Author Response: Sufficient Evidence for Lymphatics in the Developing and Adult Human Choroid?

In response to Heindl et al.,[1] we thank the authors for their continued interest in our work. A number of issues raised by these authors require further consideration.

Our study[2] provided substantial ultrastructural and immunohistochemical evidence using lymphatic markers for the existence of early absorption lymphatic capillaries located on the external side of the fenestrated vessels of the choriocapillaris, an ideal juxtaposition for the recirculation of extracellular fluid, as well as being a strategic location for immunologic surveillance. The presence of the lymphatic-like system we described in the human choroid thus provides an anatomical basis for antigen presentation in the posterior segment of the eye, as well as a pathway from the eye to the sentinel lymph nodes, similar to that described for the anterior uveal lymphatic vessels.[3]

Schroedl et al.[4] proposed in their original consensus statement on the immunohistochemical detection of ocular lymphatic vessels that "the use of markers in ultrastructural analysis is recommended (except for ocular regions where existence of lymphatics is well established as in conjunctiva and inflamed cornea)." Further, Heindl et al.[1] commented on our study of the developing and adult human choroid[2] that "despite possible postmortem tissue alterations, numerous previous studies successfully applied different detection systems for ultrastructural investigations using ocular human donor tissue." However, a recent paper from this group of authors investigating lymphatics in the human iris and ciliary body[5] does not provide ultrastructural evidence, let alone immunogold transmission electron microscopy (TEM), to support their conclusion that "various structures in the anterior uvea were immunoreactive for several lymphatic markers, while a classical lymphatic system was not detectable."

The ultrastructural characteristics of lymphatics presented in our study are well defined and compelling. Recent studies have demonstrated, for the first time, the existence of lymphatics; in the central nervous system (CNS), citing our work and our criteria for ultrastructural characterization of lymphatics and similarly using the presence of anchoring filaments associated with lymphatic capillaries as the defining ultrastructural criteria for the identification of lymphatics.[6]

Considering that the capillaries of the choroid are leaky, and a constant outflow of plasma proteins into the stroma of the choroid is expected, questions related to where this fluid goes to and how the interstitial ("lymph") pressure within the choroid is managed are relevant for ocular homeostasis. As such, a major aim of our study was to use immunohistochemical and ultrastructural approaches to discover the most compelling evidence we could for lymphatic-like structures in the human choroid. Numerous studies to date have struggled to identify a system of "true lymphatics" in the anterior and posterior eye,[7–9] although there is now functional[10–12] and anatomical evidence in support of lymphatic-like systems within the human anterior angle,[13] iris, and ciliary body,[14] and in our own study of the choroid.[2]

Further, the relative scarcity of these lymphatic structures in normal human tissue, which has prevented their previous identification, indicates the value of detailed and extensive studies such as ours for exploring the presence of a lymphatic-like system in the choroid.

A major point of contention relates to the concept of whether there are classical/typical lymphatics within the human choroid. The choroidal vasculature is known to be significantly different to the vasculature in the retina, where the choroidal vessels display a highly fenestrated phenotype, and the retinal vasculature displays barrier properties similar to those observed in the brain.[15]

For example, there are three well-described and recognized types of vascular capillaries in the human body: continuous capillaries, which have no fenestrae, and microvesicles that transport macromolecules directly across the cell in either direction; fenestrated capillaries with no diaphragm, which allow macromolecules with a size up to 100 nm to have direct access to cells (as in the liver); and fenestrated capillaries with a diaphragm, which have transendothelial channels (diaphragms) that can allow passage of large and small molecules, as observed in the choroid.[16] Their existence in contrast to the blood vessels with tight endothelial properties found in the human retina requires a system of fluid return as provided by lymphatics in the choroid.

The expectation that lymphatics are always of a classical/typical appearance is inconsistent with observations from recent studies. For example, this is clearly evidenced by the work of Iliff et al.,[17] who demonstrated the existence of a lymphatic-like system (referred to as the glymphatics) within the brain, which is dependent on the function of the water channel protein Aquaporin 4.

The possibility of a nonclassical lymphatic endothelium that does not consistently display a set phenotype is also not without precedent, based on observations within the vascular system. A growing body of evidence supports the heterogeneity of endothelial cells in normal tissue and in tumors, providing support for this remarkable capacity for endothelium to express different markers depending on the tissue context and microenvironment.[18] Nolan et al.[19] reported tissue-specific molecular signatures for microvascular endothelium and clearly showed heterogeneity associated with maintenance and repair in various body organs. A similar situation for lymphatic endothelium has also been reported,[20] indicating that a limited panel of phenotypic markers does not perhaps identify the biology of lymphatics (or vasculature).

Taken together, these observations indicate the possibility that not only classical/typical lymphatics can occur and that a limited definition does not serve ongoing research assessing the presence of lymphatics within tissues previously considered devoid of lymphatic drainage. A growing body of evidence (as discussed) supports the likelihood of our observations in choroid being confirmed by other research groups.

Baluk et al.[21] note that the most useful markers for microscopic imaging of lymphatic vessels are lymphatic vessel endothelial hyaluronan receptor (LYVE)-1, VEGFR3, Prospero homeobox (Prox)-1, and podoplanin. In their letter, Heindl et al.[1] state that, in our study, "the only lymphatic endothelial cell marker used in whole mounts was VEGFR-3." This misrepresents our results, as we investigated other lymphatic endothelial surface markers, including D2-40 and LYVE-1 in choroidal whole mounts (our Figs. 3, 5). Further, we found endomucin immunolabeled vascular structures in the human choroid that were not consistent with the known structure of venous or capillary vessels. Because endomucin also labels lymphatic endothelial cells,[22] we reasoned these structures must be endomucin$^+$ lymphatic vessels. Two characteristics distinguish the presumed lymphatic VEGFR-3$^+$ capillaries from the honeycomb-like lobular pattern of the choriocapillaris: the

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Investigative Ophthalmology & Visual Science

lymphatic VEGFR-3$^+$ capillaries are located just external to the CD34$^+$ choriocapillaris, and the lymphatic vessel lumens are slightly wider than the choriocapillaris, and their frequency is far rarer than choriocapillaris.

A potential outcome envisaged from our study was to stimulate further investigations of the choroid with in vivo imaging techniques such as enhanced depth imaging-optical coherence tomography (EDI-OCT) and swept-source OCT. As the sensitivity of the imaging modalities improves, these can provide a foundation for further research into the role of the lymphatic system in posterior eye ophthalmic diseases, especially those where inflammation or edema are involved, such as diabetic retinopathy and age-related macular degeneration. Anecdotal evidence for the possibility of these vessels has been observed by ophthalmologists who have noted the presence/visualization of tube-like structures in the choroid that are not blood vessels in fluorescein angiography.

Since the publication of our study, accumulating evidence of lymphatics in the central nervous system has also been reported.[6] Louveau et al.[6] cite our study, adopted the same ultrastructural criterion of anchoring filaments as being definitive for lymphatic endothelium, and noted a similar low frequency of occurrence where the lymphatics are embedded within the vasculature in the dura mater. Aspelund et al.[23] have also recently shown a dural lymphatic vascular system that drains brain interstitial fluid and macromolecules in the mouse.

Because the optic nerve is continuous with the dural-meningeal sheaths that encase the brain, and there is accumulating evidence of lymphatics in the anterior, mid-, and posterior eye (i.e., choroid), it is logical that this would require the existence of a lymphatic-like system in the human choroid that would drain into the dural sheath of the human optic nerve head.

The nature of the CNS lymphatics, as we originally described in the human choroid, has since been confirmed by both Louveau et al.[6] and Aspelund et al.,[23] who demonstrated that the elusive lymphatic vessel in the "CNS hides in plain sight—as they are very small and tucked behind a major blood vessel."[24]

As recently as 2006, the literature claimed as "an undisputed anatomical fact," that the brain is the only major organ that lacks a direct connection to the lymphatic system.[25] We suggest that a similar fate will meet the long held idiom that the eye is uniquely immune privileged, due to the presumed absence of lymphatics in the posterior eye.

We anticipate that as researchers are provided with further details of a lymphatic-like system in the human choroid and posterior eye, additional evidence for (or against) these structures will emerge, and we welcome ongoing dialogue with researchers in the ocular lymphatic and CNS glymphatic research community. Certainly, the letter of Heindl et al.[1] does realize a primary aim of our study, namely to stimulate research discussion and activity regarding the presence and function(s) of lymphatics in the eye and surrounding tissues.

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