Transcellular Pathways in Lymphatic Endothelial Cells Regulate Changes in Solute Transport by Fluid Stress

Valentina Triacca, Esra Güç, Witold W. Kilarski, Marco Pisano, Melody A. Swartz

Rationale: The transport of interstitial fluid and solutes into lymphatic vessels is important for maintaining interstitial homeostasis and delivering antigens and soluble factors to the lymph node for immune surveillance. Transendothelial transport across lymphatic endothelial cells (LECs) is commonly considered to occur paracellularly, or between cell–cell junctions, and driven by local pressure and concentration gradients. However, emerging evidence suggests that LECs also play active roles in regulating interstitial solute balance and can scavenge and store antigens, raising the possibility that vesicular or transcellular pathways may be important in lymphatic solute transport.

Objective: The aim of this study was to determine the relative importance of transcellular (vesicular) versus paracellular transport pathways by LECs and how mechanical stress (ie, fluid flow conditioning) alters either pathway.

Methods and Results: We demonstrate that transcellular transport mechanisms substantially contribute to lymphatic solute transport and that solute uptake occurs in both caveolae- and clathrin-coated vesicles. In vivo, intracellular uptake of fluorescently labeled albumin after intradermal injection by LECs was similar to that of dermal dendritic cells. In vitro, we developed a method to differentially quantify intracellular solute uptake versus transendothelial transport by LECs. LECs preconditioned to 1 μm/s transmural flow demonstrated increased uptake and basal-to-apical solute transport, which could be substantially reversed by blocking dynamin-dependent vesicle formation.

Conclusions: These findings reveal the importance of intracellular transport in steady-state lymph formation and suggest that LECs use transcellular mechanisms in parallel to the well-described paracellular route to modulate solute transport from the interstitium according to biomechanical cues. (Circ Res. 2017;120:1440-1452. DOI: 10.1161/CIRCRESAHA.116.309828.)

Key Words: caveolin-1 □ clathrin □ dextran □ endothelial cell □ lymphatic vessel

Lymph formation, or the uptake of interstitial fluid and solutes into lymphatic capillaries, is essential to the transport of protein and peptide antigens, cell-secreted exosomes and microvesicles, lipids, and leukocytes from the periphery to the lymph nodes and eventually back to the blood. Lymph formation in the initial lymphatics is coupled to lymph propulsion in collecting vessels via coordinated pumping by smooth muscle cells surrounding lymphangions, vessel segments separated by bicuspid valves.1 Fluid and solute transport from the interstitium to the lymphatic vessel are widely assumed to be driven by passive forces (ie, fluid convection and diffusion). Lymph formation is usually described by Starling’s law of fluid filtration, where local hydrostatic pressure differences between the interstitium and lymphatic lumen drive fluid flow across the lymphatic endothelium according to a hydraulic conductivity determined by the permeselectivity of intercellular junctions that are considered overlapping and fenestrated.1–4 A primary valve system has been described in lymphatic capillaries, based on electron microscopy studies, where loose overlapping junctions anchored to the extracellular matrix allow fluid and solute in while preventing backflow.7,8

...
Novelty and Significance

What Is Known?

- Lymphatic vessels transport fluid, solutes, antigens, and cells from peripheral tissues into lymph.
- Translymphatic transport is believed to occur primarily through gaps between lymphatic endothelial cells (LECs), created by the special conformation of their intercellular junctions.

What New Information Does This Article Contribute?

- We demonstrate that LECs use vesicular (transcellular) pathways to actively control solute uptake and transport.
- LECs can modulate solute transport in response to mechanical cues, such as transmural fluid flow, by controlling vesicular uptake.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>BEC</td>
<td>blood endothelial cell</td>
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<tr>
<td>cav-1</td>
<td>caveolin-1</td>
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<tr>
<td>CCV</td>
<td>clathrin-coated vesicle</td>
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<tr>
<td>LEC</td>
<td>lymphatic endothelial cell</td>
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<tr>
<td>P_eff</td>
<td>effective permeability</td>
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<td>TEER</td>
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On the contrary, electron microscopy studies performed decades earlier had demonstrated the abundance of vesicles inside lymphatic endothelial cells (LECs); in lacteals (chylomicon-absorbing lymphatics in the small intestine), colocalization of solutes like ferritin (≈5 nm), peroxidase (≈11 nm), and chylo-microns (≈200 nm) were observed within such vesicles but not within cell–cell junctions. This suggests that vesicular pathways could be important components of lymphatic transport.

In blood capillaries under steady-state conditions, tightly controlled intercellular junctions are impermeable to albumin and other large molecules; baseline transendothelial albumin transport relays on transcytosis, where it is taken up into cell surface caveolae (endocytosis) on the luminal side, transported across the cell, and released on the basal or abluminal side (exocytosis). It has been shown that transcytosis in blood endothelial cells (BECs) is mechanosensitive, and the loading of vesicles or their translocation between luminal and abluminal membranes is accelerated as the transmural pressure is raised. BECs respond to a sustained flow environment by directing caveolae to the cell surface, where they mediate mechanotransduction responses by adjusting endothelial cell sensitivity to mechanical stimuli, leading to increased caveolin-1 (cav-1) gene expression and density of caveolae. If transcytosis is the main transport pathway across blood capillaries in steady-state condition, under inflammatory conditions, increased vascular permeability—a process referred to as vascular leakage—mainly occurs by increased paracellular permeability through altered intercellular gap junctions, particularly vascular endothelial (VE)-cadherin. Transcellular pathways are thought to play only a minor role in vascular leakage, in response to certain types of inflammatory signals or injury.

When vascular leakage into the interstitium occurs, interstitial pressure increases, and thus, lymphatic transport must increase as well to avoid interstitial accumulation of fluid and plasma proteins. This heightened transmural flow acts as an immediate cue for signaling injury or inflammatory conditions to the lymphatic endothelium, driving changes in its transport functions to modulate fluid and immune cells trafficking to the draining lymph node. In addition to fluid flow, inflammatory cytokines and signaling molecules such as tumor necrosis factor (TNF)-α, interleukin-6, interleukin-1β, interferon-γ, and lipopolysaccharide have been reported to alter the effective LEC permeability to fluid and solutes.

Although there is evidence that transcytosis is an important route for solute transport across the blood endothelium, vesicular transport in lymphatic endothelium has been far less explored, and recent studies are challenging the view that the lymphatic endothelium is a passive exchange barrier. A recent study used adrenomedullin to stabilize intercellular junctions to tighten the lymphatic endothelial barrier and demonstrated that while the paracellular route contributed to VEGFA-induced permeability increase, it was only a minor contributor to baseline permeability. We have recently shown that extracellular solute can be taken up into LECs, processed intracellularly, and loaded onto major histocompatibility complex class I or II molecules for antigen presentation on the cell surface. However, the extent to which intracellular solute can be transcytosed, as opposed to being sorted through endocytic compartments for degradation or processed at the Golgi, is unknown.

Here, we asked whether vesicular transport is important in regulating transendothelial transport. To this end, we performed both in vivo and in vitro studies to assess the possibility that LECs can exploit the transcellular route for solute transport. Using a simplified in vitro model of the lymphatic barrier, we showed that transmural flow stimulation on LECs results in an increase in cytoplasmic vesicles and solute uptake. Furthermore, we demonstrated that the increased effective permeability (P_eff) caused by transmural flow, which we
identified earlier, could be substantially inhibited by blocking dynamin-dependent pathways. On the basis of these findings, we suggest that transcytosis could be exploited by LECs in lymph formation and that LEC modulation of lymph formation after mechanical stimuli could be achieved by modulating transcellular pathways in parallel to paracellular mechanisms.

Methods
Full methods are provided in the Online Data Supplement.

Results
Lymphatic Endothelial Cells Actively Scavenge Albumin From the Interstitium
To determine the extent to which dermal LECs take up solutes from the interstitial space, we injected AF647-albumin in the dermis of the ear and, after 2 hours, euthanized the mouse and performed whole-mount immunostaining on the ear tissue for LYVE-1 and podoplanin to identify lymphatic vessels. Untreated ear tissues were also stained for endogenous
albumin, LYVE-1 and CD31. We found that LECs within dermal lymphatic vessels contained abundant intracellular albumin, both endogenous and exogenous (Figure 1A and 1B). Using flow cytometry, we examined the effect of injected albumin dose (0.05, 0.5, or 5 μg) on uptake by LECs (CD45−CD31+GP38+) compared with uptake by dermal dendritic cells (CD45−CD11b−CD11c+). With increasing albumin dose, higher fractions of each cell type were albumin-positive (Figure 1C), although interestingly, the mean fluorescence intensity of intracellular albumin, which correlates with the amount of albumin taken up per cell, was similar in LECs compared with dermal dendritic cells for all doses tested (Figure 1D). These results highlight the scavenging capacity of dermal LECs.

To ensure that exogenous AF647-albumin is not broken down by LECs, we pulsed LECs with AF647-albumin and run

![Image](http://circres.ahajournals.org/)

**Figure 2.** In vitro, lymphatic endothelial cells take up solute into both caveolin-1+ and clathrin+ vesicles under steady-state conditions. A, Representative confocal images of lymphatic endothelial cells (LECs) after 1-h incubation with 10 μg/mL of fluorescently labeled albumin (red, top row) or dextran 70 kDa (red, bottom row) and immunostained for cav-1 (left) or clathrin (right) and nuclei (DAPI [4',6-diamidino-2-phenylindole], gray). Bar=5 μm. Insets show magnified region from each image indicated by arrows. B, Quantification of albumin and dextran colocalization with caveolin-1 (cav-1) and clathrin. **P<0.01. C, Live cell imaging in cultured LECs showing albumin (red) colocalization with intracellular vesicles (plasma membrane [PM], gray, top row) and early endosomes (EE, green, bottom row) at indicated times after introduction of 15 μg/mL of albumin (red). Bar=5 μm. D, Dynamics of albumin (left) and dextran (right) uptake, quantified as numbers of solute+ voxels colocalized with EE per cell over time.
the cell lysate on a SDS-PAGE gel. Imaging the gel with a near-infrared fluorescence detection system, we found the fluorescent signal in correspondence with the molecular weight of albumin, suggesting that AF647-albumin is not broken down by LECs (Online Figure I).

To determine whether there is directionality in albumin transport by LECs, we developed an in vitro assay where LECs are cultured on a transwell insert and AF594-albumin and AF-647 albumin are placed in the 2 different chambers of the insert (Figure 1E). To avoid concentration gradients, the 2 albumin solutions had the same concentration. Transendothelial transport was measured in both apical to basolateral and basolateral to apical directions, and the $P_{\text{rot}}$ was computed, showing that albumin permeability in the basolateral (facing the interstitial space) to apical (facing the vessel lumen) direction is higher than that in the opposite direction (Figure 1F).

### Solute Uptake Occurs by Both Caveolae- and Clathrin-Coated Vesicles

Several pathways for solute endocytosis are known, including uptake into clathrin-coated vesicles (CCVs), which involves receptor ligation, and uptake into caveolae, which require cav-1 for their formation.41 In BECs, albumin uptake and transcytosis are thought to occur primarily via caveolae,42 although recent studies have demonstrated that caveolin-independent pathways can play important roles. For example, in rat pulmonary microvascular BECs, 65% of albumin uptake persisted in the absence of cav-1 and involved CCVs that resulted in endosomal accumulation of albumin.43 Additionally, insulin transcytosis in BECs was recently shown to be clathrin dependent and cav-1 independent.44

To determine whether albumin uptake in LECs involved caveolae or CCV, we first examined albumin colocalization with cav-1 and clathrin and compared it to that of neutral 70 kDa dextran, a macromolecule similar in size to albumin but which cannot activate receptor-mediated endocytosis mechanisms. After 2-hour exposure, LECs contained abundant intracellular albumin that colocalized with cav-1, the primary structural component of endothelial caveolae, as well as with clathrin (Figure 2A); image quantification demonstrated roughly equal colocalization between these 2 compartments (Figure 2B). Intracellular dextran was also found colocalized with both proteins, although unsurprisingly, dextran colocalized much more strongly with cav-1 compared with clathrin (Figure 2A and 2B).

To visualize the dynamics of solute uptake, spinning disk confocal microscopy was used with a fluorescent reporter for early endosomes and a live cell dye for plasma membrane with fluorescent albumin (Figure 2C; Online Movie II). Image quantification revealed that solute colocalization with early endosomes increased linearly with time during 120 minutes for both dextran and albumin. Interestingly, the rate of solute accumulation into early endosomes was $\approx 50$ times greater with albumin compared with that with dextran (Figure 2D), despite similar extracellular concentrations and fluorescence intensities; this is consistent with dextran uptake being limited to receptor-independent endocytosis and with previous studies in epithelial cells suggesting that albumin, but not dextran, is mainly directed to early endosomes.45,46

Caveolae and CCVs both require dynamin, a large molecular weight GTPase, for their formation.44–46 In BECs, albumin transcytosis is initiated after albumin binds its receptor gp60 to induce clustering and phosphorylation of cav-1 and dynamin49 and involves several signaling molecules such as phosphoinositide 3-kinase and cytoskeletal components like microtubules for intracellular trafficking.47,48 To first determine the extent to which solute uptake into LECs was mediated by caveolae, we treated LECs with the dynamin inhibitor dynasore50 and used spinning disk confocal microscopy to live-image the intracellular uptake of fluorescently labeled albumin or dextran. We found that inhibiting dynamin largely prevented intracellular accumulation of both albumin and dextran (Figures 3A and 3B; Online Figure II), implicating vesicular involvement dominates both types of solute uptake by LECs. The small amount of solute uptake that was dynamin independent was likely because of micropinocytosis.40

In a pulse-chase assay to examine solute release after loading, LECs were preloaded with fluorescently labeled solute, rinsed, and treated with (1) dynasore, (2) LY294002, a phosphoinositide 3-kinase inhibitor51,52; (3) nocodazole,53 an inhibitor of microtubule polymerization; or (4) dimethyl sulfoxide control. All 3 of these inhibitors similarly decreased the release of albumin and dextran previously loaded inside LECs (Figure 3C and 3D), suggesting that solute trafficking and release involve both dynamin and phosphoinositide 3-kinase and rely on microtubule dynamics. Dynasore was the most effective at inhibiting the release of both solutes previously loaded inside LECs compared with LY294002 and nocodazole.

### Both Transcellular and Paracellular Pathways Contribute to Steady-State Transendothelial Transport of Albumin Across Lymphatic Endothelial Cells

Considering that albumin was both taken up and released by LECs via dynamin-dependent vesicular mechanisms, we next asked whether transcytosis played a major role in overall transendothelial albumin transport compared with the paracellular route, generally considered to be the dominant mechanism of lymph formation. To address this question, we developed a live cell imaging assay to simultaneously measure the dynamics of solute uptake versus transendothelial transport (also referred to as $P_{\text{cat}}$) across a confluent monolayer of LECs (Figure 4). In this assay, LECs were cultured on the bottom side of a light-impermeable porous culture insert membrane, and after reaching confluence, fluorescently labeled albumin was loaded in the top chamber, and the cultures were placed in an incubated microscope for live imaging fixed on the LEC focal plane (Figure 4A). From the raw image histograms, the extracellular solute concentration in the bottom chamber (ie, transported solute) could be computed (see Methods section), whereas the intracellular uptake could be quantified by subtracting the fluorescence intensity in the bottom chamber from the raw image (Figure 4B and 4D).

We then treated LECs with either dynasore or adenomedullin, which effectively tightens the endothelial barrier by stabilizing intercellular junctions through...
reorganization of the junctional protein ZO-1 and the adherens protein VE-cadherin, thereby inhibiting paracellular transport. LY294002 and filipin III were also used to inhibit the transcellular component of transport and $P_{\text{eff}}$ to albumin, and dextran was measured (Online Figure III). The effects of adrenomedullin on stabilizing cell–cell junctions was confirmed by an increase of transendothelial electric resistance (TEER) of the LEC monolayer (Figure 5A) and by the reorganization of the VE-cadherin and CD31 complex at the junction level (Figure 5B). Neither TEER nor junctional organization was affected by dynamin inhibition (Figure 5A and 5B; Online Figure IV), but when observing the dynamics of intracellular albumin uptake by LECs, only dynasore, but not adrenomedullin, led to a reduced uptake rate (Figure 5C and 5D). These results verified that adrenomedullin mainly inhibited the paracellular transport pathway, whereas dynasore mainly

![Figure 3. Solute uptake and release by lymphatic endothelial cells are dynamin-dependent processes. A. Intracellular uptake dynamics of albumin and dextran 70 kDa by lymphatic endothelial cells (LECs) after treatment with dynasore or dimethyl sulfoxide vehicle control (Ctrl), quantified from time-lapsed spinning disk confocal images as numbers of solute+ voxels colocalized with early endosomes (EE) per cell. B. Solute uptake rates after treatment with dynasore or vehicle control. C. Dynamics of solute release after loading with fluorescently labeled albumin or dextran. Active transport inhibitors were present only during the release phase; LY294002, an inhibitor of class I phosphoinositide 3-kinase; and nocodazole, a microtubule-disrupting agent. D. Total release of preloaded intracellular albumin and dextran in the presence of inhibitors over 48 h (% of release over total release of untreated samples). *$P<0.05$; **$P<0.01$ compared with Ctrl.](http://circres.ahajournals.org/doi/fig/10.1161/CIRCRESAHA.116.307654)
Inhibited the transcellular pathway. Interestingly, however, both treatments similarly reduced transendothelial transport (Figure 5E and 5F), suggesting that both paracellular and transcellular pathways are exploited by LECs and important for abluminal-to-luminal albumin transport.

Transmural Fluid Flow Stimulates Increased Solute Uptake and Transendothelial Transport

Previously, we reported that transmural flow could strongly regulate the $P_{\text{eff}}$ of LECs, both in vitro (after pretreatment with flow) and in vivo (after accounting for increased convection).25 Because vesicular (dynamin-dependent) transport strongly affected steady-state LEC $P_{\text{eff}}$ (Figure 5F), we next asked whether this component of solute transport was influenced by transmural flow. After pretreating the LECs to transmural flow (0, 0.1, 1, and 10 μm/s) for 16 hours, we observed substantial increases in transendothelial transport of both albumin and dextran with increasing flow velocities (Figure 6A). To determine the extent to which this increase was caused by transcellular versus paracellular transport pathways, we pretreated LECs with flow in the presence of dynasore or adrenomedullin and then measured $P_{\text{eff}}$ under static conditions for 4 hours. As before (Figure 5F), both dynasore and adrenomedullin decreased baseline $P_{\text{eff}}$ to a similar degree (Figure 6B). However, when comparing flow versus static conditions, dynasore was more effective than adrenomedullin at reducing flow-enhanced $P_{\text{eff}}$. When considering that the contribution of either pathway to the total $P_{\text{eff}}$ should hypothetically be additive, because they operate in parallel, we calculated the dynamin-dependent and
adrenomedullin-dependent components of $P_{\text{eff}}$ and found that both components were increased significantly in flow versus static conditions (Figure 6C). When comparing the ratios of static versus flow permeability for both components, we found that the effect of dynasore was significantly higher than the effect of adrenomedullin (Figure 6D). Therefore, these data suggest that while both paracellular and transcellular pathways are important in steady-state transendothelial transport, in flow conditions, it is the transcellular pathway that accounts mostly for the flow-enhanced $P_{\text{eff}}$ seen here and previously.25

Uptake and transendothelial transport experiments were repeated with human serum albumin to match the species of albumin with the cell type, and the results obtained were similar to the results obtained with bovine serum albumin (Online Figure V).

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Figure 5. Transcellular and paracellular transport can be selectively modulated in lymphatic endothelial cells. A, Changes in transendothelial electric resistance (TEER) of lymphatic endothelial cell (LEC) monolayers treated with dynasore (Dyn), adrenomedullin (AM), or the combination of both. **$P<0.01$. B, Confocal images showing altered cell–cell junctions of LECs treated with AM after immunostaining for CD31 (green) and vascular endothelial (VE)-cadherin (red). Bar=10 μm. C, Dynamics of albumin uptake by LECs treated with Dyn, AM, or the combination of both. D, Albumin uptake rate. E, Transendothelial transport of albumin across LECs treated with Dyn, AM, or the combination of both. F, Effective permeability ($P_{\text{eff}}$) of LECs treated with Dyn, AM, or the combination of both. **$P<0.01$ compared with vehicle control.

Figure 6. Pretreatment of lymphatic endothelial cells with transmural flow increases transendothelial transport via dynamin-dependent mechanisms. A, Effective permeability ($P_{\text{eff}}$) of lymphatic endothelial cells (LECs) to albumin and dextran, defined as solute flux over time, after 16 h of flow preconditioning at different flow rates (0.1, 1, 10 μm/s). In all cases, $P_{\text{eff}}$ measurement was done under static conditions. *$P<0.05$, **$P<0.01$ compared with static control condition. B, $P_{\text{eff}}$ after preconditioning LECs with 0 or 1 μm/s transmural flow and treatment with dynasore (Dyn) or adrenomedullin (AM). **$P<0.01$ compared with static control condition, #P<0.05, ##P<0.01. C, Dyn-dependent (Dyn-dep) and AM-dependent (AM-dep) components of $P_{\text{eff}}$ in LECs pretreated with static or flow conditions. **$P<0.01$ compared with the corresponding static component. D, Ratio of $P_{\text{eff}}$ components under flow vs static conditions. **$P<0.01$. 
We then stimulated LECs in static conditions with TNF-α to see whether soluble inflammatory mediators could also modulate the transcellular component of transendothelial transport. As for flow experiments, LECs were treated with TNF-α in combination with dynasore or adrenomedullin, and $P_{\text{eff}}$ was measured. Both components of transport contributed to the enhanced permeability mediated by TNF-α (Online Figure VIA and VIB). We also tested the effect of TNF-α on albumin uptake by LECs, finding that TNF-α stimulation results in the increase in albumin uptake rate by LECs (Online Figure VIC).

We next measured the intercellular junction gap from transmission electron microscopy images of LECs cross-sections and found no difference between LECs cultured in static condition or under flow (Figure 7A and 7B), while we observed a reduction in junction length, calculated as the width of junction overlap from CD31 staining, after flow treatment, consistent with the increase of paracellular permeability after flow (Figure 7C and 7D). When we immunostained LEC monolayers for cav-1 and clathrin in LECs after 16-hour static versus flow (1 μm/s) conditions, we found that the changes

Figure 7. Transmural flow preconditioning results in increased expression of vesicular markers but not intercellular junction width. A, Cross-sections of LEC monolayers cultured in static vs flow conditions, imaged by transmission electron microscopy (TEM). Bar=500 nm. B, Cell–cell junction width measured from TEM images. C, Confocal images of LECs after static or flow conditioning, immunostained for CD31 (green). Bar=20 μm. D, Average junction length after static or flow conditioning. **$P<0.01$. E, Confocal images of lymphatic endothelial cells (LECs) after static or flow conditioning, immunostained for phalloidin (green) and caveolin-1 (cav-1; red, top) or clathrin (red, bottom). Bar=10 μm. F, Quantification of cav-1 and clathrin fluorescence intensity. G, Albumin and dextran uptake by LECs. *$P<0.05$, **$P<0.01$ compared with static control condition. H, Transendothelial electric resistance of LEC monolayers after static or flow preconditioning.
in dynamin-dependent transcellular permeability mirrored an increase in vesicle density with flow (Figure 7E and 7F). Consistent with this, we found that flow-pretreated LECs could take up 2 to 3 times more albumin or dextran compared with static conditions (Figure 7G). No changes in TEER were observed after flow treatment (Figure 7H). Together, these results suggest that transmural fluid flow upregulates caveolae and CCVs in LECs, thereby increasing solute uptake and transcytosis.

**Discussion**

Transport across the lymphatic endothelium has been described by the primary valve system hypothesized by Schmid-Schönbein et al. and by the evidence collected in the work of Trzewik et al. Lymph formation is regulated by intercellular junctions that open and close according to phases of compression and expansion of the initial lymphatics. Lymphatic capillaries have in fact been described as having button-like, loose overlapping cell–cell junctions, and this architecture is likely responsible for the assumption that paracellular transport dominates lymph formation. Nevertheless, the lymphatic endothelium has been shown to be rich in vesicles, and tracer particles injected into the interstitial space have been identified within vesicles of initial lymphatic vessels. In addition, we have previously shown that LECs can take up extracellular protein from the interstitial space and process it intracellularly for loading onto their cell–cell junctions. On the contrary, lymph has similar composition and thus osmotic pressure as interstitial fluid, and hydrostatic pressure differences are small, obviating the need for as much of a barrier to fluid transport in lymphatic capillaries as in blood vessels, mirrored by the junctional structure in the different cell types (loose button-like junctions in LECs versus tight junctions in BECs). Furthermore, in BECs, transcytosis and paracellular permeability are coregulated through a signaling pathway—linking dynamin, Rac, and actin. The inhibition of transcytosis by dynamin blockade increases paracellular leakage concomitantly with the loss of cortical actin from the plasma membrane and the displacement of active Rac from the plasmalemma. However, we did not observe any change in TEER after dynasore treatment, suggesting differential regulation of paracellular permeability in LECs versus BECs that is likely because of the different conformations of cell–cell junctions.

Given that pretreating LECs with transmural flow increases their $P_{\text{eff}}$ as we previously showed, we next asked whether this was because of an increase in paracellular pathways as would be the case with blood vascular leakage during inflammation. However, we found that dynamin inhibition had a stronger effect than junction stabilization in reversing the flow-dependent increase in $P_{\text{eff}}$. This was correlated with increased presence of intracellular vesicles (both CCV and caveolae) with fluid flow, indicating that transcytosis was dominating the flow-mediated increase in $P_{\text{eff}}$. This is consistent with a previous study demonstrating that luminal shear stress caused a transient increase in TEER across LECs, suggesting that paracellular junctions may become stronger or tighter with fluid flow, although the type of flow (luminal versus transmural) was different in our study. We also looked at TEER across LECs after flow, and we found no differences, whereas we observed a reduction in junction width overlap, which is inversely correlated to paracellular permeability, suggesting an effect of flow also on the paracellular component of transport. Our results show that under fluid flow stimulation LECs react by upregulating both components of transport, even though the effect was stronger on the transcellular component in our in vitro model. This could suggest that the increased lymphatic transport after inflammatory and mechanical stimulation could be due not only to the previously described disruption of junctional integrity but also to the active modulation of the transcellular route by LECs.

It is important to note that our in vitro model of the lymphatic endothelium cannot necessarily recapitulate all aspects of the in vivo barrier function. In vivo, lymphatic capillaries exhibit button-like junctions between cells, whereas collecting vessels display zipper-like junctions. Although in vitro, the LECs used here (isolated from the human skin) present a heterogeneous phenotype that express both button-like and zipper-like junctions. Thus, if we assume that button-like junctions are more permeable to solutes, our in vitro measurements...
could overestimate the relative importance of transcellular transport compared with paracellular. On the contrary, we previously showed that cell–cell junctions in LECs seem more button like when cultured under transmural flow, and here, we found that the transcellular (dynamin-dependent) component was increased most substantially under flow (Figure 6), suggesting that the degree of button-like versus zipper-like junctions may not be as critical to solute transport as it is to cellular transmigration. Furthermore, we observed intracellular solute in LECs in vivo, highlighting that this transport route might be relevant in vivo. Thus, despite the potential limitations of the in vitro model, our major conclusions that transcellular solute transport (1) is an important component of lymph formation and (2) is actively modulated by transmural flow and other stimuli are maintained. To quantify the relative contributions of transcellular versus paracellular solute transport in vivo, a means of selectively inhibiting vesicular uptake in LECs (but not blood endothelium) is needed in the future. This will allow evaluating the real effect of transcellular mechanisms in the in vivo situation, where paracellular transport is likely to be favored by the stimuli of compression and expansion given by external forces.

Another implication of active transport in LECs is related to the recent evidence that points at new functions of LECs in promoting peripheral immune tolerance. We recently showed that a foreign antigen expressed by implanted tumor cells could be taken up and cross-presented by tumor-associated LECs, which, when isolated, could drive dysfunctional activation of cognate CD8+ T-cells and promote tumor progression. Another study from our group has shown that LECs can actively scavenge proteins to directly suppress T-cell activation, either alone or in combination with activated dendritic cells. In this context, active transport mechanisms would allow the lymphatic endothelium to modulate the uptake of tissue antigens and regulate their presentation on LECs to regulate adaptive immunity. In addition, by selectively and actively regulating the rate of transport of protein and peptide solutes (ie, antigens) to the lymph node, LECs may help to fine-tune antigen-specific immune responses.

In conclusion, our findings suggest that LECs actively mediate transendothelial permeability through vesicle formation and transcytosis and that the transcellular pathway is a major route for solute uptake, at least for albumin and dextran, into lymph. In addition, our data suggest that increased lymphatic permeability seen in inflammation is partly because of enhanced transcellular transport, unlike vascular leakage in blood capillaries, which is mediated mostly through disruption of intercellular junctions and thus paracellular transport. Finally, not all endocytosed solute is transcytosed across the LECs, but some remains in the cell and may respond to inflammatory cues such as transmural flow, by upregulating vesicle formation, albumin uptake, and transcellular transport. This has important consequences for the maintenance of tissue homeostasis, the resolution of inflammation, and the modulation of antigen-specific immune responses.

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Disclosures

None.

References


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Detailed Methods

Animals and cell lines
Primary human microvascular LECs were isolated from neonatal foreskin as previously described,\textsuperscript{1} cultured in endothelial cell basal medium (EBM; GE Healthcare, Little Chalfont, UK) supplemented with 20% FBS, 2 mM L-glutamax, 25 μg/ml cAMP, and 10 μg/mL hydrocortisone (all Life Technologies, Carlsbad, CA USA), and used at passages 7-9. The purity of the cell line was confirmed by positive staining for Prox1, LYVE-1, and podoplanin, and negative staining for PAL-E (data not shown). GFP-expressing LECs were obtained after transduction with pRRLSIN.cPPT.PGK-GFP.WPRE (a gift from Didier Trono, Addgene plasmid # 12252).

Eight- to ten-week-old female BALC/cByJ mice (Charles River Laboratories, Orleans, France) were used. Animals were housed in conventional facilities and all procedures were approved by the Office Vétérinaire Cantonale Vaud, Switzerland.

Immunostaining and flow cytometry
Cells cultured on transwell inserts (Corning Inc., Corning, NY) or on Lab-Tek® Chamber Slides™ (Thermo Fisher Scientific, Rochester, NY) were fixed in 2% PFA for 15 min, permeabilized with 0.02% saponin for 1 h at room temperature, incubated with mouse anti-human clathrin, rabbit anti-human cav-1, mouse anti-human VE-cadherin (all Abcam, Cambridge, UK) or FITC-conjugated CD31 primary antibodies (Enzo Life Sciences Inc., Farmingdale, NY) at 4°C overnight. Alexa Fluor 647 Phalloidin (Life Technologies) was used to stain F-actin. Secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 568/594/647 (Life Technologies) were used for detection. Slides were mounted in DAPI (4',6-diamidino-2-phenylindole)-containing Vectashield (Vector Laboratories Inc., Burlingame, CA) and imaged with a confocal microscope (LSM 700, Zeiss, Oberkochen, Germany). Image processing and quantification were performed using ImageJ (NIH, Bethesda, MD USA) and Imaris 7.1 (Bitplane AG, Zürich, CH).

Whole mount staining of mouse ears was performed as described previously\textsuperscript{2} 2 h after intradermal injection of Alexa Fluor 647-conjugated bovine serum albumin (AF647-albumin, 5μg in 1μL) in the dorsal ear dermis. Goat anti-mouse albumin polyclonal antibody (Abcam), goat anti- mouse podoplanin (R&D Systems), rabbit anti-mouse LYVE-1 (ReliaTech GmbH) and rat anti-mouse CD31 (BD) were used. The tissue was mounted on a glass slide and imaged using a confocal microscope (Leica SP5 with 63× lens, NA 1.40, Leica Microsystems, Wetzlar, Germany). Image stacks were analyzed using Imaris 7.1.

For flow cytometry analysis, mice were anesthetized with a humidified mixture of oxygen and isoflurane and the dermis of the dorsal ear was injected with 1μL AF647-albumin in Ringer buffer at concentrations of 0.05, 0.5, or 5mg/mL. Mice were sacrificed 2 h after the injection and ear tissue digestion was performed as described in Vigl et al.\textsuperscript{3} Cells were then stained for gp38, CD31, CD45, CD11c, CD11b and analyzed by flow cytometry (CyAn ADP Flow Cytometer, Beckman Coulter, Pasadena, CA). Data analysis was performed using FlowJo (v9.2, Tree Star Inc., Ashland, OR).

Cell uptake and cell release assay
Cells were treated with a panel of active transport inhibitors at the following concentrations: Dynasore (62.5μM), a dynamin inhibitor\textsuperscript{4}; LY294002 (500nM),\textsuperscript{5, 6} and Nocodazole (10nM), an inhibitor of microtubule dynamics\textsuperscript{7} (all from Sigma-Aldrich, St. Louis, MO USA). For solute uptake studies, cells were seeded on collagen-coated 96-well plates until confluence was reached. Cells were then treated with the indicated inhibitor for 45 min before loading with 0.5mg/mL AF647-albumin and 0.5mg/mL Texas Red-conjugated dextran 70 kDa (TR-dextran, Life Technologies) for 2 h. After thorough rinsing, cells were lysed, and their fluorescence was measured in a Safire 2 plate reader (Tecan, Maennedorf, CH). For albumin and dextran release studies, cells were seeded on collagen-coated 96 well plates, grown to confluence, and then pulsed with 0.5mg/mL AF647-albumin or TR-dextran for 24 h. Cells were rinsed...
thoroughly and treated with the inhibitor solutions in 2% serum. Medium was sampled at 2, 6, 24, and 48 h and measured in a plate reader to determine albumin and dextran release kinetics.

**Double-color albumin transendothelial transport assay**

Lymphatic endothelial cells (LECs) were seeded on 0.4μm pore size transwell inserts (Corning Inc.), either on top of the insert membrane or on the opposite side, and grown to confluence. An assay was designed to compare albumin transport from the basolateral side to the apical side of LECs and vice versa. AF594-albumin and AF-647-albumin were loaded in the two chambers of the insert, one in the top chamber and one in the bottom chamber. The concentration of the two tracers was the same (10μg/mL) to avoid the effect of concentration gradients. The assay was repeated exploring different combinations, as shown in Fig. 1E and transendothelial transport was measured by sampling media from the top and the bottom chamber 4 h after adding albumin, and by estimating $P_{eff}$ as in the endothelial permeability assay.

**Junction width measurement**

LECs were seeded on 0.4μm pore size transwell inserts (Corning Inc.) and grown to confluence. Cells were then preconditioned with flow or treated with Dynasore or vehicle control (DMSO). Cells were fixed and monolayers stained with FITC-conjugated CD31 primary antibodies (Enzo Life Sciences Inc.) and DAPI. Cells were imaged with a confocal microscope (20x objective, LSM 700, Zeiss) and image processing was performed with ImageJ (NIH). Area of pixels positive for CD31 and total length of junctions were calculated and average junction width (overlap) was computed as the ratio between the area of positive pixels and the overall length of the junctional network.

**SDS-PAGE**

LECs were seeded on 0.4μm pore size transwell inserts (Corning Inc.) and grown to confluence. 10μg/mL AF647-albumin were added to the upper chamber and cells were incubated for 4 hours. Cells were lysed and cell lysates were run on a mini-PROTEAN TGX precast gel (Bio-Rad Laboratories, Hercules, CA). Medium from the lower chamber was also collected and run on the gel. The gel was then imaged with the near-infrared detection system Odyssey CLx (LI-COR Biosciences, Lincoln, NE).

**Live imaging of cell uptake and transendothelial transport**

Cells were seeded on 3μm pores FluoroBlok™ Inserts (BD Biosciences, San Jose, CA) and grown to confluence. Cells were then cultured in medium containing 2% FBS and then treated with Dynasore, to block vesicle formation, and adrenomedullin (AM, 100nM, American Peptide Company Inc., Sunnyvale, CA USA), to stabilize intercellular junctions. AF647-albumin was loaded in the upper chamber at a concentration of 50μg/mL. The porous membrane inserts were then placed in the live cell imaging instrument (Cell-IQ®, CM Technologies, Tampere, Finland) and imaged every 90 min for 24 h. Images were then processed with ImageJ; dynamic threshold was used to separate the background signal (correlating to transendothelial transport of solute) from the intracellular fluorescence (correlating to solute uptake; Online Video I).

Spinning disk confocal microscopy was used to perform high resolution analysis of cell uptake. Cells were seeded on a Lab-Tek® II Chambered Coverglass (Thermo Fisher Scientific) and incubated overnight with CellLight® Early Endosomes-GFP, BacMam 2.0 (Life Technologies). Just before imaging, cells were incubated for 5 minutes with CellMask™ Deep Red Plasma membrane Stain (Life Technologies). Cells were then rinsed with warm media and imaged with a spinning disk confocal microscope (CSU-W1 scanning unit, Yokogawa Electric Corporation, Tokyo, Japan; PCO Edge sCMOS B/W camera, PCO AG, Kelheim, Germany), equipped with 37°C chamber and CO2 supply. 15μg/mL of albumin or dextran were added to the media right before imaging and time lapse images were taken for 2 h. Image stacks were analyzed using Imaris 7.1.
**Endothelial permeability assay**

LECs were seeded on 0.4µm pore size 12mm transwell inserts (Corning Inc.) at 2×10^5 cells per insert. Cells were incubated for 48 h in order to obtain a completely confluent monolayer. After 16 h of pretreatment with transmural flow (0, 0.1, 1 or 10µm/sec), imposed with a pressure head predetermined to drive the desired velocity, flow was stopped by normalizing the transendothelial pressure differential. Dynasore (62.5μM), AM (100nM), or vehicle control (DMSO) were added to the medium (EBM with 2% FBS) during flow preconditioning. 10µg/mL TR-Dextran and 10µg/mL AF647-albumin in phenol-red free DMEM (GE Healthcare) with 2% FBS and 1%HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) were added to the upper chamber of the insert. Following 4 h in static conditions, medium from each side (apical and basolateral) was measured for fluorescence using a Safire 2 plate reader (Tecan) and calibrated to a standard curve to estimate $P_{eff}$ (µm/sec) as previously described. For transendothelial electric resistance (TEER) measurements, cells were treated with Dynasore, AM, or vehicle control for 30 minutes and TEER was measured with a Millicell®-ERS instrument (Millipore Corporation, Billerica, MA USA).

**Human serum albumin permeability and uptake**

Experimental assays were conducted in the same way described for bovine serum albumin. Human serum albumin (HSA) Cy5 labeled (Nanocs Inc., New York, NY) was used at 100µg/mL.

**Additional inhibitors and inflammatory mediators**

Filipin III was purchased from Sigma-Aldrich and used at 500ng/mL. Recombinant mouse TNFα (Biolegend, San Diego, CA) was used at 100ng/mL unless differently specified. Cells were stimulated with TNFα for 24 h in serum reduced medium before performing the experimental assays.

**Transmission Electron Microscopy**

Cells preparation for transmission electron microscopy (TEM) was done as previously described. Briefly, cells were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde, postfixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide and then stained with 1% uranyl acetate. After dehydration with graded ethanol series, samples were embedded in Durcapan. Sections of 50 nm were obtained with a diamond knife before mounting them on grids for imaging with the Philips CM10 Electron Microscope (FEI Company, Hillsboro, OR) operating at 80 kV.

**Statistical analysis**

All data shown are expressed as mean ± SD from at least 3 independent experiments. Comparisons of parameters among multiple groups were made by one-way ANOVA (analysis of variance), followed by Tukey’s test. When only two groups were compared, a Student’s t-test was used. Values of $P < 0.05$ were considered statistically significant.

**References for Detailed Methods**

Supplemental Material

Online Figure I. AF647 fluorophore is still conjugated to albumin after albumin uptake by lymphatic endothelial cells (LECs). SDS-PAGE of AF647-albumin after albumin uptake and transport by LECs. LECs were cultured on transwell inserts and 10μg/mL AF647 albumin (Alb stock) was loaded in the upper chamber. 4 hours after loading, LECs were lysed and lysate was run on the gel (LEC lysate 1, 2 and 3 from 3 independent experiments). Medium from the lower chamber was also collected and diluted 1/10 in lysis buffer (LEC medium 1, 2 and 3), before running it on the gel. The gel was then imaged with the near-infrared fluorescence detection system Odyssey CLx (LI-COR Biosciences, Lincoln, NE USA).
Online Figure II. Dynasore treatment results in reduced uptake of albumin and 70kDa dextrans. Lymphatic endothelial cells cultured on transwell inserts were treated with 62.5µM Dynasore or vehicle control (DMSO) for 45 minutes. Cells were then loaded with albumin (red) and dextran 70kDa (green) for 2 h, washed, fixed and imaged with a confocal microscope. Scale bar=5µm.
Online Figure III. Effect of active transport inhibitors on lymphatic endothelial cells (LECs) permeability. (A) Effective permeability ($P_{eff}$) of LECs to albumin and dextran, defined as solute flux over time, after treatment with 500nM LY94002. (B) Effective permeability ($P_{eff}$) of LECs to albumin and dextran, defined as solute flux over time, after treatment with 500ng/ml Filipin III. *$P<0.05$, **$P<0.01$. 
**Online Figure IV. Dynasore treatment does not affect lymphatic endothelial cell junction width.**

Lymphatic endothelial cells cultured on transwell inserts were treated with 62.5µM Dynasore (Dyn) or vehicle control (DMSO) for 20 hours. Cells were then fixed and stained with FITC-conjugated CD31 antibodies. The average junction width was calculated as the ratio between the area of CD31 positive pixels and the length of the overall junctional network.
Online Figure V. Uptake and transport of human serum albumin (HSA) by lymphatic endothelial cells (LECs). (A) Cy5 labeled HSA taken up by LECs. Scale bar=100µm. (B) Effective permeability ($P_{eff}$) of LECs to HSA after preconditioning with 0 or 1µm/s transmural flow and/or treatment with Dynasore (Dyn) or adrenomedullin (AM). $P_{eff}$ measurement was done under static conditions. *$P<0.05$, **$P<0.01$ compared to static control condition, #$P<0.05$, ###$P<0.01$ comparing indicated groups.
Online Figure VI. Transport and uptake of albumin by lymphatic endothelial cells (LECs) after stimulation with TNFα. (A) Effective permeability ($P_{eff}$) of LECs to bovine serum albumin after preconditioning with 100ng/ml TNFα and/or treatment with Dynasore (Dyn) or adrenomedullin (AM). $P_{eff}$ measurement was done under static conditions. **$P<0.01$ comparing indicated groups. (B) Dyn-dependent (Dyn-dep) and AM-dependent (AM-dep) components of $P_{eff}$ in LECs pre-treated with TNFα. *$P<0.05$ comparing indicated groups. (C) Albumin uptake rate by LECs treated with 10 or 100ng/ml TNFα. **$P<0.01$ compared to ctrl.
**Video Legends**

**Online Video I: Live imaging of solute uptake and transendothelial transport:** LECs are seeded on the bottom of 3μm pores FluoroBlok™ Inserts (BD Biosciences, San Jose, CA) and grown until confluence is reached. Fluorescent albumin (gray) is added in the top chamber and the insert membrane is imaged with an inverted objective (10x Nikon objective mounted on the Cell IQ Imaging System, CM Technologies). The movie shows the original fluorescence corresponding to albumin that crosses the insert membrane over time (left panel, time in h shown in top left corner), fluorescence in the bottom well corresponding to transported albumin (central panel) and intracellular albumin obtained after Gaussian blur subtraction from raw images (right panel). Scale bar=100μm.

**Online Video II: Live imaging of albumin taken up by LECs stained with CellLight® Early Endosomes-GFP:** Spinning disk confocal live microscopy of a lymphatic endothelial cell stained with CellLight early endosomal marker (green) and loaded with fluorescent albumin (red). Images processed with Imaris 7.1. Scale bar=20μm, frame interval=2min.