Infection-induced lymphatic zippering restricts fluid transport and viral dissemination from skin

Madeline J. Churchill¹, Haley du Bois², Taylor A. Heim³, Tenny Mudianto³, Maria M. Steele²,³, Jeffrey C. Nolz¹, and Amanda W. Lund⁴,⁵

Lymphatic vessels are often considered passive conduits that flush antigenic material, pathogens, and cells to draining lymph nodes. Recent evidence, however, suggests that lymphatic vessels actively regulate diverse processes from antigen transport to leukocyte trafficking and dietary lipid absorption. Here we tested the hypothesis that infection-induced changes in lymphatic transport actively contribute to innate host defense. We demonstrate that cutaneous vaccinia virus infection by scarification activates dermal lymphatic capillary junction tightening (zippering) and lymph node lymphangiogenesis, which are associated with reduced fluid transport and cutaneous viral sequestration. Lymphatic-specific deletion of VEGFR2 prevented infection-induced lymphatic capillary zippering, increased fluid flux out of tissue, and allowed lymphatic dissemination of virus. Further, a reduction in dendritic cell migration to lymph nodes in the absence of lymphatic VEGFR2 associated with reduced antiviral CD8+ T cell expansion. These data indicate that VEGFR2-driven lymphatic remodeling is a context-dependent, active mechanism of innate host defense that limits viral dissemination and facilitates protective, antiviral CD8+ T cell responses.

Introduction

The peripheral vasculature, blood and lymphatic, provides the critical interface between pathogen and host. While blood vessels allow hematopoietic and effector molecule access to tissue, lymphatic vessels coordinate innate and adaptive immune cross talk through dendritic cell (DC) and soluble antigen transport to draining lymph nodes (dLNs; Steele and Lund, 2021). The current dogma is that lymphatic vessels passively transport (“drain”) antigen and pathogens to LNs. However, we recently reported reduced lymph flow to dLNs following cutaneous vaccinia virus (VACV) infection by scarification (Loo et al., 2017), raising important questions as to how changes in transport might directly impact host defense and immunity.

Viral dissemination depends on multiple pathogen-specific strategies to hijack and evade host defense mechanisms. Pathogens can rapidly disseminate to dLNs after intradermal injection (Kastenmüller et al., 2012; Reynoso et al., 2019), which may indicate that lymphatic capillaries promote passive virion transport. We and others recently reported, however, that when VACV is applied by scarification, it is not detected in dLNs despite high skin viral titers (Khan et al., 2016; Loo et al., 2017). Together with the observed reduction in fluid transport, this may indicate that lymphatic vessels have the ability to actively restrict fluid and virion transport out of skin.

Lymphatic capillaries are lined by a single layer of oak leaf-shaped lymphatic endothelial cells (LECs) that are connected by discontinuous, button-like junctions, allowing for rapid vessel distension in response to increased interstitial fluid pressure (Baluk et al., 2007). This open and responsive structure (Baluk et al., 2007; Yao et al., 2012; Dejana et al., 2009) mediates rapid fluid uptake (Swartz et al., 1999) and permits integrin-independent paracellular leukocyte transendothelial migration (Lämmermann et al., 2008; Pfliege and Sixt, 2009). In contrast, continuous junctions, termed zippers, found in collecting vessels are associated with reduced permeability. Both buttons and zippers are composed of the same adherens and tight junction molecules (Leak, 1970), and exhibit plasticity during development (Zheng et al., 2014), postnatal lymphangiogenesis (Yao et al., 2012), and inflammation (Yao et al., 2010). The plasticity of these junctions that define the lymphatic barrier may therefore provide a mechanism through which dermal lymphatic vessels can restrict transport in the setting of infection. Here we tested the hypothesis that the lymphatic vasculature actively...
remodels in the context of viral infection to restrict viral dissemination from skin.

Results and discussion

Dermal lymphatic vessels undergo infection-induced zippering

The loose structure of lymphatic capillaries has driven the dogma that pathogen and antigen are passively transported to dLNs following peripheral tissue challenge. Indeed, when pathogens, particulates, and soluble antigens are administered by injection, they rapidly reach LNs (Gerner et al., 2015) and are either captured by subcapsular sinus macrophages (Junt et al., 2007; Moseman et al., 2012) or gain access to the paracortical conduits (Reynoso et al., 2019). VACV applied by scarification (5 × 10⁶ PFU), however, does not disseminate to dLNs 5 dpi post infection (dpi), in contrast to the rapid dissemination seen after intradermal injection (Fig. 1, A and B). This raises the possibility that, in the absence of elevated interstitial fluid pressure, viral dissemination could be limited in the tissue itself. We therefore sought to test the hypothesis that the dermal lymphatic vasculature might directly prevent virion spread by restricting fluid flow.

The potential mechanisms by which lymphatic vessels regulate fluid transport remains largely unknown, but a previously described process of junctional tightening, termed zippering (Yao et al., 2012), could serve to alter the passive fluid transport properties of lymphatic capillary networks (Kulkarni et al., 2011). We previously reported that VACV scarification does not induce a significant lymphangiogenic response in skin but activates a type I IFN-dependent loss of naive blunt-ended morphology and reduced fluid flow (Loo et al., 2017). To carefully evaluate junctional dynamics in dermal lymphatic capillaries, we performed whole-mount imaging at multiple time points after infection. Using LYVE-1 and vascular endothelial (VE)-cadherin to visualize LEC junctions, we found that, 5 dpi, when skin viral titers peak (Fig. 1 C) and significant skin thickening is evident (Fig. 1 D), LYVE-1-expressing dermal lymphatic capillaries lose their blunt-ended morphology and...
punctate VE-cadherin (buttons) and instead exhibit continuous VE-cadherin (Fig. 1 E; zippers). To quantify conversion from button to zipper junctions, we measured the average surface area of discrete VE-cadherin structures in individual dermal lymphatic capillaries (Fig. S1, A and B). Lymphatic capillaries in skin 5 dpi were smaller in diameter (Fig. S1 C), and their interendothelial junctions exhibited a twofold increase in area, relative to naive (Fig. 1 F), consistent with an increased proportion of zippered junctions. Lymphatic zippering and ear thickness were not activated by mock scarification, heat-activated virus, or intradermal injection (Fig. S1, D–F), together indicating that active viral replication in the context of wounding is required.

To confirm that the lengthening of VE-cadherin interendothelial staining was associated with junctional tightening, we performed transmission EM and observed increased electron density along lymphatic junctions in infected skin (Fig. 1 G). Junctions began zipperig as early as 1 dpi, continued to zipper through day 10 and returned to a naive-like state 30 dpi (Figs. S1 G and 1 H). Our data, therefore, indicate that cutaneous lymphatic capillaries undergo a zipperig response to infection similar to what has been described in the trachea following Mycoplasma pulmonis infection (Yao et al., 2012) and in the intestinal lacteal (Suh et al., 2019; Zhang et al., 2018). How these changes in lymphatic junctional morphology impact host defense and immunity, however, remains unknown.

Dermal lymphatic zipperig requires vascular endothelial growth factor A (VEGFA)/vascular endothelial growth factor receptor 2 (VEGFR2) signaling

In the small intestine, VEGFA-dependent activation of lymphatic endothelial VEGFR2 is sufficient to activate lacteal zipperig (Zhang et al., 2018), suggesting that pathophysiological processes that alter local VEGFA bioavailability could impact regional lymphatic vessel uptake. Although VEGFC activation of VEGFR3 is also implicated in lacteal zipperig (Suh et al., 2019), the absence of a lymphangiogenic response in VACV-infected skin and undetectable levels of VEGFC (Loo et al., 2017) suggested a VEGFC-independent mechanism. We found that VEGF is elevated in skin 5 dpi (Fig. 2 A) and expressed by keratinocytes (Fig. 2 B; 5 dpi), and that VEGFR2 is expressed by dermal LECs (CD45−CD31+gp38−; Fig. 2, C and D). Given that VEGFR2 has an established role in regulating VE-cadherin protein dynamics via RhoA/Rho-associated protein kinase signaling–mediated cytoskeletal dynamics (Zhang et al., 2018, 2020), we asked whether VEGFA/VEGFR2 signaling was necessary for infection-induced lymphatic capillary zipperig. Administration of blocking antibodies against VEGFR2 (αR2) did not affect skin viral titers 5 dpi (Fig. 2 E) but did result in rapid reduction in ear thickness 3 dpi, leading to an almost 25% reduction in thickness by 5 dpi (Figs. 2 F and S1 H). Strikingly, αR2 (Fig. 2, G and H) treated mice maintained naïve-like button junctions in their dermal lymphatic capillaries, and αR2 restored naïve capillary diameter (Fig. S1 C). We saw similar changes as a function of VEGFA blockade, suggesting that the role of VEGFR2 in lymphatic zipperig is ligand dependent (Fig. S1, I–L). Interestingly, in contrast to what we see here, specific activation of VEGFR2 by VEGFE transduction in mouse dermis resulted in vessel dilation (Wirzgeniet al., 2007), and VEGFR2 activation during chronic cutaneous Leishmania major infection activated lymphangiogenesis (Bowl et al., 2021; Weinkopf et al., 2016), perhaps suggesting that the concentration, context, or duration of growth factor signaling impacts the lymphatic response.

To dissect the lymphatic-intrinsic effects of VEGFR2, we crossed inducible Prox1−/− CreERT2 + with mice bearing a floxed Vegfr2 allele (Vegfr2credi/cre) enabling specific, inducible loss of VEGFR2 in mature, adult lymphatic vessels (Vegfr2ΔProx1; Fig. S2, A–C). While VEGFR2 is required for lymphatic vessel development (Delling et al., 2013), it is thought to be dispensable for postnatal lymphangiogenesis (Zarkada et al., 2015). We found that the dermal capillary and collecting networks in mice with postnatal, lymphatic-specific VEGFR2 loss maintained similar density (Fig. S2 D), though we cannot completely exclude the possibility that loss of VEGFR2 impacts functional lymphatic homeostasis, particularly if mice were housed for longer periods of time (>17 d after tamoxifen). In our experiments, lymphatic capillaries in naive skin maintained a similar diameter (Fig. S2 E), blunt-ended capillary morphology, and exhibited no quantifiable difference in button-like junctions (Fig. S2, F and G). After infection, however, skin viral titers were elevated in Vegfr2ΔProx1 mice 5 and 7 dpi (Fig. 3 A) and ear thickness decreased through the first 7 dpi (Fig. 3 B), although no change in epidermal or dermal thickness was noted (Fig. 3, C and D). Importantly, dermal lymphatic capillaries in Vegfr2ΔProx1 skin failed to zipper their interendothelial junctions following VACV infection (Fig. 3, E and F) and maintained a naive capillary diameter (Fig. S2 H). These data establish a lymphatic intrinsic role for VEGFR2 in the transition from button to zipper, and for the first time provide a model to specifically investigate the functional relevance of dermal lymphatic zipperig in transport and host defense.

Dermal zipperig reduces fluid transport and restricts viral dissemination to LNs

We predicted that one consequence of lymphatic capillary zipperig would be reduced fluid transport and cutaneous edema. To specifically assay fluid transport out of infected skin, we quantified Evans blue in dLNs after intradermal injection 5 dpi. Both αR2 blockade (Fig. S3 A) and lymphatic-specific Vegfr2 knockout (Fig. 3 G) enhanced tracer transport to dLNs when compared with isotype and littermate controls, respectively. These data indicate that VEGFR2-dependent lymphatic capillary zipperig can directly restrict fluid and solute flux out of infected tissue and contribute to edema.

Interestingly, edema facilitates the retention of virus at the infection site and alters innate recruitment and viral control (Pingen et al., 2016). Similarly, our data indicate that lymphatic zipperig and reduced fluid transport are associated with viral retention in skin (Fig. 1 A). These observations support the hypothesis that lymphatic-dependent control of fluid flow, or changes in paracellular size exclusion, may directly restrict viral dissemination under pathophysiological levels of interstitial fluid pressure. We therefore quantified the amount of virus present in dLNs 5 dpi in either antibody-treated or Vegfr2ΔProx1

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mice. Whereas LN virus remained below the limit of detection in isotype-treated and Vegfr2 WT mice, inhibition of lymphatic-specific VEGFR2 signaling enabled consistent detection of viable virus in dLNs (Fig. 3, H and I; and Fig. S3 B). VACV virions (300–500 nm) are of a size predicted to passively transport through naive lymphatic capillaries (Rohner and Thomas, 2016) and are similar in size to chylomicrons (75–600 nm), which require open button junctions for efficient uptake in the intestinal lacteal (Zhang et al., 2018). Therefore, the tightening of lymphatic junctions might exclude virions from lymph, similar to chylomicron exclusion from the lacteal. Interestingly, we did not detect systemic virus (ovary), likely owing to the protective function of the subcapsular macrophage (Moseman et al., 2012; Iannacone et al., 2010; Junt et al., 2007), therefore indicating that multiple overlapping mechanisms along the lymphatic hierarchy protect against viremia.

While our data support the hypothesis that zippering excludes passive uptake of large viral particles, it is also possible that DCs actively transport virus to LNs. To directly test if DCs are required to carry viable virus to LNs, we infected UBC:CreERT2;Ccr7fl/fl knockout mice in the presence of VEGFR2 blockade and demonstrate that viral dissemination to LNs is independent of CCR7-mediated cell transport (Figs. S3 D and 3 J). These data support the hypothesis that fluid transport facilitated by an open lymphatic structure, mediates the movement of viable virions from infected skin to LNs. We therefore propose a mechanism whereby lymphatic capillaries physically exclude intact viral virions from passive transport to LNs. Similar mechanisms have been proposed in encephalitic flavivirus infection, where IFN-λ reduces blood–brain barrier permeability and viral transport (Lazear et al., 2015). These data also add to recent findings that viruses may specifically subvert the lymphatic vasculature to disseminate via lymph (Phillips et al., 2021), together indicating that the lymphatic vasculature is a critical innate barrier to viral dissemination from peripheral tissue.

**VEGFR2-dependent lymphatic remodeling promotes CD8+ T cell priming and cutaneous viral control**

VACV infection by scarification leads to superior immune protection over alternative routes of administration (Liu et al., 2010). We therefore next asked whether scarification-induced lymphatic capillary zippering affects the generation of protective immune responses. Despite a reduction in fluid transport following VACV scarification and the inhibition of DC migration by replication competent VACV (Aggio et al., 2021), DCs do access dLNs and drive potent protective immune responses (Loo et al., 2017). We sought to quantify the impact of VEGFR2 signaling on DC migration. Using Kaede-tg mice (Tomura et al., 2010), we photoconverted infected ears 4 dpi in mice treated with αVEGFR2 or isotype control and quantified the number of Kaede-red migratory DCs present in the dLN 24 h later (Fig. 4 A). Surprisingly, we saw a significant decrease in the number of Kaede red+ migratory DCs present in the dLN 24 h later (Fig. 4 A). Surprisingly, we saw a significant decrease in the number of Kaede-red migratory DCs in αVEGFR2-treated mice compared with controls (Fig. 4 B), suggesting that VEGFR2-dependent lymphatic remodeling contributed to the efficiency of DC migration after infection.

This reduction in DC migration led us to interrogate the impact on LN anti-viral CD8+ T cells. Total lymphocyte counts in LNs draining VACV infected skin were reduced with VEGFR2...
blockade (Fig. S3 F) as well as in Vegfr2ΔProx1 mice relative to controls (Fig. 4 C). Moreover, we noted that αVEGFR2 treatment reduced the expansion of endogenous H2-Kd B8R20–27-specific (Fig. S3 F) and OT-1 TCR-tg CD8+ T cells (SIINFEKL, H2Kb-OVA257–264) in mice infected with VACV-OVA257–264 (Fig. S3 G). We therefore tracked the H2-Kd B8R20–27-specific CD8+ T cell response 5 dpi in Vegfr2ΔProx1 mice and found a threefold reduction in B8R-specific CD8+ T cells at this time point (Fig. 4, D and E), indicating that VEGFR2-induced lymphatic remodeling and changes in afferent lymphatic transport tune antigen presentation and T cell expansion in vivo.

In addition to the impact of VEGFR2 signaling on dermal lymphatic capillary zippering, however, VEGFA/VEGFR2 is implicated in inflammation-induced LN lymphangiogenesis, which is required for LN expansion and boosts DC migration (Angeli et al., 2006). To determine whether VEGFR2 was necessary for infection-induced LN lymphangiogenesis (Gregory et al., 2017), we pulsed mice with BrdU 0–5 dpi. We indeed saw a 50% reduction in LN LEC proliferation in the absence of VEGFR2 (Fig. 4, F and G), which could restrict LN expansion. To understand whether transport of antigen to the LN via the afferent lymphatic vasculature or LN expansion was most limiting in our model, we evaluated T cell expansion after intradermal VACV injection. Here the rapid delivery of virus directly to the LN would bypass changes in afferent lymphatic transport. Interestingly, however, intradermal VACV delivery normalized total LN cellularity and the expansion of VACV-specific CD8+ T cells (Fig. 4, H–J). We therefore propose that the surprising immune phenotype we observed here is due to afferent transport phenomena, likely a combination of changes in the dermal capillary and LN lymphatic structure. Taken together, we suggest that VEGFR2-dependent lymphatic remodeling impacts the efficiency of DC movement to the LN and contributes to the quantity of the antiviral CD8+ T cell response.

While we have previously shown that lymphatic vessels are necessary for efficient CD8+ T cell activation and antiviral
protection (Loo et al., 2017), we lacked a physiologically relevant and cell-specific model to investigate the impact of lymphatic transport on host physiology. Through the use of rigorous in vivo systems, our data indicate that the active management of in vivo lymphatic transport during infection has direct implications for innate host defense and the efficiency of adaptive immune responses. We therefore suggest that the lymphatic vasculature may be a barrier to pathogen spread and a more active component of cutaneous immune surveillance than previously appreciated. Finally, these data further support current efforts to modify the regional lymphatic vasculature for vaccine response (Sasso et al., 2021) and motivate future studies to investigate the effects of different lymphatic transport on effector molecule, leukocyte, and metabolite accumulation in inflamed peripheral tissue.

**Materials and methods**

**Mice, in vivo antibodies, and reagents**
Pathogen-free mice were obtained from Charles River Laboratories. Tg(TcraTcrb)1100Mjb/J (OT-I mice, stock no. 003831) and Vegfr2fl/fl (stock no. 018977) mice were obtained from The Jackson Laboratory. Breeding was maintained at Oregon Health and Science University (OHSU) and New York University (NYU) in specific pathogen-free facilities. Vegfr2fl/fl mice were crossed in-house with Prox-1:CreERT2 mice provided by V.H. Engelhard (University of Virginia, Charlottesville, VA) in agreement with T. Makinen (Uppsala University, Uppsala, Sweden) to generate Vegfr2fl/fl,Prox-1:CreERT2 mice. UBC:CreERT2;Ccr7fl/fl were provided by Susan R. Schwab (NYU Grossman School of Medicine, New York, NY). Cre was induced by administering tamoxifen in corn oil by i.p. injection for 5 d (75 mg/kg), and mice rested for 1 wk before experimentation. In vivo blocking antibodies for mouse VEGFR2 (InVivoMAb DC101; BioxCell, 212.5 µg, day 0 and 3) were injected i.p. VEGFA blocking antibody (2G11-2A05; BioLegend, 50 µg, days 0 and 3) was administered i.v. BrdU was administered to mice i.p. (2.0 mg/mouse) on the day of infection and maintained in drinking water (0.8 mg/ml) for 5 d. For all in vivo studies, age- and sex-matched 8–20-wk-old mice were used with at least three to five mice per group, and all experiments were repeated at least twice. Littermate, tamoxifen-induced controls or isotype controls were used in all experiments. All animal procedures were approved and performed in accordance with the Institutional Animal Care and Use Committee at OHSU and NYU Langone Health.
VACV propagation and infection
VACV and VACV-OVA<sub>257-264</sub> (VACV-OVA) were propagated in BSC-40 cells using standard protocols. Mice were infected cutaneously by 25 pokes with a 29-G needle after administration of 5 × 10<sup>6</sup> PFU VACV in 10 µl PBS to the ventral side of the ear pinna (scarification). Ear thickness was measured by digital calipers. Intradermal injections were performed with a Hamilton syringe inserted in the tip of the ear (5 × 10<sup>6</sup> PFU VACV in 10 µl PBS).

Titering virus and plaque assays
Ears, LNs, and ovaries were harvested, snap frozen with liquid nitrogen, and homogenized in 0.5–1 ml of RPMI 1640/1% FBS with a handheld tissue homogenizer. Homogenized tissue was subjected to three rounds of freeze-thaw with vortexing and serially diluted. Serially diluted homogenized tissue was applied to BSC-40 monolayers for 72 h with an agarose overlay. A neutral red agarose overlay was added to aid in visualizing viral plaques. Plaques were enumerated 24 h later, and PFU values were calculated.

Microlymphangiography
Mice were administered 1 µl of 1% Evans blue by injection with a Hamilton syringe to the tip of the ear. Ears and LNs were harvested 30 min after injection and eluted in formamide for 24 h at 55°C to extract the Evans blue dye. Supernatants were collected and absorbance at 620 nm was measured.

DC trafficking
For DC trafficking in Kaede mice, ears were photoconverted at 55°C to extract the Evans blue dye. Supernatants were collected 30 min after injection and eluted in formamide for 24 h at 37°C and then stained with anti-BrdU (FITC, i:25) for 20 min at RT.

Immunohistochemistry
Mice were euthanized by CO<sub>2</sub> asphyxiation, and ears were harvested, then placed in formalin for 48 h. Ears were then processed and embedded in paraffin, and 6-µm sections were cut. Tissue was then processed and stained by the histology core. H&E stained sections were imaged on a Keyence BX-X810 microscope.

RNA<sub>scr</sub>
Formalin-fixed paraffin-embedded 5 µm sections were baked overnight at 60°C. Tissue dehydration and RNA in situ hybridization was performed according to manufacturer instructions using RNA<sub>scr</sub> 2.5 HD Assay-RED kit and manufacturer-designed probe Mm-Vegfa-01 (#436961). Sections were imaged on a Keyence BX-X810 microscope.

ELISA
Ears were harvested from euthanized mice and snap frozen in liquid nitrogen. Samples were then homogenized using a handheld tissue homogenizer in 1 ml of radioimmunoprecipitation assay buffer. Protein concentrations were determined by Thermo Fisher Scientific BCA assay kit, and 50 µg of protein was loaded into the precoated VEGFA ELISA kit (R&D Systems). VEGFA protein levels were determined by completing the ELISA assay per manufacturer’s protocol.

Whole-mount imaging of lymphatic vessels within ear skin
Ears were harvested, ventral and dorsal sides were separated, and cartilage was carefully removed before being placed immediately into ice-cold Zn fixative (BD Pharminogen) with 1% Triton-x for 48 h at 4°C on a tabletop shaker. Ears were then washed in PBS for 6 h, followed by 2 h in 2.5% BSA in PBS. Tissue was incubated with 1:400 LYVE-1 (biotinylated anti-mouse, 13-0443-02; eBioscience) and 1:200 VE-cadherin-Alexa Fluor 647 (rat anti-mouse, clone 11D4.1, BDB562242; Thermo Fisher Scientific) in 1.25% BSA in PBS for 24 h on a tabletop shaker at 4°C. Samples were washed with PBS + 0.1% Tween overnight at 4°C. Secondaries were then applied (streptavidin-AF488, 405235; BioLegend) in PBS for 24 h and then washed with PBS + 0.1% Tween overnight. Tissue was dehydrated in 70% and 100% ethanol sequentially for 5 min, then samples were cleared with 2:1 benzyl benzate/benzyl alcohol and mounted on glass slides. Whole-mount ear samples were blinded to imager and imaged with an LSM-880 confocal microscope (Zeiss) using a 20× objective at 2× zoom for individual capillaries and 0.6× zoom for wide fields. At least 10 lymphatic capillaries were imaged from each animal.

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Image analysis of lymphatic capillary junctions

Maximum projections of whole-mount z-stacks were converted into TIFF files using Zen Black (Zeiss). Images were blinded to the analyzer. The area of each VE-cadherin positive structure (cell junction) within single LYVE-1+ lymphatic capillaries was determined using the 3D Objects counter feature in Fiji (ImageJ; National Institutes of Health), irrespective of capillary area. The average size of junctions per capillary was calculated from each image and at least 10 capillaries averaged from each animal. Individual button junctions (punctate and not colocalized with LYVE-1) manually identified in naive capillaries have an average area of 0.53 ± 0.37 µm², and zipper junctions are 3.5 ± 1.5 µm² in area (Fig. S1, A and B).

EM

Ears were harvested, the dorsal and ventral sides of the ear were separated, and cartilage was removed. Ear pieces were placed in 1% PFA for 24 h and then into PBS. Ear pieces underwent EM processing using a Biowave (Pelco BioWave; Ted Pella [Walker et al., 2012]). Tissue was then placed in 1:1 propylene oxide:EPON resin for 6 h or until it sank to the bottom to allow for better infiltration of the resin. Tissue was then placed in EPON resin overnight, followed by carefully placing tissue pieces onto ACLAR sheets with more EPON Spur resin, and then was allowed to solidify overnight (18 h) in an oven set to 60°C. 60-nm sections were cut using a ultramicrotome (Leica) with a diamond knife (Diatome) and counterstained with uranyl acetate and lead citrate. Lymphatic capillaries were imaged using a 1400 Series Transmission Electron Microscope (JEOL) and photographed with a digital camera (AMT) at 1,600×, 5,000×, and 8,000×.

Statistics

Data were plotted and statistical significance was calculated in Prism (GraphPad) using parametric or nonparametric Student's t tests and one- and two-way ANOVA for multiple pairwise testing as indicated.

Online supplemental material

Fig. S1 provides the rationale for lymphatic junction analyses and data demonstrating the zipper response to heat-inactivated and intradermally injected virus. Representative images of lymphatic capillary zipperings are shown over time, and viral titer, ear thickness, and zippering are reported with and without VEGFA blockade. Fig. S2 reports characterization of the lymphatic conditional Vegfr2 knockout mouse (Proxl:CreERT2;Vegfr2fl/fl), including confirmation of VEGFR2 loss, lymphatic morphology, and junctional analysis. Fig. S3 reports the effect of VEGFR2 antibody blockade on fluid transport and viral dissemination, confirmation of CCR7 loss in UBC:CreERT2;Ccr7fl/fl mice, and VEGFR2 antibody effects on LN counts and both endogenous and exogenous CD8+ T cell responses.

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Infection-dependent dermal lymphatic capillary zippering. (A) Representative whole-mount images of naive lymphatic capillary in murine dermis. LV, lymphatic vessel; BV, blood vessel; B, button; Z, zipper. Scale bar = 20 μm. (B) Surface area of individual VE-cadherin–positive structures manually classified as either buttons (punctate and LYVE-1−) or zippers (lengthened and LYVE-1+). Student’s t test; each point represents a single junction. (C) Quantification of capillary diameter in skin 5 dpi with VACV (5 × 10^6 PFU, scarification) treated with either αVEGFR2 (αR2) antibody or isotype control (Iso). One-way ANOVA. (D) Ear thickness measurements in ear skin infected with live (scarification, s.s.; intradermal, i.d.) or heat-inactivated (HI) VACV compared with naive (N) 5 dpi. One-way ANOVA. (E) Representative whole-mount images (maximal projection) of dermal lymphatic vessels in HI or live (s.s. and i.d.) VACV-infected skin 5 dpi. Scale bar = 20 μm. (F) Junctional analysis (average surface area) of dermal lymphatic capillaries from E. One-way ANOVA. Each point represents an individual mouse. (G) Representative whole-mount images of dermal lymphatic capillaries of VACV-infected skin over time. Scale bar = 20 μm. (H) Ear thickness over time in WT mice treated with αR2 antibody or isotype control. One-way ANOVA. (I) Skin viral titers (PFU) 5 dpi of mice treated with VEGFA (αVA) blocking antibody or isotype control. (J) Ear thickness 5 dpi from I. Student’s t test. (K) Representative dermal lymphatic capillaries in mice treated with αVA antibody 5 dpi. Scale bar = 20 μm. (L) Average junctional area from K. Student’s t test. Error bars define SEM; all experiments performed at least twice. *, P < 0.05; **, P < 0.01; ***, P < 0.01; ****, P < 0.0001.
Figure S2. Lymphatic-specific loss of VEGFR2. Prox1:CreERT2 mice were crossed with Vegfr2fl/fl to generate lymphatic-specific VEGFR2 knockout animals (Vegfr2ΔProx1). (A) Representative flow plots of VEGFR2 expression in blood (CD45−CD31−gp38−, BECs), lymphatic (CD45−CD31+gp38+, LECs), skin, and LN endothelial cells in Vegfr2ΔProx1 and Vegfr2WT littermate controls. (B and C) Quantification of surface expression of VEGFR2 on dermal BECs (left) and LECs (right; B) and LN BECs (left) and LECs (right; C) from Vegfr2ΔProx1 and Vegfr2WT littermate controls 1 wk after tamoxifen induction. Student’s unpaired t test. FMO, fluorescence minus one. (D) Whole-mount images of lymphatic capillaries and precollector and collector vessels in naive mouse dermis of Vegfr2ΔProx1 or Vegfr2WT littermate controls rested for 2 wk after tamoxifen induction. Red arrowheads indicate collecting vessels and presence of valves. Scale bar = 200 μm. (E) Lymphatic capillary diameter in naive Vegfr2ΔProx1 or Vegfr2WT littermate controls. Scale bar = 20 μm. (F) Representative whole-mount images of lymphatic capillaries Vegfr2ΔProx1 or Vegfr2WT littermate controls 5 d after 5 × 106 PFU of VACV scarification. Student’s t test. Each point represents an individual mouse; all experiments performed at least twice. **, P < 0.01; ****, P < 0.0001.
Figure S3. VEGFR2 blockade impacts transport and T cell priming during VACV infection. C57Bl/6 mice were infected with $5 \times 10^6$ PFU of VACV by skin scarification and administered either isotype control or VEGFR2 antibody (αR2) 0 and 3 dpi. (A) Evans blue (EB) transport to dLNs. Student’s unpaired t test. (B) dLN viral titers. Student’s unpaired t test. (C) Flow histogram demonstrating CCR7 loss following tamoxifen induction in UBC:CreERT2, Ccr7fl/fl (Ccr7iUBC) mice relative to Cre− littermate controls (Ccr7WT). FMO, fluorescence minus one. (D and E) Total lymphocyte count (D) and total number of H2-Kd B8R26-27-specific CD8+ T cells (E) 5 dpi. (F) Total number of TCR-Tg OT-I Thy1.1+CD8+ T cells (transfer 15,000, day 0) 5 dpi with VACV-expressing OVA257-264 in mice treated with αR2 or isotype control. Student’s t test. Error bars define SEM. One point represents an individual mouse; all experiments performed at least twice. *, $P < 0.05$; **, $P < 0.01$. 

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