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OPEN INTERFACES IN INITIAL LYMPHATICS: A METHODOLOGICAL ARTIFACT?

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ABSTRACT

Castenholz, Hauck, and other authors – including myself–have previously described initial lymphatics and the existence of open interfaces in tongue, mesentery as well as in uterus. These structures were supposed to represent an additional entrance into the initial lymphatic pathway and the open interfaces were proposed to act as pressure relief valves in some organs in case of increased volumes of tissue fluid. The methodological approaches used by these authors were interstitial and retrograde fillings of the lymphatic system, and often an endothelium-lined structure was seen connecting initial lymphatics with the interstitial space and ending free in connective tissue. Further research of my group in basic lymphology has led to new and surprising insights, and we now demonstrate that most of these so called "open-interfaces" are methodological artifacts.

Keywords: lymphatic ultrastructure, uterus, endometrium, initial lymphatics, open-interfaces, precollector, collector, guinea pig

Multiple investigators have previously described the initial lymphatic structures (1-8). These and other authors have also claimed that open interfaces are a part of this system and can be used as an entry point of fluid into the lymphatics. Such structures have also been proposed to act as pressure relief valves in some organs in response to increased amounts of tissue fluid. Continuing research in basic anatomical and physiological lymphology has led us to new surprising insights demonstrating that most of these structures are methodological artifacts.

MATERIAL AND METHODS

We examined rats (Wistar strain, Hannover) and guinea pigs from our own breeding colony on different days of the estrus cycle or pregnancy. In addition to previously described methods for depicting the lymphatic vessels (6), we used LYVE-1 (Lymphatic vessel endothelial receptor 1; Biomol) as an immunohistochemical marker to demonstrate lymphatics. For immuno-histochemistry, sections were fixed in formaldehyde (4%), paraffin embedded, and sectioned at 7µm. Following removal of paraffin, sections were hydrated through graded alcohols and antigens unmasked with a pH 7.2 buffer in a microwave. We followed the primary antibody (dilution 1:1000) with a polyclonal anti-rabbit secondary antibody (Biomol) and used the NOVADetect Anti-Polivalent HRP/DAB Kit (Dianova) with 3,3-Diaminobenzidin (DAB). We counterstained with hematoxylin and analyzed by light microscopy.

RESULTS

The retrograde filling method with silver nitrate (5,6) at the endometrial base (for

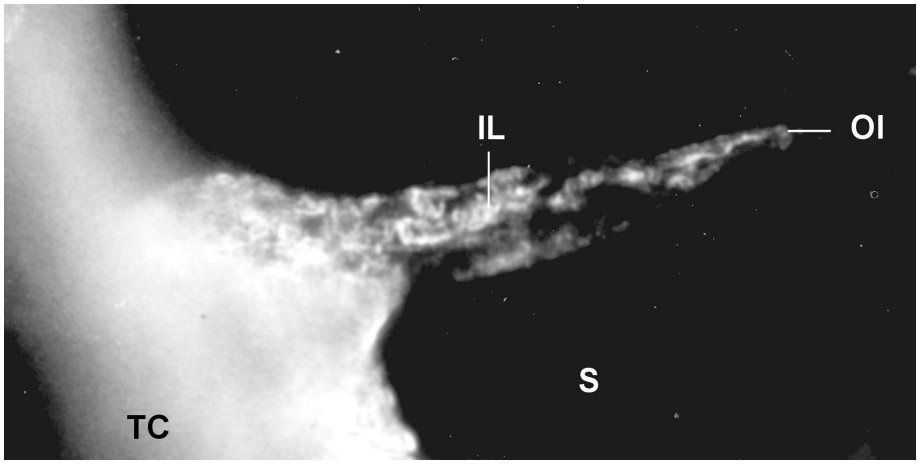

Fig. 1: Initial lymphatics (IL) and so called open-interface-formations (OI) at the endometrial base. S = stroma, TC = tunica muscularis circularis. Dark field microscopy, silver technique, Cavia, Östrus.

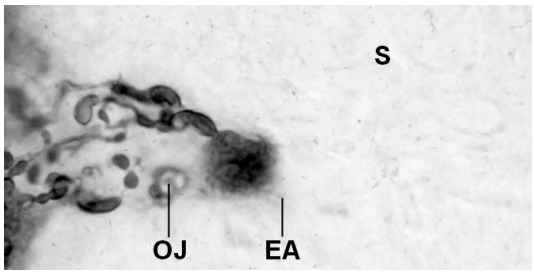

Fig. 2: So called open-interface-formation at the endometrial base. EA = endothelial cells, OJ = open-junction-formation, S = uterine stroma. Light microscopy, silver technique, Cavia, Östrus.

example) often demonstrated the so called "open-interface-formation" structure. These formations appeared to arise from lymphatics of the uterine stratum vasculosum, then branched and tapered finally ending in the endometrium *(Figs. 1,2)*. Using dark field and higher magnifications, we found a distinct space between the endothelial cells which exited with a prolongation of endothelial cells into the interstitial space of the endometrium. Until now, no lymphatics could be detected in any other region of the endometrium.

Nevertheless, a new examination using the silver method demonstrated initial lymphatics in the whole endometrium *(Figs. 3-6)*. These vessels originated in the luminal

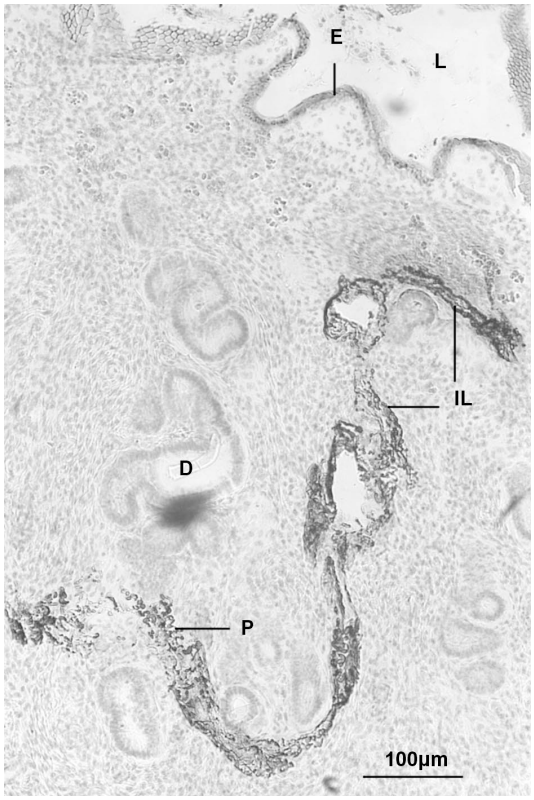

Fig. 3: Initial lymphatic and precollector running from the upper third to the endometrial base. IL = initial lymphatic, P = precollector, D = uterine gland, E = endometrial epithelium, L = uterine lumina. Light microscopy, silver technique, counter staining with nuclear fast red, Cavia.

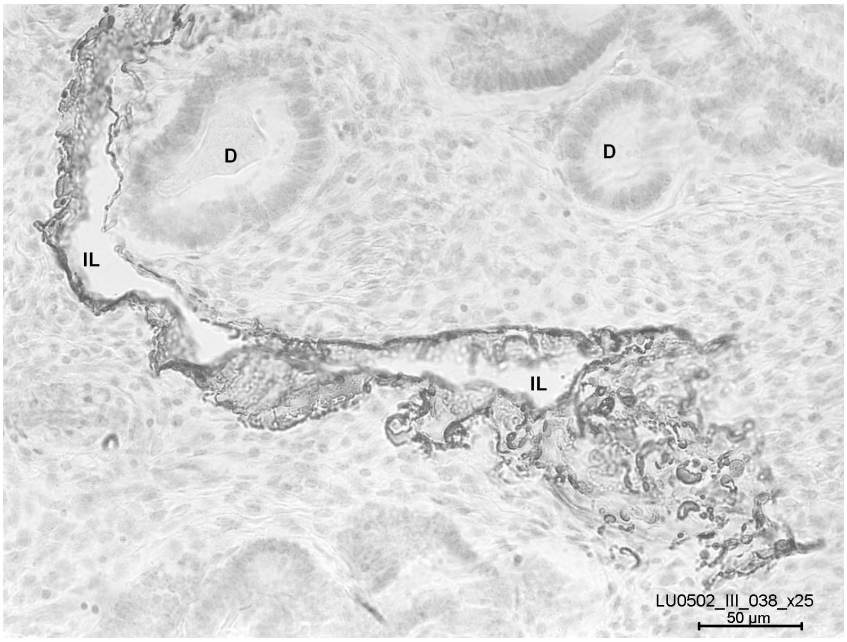

Fig. 4: Initial lymphatic close to a uterine gland. IL = initial lymphatic, D = uterine gland. Light microscopy, silver technique, counter staining with nuclear fast red, Cavia.

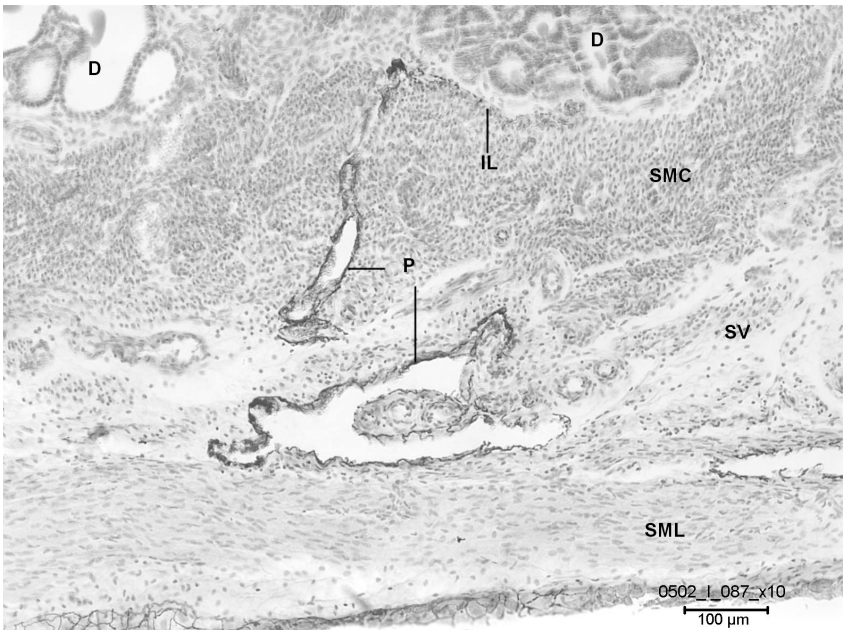

Fig. 5: Initial lymphatic and precollector at the endometrial base, parallel to the stratum muscularis circulare. IL = initial lymphatic, P = precollector, D = uterine gland, SMC = stratum muscularis circulare, SML = stratum muscularis longitudinale, SV = stratum vasculare. Light microscopy, silver technique, counter staining with nuclear fast red, Cavia.

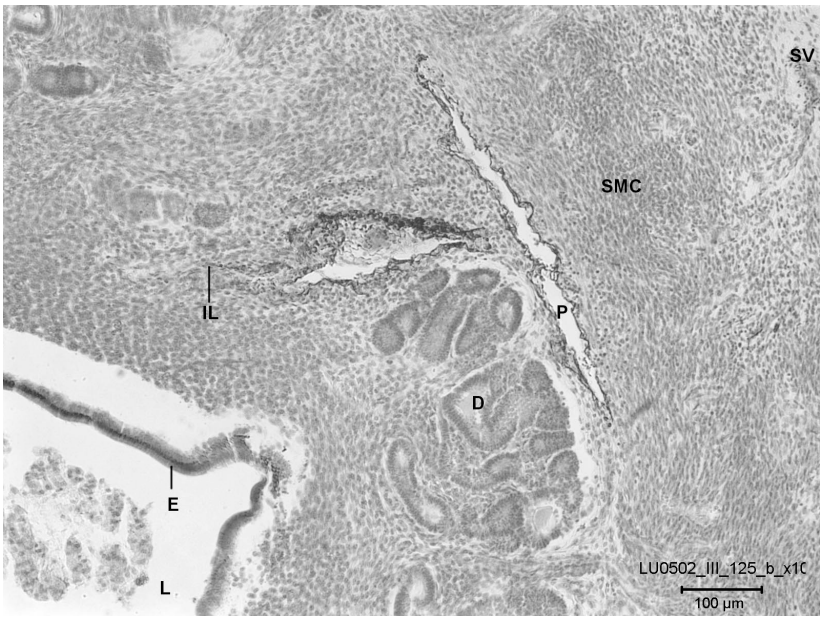

Fig. 6: Initial lymphatic in the upper third of the endometrium. IL = initial lymphatic, D = uterine gland, E = endometrial epithelium, L = uterine lumina, P = precollector, SMC = stratum muscularis circulare, SV = stratum vasculare. Light microscopy, silver technique, counter staining with nuclear fast red, Cavia.

part of the endometrium or beneath the uterine glands. These structures continued in the endometrium (sometimes close to the glands) despite the applied retrograde filling method *(Fig. 4)*. Cell borders of the endothelium were characteristic for initial lymphatics. Therefore, these lymphatics are present in the endometrium and in close connection to the uterine glands, too, with continuity to precollectors in the stratum vasculosum. Immunohistochemical demonstration with LYVE-1 also revealed these initial lymphatics in the endometrium *(Figs. 7,8)*.

Thus, in a series of sections one can see continuity between these newly demonstrable initial lymphatics in the endometrium and precollectors of the inner tunica vascularis with its radial course. In the outer tunica vascularis, the sometimes coiled precollectors are preferentially in ring formation between the stratum musculare circulare and longitudinale *(Fig. 9)*. The diameter of the precollectors is up to 300µm and more. Between the endothelial cells, a smaller number of open-junction-systems are visible compared to the initial lymphatics. In the mesometrial triangle, a large precollector runs in a longitudinal direction leading to the collectors *(Fig. 10)* and than to the truncus utero-ovaricus.

An unusual situation exists in the perimetrium. In the ringlike formed precollectors, small vessels drained lymph from the arcades of initial lymphatics of the perimetrium, arising from and leading back to the precollector. These initial vessels lie directly under the perimetrial epithelium *(Fig. 11)*.

DISCUSSION

Immunologically, the uterus is a privileged site that avoids rejection of the embryo. A key to understanding this phenomenon is the way the allogenic embryo is processed. One barrier seems to be the endometrial epithelium and basal membrane,

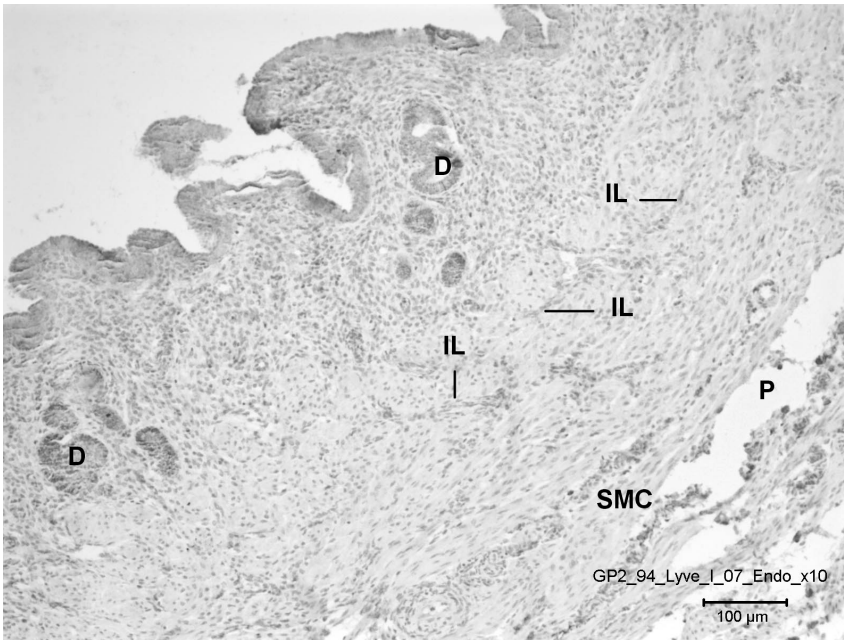

Fig. 7: Initial lymphatics in the endometrium and precollector in the Tunica vascularis, parallel to the stratum muscularis circulare. IL = initial lymphatic, P = precollector, D = uterine gland, SMC = stratum muscularis circulare. Light microscopy, LYVE-1 marker, counter staining with hematoxylin, Cavia.

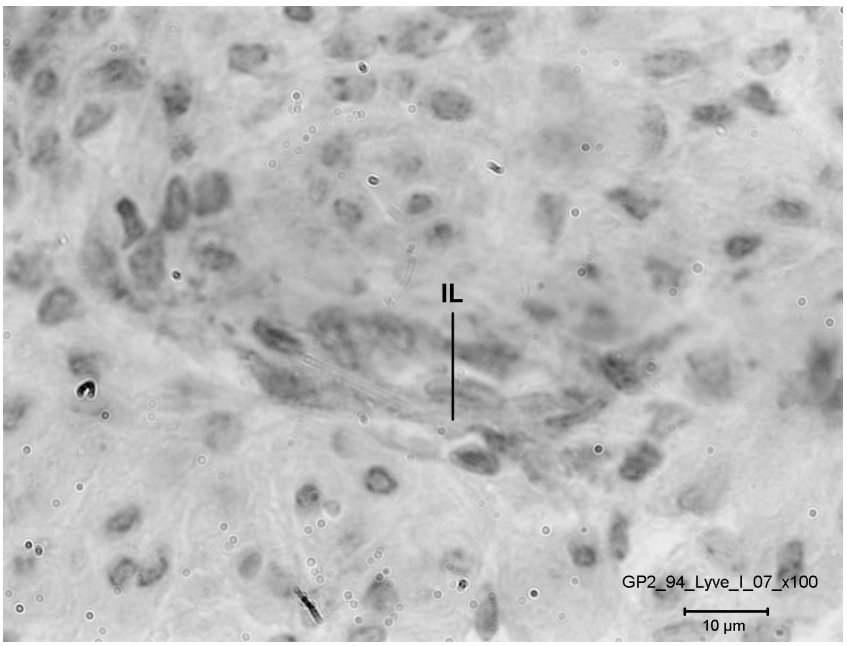

Fig. 8: Initial lymphatic in the endometrium. IL = initial lymphatic. Light microscopy, LYVE-1 marker, counter staining with hematoxylin, Cavia.

which prevents transport of cells and harmful substances to the endometrial stroma. Another reason could be a specific adaptation of the endometrial lymphatics. In non-invasive growth of the placenta (for example in horse and sheep), there are regularly lymphatics found in the endometrium. Until now, we thought that in invasive implantation, there are no lymphatics in the upper two thirds of the endometrium. In the published literature, there are different statements on this phenomenon, most of them negating the existence of lymphatics in the upper endometrium in rats and guinea pigs (9-14). An exception is the regular appearance of endometrial lymphatics in humans, who exhibit invasive implantation (15).

In rats and guinea pigs the question arises as to how the interstitial fluid would be drained. Until recently, it was thought that the pathway is via tissue channels and then via open-interface-formations at the basal endometrium to the lymphatic system. Such open-interface-formations were postulated by Hauck (1,2,16,17). Studies by our team in other organs like tongue and mesentery (18) seemed to verify the regular occurrence of open-interface-formations. Still other authors (5-7,12-14,18-26) described such structures corresponding to these open-interface structures. In their function as pressure relief valves, greater amounts of tissue fluid such as during oestrus would be transported to the initial lymphatics. So the open-interface-formation was previously characterized as an additional mechanism capable of responding to higher lymphatic loads.

Prior methodological approaches to demonstrate lymphatics were by interstitial and retrograde filling of the lymphatic system. As a result of this method with silver nitrate, a dark lined lymphatic endothelium is clearly visible at all magnifications up to TEM. Often an endothelium-lined structure was seen connecting initial lymphatics with the interstitial space which ended freely in connective tissue of the endometrium *(Fig. 2)*.

But there are problems with retrograde

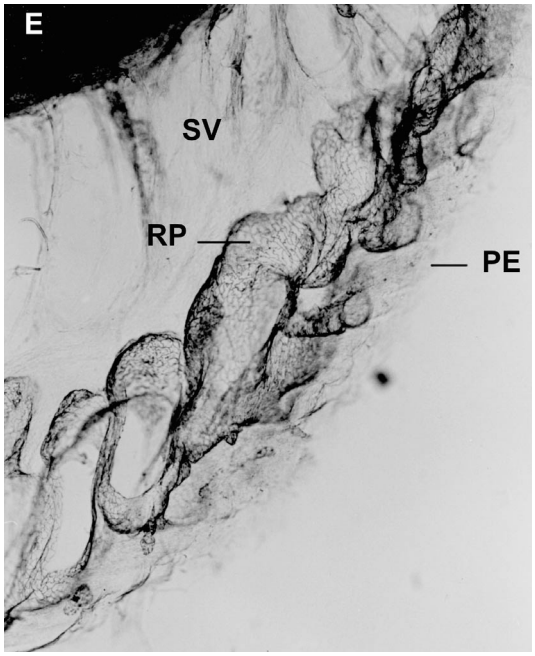

Fig. 9: Ring formed precollector in the outer zone of the tunica vascularis with typically coiled arrangement. E = endometrium, PE = Perimetrium, RP = ring precollector, SV = inner zone of stratum vascularis. Dark field microscopy, silver technique, Cavia.

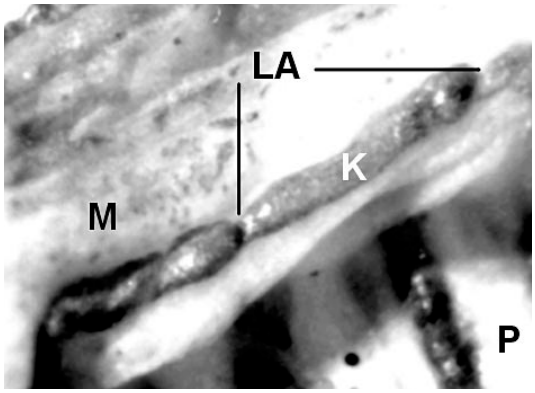

Fig. 10: Collector in the Mesometrium. K = collector, M = mesometrium, LA = lymphangion, P = perimetrium. Macro photo, silver technique, Cavia.

fillings. We are now able to show that most of these presumed structures are artificial and dependent on this applied methodology. In serial sections of endometrium in guinea pigs

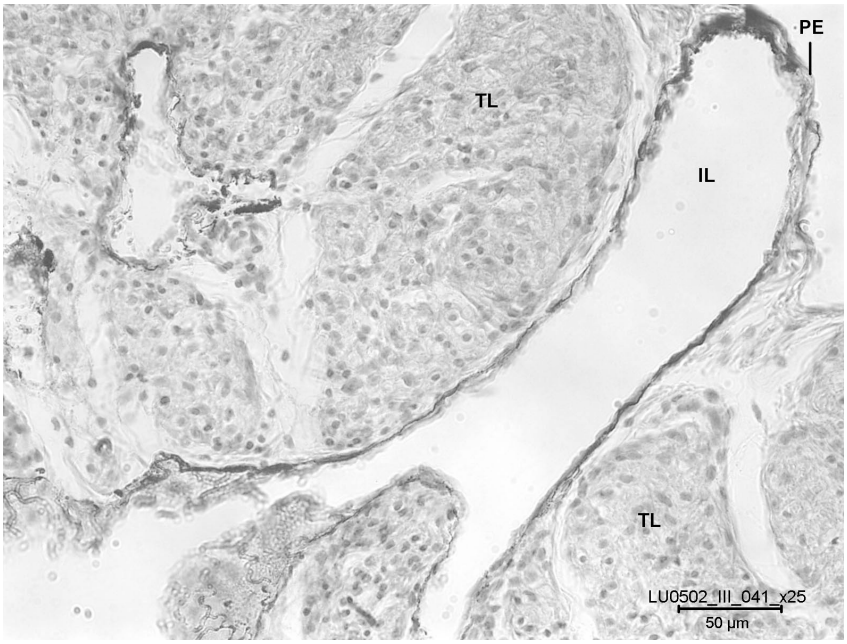

Fig. 11: Perimetrial loop of an initial vessel between bundles of longitudinal musculature of the tunica muscularis longitudinale, adjacent under the Perimetrium. IL = initial lymphatic, TL = tunica muscularis longitudinale, PE = Perimetrium. Light microscopy, silver technique, Counter staining with nuclear fast red, Cavia.

and rats, we have demonstrated continuity between clearly marked initial lymphatics in the endometrium and precollectors in the stratum vasculosum *(Fig. 3-8)*. In these cases, retrograde filling of the lymphatics of the endometrium successfully overcame the tissue and vessel pressure. Apparently, the normally applied pressure for interstitial perfusion is too small for this approach, and this leads to stagnation of the contrast fluid at the base of the endometrium. Support for this explanation comes from the black plug between the endothelium cells shown in *Fig. 2*. Endothelial cells seem to be above this structure but here, without contrast. Prior investigations from Castenholz and from Zöltzer (3,5-7,22-26) described only a small part of this connecting system as obviously tapering in the base of endometrium. These so-called "open interfaces" were thus methodological artifacts due to the retrograde filling method. The lymphatics are present in the endometrium of rats and guinea pigs up to 200µm under the endometrial epithelium and in close connection to the uterine glands (*Figs. 3-8)*. This arrangement is demonstrable with the silver method and its clear cut cell-borders and with the immunohistochemical method using LYVE-1 marker.

Therefore, the observation that in invasive implantation, there are no lymphatics in the endometrium of rats and guinea pigs is in error. Failure of lymphatic supply in the endometrium is no longer a viable explanation for inhibition of the allogenic embryo. We should now revisit the papers of Fabian (27-29), describing a dense network of lymphatics in rat uterus beneath the endometrial epithelium. Nevertheless, she used patent blue V, which does not define the endothelial lining and so the demonstrated structures could also have been tissue channels.

With regard to the initial lymphatics lying directly beneath the perimetrial epithelium *(Fig. 11)*, their function is unclear. The lymphatic load in this region is not high;

but it is possible that there is communication between these vessels and the peritoneal space via the perimetrium. It is conceivable, therefore, that fluid and macromolecules are resorbed from the peritoneal space.

CONCLUSION

Most of the formerly so-called "open-interface-structures" are likely methodological artifacts resulting from the retrograde filling method. Furthermore, a lymphatic vessel system does exist in the endometrium of guinea pigs and rats. This system is drained via precollectors of the uterine stratum vasculosum to collectors and then to the truncus-utero-ovaricus. This study demonstrates that the hypothesis that there are no lymphatics in endometrium, even in invasive implantation, is wrong, and inhibition of the rejection response against the allogenic embryo cannot be explained by the failure of lymphatics in the endometrium.

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