LYVE-1–Positive Macrophages Are Present in Normal Murine Eyes

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PURPOSE. A functioning lymphatic system is necessary not only to permit the organism to mount a rapid and effective immune response but, even more so, to maintain tissue fluid homeostasis. However, no clear evidence of lymphatic vessels draining intraocular and orbital tissues—retina, choroid, sclera, and extraocular muscles—exists.

METHODS. Ocular tissue flatmounts from normal or enhanced green fluorescence protein (EGFP) chimERIC mice were immunoNostained for lymphatic endothelial hyaluronan receptor (LYVE-1, a routinely used lymphatic endothelial marker), podoplanin, Flt4/VEGFR3, Sca-1, CD11b, or F4/80 and were observed by confocal microscopy. Single-cell suspensions from ocular tissues were also prepared and were analyzed by flow cytometry.

RESULTS. Lymphatic vessels were detected in the posterior regions of the extraocular muscles and the connective tissues of the extraocular muscle cones in the normal mouse. No typical lymphatic vessels were found within the eye. A large population of LYVE-1$^+$ nonendothelial cells, distributed as single cells, was detected in all ocular tissues except the central cornea. These cells also express another lymphatic endothelial cell marker, Flt4/VEGFR3, but not podoplanin, and they have hyaluronan-binding ability. Bone marrow chimera studies indicated that the LYVE-1$^+$ cell populations are bone marrow derived and have a slow turnover in ocular tissues (3–6 months). Phenotype analysis revealed that nonendothelial LYVE-1$^+$ cells in the sclera, choroid, and iris included CD11b$^+$F4/80$^+$ macrophages, CD11b$^+$F4/80$^+$ macrophages, and CD11b$^+$F4/80$^+$ bone marrow–derived cells. All LYVE-1$^+$ cells in the retina were CD11b$^+$F4/80$^+$ macrophages. Cells in the limbus and the iris root also express Sca-1, suggesting that they are hematopoietic lymphatic vessel progenitor cells.

CONCLUSIONS. These observations suggest that a lymphatic system exists for the transport of immune cells and fluids from the posterior segment of the eye, that ocular tissues are rich in bone marrow–derived LYVE-1$^+$ macrophages under normal physiological conditions, and that a subpopulation of these cells may represent resident precursor cells necessary for the de novo formation of ocular/orbital lymphatic vessels in pathologic conditions. (Invest Ophthalmol Vis Sci. 2007;48: 2162–2171) DOI:10.1167/iovs.06-04783

The lymphatic system is central to the effective development of an immune response and to the maintenance of tissue fluid homeostasis. In the immune system, tissue-resident dendritic cells (DCs) transport captured antigens to draining lymph nodes by way of lymphatic vessels and present antigen to T and B cells in the lymph nodes. In some circumstances, soluble antigens can also drain directly into lymph to access lymph node DCs for presentation. Antigen presentation in the lymph node may induce immune tolerance or immune activation. Lymphatics form a secondary circulatory system that collects tissue fluids and macromolecules through the afferent vessels and processes them in the lymph nodes for recirculation to the bloodstream through the thoracic duct. The lymphatic system is therefore essential for the maintenance of normal interstitial fluid balance. During inflammation, new lymphatic vessels are formed (lymphangiogenesis) to remove excess interstitial fluid caused by increased vascular permeability. Failure to clear interstitial fluid effectively leads to tissue edema.

In the human eye and the monkey eye, lymphatic vessels have been detected at the limbus and conjunctiva of the ocular surface and in the lacrimal gland and the dura mater of the optic nerve. No lymphatics have been observed in the central cornea or the retina. Corneal and retinal tissues are considered immunoprivileged tissues. Although lymphatics have been detected in the choroid of the avian eye, such structures have never been detected in the choroid of rodent eyes or human eyes. In rodent eyes and human eyes, lymphatic vessels are also thought to be absent from other ocular tissues, including ocular muscles, sclera, and ciliary body, though these tissues contain large numbers of macrophages and DCs. How immune responses and tissue fluid homeostasis are controlled in pathophysiological conditions in these ocular tissues is unclear. A previous study has shown that antigen-presenting cells (APCs) in the anterior uveal tract did not appear to migrate in significant numbers to the draining lymph nodes. However, soluble antigen injected into the ocular compartments was observed to traffic from the anterior or posterior chamber to the submandibular and cervical lymph nodes, indicating that a fluid transport pathway exists from the intraocular compartment to the regional lymph nodes.

A well-recognized afferent drainage pathway is known to exist from the anterior and posterior chambers to the aqueous venous plexus through the trabecular meshwork. In fact, this pathway is considered to serve the oculosplenic axis for the induction of anterior chamber-associated immune deviation (ACAID), an altered or “deviated” immune response to soluble antigens injected into the anterior chamber of the eye. A minor drainage pathway has also been described through the ciliary body into the suprachoroidal space and from there to the uveovortex or uveoscleral drainage vessels, and this pathway may allow soluble antigens from vitreous or aqueous to enter regional lymph nodes. However, lymphatic vessels have never been detected in the suprachoroidal space in rodent or human eyes. In rodents, the conjunctiva extends

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further toward the posterior than in humans; therefore, it is possible that antigens may also be able to drain from the suprachoroidal space or sclera into the conjunctival lymphatics. Even so, this cannot explain the fact that antigens are able to migrate from anterior chamber to deep-cervical lymph nodes.\(^\text{16}\)

Improved techniques in ocular tissue flatmount preparations, availability of specific lymphatic endothelial antibodies, and more advanced confocal microscopes provide new opportunities for the detection of rare lymphatic cells within complex ocular tissue. To further understand the trafficking of intraocular immune cells and antigens in physiopathologic conditions, we revisited the issue of ocular and orbital lymphatics by systematically examining the distribution of lymphatic vessels in discrete ocular tissues. Each ocular tissue was carefully dissected, and lymphatic vessels were identified using lymphatic endothelial cell-specific markers, including lymphatic endothelium hyaluronan receptor (LYVE-1)\(^\text{22,23}\) and podoplanin.\(^\text{24}\) Flatmounts of ocular tissues were examined by confocal scanning laser microscopy. Our results showed that in addition to the limbus, conjunctiva, and optic nerve sheath, lymphatic vessels also occur in the posterior region of extraocular muscles and in the connective tissue of the extraocular muscles. More important, we observed a large population of LYVE-1 cell representing nonendothelial cells in almost all ocular tissues. With the use of enhanced green fluorescence protein (EGFP) transgenic mice, we demonstrate that these cells are bone marrow derived and have a slow turnover in normal ocular tissues. Further studies revealed that ocular nonendothelial LYVE-1 cells were phenotypically heterogeneous. LYVE-1 cells in the choroid, iris, and retina coexpress F4/80 and CD11b, indicating they are a subpopulation of macrophages similar to those observed previously in the central cornea.\(^\text{25}\) Approximately 30% of LYVE-1 cells in the limbus express CD34 and Sca-1, suggesting that these cells probably represent ocular lymphatic vessel progenitor cells.

**Materials and Methods**

**Animals**

Eight- to 12-week-old C57BL/6 mice were obtained from the Medical Research Facility at the Medical School of University of Aberdeen. Homozygous C57BL/6 mice expressing EGFP under the control of a chicken β-actin promoter and CMV enhancer were obtained from Masaru Okabe (Osaka University, Osaka, Japan) and maintained in the Medical Research Facility at the University of Aberdeen. All procedures conformed to the regulations of the Animal License Act (UK) and to the ARVO Statement for the use of Animals in Ophthalmic and Vision Research.

**Bone Marrow Chimera**

Adult 8- to 12-week-old C57BL/6 mice (n = 6) were irradiated with 8 Gy γ-ray. Ten million bone marrow cells from homozygous EGFP C57BL/6 mice were transplanted through the tail vein into irradiated mice. Eight weeks to 6 months after bone marrow transplantation, bone marrow, blood, spleen, and lymph node cells were harvested and analyzed by flow cytometry to confirm the successful engraftment of EGFP\(^\text{+}\) hematopoietic stem cells and the resultant reconstitution of the immune system. Ocular tissue flatmounts were prepared for confocal microscopy.

**Antibodies**

The following primary antibodies were used: allophycocyanin (APC)-conjugated rat anti-mouse CD51 (PECAM-1, MECL13.5), PerCP-Cy5.5-conjugated rat anti-mouse CD11b (M1/70), PerCP-Cy5.5-conjugated rat anti-mouse CD45/B220 (RA3 to 6B2), APC-conjugated hamster anti-mouse CD11c (HL3), APC-conjugated rat anti-mouse Ly-6G and Ly-6C (Gr1, RB6–8C5), fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD45 (LC, Ly5, 30-F11), FITC-conjugated rat anti-mouse Ly6A/E (Sca-1, D7), R-phycocerythrin (PE)-conjugated rat anti-mouse major histocompatibility complex (MHC) class II (M5/114), R-PE conjugated anti-mouse CD44 (IM7), purified rat anti-mouse CD16/CD32 (Fcy III/II receptor, 2.4G2) (all from BD Biosciences, Oxford, UK); FITC-conjugated rat anti-mouse CD34 (MEC14.7) and biotinylated anti-mouse F4/80 (C1:A3–1) (Serotec, Oxford, UK); goat anti-LYVE (S-20; Santa Cruz Biotechnology Inc., Santa Cruz, CA); and biotinyl-hyaluronan (Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK). Secondary antibodies were PE-conjugated streptavidin and APC-conjugated streptavidin (BD Biosciences); PE-conjugated anti-hamster IgG (Serotec); and FITC-conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA). Isotype controls included rat IgG2a, rat IgG2b, and hamster IgG (all from BD Biosciences).

**Immunostaining of Ocular Tissue Flatmounts**

Ocular tissues were dissected and processed as previously described\(^\text{26}\) with slight modification. Briefly, the conjunctiva and cornea were carefully removed in ice-cold phosphate-buffered saline (PBS). The crystalline lens and vitreous were discarded. The iris, retina, and choroid/sclera were then dissected. The conjunctiva, cornea, retina, iris, and choroid/sclera tissues were then fixed in 100% ethanol for 30 minutes at 4°C, followed by 5 minutes of 100% ice-cold acetone treatment. To remove the choroid from the sclera, the fixed choroid/sclera tissue was cut into quadrants. A flat, rounded blade was gently inserted into the tissue plane between the choroid and sclera and slowly advanced to separate the choroidal and scleral tissues.

For ocular tissue flatmount staining, tissue pieces were first permeabilized with 5% (wt/vol) bovine serum albumin (BSA) with 0.3% (vol/vol) Triton in PBS at room temperature for 2 hours and then blocked with anti-CD16/32 (Fcy R) and 5% rabbit serum for 30 minutes. Samples were then incubated with primary antibodies (1:20 to 1:50) or isotype control antibodies at 4°C overnight. After thorough washing, the samples were incubated with directly conjugated secondary antibodies (1:100) at room temperature for 2 hours. All antibodies were diluted in PBS containing 1% BSA. After thorough washing, samples were gently spread flat on microscope slides and covered with antifade medium (Vectashield; Vector, Burlingame, CA). Flatmount ocular tissues were examined with a confocal microscope (LSM510 META: Carl Zeiss Meditec, Göttingen, Germany).

Z-stack images were taken from the whole thickness of ocular flatmount preparations. Images were then reconstructed (Image Pro Plus; Media Cybernetics, MD).

**Single-Cell Suspension of Ocular Cells**

Ocular single-cell suspension was used for flow cytometry studies. To obtain ocular single-cell suspension, 18 dissected normal mouse eyes (exclusive of central cornea, crystalline lens, vitreous, and retina) were cut into small pieces and incubated in DMEM containing 0.2% (wt/vol) collagenase A and 5% (vol/vol) fetal calf serum (FCS) for 16 hours with rotation (70 rpm) at 37°C. The solution was filtered through a 70-μm cell strainer, and the filtrate was collected. After washing, single cells were suspended in fluorescence-activated cell sorter (FACS) buffer (1% BSA/PBS/10 mM NaH2PO4), and aliquots were prepared for further staining and flow cytometric analysis.

**Flow Cytometry**

To analyze the phenotype of LYVE-1-positive ocular cells, aliquots of ocular single-cell suspensions were first blocked with 5% normal rat/rabbit serum for 30 minutes and then stained with rabbit anti-mouse LYVE-1 (1:50) in combination with hamster anti-mouse podoplanin or directly conjugated monoclonal antibodies for mouse CD45, CD11b, CD31, CD34, CD11c, and MHC class II molecules for another 30
minutes. After washing, samples were incubated with FITC-conjugated anti-rabbit IgG (1:100) and PE-conjugated anti-hamster IgG (1:100) for 30 minutes. Samples were kept on ice throughout the experiment. All antibodies were diluted in PBS containing 1% BSA. Negative controls and single fluorochrome controls were performed to allow accurate compensation. Monochrome-isotype control antibodies were used to ensure the specific staining of each antibody. All samples were analyzed by flow cytometry (CellQuest Pro software; BD Biosciences).

To detect the hyaluronic acid (HA)–binding ability of LYVE-1–positive ocular cells, ocular single-cell suspensions were incubated with biotinylated HA in 1% BSA/PBS at 37°C for 30 minutes. After washing, samples were then stained with rabbit anti–mouse LYVE-1 on ice, followed by APC-streptavidin and FITC-anti–rabbit secondary antibodies, and were analyzed by flow cytometry as described.

For analysis of bone marrow, blood, lymph node, and spleen cells after bone marrow transplantation, 1 x 10⁴ cells from each sample were blocked with 5% normal rat serum and staining with anti–mouse CD3, CD45, CD11c, CD11b, Gr1, and B220 and were examined by flow cytometry.

**RESULTS**

LYVE-1⁺ Lymphatic Vessels in Normal Mouse Ocular Tissues

A rich network of lymphatic vessels was detected in the limbus (Fig. 1A) and conjunctiva (Fig. 1B). Interestingly, LYVE-1⁺ lymphatic vessels were also detected in the posterior part of extraocular muscles (Fig. 1C), the connective tissue of the extraocular muscle cone (Fig. 1D), and the optic nerve sheath (Fig. 1E). Lymphatic vessels were detected as LYVE-1⁺ and gp36/podoplanin⁺ (Fig. 1F). No lymphatic vessels were detected in the sclera, anterior part of extraocular muscle, choroid, iris, ciliary body, or retina, though some of these tissues also contained a discrete population of LYVE-1⁺ podoplanin⁻ cells.

Expression of LYVE-1 by Ocular Nonendothelial Cells

Previously, LYVE-1 was detected in conjunctival nonendothelial cells. In this study, we also found a large population of LYVE-1⁺ nonlymphatic endothelial cells in the conjunctiva that were negative for gp36/podoplanin (Fig. 1F). Surprisingly, these LYVE-1⁺ podoplanin⁻ nonendothelial cells were also detected in almost all other ocular tissues, including limbus (Fig. 2A), conjunctiva (Fig. 2B), sclera (Fig. 2C), extraocular muscles (Fig. 2D), para-optic nerve tissues (Fig. 2D), optic nerve sheath (Fig. 2F), iris (Fig. 2G), choroid (Fig. 2H), ciliary body (Fig. 2I), and retina (Fig. 2J). Staining with secondary antibody alone or with rabbit serum instead of LYVE-1 primary antibody did not reveal any positive staining (Fig. 2K). The density of the LYVE-1⁺ cells varied in different microanatomic locations (Fig. 2M). In ocular tissues of the outer layer, including limbus, conjunctiva, sclera, and ocular muscles, most LYVE-1⁺ cells were elongated and displayed a particular orientation—that is, they were parallel to lymphatic vessels in the limbus and conjunctiva (Figs. 2A, 2B) or parallel to ocular muscle fibers in the sclera surface (Fig. 2D) or the surrounding optic nerve (Fig. 2E). Some of the elongated cells could be seen connecting to each other and forming tubelike structures (Fig. 2L). Reconstruction of Z-stack images did not show a complete vessel structure. A few LYVE-1⁺ cells in the outer layers of the ocular tissues had irregular or dendriform shapes (Figs. 2B–E). In the

**FIGURE 1.** LYVE-1⁺ lymphatic vessels in ocular tissues. Flatmount ocular tissues were stained with anti–mouse LYVE-1 alone (A–E) or anti–mouse LYVE-1 and anti–mouse podoplanin (F) and were observed by confocal microscopy. LYVE-1⁺ lymphatic vessels are seen at the limbus (A), in the conjunctiva (B), in the posterior region of the extraocular muscle (C), in the connective tissue of extraocular muscle cone (D), and in the optic nerve sheath (E). (F) Double staining of a conjunctival flatmount with LYVE-1 and podoplanin shows that lymphatic vessels are double positive for LYVE-1 and podoplanin, whereas nonendothelial LYVE-1⁺ cells are negative for podoplanin. All images shown are two-dimensional reconstructions of a series of Z-stack confocal images.
iris, ciliary body, and retina, all LYVE-1\(^+\) cells had a dendriform shape (Figs. 2G, 2I, 2J). The number of LYVE-1\(^+\) cells varied in different tissues, with the highest density in the limbus and conjunctiva (Fig. 2M).

**Phenotype of Ocular Nonendothelial LYVE-1\(^+\) Cells**

To analyze the phenotype of ocular LYVE-1\(^+\) cells, single-cell suspensions were prepared (see Materials and Methods) and were stained with different cell surface markers. Flow cytometry analysis showed that approximately 30\% of ocular cells collected in this way were LYVE-1 positive (Fig. 3A). When LYVE-1\(^+\) cells were gated, 28\% of LYVE-1\(^+\) cells expressed CD45, the leukocyte common antigen, 38\% cells were CD11b\(^+\), and 40\% cells were MHC class II\(^+\). No LYVE-1\(^+\) cells expressed CD11c\(^+\) (Fig. 3B). In addition, 30\% of LYVE-1\(^+\) cells were double positive for CD31, and approximately 46\% were double positive for CD34 (Fig. 3B). Almost all LYVE-1\(^+\)CD11b\(^+\)
cells expressed CD34 (Fig. 3C); however, approximately 20% of LYVE-1+CD34+ cells were negative for CD11b (Fig. 3C).

Previous studies have shown that a subset of CD11b+ F4/80+ macrophages also expresses the LYVE-1 molecule.25,26 Flow cytometry showed that 38% of ocular LYVE-1+ cells were CD11b+ cells. To further characterize these ocular LYVE-1+CD11b+ cells, ocular tissue flatmounts were triple labeled with CD11b, F4/80, and LYVE antibodies. Most LYVE-1+ cells in the limbus and conjunctiva were stained weakly positive for CD11b, but few cells were positive for F4/80 (Figs. 4A, 4B). In the sclera 75.48%, 14.33%, and 64.43% of them coexpressed F4/80/CD11b+/LYVE-1+. In the choroid, 23.07%, 19.13%, and 3.93% of them were positive for CD11b, F4/80, and LYVE antibodies. Most LYVE-1+ cells in both CD11b and F4/80, and these cells had a highly ramified morphology compared with the F4/80+Sca-1+ cells (Fig. 4E). All LYVE-1+ cells in the retina were positive for both CD11b and F4/80 (Fig. 4F).

Coexpression of CD34 in nonendothelial LYVE-1+ cells suggested that this subset of LYVE-1+ cells might be lymphatic progenitor cells. To further confirm this, flatmount ocular tissues were double stained with LYVE-1 and Sca-1, a mouse hematopoietic stem cell marker. Results showed that nonendothelial LYVE-1+ cells in the limbus (Fig. 5A) and the root of iris (Fig. 5B) also expressed Sca-1. These LYVE-1+Sca-1+ cells were smaller than the LYVE-1+Sca-1+ cells and exhibited a more rounded morphology (Figs. 5A, 5B). These smaller LYVE-1+ cells in the limbal area were dual positive for CD34 (data not shown). Most nonendothelial LYVE-1+ cells in the conjunctiva (Fig. 5C), choroid, sclera (Fig. 5D), and extraciliary muscles expressed no or very weak Sca-1.

Examination of ocular tissue with other lymphatic endothelial cell markers indicated that these nonendothelial cells were negative forgp36/podoplanin (Figs. 1F, 5E), though most of the LYVE-1+ cells were also positive forVEGFR3/Fli1 (Fig. 5F).

The Origin and Turnover of Ocular Nonendothelial LYVE-1+ Cells

To identify whether the ocular nonvascular LYVE-1+ cells are bone marrow derived, EGFP bone marrow cells were adaptively transferred into lethally irradiated cognate C57BL/6 mice. Eight and 14 weeks later, ocular tissue flatmounts were prepared and double stained with LYVE-1. Flow cytometry analysis showed that the extent of EGFP reconstitution in the bone marrow, blood, and spleen ranged from 86% to 99% for each of the CD11b+, CD11c+, GR1+, and B220+ leukocyte subsets (Figs. 6A, 6B), indicating complete acceptance of the bone marrow transplant and chimera establishment in the recipient mice. EGFP+ bone marrow–derived cells were detected in all ocular tissues of the recipient mice, including the cornea, conjunctiva, iris, ciliary body, choroid, sclera, and retina (Figs. 7A–J). Eight weeks after bone marrow transplantation, approximately 15% to 30% of LYVE-1+ cells were EGFP+ cells in the limbus (Fig. 7A), conjunctiva (Fig. 7B), sclera (Fig. 7C), extraocular muscles (Fig. 7D), and retina (Fig. 7E), whereas in the choroid, though all EGFP+ cells had the same morphology as the endogenous LYVE-1+ cells, most EGFP+ cells were LYVE-1+ (Fig. 7E). Fourteen weeks after transplantation, approximately 60% to 70% of the LYVE-1+ cells in the limbus, conjunctiva, sclera, and retina were double positive for EGFP. The number of LYVE-1+ and EGFP double-positive cells also increased in the choroid (data not shown). Six months after bone marrow transplantation, almost all LYVE-1+ cells were double positive for EGFP in conjunctive, extraocular muscle (Fig. 7G), choroid (Fig. 7H), and sclera (Fig. 7I). None of the lymphatic vascular endothelial LYVE-1+ cells were positive for EGFP (Fig. 7J), indicating zero turnover in established lymphatic vessels.

Ocular LYVE-1+ Cells Have HA-Binding Ability

As one of the main HA receptors, LYVE-1 molecules have considerable HA-binding capacity.22,23 To evaluate whether LYVE-1 molecules expressed on nonlymphatic vascular cells have HA-binding properties, ocular single-cell suspensions were incubated with biotinylated HA and then double stained with LYVE-1. Flow cytometry analysis showed that all LYVE-1+ ocular cells were double positive for HA, indicating binding of HA to their surfaces (Figs. 8A, 8B). Ocular single-cell suspensions also contained a certain population of HA+LYVE-1+ cells (Figs. 8A, R1). The binding of HA in LYVE-1+cells could be mediated by alternative receptors such as CD4429 or the receptor for HA-mediated motility (RHAMM).30

FIGURE 3. Phenotype of ocular nonendothelial LYVE-1+ cells examined by flow cytometry. Single-cell suspensions of ocular tissues were prepared and stained for LYVE-1, CD11b, CD49, CD11c, MHC II, CD34, and CD31. Samples were then analyzed by flow cytometry. (A) Dot plot image shows four populations of ocular cells: LYVE-1+, CD11b+, CD11b+LYVE-1+, and CD11b+LYVE-1+ cells. Approximately 30% of cells in the sample are LYVE-1 positive by the coexpression of CD45, CD11b, CD11c, MHC II, CD34, and CD31. The x-axis represents the mean fluorescence intensity, and the y-axis represents the cell number. Shaded histograms represent the isotype controls. (B) Histograms of ocular LYVE-1+ cells showing the coexpression of CD45, CD11b, CD11c, MHC II, CD34, and CD31. The x-axis represents the mean fluorescence intensity, and the y-axis represents the cell number. Shaded histograms represent the isotype controls. (C) Coexpression of CD34 and CD11b by ocular LYVE-1+ cells.
FIGURE 4. Expression of macrophage markers by ocular nonendothelial LYVE-1\textsuperscript{+} cells. Ocular tissue flatmounts were stained with anti-mouse LYVE-1 (green), CD11b (red), and F4/80 (blue) and then were observed by confocal microscopy. (A) Limbus, (B) conjunctiva, (C) sclera, (D) choroid, (E) iris, (F) retina. Three subsets of LYVE-1\textsuperscript{+} cells can be seen: LYVE-1\textsuperscript{+}CD11b\textsuperscript{+}F4/80\textsuperscript{+} cells (arrowheads), LYVE-1\textsuperscript{+}CD11b\textsuperscript{−}F4/80\textsuperscript{+} cells (asterisks), and LYVE-1\textsuperscript{+}CD11b\textsuperscript{−}F4/80\textsuperscript{−} cells (arrows). In addition, CD11b\textsuperscript{−}F4/80\textsuperscript{−}LYVE-1\textsuperscript{+} macrophages were seen in the choroid (triangle). All images shown are two-dimensional reconstructions of a series of Z-stack images.
Figure 5. Phenotype of ocular non-endothelial LYVE-1⁺ cells examined by confocal microscopy. (A–D) Ocular tissue flatmounts were stained with anti-mouse LYVE-1 (red) and Sca-1 (green). Small LYVE-1⁺ cells (arrows) were stained positive for Sca-1 in a normal mouse limbus (A) and the root of iris (B) but not the conjunctiva (C) and extraocular muscles/sclera (D). (E, F) Flatmount of extraocular muscle was stained with anti-mouse LYVE-1 (red) and podoplanin (E, green) or Flt4 (F, green) and were observed by confocal microscopy. None of the LYVE-1⁺ cell expresses podoplanin (E). Most of the LYVE-1⁺ cell are dual positive for Flt4. All images shown are two-dimensional reconstructions of a series of Z-stack images.
DISCUSSION

In the orbit, lymphatics have previously been detected in the lacrimal gland and the dura mater of the optic nerve in human\(^8,9\) and in the Harderian gland in rodents.\(^3,1\) In this study, using LYVE-1 as a lymphatic endothelial cell marker, we provide the first evidence of the existence of formed lymphatic vessels in the posterior part of the extraocular muscles and in the connective tissue of the extraocular muscle cone in mice. The presence of lymphatic vessels in these regions provides a unique pathway for the movement of ocular/orbital tissue fluid and also for the trafficking of immune cells. It is well recognized that some level of circulation of intraocular fluids occurs through the suprachoroidal space. It has been suggested that...
Our observations suggest that ocular antigens to travel to the deep cervical lymph nodes, as observed by Camelo et al. Our observations suggest that ocular antigens may first track to the suprachoroidal space from the eye to the posterior ocular lymphatic vessels and from there to the deep cervical lymph nodes.

Another important observation of this study was the identification of a novel population of nonendothelial LYVE-1+ cells in all ocular tissues except the central cornea. These cells are bone marrow derived and have a slow turnover within the ocular tissues. Their phenotype varies in different ocular tissues. In the normal retina, iris, and choroid, these nonendothelial LYVE-1+ cells coexpress F4/80 and CD11b, similar to previously observed LYVE-1+ cells in the inflamed cornea. Malignant tumors and excised wounds. The second population of ocular nonendothelial LYVE-1+ cells are Sca-1+ and CD34+ cells localized at the limbal area and the root of the iris. They may represent ocular lymphatic progenitor cells. In the choroid, sclera, and extraocular muscles, a third population of LYVE-1+ CD11b+ F4/80+ and LYVE-1+ CD11b+ F4/80- cells was also observed. Whether the different subsets of ocular nonendothelial LYVE-1+ cells represent various differentiation stages of the same cells or different subsets of bone marrow-derived ocular nonendothelial LYVE-1+ cells with different functions is unclear and was outside the scope of this study.

The function of the ocular nonendothelial LYVE-1+ cells is unclear. LYVE-1 is one of the main HA receptors, but its biological function is not fully elucidated. It has been speculated that LYVE-1 may function as an HA transporter. HA is one of the main extracellular matrix molecules, and it is critical for the maintenance of interstitial fluid homeostasis, tissue remodeling, and leukocyte migration. Tissue HA is normally degraded, and HA oligosaccharides recirculate through the lymphatic system into the blood to be fully digested in the liver. LYVE-1+ nonendothelial cells observed in this study have strong HA-binding ability. They may function as HA transporters and carry HA and other interstitial solutes to orbital lymphatics in the conjunctiva, extraocular muscles, and periocular space. The specific alignment of LYVE-1+ cells in the anterior part of extraocular muscles (Fig. 2D) and the scleral surface of the posterior pole (Fig. 2E) may be indicative of cell migration toward the nearby lymphatics themselves or may transport molecules such as HA to the nearby lymphatics.

Another possible function of these nonendothelial LYVE-1+ cells may involve ocular lymphangiogenesis. Lymphangiogenesis is a well-recognized phenomenon on the ocular surface in pathologic conditions. However, the detailed mechanism is not fully understood. Previous studies have shown that vascular endothelial growth factor (VEGF) and its receptor (VEGFR) pathways play important roles. Recent studies suggest that LYVE-1+ CD11b+ F4/80+ macrophages play important roles in inflammation-induced corneal lymphangiogenesis. A dual role (two-way mechanism) of macrophages in lymphangiogenesis has recently been proposed. During inflammation, macrophages can be recruited from the bone marrow through the VEGF-A pathway and can be reprogrammed to produce large amounts of lymphangiogenesis factors VEGF-C/VEGFR3 into the inflammatory tissue and can induce local sprouting of preexisting lymphatic endothelial cells. Alternatively, recruited macrophages can also form cell aggregates and then physically integrate into sprouting lymphatic vessels. Although it is unknown whether the ocular nonendothelial LYVE-1+ cells observed in this study are capable of producing lymphangiogenic growth factors such as VEGF-C, they do express VEGFR3/Flt4, a receptor for VEGF-C. Our observation suggests that the lymphangiogenic LYVE-1+ CD11b+ F4/80+ macrophages are present in ocular tissue in normal physiological conditions and may provide a good source for rapid development of new lymphatic vessels in response to inflammatory challenge by direct integration into new lymphatic vessels, by release of lymphangiogenic growth factors, or both.

In summary, in this study we have demonstrated that in addition to the limbus and the conjunctiva, lymphatic vessels are present in the posterior part of the extraocular muscles and the connective tissue of the extraocular muscle cone. In addition, we have shown that LYVE-1+ cells are expressed in a large population of nonendothelial cells in all ocular tissues except the central cornea in normal physiological conditions. These nonendothelial LYVE-1+ molecules are bone marrow derived and have HA-binding ability. Cells in the limbus and the root of the iris also express Sca-1, suggesting that they are hematopoietic lymphatic progenitor cells, whereas LYVE-1+ cells in the choroid and retina are CD11b+ F4/80+ macrophages. Their roles in ocular lymphangiogenesis and ocular fluid transport remain to be elucidated.

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References


