Video Article

Isolation of Human Lymphatic Endothelial Cells by Multi-parameter Fluorescence-activated Cell Sorting

Zerina Lokmic[1,2], Elizabeth S. Ng[1,3], Matthew Burton[1], Edouard G. Stanley[1,2,3], Anthony J. Penington[1,2], Andrew G. Elefanty[1,2,3]

[1]Murdoch Childrens Research Institute, The Royal Children#s Hospital

[2]Department of Paediatrics, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne

[3]Department of Anatomy and Developmental Biology, Faculty of Medicine, Nursing and Health Sciences, Monash University, Clayton

Correspondence to: Zerina Lokmic at zerina.lokmic@mcri.edu.au

URL: https://www.jove.com/video/52691
DOI: doi:10.3791/52691

Keywords: Medicine, Issue 99, lymphatic endothelial cell, lymphatic malformation, flow cytometric sorting, cell culture, cell surface markers

Date Published: 5/1/2015

Citation: Lokmic, Z., Ng, E.S., Burton, M., Stanley, E.G., Penington, A.J., Elefanty, A.G. Isolation of Human Lymphatic Endothelial Cells by Multi-parameter Fluorescence-activated Cell Sorting. *J. Vis. Exp.* (99), e52691, doi:10.3791/52691 (2015).

Abstract

Lymphatic system disorders such as primary lymphedema, lymphatic malformations and lymphatic tumors are rare conditions that cause significant morbidity but little is known about their biology. Isolating highly pure human lymphatic endothelial cells (LECs) from diseased and healthy tissue would facilitate studies of the lymphatic endothelium at genetic, molecular and cellular levels. It is anticipated that these investigations may reveal targets for new therapies that may change the clinical management of these conditions. A protocol describing the isolation of human foreskin LECs and lymphatic malformation lymphatic endothelial cells (LM LECs) is presented. To obtain a single cell suspension tissue was minced and enzymatically treated using dispase II and collagenase II. The resulting single cell suspension was then labelled with antibodies to cluster of differentiation (CD) markers CD34, CD31, Vascular Endothelial Growth Factor-3 (VEGFR-3) and PODOPLANIN. Stained viable cells were sorted on a fluorescently activated cell sorter (FACS) to separate the $CD34^{Low}CD31^{Pos}VEGFR\text{-}3^{Pos}PODOPLANIN^{Pos}$ LM LEC population from other endothelial and non-endothelial cells. The sorted LM LECs were cultured and expanded on fibronectin-coated flasks for further experimental use.

Video Link

The video component of this article can be found at https://www.jove.com/video/52691/

Introduction

A major function of the lymphatic vascular system is to absorb lymph, an excess interstitial fluid containing lipids, proteins and cellular components, and conduct it to the blood venous system. A network of lymphatic capillaries directs lymph to the lymph nodes where it is screened for presence of foreign antigens, an important process in immune surveillance and deployment of white blood cells to neutralize foreign antigens.

The uni-directional lymphatic system starts in tissues with the initial lymphatic capillary, a unique structure with a discontinuous single layer of thin-walled flat endothelial cells with specialized cell junctions that permit lymph entry[1,2]. These capillaries are attached to the neighboring connective tissue matrix via anchoring filaments to prevent vessel collapse in presence of increased interstitial pressure[3]. The initial lymphatic capillaries empty into collecting lymphatic capillaries that coalesce into larger lymphatic vessels or veins. In comparison to initial lymphatic capillary vessels, collecting lymphatic vessels have thicker vessel walls, paired lymphatic valves and are encased by a discontinuous basement membrane in which a few smooth muscle cells are embedded[4]. Coordinated opening and closure of lymphatic valves and contraction of smooth muscle cells facilitates flow of lymph[3]. In humans, the lymphatic veins from various regions of the body join to form lymphatic trunks which merge to form two lymphatic ducts: the thoracic duct and the right lymphatic duct. The thoracic duct drains lymph from the left side of the body and from the right side below the chest while the right lymphatic duct drains lymph from the right arm and right side of the head, neck, and thorax. Both ducts conduct lymph into the subclavian veins in the neck[5].

Disorders of the lymphatic system are broadly grouped into acquired and congenital (**Table 1**). Examples of acquired conditions are lymphangitis and secondary lymphedema. Lymphangitis is an inflammation of a lymphatic vessel due to bacterial infection. The affected lymphatics dilate and fill with exudate containing polymorphonuclear cells. In skin, these lymphatics are visible as red, painful subcutaneous streaks often accompanied by enlargement of the associated draining lymph node (lymphadenitis)[6]. Secondary lymphedema arises as a consequence of damage or obstruction to the lymphatic vessel or lymph node obstruction. This leads to chronic progressive swelling due to accumulation of lymph distal to the damage or obstruction. In developed countries, secondary lymphedema is most commonly associated with malignancy where metastasizing tumors obstruct lymphatic vessels or regional lymph nodes, or as a consequence of anti-cancer therapy following surgical removal of lymph nodes, post-irradiation fibrosis and post-inflammatory thrombosis and scarring[7]. In other parts of the world, secondary lymphedema may be secondary to lymphatic obstruction caused by parasitic worms such as *Wuchereria bancrofti*[6].

Disorders of Lymphatic Vascular System			
Acquired	**Congenital**		
Lymphadenitis Secondary lymphedema	**Primary Lymphedema**[10]	**Sporadic Lymphatic Malformations**[13]	**Lymphatic Malformations Associated with Syndromes**[13]
	e.g. Milroy Syndrome Meige Syndrome	**Simple**: Lymphatic malformations	*e.g.* Klippel-Tranaunay Syndrome Parks Weber Syndrome Sturge-Weber Syndrome
		Combined: Capillary-lymphatic malformations Capillary-lymphatic-venous malformation Capillary-lymphatic-arteriovenous malformation Capillary-lymphatic venous-arteriovenous malformation	

Table 1. Overview of the disorders of lymphatic vascular system.

Congenital disorders of the lymphatic system include primary (idiopathic) lymphedema thought to be caused by genetic mutations, lymphangiectasia and anomalies of the lymphatic system[8,9]. Primary lymphedema can be sporadic presumably caused by *de novo* mutations, or inherited. Lymphatic disorders can also be isolated or comprise part of a more generalized syndrome[10]. In the pediatric population, 97% of lymphedema is sporadic with abnormalities in lymphatic vessel structure that impair regional lymph drainage[11]. Milroy disease is an example of primary lymphedema caused by mutation in the VEGFR-3 gene evident at birth or soon after[12]. Although mostly familial condition, the Milroy disease can also be identified in infants without family history of Milroy disease[32]. The severity of any lymphedema is dependent on the amount of lymph production and ability to transport lymph back to venous circulation[6].

Based on clinical presentation and *in situ* endothelial cell proliferation, anomalies of the lymphatic system are classified as lymphatic tumors or lymphatic malformations[13]. Kaposiform lymphangiomatosis is an example of an LEC tumor[14]. Lymphatic malformations are thought to arise during embryonic development and grow in proportion to the child[15,16]. They rarely regress but can remain asymptomatic until trauma or infection precipitates rapid growth leading to clinical complications. The orderly structure of lymphatic network and conduction of lymph from the tissue to venous circulation described above is perturbed in lymphatic malformations which consist of localized collections of abnormal cystic structures filled with lymphatic fluid. While there is no clinical or experimental evidence that these cystic vessels are connected to the lymphatic circulation or that they contain functional lymphatic valves, their lymphatic identity is confirmed by expression of range of lymphatic cell markers such as PODOPLANIN, CD31, Lymphatic Vessel Endothelial Receptor 1 (LYVE-1), Prospero homeobox protein 1 (PROX-1) and VEGFR-3[15,17,18]. These cystic structures can be either small (microcystic) or large (macrocystic), but most lymphatic malformations contain both microcystic and macrocystic components (**Figure 1**)[16]. Following surgery, injection sclerotherapy and/or radiofrequency ablation the lymphatic malformations often reoccur.

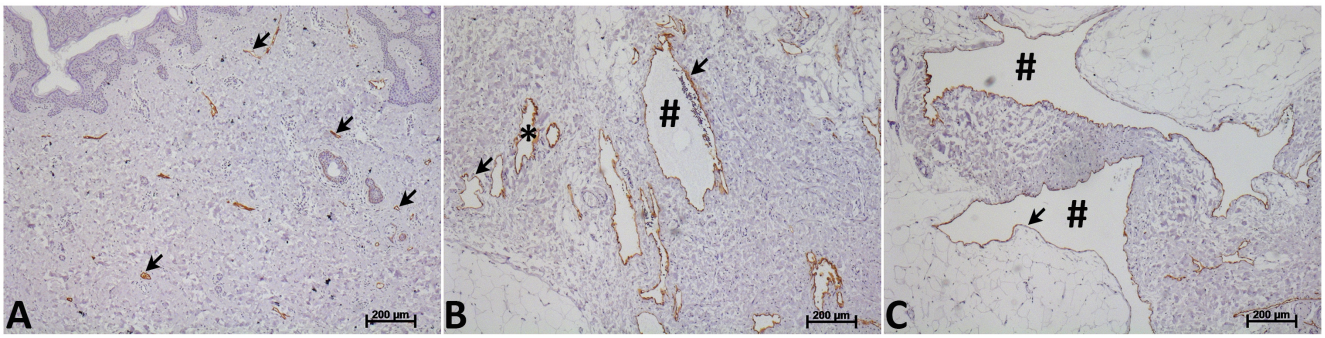

Figure 1. Morphology of human lymphatic vessels and lymphatic malformations. Normal human lymphatic (**A**) and lymphatic malformation vessels (**B** and **C**) labelled with antibody to PODOPLANIN (brown label, arrow). Human lymphatic malformation vessels are characterized by marked dilation and considerable variation in lumen size. These localized abnormal cystic structures can be either small (microcystic, *****) (**B**) or large (macrocystic, **#**) (**C**). Most lymphatic malformations contain both microcystic and macrocystic components. Please click here to view a larger version of the figure.

Some investigators have suggested that lymphatic malformations represent a developmental disorder of lymphatic vasculature in which the LECs do not have abnormal growth potential but instead have failed to connect to the normal circulation[19]. However, we have found that the LM LECs proliferate faster and are more resistant to apoptosis than foreskin LECs[15] suggesting that there is a primary defect in the LM LECs. When LM LECs are implanted in a mouse xenograft model, they form structures reminiscent of lymphatic malformations[15]. This supports a hypothesis that lymphatic malformations may be caused by one or more somatic mutations arising in LM LECs during fetal development. Indeed, recent reports have identified one such mutation in the p110α catalytic subunit of Phosphoinositide-3-Kinase (*PIK3CA)* gene[20].

Given the advances in DNA sequencing technology, relevant mutations could be more readily identified in isolated LM LECs, guiding future studies of these conditions. The isolation of viable LECs would facilitate comparisons between abnormal and normal LECs in assays such as migration, proliferation, tube forming ability and survival in response to reduced nutrient availability or pro-apoptotic agents[15]. Isolated LECs

would further enable us to perform cell-specific gene expression and proteomic studies, to delineate new LEC subpopulations and discover novel pharmacological agents suitable for clinical management of lymphatic malformations.

We have previously published a LEC isolation method based on magnetic bead separation of LECs from neonatal foreskin and lymphatic malformations[15]. We reported a strategy of separating normal and diseased LECs from vascular endothelial cells based on the absence of CD34 expression, followed by subjecting $CD34^{Neg}$ cell fraction to positive selection for CD31. However, this method was hampered by the presence of residual non-endothelial cells. This was independent of removing epidermis prior to subsequent connective tissue digestion. These contaminants generally proliferated more rapidly and thus eventually overgrew the endothelial cell cultures despite subsequent attempts to repeat LEC isolation. Indeed, an initial contamination of non-endothelial cells as low as 2% to 5% was sufficient to overwhelm the LEC population[15]. This prompted us to explore fluorescently activated cell sorting method as an option to improve LEC cell yield and purity. In addition, we used multi-parameter sorting to enhance the specificity of the LEC populations, adding VEGFR-3 and PODOPLANIN to the selection markers to identify $CD34^{Low}CD31^{Pos}$VEGFR-$3^{Pos}PODOPLANIN^{Pos}$ LECs.

The rationale for selecting these markers was based on the reports that while LECs and blood vascular endothelial cells have many cell surface markers in common such as CD31, LECs show phenotypic variation in their expression of CD34, PODOPLANIN and VEGFR-3 cell surface marker when compared to blood vascular endothelial cells[21-23]. CD31 is a 130 kDa transmembrane glycoprotein also known as platelet endothelial cell adhesion molecule 1 (PECAM-1). It is considered to be a pan-endothelial cell marker since it is expressed on all types of blood and lymphatic vessels[21,24,25]. CD34 is 110-kDa transmembrane glycoprotein present on most hematopoietic progenitor and stem cells, vascular endothelial cells and some lymphatic vessels[26].

VEGFR-3, the receptor for vascular endothelial growth factors C and D, is initially present on the developing veins in the mouse embryo, but following lymphatic specification regulated by the transcription factors SRY-related HMG-box (SOX)-18, **c**hicken **o**valbumin **u**pstream **p**romoter **t**ranscription **f**actor 2 (COUPTF-II) and PROX-1, VEGFR-3 venous expression is lost and it becomes restricted to embryonic LECs[25,27]. PODOPLANIN, a 38 kDa membrane mucoprotein, is first noted on lymphatic vessels at approximately embryonic day 11 (~E11.0) of mouse embryonic development [28] and whilst it is strongly expressed by microvascular lymphatic vessels, PODOPLANIN expression by macrocystic lymphatic endothelium in lymphatic malformations is more variable[15]. Flow cytometry experiments suggest that at least some $CD34^{High}CD31^{Pos}$ endothelial cells express the lymphatic marker PODOPLANIN[29]. Although systematic evaluation of LYVE-1 and PODOPLANIN staining in human lymphatic malformations showed that both are effective at staining lymphatic malformation endothelium[30], in normal tissues, LYVE-1 was reported to be strongly present in the initial lymphatic capillary endothelium but reduced and even absent in the collecting lymphatic endothelium[31]. As our aim is to isolate both the initial and collecting lymphatic endothelial cells we have opted not to use LYVE-1 as part of our cell selection strategy. Finally, the decision to employ these markers was also based on the availability of antibodies that are used diagnostically for labelling lymphatic vessels for microscopic imaging, a feature that would permit correlation between flow cytometry and immunofluorescent studies.

This article will describe the tissue digestion method, cell staining and FACS settings required for successful isolation of $CD34^{Low}CD31^{Pos}$VEGFR-$3^{Pos}PODOPLANIN^{Pos}$ LECs as well as $CD34^{High}CD31^{Pos}$VEGFR-$3^{Pos}PODOPLANIN^{Pos}$ endothelial cells from foreskin and lymphatic malformation tissue.

Protocol

Ethics statement: Ethical approval for collection of lymphatic malformation and foreskin tissues was obtained from the Human Research Ethics Committees at the Royal Children's Hospital, Melbourne, Australia. Signed consent was received from patients' parents prior to surgery. Tissue samples were collected from patients diagnosed with LMs undergoing surgical procedures as part of their clinical management and patients undergoing elective circumcision. All experiments were performed in accordance with guidelines of the National Health and Medical Research Council, Australia.

1. Preparation of Cell Suspension from Foreskin and Lymphatic Malformation Tissues

1. Buffers and Media Preparation.
    1. Prepare complete endothelial cell media using commercially available EGM-2 MV Bullet Kit by warming EGM-2 media and gently thawing the kit components in 37 °C water bath. In class II biosafety cabinet, aseptically add each component to the EGM-2 media. Once all components are added to the media, refer to this media as 'complete endothelial cell media'. Use sterile 50 ml pipette to mix the contents prior to use.
    NOTE: All steps pertaining to cell culture are performed in a class II biohazard cabinet. All solutions are stored at 4 °C as per manufacturers' instructions and are warmed to room temperature prior to use. The complete endothelial cell media, cell culture buffers and enzyme solutions are warmed at 37 °C for 20 min prior to use.
    2. Supplement complete endothelial cell media with 50 ng/ml VEGF-C. Aliquot the supplemented endothelial cell media into 50 ml sterile tubes and store at 4 °C until use. The media is stable for at least 4 weeks when stored at 4 °C.
    3. Prepare calcium and magnesium free phosphate-buffered saline (PBS) by dissolving 8.752 g NaCl, 1.416 g $Na_2HPO_4.2H_2O$ and 0.395 g KH_2PO_4 in 1,000 ml of water. Adjust pH to 7.4. Filter sterilize PBS using 0.22 µm filter and store at 4 °C. Prior to use add antibiotic/antimycotic solution (used at 1:100).
    4. To prepare human fibronectin, dissolve 1 mg of fibronectin in 10 ml of sterile water. Store reconstituted solution in 100 µl aliquots at -20 °C. On the day of use add 10 ml of sterile PBS to 100 µl of fibronectin aliquot (to give a final working concentration of 10 µg/ml).
    5. For the enzyme media, prepare a solution containing 0.04% Dispase II, 0.25% Collagenase II and 0.01% DNase I in sterile PBS. First, weigh dispase and collagenase, place in a sterile 50 ml tube, add the required volume of PBS and incubate for 30 min with shaking at 37 °C to dissolve. Once dissolved, filter sterilize (0.22 µm filter) dispase/collagenase solution in biosafety hood. Aseptically add the DNase I. Store the enzymatic solution at 37 °C until use.

NOTE: DNase I is a commercial cell culture grade reagent available as sterile lyophilized powder.

2. Preparation of Cell Suspension from Foreskin and Lymphatic Malformations

1. Weigh a 50 ml tube containing 10 ml of DMEM/2% antibiotic antimycotic solution. This tube will be used for tissue collection.
2. Following surgical removal of lymphatic malformation and foreskin tissues, aseptically add tissue specimen to the tube and transfer on ice to the cell culture laboratory.
3. Weigh the tube containing the tissue and calculate tissue weight. Use this weight to calculate the volume of enzymatic solution needed to digest the tissue.
4. In a Class II biosafety cabinet, use sterile forceps to transfer the tissue and media into a 100 mm tissue culture dish. Use sterile scissors to finely mince the tissue into ~1 mm^3 pieces.
5. Transfer minced tissue mixed with media into a 50 ml tube containing an additional 10 ml of sterile DMEM/2% antibiotic antimycotic solution. Mix by inversion to re-suspend the tissue. Centrifuge the sample at 300 x g for 5 min.
6. Remove the supernatant. Based on tissue weight, add 1 ml of pre-warmed (37 °C) collagenase II/DNase I/ Dispase II digestion solution per 100 mg of minced tissue. Generally, use 10 ml of enzyme solution for approximately 1 g of minced tissue.
7. Incubate the tissue in a 37 °C incubator for 20-90 min with constant shaking at 200 rpm. At the end of this period, ensure that nearly all of the tissue is digested into fine fragments.
   NOTE: Exposure of the cells to collagenase and dispase at the time of isolation from the primary tissues influences cell survival. We have found empirically that most neonatal foreskin samples optimally require 20-30 min of digestion, whereas fibrotic lymphatic malformation samples may require 60-90 min of digestion. LM LEC yields are influenced by the amount of fibrosis present, more fibrous tissue yielding fewer cells, possibly due to deleterious effects of prolonged exposure to collagenase II and dispase II.
8. Following digestion, transfer the tube to the biosafety cabinet and pass the digested tissue solution through a 70 µm strainer placed into a sterile 50 ml tube. Using 3 ml syringe piston with rubber plunger, grind down the remaining tissue until only small traces of extracellular matrix are observed.
9. Wash the cell strainer with a volume of endothelial cell medium equivalent to the volume of dissociating enzyme medium to recover cells stuck to sieve and to inactivate the enzymes. For example, for 2 ml of dissociating enzyme medium to digest the minced tissue, add 2 ml of endothelial cell medium to inactivate the enzymes.
10. Centrifuge the cell solution at 300 x g for 5 min and aspirate the supernatant. R>e-suspend the cells in 10 ml of PBS. Centrifuge cells at 300 x g for 5 min, remove the supernatant then repeat cell wash twice. >Resuspend the cell pellet in 20 ml of endothelial cell medium. Count cells using trypan blue then seed 2 x 10>^{6} cells in 150 cm>^{2} flask pre-coated with fibronectin in a final volume of 20 ml of endothelial cell medium. Culture at 37 °C in a 5% $CO>_2$ in humidified air incubator.>
    NOTE: It is expected that 75%-90% of nucleated cells survive tissue digestion as estimated by trypan blue exclusion. The initial cell count reflects all nucleated cells in the cell suspension (*i.e.* keratinocytes, leukocytes, macrophages, connective tissue cells and blood vessel cells). The cell count at 5-7 days reflects cells that have attached, survived and proliferated during cell culture. Coating tissue culture flasks with fibronectin also improves subsequent LEC and LM LEC survival.
11. After overnight incubation, wash away unbound cells in three washes with PBS/1% antibiotic-antimycotic solution and add fresh endothelial cell medium. Carry out three washes to effectively remove unbound cells and red blood cells present in the flask. Change media every second day. Cells will be ~80% confluent after 5-7 days.

3. Antibody Staining of Cells for Lymphatic Endothelial Cell Surface Markers for Flow Cytometry

1. After the cells have reached 80% confluence, aspirate medium and rinse cells with PBS.
2. Detach adherent cells by incubating cells with 7 ml of cell detachment solution such as Accutase per 150 cm^2 flask for 5-7 min at 37 °C.
3. Harvest detached cells, inactivate the cell detachment solution by adding 3 volumes of endothelial cell medium and transfer the cell suspension to a new sterile tube.
4. Centrifuge the cells at 300 x g for 5 min.
5. Aspirate supernatant and re-suspend the cells in 5 ml of PBS. Centrifuge cells at 300 x g for 5 min, remove the supernatant then repeat cell wash. Re-suspend the cells in 5 ml of PBS.
6. Count the number of viable cells. Mix 10 µl of cell suspension with 90 µl of 0.4% trypan blue. Transfer 10 µl of this suspension to hemocytometer for counting.
   NOTE: The final cell yield will depend on the sample size processed. The usual cell yield per 150 cm^2 flask ranges from 7 x 10^5 to 1.2 x 10^6 of viable cells.
7. Prepare the antibody solutions for cell staining.
   NOTE: The following dilutions were titrated for staining 1 x 10^6 in a staining volume of 100 µl.
   1. Dilute PE conjugated mouse anti human VEGFR-3 (1:50); PE-Cy7 conjugated mouse anti human conjugated CD34 (1:200); APC-conjugated mouse anti human CD31 (1:100) and Alexa 488-conjugated rat anti human PODOPLANIN (1:200) in total volume of 100 µl sterile 5% FBS/PBS solution per tissue sample. Following preparation of antibody solution, keep on ice until use. Similarly prepare diluted isotype control antibody mixture to facilitate setting of flow cytometry gates.
8. To reduce nonspecific binding of the antibodies, centrifuge cells at 300 x g for 2 min, remove supernatant then suspend the cells in 100 µl sterile 5% FBS/PBS solution per sample to be stained. Incubate cells on ice for 20 min at 4 °C.
9. Centrifuge cells at 300 x g for 5 min, remove supernatant then re-suspend cells in the conjugated antibody cocktail. Also stain a smaller aliquot of cells (1 x 10^5) in 100 µl isotype control antibody mixture. Incubate cells on ice for 20 min.
10. To remove the unbound antibody, add 2 ml of 2% FBS/PBS solution and centrifuge the cells at 300 x g for 5 min. Aspirate the supernatant and repeat the wash.

11. Resuspend the cell pellet for the isotype control and the antibody stained sample to be sorted in 300 µl of 0.5 mg/ml propidium iodide/2% FBS/PBS solution. Place tubes on ice until sorting.

4. Cell Sorting

1. Use the following materials to set up the instrument; alignment beads such as commercially available fluorescent particles, phosphate-buffered saline based sheath fluid and drop delay beads, such as Accudrop fluorescent beads.
2. Isolate the purified human LECs on a multi-parameter fluorescence-activated cell sorting instrument. Set up the instrument and align as per manufacturer's recommendations. In addition, run single stain compensation controls with each sort to generate the compensation matrix and to thus eliminate substantial bleed through of the emission of fluorochromes such as PE into the PE-Cy channel.
   NOTE: A higher proportion of the FACS sorted cells survive the isolation if a 100 µm nozzle is used and cells are sorted into endothelial cell medium.

5. Cell Culture Post FACS Sorting

1. Following sorting, centrifuge cells at 300 x g for 5 min. Aspirate the supernatant and resuspend the cells in 5-10 ml of media (depending on the flask used to seed the cell). If less than 50,000 cells are sorted, the cells are cultured in fibronectin coated 25 cm^2 flask.
2. Otherwise culture the cells in 75 cm^2 fibronectin-coated flask at 37 °C in a 5% CO_2, air humidified incubator. Change media after 2 days.
   NOTE: Cell media is changed every second day because prolonging the time between media changes appears to favor survival of the non-endothelial cells. We validate the lymphatic endothelial cell phenotype using immunohistochemical detection of markers: PROX-1, VEGFR-3, Podoplanin, CD34 and CD31 when the cells are first split for further expansion[15].

Representative Results

Following initial tissue digestion, after 24 hours in culture of unfractionated samples, distinct endothelial cell colonies can be observed (**Figure 2A**) together with fibroblast-like cells and smooth muscle cells. Following sorting and after 24 hours in cell culture, the $CD34^{Low}CD31^{Pos}VEGFR\text{-}3^{Pos}Podoplanin^{Pos}$ cells attach and show typical cobblestone morphology (**Figure 2B** and **C**). Using the FACS method described above, we are able to purify cells up to 99.8% purity and distinguish them from $CD34^{High}CD31^{Pos}VEGFR\text{-}3^{Pos}Podoplanin^{Pos}$ endothelial cells. Representative results of gating strategy and sorting are presented in **Figure 3**. This has resolved the issues experienced when using magnetic-bead isolation method. To date, we have passaged these cells up to passage 13. After this passage, the LM LECs and foreskin LECs start to senescence, accompanied by morphological changes and reduced cell division.

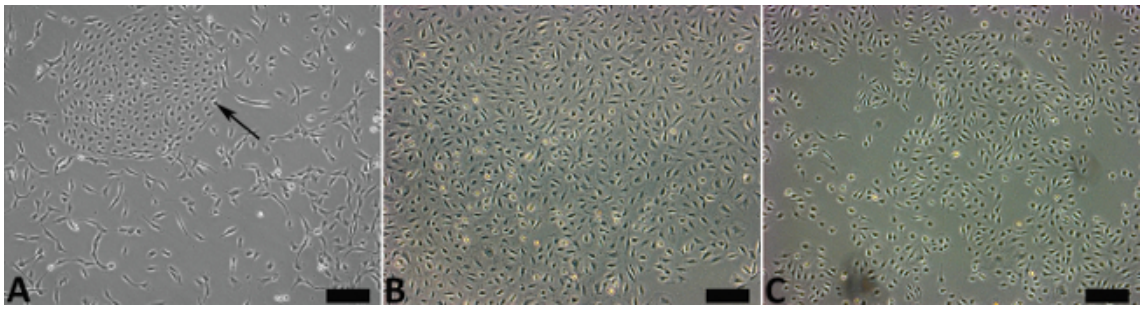

Figure 2. Foreskin LECs and LM-LEC. Twenty-four hours after enzymatic digestion, unsorted cells contain both endothelial (arrow) and non-endothelial cells (**A**). Following $CD34^{Low}CD31^{Pos}$ $VEGFR\text{-}3^{Pos}Podoplanin^{Pos}$ FACS cell isolation, foreskin LECs (**B**) and LM-LECs (**C**) are devoid of non-endothelial cells and maintain cobblestone morphology. Please click here to view a larger version of the figure.

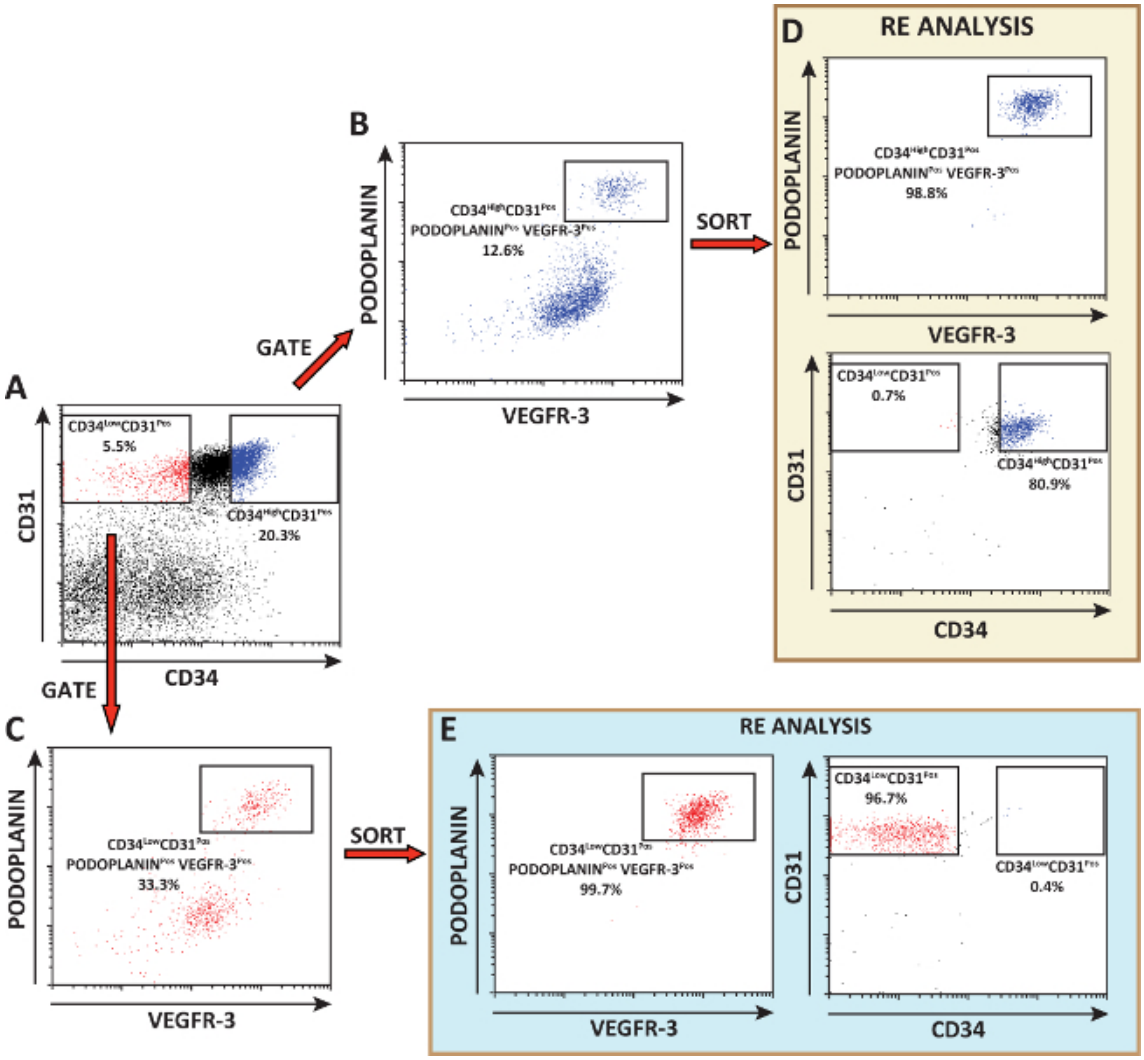

Figure 3. Fluorescence-activated cell sorting gating strategies for cell sorting of vascular and lymphatic endothelial cells. (**A**) Live cells are first gated on CD34 and CD31 expression, followed by VEGFR-3 and PODOPLANIN gating to sort vascular (**B**) and lymphatic endothelial (**C**) cells respectively. (**D**) Re-analysis of sorted $CD34^{High}CD31^{Pos}VEGFR\text{-}3^{Pos}PODOPLANIN^{Pos}$ cells shows >98% vascular endothelial cell phenotype. (**E**) LEC cells sorted on $CD34^{Low}CD31^{Pos}VEGFR\text{-}3^{Pos}PODOPLANIN^{Pos}$ phenotype are also >98% pure. Please click here to view a larger version of the figure.

Discussion

LECs play an important role in maintaining fluid homeostasis, immune response to foreign antigens and absorption and transport of some nutrients. LEC homeostasis can be affected by disease processes such as bacterial infections and tumor metastasis, but LECs can also develop somatic mutations that result in formation of dysfunctional lymphatic vessels and significant morbidity for affected patients. To gain more understanding of lymphatic malformation etiology through *in vivo* implantation and *ex vivo* experimentation, and discover new treatment options, we first developed an LEC isolation method based on magnetic bead selection strategy using $CD34^{Neg}CD31^{Pos}$ selection strategy[15]. However, the resulting cultures often contained cells other than endothelial cells that were difficult to remove. Subsequently, the method was refined by using FACS to sort LECs that express $CD34^{Low}CD31^{Pos}VEGR3^{Pos}PODOPLANIN^{Pos}$ phenotype. This approach resulted in relatively homogenous LEC cultures containing <0.5% of non- $CD34^{Low}CD31^{Pos}VEGR3^{Pos}PODOPLANIN^{Pos}$ cells on post-sorting check. In practical terms, the advantage of sorting cells by FACS is that reduction of non- LECs presence to <0.5%. Cultured LECs and LM LECs have a typical cobblestone morphology and become senescent around passage 13.

In this study we have described a flow cytometry based protocol for the isolation and culture of highly enriched LECs from normal and abnormal tissues based on their expression of a suite of cell surface markers ($CD34^{Low}CD31^{Pos}VEGR3^{Pos}PODOPLANIN^{Pos}$). Cells were sorted from primary tissues following 5-7 days of *in vitro* expansion, a step that resulted in substantial enrichment of LEC compared to their frequency in tissues at the time of isolation. During surgery we obtain tissues ranging from several micrograms to tens of grams in weight. This tissue can vary greatly in its composition with respect to volume of lymphatic malformation vessels present, tissue scarring and presence of lymphatic malformation cyst thrombi. All these components will influence how many cells can be isolated from the diseased tissue. Hence the starting cell number can range from few thousand cells to several million cells. Similarly, this will influence the composition of the cell suspension and starting cell numbers meaning that the number of sorted cells will also differ from one sample to another.

The frequency of LECs in unsorted foreskin samples following 5-7 days in culture ranges from 0.52% to 4.7%, whereas LM LECs range from 0.8% to 12.43%. The LEC cultures comprised >99% $CD34^{Low}CD31^{Pos}VEGR3^{Pos}PODOPLANIN^{Pos}$ cells after post-sorting re-analysis and maintained a typical cobblestone morphology in culture until senescence at passage ~13 without overgrowth by non-endothelial elements.

It is necessary to expand cells *ex vivo* following initial tissue digestion and sorting because the yield of either foreskin or lymphatic malformation cells is generally low. Thus, the prior expansion of lymphatic endothelial cells *in vitro* allows the generation of the ~$2x10^6$ cells needed to seed a mouse chamber. This requires an *in vitro* expansion stage of 5-7 days. While we accept that cell culture may induce phenotypic changes in the primary cells, we have shown that these cultured cells retain their capacity to form lymphatic malformation-like structures in a mouse xenograft model[15].

There are several critical steps within the protocol that determine the degree of success when sorting cells by FACS. The most critical step is the time of exposure of the cells to collagenase and dispase during isolation from the primary tissues as described in step 2.7. This is not only influenced by the amount and type of tissue present but also by the batch of enzymes used. In addition, fluorescently labelled antibodies used to characterize the cell populations need to be titrated and preferably monoclonal. We tested several different cell detachment solutions such as trypsin/EDTA, EDTA, TrypLE Select and cell detachment solution. We found that EDTA had to be used for a prolonged period of time to detach cells and residual cell clumps often remained. In contrast, single cell suspensions were generated within 3-5 minutes of incubation in Trypsin/EDTA, cell detachment solution and TrypLE Select. We found no substantial difference in the expression of CD31, CD34, Podoplanin and VEGFR-3 following treatment with any of the enzyme solutions. Single stained samples should also be done with every sorting experiment to ensure that fluorochrome emission spectra do not overlap and therefore give incorrect readings. These critical steps also constitute steps where modifications to the cell protocol might be required or troubleshooting during the flow cytometry sorting.

When compared to our previously published magnetic bead isolation of LECs [15], the advantage of isolating LECs by FACS includes the following: more rapid isolation of cells with high purity and identification of discrete cell subsets and rare populations within a heterogeneous sample. The major limitations of FACS-based LEC and LM LEC isolation revolves around limited number of cells available for validation following cell sorting results. In addition, while every attempt is made to standardize our assays and instrument set-up, these same parameters may not necessarily be reproducible in other laboratories. Therefore, some variability in the results may occur. In addition, one of the issues that lymphatic biologists face is the absence of cell specific markers that would differentiate between initial capillary LEC markers and collecting capillary LEC markers. At this stage, we have not yet identified LEC markers that would distinguish between healthy LECs and LM LECs. Since lymphatic malformation tissue also contains normal-looking lymphatic vessels (**Figure 1A**), the resulting $CD34^{Low}CD31^{Pos}VEGR3^{Pos}PODOPLANIN^{Pos}$ will contain a proportion of normal LECs as well as those of the diseased phenotype. Future studies examining LEC and LM LEC gene expression and proteomics may be able to direct us towards more specific LM LEC markers that could distinguish LM LECs from LECs in the same tissue.

In the future, we expect to utilize FACS and these new LEC markers in attempt to identify rare subsets of LEC populations both in foreskin and lymphatic malformations. This would allow us to isolate these cell populations and study their role in the development of the lymphatic vascular system and lymphatic malformations.

Isolating LECs and LM LECs based on $CD34^{Low}CD31^{Pos}VEGR3^{Pos}PODOPLANIN^{Pos}$ significantly reduced non-endothelial cell contamination. Furthermore, the FACS technology allows us to separate LECs into subtypes based on the expression of CD31, PODOPLANIN and VEGFR-3 cell surface markers and to understand this in the context of cell lineage development. For diseased LECs, this allows for cell-specific studies that will shed further light on genes causing LEC diseases and LEC capacity to respond to various cell stimuli *in vitro* and in animal xenograft models.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors would like to acknowledge the Baker Foundation and the Royal Children's Foundation 'Women in Science Fellowship' support of Zerina Lokmic. Andrew G. Elefanty and Edouard G. Stanley are NHMRC Senior Research Fellows. Work in their laboratories was supported by NHMRC and Stem Cells Australia.

References

1. Baluk, P., *et al.* Functionally specialized junctions between endothelial cells of lymphatic vessels. *J Exp Med.* **204**, (10), 2349-2362 (2007).
2. Yao, L. C., Baluk, P., Srinivasan, R. S., Oliver, G., McDonald, D. M. Plasticity of button-like junctions in the endothelium of airway lymphatics in development and inflammation. *Am J Pathol.* **180**, (6), 2561-2575 (2012).
3. Zawieja, D. C. Contractile physiology of lymphatics. *Lymphat Res Biol.* **7**, (2), 87-96 (2009).
4. Witte, M. H., *et al.* Structure function relationships in the lymphatic system and implications for cancer biology. *Cancer Metastasis Rev.* **25**, (2), 159-184 (2006).
5. Andrade, M., Jacomo, A. Anatomy of the human lymphatic system. *Cancer Treat Res.* **135**, 55-77 (2007).
6. Kumar, V., Abbas, A. K., Aster, J. C., Fausto, N. Ch. 2. *Robbins & Cotran Pathologic Basis of Disease.* 43-78 Elsevier Saunders (2009).
7. Shayan, R., Achen, M. G., Stacker, S. A. Lymphatic vessels in cancer metastasis: bridging the gaps. *Carcinogenesis.* **27**, (9), 1729-1738 (2006).
8. Feins, N. R., Greene, A. K. Ch. 29. *Mulliken & Young's Vascular Anomalies Hemangiomas and Malformations.* Mulliken, J. B., Burrows, P. E., Fishman, S. J. 1059-1076 University Press Oxford (2013).

9. Ch. 14. *Mulliken & Young's Vascular Anomalies Hemangiomas and Malformations.* Mulliken, J. B., Burrows, P. E., Fishman, S. J. **14**, 562-594 Oxford University Press (2013).
10. Bellini, C., Hennekam, R. C. Clinical disorders of primary malfunctioning of the lymphatic system. *Adv Anat Embryol Cell Biol.* **214**, 187-204 (2014).
11. Schook, C. C., *et al.* Primary lymphedema: clinical features and management in 138 pediatric patients. *Plast Reconstr Surg.* **127**, (6), 2419-2431 (2011).
12. Brouillard, P., Boon, L., Vikkula, M. Genetics of lymphatic anomalies. *J Clin Invest.* **124**, (3), 898-904 (2014).
13. *International Society for the Study of Vascular Anomalies (ISSVA) classification.* Available from: www.issva.org/classification.com (2014).
14. Croteau, S. E., *et al.* Kaposiform lymphangiomatosis: a distinct aggressive lymphatic anomaly. *J Pediatr.* **164**, (2), 383-388 (2014).
15. Lokmic, Z., *et al.* Isolation of human lymphatic malformation endothelial cells, their in vitro characterization and in vivo survival in a mouse xenograft model. *Angiogenesis.* **17**, (1), 1-15 (2014).
16. Smith, R. J. Lymphatic malformations. *Lymphat Res Biol.* **2**, (1), 25-31 (2004).
17. Chen, E. Y., *et al.* Similar histologic features and immunohistochemical staining in microcystic and macrocystic lymphatic malformations. *Lymphat Res Biol.* **7**, (2), 75-80 (2009).
18. Norgall, S., *et al.* Elevated expression of VEGFR-3 in lymphatic endothelial cells from lymphangiomas. *BMC cancer.* **7**, (2), 75-80 (2007).
19. Garzon, M. C., Huang, J. T., Enjolras, O., Frieden, I. J. Vascular malformations. *Part I. J Am Acad Dermatol.* **56**, (3), 353-370 (2007).
20. Osborn, A., Dickie, P., Adams, D., Dickie, B. *20th International workshop on Vascular Anomalies.* ISSVA Melbourne, Australia (2014).
21. Baluk, P., McDonald, D. M. Markers for microscopic imaging of lymphangiogenesis and angiogenesis. *Ann N Y Acad Sci.* **1131**, 1-12 (2008).
22. Lokmic, Z., Mitchell, G. M. Engineering the microcirculation. Tissue engineering. *Part B, Reviews.* **14**, (1), 87-103 (2008).
23. Lokmic, Z., Mitchell, G. M. Visualisation and stereological assessment of blood and lymphatic vessels. *Histol Histopathol.* **26**, (6), 781-796 (2011).
24. Hagerling, R., *et al.* A novel multistep mechanism for initial lymphangiogenesis in mouse embryos based on ultramicroscopy. *EMBO J.* **32**, (5), 629-644 (2013).
25. Pollmann, C., Hagerling, R., Kiefer, F. Visualization of lymphatic vessel development, growth, and function. *Adv Anat Embryol Cell Biol.* **214**, 167-186 (2014).
26. Breiteneder-Geleff, S., *et al.* Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. *Am J Pathol.* **154**, (2), 385-394 (1999).
27. Kaipainen, A., *et al.* Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci U S A.* **92**, (8), 3566-3570 (1995).
28. Schacht, V., *et al.* T1alpha/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. *EMBO J.* **22**, (14), 3546-3556 (2003).
29. Kriehuber, E., *et al.* Isolation and characterization of dermal lymphatic and blood endothelial cells reveal stable and functionally specialized cell lineages. *J Exp Med.* **194**, (6), 797-808 (2001).
30. Florez-Vargas, A., *et al.* Comparative analysis of D2-40 and LYVE-1 immunostaiing in lymphatic malformations. *Lymphology.* **41**, (3), 103-110 (2008).
31. Makinen, T., *et al.* PDZ interaction site in ephrinB2 is required for the remodeling of lymphatic vasculature. *Genes Dev.* **19**, (3), 397-410 (2005).
32. Connell, F. C., *et al.* Analysis of coding regions of VEGFR3 and VEGFRC in Milroy disese and other primary lymphoedemas. *Hum Genet.* **124**, (6), 625-631 (2009).

