Lymphatic Markers in the Adult Human Choroid

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Submitted: August 5, 2015 Accepted: October 15, 2015


PURPOSE. Reports of lymphatics in the posterior human uvea are contradictory. We systematically analyzed the choroid by combining various lymphatic markers, following recently established guidelines for the immunohistochemical detection of ocular lymphatics.

METHODS. Human choroids were prepared for flat mount serial cryosectioning. Sections were processed for immunohistochemistry of the lymphatic markers LYVE-1, PDLPN, PROX1, FOXC2, VEGFR3, CCL21, and combined with α-smooth muscle-actin and 4’6-diamidino-2-phenylindole (DAPI). Single, double, and triple marker combinations were documented using confocal microscopy. Messenger RNA analysis for CCL21, FOXC2, LYVE-1, PDLPN, PROX, and VEGFR3 was performed in choroid and skin.

RESULTS. In the choroid, CCL21 immunoreactivity was detected in choroidal blood vessels, intrinsic choroidal neurons, and numerous small cells of the choroidal stroma. These small cells were not colocalized with PROX1 and PDLPN, while a subpopulation of cells showed immunoreactivity for CCL21 and LYVE-1, and very occasionally PDLPN-only+ cells were detected. Nuclei positive for PROX1 were never detected in the choroid, and vessel-like structures immunoreactive for LYVE-1, PDLPN, or CCL21 (other than blood vessels) were never observed. Immunoreactivity of VEGFR3 was absent in the majority of choroidal blood vessels, but present in choriocapillaris, while other structures positive for VEGFR3 were not detected. Nonvascular smooth muscle cells were lacking VEGFR3-immunoreactivity. Messenger RNA analysis detected all lymphatic markers investigated and confirmed immunohistochemical results.

CONCLUSIONS. By combining several lymphatic markers, single cells expressed these markers, but classical lymphatic vessels were not detected in the human choroid. Therefore, the healthy adult human choroid must be considered alymphatic, at least with the markers applied here.

Keywords: eye, lymphatics, immunohistochemistry, confocal microscopy

I t generally is accepted that the eye represents an alymphatic organ,1 at least under physiological conditions, and that the absence of lymphatics leads to the so-called “immune privilege of the eye,” thereby representing an organ where foreign tissue grafts receive longtime or even indefinite survival.2,5 With the recent availability of lymphatic markers, this general opinion changed, and while conjunctival lymphatics generally are recognized,4 it has been demonstrated that lymphangiogenesis can take place in the cornea, especially during inflammatory conditions.5,6 On the other hand, intraocular lymphatics are detectable when the scleral border is degraded, as a result of tumor7–9 or trauma.9 However, the situation in the posterior parts of the eye is less clear. Few reports about putative lymphatics in the choroid of humans and nonhuman primates exist, based mainly on ultrastructural investigations,10,11 while others failed to demonstrate these possible lymphatics on the ultrastructural level.12–18

The introduction of lymphatic markers, such as LYVE-1, podoplanin, or PROX1, therefore, represents a useful tool in further large-scale analysis of choroidal lymphatics.19,20 By applying these markers, first studies were unable to demonstrate lymphatic vessels in the human choroid; instead, numerous macrophages with lymphatic phenotype, that is, those expressed lymphatic markers, were found.21

One of the most important problems in research with lymphatic markers is that to date a single exclusive lymphatic marker is not yet available,20,22,23 and, therefore, only the combination of several lymphatic markers allows for reliable conclusions to be drawn. This fact led to a Consensus Statement24 on how to proceed with lymphatic research in the inner eye when applying immunohistochemistry, moreover since recently published data regarding human choroidal lymphatics are contradictory.21,25 Therefore, we systematically analyze adult human choroid with combinations of various lymphatic markers, in accordance with the demands of the Consensus Statement.24 We present adequate negative and positive controls in detail, and we perform an analysis of choroidal mRNA content of markers used.
Lymphatics in Human Choroid

Materials and Methods

Specimens

The study adhered to the tenets of the Declaration of Helsinki. Human tissue was obtained from the cornea-donor program of the University Eye Clinic Salzburg (Salzburg, Austria) and showed no signs of pathological alterations (n = 8 from seven different donors of both sexes; 45 to 78 years; postmortem time, 7 to 26 hours). After corneal removal, eyes were opened further at the ora serrata and fixed by immersion in PBS containing 4% PFA (2 hours at room temperature, RT). The choroid was dissected free, transferred into PBS containing 15% sucrose (12 hours at 4°C), embedded in tissue embedding medium (Slee Technik, Mainz, Germany) and frozen at −80°C by using liquid nitrogen-cooled methylbutane and stored at −20°C for further processing. Human skin samples were obtained during routine plastic surgery from the University Clinic of Dermatology, Salzburg, following patient’s informed consent and treated identical as described above.

Immunohistochemistry

Sections of choroidal flat mount preparations were achieved in a cryostat (HM 550, Microm, Walldorf, Germany) in a way that choroids were flat frozen on the cutting device.20 Skin cross-sections and choroidal serial sections of 16 μm were collected on adhesion slides (Superfrost Plus; Thermo Scientific, Wien Austria) and air-dried for 1 hour at RT. After a 5-minute rinse in tris-buffered saline (TBS; Roth, Karlsruhe, Germany) slides were incubated for 1 hour at RT in TBS containing 5% donkey serum (Sigma-Aldrich, Wien, Austria), 1% BSA (Sigma-Aldrich), and 0.5% Triton X-100 (Merck, Darmstadt, Germany). Followed by a 5-minute rinse, slides were further incubated for single and double immunohistochemistry of the markers listed in Table 1 (in TBS, containing 1% BSA and 0.5% Triton X-100, 12 hours at RT). After a rinse in TBS (four times 5 minutes) binding sites of primary antibodies were visualized by corresponding Alexa488/-, or Alexa555-tagged antiserum (1:1000; Invitrogen, Karlsruhe, Germany) in TBS, containing 1% BSA and 0.5% Triton X-100 (1 hour at RT) followed by another rinse in TBS (four times 5 minutes). Some of the slides received an additional nuclear staining using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). For that, slides were incubated 10 minutes (1:4000, stock 1 mg/mL; VWR, Vienna, Austria) followed by a rinse in PBS (three times 5 minutes). All slides were embedded in TBS-glycerol (1:1 at pH 8.6). All antibodies used were generated against human epitopes and before the experiments in the choroid, the reliability of these antibodies has been tested in human skin sample experiments and resulted in appropriate immunoreactivity, that is, these detected lymphatic endothelial cells (PPDN, LYVE-1, VEGFR3, CCL21) or their nuclei (PROX1, FOXC2).

TABLE 1. Antibodies Used in this Study

<table>
<thead>
<tr>
<th>Protein, Full Name</th>
<th>Protein, Abbreviation</th>
<th>Antibody Rised in</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prospero homeobox protein 1</td>
<td>PROX1</td>
<td>Rabbit</td>
<td>Novus Biologicals, Cambridge, UK</td>
<td>1:200</td>
</tr>
<tr>
<td>Prospero homeobox protein 1</td>
<td>PROX1</td>
<td>Mouse</td>
<td>Acris, Herford, Germany</td>
<td>1:500</td>
</tr>
<tr>
<td>Chemokine ligand 21</td>
<td>CCL21</td>
<td>Goat</td>
<td>R&amp;D, Abingdon, UK</td>
<td>1:100</td>
</tr>
<tr>
<td>Forkhead box protein C2</td>
<td>FOXC2</td>
<td>Goat</td>
<td>Abcam, Cambridge, UK</td>
<td>1:200</td>
</tr>
<tr>
<td>Lymphatic vessel endothelial hyaluronan receptor 1</td>
<td>LYVE-1</td>
<td>Rat</td>
<td>Acris, Herford, Germany</td>
<td>1:50</td>
</tr>
<tr>
<td>Podoplanin</td>
<td>PDPN</td>
<td>Mouse</td>
<td>Bio-Rad Abs, Düsseldorf, Germany</td>
<td>1:50</td>
</tr>
<tr>
<td>Vascular endothelial growth factor receptor 3</td>
<td>VEGFR3</td>
<td>Rabbit</td>
<td>ReliaTech, Wolfsburg, Germany</td>
<td>1:50</td>
</tr>
<tr>
<td>a-Smooth-muscle actin</td>
<td>ASMA</td>
<td>Goat</td>
<td>Acris, Cambridge, UK</td>
<td>1:200</td>
</tr>
</tbody>
</table>

Further, a hepatocellular carcinoma assay (HEPG2) was used to test for the reliability of PROX1, receiving an identical immunohistochemical protocol. Additionally, to avoid possible cross-reactions in experiments with two antibodies, interval incubations have been performed (i.e., first and second antibody for epitope one, followed by incubation with first and second antibody for epitope two). To avoid misinterpretation due to unspecific background fluorescence caused by collagen or other autofluorescent signals, the same epitopes also have been detected with different color-coded secondary antibodies. Negative controls were performed by omission of the primary antibodies during incubation and resulted in absence of immunoreactivity.

Documentation

To document double and triple label immunohistochemistry, a confocal laser scanning unit (Axio ObserverZ1 attached to LSM710; ×20 dry or ×40 and ×60 oil immersion objective lenses, with numeric apertures 0.8, 1.30, and 1.4, respectively; Zeiss, Göttingen, Germany) was used. Sections were imaged using the appropriate filter settings for Alexa555 (568 nm excitation, channel 1, coded red), Alexa488 (488 nm excitation, channel 2, coded green), and DAPI (345 nm excitation, coded blue), and up to three channels were detected simultaneously. All images presented here represent confocal images in single optical section mode. Negative controls were retrieved with identical laser settings as used in corresponding immunohistochemical experiments.

Molecular Biology, Quantitative (q) RT-PCR

To confirm the immunohistological findings, mRNA levels of lymphatic endothelial marker genes in human choroid (n = 3, male and female, age 74–78, postmortem times 7.5–26 hours) were analyzed. Tissue was homogenized with the TissueRuptor (Qiagen, Hilden, Germany) and mRNA was isolated with TRI reagent (Sigma-Aldrich) according to the manufacturer’s instructions. Two μg of RNA were digested with the Dnase I Kit (Sigma-Aldrich), and subsequently used for cDNA synthesis (iScriptcDNA synthesis kit; BioRad, Vienna, Austria). Quantitative PCR (qPCR) was performed with the SsoFastEvaGreen Supermix (BioRad) for the genes coding for selected markers of lymphatic endothelial cells (for primer sequences see Table 2). The gene coding for hypoxanthine-guanine-phosphoribosyltransferase (HPRT1) was used as reference. Each reaction was run in triplicates on the CFX96 (C1000 Thermal Cycler; BioRad) with a two-step protocol (95°C, 5 minutes, 50 cycles: 95°C 5 seconds, 60°C 20 seconds) and melt curve. A genomic DNA control (PrimePCR DNA Contamination Control SYBR Green Assay; Human; BioRad), and no template control was included in every RT-PCR experiment. cDNA prepared from human skin (n = 4) was used as positive control.
RESULTS

Controls: Cell Assays and Skin

Since it is known that the hepatocellular carcinoma cell line HEPG2 is constitutively expressing PROX1,27 this cell assay was used to test for the reliability of our PROX1 antibody. Indeed, PROX1-immunoreactivity was detected in nuclei only, as verified by nuclear DAPI-labeling (Fig. 1A), and was further absent in corresponding negative controls (Fig. 1B). In human skin samples, PROX1-immunoreactivity again revealed a clear nuclear expression when applying DAPI (Fig. 1C), and nuclei displayed a typical single or double lined “pearl-string-pattern” (Fig. 1C). Double labeling experiments with PROX1 and FOXC2 revealed an overlap of both nuclear markers, with PROX1 being more consistent in its expression pattern (Fig. 1D). For that reason, FOXC2 was excluded in further choroidal immunohistochemical analysis. Double labeling experiments with PROX1 and VEGFR3 (Fig. 1E), and also PROX1 and PDNP (Fig. 1F) revealed PROX1-positive nuclei surrounded by the corresponding lymphatic endothelial surface markers, and identical results were retrieved when combining PROX1 with CCL21 (Fig. 1G). In controls applying VEGFR3 and ASMA, a clear distinction between labeled structures was evident, and an overlap was not detectable (Fig. 1H).

Choroid: Single Labeling Experiments

Immunoreactivity of CCL21 was detected in cells tangentially surrounding blood vessels of the choroidal stroma, from size and shape most likely representing vascular smooth muscle cells (Fig. 2A), but immunoreactivity was absent in corresponding negative controls (Fig. 2B). Further, immunoreactivity was detectable in numerous small cells throughout the choroidal stroma (Fig. 2C), but lacking in corresponding negative controls (Fig. 2D). CCL21 immunoreactivity was present in intrinsic choroidal neurons (Fig. 2E), as identified by size and shape, and further by the presence of lipofuscin-granules located within the cytoplasm. Again, CCL21-immunoreactivity was absent in corresponding controls (Fig. 1F).

CCL21-immunoreactive structures with vessel-like appearance or forming lines were never observed.

Choroid: Double Labeling Experiments

CCL21-immunoreactive cells of the choroidal stroma were lacking PROX1-immunoreactive nuclei (Figs. 3A, 3B), and corresponding controls revealed absence of immunoreactivity (Fig. 3C). When localizing CCL21 and LYVE-1, some cells of the choroidal stroma were immunoreactive for both markers, with CCL21 displaying a granular cytoplasmic localization and LYVE-1 a rather membrane-bound localization (Figs. 3D, 3E), while a subpopulation of rather small round to ovaloid cells were immunoreactive for CCL21 only (Fig. 3E). Corresponding negative controls revealed absence of immunoreactivity (Fig. 3F). LYVE-1-immunoreactive structures with vessel-like appearance or forming lines were never observed.

The aforementioned small, round to ovoid CCL21-immunoreactive cells were lacking PDNP-immunoreactivity (Fig. 3G), and only very occasionally larger polymorph cells were detectable displaying immunoreactivity for podoplanin only (Fig. 3H). Corresponding negative controls were lacking immunoreactivity (Fig. 3I). PDNP-immunoreactive structures with vessel-like appearance or forming lines were never observed. A combination of CCL21 immunohistochemistry and transillumination in the confocal microscope revealed that CCL21+ cells were not representing choroidal melanocytes, but contained melanin-like granules (Fig. 3J). Double labeling experiments with PROX1 and VEGFR3 were applied in serial sections of 3 choroids of three different donors. In none of the investigated specimens were PROX1 positive nuclei detectable displaying a single or double lined “pearl-string-pattern,” and further dispersed PROX1-immunoreactive cells within the choroidal stroma were not identified (Fig. 3K). Single VEGFR3-immunoreactive cells were not detected. Corresponding negative controls displayed absence of immunoreactivity (Fig. 3L).

Since VEGFR3-immunoreactivity was detected in human iris smooth muscle cells, we asked if this also is the case in the choroid. For that, double labeling experiments with VEGFR3 and ASMA were performed. Nonvascular smooth muscle cells of the choroidal stroma displayed absence of VEGFR3-immunoreactivity (Figs. 4A, 4B). Choroidal arteries and veins, as identified by size and pattern of vascular smooth muscle cells, were lacking VEGFR3-immunoreactivity (Figs. 4B, 4C). However, some vessels displayed a rather high autofluorescence, thus, an absence of VEGFR3-immunoreactivity could not be unequivocally determined in these cases (Fig. 4C). Nevertheless, in all of these cases, vessels were surrounded by ASMA-positive cells. “Feeding-vessels” of the choriocapillaris (Fig. 4D) as well as choriocapillaris itself (Fig. 4E) displayed immunoreactivity for VEGFR3 on the endothelial side, while surrounding vascular smooth muscle cells were not colocalized for VEGFR3 (Figs. 4D, 4E). Interestingly, ASMA+ structures were following, though incomplete, the vascular bed into the choriocapillaris (Fig. 4E). Besides aforementioned results, other VEGFR3-positive structures with vessel-like appearance or forming lines were never observed. Corresponding negative controls displayed absence of immunoreactivity (Fig. 4F).

qRT-PCR of Lymphatic Markers Used

The presence of mRNA from lymphatic endothelial cell markers was assessed by qRT-PCR in RNA samples prepared from human choroids (n = 3) and skin samples (n = 4) as positive control. All tested genes (CCL21, FOXC2, LYVE-1, PDNP, PROX1, VEGFR3) were expressed in human skin, and all tested genes also were detected in human choroid (Fig. 5). Here, the expression levels were much lower compared to skin, represented by higher quantification cycle (Cq) levels.
FIGURE 1. Lymphatics in skin. (A, B) In HEPG2-cells, PROX-1-immunoreactivity (red) displays complete colocalization with nuclear DAPI-staining (blue), as seen by purple-mixed color (A), and immunoreactivity is absent in corresponding controls (B). (C) In human skin samples, PROX-1 immunoreactivity (red) was identified in nuclei (DAPI, blue) only (purple mixed color), appearing as typical single or double lined “pearl-string-pattern.” (D) PROX-1 (green) and FOXC2 (red) were colocalized in nuclei of human skin samples (yellow, arrows); however, PROX1-only nuclei were more abundant (arrowheads). (E) PROX-1-positive nuclei (red) were closely associated with VEGFR3-immunoreactivity (green), without
colocalization. (F) PROX1-positive nuclei (green) were associated closely with PDPN-immunoreactivity (red) in human skin samples, without colocalization (blue: DAPI). (G) PROX1-positive nuclei (green) were associated closely with CCL21-immunoreactivity (red) in human skin samples, without colocalization (blue: DAPI). (H) VEGFR3-immunoreactivity (red, arrowhead) is not overlapping with ASMA-immunoreactivity (green, arrows). Blue: DAPI.

**FIGURE 2.** CCL21 in choroid. (A, B) CCL21-immunoreactivity (red) was detected in cells surrounding choroidal blood vessels (A), most likely representing vascular smooth muscle cells (blue: DAPI; asterisk displays lumen of the blood vessel). Immunoreactivity was absent in corresponding controls (B). (C, D) Within the choroidal stroma, numerous cells with CCL21-immunoreactivity (red) were detectable (C), which were absent in corresponding negative controls (D). Blue: DAPI. (E, F) Intrinsic choroidal neurons (E) displayed immunoreactivity for CCL21 (red), and immunoreactivity was absent in corresponding controls (F; arrowbeads mark the cell border of a single neuron; arrow indicates lipofuscin granules).
FIGURE 3. Double labeling experiments of lymphatic markers in choroid. (A–C) CCL21-immunoreactive cells (red) in the choroidal stroma displayed absence of PROX1-nuclear immunoreactivity ([A]; closeup in [B]), while corresponding controls reveals absence of immunoreactivity (C). (D–F) Some CCL21-immunoreactive cells (red) in the choroidal stroma show close association with LYVE-1 immunoreactivity (green; [D]; [E], arrow), while other cells display immunoreactivity for CCL21 only ([E], arrowhead). Immunoreactivity was absent in corresponding controls (F). Blue: DAPI. (G–I) The majority (arrowheads in [G] and [H]) of the small CCL21-immunoreactive cells (green) do not colocalize for PDPN (red), while very occasionally larger cells display immunoreactivity for PDPN only ([H], arrows). Immunoreactivity was absent in corresponding controls.
with PDPN as the only exception. This marker reached higher levels compared to skin, however a contamination of the choroidal RNA preparations with adjacent RPE could not be excluded. Besides this, higher levels of RNA expression in choroid were reached by LYVE-1, followed by FOXC2 > PROX1 > CCL21 > VEGFR3 (Fig. 5). The housekeeping gene HPRT1 was used to check for RNA quality and quantity and showed comparable amplification in skin and choroid cDNA (mean Cq 27.27 and 27.30, respectively). It has to be mentioned that all PCR products were analyzed by agarose gel analysis and revealed the expected product size (data not shown).

**Discussion**

We analyzed adult human choroid with a combination of lymphatic markers by means of immunohistochemistry, and further via qRT-PCR. While with the marker combinations applied “classical” lymphatics were not identified, instead various cells expressing lymphatic markers were detected. All tested markers also were found in the mRNA-analysis.

Lymphatics form a network of blind-ending lymphatic capillaries for the drainage of extracellular fluid and lipid transport, and represent also an important part in the immune defense. Further, lymphatics have a major role in immune reactions following transplantation. While lymphatics are hard to detect in routine histological sections, and in many cases also are not discernible from small caliber blood vessels even for the well trained eye, the introduction of lymphatic markers allowed for a more suitable detection within a histological section. Such markers display extranuclear (CCL21, LYVE-1, PDPN, VEGFR3) or nuclear localization (PROX1, FOXC2) in lymphatic endothelial cells; despite reports of extranuclear PROX1 localization, this is not the case for lymphatic endothelium, as reported earlier, and also confirmed in our control study. Still, the interaction of mentioned markers in physiological and pathological conditions is not yet understood. While the transcription factor

![Figure 4](image-url)
**PROX1** is a master control gene in lymphatic development, retaining its activity in adulthood. VEGFR3 is the membrane bound target of VEGF-C and D and, thus, also responsible for lymphangiogenesis. Interestingly, VEGFR3 controls the expression of the transcription factor **PROX1** in a feedback loop. Other surface markers comprise podoplanin and LYVE-1, however, podoplanin, originally discovered in renal podocytes, also is expressed in various cell types other than lymphatic endothelium, as is LYVE-1. This highlights an important problem in lymphatic research: a single exclusive lymphatic marker is not available to date; thus, making it difficult to unequivocally determine the presence/absence of lymphatics by applying a single-marker strategy, especially in an hitherto alymphatic organ, as it is the case in the inner eye and also choroid. The combination of several markers, with at least two markers simultaneously applied, appears evident here. A further problem is the reliability of markers used for the detection of a structure that per se does not exist. For that, adequate positive controls are indispensable, and this is why skin controls have been included and presented in this study. Both parameters (i.e., several markers/adequate controls) were demanded recently in a Consensus Statement for the immunohistochemical application of lymphatic markers in ocular research, and were consequently applied here.

When immunohistochemically detecting lymphatic capillaries, these either form a double lined structure bordering a lumen if tangentially hit, or a single lined structure if the lumen is compressed due to tissue shearing artefacts (or due to tumor masses in pathological conditions), or if applying nuclear markers a single or double lined “pearl-string pattern” is evident, as shown in our controls, and also presented earlier. In none of our experiments, these aforementioned patterns have been observed in our choroidal tangential serial sections when applying four different marker combinations. Therefore, we concluded an absence of lymphatics in the adult human choroid, and this also matches with the absence of a transcellular lymphatic drainage. This, however, does not necessarily mean a general absence of choroidal lymphatics: if those exist, their lymphatic phenotype might differ from a generally presumed one, as it has been shown for the Schlemm’s Canal, for example. Further, embryological studies also might lead to different results, as markers change during maturation, but again available data are controversial.

While the typical appearance of lymphatic vessels as described above was not observed, instead various cells expressing lymphatic markers have been detected and will be discussed further. Marker CCL21 is one of the most well studied chemokine ligands in lymphatic endothelial cells. As already mentioned with other lymphatic markers (see above), CCL21 expression unfortunately is not restricted to lymphatic endothelial cells, but also detected in primary and secondary lymphoid organs in high endothelial venules, fibroblastic reticular cells, and medullary thymic epithelial cells. Together with its receptor CCR7, CCL21 represents one of the key players for B-cell, naïve T-cell, and dendritic cell trafficking via lymphatic vessels into draining lymph nodes. While in vitro experiments revealed an enhancement of dendritic cells in the induction of cytotoxic T-lymphocytes, the role for other peripheral cells, that is, cells not situated in lymph nodes, is less clear, especially in the alymphatic environment of the eye. Of interest in this sense is that CCL21 also is present in another alymphatic environment, namely the CNS, and detected at low levels in normal conditions, but elevated levels in inflammatory conditions.

We detected numerous cells immunoreactive for CCL21 and LYVE-1, clearly discernible from choroidal melanocytes. Since earlier studies demonstrated that the majority of LYVE-1-positive cells in human choroid and anterior uvea also colocalize for the macrophage marker CD68, it is most likely that the observed CCL21+/LYVE-1+ cells represent macrophages. Since CCL21 generally is not expressed on macrophages, we, therefore, interpreted this as a special requirement in an alymphatic environment. In this sense, it is important to mention that macrophages take an important part in the formation of lymphatic vessels: not only via upregulation of lymphangiogenic factors VEGF-C/VEGF-D in inflammatory conditions, thus inducing local sprouting of preexisting lymphatic endothelial cells, but also via transdifferentiation into lymphatic endothelial cells. Moreover, macrophages also

![Figure 5. Quantitative RT-PCR analysis of mRNA isolated from human donor tissue. RT-PCR results are shown as Cq values (i.e., the higher the value, the lower the mRNA amount), data are mean ± SEM of the n indicated, performed as technical triplicates. Human HPRT1 gene expression was analyzed as reference, and is almost equally expressed in both tissues.](image-url)
are crucial for the maintenance of lymphatic vessels.\textsuperscript{56} It is well known that the choroid contains a high number of resident macrophages.\textsuperscript{57} However, while their relationship in choroidal neovascularization is established,\textsuperscript{58} their contribution in the possible formation of choroidal lymphatics in inflammatory conditions must be established.\textsuperscript{59} A (theoretical) heterogeneity in the lymphangiogenic response in human should be considered, as demonstrated in animal experiments.\textsuperscript{59}

The subpopulation(s) of CCL21\textsuperscript{+} cells lacking PDLPN/LYVE-1/PROX1 must be determined in upcoming studies. Interestingly, they do not colocalize for PDLPN, as seen in lymph nodes,\textsuperscript{60} and most likely, these represent leucocytes.\textsuperscript{61} Of interest are the few observed PDLPN+/CCL21\textsuperscript{+} cells, since there are hints that PDLPN might regulate the availability of CCL21.\textsuperscript{62,65}

In our study, CCL21 has been detected in vascular smooth muscle cells of the choroid. An earlier study reported CCL21 in human aortic vascular smooth muscle cells,\textsuperscript{62,63} and in vitro experiments suggested that inflammatory conditions are able to alter the vascular smooth muscle cell phenotype into lymphoid tissue organizer cells\textsuperscript{64}, thus contributing to the lymphoid homeostasis. If this also is the case in the posterior uvea remains unknown as yet, and of special interest will be to investigate this situation further under inflammatory conditions.

Expression of CCL21 also is a known feature in neurons and glial cells of the central and peripheral nervous system,\textsuperscript{65,66} and the clustered cells with smooth contoured outline containing lipofuscin granules were identified unequivocally as intrinsic choroidal neurons.\textsuperscript{67–69} While in the central nervous system, CCL21 has been detected in injured neurons only,\textsuperscript{70} the neuronal CCL21-immunoreactivity observed herein might be caused by unknown neuronal stress, but we also cannot exclude postmortal tissue alterations leading to CCL21 expression. If CCL21 will serve as a tool to study intrinsic choroidal neurons in pathological conditions must be clarified.

A further lymphatic endothelial marker investigated more thoroughly in this study is VEGFR3, and, as already mentioned above, VEGFR3 represents the target for VEGFC and D and, thus, is responsible for lymphangiogenesis. If and how VEGFR3 also is involved in lymphvasculogenesis,\textsuperscript{71} and if this also can take place in the eye/choroid under certain conditions, remains to be established. While VEGFR3 positive cells are known\textsuperscript{72} and also found in the anterior uvea,\textsuperscript{52} these were not present in the choroid, and we interpret this difference in the different functional needs of anterior (accommodation/aqueous humor production) and posterior uvea (retinal supply). We present in the choroid, and we interpret this difference in the different functional needs of anterior (accommodation/aqueous humor production) and posterior uvea (retinal supply). We therefore are the few observed PDLPN+/CCL21\textsuperscript{+} cells, since there are hints that PDLPN might regulate the availability of CCL21.\textsuperscript{62,65}

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Expression of CCL21 also is a known feature in neurons and glial cells of the central and peripheral nervous system,\textsuperscript{65,66} and the clustered cells with smooth contoured outline containing lipofuscin granules were identified unequivocally as intrinsic choroidal neurons.\textsuperscript{67–69} While in the central nervous system, CCL21 has been detected in injured neurons only,\textsuperscript{70} the neuronal CCL21-immunoreactivity observed herein might be caused by unknown neuronal stress, but we also cannot exclude postmortal tissue alterations leading to CCL21 expression. If CCL21 will serve as a tool to study intrinsic choroidal neurons in pathological conditions must be clarified.

A further lymphatic endothelial marker investigated more thoroughly in this study is VEGFR3, and, as already mentioned above, VEGFR3 represents the target for VEGFC and D and, thus, is responsible for lymphangiogenesis. If and how VEGFR3 also is involved in lymphvasculogenesis,\textsuperscript{71} and if this also can take place in the eye/choroid under certain conditions, remains to be established. While VEGFR3 positive cells are known\textsuperscript{72} and also found in the anterior uvea,\textsuperscript{52} these were not present in the choroid, and we interpret this difference in the different functional needs of anterior (accommodation/aqueous humor production) and posterior uvea (retinal supply). We then were addressing the question if the nonvascular\textsuperscript{54} or vascular smooth muscle cells of the choroid express VEGFR3, as demonstrated for the anterior uvea.\textsuperscript{52} As our results showed, these are absent, and only vessels of the choriocapillaris expressed VEGFR3-immunoreactivity on the endothelial side. This matches with earlier results demonstrating VEGFR3 in fenestrated capillaries,\textsuperscript{75} including the human choriocapillaris.\textsuperscript{76} While a strong polar expression was not evident in our results, probably caused by the different tissue orientation or detection method used, choriocapillaris feeder vessels, that is, vessels directly entering the choriocapillaris from the suprachoroidal/scleral side, also were displaying VEGFR3 immuno-reactivity. These vessels still might be influenced by the RPE complex, as proposed earlier, and also could be part of the CNV-development cascade.\textsuperscript{76} Of interest in this sense is the observed incomplete ASMA coverage of the choriocapillaris, displaying a rather venous phenotype. These ASMA\textsuperscript{+} structures could derive from pericytes, as these can be “positioned longitudinally in a polar fashion along the microvessel.”\textsuperscript{77}

While pericytes express ASMA, depending on their location in the vascular bed, several investigators have shown that this is not the case in pericytes surrounding capillaries.\textsuperscript{78–80} Given that the pericyte coverage in the adult human choriocapillaris is almost absent,\textsuperscript{81} detected ASMA\textsuperscript{+} structures, therefore, might also represent remnants of true vascular smooth muscle cells, originating from larger, and more suprachoroidally situated vessels. This, however, has to be proven in upcoming studies. Nevertheless, it would be of interest if these observed smooth muscle fibers contribute to changes in capillary lumen and, hence, choriocapillaris blood flow and also which parameters might be involved in controlling lumen changes. Since the choriocapillaris lacks innervation from the autonomic nervous system,\textsuperscript{82,83} nitric oxide or neuro(peptide)receptors may be suitable candidates.\textsuperscript{68,84,85}

Messenger RNA expression was detected successfully for all lymphatic markers analyzed, and matches with the immunohistochemical results. However, choroidal PDLPN reached a rather high mRNA level compared to skin, and we probably were not successful in the total removal of RPE from the choroid before the RNA analysis, and therefore a PDLPN “contamination” of RPE origin\textsuperscript{80} in the choroidal sample might lead to the high RNA amount observed. Besides this, only LYVE-1 is expressed at higher levels in the human choroid, which is in line with results of earlier immunohistological studies\textsuperscript{81} and also this study.

Taken together, we adhered to the demands of a Consensus Statement regarding the use of lymphatic markers in ocular research, and were unable to detect lymphatic vessels with the marker combinations used. Therefore, we considered the choroid and the inner human eye as alymphatic, at least under physiological conditions. While the characterization of observed cells expressing lymphatic markers is beyond the scope of this study, it will be important for future studies and especially in pathological conditions, where expression levels/cell types/phenotypes might be altered. Their contribution to ocular homeostasis is unknown yet.

Acknowledgments

Supported by grants from the Research Fund of the Paracelsus Medical University (R15-/01/042-KAS, AK-E), The Lotte Schwarz Endowment for Experimental Ophthalmology and Glaucoma Research, The Fuchs-Foundation for Research in Ophthalmology, and the German Research Foundation (FOR 2240 “LymphAngiogenesis and Cellular Immunity in Inflammatory Diseases of the Eye”) (LMH). HE 67/3-2/1 and HE 67/3-3/1 (LMH), GERO Program University Hospital of Cologne (LMH). The authors alone are responsible for the content and writing of this paper.

Disclosure: F. Schrödl, None; A. Kaser-Eichberger, None; A. Trost, None; C. Strohmaier, None; B. Bogner, None; C. Runge, None; K. Motloch, None; D. Bruckner, None; M. Laimer, None; L.M. Heindl, None; H.A. Reitsamer, None

References

Lymphatics in Human Choroid


