HHS Public Access

Author manuscript

Cytometry A. Author manuscript; available in PMC 2021 November 16.

Published in final edited form as:

Cytometry A. 2021 March ; 99(3): 251–256. doi:10.1002/cyto.a.24292.

Comprehensive Phenotyping of Endothelial Cells Using Flow Cytometry 1: Murine

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Abstract

The endothelium forms a selective barrier between circulating blood or lymph and surrounding tissue. Endothelial cells play an essential role in vessel homeostasis, and identification of these cells is critical in vascular biology research. However, characteristics of endothelial cells differ depending on the location and type of blood or lymph vessel. Endothelial cell subsets are numerous and often identified using different flow cytometric markers, making immunophenotyping these cells complex. In part 1 of this two part review series, we present a comprehensive overview of markers for the flow cytometric identification and phenotyping of murine endothelial subsets. These subsets can be distinguished using a panel of cell surface and intracellular markers shared by all endothelial cells in combination with additional markers of specialized endothelial cell types. This review can be used to determine best markers for identifying and phenotyping desired murine endothelial cell subsets.

Keywords

Murine; endothelial cell; phenotyping; immunophenotyping; flow cytometry; endothelial cell subset

Introduction

Endothelial cell function and morphology are primarily determined by location. The plasticity of endothelial cells allows change of function and phenotype depending on the microenvironment. Proper identification and understanding of specific endothelial cell phenotypes grant valuable insight into tissue-specific physiology and pathology. As endothelial dysfunction is increasingly used as a diagnostic tool, the importance of accurately detecting targeted endothelial cell phenotypes is key to unlocking further endothelium-based medical diagnostics and research. Flow cytometric phenotyping of endothelial cells begins with selecting endothelial cell-specific markers. Combining pan-endothelial antigens with one or more dump channel markers allows for the gating of all endothelial cells that can be further analyzed and segregated into subsets. Commonly used

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pan-endothelial antigens and dump channels are listed in Table 1. Listed dump channel markers can be substituted for any other established cell-specific antigen.

Organ-Specific Endothelial Phenotypes

Differentiation of the endothelium into specialized endothelial cells with organ-specific heterogeneity allows for further flow cytometric profiling. Lymphatic endothelial cells, for example, are functionally distinct from blood vessel endothelial cells and can be recognized by the expression of markers-such as CD90-absent from the blood endothelium in mice [12]. Specific identifiable properties of specialized endothelial cells include loose junctions and lack of a basement membrane in the lymph, large and diaphragm-free fenestrae in the liver sinusoids, diaphragmed fenestrae in the renal peritubular capillaries, highly fenestrated islet capillaries in the pancreas, tight junctions forming a continuous barrier in the brain, and a lack of Weibel-Palade bodies in the alveolar capillaries [3456]. The unique characteristics of these endothelial cells enable the use of tissue-specific markers listed in Table 2 for identification of organ-specific phenotypes.

Characterization of described endothelial subsets should always follow a validated gating strategy. Figure 1 demonstrates an example gating strategy to identify three main murine pulmonary endothelial subsets. After excluding dead cells via a live/dead dye, an initial gate of $CD45^{-}/Sca\text{-}1^{+}$ selects all endothelial cells. The endothelial subsets can then be differentiated based on expression of CD90.2 and vWF. Alveolar capillary, lymphatic, and non-alveolar vascular endothelial cells are identified as $CD90.2^{-}/vWF^{-}$, $CD90.2^{+}/vWF^{-}$, and $CD90.2^{-}/vWF^{+}$, respectively.

Endothelial Cell Subsets within Organs

Further specialization into various endothelial cell subsets can be identified using a combination of cell surface markers and intracellular markers. Combining constitutive endothelial markers with those listed in Table 3 permit endothelial cells to be segregated by vessel size, vessel type, and stemness.

An identifiable subset of note is the high endothelial venule (HEV). HEVs are highly specialized post-capillary venule endothelial cells involved in lymphocyte trafficking [7]. A combination of lymphatic endothelial markers with HEV-specific markers is used to phenotype HEVs, which can be further segregated into peripheral lymph HEVs and mucosal HEVs using markers MECA-79 and MECA-367 [891011].

Inducible Markers

Cytokine and chemokine activation of injured endothelium leads to phenotypic changes via the induction of endothelial activation markers. These upregulated molecules play a pivotal role in the inflammatory response mainly by promoting leukocyte recruitment. Upregulation of inducible markers like CD54 (I-CAM), CD62P (P-Selectin), and CD142 [12] can be used to identify injured or dysfunctional endothelial cells [131415]. The inducible markers for endothelial cells are listed in Table 3.

Endothelial injury can also affect the specificity of PROX-1. The nuclear marker PROX-1, specific to the lymphatic endothelium, is found in murine liver sinusoidal endothelial cells following injury [16]. PROX-1 is critically involved in the trans-differentiation of blood ECs into lymphatic vessels via inducing proliferation, migration and supporting survival. PROX-1 promotes upregulation of FGFR-3 and lymphatic EC markers such as and VEGFR-3 while downregulating blood EC markers [1617181920211222].

Important Considerations

Just as the endothelium is diverse and highly specialized, certain flow cytometric markers have opposing or differential expression depending on the type of endothelial cell being analyzed. Specialized markers include CD117, CD133, PV-1, eNOS, and vWF. Both vWF and CD105, widely used pan-endothelial markers, are not expressed on the lymphatic endothelium [232425]. Expression of vWF is also absent from the alveolar endothelium and can be used to differentiate alveolar ECs from the rest of the pulmonary endothelium [26]. The hematopoietic marker CD117 can be used as a dump channel in endothelial panels but is expressed on endothelial colony forming cells (ECFCs) and murine pancreatic islet endothelial cells [272829]. Endothelial expression of progenitor marker CD133 is limited to bone marrow-derived ECFCs and murine endothelial cells in the CNS [303132]. Expression of diaphragmed fenestrae marker PV-1 is lost as fetal liver sinusoidal and renal glomerular endothelial cells shed their diaphragms during maturation [3334]. It is also important to carefully verify negative markers as truly negative for desired phenotypes, especially if there is no overall consensus or if markers are only published by one group. Single-cell transcriptomics technology reveals more profound organ and site-specific heterogeneity among endothelial cells [35]. Development of antibodies against the proteins defining these newly identified endothelial subsets will most definitely expand the flow cytometric endothelial phenotypes.

Acknowledgements

The authors thank the LRI Flow Cytometry Core technical staff for assistance with instrument QC and setup.

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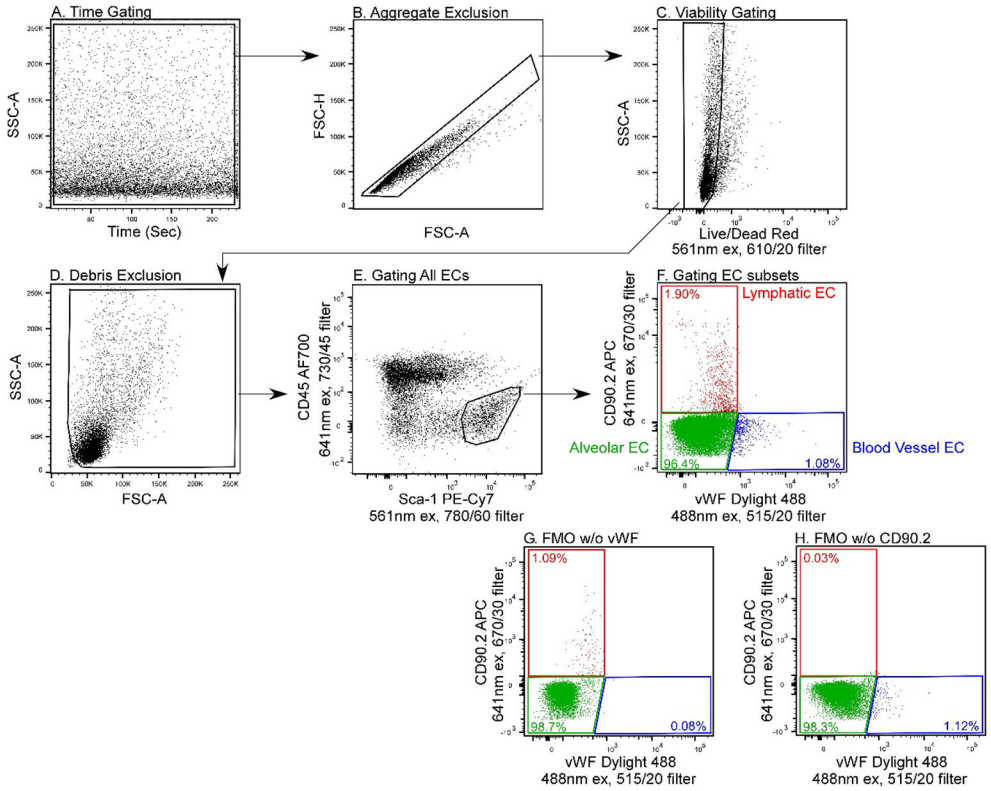

Figure 1. Example Gating Strategy for Identification of Murine Pulmonary Endothelial Subsets

Artifact exclusion included time gating to identify and exclude fluidic disturbances (A), removal of aggregates (B), selection of live cells based on uptake of a live/dead dye (C), and exclusion of debris based on low forward scatter (D). Unlike white blood cells, the FSC/SSC of endothelial cells is not characteristic. The best practice is to set the FSC threshold to acquire all cells and gate endothelial cells based on their specific antigen expression patterns. Pan-endothelial cells are selected as CD45−/Sca-1+ (E) then, based on expression of CD90.2 and Von Willebrand Factor lymphatic ECs (CD90.2+/vWF−), alveolar ECs (CD90.2−/vWF−), and non-alveolar ECs (CD90.2−/vWF+) were differentiated (F). Fluorescence minus one (FMO) controls containing all of the fluorophores in the panel except vWF (G) and all fluorophores except CD90.2 (H) were used to create the EC differentiation gates shown in plot F.

Table 1.

Murine Pan-Endothelial Cell Markers/Probes and Dump Channels

Pan-Endothelial Markers	*References*
Cell Surface	
CD31 (PECAM-1)	363738
CD105 (Endoglin)	2324
CD144 (VE-cad)	39
CD146 (P1H12, MCAM, MUC18, S-endo-1)	40
CD202b (Tie-2)	38414243
CD309 (VEGFR-2, KDR, Flk-1)	3844
VEGFR-1 (Flk-1)	2945
Tie-1	384142
Intracellular	
Dil-Ac-LDL Uptake	46
vWF	2526
eNos	4748
Dump Channels	
E-Cad (Epithelial Cells)	49
CD42 (Platelets)	13
CD45 (Hematopoietic Cells)	33
CD117 (c-kit) (Hematopoietic and Progenitor Cells)	272850
CD133 (Stem Cells)	30
CD326 (EpCAM) (Epithelial Cells)	514939

Framed markers are used for both murine and human panels.

Table 2.

Murine Organ-specific Endothelial Cells

	Markers	
	Positive	**Negative**
Lymphatic	**CD90** [12], **LYVE-1** [4], **VEGFR-3 (Flt-4)** [7], PROX-1 [22]	**vWF** [25], **PV-1 (PAL-E, PLVAP, MECA32)** [52]
Liver Sinusoidal	LYVE-1 [46]	**PV-1 (PAL-E, PLVAP, MECA32)** [34], PROX1* [16]
Renal Peritubular Capillary	**PV-1 (PAL-E, PLVAP, MECA32)** [33]	PDGFR-β [33], FSP-1 [33]
Central Nervous System	CD133 [32], Sca-1 [32]	**PV-1 (PAL-E, PLVAP, MECA32)** * [5354]
Spleen	LYVE-1 [4], **PV-1 (PAL-E, PLVAP, MECA32)** [52]	
Pancreatic Islet	CD117 (c-kit) [32]	
Pulmonary	Sca-1 [55]	
Alveolar Capillary	Sca-1 [55]	**vWF** [26]

Markers indicated with * are positive following injury. Markers in bold are the most commonly used to discriminate endothelial cells belonging to each listed district. Framed markers are used for both murine and human panels.

Table 3.

Murine Endothelial cell phenotypes segregated by vessel size, vessel type, stemness, and inducibility

	Markers	
	Positive	**Negative**
Vessel Size		
Large Vessel	CD157 [56], Helix Pomatia Lectin (HPA) [5758]	Griffonia Simplicifolia Lectin (GS-1) [5758], Glycine Max Lectin (SBA) [5758]
Microvascular	Griffonia Simplicifolia Lectin (GS-1) [5758], Glycine Max Lectin (SBA) [5758]	CD157 [56], Helix Pomatia Lectin (HPA) [5758]
Stemness		
E-SP	CD34 [59], **CD157** [605661], **CD200** [6056], **Sca-1** [5962]	
ECFC	**CD117 (c-kit)** [29], **CD133** [31]	PDGFR-β [3363]
Endothelial Stem Cell	**CD157** [605661], CD200 [6056], **Procr** [6465], **Sca-1** [64]	PDGFR-β [3363]
Lymph Node High Endothelial Venules (HEVs)		
Mucosal	**VEGFR-3 (Flt4)** [7], **MECA367 (MAdCAM-1)** [89]	**MECA-79** [89]
Peripheral Lymph Node	**VEGFR-3 (Flt4)** [7], **MECA-79** [89]	**MECA367 (MAdCAM-1)** [89]
Other EC Subsets		
Arterial	**Ephrin β2** [6667] , Depp [68]	**Ephrin β4** [6667]
Venous	**Ephrin β4** [66676970]	**Ephrin β2** [66676970], Depp [68]
Inducible Markers	CD54 (I-CAM) [14], CD62P (P-Selectin) [13], CD142 [1213]	

Markers in bold are the most commonly used to discriminate endothelial cells belonging to each listed district. Framed markers are used for both murine and human panels.