Transmural Flow Modulates Cell and Fluid Transport Functions of Lymphatic Endothelium

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Rationale: Lymphatic transport of peripheral interstitial fluid and dendritic cells (DCs) is important for both adaptive immunity and maintenance of tolerance to self-antigens. Lymphatic drainage can change rapidly and dramatically on tissue injury or inflammation, and therefore increased fluid flow may serve as an important early cue for inflammation; however, the effects of transmural flow on lymphatic function are unknown.

Objective: Here we tested the hypothesis that lymph drainage regulates the fluid and cell transport functions of lymphatic endothelium.

Methods and Results: Using in vitro and in vivo models, we demonstrated that lymphatic endothelium is sensitive to low levels of transmural flow. Basal-to-luminal flow (0.1 and 1 μm/sec) increased lymphatic permeability, dextran transport, and aquaporin-2 expression, as well as DC transmigration into lymphatics. The latter was associated with increased lymphatic expression of the DC homing chemokine CCL21 and the adhesion molecules intercellular adhesion molecule-1 and E-selectin. In addition, transmural flow induced delocalization and downregulation of vascular endothelial cadherin and PECAM-1 (platelet/endothelial cell adhesion molecule-1). Flow-enhanced DC transmigration could be reversed by blocking CCR7, intercellular adhesion molecule-1, or E-selectin. In an experimental model of lymphedema, where lymphatic drainage is greatly reduced or absent, lymphatic endothelial expression of CCL21 was nearly absent.

Conclusions: These findings introduce transmural flow as an important regulator of lymphatic endothelial function and suggest that flow might serve as an early inflammatory signal for lymphatics, causing them to regulate transport functions to facilitate the delivery of soluble antigens and DCs to lymph nodes. (Circ Res. 2010;106:920-931.)

Key Words: CCL21 ▪ ICAM-1 ▪ inflammation ▪ lymphedema ▪ in vitro ▪ overhydration

Immune functions of lymphatic endothelium include the transport of interstitial fluid to the lymph node, which provides constant sampling of peripheral antigens to antigen-presenting cells such as dendritic cells (DCs), macrophages, and B cells residing in the lymphatic endothelium, and cell transport from the periphery to the lymph node. These processes are important both for inducing an adaptive immune response as well as for maintaining tolerance to self-antigens. Despite its importance, the active regulation of fluid and cell transport by lymphatic endothelium is largely unexplored. We asked how the lymphatic endothelium might sense and respond to its local physical environment to regulate these functions, particularly with respect to inflammation and tissue injury.

Arguably, the first events in tissue injury and inflammation include the rapid release of mediators that increase the permeability of the local blood vessels, which leads to influx of plasma fluid and proteins into the interstitium, elevated interstitial fluid pressure, and increased lymph flow. This is followed by the release of inflammatory cytokines such as tumor necrosis factor (TNF)-α. Recently, several inflammatory cues including TNF-α, TNF-β, and interleukin-1, as well as pathogenic signals such as bacterial lipopolysaccharide (LPS), were shown to influence immune cell traffic into lymphatics by modulating lymphatic endothelial expression of intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule-1 (VCAM-1), and selectin (endothelial adhesion molecule 1 or CD62E), which are used by DCs for transmigration into lymphatics. Whereas the expression of such inflammatory cytokines following tissue injury can take several hours, heightened lymph flow increases almost immediately. We hypothesized that heightened transmural flow could act as an immediate cue for signaling injury or inflammatory conditions to lymphatic endothelium, driving changes in lymphatic endothelial transport functions to modulate fluid and DC trafficking to the draining lymph node. This could potentially act alone or in concert with inflammatory cytokines. Other recent evidence points to fluid shear stress as a modulator of nitric oxide release and lymphatic pump function in contractile lymphatics, and intraluminal shear stress is well known to

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regulate leukocyte adhesion and transmigration events in blood endothelium.\(^{15,16}\) However, the role of transmural flow on lymphatic endothelium and its transport functions has not been examined to date, and whereas leukocytes exiting blood vessels experience a high-shear environment before and during their transmigration, leukocytes entering lymphatics do not experience such conditions in the basal interstitium. Because lymphatic capillaries play a different role in inflammation than blood capillaries, their functional responses to environmental cues need to be separately investigated.

Here, using both in vivo and in vitro systems, we demonstrate that transmural flow can stimulate lymphatic endothelium to increase fluid transport and DC transmigration in part by increasing the DC chemoattractant CCL21 and the DC adhesion molecules ICAM-1 and E-selectin, and by decreasing the lymphatic junctional adhesion molecules PECAM-1 and vascular endothelial (VE)-cadherin. Based on these findings, we suggest that lymphatic flow is a key mediator of lymphatic function, particularly with respect to DC recruitment and trafficking to the lymph node.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Cell Lines**

Human microvascular lymphatic endothelial cells (LECs) were isolated from neonatal foreskin and cultured as described\(^{17}\); details are given in Online Data Supplement. DCs were derived from peripheral blood mononuclear cells of healthy human donors as described\(^{18}\) and matured by increasing the DC chemoattractant CCL21 and the DC adhesion molecule ICAM-1 and vascular endothelial (VE)-cadherin. Based on these findings, we suggest that lymphatic flow is a key mediator of lymphatic function, particularly with respect to DC recruitment and trafficking to the lymph node.

**DC Transmigration Assay**

Details are given in the Online Data Supplement. Briefly, LECs were cultured to confluence on the underside of collagen-coated 8-µm-pore culture inserts. Cell tracker green-labeled human DCs, suspended in 1.8 mg/mL type I collagen with 10% Matrigel, were added to the inserts and transmural flow velocities of 0.1 or 1 µm/sec were imposed via a medium pressure head between upper and lower chambers. After 12 hours, transmigrated DCs were counted. To assess the roles of ICAM-1, E-selectin, or VCAM-1, LECs were preincubated for 1 hour with the appropriate neutralizing antibody that was maintained in the gel and medium for the duration of the experiment. In some experiments, 100 ng/mL TNF-α (R&D Systems) was added. We estimated transmural flow velocities in vitro by measuring flow rates through the inserts at different pressure heads and dividing by the insert cross-sectional area.

**In Vivo Overhydration and Adoptive Transfer**

C57BL/6 CD45.1 and BALB/c mice were subjected to overhydration for 24 hours by SC injection with saline (10% to 15% of body mass). Mouse weight was measured every 2 to 3 hours, and additional saline was injected when necessary to maintain the 10% to 15% increase in body mass. The overhydration was performed for 24 hours before conductance measurements or DC transfer. Splenic DCs from C57BL/6 CD45.2 donors were isolated using magnetic sorting with CD11c microbeads (MACS Miltenyi Biotech). Bone marrow DCs from eGFP-BALB/c donors were isolated as described.\(^{19}\) Cells (5×10⁵) in 10 µL were injected intradermally into each foot. Overhydration was maintained for another 12 hours then draining lymph nodes were isolated and the total number of GFP DCs (in BALB/c mice) or CD45.1⁺CD11c⁺ cells (in C57BL/6 mice) was measured by flow cytometry.

**Lymphatic Conductance**

The functional uptake of an injected dextran solution into the initial lymphatics in the tail, as defined by the volume of fluid drained by lymphatics per tissue volume, applied pressure, and time (yielding units of mm Hg⁻¹ min⁻¹), was determined as described previously\(^{20,21}\) and detailed in the Online Data Supplement. This method allows one to differentiate the tissue hydraulic conductivity from the lymphatic conductance and treat them independently from each other.

**Mouse Tail Lymphedema Model**

Lymphedema was induced as described previously.\(^{20,22}\) Briefly, a circumferential incision was made through the dermis close to the tail base to sever dermal lymphatic vessels without disturbing major blood vessels. The edges of this incision were then pushed apart with forceps, creating a ~2-mm gap to delay wound closure. Tissue was excised after 7 days for CCL21 determination of CCL21 expression associated with lymphatic capillaries (LYVE-1⁺ structures).

**Permeability Assay**

LECs were seeded on the bottom of 0.4 µm pore size inserts (Costar) as described above and cultured for 24 hours. After 12 hours of pretreatment with 0.1 or 1 µm/sec transmural flow, flow was stopped by normalizing pressure and 10 µg/mL LPS (Invitrogen) for 48 hours.

**Small Interfering RNA Transfection of DCs and LECs**

Small interfering (si)RNA transfection of DCs and LECs was performed using a Nucleofector kit (Amaxa, Lonza) according to the protocol of the manufacturer. Control siRNA and siRNA targeting CCR7 on DCs and ICAM-1 on LECs were used (Santa Cruz Biotechnology). Knockdown efficiency was tested for CCR7 in DCs and ICAM-1 in LECs by flow cytometry after 48 hours and following 12 hours of stimulation with 10 ng/mL TNF-α.

**Protein and Gene Expression**

CCL21 secretion from LECs was evaluated by ELISA (R&D Systems) after the collagen/Matrigel matrix was removed and digested in Collagenase D (Roche). Gene expression was evaluated after TRIzol (Invitrogen) extraction from cells in the matrix when required, cell lysis with RNA lysis-binding solution (Ambion) and

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**Non-standard Abbreviations and Acronyms**

- DC: dendritic cell
- ICAM: intercellular adhesion molecule
- LEC: lymphatic endothelial cell
- LPS: lipopolysaccharide
- PECAM: platelet/endothelial cell adhesion molecule
- siRNA: small interfering RNA
- TNF: tumor necrosis factor
- VCAM: vascular cell adhesion molecule
- VE: vascular endothelial
RNA extraction (RNAsous-Micro; Ambion); cDNA synthesis was performed with iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Gene expression was normalized to GAPDH and then to the static control for each experiment. Primer sequences are given in the Online Data Supplement.

Flow Cytometry and Immunostaining
DCs were isolated from their matrix with collagenase D and analyzed by flow cytometry (CyAN; Dako) using FlowJo software (Tree Star Inc). For immunostaining, cultured cells or tail skin cryosections were fixed in 2% paraformaldehyde, and incubated with primary and secondary antibodies (detailed in the Online Data Supplement). Actin was visualized using AlexaFluor-647 phalloidin (Invitrogen). Sections were mounted in DAPI-containing Vectashield (Vector Laboratories) and viewed under a LSM 510 META microscope (Zeiss). Image quantification was done in Matlab (Mathworks) and Metamorph 6.3 (Molecular Devices). Huygens (Scientific Volume Imaging) and Imaris (version 6, Bitplane) were used for isosurface rendering as described.23

Statistical Analysis
Kruskal–Wallis with Tukey post tests or Mann–Whitney U tests (when comparing only 2 groups) were used; \( P \leq 0.05 \) was considered significant. Means±SD are shown in all bar graphs; medians±95% confidence intervals are shown in box plots.

Results
Transmural Flow Regulates the Fluid Transport Function of Lymphatic Endothelium
To determine whether transmural flow regulates fluid transport and influences DC migration into lymphatic vessels (Figure 1a), we used an in vitro 3D model that recapitulates both the migratory environment of the interstitium and the basal-to-apical fluid flow and DC transmigration directions (Figure 1b). Whereas transmural flow across lymphatic endothelium has not been measured, interstitial flow has been measured in normal (noninjured) tissues and found to be on the order of 0.1 m/sec,24 which is likely to be within the same order of magnitude as bulk-averaged transmural lymph flow velocities (but not actual velocities in cell–cell junctions). In acute inflammation, lymph flow can increase by an order of magnitude or more.6–8 Therefore, we chose to...
compare bulk transendothelial flow velocities of 0, 0.1, and 1 μm/sec to represent static, low flow, and high flow conditions, respectively, which very roughly correlates with measured interstitial flow velocities in normal versus inflamed tissue24-25 (noting that there is likely to be a large range depending on the animal, tissue, state of activity, type of inflammatory agent, etc).

We found that after 12 hours of 0.1 or 1 μm/sec transmural flow preconditioning, the permeability of lymphatic endothelium to 3-kDa dextran was increased (Figure 1c); we note that the permeability was measured under static conditions for 3 hours in all cases. The presence of mature DCs did not affect this permeability. Because TNF-α is known to increase blood endothelial permeability,26,27 we compared the flow effects on LECs with those of TNF-α and found that the flow-enhanced permeability was of a similar magnitude as that attributable to 100 ng/mL TNF-α (Figure 1c). In vivo, lymphatic conductance was nearly doubled after 24 hours of overhydration (Figure 1d). To further assess the impact of transmural flow on lymphatic permeability, we looked at flow-induced changes to Aquaporin-2 channels, which regulate water flux in renal epithelial cells and which are abundant on LECs. Following 1 μm/sec transmural flow for 12 hours, we observed significantly more aquaporin-2 protein expression in LECs (Figure 1e and 1f). These data suggested that lymphatic transport of water and solutes is sensitive to transmural flow.

Flow Downregulates and Delocalizes VE-Cadherin and PECAM-1

Lymphatic cell–cell junctions are composed of vascular endothelial (VE)-cadherin and platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31), along with other tight junctional molecules including occludin and zona occludens-1 (ZO-1).21 In lymphatic capillaries, VE-cadherin appears as discontinuous “buttons” that pin down overlapping lymphatic endothelial “flaps” expressing PECAM-1.23 In blood endothelium, VE-cadherin and PECAM-1 both help regulate leukocyte extravasation.28-30 Importantly, PECAM-1 and VE-cadherin have been shown, together with VEGFR-2, to comprise a flow mechanosensory complex in blood endothelium, and vessels of PECAM-1−/− mice do not activate NF-κB in response to disturbed flow.31 We therefore wondered whether slow transmural flow could affect these junctional molecules in lymphatic endothelium.

In vitro, we found that the expression patterns of VE-cadherin and PECAM-1 in LEC junctions changed dramatically in response to transmural flow (Figure 2a). In static conditions, VE-cadherin expression appeared continuous along cell–cell borders, and PECAM-1 was present on overlapping cell–cell surfaces (Figure 2a, yellow arrow). In contrast, under flow conditions, these junctions appeared disorganized (Figure 2a), and the expression of VE-cadherin and PECAM-1 decreased both at the protein (Figure 2b) and gene (Figure 2c) levels. Flow also caused the two molecules to become spatially delocalized in both the x-y plane (Figure 2d) as well as in the z direction (Figure 2e and 2f), where VE-cadherin localized on the basal side relative to PECAM-1 (Figure 2a and 2f). In vivo, overhydration triggered decreases in expression of lymphatic (LYVE-1−)–associated VE-cadherin and PECAM-1 as compared to those in control conditions (Figure 2g; Online Figure I, A). In contrast, we found no changes in expression of ZO-1 or occludin (Online Figure I, B through D). Furthermore, expression of β-catenin, which binds the cytoplasmic domains of PECAM-1 and VE-cadherin32 and could also be triggered by mechanical stress,31 was unchanged (Online Figure I, D). Therefore, of the junctional molecules examined, only PECAM-1 and VE-cadherin were strongly affected by transmural flow. This was consistent with the increases in permeability observed (Figure 1), because the organization of VE-cadherin is strongly correlated with vascular permeability in blood endothelium.32

Transmural Flow Regulates DC Migration Across Lymphatic Endothelium

We next asked whether transmigration of DCs across lymphatic endothelium was influenced by transmural flow, using our in vitro model (Figure 1b) along with the in vivo model of adoptive transfer of DCs into overhydrated mice. In both cases, 12 hours was determined to be optimal for observing sufficient numbers of transported DCs (Online Figure II and data not shown). In vitro, transmigration of mature DCs (CD11c+CD86+CCR7+) was higher in 1 μm/sec flow versus static conditions (Figure 3a). This enhancement (∼3-fold) was similar to that driven by TNF-α, but together, flow and TNF-α synergized to increase DC transmigration 5-fold (Figure 3a). In vivo, 4 times more adoptively transferred DCs migrated to lymph nodes in overhydrated mice as compared to in control mice (Figure 3b); however, we note that in addition to increased lymphatic flux, overhydration may cause other changes that could directly affect DC. Interestingly, although DCs were frequently observed at the junctional convergence between 2 or 3 adjoining cells (Figure 3c), as previously reported,33 we infrequently observed in vitro DCs transmigrating through the LEC cytoplasm (Figure 3d) only under flow conditions. Taken together, these data indicate that the elevated lymph drainage facilitates DC transmigration into lymphatic vessels and that both transcellular and paracellular routes may be used, at least in cultured LECs.

CCL21 Expression and Secretion From Lymphatic Endothelium Is Flow-Dependent

One possible mechanism of flow-enhanced DC trafficking is the modulation of relevant chemokines in response to flow stimulation. Chemokine (C-C motif) ligand 21 (CCL21, SLC, or 6Ckine) is secreted by lymphatic endothelium to attract mature DCs that express its receptor, C-C chemokine receptor type 7 (CCR7).34 In static culture, LEC expression of CCL21 protein was very low, but transmural flow visibly and significantly enhanced CCL21 protein, as determined by immunostaining and ELISA (Figure 4a through 4c). In vivo, lymphatic vessels in 7-day lymphedematous skin, which experience greatly reduced flow,20,21 showed virtually no CCL21 expression compared to skin of control and overhydrated mice (Figure 4d and 4e). At the mRNA level, we found time-dependent expression profiles of CCL21, with increased
at 6 hours followed by a decrease at 12 hours (Figure 4f). Furthermore, both LECs and CCR7 signaling were required for the flow-enhanced DC transmigration shown earlier (Figure 1b), because blocking CCR7 or removing LECs reduced DC transmigration to levels seen in static conditions (Figure 4g).

We note that small increases in LEC-independent DC transmigration were observed under flow conditions (Figure 4g), reminiscent of flow-induced “autologous chemotaxis” previously described,35 but because autologous CCL19 chemokine secretion by DCs was quite low (Figure 4h) and CCR7 expression was unchanged by flow (Figure 4i), we

Figure 2. Transmural flow downregulates and delocalizes VE-cadherin and PECAM-1 on lymphatic endothelium. a, Immunostaining for VE-cadherin (red) and PECAM-1 (green) with 3D reconstruction of LEC junctions in vitro after 12 hours. In static conditions, PECAM-1 appears on overlapping portions of LECs surrounded by VE-cadherin (yellow arrows); in flow conditions, PECAM-1 and VE-cadherin appear delocalized and downregulated, with VE-cadherin localizing to the basal side (lower right). Bar, 20 μm. b, Image quantification shows downregulation of VE-cadherin and PECAM-1 with 1 μm/sec flow after 12 hours. c, Quantitative PCR for VE-cadherin and PECAM-1 in LECs after 12 hours of treatment with transmural flow or with DCs. d and e, Colocalization of VE-cadherin and PECAM-1 in the x-y plane (d) and z plane (e). f, Relative distributions of VE-cadherin and PECAM-1 show that VE-cadherin becomes localized to the basal surface on flow activation. g, Quantification of in vivo immunostaining for VE-cadherin and PECAM-1 (associated with LYVE-1⁺ lymphatic structures) after 24 hours of overhydration (OH).
concluded that the flow-enhanced DC transmigration across LECs was attributable mainly to LEC activation by flow rather than direct effects on the DCs themselves. To further support the hypothesis that flow-enhanced DC transmigration was LEC CCL21-dependent, we silenced CCR7 in DCs (with $60\%$ knockdown, Figure 4j) and found corresponding knockdown of flow-enhanced effects (Figure 4k) but no effects on static transmigration levels. Similarly, selective inhibition of CCL21 from LECs following pretreatment with neutralizing antibodies significantly reduced DC transmigration only in flow conditions (Figure 4l), implying that flow-enhanced DC transmigration was at least partly mediated by LEC-derived CCL21, which increases with flow.

ICAM and E-Selectin Are Regulated by Flow to Facilitate DC Transmigration

Although CCL21 secretion provides directional cues for the DC to migrate toward the lymphatic vessel, adhesion molecules may also be required for transmigration into the vessel. ICAM-1, VCAM-1, and E-selectin were previously found to be upregulated on TNF-$\alpha$-stimulated LECs to facilitate DC transmigration across inflamed lymphatic endothelium, suggesting that lymphatic capillaries may sense and respond to inflammatory cytokines via upregulation of adhesion molecules. In vitro, we found that $1\ \mu m/sec$ transmural flow upregulated protein expression of ICAM-1, VCAM-1, and E-selectin after 12 hours (Figure 5a and 5b). In vivo, only

Figure 3. DC transmigration across lymphatic endothelium is increased by transmural flow and occurs through both transcellular and paracellular routes. a, In vitro DC transmigration across cultured LECs after 12 hours of transmural flow and $100\ ng/mL$ TNF-$\alpha$ treatment. b, In vivo DC migration to the draining lymph node (LN) 12 hours after adoptive transfer in control vs overhydrated mice. White and black circles represent data from BALB/c and C57BL/6 mice, respectively. c and d, Paracellular (c) and transcellular (d) transmigration routes of DCs were seen in vitro. Top row, Confocal images of DCs (green, CD11c) in the process of transmigration across LECs (violet, phalloidin; red, VE-cadherin). Right and bottom insets to the first row of images show cross-sections in the y and x directions, respectively, with dotted lines indicating the insert membranes. Bar, $10\ \mu m$. Bottom row, Scanning electron micrographs show DC migration through both paracellular (left) and transcellular (right) pathways. Bars: $10\ \mu m$ (left); $4\ \mu m$ (right).
Figure 4. Transmural flow increases CCL21 secretion by lymphatic endothelium. a, Representative confocal images show CCL21 (green) expressed by LECs after 12 hours of exposure to 0 (static), 0.1, and 1 μm/sec flow; red, VE-cadherin. Bar, 20 μm. b, Image quantification of CCL21 protein in cells exposed to 12 hours of flow vs static conditions. c, CCL21 protein measured by ELISA after 24 hours of culture. d, Representative images of lymphatic vessels (red, LYVE-1) and CCL21 (green) in lymphedematous (LE), control, and overhydrated (OH) skin. Arrows indicate lymphatic vessels. Bar, 50 μm. e, Quantification of CCL21 staining in cultured LECs after 12 hours of transmural flow. f, Real-time PCR expression after 6 and 12 hours of flow treatment. g, DC transmigration across a LEC monolayer with control IgG or anti-CCR7 blocking antibodies. h, CCL19 gene expression by DCs in 3D cultures after 12 hours of static or flow conditions. i, Representative histogram from flow cytometry showing no differences in CCR7 expression by DCs in static (dotted line) and 12 hours of flow (1 μm/sec (solid line)) conditions; shaded area shows the negative control. j, Real-time PCR for CCR7 expression in DCs after 24 hours of siRNA transfection. k, DC transmigration across a LEC monolayer following DC transfection with control or CCR7 siRNA. l, CCL21 blocking inhibits the flow-enhanced DC transmigration across LECs. *P<0.05 compared to static controls.
ICAM-1 and E-selectin protein levels were significantly increased on lymphatic endothelium after 24 hours of overhydration when compared to control conditions (Figure 5c and Online Figure III); again, we note that overhydration may cause other tissue changes as well as increased fluid flow. In addition, quantitative PCR revealed time-dependent gene expression profiles (Figure 5d); ICAM-1 and VCAM-1 were strongly increased after 6 hours of flow but decreased to baseline levels at 12 hours, whereas E-selectin was increased at both 6 and 12 hours but only at 1 μm/sec flow.

Moreover, when comparing protein and gene expression in cultured LECs under flow and in the presence or absence of...
mature DCs, we observed that, together, flow and DCs caused a synergistic increase in ICAM-1 and E-selectin compared to either flow or DCs alone (Figure 5d, black bars). VCAM-1 expression was not changed upon addition of DCs in either static or flow conditions. ICAM-1 and VCAM-1 were recently shown to be involved in migration of T cells in the basal to apical direction of lymph node-associated lymphatics. However, in vitro, all were expressed on both the apical and basal sides of the LEC monolayer (Figure 5e).

To determine whether the flow-enhanced DC migration was dependent on this upregulation of adhesion molecules, we selectively blocked ICAM-1, E-selectin, and VCAM-1 in vitro. Blocking either ICAM-1 or E-selectin alone completely inhibited the flow-enhanced increase in DC migration, whereas blocking VCAM-1 had no significant effect (Figure 6a). Interestingly, DC transmigration under static conditions was not reduced following blocking antibody treatment (Figure 6a), consistent with recent findings that DC transmigration into lymphatics could be integrin-independent under steady-state conditions.

To determine whether the neutralizing antibodies affected LEC permeability, we measured permeability after 12 hours of flow with concurrent blocking of ICAM-1, E-selectin, or VCAM-1 and found no differences in $P_{\text{eff}}$ between any groups (Figure 6b). Furthermore, because DCs also express ICAM, we blocked ICAM on DCs in the absence of LECs and found this to have little effect on transmigration (Figure 6a). In contrast, when ICAM-1 was knocked down by 40% in LECs (Figure 6c), flow-enhanced DC transmigration was also decreased by 40% (Figure 6d).

Finally, we assessed whether interstitial flow could influence DC expression of lymphocyte function-associated antigen-1 (LFA-1 or CD11a), macrophage-1 antigen (MAC-1 or CD11b), CD44, and very-late antigen-4 (VLA-4 or CD49f), which are the DC-expressed ligands for ICAM-1, E-selectin, and VCAM-1, respectively, as well as CD51, which is involved in PECAM-1 interactions; we found no flow effects on surface expression by flow cytometry (Figure 6e) or gene expression (Figure 6f). Together, these findings suggest that flow-mediated, ICAM-1– and E-selectin–dependent DC transmigration primarily results from changes in the lymphatic endothelium in response to flow.

**Discussion**

Fluid drainage into lymphatic capillaries can change rapidly and dramatically on tissue injury or inflammation, and our results demonstrate that the lymphatic endothelium is highly sensitive to this biophysical cue in regards to its fluid and cell transport functions. This likely has the effect of optimizing the delivery of fluid (and presumably soluble antigens), as
well as DCs to the draining lymph node after injury or inflammation. Specifically, transmural flow across a lymphatic endothelium led to (1) increased CCL21 secretion, which guides DCs to the lymphatics and lymph nodes\(^\text{37,38}\); (2) reorganization and downregulation of PECAM-1 and VE-cadherin, which was consistent with increased lymphatic permeability; and (3) upregulation of ICAM-1 and E-selectin (and to a lesser extent VCAM-1), which facilitate DC transmigration into lymphatic vessels under flow conditions. Moreover, the flow enhancement of DC migration was dependent on CCR7, E-selectin, and ICAM-1.

Following injury or inflammation, the way that local lymphatic capillaries respond may be relevant to the type of immune response subsequently triggered. For example, after subcutaneous antigen delivery, it was shown that MHCIIC presentation of peptide on DCs in the draining lymph node came in two discrete stages: first from the lymph node-resident DCs that acquired antigen within the lymph node, and second the DCs that had migrated there from the injection site.\(^\text{1}\) Furthermore, a humoral response can be initiated by lymph node-resident B cells that uptake antigen directly in the node follicles rather than by migrating B cells or exposure to DCs.\(^\text{2}\) These highlight the importance of lymph flow, which delivers antigen to the lymph node, to adaptive immunity. Whereas increased levels of cytokine production is observed from 4 hours, peaking at 16 hours after challenge in a skin lesion model,\(^\text{39}\) increased interstitial flow is an immediate response to certain types of peripheral inflammation.\(^\text{4}\) Our data show that both fluid and cell transport are enhanced on increased flow, and because flow can increase immediately, it is likely to be an important mediator of early responses to infection.

Recent work has begun to hint at the role of flow on lymphatic endothelial function. Lymphangiogenesis in the adult requires interstitial flow,\(^\text{40}\) and VEGF has a reduced ability to rescue lymphatic function in tissue with poor interstitial flow.\(^\text{41}\) Fluid stagnation in secondary lymphedema causes lymphatic hyperplasia and decreased Langerhans DC homing to the lymphatics.\(^\text{21}\) In the lymph node, flow upregulates CCL21,\(^\text{42}\) presumably to help orchestrate the interactions between naïve T cells and mature DCs, which both express its receptor CCR7. Without flow, CCL21 silences the activity of integrin lymphocyte function associated antigen-1 (LFA-1 or CD18:CD11a), which is expressed by antigen-presenting cells and binds ICAM-1, and this silencing is released after the induction of flow.\(^\text{7}\) Finally, tumor cells may use interstitial flow to home to draining lymphatics.\(^\text{35}\) Our finding that CCL21 is regulated with flow is consistent with other reports of increased CCL21 expression by lymphoid tissues following viral and bacteria infection, peripheral inflammation, or tumor invasion,\(^\text{43}-\text{45}\) all of which may be accompanied by increased lymph flow.\(^\text{68,46-48}\)

Lymph flow itself is largely regulated by vascular permeability, and increased lymph flow can be caused by the rapid increase in vascular permeability that occurs in acute inflammation or by the leaky angiogenic vessels in rapidly growing tumors. Molecules associated with regulation of vascular permeability are adherens and tight junctional proteins, particularly VE-cadherin,\(^\text{32}\) and blocking VE-cadherin causes junctional redistribution and results in elevated permeability.\(^\text{49,50}\) We observed that elevated lymphatic permeability induced by transmural flow coincided with VE-cadherin and PECAM-1 downregulation and delocalization on lymphatic endothelium. Furthermore, whereas TNF-\(\alpha\) has also been shown to increase vascular permeability,\(^\text{51}\) flow greatly increased this effect in an apparently additive manner (Figure 1c). Finally, it is possible that the VE-cadherin and PECAM-1 redistribution that we saw with flow directly resulted from the mechanical stimulus itself, because these contribute to a mechanosensory complex that regulates the well-described shear stress effects on vascular endothelium.\(^\text{31}\)

The importance of flow-dependent adhesion and transendothelial migration has been widely studied on blood endothelium, but in blood, leukocytes require arrest and firm adhesion against high shear stresses before transmigrating from the lumen to the extracellular matrix, whereas in lymphatics, the transmigration occurs from the matrix into the vessel lumen. In the blood, E-selectin tethers leukocytes, whereas ICAM-1 and VCAM-1 promote stable adhesion of leukocytes to the apical endothelial membrane surface.\(^\text{52,53}\) This firm binding to ICAM-1 and VCAM-1 then allows the adherent leukocytes to crawl toward intercellular junctions,\(^\text{54}\) where additional interactions with the homophilic adhesion molecules PECAM-1 and CD99 and junctional molecules including junctional adhesion molecule 1 and VE-cadherin promote diapedesis.\(^\text{55}\) In lymphatics, ICAM-1 and VCAM-1 expression on lymphatic vessels has been reported after TNF-\(\alpha\), LPS, interleukin-1, and TNF-\(\beta\) stimulation.\(^\text{9,10}\) Our findings that transmural flow, in the absence of inflammatory cytokines, can upregulate these same molecules points to flow as a biophysical cue of acute inflammation. Furthermore, our demonstration (using blocking antibodies, Figure 6a) that ICAM-1 and E-selectin were only required for flow-enhanced DC transmigration, but not DC transmigration under static conditions, indicates that the flow-induced up-regulation of these molecules on LECs is mechanistically responsible for the enhanced DC transmigration seen under flow conditions. This is consistent with observations of integrin-independent DC transmigration into lymphatics in steady-state conditions.\(^\text{33}\)

These results have important implications for lymphedema as well as for injury and inflammation. As the converse of heightened flow rates in inflammation, lymphedema is characterized by dysfunctional lymphatic transport that leads to an accumulation of interstitial fluid in the affected limb. Edematous tissues have exhibited impaired immune cell scavenging and susceptibility to infection.\(^\text{56}\) Our findings suggest that the impaired DC trafficking noted in lymphedema\(^\text{21}\) are likely also dependent on the reduced interstitial flow velocities resulting from downstream lymphatic insufficiencies.

In conclusion, by implicating lymphatic fluid flow as a regulator of lymphatic function, our findings reveal that lymphatic flow is a key mediator of interactions between lymphatic endothelium and DCs. Because increased lymphatic drainage attributable to blood vessel hyperpermeability occurs immediately on tissue injury and in acute inflammatory events, our data hint at the possibility that the interstitial fluid flow increase acts as a preconditioning mechanism that
affect lymphatic endothelial cell function with respect to immune cell trafficking.

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Disclosures
None.

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**Novelty and Significance**

**What Is Known?**

- Lymphatic transport of dendritic cells and fluid from the periphery to the lymph node is important in injury and inflammation.
- Lymphatic drainage (fluid flow) can be rapidly increased on injury or inflammation.
- There is no knowledge to date on whether or little understanding how fluid flow can modulate lymphatic transport functions.

**What New Information Does This Article Contribute?**

- This is the first demonstration that lymphatic endothelium is sensitive to transmural fluid flow and modulates fluid and dendritic cell transport functions in response.
- This article introduces the concept that biophysical aspects of inflammation (ie, elevated transmural flow from blood to lymphatic capillaries) can directly regulate lymphatic biology, even in the absence of inflammatory mediators.

Lymphatic capillaries collect interstitial fluid and dendritic cells from the periphery and deliver them to the lymph nodes for immune surveillance and tolerance maintenance. Lymphatic drainage can increase rapidly on injury and inflammation, and we hypothesized that fluid flow could act as biophysical mediator of immunologic transport functions of lymphatic capillaries. We demonstrate that lymphatic endothelium is exquisitely sensitive to transmural flow, modulating both fluid and cell transport functions even in the absence of inflammatory cytokines. Flow increased lymphatic permeability and dendritic cell transmigration, which were coincident with changes in gene and protein expression of related factors. These data provide the first evidence that lymphatic endothelium can regulate its transport functions in response to biomechanical cues. They also show for the first time that transmural flow can, in the absence of inflammatory cues like tumor necrosis factor-alpha, drive expression of dendritic cell adhesion molecules like intercellular adhesion molecule-1 and chemokines like CCL21 by lymphatic endothelium. This new concept of flow-mediated lymphatic activation highlights how sensitive the lymphatic capillaries are to their extracellular environment and how they use multiple cues to sense inflammation and danger, modulating their transport functions accordingly to regulate the delivery of antigens and immune cells to the lymph nodes.
Transmural Flow Modulates Cell and Fluid Transport Functions of Lymphatic Endothelium

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Supplementary Material

DETAILED METHODS

Cell Lines. Human microvascular LECs (defined as CD31⁺ endothelial cells positive for the lymphatic-specific markers Prox-1, LYVE-1, and podoplanin, while negative for blood-specific PAL-E) were isolated from neonatal foreskin as described, cultured in endothelial cell basal medium (EBM; Cambrex BioScience) supplemented with 20% FBS, 2 mM L-glutamax, 25 µg/ml cAMP, and 10 µg/mL hydrocortisone (all Invitrogen), and used at passages 7-9. DCs were derived from peripheral blood mononuclear cells (PBMCs) of healthy human donors as described and matured with 0.2 µg/ml LPS (Invivogen) for 48 h.

Mice. 8-10 week old female C57BL/6 and BALB/c mice were obtained from Charles River Laboratories. Transgenic CD45.1 mice on a C57BL/6 background and eGFP-BALB/c mice were obtained from Jackson Laboratories. All experimental procedures were approved by the Office Vétérinaire Cantonale Vaud, Switzerland.

DC Transmigration assay. LECs were seeded on the undersides of 12mm, 8µm pore culture inserts (Millipore) coated with type I collagen (50µg/ml, BD Biosciences) at 10⁵ cells/insert. After 2h at 37°C the wells were turned and cultured in EBM medium for 24h. Human DCs (10⁶ cells/ml) labeled with Cell tracker green (1 µM, Invitrogen) were suspended in type I collagen (1.8 mg/ml) with 10% growth factor reduced Matrigel (BD Biosciences), and this mixture was added to the inserts (100µl each). After 30 min, a pressure head of 0.1 or 1 cm culture medium (basal EMB with 2% FBS and 0.1%BSA) was imposed to create average flow velocities of 0.1 or 1 µm/s, respectively. After 12h at 37°C, the gel was removed with a cotton swab, inserts were fixed in 2% PFA, and transmigrated DCs were counted. To assess the roles of ICAM-1, E-selectin, or VCAM-1 on DC transmigration, LECs were pre-incubated for 1h with the appropriate neutralizing antibody (see online supplementary methods); blocking antibodies were maintained in the gel and medium for the duration of the experiment. In some experiments, 100 ng/ml TNF-α (R&D Systems) was added to the culture.

In vivo overhydration. C57BL/6 CD45.1 and BALB/c mice were subjected to overhydration by s.c. injection with saline (10-15% of body mass). Mouse weight was measured every 2h-3h, and additional saline was injected when necessary to maintain the 10-15% increase in body mass.

Lymphatic conductance. The functional uptake of an injected dextran solution into the initial lymphatics in the tail, as defined by the volume of fluid drained by lymphatics per tissue volume, applied pressure, and time (yielding units of mmHg⁻¹min⁻¹), was determined as described previously. This method allows one to differentiate the hydraulic conductivity from the lymphatic conductance and treat them independent from each other. Briefly, a 30-gauge needle catheter containing 0.9% NaCl with 1% FITC-conjugated dextran (70 kDa, Sigma) was placed intradermally into the tip of the tail of an anesthetized mouse situated under a stereomicroscope (MZ16FA, Leica) on a temperature-controlled stage. The catheter was attached to a pressure reservoir that allowed controlled, slow infusion into the tail and uptake into the lymphatic capillaries without tissue swelling. The fluorescent dextran, once in the tail, could either travel through the interstitial space (linearly with pressure change) or get taken up and transported by the lymphatic capillaries. The infusion flow rate was continually monitored; then pressures were changed and flow rates monitored for 30 minutes per pressure setting (45, 50, 55, and 60 cm H₂O). By comparing the flow rate and the moving diffusive fluorescence front with pressure, the lymphatic conductance was calculated. Measured values in overhydrated mice were then normalized to their littermate controls.
**Antibodies.** For function blocking in vitro, mouse anti-human ICAM-1, VCAM-1, E-selectin, or mouse IgG were used (all R&D Systems). For cultured human cells, the following monoclonal anti-human antibodies were used: ICAM-1, E-selectin, and VCAM-1 (R&D Systems), VE-cadherin (BD Biosciences), CD11c (Serotec), and FITC-conjugated PECAM-1 (Ancell); goat anti-human CCL21 (R&D Systems), mouse anti-human Aquaporin2 (Chemicon), and rat anti-human Podoplanin (Invitrogen), rabbit anti-human ZO-1 (Abcam). For mouse skin sections, rabbit anti-mouse LYVE-1 (ReliaTech), goat anti-mouse CCL21, ICAM-1, and VCAM-1 (R&D Systems), rat anti-mouse VE-cadherin, E-selectin, and PECAM-1 (BD Pharmigen) were used. Alexa Fluor 488 or Alexa Fluor 567/594/647 conjugated IgGs (Invitrogen) were used for secondary antibodies. For flow cytometry, PE-Cy7-conjugated rat anti-human CCR7 (BD Biosciences) and the following anti-human antibodies were used: CD11c-APC (eBioscience), PE-conjugated (BD Biosciences), and FITC-conjugated ICAM-1, CD11a, CD11b, CD44, CD49d and CD51 (Ancell). For each antibody, the relevant isotype controls were used as negative controls.

**Immunostaining.** Cultured cells or tail skin cryosections were fixed in 2% PFA for 15 min, incubated in primary antibodies, at 4°C overnight, washed and incubated secondary antibodies. Actin was visualized using Alexa Fluor 647 phalloidin (Invitrogen). Sections were mounted in DAPI-containing Vectashield (Vector Laboratories) and viewed in a LSM 510 META microscope (Zeiss). Image quantification was done in Matlab (Mathworks) and Metamorph 6.3 (Molecular Devices).

**Real-time PCR.** Forward and reverse primer sequences are: CCL-21, AGCCTCTTAAGGTTCGTTG and GTCCGCTTCGACACCTTG; CCR-7, CCGAGACCACCAACCTTC and AGTCATTGCAGCTCTCCCTATCC; ICAM-1, ACCTATGGCAACGACTCCTTCT and GTTGCTCTGCTCTGCTCTCC; E-selectin, TGTGAGCAGGGGAGTGAAG and TGGTGACAGCATCCTATCC; VCAM-1, CGAATGGGAGAAGGCTGACC and ACCAGGCTCTGTCATGTACC; VE-cadherin, ACCAGGCTCTGTCATGTACC and GCTTCCACCAACAGCTATCC; PECAM-1, GTGTGAGAATGTAATTGCCATTCC and CTCCAGACTCCACCACCCTTACTTG; CD11a, AGCCAAGTACCCCAAGAGGAAG and AGCCAACATCTGCCAAGCCATC; CD11b, TGGGAGCTTGATGCTCTGCTCTGCTTG; CD49d, CTGCCCCACCCGCTCTGATGCTC and CTCCAGACAAAGTGTGCTGCTATCCAGG; CD51, ACCCTGAGCTGGAAGATGTGG and CACTGAGATGGAGCATTGGCCTTG; ZO-1, AAACGCTATGAACTCCATCC and AATCCTAACTGACACTGAACT; Occludin, GCTTCCATTAACCTTCGCTGTTG and TCTTTGACCCCTTGGCTCTCCTC; beta catenin, CCAGCCGACCCAAGAAG and CGAATCAATCCACAGTAGCC

**Statistical analysis.** Kruskal-Wallis with Tukey post-tests or Mann-Whitney U-tests (when comparing only two groups) were used; P≤0.05 was considered significant. Mean ± s.d. are shown in all bar graphs; median ± 95% confidence intervals are shown in box plots.

**References**


**Figure legends**

**Supplementary Figure I.** Effects of transmural flow on lymphatic endothelial cell (LEC) expression of junctional molecules. a, VE-cadherin and PECAM-1 (green) colocalization with lymphatic vessels (LYVE-1, red) in normal and overhydrated (OH) mouse skin, bar = 50µm. b, zona occludens (ZO)-1 expression (green) c, Image quantification from of zona occludens immunostaining d, Gene expression from real-time PCR of ZO-1, occludin, and β-catenin on LECs show no differences between static and flow conditions.

**Supplementary Figure II.** Flow-induced DC transmigration is time-dependent. Flow enhance DC transmigration is optimal after 12h of flow, at early time point basal level of transmigration in static and in flow condition is low and the effect of flow is weak.

**Supplementary Figure III.** Lymphatic expression of ICAM-1, E-selectin, and VCAM-1 is regulated by lymph drainage in vivo. In mouse tail skin of control, and overhydrated (OH) mice, lymphatic vessels, indicated with arrows (red, LYVE-1) shows higher expression of ICAM-1, and E-selectin(green), VCAM-1 (in green) was not affected by increased flow. Bar, 50µm.
Supplementary Figure I Increased interstitial flow regulation of junctional molecule expression in vitro and in vivo.  a, VE-cadherin and PECAM expression in vivo VE-cadherin and PECAM-1 in green, LYVE-1 in red, arrows are showing the lymphatic vessels, scale bar 50 µm. b, ZO-1 (in green) expression of cultures LECs, scale bar 20 µm. c, Quantification of the images, showing no differences between static and flow condition. d, normalized gene expression of tight junctional molecules ZO-1, Occludin and β-catenin
**Supplementary Figure II.** Flow induced DC transmigration is time dependant. Flow enhance DC transmigration is optimal after 12h of flow, at early time point basal level of transmigration in static and in flow condition is low and the effect of flow is weak.
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