8.1.1; no.127407), anti-CD45 (30-F11; no. 103107), anti-CD11c (N418; no. 117307), anti-CD31 (MEC13.3; no. 102509), anti-CD11b (M1/70; no. 101211), and...
anti-Ly6C/6G (RB6-8C5; no. 108422) (all from BioLegend), and anti-Ki67 (SolA1; no. 2040334, Invitrogen). Nonspecific staining was reduced with Fc receptor block (rat monoclonal anti-CD16/CD32; no. 14-0161-85, eBioscience). DAPI viability dye was also used to exclude dead cells. Single-stain compensation samples were created using UltraComp eBeads (no. 01-2222-42, Affymetrix Inc.). Flow cytometry was performed using a BD Fortessa flow cytometry analyzer (BD Biosciences, San Jose, CA), and data were analyzed with FlowJo software (Tree Star, Ashland, OR). CD31 and PDPN double-positive cells from CD45-negative population were sorted using BD Aria 6 cell sorter.

**Enzyme-linked immunosorbent assay**

Protein isolated from carefully dissected mouse tissues was analyzed by ELISA to measure concentrations of cytokines and growth factors. The following ELISA kits were used according to the respective manufacturer’s protocol: VEGF-C (no. 028842) from United States Biological (Salem, MA) and VEGF-A (no. BMS619) from eBioscience.

**Western blotting and quantitative real-time PCR**

Flow cytometry was used to isolate CD45−CD31+PDPN+ primary mouse LECs. Human dermal LECs (PromoCell, Heidelberg, Germany) were cultured in endothelial cell growth medium-MV-2 (PromoCell), and 3 × 10^5 cells were seeded in a six-well culture plate. After 2 days, the cells were washed with ice-cold PBS and treated with rhVEGF-C (500 ng/ml; R&D Systems) or IL-1β or IL-12 (PeproTech, NJ, USA) for 30 min. Mouse and human LECs were lysed with radioimmunoprecipitation assay lysis buffer containing Halt protease and phosphatase inhibitor single-use cocktail (Thermo Fisher Scientific). The lysates were centrifuged at 13,000 g for 10 min at 4°C, after which the protein concentration was measured using the BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. A total of 3 to 8 μg of protein was separated by NuPAGE 4 to 12% bis-tris gel (Thermo Fisher Scientific) and transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% skim milk in tris-buffered saline containing 0.1% Tween 20 (TBST) at room temperature for 1 hour and then incubated with the following antibodies in 0.5% skim milk in TBST at 4°C overnight: rabbit monoclonal anti-PTEN (1:1000; no. ab32199) from Abcam; rabbit monoclonal anti–phospho-AKT (1:1000; no. 4060); mouse monoclonal anti–total AKT (1:1000; no. 2920S), phospho-ERK1/2 (1:1000; no. 9108S), mouse monoclonal anti–total-ERK1/2 (1:1000; no. 4696S), and mouse monoclonal anti–β-actin (1:1000; no. 3700S) from Cell Signaling Technology (Danvers, MA); rabbit polyclonal anti–phospho-VE-cadherin (Tyr^685, 1:100; no. CP19981) from ECM Biosciences (Versailles, KY); mouse monoclonal anti–total VE-cadherin (1:100; no. 14-1449-82) from Thermo Fisher Scientific; and mouse monoclonal anti–glyceraldehyde-3-phosphate dehydrogenase (1:2000; no. MAB374) from Millipore (Burlingame, MA). After washing three times with TBST, the membrane was incubated with horseradish peroxidase–conjugated secondary antibody in TBST at room temperature for 1 hour. After three additional washes with TBST, immunoreactive bands were detected with enhanced chemiluminescence Western blotting substrate (Thermo Fisher Scientific). The intensities of protein bands were quantitated using ImageJ software and expressed as the ratio of phosphorylated to nonphosphorylated protein for VE-cadherin protein.

For real-time RT-PCR, total RNA was extracted from footpad skin using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. The RNA (1 μg) was reverse-transcribed into cDNA using the Maxima H Minus cDNA Synthesis kit (Thermo Fisher Scientific). Quantitative real-time RT-PCR was performed with the following primers: IL-12a (QT01048334), IL-1β (QT01048355), CCL2 (QT00167832), IL-10 (QT00106169), IL-27 (QT00143017), tumor necrosis factor–α (TNF-α) (QT00104006), P-selectin (QT00106379), PTEN (QT00141568), Proxl (QT01070615), VEGFR3 (QT00099064), PDPN (QT01552257), CCL21 (QT00284753), VEGFR2 (QT00097020), and Ki67 (QT00247667) purchased from Qiagen (Hilden, Germany) using Viia 7 Real-Time PCR System with a 384-well block (Applied Biosystems, Waltham, MA).

**VEGF-C injection studies**

Two microliters of rhVEGF-C (0.1 mg/ml; catalog no. 9199-VC/CF, R&D Systems) or recombinant mouse VEGF-C (carrier-free) (0.1 mg/ml; catalog no. 775104, BioLegend) was injected intradermally to ear skin (for functional analysis and UVR irradiation experiments) or footpad (to assess inflammation) with a Hamilton syringe every other day for 8 and 11 days for UVR experiment before tissue analysis. Control animals were injected with the same volume of sterile PBS at the same time points.

**Lymphatic function analysis**

Lymphatic function was assessed using both Evans blue dye and FITC-conjugated lectin. Evans blue dye (4%, 5 μl; Sigma-Aldrich) was injected intradermally into tip of the ear, and images were taken to identify leaky spots and visualize the lymphatic drainage pattern. Twenty-four hours after injection, ears were harvested, and the remaining Evans blue was extracted by soaking the ear skin in formamide for 36 hours, after which they were centrifuged. The concentration of the extracted Evans blue was measured by a spectrophotometer at a wavelength of 620 nm.

Ear skin was used to test lymphatic function with FITC-conjugated lectin (no. FL-1171, Vector Laboratories). Using an ultrafine Hamilton syringe needle, 5 μg (1 μg/μl) of lectin was intradermally injected. Five minutes later, the ears were harvested and underwent immunofluorescent staining to identify the capillary and collecting lymphatic vessels draining the lectin.

**DC migration assay by FITC painting**

Skin DC migration through lymphatic vessels was assessed using a modification of a previously reported method (60). Briefly, 8% type I isomer FITC (5 mg/ml; Sigma Aldrich) was diluted in a 1:1 mixture of acetone and dibutylphthalate (Sigma Aldrich). A total of 10 or 20 μl of the solution was painted on each side of the mouse ear or footpad, respectively. The mice were sacrificed at 8 and 18 hours, and the draining cervical or popliteal lymph nodes after ear or footpad painting, respectively, were collected. Single-cell suspensions were obtained from these lymph nodes by enzymatic digestion to allow for flow cytometric analysis of FITC+CD11c+ DCs per the protocol described above.

**In vitro LEC proliferation assay**

Mouse primary LECs were sorted from single-cell suspension of lymph nodes collected from control and LECPTEN mice. A total of 0.5 × 10^6 cells were plated into each well of a 96-well plate in 100 μl of LEC culture medium and cultured for 48 hours. Cell proliferation...
was measured using a CyQUANT MIT assay kit (catalog no. V13154, Invitrogen, OR) as per the manufacturer’s instructions.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Normal distribution of all the datasets was checked using the Shapiro-Wilk normality test. Unpaired Student’s t test was used to compare differences between two groups, and one- or two-way analysis of variance (ANOVA) was used for multiple groups. Data are presented as mean ± SD unless otherwise noted, and P < 0.05 was considered significant.

**SUPPLEMENTARY MATERIALS**

stke.sciencemag.org/cgi/content/full/14/695/eabc0836/DC1

**REFERENCES AND NOTES**


Acknowledgments: We thank R. Yevgeny and S. Fujisawa of the Molecular Cytology Core at MSK for help with microscopy and imaging, as well as the Flow Cytometry Core facility at MSK for assistance in cell sorting. Funding: This research was funded in part through the Emerson Collective cancer research fund, ID: 691032 (to R.P.K.); NIH/NCI Cancer Center support grant P30 CA008748 and NIH/NHLBI research grant HL111130 (to B.J.M.); NIH/NIAID RO1 A1079178-06A1 and Novel Research grant from Lupus Research Alliance (to T.T.L.); and NIH/P30 CA008748 and NIH/NHLBI research grant HL111130 (to B.J.M.); NIH/NIAID RO1 A1079178-06A1 and Novel Research grant from Lupus Research Alliance (to T.T.L.); and NIH/NIAMS T32AR071302-01 (to N.S.).

This research was funded in part through the Emerson Collective cancer research fund, ID: 691032 (to R.P.K.; NIH/NCI Cancer Center support grant P30 CA008748 and NIH/NHLBI research grant HL111130 (to B.J.M.); NIH/NIAID RO1 A1079178-06A1 and Novel Research grant from Lupus Research Alliance (to T.T.L.); and NIH/NIAMS T32AR071302-01 (to N.S.). Author contributions: R.P.K. and B.J.M. conceived the concept and designed the experiments. R.P.K. developed the methods. R.P.K., H.J.P., J.E.B., and J.S. performed all the experiments. R.P.K. and B.J.M. analyzed the data. N.S. and T.T.L. helped with UVRI experiments. S.O. made the FLT4-CreERT2 mice. R.P.K., C.L.L., T.T.L., and B.J.M. prepared and edited the manuscript. All authors reviewed and approved the manuscript.

Competing interests: B.J.M. is an inventor on a patent application (US20210069164A1) held by Memorial Sloan Kettering Cancer Center that covers the composition and methods for treatment of edema. B.J.M. is also an inventor on a patent application (US10548858B2) held by Memorial Sloan Kettering Cancer Center that covers inhibition of sphinogine 1-phosphate receptor for treatment and prevention of lymphedema. The other authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. FLT4-CreERT2 mice are available from S.O. under a material transfer agreement with CNIO, Spain.

Submitted 5 April 2020
Accepted 24 June 2021
Published 10 August 2021 10.1126/scisignal.aeb0836

Lymphatic-specific intracellular modulation of receptor tyrosine kinase signaling improves lymphatic growth and function

Raghu P. Kataru, Jung Eun Baik, Hyeung Ju Park, Catherine L. Ly, Jinyeon Shin, Noa Schwartz, Theresa T. Lu, Sagrario Ortega and Babak J. Mehrara

DOI: 10.1126/scisignal.abc0836

Less leaky lymphatics
The lymphatic system enables drainage of excess fluid from tissues and immune cell migration during inflammatory responses. Attempts to increase lymphatic vessel density have generally relied on the exogenous administration of the lymphangiogenic growth factor VEGF-C, which yields leaky and less functional lymphatics and off-target inflammation. Kataru et al. generated mice with a lymphatic endothelial cell–specific deficiency of the lipid phosphatase PTEN, reasoning that the absence of PTEN would promote signaling downstream of VEGFR3, a key receptor for VEGF-C (see also the Focus by Künnapuu and Jeltsch). These mice had an expanded lymphatic vessel network that was not leaky and that contributed to improved resolution of inflammation, compared to control mice injected with VEGF-C. These results suggest that a better approach to increasing lymphangiogenesis may be to enhance signaling downstream of VEGFR3.