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An *in Vivo* Method for Visualizing Flow Dynamics of Cells within Corneal Lymphatics

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Abstract

Background: Monitoring the trafficking of specific cell populations within lymphatics could improve our understanding of processes such as transplant rejection and cancer metastasis. Current methods, however, lack appropriate image resolution for single-cell analysis or are incompatible with *in vivo* and longitudinal monitoring of lymphatics in their native state. We therefore sought to achieve high-resolution live imaging of the dynamic behavior of cells within lymph vessels in the rat cornea.
Methods/Results: Inflammatory angiogenesis was induced by suture placement in corneas of Wistar rats. Pre- and up to 3 weeks post-induction, corneas were noninvasively examined by laser-scanning *in vivo* corneal confocal microscopy (IVCM) using only endogenous contrast. Lymph vessels and the cells harbored therein were documented by still images, real-time video, and 3D confocal stack reconstruction of live tissue. *In vivo,* conjunctival and corneal lymphatics were morphologically distinct, those with corneal location being one-quarter the diameter of those in the conjunctiva ($p < 0.001$). Cells were recruited to initially empty pre-existing lymph vessels during the first day of inflammation and maintained a dense occupation of vessels for up to 7 days. A diverse population of cells (diameter range: 1.5–27.5 μm) with varying morphology was observed, and exhibited variable flow patterns and were transported singly and in clusters of at least 2–9 adherent cells.
Conclusions: The *in vivo* microscopic technique presented enables lymph vessels and cell trafficking to be studied in high resolution in a minimally-perturbed physiologic milieu.

Introduction

The lymphatic system is composed of a network of nodes and vessels that are essential for the maintenance of fluid homeostasis and for immune surveillance in tissues and organs. The stimulated growth of new lymph vessels—lymphangiogenesis—often occurs in pathologic situations, and is implicated in cell-mediated phenomena such as the immune rejection of transplanted tissue[1] and tumor cell metastasis.[2,3] Lymphatic research has in recent years accelerated due to the development of lymphatic-specific markers (LYVE-1, Prox-1, podoplanin, and VEGFR3), robust *in vivo* lymphangiogenesis assays,[4–6] and new methods for the visualization of organism, tissue, and vessel-level lymphatics.[5,7–10] Few studies, however, have directly analyzed the cell populations present within lymphatics or their dynamic motion.[10,11] More commonly, cell populations are analyzed indirectly by *ex vivo* flow cytometric analysis after collecting cells *en masse* by cannulation or adnectomy.[1,12,13] Direct visualization and analysis of cells flowing within the lymph has not been achieved in current migration assays,[14] limiting our ability to understand the crosstalk between various cell populations and lymphatics during their migration to the lymph nodes. In particular, a small animal model for monitoring cell migration through afferent lymphatics is lacking.[14] The importance of lymphatic diseases, antigen presentation to T-cells, and dissemination of tumor cells all underscore the need for direct imaging of cell transport within afferent lymph vessels. The likely contribution of multiple, heterogeneous cell types to immune cell traffic,[12,14,15] moreover, necessitates techniques capable of simultaneously detecting and distinguishing various cell populations *in vivo*.

Recently, we reported *in vivo,* label-free imaging of lymph vessels in the rat cornea,[11] where it was additionally shown that cells transported within the lymph could be monitored in real time. Imaging and analysis of the time-dependent mobilization, transport, and flow characteristics of these cells along the pathway towards the regional lymph nodes, however, was not undertaken. Moreover, the cells observed within a small number of newly-formed corneal lymphatics appeared to have homogeneous size and morphology. The goal of this study was therefore to characterize the detailed

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morphology, flow characteristics, and time-dependent changes in the cell population transported within a larger sampling of both corneal and pre-existing limbal-conjunctival lymph vessels. It was found that lymph vessels harbor a morphologically heterogeneous cell population that is rapidly mobilized and sustained during inflammation. Cells are transported either singularly or in adherent groups in the limbus and conjunctiva with flow directed by valves, while cells within new corneal lymphatics exhibit varied flow rates and patterns. The *in vivo* confocal imaging technique enables live lymphatics and cells to be studied in real time, in 3D, or in high-resolution live histologic sections. This imaging technique therefore presents the possibility to characterize the direct effect of external stimuli or agents on the cells transported within the lymph longitudinally, and could thereby provide insights into cell-mediated processes such as tissue rejection and metastasis.

Methods and Materials

Rat model of suture-induced inflammatory corneal neovascularization

Animal investigations in this study were conducted according to guidelines for the Use of Animals in Ophthalmic and Vision Research from the Association for Research in Vision and Ophthalmology (ARVO) and with approval from the Linköping Regional Animal Research Ethics Committee (Protocol 73-09). All procedures were performed under general and local anesthesia, and all efforts were made to minimize suffering. Twelve- to 16-week-old male Wistar rats weighing 200 to 400 g (Scanbur AB, Sollentuna, Sweden) were anaesthetized using intraperitoneal injection of dexdomitor and xylazine (20 mg/kg body weight and 75 mg/kg body weight, respectively), and topical tetracaine 0.1% eye drops were instilled. Under an operating microscope, a single 10-0 nylon suture was placed through the corneal stroma approximately 1.5 mm from the temporal limbus at the 9 o'clock position in the right eye. Suture placement was chosen to limit neovascularization to a well-defined area and to facilitate access to the vessels by the *in vivo* confocal microscope. At day 7 after suture placement, the cornea was fully vascularized from the limbus to the suture, as previously depicted.[16] Both prior to, and at 1, 4, 7, 14, and 21 days after the initial surgery, rats were anesthetized and laser scanning in vivo confocal microscopy (IVCM; HRT3-RCM, Heidelberg Engineering, Heidelberg, Germany) of the corneas was performed.

Laser-scanning in vivo *confocal micoscopy (IVCM)*

Corneas from anesthetized rats and a patient suffering from a corneal inflammatory condition were examined with IVCM.[17] In order to avoid pressure-induced artifacts that could influence vessel diameter and cell movements, the IVCM objective was carefully adjusted on the cornea.[11] The equipment and IVCM procedure have been described in detail elsewhere,[16] but briefly, a 63×/0.95 NA water-immersion objective (Zeiss) is placed, via a lubricant eye gel, in contact with a locally anesthetized cornea, and provides an *en face* view of a 400×400 μm corneal area at a selectable corneal depth. The microscope uses a focused red laser diode (670 nm wavelength) to scan the focal region to provide a darkfield image from light directly backscattered by tissue structures (endogenous scatter imaging). The microscope is approved for human clinical use in the cornea[17] and is known to be a noninvasive technique that does not cause corneal irritation or inflammatory reaction. With IVCM it is possible to identify and follow the same structures and thereby observe dynamic processes in live tissue. The technique, however, is limited to transparent tissues, and imaging in a nonfluorescent mode.

Acquisition and 3D rendering of ultrafine IVCM image stacks

To enable detailed analysis of cell localization within corneal lymph vessels, high resolution *in vivo* image stacks of corneal lymph vessels were acquired. Consecutive depth images were acquired using an optional motor-driven axial focusing unit for the IVCM to avoid motion-induced artifacts. The motor-driven focusing also enables confocal depth scanning at a variable axial speed. By this mechanism, an ultrafine confocal axial image spacing of 0.5 μm was achieved by acquisition of 60 consecutive confocal image frames through a focusing depth of 30 μm at a rate of 30 frames/sec. The stack of raw confocal images is rendered into a 3D model using the 3D opacity tool option in Volocity Visualization software (Volocity 6.0, Perkin Elmer Inc., Waltham, MA). Slight lateral motion of the images (due to animal respiration) was autocorrected using the image alignment tool in Volocity, resulting in smooth lymphatic vessel boundaries when viewed in cross-section using the XYZ tool. From the 3D model, any desired viewpoint, x-z, or y-z cross section could be viewed.

Analysis of corneal and conjunctival lymph vessel lumen and cell diameter

To quantify lymph vessel lumen and cell diameter, individual still IVCM image frames of lymph vessels with an established flow were used. Lymphatics were distinguished from blood vessels by their specific morphology, including a thin vessel wall with a varying diameter, and a dark lumen harboring only highly scattering leukocytes moving with variable speed.[11] Conjunctival lymphatics and their cells were measured prior to and up to 14 days after stimulated inflammation. Corneal lymphatics and their cells were measured in inflamed corneas when they could be detected by IVCM, from 4 to 14 days after stimulation. Measurements were performed using the line tool in ImageJ (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Lumen diameter was measured in each vessel segment of differing vessel diameter. Leukocyte diameter was measured once per cell and only for cells with distinct boundaries. Lumen diameter in corneal and conjunctival lymphatics was compared using the Mann-Whitney Rank Sum Test for non-normally distributed data, using commercial statistical software (SigmaStat 3.5, Systat Software Inc., Chicago, Il).

Results

Corneal and conjunctival lymph vessel morphology differ significantly in vivo

Lymph vessel morphology differed substantially *in vivo* depending on vessel location. Lumen diameter of pre-existing mature conjunctival and limbal lymphatics (mean diameter 108.7 μm, $N=17$ measurements, 6 corneas) was significantly

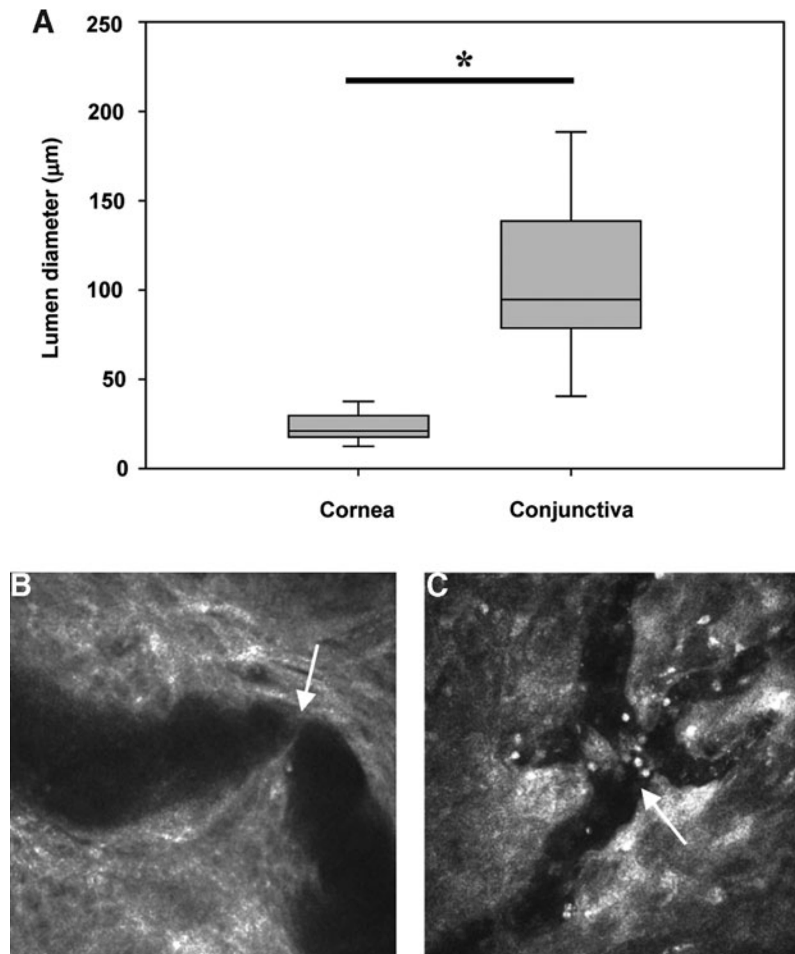

FIG. 1. Lymph vessel size and morphology differs with location. **(A)** *In vivo* measurements of lymph vessel lumen diameter indicated that conjunctival lymphatics were significantly larger than inflammation-induced corneal lymphatics (*$p < 0.001$). **(B)** Conjunctival lymphatics consist of large balloon-like segments, often connected at acute angles (*arrow*). **(C)** Newly formed corneal lymphatics triggered by inflammation are smaller, less segmented, and often exhibit junctions (*arrow*) where three of more vessel branches come together. Images are $400 \times 400\ \mu$m.

greater ($p < 0.001$, Mann-Whitney) than that of the newly-formed inflammation-induced corneal lymphatics (23.7 μm, $N = 65$, 8 corneas, Fig. 1A). Conjunctival lymphatics were also more visibly divided into segments (termed lymphangions[14]) often oriented at acute angles to one another (Fig. 1B). Lumen diameter of adjacent lymphangions varied. Corneal lymphatics exhibited lumen diameter variation to a lesser degree, and did not exhibit the bulb-like lymphangions seen in the conjunctiva. Newly formed corneal lymph vessels instead had junctions where three or more lymph vessel segments came together (Fig. 1C), while such junctions were absent in the conjunctiva. In rare cases, large, fluid-filled conjunctival lymphatics were visible endogenously in a standard operating microscope (Fig. 2). These lymphatics were distributed circumferentially in the conjunctiva, and were visibly connected to existing lymphatics in the limbus.

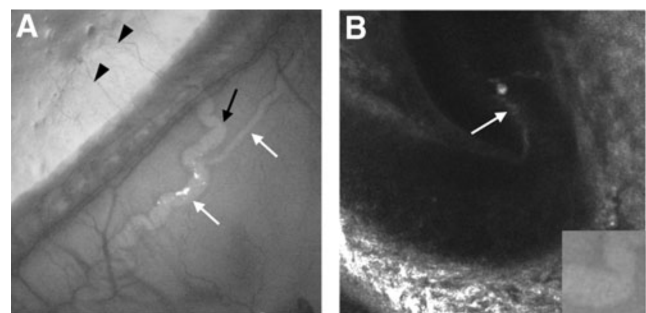

FIG. 2. Macroscopic and microscopic appearance of endogenously visible lymph vessels in the murine conjunctiva. **(A)** Under an operating microscope, the conjunctival lymphatic is clearly visible in the live murine cornea (*white arrows*). In this case, stimulated corneal angiogenic vessels are additionally visible (*arrowheads*). **(B)** *In vivo* confocal microscope appearance of a region of the conjunctival lymphatic (*black arrow in* **A**) where two segments adjoin at an acute angle. This region contained a lymphatic valve (*white arrow in* **B**), which was observed actively diverting cells (see Supplementary Video S1; supplementary material is available online at www.liebertonline.com/lrb) *Inset:* appearance of the same region of the lymphatic in the same orientation, under the operating microscope.

Lymph vessels harbor a population of cells with heterogeneous size, morphology, and density

In vivo imaging of murine lymphatics in inflamed corneas in real time over a period of several minutes revealed a cell population within the lymph fluid made visible by

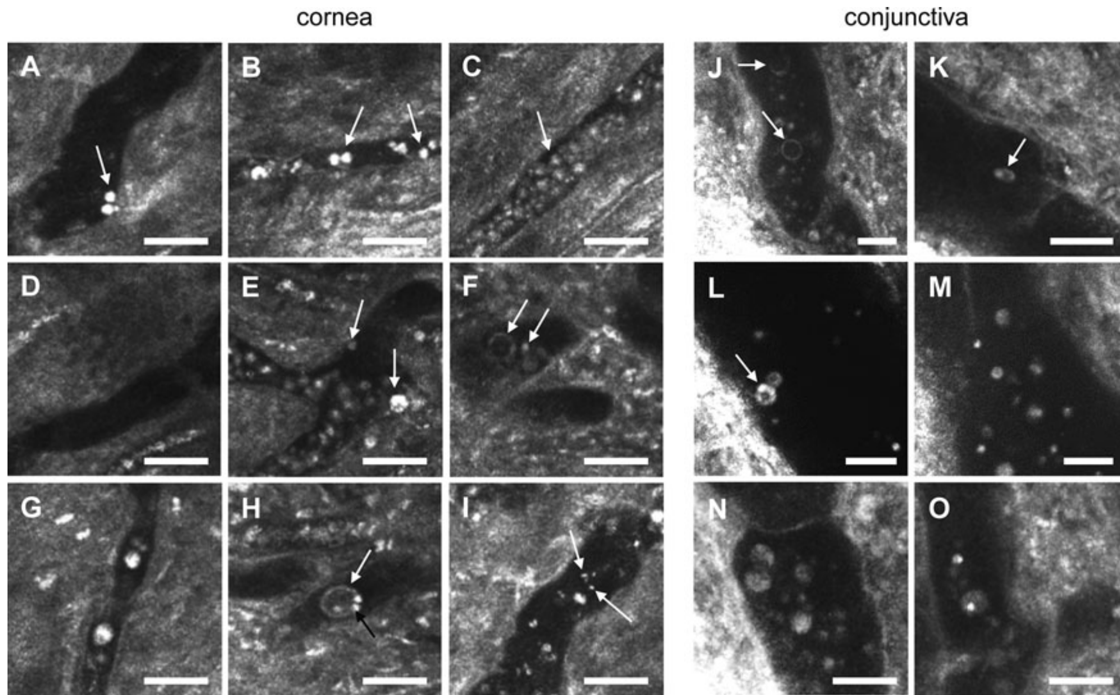

FIG. 3. *In vivo* examination of cells contained within lymphatics during inflammation reveals a heterogeneous cell population. Cells in corneal lymphangiogenic vessels (**A—I**) 14 to 21 days after stimulus, and cells in pre-existing conjunctival lymphs (**J—O**) 1 to 14 days after stimulus. Lymph vessels contained a population of uniformly-sized, highly light-scattering cells as reported previously (*arrows,* **A**, **B**). Additionally, some lymphatics were tightly packed with cells of variable intensity (*arrow*, **C**). Other lymph vessels appeared cell-free under observation periods of up to a few minutes **(D)**. Lymphatics often contained cells of various sizes and intensity (*arrows*, **E**, **F**). Very large cells were observed (**H**, *white arrow*) attached to smaller, more optically dense inclusions (**H**, *black arrow*), and sometimes very small cells or particulate matter was observed (**I**, *arrows*). A similar heterogeneity of cell size and morphologic appearance was observed in conjunctival lymphatics. In some cases, cell walls were clearly discernible (*arrows* in **J—L**). Scale bars are 50 μm.

endogenous light scattering.[11] Cells were visible in both corneal and conjunctival lymphatics, and had varying size, morphology, and reflectivity (Fig. 3). Cell localization within the lymph vessels (and not attached to the outer vessel wall) was confirmed by motion of the cells in the lymph fluid (see Section on cell dynamics) and by 3D imaging. The density of cells within both corneal and conjunctival lymphatics varied widely. Some vessel regions contained no visible cells, while others were sparsely populated with cells, and some newly formed corneal lymphatics had a high density of cells packed tightly within the vessel lumen (Fig. 3).

Cell size ranged from very small particle-like features only 1–2 μm in diameter up to large cells over 25 μm in diameter, with clearly discernible cell wall and subcellular features. In 12 different corneas, 175 cells observed *in vivo* in corneal and conjunctival lymph vessels were measured, and had a diameter ranging from 1.5 to 27.5 μm (Fig. 4). Previously, we reported leukocyte diameter within inflammation-induced corneal blood vessels, where 52 leukocytes from blood vessels in five different corneas were measured, with a diameter ranging from 5.2 to 11.6 μm.[11]

Inflammatory cells mobilize rapidly in limbal lymphatics

After stimulation of inflammation by placement of a suture in the corneal stroma, imaging by IVCM enabled a single lymph vessel located at the corneal limbus to be monitored *in vivo* longitudinally over a period of several days. Inflammatory cells were rarely observed for several hours after stimulation, but by 24 h a large number of cells were present within the vessel (Fig. 5). The high density of cells was sustained to at least 7 days. Inflammatory cells with heterogeneous morphology flowed within these lymphatics (Supplementary Videos S2, S3; Supplementary material is

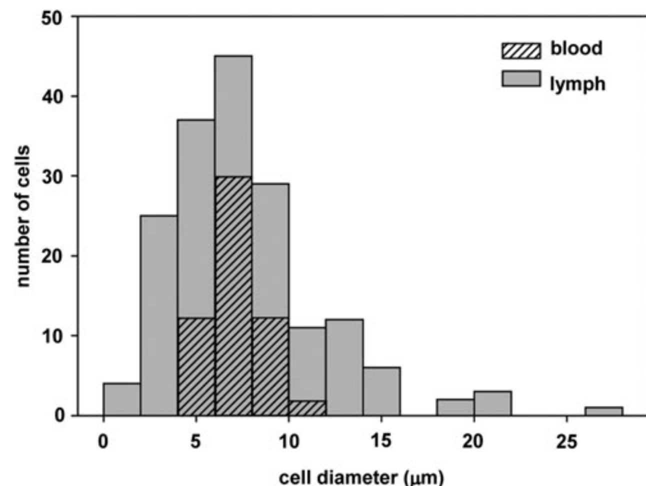

FIG. 4. Cells within lymphatics are larger than leukocytes found within blood vessels. *In vivo* cytometry of cell diameter within blood (erythrocytes excluded) and lymph vessels indicates a population of large cells (>15 μm diameter) located within lymphatics.

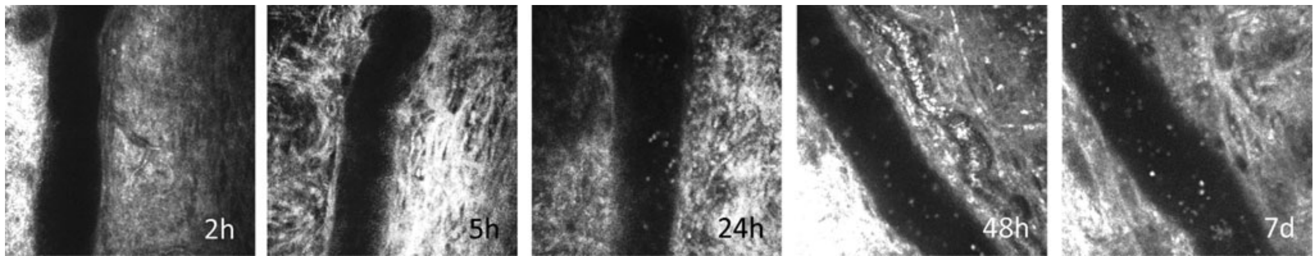

FIG. 5. Time-series images of inflammatory cell mobilization to a limbal lymphatic vessel at various times after stimulation of inflammation. Virtually no cells were observed in the lymph vessel at 2 and 5 h after suture placement. A large influx of immune cells, however, was apparent by 24 h, and was sustained within the lymphatic to at least 7 days. Note that the same lymph vessel is depicted at all time points, but at slightly different locations. Images are $400 \times 400\ \mu$m.

available online at www.liebertonline.com/lrb). By contrast, a sparse population of cells more homogeneous in appearance flowed within native, noninflamed limbal lymph vessels (Supplementary Video S4). In limbal lymph vessels in both unperturbed and inflamed corneas, cells flow was often in the form of aggregated 'clusters' of two to eight or more cells (Fig. 6). Clusters consisted of cells with similar size and reflectivity, and have been observed in the limbal region in human subjects by IVCM (Fig. 6E, F).

Dynamic flow characteristics of cells in corneal lymphatics

In inflammation-induced corneal lymphatics, cells exhibited a variable dynamic behavior. In some cases, cells appeared to be stationary within the observation period of seconds to minutes (Supplementary Video S5). In other cases, moving cells flowed past still ones, with some moving cells 'jarring loose' stationary cells, thereby setting them in motion (Supplementary Video S6). In other lymphatics, cells appeared to be suspended in the lymph, slowly rocking back and forth, while other cells appeared to flow unimpeded at a different depth (Supplementary Video S7). In one inflamed cornea, large transparent cells with strongly reflecting cell borders and optically-dense inclusions were observed moving alongside smaller, more uniformly reflective cells (Supplementary Video S8).

3D rendering of lymphatics and lumen cross-section visualization in vivo

In vivo imaging enables cells within corneal lymph vessels to be analyzed by 3D rendering of ultrafine IVCM image stacks (Fig. 7A). The resulting high-resolution images (Fig. 7B, C) represent histologic optical cross-sections of lymph vessels *in vivo*, in which the vessels contain lymph fluid and cells in a native, unaltered state. This *in vivo* histology enables investigation of the vessel lumen without artifacts such as shrinkage and collapse of vessels due to fixation, embedding, and sectioning. By selecting specific x-z and y-z optical sections from 3D image stacks, cells are localized within the lymph vessel lumen in three dimensions. The resulting cross-sections can resolve cells appearing to overlap in the *en face* x-y plane, but are distinct at different depths (Fig. 7B). The technique was also used to locate weakly light-scattering cells not contained within the lumen but instead localized to the lymphatic vessel wall (Fig. 7C).

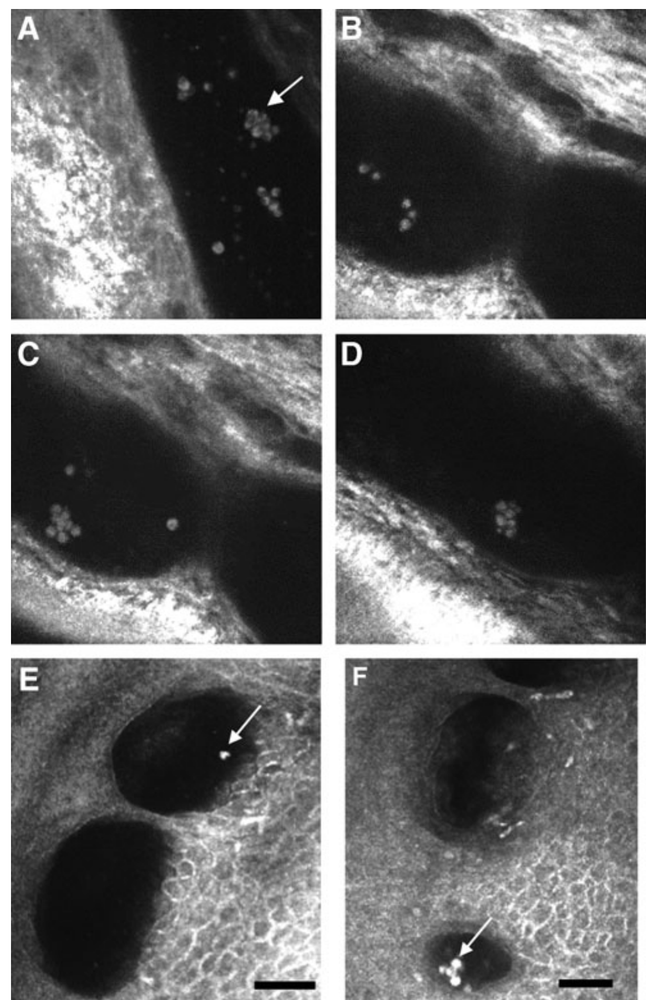

FIG. 6. Cells in limbal lymph vessels can be transported in clusters. **(A)** Four days after stimulus in an inflamed cornea, a limbal lymph vessel contains individual and clustered (*arrow*) cells, with clusters containing cells of similar morphology. **(B—D)** Limbal lymph vessels in native, untreated corneas also exhibit clustered cells (see also Supplementary Video S4). **(E, F)** In a human subject, limbal lymph vessels contained either single or clusters of cells (*arrows*). Images **(A—D)** are $400 \times 400\ \mu$m, in images **(E, F)** scale bars are $50\ \mu$m.

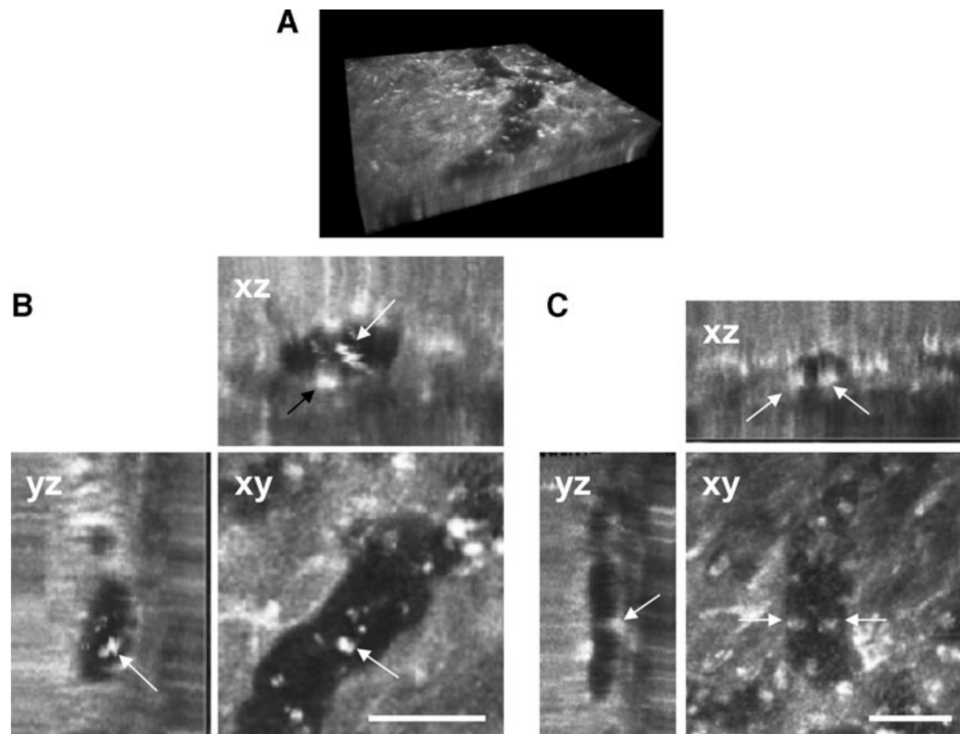

FIG. 7. Localization of cells in or around corneal lymph vessels is enabled by *in vivo* histology-type optical tissue sections. **(A)** 3D volume containing corneal lymph vessels, rendered from an *in vivo* confocal image stack with ultrafine axial spacing. The volume can be viewed from any desired orientation in 3D space. **(B)** Example of optical sectioning. A feature in the x-y plane (*arrow*) is noted in y-z and x-z planes to be two separate cells located at different depths (*white arrows*). Another cell visible in the x-z plane (*black arrow*) is localized to the lymphatic vessel wall, not the lumen. The x-y and y-z planes are high resolution optical cross-sections of a live lymph vessel, enabling the lymph vessel lumen to be examined without tissue processing artifacts. **(C)** Optical sectioning of a corneal vessel reveals that diffuse cellular features in the x-y plane (*arrows*) do not lie within the lymph vessel lumen. From y-z and x-z optical sections, the cells are localized to the posterior lymph vessel wall (*arrows*). Scale bars are 50 μm.

Discussion

Despite increased attention to the role of lymph vessels in pathologies such as lymphedema, transplant rejection, and tumor metastasis, there remains a dearth of information regarding the direct transport of cells within peripheral lymphatics. Collection of cells from the lymph fluid and subsequent *ex vivo* analysis provides evidence for trafficking of specific cell types during an immune response and information about the composition of the cell populations present within lymphatics.[1,12,13,19] The presence of a large population of resident immune cells in lymph nodes,[13] however, limits the specificity of this technique, while its invasiveness precludes longitudinal observation in single organisms. Moreover, information about the actual density of cells transported within the peripheral lymph, local tissue changes in lymphatic cell populations, and the *in vivo* morphologic characteristics of cells is lost when the cells are physically separated from lymph vessels.

The IVCM imaging approach presented herein enables *in vivo*, real-time, and repeated monitoring of lymph vessel characteristics as well as the cells within afferent lymphatics. The newly formed corneal lymph vessels appeared smaller and had a more uniform diameter than established lymphatics in the limbus and conjunctiva. It is suspected that the densely packed collagen lamellae within the cornea proper may limit the size of the corneal lymphatics, in contrast to limbal and conjunctival lymphatics, which can expand to greater size in this less dense tissue.

Immune cells within the newly formed lymphangiogenic corneal vessels are by definition nonresident, and according to the requirement of one-way lymphatic flow,[20] all cells present within corneal lymphatics have transmigrated across lymphatic vessel endothelium from the surrounding stromal tissue. Monitoring cells within corneal lymphatics is therefore a highly sensitive method to assess the migration of cells prior to, during, and after an immune response. With IVCM, a heterogeneous cell population was observed *in vivo*, similar to *ex vivo* studies of peripheral lymph where different non-lymphoid macrophage and dendritic cell populations were documented.[12,19] Interestingly, subpopulations of macrophage and dendritic-lineage cells harvested from lymphatics appear morphologically distinct,[12,19] suggesting the possibility of cell identification by morphology in our *in vivo* model. Alternatively, immunofluorescent identification of isolated cells within labeled corneal lymph vessels has been demonstrated;[9,10] however, poor image contrast and resolution and antigen selectivity in fluorescence is insufficient to reveal the detailed morphology of cells at the level provided by IVCM.

In vivo imaging provided some initial insights into the dynamic behavior of cells within lymph vessels. Immune cells are rapidly recruited into lymphatics and maintain a strong presence during inflammation. Cells also had a range of motility,

physical cell-to-cell interactions, and can be transported in clusters of adherent cells, the significance of which is unknown. The presence of cells within native, noninflamed lymphatics suggests a constant, low-level flux of extravascular cells into afferent conjunctival lymphatics in the healthy eye. This population of cells—the maturity or antigen-presenting capability of which is unknown—warrants further examination. Within corneal lymphangiogenic vessels, stationary cells became dynamic by the force of impact from other cells. Other cells appeared loosely adherent to vessel walls, while still others appeared completely stationary within the observation period. These observations suggest that lymph flow is not the sole determinant of cell transport. Cell adhesion to vessel walls and to other cells is likely an important contributing factor. Further investigation is required to determine the molecular basis of adhesion and clustering of cells within lymphatics, in particular the role of adhesion molecules, such as those known to facilitate cell transmigration across lymphatic endothelium.[21–23] Additionally, the physiologic relevance of adherent cells in the delivery of antigen to regional lymph nodes remains to be elucidated. Of interest, it is still unknown whether all cells entering peripheral lymph vessels are destined to reach the regional lymph node; long-dwelling cells could potentially die or migrate out of the lymph.[9,14] In this context, clustered cells may resist transmigration out of the lymph and survive to reach the regional lymph node. Longer observation periods and examination of a large number of lymph vessels by IVCM may aid in investigating this question. It is interesting to note, however, that cell velocities within lymphatics observed in the present study are substantially greater than those observed in a recent study by Steven et al. using intravital two-photon imaging.[9] The maximum velocity reported in that study was 224 μm/min, whereas the videos in the present study indicate velocities up to hundreds of microns per second. Only three intravascular cells, however, were observed in the study by Steven et al.,[9] and only cells with specific fluorescent characteristics were tracked. The rapid cell transport within lymphatics observed by the IVCM technique is evidence of a highly sensitive afferent arm of the immune system.

The possibility of live imaging of cell dynamics within afferent lymphatics has been demonstrated. The strength of IVCM is the use of endogenous contrast for high-resolution *in vivo* cell imaging, avoiding the use of dyes that pre-select only a subset of cells and tend to obscure cell morphology. Moreover, the cornea model is ideal for high-resolution cell imaging due to transparency and superficiality of the tissue. Spatial separation of lymphangiogenic from pre-existing lymphatics in the cornea additionally aids in the isolation of cells having migrated across the lymphatic endothelium. The IVCM technique could be further developed to study cells *in vivo* in a cytometric manner by imaging the flow in single lymph vessels for longer periods of several minutes up to hours. Additionally, cell migration dynamics could be examined and compared under different pathologies or in response to a disruption of targeted cell populations or molecular signaling pathways.[4,13] The method could therefore provide a direct indicator of the effect of potential therapeutic approaches on lymphatic vessel cell uptake and trafficking.

Author Disclosure Statement

No competing financial interests exist.

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