Expression of Podoplanin and Other Lymphatic Markers in the Human Anterior Eye Segment

Kerstin Birke,\textsuperscript{1} Elke Lütjen-Drecoll,\textsuperscript{1} Dotscho Kerjaschki,\textsuperscript{2} and Marco T. Birke\textsuperscript{1}

PURPOSE. To investigate the expression of lymphatic endothelial cell (LEC) markers in tissues of the anterior eye segment.

METHODS. Sections of human anterior segments from eight eyes of eight donors (37–100 years) were stained for Vegf-R3, Prox-1, Lyve-1, Pdpn, Pcx, CCR7, Ccl19, Ccl21, and CD68. Pdpn localization was additionally analyzed by immunogold labeling on sections of five eyes. Expression of LEC markers and chemokine receptor/ligands was analyzed by RT-PCR in iris and trabecular meshwork (TM) tissue from three eyes and eight human TM (hTM) cell cultures.

RESULTS. Vegf-R3 and Prox-1 stained no structures in the anterior segment. Lyve-1 stained single dendriform cells in the ciliary body, the TM, and the iris. Pdpn stained all trabecular cells, the cells of the trabeculum ciliare, and the anteriormost perimysium cells of the ciliary muscle. Schlemm’s canal endothelium was not stained but reacted to a podocalyxin antibody. In the iris stroma, single dendriform cells were stained; at the anterior surface, almost all cells were Pdpn\textsuperscript{-}. Few stromal cells were Pdpn/Lyve\textsuperscript{+}, but several anterior surface cells were Pdpn/Ccl21\textsuperscript{-}. Solitary CCR7\textsuperscript{+} cells were observed there, too. IF results were confirmed by PCR, but Prox-1 was detected in TM and iris. Cultured hTM cells displayed partial Pdpn/Ccl21 colocalization.

CONCLUSIONS. Coexpression of Pdpn and Ccl21 at the anterior iris surface and in the chamber angle suggests the constitutive expression of a chemokine gradient guiding APCs through the anterior chamber. The more pronounced expression of Pdpn in the TM could favor egression of APCs by way of the conventional outflow. (Invest Ophthalmol Vis Sci. 2010;51:344–354) DOI: 10.1167/iovs.08-3507

The lymphatic system is a highly specialized vessel system that fulfills critical functions within the body. On the one hand, lymphatics take up excess interstitial fluid and macromolecules from peripheral tissues and feed them back into the circulatory system.\textsuperscript{1,2} On the other hand, lymphatic vessels serve as migration routes for lymphocytes and APCs from the periphery toward secondary lymphatic organs, implying that a central role in the regulation and mediation of immunologic responses.\textsuperscript{1–3}

In the eye, lymphatic vessels with a continuous endothelial lining do not exist. Excess fluid and macromolecules are transported from the ocular tissues to the venous system via the aqueous humor.\textsuperscript{4–8} Moreover, the aqueous humor serves as a transport medium for bone marrow-derived cells of the immune system, such as antigen-presenting cells (APCs), so the aqueous humor drainage system substitutes the functions of lymphatic vessels in the inner eye. APCs that patrol the anterior chamber enter the anterior eye segment mainly through the iridal vasculature.\textsuperscript{9–12} APCs have been detected in tissues of the chamber angle and within the ciliary muscle, implying that they leave the anterior chamber together with aqueous humor via the conventional and the uveoscleral outflow pathways.\textsuperscript{13,15} The transport of fluid through both outflow routes is passively driven by the pressure difference between the intraocular pressure and the venous pressure within the draining venules. This might be sufficient for cellular transport, but it is tempting to speculate about the existence of a supportive guidance mechanism that actively conveys and regulates APC migration. This assumption becomes even more equitable against the background that the choice of outflow route—conventional versus uveoscleral—is discussed to have an important impact on ocular immune privilege, a phenomenon important for lifetime protection of the eye.\textsuperscript{16–19}

The human eyes used in this study were donated to the Institute of Anatomy Erlangen. Methods of securing human tissue were humane, included proper consent and approval, and complied with the Declaration of Helsinki.

Altogether, 23 eyes from 13 donors with no history of ocular disease were studied. Donor ages ranged from 37 to 100 years.

MATERIALS AND METHODS

Human Donor Eyes

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**Table 1. List of Antibodies**

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<th>Antibody</th>
<th>Abbreviation</th>
<th>Application</th>
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<td>IF (tissue)</td>
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<td>Abcam, Cambridge, MA</td>
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<td>Gift of Dontscho Kerjaschki</td>
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**Histologic Processing**

Fifteen donor eyes from 13 donors were used for immunofluorescence analysis. Mean postmortem time until fixation was 15 ± 3 hours. The eyes were bisected at the equator and immersion-fixed in 4% (vol/vol) paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2) for at least 24 hours. For paraffin sections, eyes were cut in quadrants, dehydrated in ascending ethanol series and isopropanol, and embedded in paraffin in an embedding machine (Microm-Heidelberg, Heidelberg, Germany). Twelve-micrometer paraffin sections were cut (Histostride 200R; Leica, Wetzlar, Germany) and mounted on coverslips (Superfrost; Menzel Glaser, Braunschweig, Germany). Twelve-micrometer paraffin sections were deparaffinized and rehydrated in xylol and a descending series of ethanol and rinsed in PBS. Cryosections, fixed quadrants were washed in PBS and embedded in tissue-freezing medium (Jung; Leica, Wetzlar, Germany); 12-μm cryosections were then cut with a cryostat (CM 3050S; Leica).

**Human TM Cell Cultures**

Explant cultures of human TM (hTM) cells were obtained from our collaborators at the Eye Department Erlangen. Methods of securing human tissue were humane, included proper consent and approval, and complied with the Declaration of Helsinki. Monolayer cultures were established from eyes of five human donors without any history of eye diseases and obtained 4 to 8 hours postmortem. Cultures were grown in 2 mL complete Ham’s F-10 medium (10% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin [all from Gibco-Life Science Technology, Karlsruhe, Germany]). The medium was changed every second day, and cells were passaged in a split ratio of 1:2 using 50 U/mL penicillin, and 50 μg/mL streptomycin (Gibco-Life Science Technology). The medium was changed every second day, and cells were passaged in a split ratio of 1:2 using 50 U/mL penicillin, and 50 μg/mL streptomycin (Gibco-Life Science Technology).

**Immunofluorescence Procedure**

Paraffin sections were deparaffinized and rehydrated in xylol and a descending series of ethanol and rinsed in PBS. Cryosections were air-dried before the staining procedure. Cultured hTM cells were grown on microscope chamber slides. The cells were washed three times with PBS, then fixed in methanol for 4 minutes and air dried. Sections or hTM cells were blocked in 1% (wt/vol) bovine serum albumin (BSA) and 0.2% Triton X-100 (Table 1 lists suppliers and dilutions) and were incubated overnight at RT. After three rinses with PBS, sections were incubated with the corresponding secondary antibodies (Table 1) diluted in PBS for 1 hour at RT. After three rinses with PBS, the sections were mounted with glycerol gelatin (Kaiser’s; Merck KgaA, Darmstadt, Germany), and hTM cells were mounted with fluorescent mounting medium (Dako, Glostrup, Denmark) containing 4’,6-diamidino-2-phenylindol to counterstain the cell nuclei.

**Immunoelectron Microscopy**

Five donor eyes from five donors were used for immunoelectron microscopy analysis. Specimens of the chamber angle region and the iris, respectively, were prepared and fixed in 4% (vol/vol) PFA in PBS for 2 hours at 4°C. The fixed tissue samples were then immersed in 4% (wt/vol) sucrose in PBS for at least 24 hours and were subsequently frozen in liquid nitrogen. For pre-embedding immunocytochemistry, 25-μm cryostat sections were cut and mounted on coverslips (Thermo Scientific). Table 2. Primer Sequences and PCR Conditions

<table>
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<tr>
<th>Gene</th>
<th>Primer Sequence (5’-3’)</th>
<th>nt Position</th>
<th>Annealing (°C)</th>
<th>Cycles</th>
<th>Product Size (bp)</th>
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<td>471</td>
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<td>841</td>
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<td>CCL21</td>
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<td>β-Actin</td>
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<td>260–805</td>
<td>58</td>
<td>27</td>
<td>545</td>
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Fwd, forward; Rev, reverse.
Cryosections were blocked in low-fat milk powder (Blotto; Santa Cruz Biotechnology) for 30 minutes at RT. The anti-Pdpn antibody (Table 1) was applied in PBS/0.2% (wt/vol) BSA and incubated overnight at 4°C. After six rinses with PBS/2% (wt/vol) BSA, the sections were incubated overnight at 4°C with ultra-small gold-conjugated anti-mouse F(ab′)2 in PBS/0.2% (wt/vol) BSA. Sections were then rinsed five times with PBS/0.2% (wt/vol) BSA and two times with PBS and were fixed with 2.5% (vol/vol) glutaraldehyde in PBS for 2 hours at 4°C. Silver enhancement was then performed (R-Gent SE-EM; Aurion, Wageningen Netherlands) for 1.5 hours in darkness. Sections were postfixed with 0.5% (wt/vol) OsO4 in PBS for 15 minutes and embedded in Epon. Ultrathin sections of the specimens were cut and examined in an electron microscope (EM 902; Zeiss, Oberkochen Germany). Apart from the omission of the primary antibody, the control sections were treated the same way. No control section showed labeling with gold particles.

**RNA Isolation, Reverse Transcription, and Polymerase Chain Reaction**

TM and iris tissue were prepared from three eyes from three donors. Samples were homogenized, and total RNA was extracted with the phenol-chloroform method using reagent (TRIzol; Invitrogen, Karlsruhe, Germany). Structural integrity of the RNA samples was confirmed by electrophoresis in 1% Tris-acetate-EDTA (TAE)-agarose gels. Yield and purity were determined photometrically. First-strand complementary DNA (cDNA) for PCR was prepared from total RNA by reverse transcription using a reverse transcription kit (SuperScriptIII; Invitrogen).

**FIGURE 1.** Overview of Pdpn staining in the anterior eye segment. There was intense staining for Pdpn in the entire TM, including the trabeculum ciliare (TC), at the muscle tips, at the anterior iris surface, and within the iris (I) stroma. S, sclera; C, cornea; AC, anterior chamber; SC, Schlemm’s canal; CB, ciliary body.

**FIGURE 2.** Staining and PCR expression analysis of LEC markers in the TM. (A) Higher magnification of the TM showed that all TM cells were Pdpn+.* (B) Single Lyve-1+ cells (arrowheads) were detected within the intertrabecular spaces. Staining for VEGF-R3 (C; small speckles are due to the autofluorescence of lipofuscin granules) and for Prox-1 (D) was negative. (E) Pdpn and Prox-1 mRNA expression were detected by PCR. Lyve-1 and Vegf-R3 mRNAs were not detectable.
Linear range of the gene-specific PCRs and functionality of primers were tested before the experiments on cDNA prepared from human lymph nodes. Amplificates of correct sizes were always obtained, even on lymph node material with extended postmortem times. PCRs were performed with a temperature profile as follows: 30 seconds denaturation at 94°C, 30 seconds annealing at primer-specific temperatures (Table 2), and 45-second primer extension at 72°C. Product-specific cycle numbers are shown in Table 2. PCR products were size fractioned in 1% TAE-agarose gels and ethidium bromide (EtBr) stained, and signal intensities were quantified with specialized software (BioDocAnalyze; Whatman Biometra biomedizinische Analytik GmbH, Göttingen, Germany). β-Actin PCR served as an internal control and was considered for quantification.

**RESULTS**

**Control Stainings for LEC Markers and Ccl5**

Antibodies directed against LEC markers Pdpn, Vegf-R3, Prox-1, and Lyve-1 were tested in IF stainings on control tissues before they were used on anterior chamber sections. Positive controls were sections of PFA-fixed, paraffin-embedded, or cryoembedded human lymph nodes and corneal sections with intact bulbar conjunctiva. All antibodies stained vessel-like structures in human lymph nodes and the endothelial cell lining of lymphatic vessels in the bulbar conjunctiva on cryoembedded tissue with short postmortem times (<4 hours). Lyve-1 and Pdpn additionally stained solitary dendriform cells within the lymph node, as described. On paraffin-embedded tissues, clear staining signals were obtained for Pdpn only. On tissues with extended postmortem times (>4 hours), comparable to that of the donor eyes, signals for Prox-1 and Vegf-R3 became strongly reduced to nondetectable. None of these structures was labeled by a podocalyxin (Pcx) antibody, a marker for vascular capillary endothelium, thus ensuring the lymphatic nature of the described vessel structures. Blood vessels within the lymph nodes, however, were intensely stained. Results of control stainings for Ccl19, Ccl21, and CCR7 were identical and were obtained on cryoembedded tissues with short postmortem times only.

**Overview of LEC Markers in the Anterior Eye Segment**

None of the LEC markers stained vessel-like structures in the AC. Vegf-R3 and Prox-1 did not stain any tissue structures or cells in the entire anterior eye segment. Staining for Lyve-1 was seen on solitary dendriform cells throughout the entire uvea and TM. Staining for Pdpn, in contrast, was intense on almost all cells of the tissues lining the anterior chamber, namely the TM, including the trabeculum ciliare in front of the ciliary muscle, and the iris (Fig. 1).

**Trabecular Meshwork**

Trabecular cells throughout all layers of the TM were intensely stained for Pdpn (Figs. 1, 2A). In sagittal sections, Pdpn staining was seen anteriorly up to the transition zone of the TM into the cornea. Corneal endothelial cells and keratocytes remained unstained (Fig. 1). Posteriorly, the Pdpn signal disappeared abruptly at the scleral spur, and scleral spur cells as well as scleral fibroblasts were not stained (Fig. 1). Tangential sections parallel to the inner wall of Schlemm’s canal showed that all TM cells throughout the entire circumference were Pdpn⁺.

Lyve-1 staining, in contrast, was detected on some sections of the TM only. TM cells themselves were not stained, but, when present, solitary cells of dendriform shape sitting within the intertrabecular spaces of the TM were Lyve-1⁺ (Fig. 2B). Vegf-R3 and Prox-1 did not stain any cell in the TM (Figs. 2C, D).

**PCR expression analysis of TM tissue confirmed the presence of Pdpn mRNA and the absence of Lyve-1 and Vegf-R3 expression. In contrast to the staining results, amplificates of the Prox-1 mRNA were obtained, indicating low-level expression of this factor at least on the transcriptional level (Fig. 2E).**

On the ultrastructural level, immunogold labeling for Pdpn confirmed that all trabecular cells were immunoreactive to Pdpn and that even the subendothelial TM cells, often in contact with the inner wall endothelium, were labeled (Fig. 3A). Moreover, it revealed that labeling was restricted to the cell membrane of the TM cells. Endothelial cells covering the inner and outer walls of Schlemm’s canal, in contrast, remained completely unlabeled (Fig. 3A). They were reactive to anti-Pcx antibody, a marker for blood vessel endothelial cells, which did not stain the TM cells (Fig. 3B).

PCR expression analysis of TM tissue samples demonstrated expression of the chemokine ligands Ccl19 and Ccl21, whereas the corresponding receptor, CCR7, was not detectable (Fig. 4A). Like intact TM tissue, in vitro-cultured human TM cells also stained intensely for Pdpn. In addition, they stained for Ccl21, but the staining was less intense (Fig. 4B). Overlay of the staining signals revealed that Pdpn and Ccl21 signals colocalized at certain areas of the cell membrane (Fig. 4B). None of the TM cultures stained for Ccl19 or CCR7. PCR analysis confirmed the staining result of Pdpn and Ccl21 because both were expressed in cultured TM cells. In addition, slightly decreased expression of Ccl21 compared with TM tissue was
detected. Like TM tissue, cultured TM cells also expressed Ccl19 mRNA (Fig. 4C).

Ciliary Body and Ciliary Muscle
Staining for Pdpn was observed in the region of the trabeculum ciliare, the connection between the uveal TM and the iris root, at the anterior part of the CM (Figs. 1, 5A). Thin line–like staining surrounded the interstitial spaces within the anteriormost tips of the ciliary muscle (Fig. 5A). The ciliary muscle itself and the ciliary processes, including their epithelia, were not stained. Sparse, single Pdpn+ dendriform cells were detected within the ground plate of the ciliary body (Fig. 1). Lyve-1+ cells were present within the ground plate of the ciliary body and also within the interstitial spaces of the ciliary muscle (Fig. 5B).

Electron microscopic investigations disclosed flat cells with thin cytoplasmic processes (i.e., trabecular cells of the trabeculum ciliare and perimysium cells of the anterior ciliary muscle tips) to account for Pdpn labeling (Fig. 5C). Ciliary muscle cells themselves were not immunoreactive (Fig. 5C).

Iris
Intense staining for Pdpn was observed on all cells at the anterior surface. In addition, Pdpn+ dendriform cells were detected throughout the entire stroma, displaying a homogeneous distribution (Figs. 1, 6A). Lyve-1+ dendriform cells were observed throughout the entire iris stroma (Fig. 6B), but they appeared to be more concentrated close to vessels or even within the adventitial sheath of the vessels. Double staining with Pcx confirmed that numerous Lyve-1+ cells were located adjacent to the Pcx+ vascular endothelium (Fig. 6C). Overall, the number of Lyve-1+ cells increased toward the iris root, whereas only low numbers of Lyve-1+ cells were detected in the region of the pupillary sphincter. Double staining for Pdpn and Lyve-1 revealed that only a small number of the dendriform cells within the stroma, but also at the anterior surface, expressed both markers (Fig. 6D). Most of the dendriform cells were stained solely for Pdpn or Lyve-1. Further discrimination of the cells by double staining with CD68 did not succeed because the quality of the material with respect to fixation and postmortem time was not adequate for the requirements of the applied antibodies. Staining signals for Vegf-R3 or Prox-1 antibody were not observable in the entire iris (Figs. 6E, F).

PCR expression analysis of human iris tissue confirmed the presence of Pdpn and Lyve-1 mRNA and the lack of Vegf-R3 mRNA, consistent with staining data. As observed in TM tissue and in the iris expression of Prox-1 mRNA was also revealed by this method (Fig. 6G).

On the ultrastructural level, immunoreactivity to Pdpn was observed at the anterior surface (Fig. 7A) and within the stroma (Fig. 7B), consistent with immunofluorescence staining data. At the anterior surface, all cells were labeled for Pdpn, and immunogold particles were restricted to the cell membranes (Fig. 7A). Within the stroma, most cells without dense...
bandlike connections between the cell membrane and the surrounding connective tissue were Pdpn immunoreactive (Fig. 7B).

Staining for Ccl21 was detected most abundantly toward the anterior surface (Fig. 8A). Double staining with Pdpn revealed that most of the Pdpn+/Ccl21+ cells were located at the anterior surface and reaching into iris crypts. Staining signals for Ccl19 were not observed. CCR7+/H11001 cells were detected only at the anterior iris surface in some sections, and some of these cells appeared to just exit the iris (Fig. 8B).

Expression of Ccl21 and CCR7 was confirmed by PCR analysis of iris tissue (Fig. 8C). In addition, signals for Ccl19 were also detected, but only at higher PCR cycles, indicating that Ccl21 was more abundantly expressed than Ccl19.

**DISCUSSION**

The intention of the presented study was to analyze the expression and distribution of a set of characteristic LEC markers in the tissues of the anterior eye segment. Concordantly to the literature, no lymph vessel-like structures were stained for any of the markers in the inner eye. However, expression of individual markers was detected either on the protein level by immunofluorescence or on the mRNA level by PCR. The markers detected by immunofluorescence were Pdpn and Lyve-1, which stained either entire structures such as the TM and anterior iris surface (Pdpn) or stained single dendriform cells distributed throughout the entire anterior segment (Lyve-1). Dendriform cells within the iris stroma were stained for Lyve-1 or Pdpn, and, to a small extent, for both markers. Expression of both markers was confirmed by PCR in TM and iris tissue; Prox-1 mRNA expression was detected in both tissues. VEGF-R3 expression was not observed, either by IF or by PCR.

In the field of lymphatics, Prox-1 and, especially, Vegf-R3 seem to play crucial roles in the early induction of lymphangiogenesis. In the proposed model of this process, Vegf-1 and -3 signals are conducted via Vegf-R3 into the cell and induce Prox-1 expression in the nucleus of LEC precursors. This transcription factor then activates the expression of downstream target genes, such as Pdpn, promoting LEC differentiation and maturation. This would demand strong Vegf-R3 and Prox-1 expression during embryonic lymphangiogenesis or lymph(neo)angiogenesis, but it also implies that Prox-1 would be a prerequisite for Pdpn expression. Indeed, during abortive corneal transplantation, a strong reactivation of Vegf-R3 and Prox-1 expression has been demonstrated in newly developing lymphatic vessels, sprouting from preexisting lymphatics, accompanying the rejection process. Our negative immunofluorescence data on Prox-1 protein expression, however, seem to conflict with the mechanistic demand.
for subsequent Pdpn expression. In addition to technical issues, such as extended postmortem time of donor material or antigen destruction attributed to fixation procedures, other possible explanations for the negative staining results might have to be taken into account. In mature lymphatics, other mechanisms ensuring the constitutive transcription of Prox-1 downstream targets might be active (e.g., chromatin remodeling at the promoter site to a constitutively transcriptional “open” state, posttranscriptional stabilization of the corresponding mRNA species, protein stabilization). Our data might indicate the downregulation of Prox-1 expression in a mature lymphatic-like tissue, and the detection of Prox-1 mRNA would still comply with the mechanistic demands. Low-level Prox-1 protein expression, therefore, just might underrun the methodical threshold of the staining technique. To our knowledge, involvement of any of these mechanisms has not yet been described in LECs; thus, future studies would be of high interest for new insight into the control of LEC marker expression.

As mentioned, we did not detect Lyve-1 expression on structural cells of ocular tissues. This finding might be reasonable when the proposed function of Lyve-1 in lymphatics is considered. Based on its capacity to bind hyaluronan (HA), Lyve-1 is thought to be important for coating the luminal surface of lymphatic vessels with HA, which might in turn serve as a docking molecule for HA-binding lymphocytes and dendritic cells. Moreover, discussions are under way that
binding to HA is the initial prerequisite for transendothelial passing of immune cells during extravasation from lymphatics into the tissue. Yet, interestingly, lymphatic collector vessels that take up immune cells leaving the tissue have been shown to be negative for Lyve-1.41,42 On the one hand, lack of Lyve-1 in the tissues of the conventional and the uveoscleral outflow would agree with this finding because both tissues in the context of APC migration out of the anterior chamber would imply the transport of excess extracellular fluid and macrophages are CD68+. In our study, reliable results for Lyve-1/CD68 double staining were not obtained, most likely because of the already mentioned technical issues with respect to postmortem times of the material and fixation requirements of the applied CD68 antibodies. Therefore, exact characterization of the phenotype was not possible, and we could not exclude that sessile iridal reticulum cells are Lyve-1+. The role of Lyve-1 in APC transport through the iris crypts toward the anterior surface will require further investigation.

The distribution of Pdpn+ cells within the iris stroma was similar to some extent. Dendriform cells were located loosely throughout the entire stroma, but distinct accumulation next to vessels was not observed. Again, exact discrimination of sessile iridal reticulum cells and migrating APCs was not possible. Immunogold labeling indicated that motile cells, which did not show tight association to the connective tissue, were Pdpn+. It is known that Pdpn renders cells motile by directly affecting the cytoskeleton45 and that tumor cells express high levels of Pdpn in the dissemination process. However, such a mechanism has not yet been described for nonmalignant, normal APCs. Recently, Pdpn was introduced as a marker for follicular dendritic cells68,69 which indicates that it is a marker for nonmigrating dendritic cells. Thus, the exact function of Pdpn for the stromal iris cells remains unclear. The double staining for Pdpn and Lyve-1 revealed that only a small number of cells were positive for both markers, indicating either the coexistence of different types of APCs or changes of the cells’ phenotype on their way through the iris, presumably reflecting different maturation states. Additional investigations of the stromal cells with respect to their marker expression and changes thereof will be required to elucidate the nature of the iridal dendriform cells.

Toward the anterior surface, the Pdpn signal became significantly stronger when compared with that of the stroma. Here almost every cell was Pdpn+ so that the entire anterior surface seemed to be built up of Pdpn+ cells. Kerjaschki et al.47 demonstrated that Pdpn binds Ccl21 with high affinity in vitro and that Pdpn and Ccl21 significantly colocalize within lymph nodes in vivo. Our observed expression of Ccl19 and Ccl21 in the iris, and especially the colocalization of Pdpn and Ccl21 signals on cells at the anterior surface, led to the speculation that Pdpn could contribute to the establishment of a chemokine gradient toward the anterior surface, the desired exit for APCs. Ccl21 and Ccl19 are the only ligands for CCR7, a surface receptor expressed by most migrating APCs.70–74 We consistently detected CCR7 expression in the iris and identified CCR7+ cells at the anterior surface. For the murine system, it was shown that in vitro-generated ocular APCs do not upregulate CCR7 on antigen uptake, as nonocular APCs do.75–77 However, CCR7 was still expressed, though at lower levels than in active, migrating APCs from other tissues.77 As a consequence, the data collected in mice do not exclude the possibility of the migration mechanism we propose. In the same study it was shown that ocular APCs express and, when activated, down-regulate CCR6,70 another chemokine receptor that, via its interaction with Ccl20, facilitates tissue entry and maintains localization of APCs within the tissue.77–79 Further studies will be required focusing on the role of this receptor/ligand interaction to elucidate the entry mechanism by which APCs access the iris.

In addition to the anterior iris surface, strong labeling for Pdpn was detected at the ocular tissues bordering the anterior eye chamber and the aqueous humor outflow routes. These tissues convey the controlled drainage of aqueous humor and thereby the transport of excess extracellular fluid and macro-molecules. Moreover, cells of the immune system, such as APCs, have been localized in both aqueous humor outflow...

**Figure 7.** Immunogold labeling for Pdpn on iris tissue. (A) Labeling for Pdpn was found at the cell membranes of all cells at the anterior surface and (B) on cells not attached to the fibrillar material within the iris stroma.
tissues. It is not known whether those cells reach the described tissues passively, driven by the flow of aqueous humor circulation toward the outflow tissue only, or whether the cells of the outflow tissues actively participate in attracting, thus directing, the immune cells. As mentioned, one of the most important mechanisms is the interaction between CCR7 and its ligands Ccl21 and Ccl19. In this context, the expression of Pdpn in the chamber angle region would be reasonable. This constitutes the entry site to the outflow routes and, thus, the exit for aqueous humor and presumably the APCs circulating within the aqueous humor. In support of this hypothesis, we demonstrated excised TM tissue specimens to express the mRNAs for Ccl19 and Ccl21 and, moreover, showed that Pdpn and Ccl21 colocalize on in vitro-cultured TM cells. Consequently, the constitution of a sink for chemokine ligands toward the outflow tissues seems possible. Moreover, the different Pdpn staining intensities at the conventional and the uveoscleral outflow routes might be suggestive for differences in the attraction capacities of both sites. The by far more pronounced expression of Pdpn in the conventional outflow tissue could be the basis for a favored migration toward the TM. This would be of pivotal significance with regard to Streilein’s postulations concerning ACAID. He claimed that the selection of the outflow route used by the APCs (use of the conventional outflow route) constitutes a crucial prerequisite for ACAID because only through the TM and Schlemm’s canal can the APCs access the blood circulation to reach the spleen.

In summary, our morphologic and initial experimental data suggest that Pdpn might have a function in constituting a chemokine gradient guiding immunocompetent cells out of the iris toward the entry sites of the outflow tissues. Hence, the anterior surface of the iris, the anteriormost portion of the uveoscleral outflow route, and especially the TM might fulfill surrogate lymphatic functions.

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**References**

4. Kaufman PL. Enhancing trabecular outflow by disrupting the actin cytoskeleton, increasing uveoscleral outflow with prostaglandins, and understanding the pathophysiology of presbyopia interrogating mother nature: asking why, asking how, recognizing the signs, following the trail. Exp Eye Res. 2008;86:3-17.


42. Jackson DG. Biology of the lymphatic marker LYVE-1 and applications in research into lymphatic trafficking and lymphangiogenesis. APMIS. 2004;112:526–538.


