Consensus Statement on the Immunohistochemical Detection of Ocular Lymphatic Vessels

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There is currently considerable controversy about existence and classification of "lymphatic vessels" in the eye. Some of the confusion is certainly caused by inappropriate use (or nonuse) of the correct immunohistochemical markers. Many experts in the field expressed the need for a consensus statement, and, in this perspective, authors offer arguments and solutions to reliably continue with immunohistochemical ocular lymphatic research.

Keywords: immune privilege, immunocytochemistry, lymphatics

The eye is an immune-privileged site where foreign tissue grafts enjoy long-term or even indefinite survival in the absence of immunosuppressive treatments.[1] A number of factors contribute to the immune-privileged nature of various sites within the eye including the absence of a "classical" ocular lymphatic system.[2] Recent advances in lymphangiogenesis research,[3] have however, shown that lymphangiogenesis can occur in the normally avascular and alymphatic cornea in inflammatory conditions,[4,5] and intraocularly, if the scleral border is compromised.[6,7] The evidence for a "classical" lymphatic system in the inner portions of the eye under physiological conditions remains controversial.[8–10] Earlier, limited ultrastructural evidence of lymphatic capillaries was found in the choroid of nonhuman primate eyes, where distinct spaces that contain amorphous material and no collagen, delineated by extremely thin and irregular cellular walls, were identified.[11] Lymphatic capillaries have also been identified in the choroid of birds.[12,13]

The application of lymphatic lineage markers LYVE-1 and Podoplanin,[8] or LYVE-1, Flt4/VEGFR-3,[14] found only LYVE-1$^+$ macrophages in the choroid. These studies concluded that there is an absence of formed lymphatic channels in the healthy, adult, human choroid[8] and in 8- to 12-week-old murine eyes,[14] with no evidence of "classical" lymphatic vessels in the healthy, adult, human choroid. However, net-like structures with a pseudovessel appearance in the human choroid have been reported,[8] and it has been suggested that lymphatic vascular precursor cells (represented as LYVE-1$^+$ macrophages) in the choroidal stroma do not form functional channels but may respond to inflammatory stimuli.[8] The field, however, may be moving rapidly. Indeed, preliminary work presented at two conferences in recent years, may suggest a system of initial lymphatic, pre-, and collector vessels close to the choriocap-

illaris in the human adult choroid and retina. Moreover, there is functional evidence for a lymphatic system in the anterior uvea[9] and in the bulbar conjunctiva.[15] Yucel[9] provided functional evidence for the existence of anterior uveal lymphatics in sheep by injecting Iodine-125 radio-labelled human serum albumin into sheep eyes that drained into head and neck lymph nodes, including the cervical, retropharyngeal, submandibular, and preauricular nodes. While it would appear inconsistent for lymphatics to be present only in the anterior eye, with no system for removal of excess interstitial fluid and proteins from the posterior eye; and no direct means for antigen presenting cells to exit the posterior eye and present antigen at sentinel lymph nodes, the existence of "classical" lymphatics within the eye remains controversial.

The main reasons for this controversy are (1) that the detection of lymphatic vessels, especially capillaries, in routine histology is not possible with enough sensitivity and specificity, even for the well-trained observer.[16] In this context, the absence of erythrocytes or the presence of cell-free homogeneous material in luminal structures, although suggesting lymphatic vessels, do not alone provide sufficient evidence to discriminate between blood and lymphatic vessels, (2) until recently, appropriate immunohistochemical markers for confident identification of lymphatic vessels were not available and lymphatic identification previously relied on positive identification of blood vessels using strategies, such as the injection of colloidal carbon into blood vessels.[17] Since then, several immunomarkers (nuclear, cytoplasmic, membrane-bound) have emerged that compliment and facilitate histologic identification of lymphatics (e.g., VEGFR3, LYVE-1, podoplanin, PROX1).[3] However, a single exclusive marker for lymphatics is not yet available.[18,19] This implies that for unequivocal identification of lymphatics, especially at locations in the eye conventionally being thought of as "alymphatic," a panel of at least two markers is required. A panel is also necessary since different markers are expressed in different tissues, and also at different sites, as seen for the vascular system.[19–21] In addition, lymphatic endothelial markers such as LYVE-1 are also expressed on cells other than lymphatic endothelium (e.g., LYVE-1 on macrophages and podoplanin on epithelial cells).[8,20,22,23] Consequently, adequate controls are essential, keeping in mind that most the currently available lymphatic markers are not intended for diagnostic purposes and, therefore, are not tested to work reliably in any experimental condition (e.g., various fixation protocols, cryo- versus paraffin-embedding, amplification methods, microwave-treatment). This is not relevant for sites where the existence of lymphatic vessels is already well established as is the case for pathologically vascularized corneas and physiologically vascularized conjunctiva, (3) although ultrastructural criteria to define lymphatic vessels exist, their unequivocal detection in certain intraocular tissues, such as choroid is challenging.[4,8] Therefore, it seems appropriate that adequate labeling (i.e., immunohistochemical electron microsopy) be demonstrated in instances where current textbook knowledge is to be modified (i.e., except cornea and conjunctiva), and (4) within the eye, possibly "atypical" lymphatic cells might exist (i.e., endothelial cells with divergent or uncommon immunohistochemical phenotypes). For example, the endothelial cells of Schlemm's canal seem to display many, but not all features of terminally differentiated lymphatic endothelial cells, including responsiveness to VEGF-C–induced lymphangiogenesis.[24,25] These vessels should be labeled appropriately, and their presence taken into account in pathological conditions where VEGF-C is over expressed, such as in melanoma.

Given the recent and ever increasing interest in the role of ocular lymphatic vessels in the healthy eye and their role in the pathogenesis of corneal graft rejection, ocular tumor recurrence and metastasis, dry eye, allergy, and glaucoma and along with the hope for new anti- or prolymphangiogenic treatment concepts for these diseases, it is essential to develop evidence-based guidelines on the criteria for identifying ocular lymphatic vessels.[25–28]

Therefore, starting from a 2014 ARVO annual meeting symposium, lymphangiogenesis researchers began to discuss the aforementioned criteria and developed the following "recommendations" for the detection of ocular lymphatics in and around the eye, in agreement with already existing recommendations regarding the use of marker panels in cancer lymphangiogenesis research.[29]

Thus, the following criteria are recommended when applying immunohistochemistry: (1) the presence/absence of erythrocytes/lymph-like fluid is insufficient to discriminate between lymphatic and blood vessels, (2) the use of more than one lymphatic endothelial marker or a marker panel is recommended for immunohistochemistry in the eye except for regions where the existence of lymphatics is already well established (i.e., physiologically in conjunctiva and pathologically in inflamed cornea), (3) the use of markers in ultrastructural analysis is recommended (again except for ocular regions where existence of lymphatics is well established as in conjunctiva and inflamed cornea), and (4) the use of appropriate control tissue and appropriate documentation for detection of blood and lymphatic vessels is recommended.

These suggested criteria should be used as guidelines when working in the (peri-)ocular lymphatic system, at the histologic, immunohistochemical, and ultrastructural level. Following these guidelines should be helpful in resolving current controversies regarding ocular lymphatics and lymphangiogenesis.

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