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FLOW CYTOMETRY-BASED ISOLATION OF DERMAL LYMPHATIC ENDOTHELIAL CELLS FROM NEWBORN RATS

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ABSTRACT

The lymphatic system plays a key role in tissue homeostasis, fatty acid transport, and immune surveillance. Pathologically, dysfunction of the lymphatic system results in edema, and increased lymphangiogenesis can contribute to tumor metastasis. Lymphatic vessels are composed of lymphatic endothelial cells (LECs) that can be identified by distinct marker molecules such as Prox-1, podoplanin, VEGFR-3 and LYVE-1. Primary LECs represent a valuable tool for the study of basic functions of the lymphatic system. However, their isolation remains a challenge, particularly if rodent tissues are used as a source. We developed a method for the isolation of rat dermal LECs from the skin of newborn rats based on sequential enzymatic digestion with trypsin and Liberase followed by flow cytometric sorting using LYVE-1 specific antibodies. Cells isolated according to this protocol expressed the lymphatic markers Prox-1, podoplanin, LYVE-1 and VEGFR-3, and displayed an endothelial-like morphology when taken into culture. These primary cells can be used for studying lymphatic biology in rat models, and the protocol we describe here therefore represents an important extension of the experimental repertoire available for rats and for modeling the human lymphatic system.

Keywords: lymphatic endothelial cell, isolation, flow cytometry, LYVE-1, rat, dermis

The lymphatic system mediates the recovery of interstitial fluid, is involved in the absorption and transport of triglycerides via chylomicrons, and plays an integral role in immune surveillance (reviewed in 1). Structurally, the lymphatic system is composed of the lymphoid organs and lymphatic vessels of different size and function. In the vascular hierarchy of the lymphatic tree, blind-ended capillaries merge into collecting ducts, which ultimately drain into the blood circulatory system through anastomoses such as the thoracic duct (1).

The lymphatic system plays an important role in several pathologic conditions. Impaired lymphatic function can for example lead to lymphedema, which remains a major clinical challenge (2). On the other hand, many human carcinomas metastasize via the lymphatic route, and tumor-induced lymphangiogenesis, the outgrowth of new lymphatic vessels from pre-existing ones, can promote metastasis to lymph nodes and organs (reviewed in 3,4). Further understanding of the molecular mechanisms that regulate lymphatic endothelial cell (LEC) identity and function will allow novel therapeutic strategies to be developed for these pathologies.

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In order to study the role of the lymphatic system in normal physiology and disease, the analysis of isolated primary lymphatic endothelial cells (LECs) is often a desirable, but not always trivial, undertaking. Several protocols for the isolation of human and murine LECs have been published to date. Most of these protocols are based on enzymatic digestion with dispase (5-9), collagenase (7-11) or trypsin (6,12,13), followed in many cases by cultivation and subsequent marker-based enrichment and isolation using magnetic beads (6,7,9,10,12).

Several different marker molecules can be used to identify and/or distinguish LECs from blood endothelial cells (BEC). Prominent examples are the prospero homeobox protein Prox-1 (14), the mucin-type transmembrane protein podoplanin (15), the trans-membrane tyrosine kinase vascular endothelial growth factor receptor (VEGFR)-3 (1), and lymphatic vessel endothelial hyaluronan receptor (LYVE)-1 (16). While Prox-1 is a highly specific marker for LECs, it is not suited for use in LEC isolation due to its nuclear localisation. On the other hand, the cell surface proteins podoplanin (5-7), VEGFR-3 (6) and LYVE-1 (10-12) have been used to successfully isolate primary LECs from human and murine tissues. In addition to these molecules, differential expression of CD34 and CD31 has also been used in the separation of LECs from BECs (5,8,9,11-13).

To date, efforts to develop methods for the isolation of primary LECs have largely focused on human and murine tissues. Nevertheless, the rat is an important animal model for the study of a variety of human diseases. We have therefore established a method for the isolation of LECs from the skin of newborn rats. Enzymatic digestion with Liberase was used to release dermal cells from neonatal rat skin, which were then stained with LYVE-1$^-$ specific antibodies and sorted with a flow cytometer to isolate LECs. LYVE-1$^+$ cells isolated according to this protocol expressed transcripts for the LEC markers Prox-1, podoplanin, LYVE-1 and VEGFR-3, and exhibited an endothelial-like morphology.

MATERIALS & METHODS

Isolation of Dermal Cells

For each preparation, four newborn rats 0-2 days old were decapitated, and their tails and legs removed. The skin of the torso was then cut through, peeled off, and placed in HBSS (-Ca^{2+}; -Mg^{2+}) containing 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml fungizone and 10 µg/ml gentamycin. The skin was then cut in small pieces and digested with trypsin (without EDTA) over night at 4°C. Dermis and epidermis were then separated, the epidermis discarded, and the dermis transferred into HBSS (-Ca^{2+}; -Mg^{2+}) containing 5% FCS. The pieces of dermis were centrifuged at 1500 rpm (460 rcf), and digested in HBSS containing 0.28 Wünsch units of Liberase (#05401119001; Roche, Mannheim, Germany) and 0.1 mg/ml DNAse (Sigma, Taufkirchen, Germany) for 30 min at 37°C. The digestion was stopped by adding an equal volume of HBSS (-Ca^{2+}; -Mg^{2+}) containing 5% FCS. The digest was then transferred into a petri dish, and the dermis pieces were gently squeezed using forceps with a thin bent tip. After transfer into 40 &m cell strainers (Becton Dickinson, Heidelberg, Germany) mounted on 50 ml Falcon tubes, the digest was then centrifuged at 1500 rpm. Fresh HBSS (-Ca^{2+}; -Mg^{2+}) containing 5% FCS was then added into the strainers, and the dermis pieces were pipetted up and down to release cells. After a further centrifugation step, the cell strainers were discarded, and the pellet within the 50 ml Falcon tube was washed once with HBSS (-Ca^{2+}; -Mg^{2+}) containing 5% FCS followed by centrifugation. The resulting pellet was resuspended in HBSS (-Ca^{2+}; -Mg^{2+}) containing 5% FCS. The cells were then counted with a hemocytometer and the percentage of vital cells was determined using trypan blue.

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FACS of Dermis-Derived LYVE-1 Positive Cells

Aliquots (100 µl) of isolated dermal cells were transferred into a round bottom 96 well plate at $4x10^7$ cells/ml, and incubated as indicated with primary anti-LYVE-1 antibodies (#103-PA50S; Reliatech, Wolfenbüttel, Germany) or with rabbit IgG isotype control antibodies (#017K7430 Sigma) at 0.1 µg/ml for 1 h at 4°C. The cells were then washed twice with HBSS ($-Ca^{2+}$; $-Mg^{2+}$) containing 5% FCS, and then incubated with anti-rabbit immunoglobulin RPE-labelled secondary antibody (Dianova, Hamburg, Germany) for 30 min at 4°C. After washing three times, the cells were resuspended in HBSS ($-Ca^{2+}$; $-Mg^{2+}$) containing 5% FCS, and were then sorted into LYVE-1^+ and LYVE-1^- fractions using a BD FACS Aria SORP flow cytometer (Becton Dickinson). Gating strategies for discrimination of doublettes were applied to exclude clumping cells from the sorting process in order to avoid contamination of the LYVE-1^+ and LYVE-1^- fractions.

Analysis of Plated Dermis-derived Lyve-1 Positive Cells

After sorting, cell viability was assessed by staining the cells with 0.5% trypan blue in PBS followed by counting using a hemocytometer. LYVE-1^+ and LYVE-1^- populations were resuspended in EGM-2 MV human lymphatic endothelial cell growth medium (Lonza, Cologne, Germany), and cultivated at 50,000 cells per mm^2 on uncoated plastic. The cells were photographed using a Zeiss Axiovert 40C microscope (Carl Zeiss, Jena, Germany) 24 hours after plating.

Semi Quantitative RT PCR

RNA was prepared from sorted LYVE-1^+ and LYVE-1^- populations using peqGOLD RNAPure (PeqLab, Erlangen, Germany). Synthesis of cDNA using Superscript III (Life Technologies, Darmstadt, Germany) was performed according to the manufacturer's recommendations. The following primers and conditions were used for amplification of the respective cDNA templates: ratProx-1_for 5' tctggcagagaccttaaaacagg 3'; ratProx-1_rev 5' tcaccaaagcattgcaggc 3' (amplifying nuleotides 1518-1718 of rat Prox1; NM_001107201.1); 95°C, 30 s, 55°C, 30 s, 72°C, 60 s; 35 cycles; ratPodoplanin_for 5' cattgaggaactgccgacct 3'; ratPodoplanin_rev 5' gggcgagaaccttccagaaa 3' (amplifying nuleotides 458-734 of rat Pdpn; NM_019358.1); 95°C, 30 s, 55°C, 30 s, 72°C, 60 s, 30 cycles; ratVEGFR-3_for 5' cacagagacctggctgctcgg 3'; ratVEGFR-3_rev 5' tgcatgatgtggcgtatggcagg 3' (amplifying nuleotides 3103-3446 of rat Flt4; NM_053652.1); 95°C, 30 s, 62°C, 30 s, 72°C, 60 s, 32 cycles; ratHprt_for 5' tggtcaagcagtacagcccc 3'; ratHprt_rev 5' acttggcttttccactttcgc 3' (amplifying nucleotides 512-712 of rat Hprt1; NM_012583.2); 95°C, 30 s, 60°C 30 s, 72°C 60 s; 28 cycles.

RESULTS

Isolation of LYVE-1^+ Cells from the Dermis of Newborn Rats

Among the markers used for the identification of LECs, Prox-1 is currently considered to be the most specific. However, Prox-1 is localized to the nucleus and can therefore not be easily used for the isolation of living cells. Accordingly, we chose to base the isolation procedure for rat LECs on LYVE-1 expression. LYVE-1 is predominantly expressed on LECs but can also be found on BECs in the liver (17) and lung (18). However, unwanted isolation of contaminating BECs can be avoided by using other tissues as a source. In the skin, LYVE-1 has been shown to be restricted to lymphatics (19). Accordingly, we prepared LECs from the skin of newborn rat pups. The skin was first digested with trypsin to allow the epidermis and dermis to be separated manually. The dermis was then digested with Liberase, a mixture of clostridial collagenases

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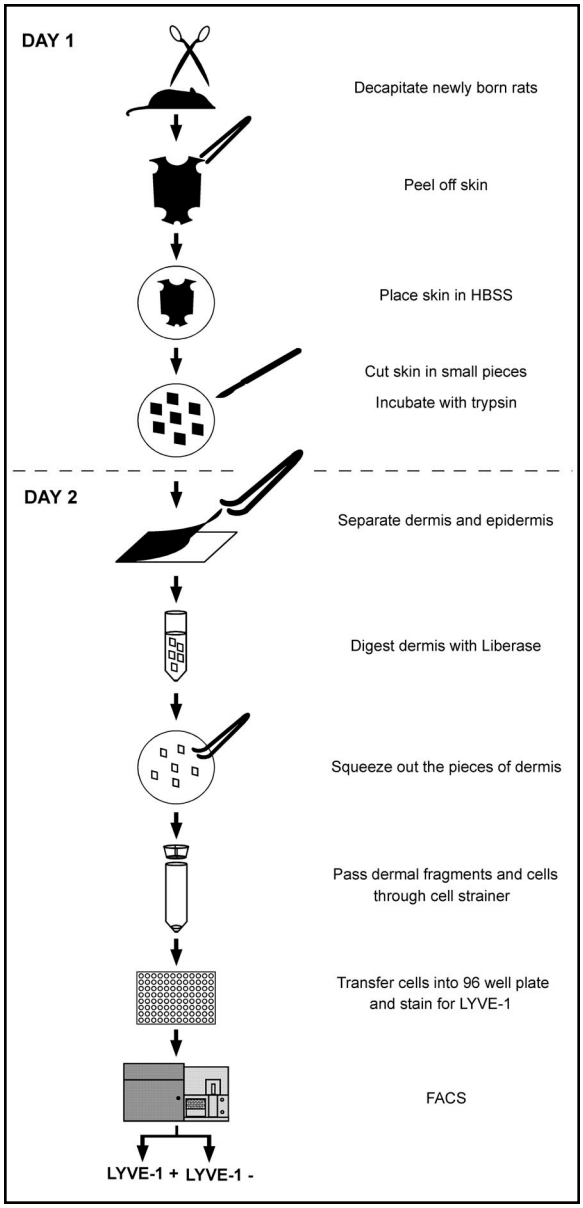

Figure 1: Scheme of the LEC isolation procedure. Newborn rats were killed by decapitation. Their skin was peeled off, and placed in HBSS. The skin was then cut into small pieces and incubated in trypsin over night at 4°C. Dermis and epidermis were then separated and epidermal fragments were discarded. Pieces of dermis were digested with Liberase, then carefully squeezed out. Dermal fragments were then loaded onto cell strainers mounted on Falcon tubes and centrifuged to obtain dermal cells, which were then stained with primary antibodies (isotype control or anti-LYVE-1) and sorted based on LYVE-1 expression.

I and II and thermolysin metalloproteinase. DNAse was also added to the digest to avoid the formation of cell clumps. After the digestion, cells were released from the tissue by mechanical trituration of the dermal pieces with forceps and by pipetting. Finally, dermal cell suspensions were obtained using cell strainers. Subsequent incubation of the cells with LYVE-1-specific antibodies allowed flow cytometric sorting of dermal LECs. A schematic overview of the procedure is depicted in *Fig. 1.*

Sorting of LYVE-1$^+$ and LYVE-1$^-$ Populations from Dermal Cell Preparations

For the sorting of LYVE-1$^+$ cells, dermal cell preparations were incubated with primary antibodies specific for LYVE-1 and RPE-labeled secondary antibodies, then analyzed and sorted with a flow cytometer. For this purpose, LYVE-1$^+$ and LYVE-1$^-$ populations were defined by regions as shown in *Fig. 2*, These regions defined the gates used to sort the cells. Unstained cells, cells stained with the secondary antibody alone, or cells stained with isotype control antibodies together with the secondary antibody were used as controls (*Fig. 2*). The proportion of LYVE-1$^+$ cells within the dermal preparations typically varied between 0.6% and 2.4% before sorting. After sorting, the LYVE-1$^+$ and LYVE-1$^-$ cell populations were re-analyzed to ensure the purity of the respective populations (*Fig. 2*). In the LYVE-1$^-$ population, a maximum of 0.1% contaminating LYVE-1$^+$ cells were typically detected, whereas 50% - 70% of the sorted LYVE-1$^+$ cells were localized within the gate defining LYVE-1 positivity (*Fig. 2*).

Characterization of Sorted LYVE-1$^+$ and LYVE-1$^-$ Populations

To characterize further the sorted LYVE-1$^+$ and LYVE-1$^-$ populations, and to confirm the lymphatic endothelial identity of the LYVE-1$^+$ cells, we assessed the

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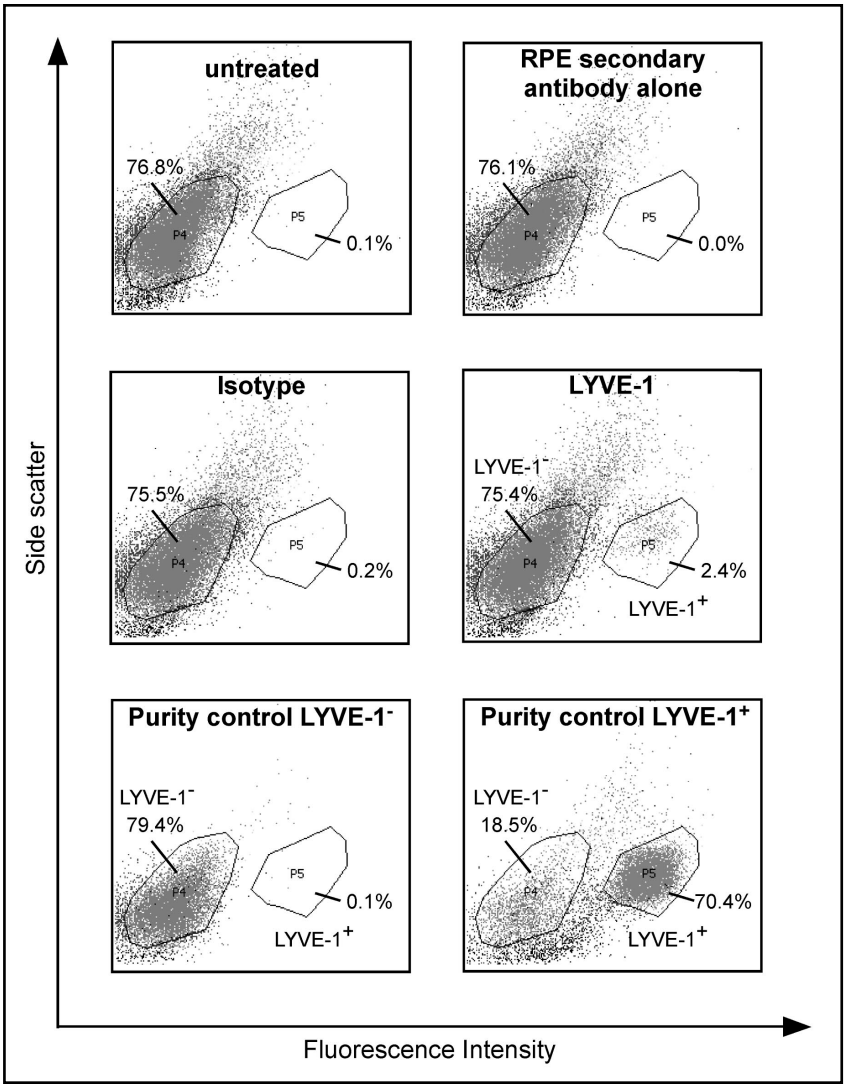

Figure 2: LYVE-1$^+$ cells can be isolated by FACS. Dermal cells were isolated from the skin of newborn rats. The cells were then incubated with either LYVE-1 specific antibodies (LYVE-1) or with isotype-matched unspecific immunoglobulins (Isotype) or were left untreated as controls, before incubation with RPE-coupled secondary antibodies (RPE) to fluorescently mark LYVE-1$^+$ cells. The cells were then analyzed and sorted with a BD FACS Aria according to LYVE-1 positivity or negativity, respectively. To this end, several regions were defined to gate specific populations. A first region (side scatter signal area vs. forward scatter signal area) excluded cellular debris, and two further regions (forward scatter signal area vs. forward scatter signal width and subsequently side scatter signal area vs. side scatter signal width) were defined to gate out doublets. Based on the "untreated" (top left panel), "RPE secondary antibody alone" (top right panel) and "isotype" controls (middle left panel) as well as cells actually stained for LYVE-1 (middle right panel), regions were defined that included LYVE-1$^-$ (P4) or LYVE-1$^+$ (P5) populations. The proportion of LYVE-1$^+$ cells before sorting typically varied between 0.6 and 2.4% of the total cells. The sort was performed according to the regions P4 and P5. The sorted fractions were re-analyzed to determine their purity (purity control LYVE-1$^+$ (bottom left panel); purity control LYVE-1$^-$ (bottom right panel)). In the depicted situation, in the sorted LYVE-1$^-$ fraction, LYVE-1$^+$ events were 0.1% of the total cells, whereas 70.4% of the events in the LYVE-1$^+$ fraction were confined to the region defining LYVE-1 positivity. Typically, a 30-fold enrichment of LYVE-1$^+$ cells was achieved.

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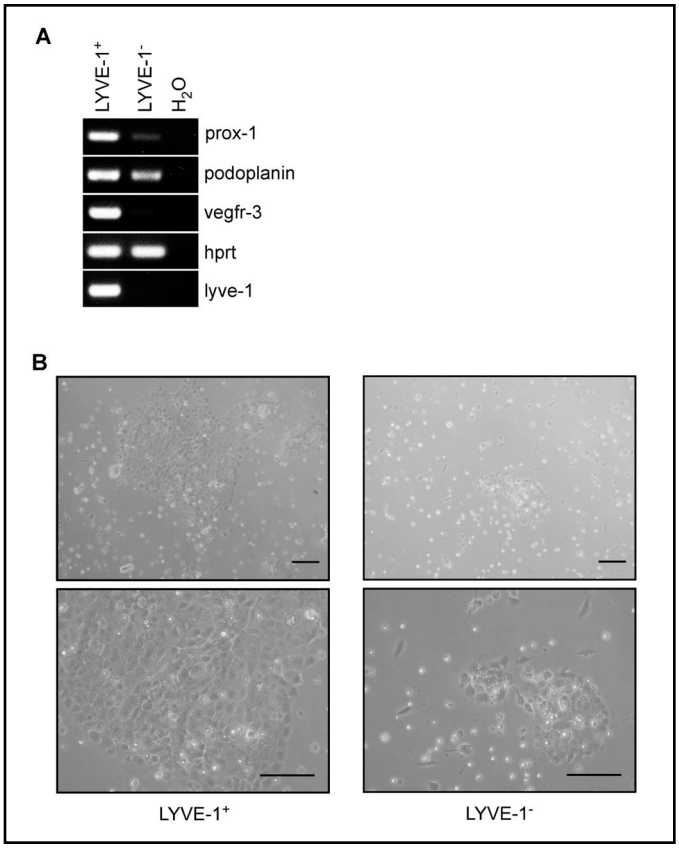

Figure 3: LYVE-1$^+$ cells sorted from dermal preparations display an endothelial-like morphology, and express transcripts of lymphatic endothelial markers prox-1, podoplanin and VEGFR-3. (A) Cells were isolated from the dermis of newborn rats and sorted based on LYVE-1 expression. RNA was isolated from LYVE-1$^+$ and LYVE-1$^-$ sorted dermal cells and transcribed into cDNA, which served as a template for semi-quantitative PCR. Primers specific for lymphatic markers lyve-1, prox-1 and podoplanin were used. Amplification of hprt ensured equal loading. As a negative control (-), the PCR was performed with water instead of cDNA. (B) Cells isolated from the dermis of newborn rats were sorted according to their LYVE-1 expression status. LYVE-1$^+$ (right panels) and LYVE-1$^-$ (left panels) sorted dermal cells were then plated on tissue culture plates and allowed to adhere, then analyzed and photographed 24 hours after plating using a Zeiss Axiovert 40c microscope with A-Plan 10x and 20x objectives, and a Canon Powershot G12 camera. Scale bar: 100 µm.

transcription of LEC markers in freshly sorted LYVE-1$^+$ and LYVE-1$^-$ populations. Transcripts of the lymphatic endothelial markers Prox-1, podoplanin, LYVE-1 and VEGFR-3 were strongly expressed in LYVE-1$^+$ cells, whereas only low amounts of the respective transcripts were detected in the LYVE-1$^-$ population (*Fig. 3A*).

In addition, LYVE-1$^+$ and LYVE-1$^-$ cells were plated after sorting. Trypan blue staining showed a comparable vitality of around 75% in both populations. The cells were then allowed to attach to either gelatin-coated or uncoated tissue culture plates, and subsequently analyzed microscopically. We found that the majority of LYVE-1$^+$ cells displayed a typical endothelial-like cobble-stone morphology, and grew out as colonies 24 hours after plating (*Fig. 3B*). In contrast, LYVE-1$^-$ cells were smaller in size and grew in fewer and much smaller colonies (*Fig. 3B*).

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Together these findings suggest that the protocol described here can be used successfully to isolate and culture viable LECs derived from the neonatal rat dermis.

DISCUSSION

Here, we describe a method for the isolation of LECs from the dermis of newborn rats. In this method, cells are released from the dermis after digestion with Liberase, and LYVE-1$^+$ cells are subsequently sorted using flow cytometry. The LYVE-1$^+$ population expresses transcripts for the lymphatic endothelial markers Prox-1, podoplanin, LYVE-1 and VEGFR-3, and exhibits an endothelial-like morphology in culture.

Most protocols for the isolation of LECs published to date are based on enzymatic digestion with dispase, a protease that cleaves fibronectin, collagen I and collagen IV (5-10), with collagenase (7-11), or with trypsin (12,13). The majority of protocols then cultivate the isolated cells (5-7,9,10,12), before using immuno-based isolation using magnetic beads (6-10,12) or FACS (5). An exception to this standard scheme is the approach of Planas-Paz and colleagues, who digested complete murine embryos, and isolated LECs directly from the resulting cell suspension by MACS (11). Similarly, we successfully stained and sorted LYVE-1$^+$ dermal LECs directly after their isolation from the skin without the need for culturing, which significantly speeds up the isolation procedure, and prevents transdifferentiation and subsequent loss of lymphatic phenotype. A similar approach has also been used in other protocols (5,8,13).

Our method for the isolation of rat dermal LECs employs LYVE-1 as a marker. LYVE-1 has also been used for the isolation of human and murine LECS (10,11). LYVE-1 is a receptor for hyaluronic acid (HA) (reviewed in 20). However, due to its sialylation status, binding of high molecular weight HA by LYVE-1 is inhibited on LECs (21,22), although small molecular fragments of HA (sHA) can bind LYVE-1 and regulate lymphangiogenesis (23). Furthermore, LYVE-1 can interact with several other molecules including PDGF and its receptor PDGFR, VEGF-A, IGFBP-3 and FGF, to regulate lymphatic flow and lymphangiogenesis (24-27).

The specificity of LYVE-1 as a lymphatic marker is strongly dependent on the context. In addition to LECs, LYVE-1 is expressed by subsets of macrophages that are associated with tumors and wounds (19), as well as by primary murine bone marrow cells (19,28), hepatic sinusoidal (17) and pulmonary blood endothelium (18). In the healthy skin, LYVE-1 is restricted to LECs (19). Thus, although we have not explicitly excluded the presence of LYVE-1$^+$ non-LECs, contamination of LYVE-1$^+$ LECs prepared from the dermis of healthy skin with other LYVE-1$^+$ non-LEC cell populations is likely to be insignificant.

Depending on their position within the hierarchy of the lymphatic tree, lymphatic vessels display distinct morphologies. In contrast to collecting vessels that possess a basement membrane and are at least partially covered by smooth muscle cells, lymphatic microcapillaries lack a basement membrane and mural cells (2). Furthermore, LECs in lymphatic microcapillaries are only loosely connected and possess intermittent button-like junctions, whereas LECs in collecting vessels are connected by zipper-like junctions (29). LYVE-1 is robustly expressed on lymphatic microcapillaries but is almost absent from collecting vessels in which LECs are in contact with mural smooth muscle cells (30-33). It is therefore likely that LECs isolated with our protocol represent mainly LECs from lymphatic microcapillaries.

We show that LYVE-1$^+$ cells isolated according to our protocol express transcripts of LEC markers Prox-1, podoplanin and VEGFR-3 (*Fig. 3A*). Prox-1 is a homeobox transcription factor that determines lymphatic identity, and regulates the expression of other molecules that maintain lymphatic characteristics (34). Podoplanin is a transmembrane sialomucin-like glycoprotein whose expression

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is regulated by Prox-1 (35,36). Podoplanin can mediate LEC adhesion as well as migration and is involved in the formation of lymphatic vessels (37). In human skin, podoplanin is strongly expressed in lymphatic capillaries and to a lesser extent in lymphatic collecting vessels (15). VEGFR-3 is a transmembrane receptor tyrosine kinase that is mainly expressed by LECs but can also be found on other cell types such as blood endothelium (38), megakaryoblasts (28), and other yet uncharacterized cells in the bone marrow (28). Upon binding to its ligands VEGF-C and VEGF-D, VEGFR-3 can induce proliferation and migration of LECs (1). While each of the LEC markers used in this study in principle has a context-dependent potential to be expressed by cell types other than LECs, the finding that all LEC markers are expressed within the LYVE-1$^+$ population prepared and sorted according to our protocol strongly supports the notion that the sorted cells are indeed derived from the lymphatic endothelium and that the protocol presented here strongly enriches for LECs.

Very weak expression of Prox-1 and VEGFR-3 was detected within the LYVE-1$^-$ population (*Fig. 3A*), likely reflecting the intrinsic but low failure rate of the flow cytometry sorting procedure, or the presence in the LYVE-1$^-$ population of LECs derived from collecting vessels. Much higher levels of podoplanin were observed in the LYVE-1 negative population even though podoplanin expression has been reported to be lower in collecting vessels than in capillaries (15). However, expression of podoplanin has been reported on several cell types other than LECs (e.g., 18,39,40). Notably, podoplanin is expressed by basal cells in the skin (41). Due to their localization, basal cells are likely to cross-contaminate the dermal preparations when the dermal and epidermal layers are separated. This probably explains the relatively high podoplanin transcript levels in the LYVE-1 negative fraction (*Fig. 3A*) and suggests that care should be taken when using podoplanin as the primary marker for LEC isolation from the skin.

The major strengths of the method we describe here are that staining with only one single marker is needed and that the cells can be sorted without previous culturing. On the other hand, magnetic bead-based sorting approaches are faster than flow cytometry-based isolation, and generally result in higher yields, although the purity of flow cytometry-sorted cells is usually better. The purity of our preparations was around 70%, which is sufficient for most applications. Higher purity could probably be achieved by including additional lymphatic markers such as murine chloride channel calcium-activated 1 (mCLCA1) (42) to further refine the sort parameters. As LECs derived from larger vessels do not express significant levels of LYVE-1, the method described here is only useful for the isolation of microcapillary LECs. Other approaches would be necessary to isolate LECs from larger lymphatic vessels.

To the best of our knowledge, this is the first description of a method for the isolation and culture of rat dermal microvascular LECs. Others have published methods for isolating LECs from rat thoracic ducts (43). LEC cultures have also been established from lymphangiomas induced in rats (44). The method we describe here therefore represents an important addition to the tools available for studying the lymphatic system in rats and for modeling of the human lymphatic system.

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