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PV-1: LEUKOCYTE TRAFFICKING MOLECULE AND VASCULAR MARKER

by

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ABSTRACT

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Title: PV-1: Leukocyte trafficking molecule and vascular marker

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The distinction between lymphatic vessels and blood vessels is a crucial factor in many studies in immunology, vascular biology and cancer biology. They both share several characteristics and perform related, though different functions. They are equally important for the performance of the human immune system with the continuous recirculation of leukocytes from the tissues via lymphatics to the blood vessels and back into the tissue presenting the link between both systems.

This study was undertaken to elucidate the differences in the gene expression between primary blood- and lymphatic endothelial cells as well as the two immortalized cell lines HMEC-1 (human microvascular endothelial cell line 1) and TIME (telomerase immortalized microvascular endothelial cell line). Furthermore, we wanted to investigate the mystery surrounding the identity of the antigen recognized by the prototype blood vascular marker PAL-E. In the last step we wanted to study whether the PAL-E antigen would be involved in the process of leukocyte migration from the bloodstream into the surrounding tissue.

Our results clearly show that the gene expression in primary blood endothelial cells (BEC), lymphatic endothelial cells (LEC) and the cell lines HMEC-1 and TIME is plastic. Comparison of a large set of BEC- and LEC datasets allowed us to assemble a catalog of new, stable BEC- or LEC specific markers, which we verified in independent experiments. Additionally, several lines of evidence demonstrated that PAL-E recognizes plasmalemma vesicle associated protein 1 (PV-1), which can form complexes with vimentin and neuropilin-1. Finally, numerous *in vitro* and *in vivo* experiments identify the first function of the protein PV-1 during leukocyte trafficking, where it acts as regulatory molecule.

Keywords: Endothelial cells, HMEC-1, TIME, PAL-E, PV-1, Leukocyte trafficking

TIIVISTELMÄ

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PV-1: Valkosolujen liikennemolekyyli ja verisuonimarkkeri

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Imusuonten ja verisuonten erottaminen toisistaan on tärkeää monissa immunologian, sekä verisuoni- ja syöpäbiologian tutkimuksissa. Näillä kahdella suonityypillä on monia yhteisiä piirteitä ja ne toteuttavat samankaltaisia, mutta kuitenkin erilaisia toimintoja. Valkosolujen jatkuva kierto verisuonista kudoksiin ja sieltä imusuonten kautta takaisin verenkiertoon yhdistää nämä kaksi järjestelmää, minkä vuoksi imusuonet ja verisuonet ovat yhtä tärkeitä ihmisen immuunijärjestelmän suorituskyvyn kannalta.

Tässä tutkimuksessa haluttiin selvittää, mitä geenien ilmentymisen eroja voidaan nähdä veri- ja imusuonten endoteelisolujen ja immortalisoidun solulinjan, HMEC-1 (human microvascular endothelial cell line 1) ja TIME (telomerase immortalized microvascular endothelial cell line) välillä. Lisäksi halusimme selvittää arvoituksen siitä, mihin antigeeniin PAL-E -vasta-aine, joka on eniten käytetty verisuoniendoteelin merkkiaine, sitoutuu. Viimeisessä vaiheessa halusimme tutkia, onko tunnistettu PAL-E-antigeeni mukana valkosolujen migraatioprosessissa verenkierrosta ympäröivään kudokseen.

Tuloksemme osoittavat selvästi, että geenien ilmentymisen muutokset eristetyissä tuoreissa verisuonten endoteelisoluissa (BEC), lymfaattisissa endoteelisoluissa (LEC) ja solulinjoissa HMEC-1 ja TIME ovat plastisia. Vertailemalla monia eri BEC ja LEC -geenimäärityksiä, pystyimme kokoamaan uuden luettelon vakaista BEC ja LEC -spesifisistä markkereista, jotka varmistettiin biologisilla kokeilla. Osoitimme myös, että PAL-E tunnistaa PV-1 (engl. plasmalemma vesicle associated protein 1) -proteiinin, joka voi muodostaa komplekseja vimentiinin ja neuropiliini-1:n kanssa. Lukuisien *in vitro* ja *in vivo* -testien perusteella saimme ensimmäistä kertaa todisteita siitä, että PV-1 osallistuu valkosolujen liikenteeseen, jossa se toimii säätelevänä molekyylina.

Avainsanat: Endoteelisolut, HMEC-1, TIME, PAL-E, PV-1, Valkosoluliikenne

TABLE OF CONTENTS

ABBREVIATIONS

3D	3-dimensional
ANG	Angiopoietin
BEC	Blood endothelial cell
CLEVER-1	Common lymphatic endothelial and vascular endothelial receptor 1
Co-IP	Co-immunoprecipitation
COLEC12	Collectin placenta 12
COUP-TFII	Chicken ovalbumin upstream promoter transcription factor 2
ECM	Extracellular matrix
EphA/B	Eph receptor class A/B
ERK	Extracellular-signal-regulated kinases
ESAM	Endothelial cell–selective adhesion molecule
FD	Fenestral diaphragm
FOXC1/2	Forkhead box transcription factor 1/2
HDMEC	Human dermal microvascular endothelial cell
HEV	High endothelial venules
HMEC-1	Human microvascular endothelial cell line 1
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule 1
JAM-A/B/C	Junctional adhesion molecule A/B/C
LBRC	Lateral border recycling compartment
LEC	Lymphatic endothelial cell
LFA-1	Leukocyte function associated antigen 1
LPAM-1	Lymphocyte peyer's patch adhesion molecule 1
LYVE-1	Lymphatic vessel hyaluronan receptor-1
MAC-1	Macrophage antigen-1
MAdCAM-1	Mucosal addressin cell adhesion molecule 1
MCAM	Melanoma cell adhesion molecule
MDS	Multidimensional scaling
NO	Nitric oxide

NRP-1/2	Neuropilin-1/2
PAL-E	Pathologische anatomie leiden endothelium
PBMC	Peripheral blood mononuclear cells
PECAM-1	Platelet endothelial cell adhesion molecule 1
PlGF	Placental growth factor
PMN	Polymorphonuclear leukocytes
PROX1	Prospero-related homeobox 1
PSGL-1	P-selectin glycoprotein ligand-1
PV-1	Plasmalemma vesicle associated protein 1
RTK	Receptor tyrosine kinase
S1P	Sphingosine-1-phosphate
SD	Stomatal diaphragm
Shh	Sonic hedgehog
TEC	Transendothelial channel
TIE	Tie receptor
TIME	Telomerase immortalized microvascular endothelial cell line
UEAI	*Ulex europaeus* agglutinin I lectin
VCAM-1	Vascular cell adhesion molecule 1
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VLA-4	Very late antigen 4
vSMC	Vascular smooth muscle cell
VVO	Vesiculo-vacuolar organelle
vWF	Von Willebrand Factor

LIST OF ORIGINAL PUBLICATIONS

This work is based on the following original publications, which are referred to in the text by Roman numerals (I-III)

I. **Keuschnigg J**, Karinen S, Auvinen K, Irjala H, Mpindi JP, Kallioniemi O, Hautaniemi S, Jalkanen S, Salmi M. Plasticity of blood- and lymphatic endothelial cells and marker identification. Manuscript submitted

II. **Keuschnigg J**, Tvorogov D, Elima K, Salmi M, Alitalo K, Salminen T, Jalkanen S. PV-1 is recognized by the PAL-E antibody and forms complexes with NRP-1. Blood. 2012 Jul 5;120(1):232-5. doi: 10.1182/blood-2012-01-406876. Epub 2012 May 24.

III. **Keuschnigg J**, Henttinen T, Auvinen K, Karikoski M, Salmi M, Jalkanen S. The prototype endothelial marker PAL-E is a leukocyte trafficking molecule. Blood. 2009 Jul 9;114(2):478-84. doi: 10.1182/blood-2008-11-188763. Epub 2009 May 6.

1. INTRODUCTION

The human vasculature consists of two distinct branches, namely the lymphatic vasculature and the blood vasculature. They are formed by endothelial cells, lining the vessel lumen through which cells, molecules and liquid are transported. They both share a common precursor and the lymphatic vasculature is formed by differentiation from the blood vasculature. This common origin and their similar function in the human body result in phenotypical and functional similarities.

However, the differences that can be found between blood endothelial cells (BECs) and lymphatic endothelial cells (LECs) are important for many processes that are specific for one vessel type or the other. Therefore, the capacity to identify the individual vessel types is a crucial factor in many studies in the fields of immunology, vascular biology, pathology and oncology. A common tool used to distinguish between blood vessels and lymphatic vessels are proteins expressed specifically in one type of vessel but not the other. These marker proteins can be visualized with antibodies conjugated to reporters such as fluorescent molecules. One such antibody used for over 20 years to identify blood vessels is PAL-E (pathologische anatomie-leiden endothelium).

One function that connects the blood vessels and the lymphatic vessels is the circulation of leukocytes through the body. They travel via the blood vasculature through the body until they exit the blood vessel and enter the surrounding tissue. From there most leukocyte types continue to the lymphatic vasculature, which transports them to the lymph nodes and finally via the thoracic duct, the largest lymphatic vessel in the human body, back to the blood vasculature.

The exit of leukocytes from the blood vessels occurs via the so called adhesion cascade. Leukocytes first form transient interactions with the endothelium, causing them to roll along the vessel wall. Activation induces the immune cells to arrest their rolling and triggers firm adhesion. Finally, leukocytes cross the endothelium via two pathways: the paracellular pathway, whereby cells cross the endothelium through gaps between adjacent endothelial cells and the transcellular pathway, which describes the migration of leukocytes through the body of one individual endothelial cell. Several molecules that can be used to identify blood vessels are also involved during this extravasation of leukocytes and while the paracellular pathway is reasonably well understood, more work needs to be done to better comprehend the transcellular pathway.

2. REVIEW OF THE LITERATURE

2.1 The vascular system

During human development the blastocyst gives rise to three different layers of cells, the endoderm, mesoderm and ectoderm. The ectoderm is the first to emerge and finally forms structures such as the nervous system and the epidermis. The innermost layer of cells is called the endoderm, which develops into the lining of the respiratory tract and the digestive tract.

The mesoderm finally, gives rise to numerous tissues. Among others it forms the kidney, muscles, connective tissues, haematopoietic cells and finally the blood- and lymphatic vasculature. This vascular system is responsible for the transport of blood, cells, gases and nutrients in the body of highly developed, multicellular organisms (Carmeliet 2005).

The building blocks of the vascular system are the endothelial cells, forming the endothelium, a monolayer of cells, which lines all blood vessels and lymphatic vessels. Endothelial cells are slightly elongated and in blood vessels they orient themselves in parallel to the blood flow to minimize shear stress. They are usually ~60 µm long, ~20 µm wide, between 0,1-10 µm thick and number ~10-60 x 10^{12} in humans (Hutttner and Gabbiani 1983). The entire surface of the human vasculature has been estimated to measure 300-1000 m^2 (Jaffe 1987; Pries et al. 2000).

2.1.1 Development of the blood vasculature

Development of the human blood vasculature starts with the differentiation of cells from the mesodermal germ cell layer into progenitor cells, the so-called hemangioblasts. These cells are thought to differentiate into pluripotent haematopoietic stem cells and angioblasts. While the haematopoietic stem cells give rise to cells of the haematopoietic lineage, the angioblast is the precursor of endothelial cells (Figure 1) (Mikkola and Orkin 2002; Cao and Yao 2011; Hirschi 2012).

In adults, endothelial cells can also develop from endothelial precursor cells or multipotent adult progenitor cells (Luttun et al. 2002; Reyes et al. 2002) as well as from angioblasts derived from mesodermal tissues (Swift and Weinstein 2009). However, the concept of adult progenitor cells is controversial. After the first purification of potential endothelial progenitor cells from peripheral human blood in 1997 (Asahara et al. 1997), these cells were considered an important mechanism for neoangiogenesis and tumor growth promotion for many years (Shaked et al. 2006; Nolan et al. 2007). Nevertheless, subsequent studies failed to confirm these findings (Hagensen et al. 2010; Kozin et al. 2010). A recent review comprehensively

summarizes the controversy surrounding adult progenitor cells (Fang and Salven 2011). The first step in the vascular development is the accumulation of hemangioblasts into so-called blood islands. These islands subsequently fuse and organize themselves into the primary capillary plexus. These steps are referred to as vasculogenesis, while subsequent stages of vascular development are summarized under the term angiogenesis (Carmeliet 2003; Adams and Alitalo 2007).

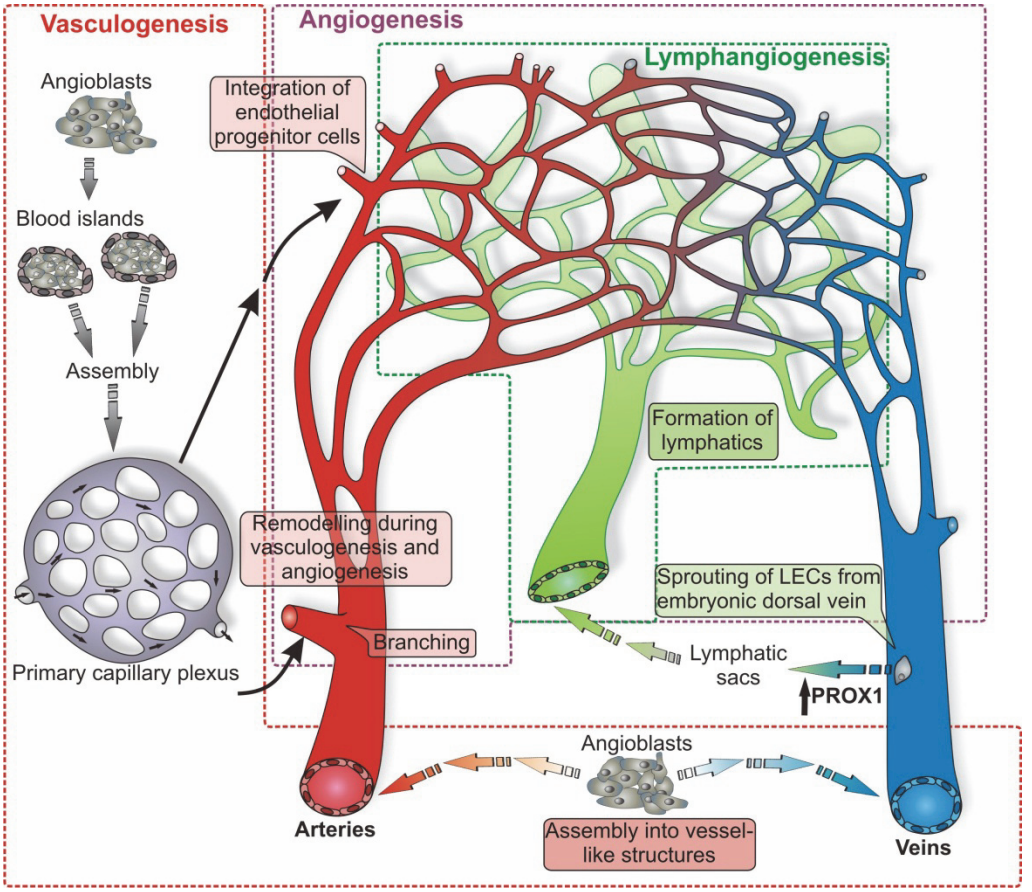

Figure 1: The development of the vascular system. Embryonal mesodermal cells differentiate into angioblasts and form blood islands, which in turn fuse to give rise to the primary capillary plexus. Both the posterior cardinal vein and the dorsal aorta are formed directly from angioblasts (endothelial progenitor cells). Up to this point, the developmental steps are summarized under the term vasculogenesis. Angiogenic remodeling of the primary capillary plexus and existing vessels as well as incorporation of progenitor cells results in the formation of a hierarchical network of blood vessels. A small number of venular endothelial cells then change their gene expression leading to the upregulation of the prototype lymphatic marker PROX1. This transcription factor then initiates the transcription of lymphatic-specific genes leading to the differentiation of venular endothelial cells into LECs, which go on to form lymphatic sacs. Remodeling, branching, sprouting and differentiation during lymphangiogenesis then lead to the formation of the lymphatic vasculature. Adopted from (Adams and Alitalo 2007).

The most important factor for most of the steps during the development of the blood vasculature is the vascular endothelial growth factor A (VEGF-A). It belongs to a family of molecules that mainly regulates the different steps during angiogenesis and vasculogenesis by binding to receptors of the VEGFR-family. Other members include proteins such as VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF) (Ferrara et al. 2003; Shibuya 2006; Adams and Alitalo 2007).

The potency of VEGF functions require the proteins to be tightly regulated. Humans have developed several redundant and combinatory mechanisms to control the VEGF family members: Differentially spliced variants exhibit varying degrees of affinity for their receptors, which can promote or inhibit VEGF functions (Adams and Alitalo 2007). For example, VEGF-A isoform 165 can bind to VEGFR-2 (also known as KDR or FLK1), inducing angiogenesis. However, binding of the same isoform to the receptor VEGFR-1 (also known as FLT1), inhibits angiogenesis. If VEGFR-1 is expressed, it exhibits higher affinity for VEGF-A than VEGFR-2. However, VEGFR-1 is a very weak kinase and hence acts as an inhibitor of angiogenesis (Ferrara et al. 2003; Shibuya 2006; Adams and Alitalo 2007).

Moreover, the presence or absence of domains in VEGF isoforms regulates the binding to the VEGF co-receptors such as neuropilin-1 and heparan sulphate proteoglycans. At the same time, the interactions of VEGF isoforms with their co-receptors cause growth factor gradients in tissues (Olsson et al. 2006; Koch et al. 2011). In addition to regulation at transcript level, VEGF family members are also proteolytically regulated at protein level (Houck et al. 1992; Tammela et al. 2005).

2.1.1.1 Arteriovenous differentiation

The differentiation and development of arteries and veins from endothelial cells starts with the capillary plexi in the embryo and is referred to as arteriovenous differentiation. Fully developed arteries and veins can be distinguished using their specific genetic expression pattern in addition to phenotypic differences such as diameter, presence/absence of valves as well as thickness and composition of the vessel wall (Adams and Alitalo 2007; dela Paz and D'Amore 2009).

In cells determined to develop into arteries, the differentiation process is initiated with the expression of the sonic hedgehog protein (Shh; Figure 2). Shh then acts on neighboring somites, inducing the expression of VEGF-A. VEGF-A subsequently binds to VEGFR-2 and its coreceptor neuropilin-1 (NRP-1) thereby activating Notch and ERK (Extracellular-signal-regulated kinases) signaling. In addition, Notch signaling is also stimulated via expression of the Notch ligand Delta-like 4 (DLL4) by the forkhead box transcription factors 1 and 2 (FOXC1/2). All these signaling networks finally lead to the expression of the arterial marker ephrin-B2.

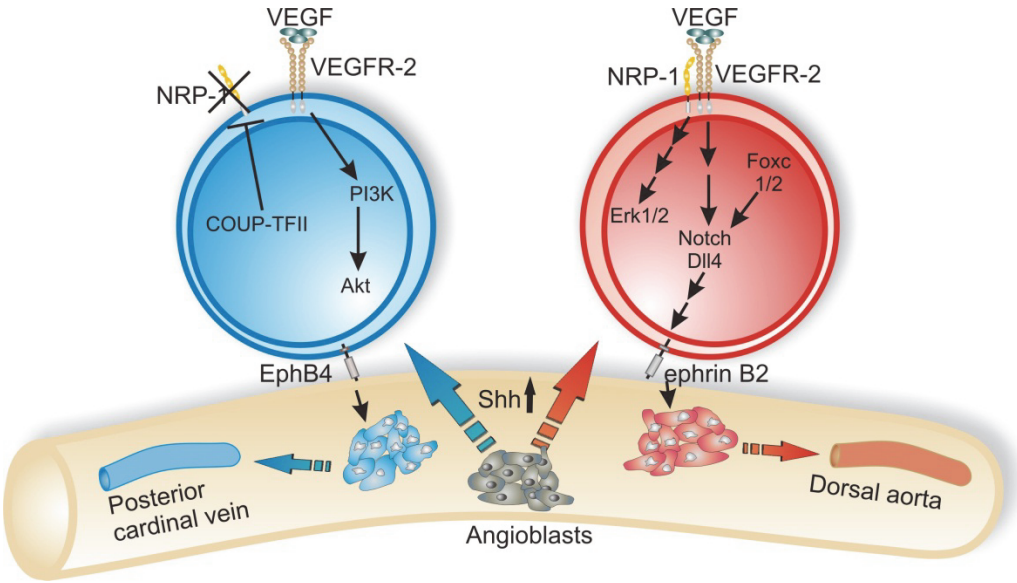

Figure 2: Arteriovenous differentiation. In embryonal angioblasts the upregulation of the gene Shh in the notochord and the neural tube causes neighboring somites to produce VEGF. In angioblasts determined to become arterial endothelial cells, VEGF binds to VEGFR-2 and its coreceptor NRP-1. VEGFR-2 signaling then leads to the induction of the Notch signaling pathway and ultimately to the expression of ephrin-B2. Subsequently, these cells form the dorsal aorta. In future venular endothelial cells COUP-TFII expression inhibits the expression of NRP-1, thereby contributing to the prevention of the induction of Notch signaling. This results in the expression of EphB4 on the surface of these cells and the formation of the posterior cardinal vein. Adopted from (dela Paz and D'Amore 2009).

In venous-fated cells on the other hand, Notch signaling is suppressed due to the expression of the nuclear orphan receptor COUP-TFII (chicken ovalbumin upstream promoter transcription factor 2). COUP-TFII contributes to the prevention of Notch and ERK activation by inhibiting NRP-1 expression. These pathways cause the expression of venous marker Ephrin-B4 (Lawson and Weinstein 2002; Lamont and Childs 2006; Adams and Alitalo 2007; dela Paz and D'Amore 2009).

2.1.2 Development of the lymphatic vasculature

The lymphatic vasculature is essential for the maintenance of tissue fluid homeostasis, immunity and dietary fat absorption. Furthermore, it plays an important role in tumor biology.

Partially permeable blood capillaries and the hydrostatic pressure generated by the human heart cause fluid to seep from blood vessels into the surrounding tissue. Reabsorption of this interstitial liquid occurs mainly in blind ending lymphatic capillaries, which then transport it via precollectors and collecting lymphatic vessels to

the thoracic duct, where it is returned to the blood circulation. The efficiency of this drainage system becomes clear when considering that in healthy adults between one to two liters of fluid containing 20-30 grams of protein per liter are returned to the blood circulation every day (Tammela and Alitalo 2010). Failure to clear the interstitium of liquid can lead to pathological conditions such as lymphedema (Achen et al. 2005; Adams and Alitalo 2007; Norrmen et al. 2011).

In addition to interstitial liquid, lymphatics also drain extravasated leukocytes and antigens to the draining lymph nodes, where an immune response is then initiated (Norrmen et al. 2011). During tumor progression cancer cells can escape from the primary tumor, enter lymphatics, travel to the draining lymph nodes and establish metastases. The presence of tumor cells in lymph nodes can therefore be important for the classification of tumors and influence the therapeutic strategies employed (Marshall et al. 2012; Shayan et al. 2012).

The development of the lymphatic vasculature starts after blood endothelial cells (BEC) have undergone arteriovenous differentiation. Venular endothelial cells express significant amounts of VEGFR-3. A subset of BECs in the central veins additionally expresses lymphatic vessel hyaluronan receptor-1 (LYVE-1). This small number of LYVE-1-expressing cells induces the expression of the transcription factor SOX18. SOX18 acts upstream and induces the expression of the lymphatic marker prospero–related homeobox 1 (PROX1) transcription factor (Figure 1) (Wigle and Oliver 1999; Oliver 2004; Alitalo et al. 2005; Jurisic and Detmar 2009; Tammela and Alitalo 2010). PROX1 finally polarizes the endothelial cells to exhibit a lymphatic phenotype and starts the lymphatic specific gene expression profile (Francois et al. 2008). Once this step has been completed, BECs downregulate their expression of VEGFR-3 and initiate their BEC-specific gene expression (Tammela and Alitalo 2010).

Lymphatic endothelial cells (LEC) on the other hand start to express neuropilin-2 (NRP-2), rendering them more responsive to VEGF-C signaling, which is necessary for sprouting of lymphatic vessels (Yuan et al. 2002). To form an independent vascular network, the lymphatic vasculature needs to separate itself from the blood vasculature. To that end LECs express podoplanin, a protein that stimulates platelet aggregation via c-type lectin-like receptor 2. This step is thought to close the connections between veins and the newly formed lymphatic vessels (Schacht et al. 2003; Tammela and Alitalo 2010).

2.1.3 Angiogenic and lymphangiogenic growth factors

Development, maintenance and function of both, the blood vasculature as well as the lymphatic vasculature depend on various growth factors. The most common angiogenic molecules belong to three families: the VEGF family of growth factors, the angiopoietins and the ephrins (Figure 3).

2.1.3.1 VEGF family of growth factors

In addition to VEGF molecules in mammals (see chapter 2.1.1 and Figure 3A), VEGF-like molecules were found in snake venom (Gasmi et al. 2000) and the parapoxvirus (Lyttle et al. 1994). Their common structural basis is formed by 8 conserved cysteine residues per molecule and association in homodimers (Ferrara and Davis-Smyth 1997; Dvorak 2002). All members of the VEGF family bind to three closely related cell surface receptors of the tyrosine kinase family termed VEGFR-1 (=Fms-like tyrosine kinase-1, Flt-1), VEGFR-2 (=fetal liver kinase, Flk-1, or kinase-insert domain receptor, KDR) and VEGFR-3 (=Fms-like tyrosine kinase, Flt-3)(Lohela et al. 2009; Koch et al. 2011).

VEGF-A, was first discovered in 1986 (Senger et al. 1986) and subsequently cloned in 1989 (Keck et al. 1989; Leung et al. 1989). It was originally characterized by its ability to induce vascular permeability and leakage as well as endothelial cell proliferation (Dvorak et al. 1999; Ferrara 1999). Hence it was initially also known under the name vascular permeability factor. VEGF-A can be found in several isoforms, distinguished by their polypeptide lengths. Differential splicing produces more than 10 VEGF-A variants with the predominant sizes being 121, 165 and 189 amino acids (Jussila and Alitalo 2002). The individual isoforms exhibit differences in their interaction with the extracellular matrix and coreceptors of the neuropilin family and can act in a synergistic or antagonistic manner (Olsson et al. 2006; Koch et al. 2011).

VEGF-A is crucial for the correct development of the vasculature in the embryo as deletion of only one allele in mice caused embryonic lethality due to impaired angiogenesis (Carmeliet et al. 1996; Ferrara et al. 1996). Further studies then showed that lack of isoforms 164 and 188 (VEGF-A in mice is one amino acid shorter than in humans) caused increased embryonic lethality, indicating that these two polypeptides cannot completely be replaced by other isoforms (Carmeliet et al. 1999). VEGF-A can bind to VEGFR-1 and VEGFR-2 (Ferrara 2001). While binding to VEGFR-2 triggers angiogenesis and growth, binding of VEGF-A to VEGFR-1 seems to counteract VEGFR-2 function (Fong et al. 1995; Shalaby et al. 1995). In addition to membrane bound forms of the VEGFR, soluble forms of VEGFR-1 (Kendall and Thomas 1993) and VEGFR-2 (Albuquerque et al. 2009) have been found. Soluble VEGFR-1 seems to act by sequestering VEGF-A, or forming non-functional, soluble VEGFR-1/2 heterodimers (Kendall and Thomas 1993; Kappas et al. 2008).

VEGF-B binds to VEGFR-1 and can form complexes with VEGF-A. This association with VEGF-A might present one form of regulation of VEGF-A function (Olofsson et al. 1996; Makinen et al. 1999). The function of VEGF-B is not yet fully understood. It is highly expressed in the developing myocardium as well as for example in muscle and bone (Aase et al. 1999). Interestingly, recent results indicate that VEGF-B might be important for the lipid metabolism of the heart (Karpanen et al. 2008; Hagberg et al. 2010).

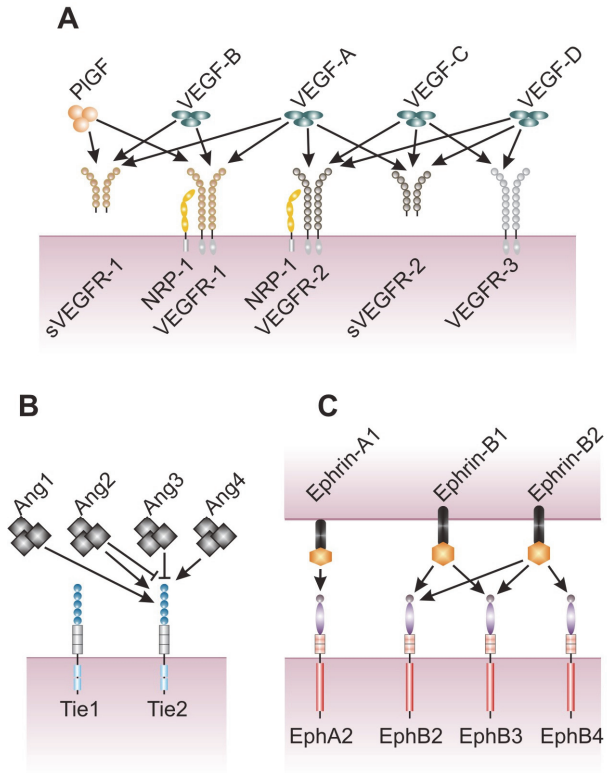

Figure 3: Vascular growth factors and their receptors. A) The main members of the VEGF-family of growth factors and their receptors are depicted. **B)** The activating and inhibiting actions of angiopoietins on their receptor Tie2 are shown. The function of the Tie1 receptor is not yet fully understood. **C)** The ephrin molecules with their respective Eph receptors important for vascular development and angiogenesis are shown here. Arrows indicate activation and ⊥ indicates inhibition of receptor signaling.

VEGF-C on the other hand shows only a limited homology of ~30% with VEGF-A_{165} (Joukov et al. 1996) and can bind to both VEGFR-2 and VEGFR-3. In addition to stimulation of angiogenesis and endothelial cell proliferation via VEGFR-2, VEGF-C can also act in a similar way on lymphatics via VEGFR-3 (Joukov et al. 1997). Similar to soluble VEGFR-1, soluble VEGFR-2 binds VEGF-C, thereby preventing the stimulation of VEGFR-3. This finally leads to reduced proliferation of lymphatic endothelial cells (Albuquerque et al. 2009). VEGF-D shows similarities with VEGF-C in that it binds to VEGFR-2 and VEGFR-3 and seems to exhibit angiogenic and lymphangiogenic properties (Achen et al. 1998; Marconcini et al. 1999; Veikkola et al. 2001).

NRP-1 is an important co-receptor for VEGFR-1 and VEGFR-2. It is a ~140 kDa transmembrane molecule that, with the help of a VEGFR molecule, can bind several growth factors of the VEGF family (Petrova et al. 1999). In addition, it was shown that in neuronal cells NRP-1 can bind semaphorin III A with the help of the co-

receptor plexin-A. This semaphorin III A signaling via NRP-1 is important for axon guidance during embryonic development, can reduce the motility of axons and endothelial cells and impairs endothelial cell sprouting (Kolodkin et al. 1997; 1999; Petrova et al. 1999; Adams and Eichmann 2010). In the context of growth factor signaling, association of NRP-1 with VEGFR-2 enhances the binding of VEGF-A to VEGFR-2 and by itself NRP-1 has been implicated in increased tumor angiogenesis *in vivo* (Otrock et al. 2007). The importance of NRP-1 for the development and function of the vasculature and axon guidance has been demonstrated by showing that knock out of NRP-1 in mice causes embryonic lethality (Kawasaki et al. 1999).

2.1.3.2 Angiopoietins

After the discovery of the VEGF family, it took several years before the next group of vascular growth factors, the angiopoietins, was identified (Figure 3B) (Davis et al. 1996; Suri et al. 1996). The 4 members angiopoietin-1 (ANG1), ANG2 and ANG3/4 bind to the Tie receptor called TIE2 (Partanen et al. 1992; Augustin et al. 2009). Both molecules can assemble in trimer, tetramer and pentamer structures, with ANG1 also able to form higher order oligomers (Kim et al. 2005). ANG1 and ANG2 both bind the TIE2 receptor, but only ANG1 can readily activate it (Davis et al. 2003). Activation of TIE2 is important for the stability and remodeling of vessels and the survival of endothelial cells (Bogdanovic et al. 2009). In addition to TIE2, a second receptor called TIE1 exists. However, the function of TIE1 is not yet fully understood.

In line with its expression in the vasculature, the Ang-Tie system exhibits important functions during processes such as sprouting angiogenesis, inflammation and vascular remodeling (Augustin et al. 2009). The Ang-Tie system also interacts with peri-vascular cells such as pericytes and vascular smooth muscle cells (vSMCs), who are known to have an important role in regulating the state of the vasculature (Stratmann et al. 1998; Sugimachi et al. 2003; Augustin et al. 2009).

Experiments using genetically modified mice suggested that there is a delicate balance between ANG1 and ANG2, where ANG1 induces a resting, quiescent endothelium, whereas ANG2 causes activation of the endothelium and responsiveness toward stimuli such as inflammatory cytokines (Fiedler et al. 2004; Pfaff et al. 2006; Augustin et al. 2009). Further studies revealed that the lack of sensitization toward inflammatory stimuli in ANG2 deficient mice lead to reduced expression of adhesion molecules and consequently to impaired firm adhesion of leukocytes (Fiedler et al. 2006). Similarly, ANG1 can act in an anti-inflammatory way by inhibiting the induction of adhesion molecule expression (such as intercellular adhesion molecule 1 and vascular cell adhesion molecule 1) by VEGF (Kim et al. 2001).

In addition, the vascular permeability reducing effects of ANG1 also involve sphingosine-1-phosphate (S1P) (Li et al. 2008), a lipid that has also been shown to be involved in immune cell trafficking by regulating lymphocyte exit from lymph nodes into efferent lymphatics (Chi 2011). Adding to these findings, a recent report demonstrated that S1P produced in pericytes strengthens the microvascular barrier function by upregulating the expression of N-cadherin and VE-cadherin and concomitantly downregulating ANG2 expression. This increase in the expression of

the adhesion molecule VE-cadherin extents the function of S1P also into the area of leukocyte trafficking (McGuire et al. 2011). N-cadherin on the other hand has been shown to be a target molecule of the TGF-β signaling pathway (Li et al. 2011), which in turn has also been implicated in pericyte/vSMC development (Oshima et al. 1996; Lan et al. 2007).

Taken together, these studies reveal an intricate network, involving endothelial cells and peri-vascular cells, which regulates the functions of the vasculature in physiological and pathological conditions.

2.1.3.3 Ephrins

The ephrins and their Eph receptor tyrosine kinases (RTK) comprise the largest family of growth factors with over 20 different receptors and ligands (Figure 3C). Based on sequence similarity and affinity for their ligands, Eph receptors have been divided into EphA (Eph class A) and EphB (Eph class B). Their ligands, the ephrins are either GPI-anchored to the plasma membrane or are transmembrane proteins. GPI-anchored ligands belong to the ephrin A family, and mainly bind to the EphA receptors. The ephrin B ligands on the other hand bind to RTKs of the EphB family (Yancopoulos et al. 2000; Brantley-Sieders et al. 2004; Kuijper et al. 2007). Binding of ephrins to their Eph receptors can trigger two kinds of signaling: Functioning as ligand for their RTKs they induce so called "forward signaling" into the cell expressing the Eph receptor. In contrast, "reverse signaling" refers to the signaling of ephrins into the cell expressing the ligand (Kullander and Klein 2002).

During development and angiogenesis, EphB4 and ephrin-B2 seem to play the most important roles, as knock-outs in mice lead to lethal defects during early angiogenesis. While EphB4 is restricted to venous endothelium, ephrin-B2 seems to be specific for arterial endothelial cells (Kuijper et al. 2007). Their exclusive expression on either arterial or venous endothelium and the severe effects of their absence indicate that these two molecules might be involved in the arteriovenous differentiation process (Wang et al. 1998; Adams et al. 1999; Gerety et al. 1999). Interestingly, EphB4 and ephrin-B2 expression have also been demonstrated on lymphatics. While ephrin-B2 seems to be expressed solely on the endothelium of collecting lymphatics, EphB4 is expressed throughout the lymphatic vasculature (Makinen et al. 2005).

Two recent publications demonstrated important roles for ephrin-B2 during angiogenic- and lymphangiogenic growth (Sawamiphak et al. 2010; Wang et al. 2010). These two studies show that ephrin-B2 is crucial for VEGF signaling via both VEGFR-2 and VEGFR-3 and is thereby involved in the regulation of both angiogenic- and lymphangiogenic growth.

2.1.4 Heterogeneity of endothelial cells

Endothelium is defined as the inner cellular lining of blood and lymphatic vasculature. However, despite their common ancestor, endothelial cells exhibit a wide variety of phenotypes and gene expression patterns. Differences between types of endothelial cells can be classified as structural- and functional heterogeneity.

With the exception of capillaries, all mature blood vessels show the same basic layered structure: endothelial cells and their basement membrane form the innermost *tunica intima.* In arteries, these endothelial cells are narrow and aligned along the blood flow, while those in veins are of a more rounded phenotype (dela Paz and D'Amore 2009). The second layer, the *tunica media*, is mainly formed by several layers of vascular smooth muscle cells (vSMCs) and elastic fibers. In arteries, this layer represents the thickest part of the vessel wall, whereas it is not as prominent in veins. The outermost *tunica adventitia* consists of collagen-rich, connective tissue and fibroblasts. In veins the *tunica adventitia* forms the biggest part of the vessel wall. Larger arteries and veins possess a specialized blood supply for the *tunica adventitia*, the vasa vasorum (Adams and Alitalo 2007; dela Paz and D'Amore 2009). The most striking difference between veins and arteries however, is the presence of valves in veins. These valves protrude into the lumen of the vessel and prevent the backflow of blood (dela Paz and D'Amore 2009).

Morphologically, blood vascular endothelium can be described as continuous or discontinuous type. Continuous endothelium can be divided into fenestrated and non-fenestrated (Aird 2007). All three types can be defined by their content of endothelial structures such as fenestrae, transendothelial channels (TEC), vesiculo vacuolar organelles (VVO) and caveolae (Stan 2007). Caveolae are bottle-shaped invaginations of the plasma membrane with a diameter of ~70 nm (Figure 4) (Stan 2005). They occur in most mammalian cell types and are not restricted to endothelial cells. However, only in endothelia can caveolae be equipped with structures called stomatal diaphragm (SD) (Bearer and Orci 1985). SDs are barriers formed by protein fibers reaching from the plasma membrane to the center of the caveolae where they interweave to create a central knob (Stan 2005).

Fenestrae are circular openings in the body of endothelial cells, connecting apical and basal membrane. They are often arranged in regular structures called sieve plates. With the exception of capillaries in renal glomeruli, fenestrae are provided with fenestral diaphragms (FD). They seem to be morphologically similar to SDs (Simionescu et al. 1974; Rostgaard and Qvortrup 1997; Stan 2007).

Transendothelial channels are similar to fenestrae in that they penetrate the entire endothelial cell, creating a pore. However, contrary to fenestrae, TECs have two SDs, one on the luminal and the other on the abluminal side of the cell (Stan 2007).

Lastly, vesiculo vacuolar organelles appear like and are difficult to distinguish from clusters of caveolae. As the name implies, they are formed by groups of

interconnected vesicles and vacuoles. Diaphragms similar to those found in caveolae are present between individual vesicles and vacuoles and can also be found at the border between VVO and extracellular space (Kohn et al. 1992).

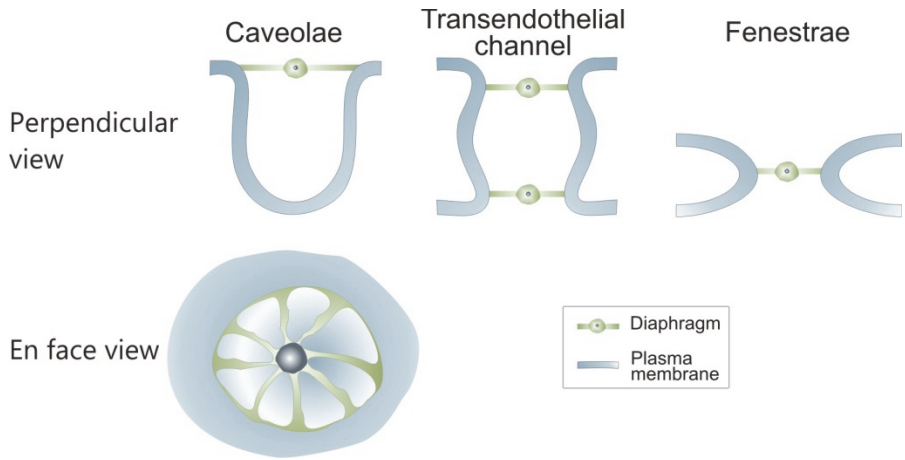

Figure 4: Endothelial structures: Schematic representations of the endothelial structure caveolae, transendothelial channel and fenestrae with their diaphragms are shown in perpendicular- and en face view.

The stomatal and fenestral diaphragms share one common structural marker, namely the protein plasmalemmal vesicle associated protein 1 (PV-1). This type II, single span transmembrane protein was first discovered on caveolae from rat lung (Ghitescu et al. 1997; Stan et al. 1999; Hnasko et al. 2002). PV-1 was characterized as a heavily glycosylated protein (~15% of its molecular mass) that forms homodimers *in situ* and possesses a short intracellular N-terminus and long extracellular C-terminal domain (Stan et al. 1999). This morphology fits to the model where PV-1 homodimers form the radial fibers creating the stomatal and fenestral diaphragms (Stan 2007). Functionally, the role of diaphragms has not yet been elucidated. A commonly proposed theory suggests a sieving function for diaphragms in endothelial structures. Passage of macromolecules and even cells could be selectively inhibited or facilitated (Aird 2007; Stan 2007).

Continuous fenestrated endothelium can be found in tissues such as glomeruli and mucosae, where filtration and transcellular transport of liquid and macromolecules play a significant role. This type of endothelium is characterized by the presence of endothelial structures such as fenestrae, TEC and caveolae (Stan 2007). Continuous non-fenestrated endothelium can be found in veins and arteries as well as capillaries of the skin, heart and lung. It is characterized by a low number of TEC, numerous caveolae and practically no fenestrae (Stan 2007). As the name implies, it also forms the almost impermeable blood-brain barrier and blood-retina barrier (Ballabh et al. 2004). Discontinuous endothelium, also known as sinusoidal endothelium, lines the

sinusoids in the liver and the bone marrow. Defined by its large gaps of 100-200 nm, it possesses few caveolae and a disorganized basement membrane (Braet and Wisse 2002; Aird 2007).

Lymphatic endothelial cells share many features with their counterparts in blood vessels; however, their distinct function requires extensive specialization. To enable the entry of cells, macromolecules and fluid from the interstitium, lymphatic capillaries are irregular, have no pericyte coverage and no SMCs. Furthermore, their basement membrane is usually missing or very rudimentary (Norrmen et al. 2011) and also their cell-cell junctions are discontinuous and spot-like (Baluk et al. 2007).

Capillary lymphatic endothelial cells form so called microvalves, overlapping endothelial folds, which prevent fluid from escaping back into the tissue from the lymphatic lumen (Trzewik et al. 2001). Anchoring filaments containing emilin-1 anchor the capillaries to their surrounding tissue and cause the microvalves to open in case of high interstitial pressure, allowing liquid to seep into the lymphatics (Danussi et al. 2008). Precollectors and collecting lymphatics on the other hand have continuous cell-cell junctions, a basement membrane and are covered with pericytes and/or SMCs (Baluk et al. 2007). Unidirectional flow of lymph is achieved by contractile SMCs and surrounding skeletal muscles (Muthuchamy and Zawieja 2008) as well as secondary, intraluminal valves that prevent lymph backflow (Alitalo et al. 2005).

Pericytes and vSMCs are two cell types that are sometimes used as synonyms, even though they differ in several aspects such as their localization in relation to the blood vessel, their morphology and to a lesser degree their gene expression (Armulik et al. 2005). However, they seem to represent two states in a continuum of cellular phenotypes and gene expression profiles and it has been suggested that pericytes and vSMC can differentiate into each other (Nehls and Drenckhahn 1993). While vSMCs form the *tunica media*, a separate layer in the vasculature, pericytes are embedded within the basement membrane (Armulik et al. 2005). VSMCs are crucial for the maintenance of regulated blood flow. Through contractions these cells control the vessel diameter and thereby the blood pressure in their underlying vessels. During vascular development they are an important factor for vessel morphogenesis and exhibit a high rate of proliferation and protein synthesis. However, within terminally differentiated adult vasculature, vSMC exhibit a slower metabolism. Nevertheless, they retain the capacity for dramatic changes in response to variations in their environment such as vascular injury (Owens 1995; Owens et al. 2004).

Pericytes on the other hand have been implicated in angiogenesis and maintenance of vessel stability. They protrude long, filopodia like cellular structures with which pericytes form focal contacts with the adjacent endothelial cells (von Tell et al. 2006). As pericytes usually form contacts with several individual endothelial cells, it has been suggested that they could serve as hubs, integrating signals from several endothelial cells (Gerhardt and Betsholtz 2003). However, their main functions seem to be vessel stabilization and regulation as well as signal relay (Gerhardt and Betsholtz 2003; Armulik et al. 2005; von Tell et al. 2006; Gerhardt and Semb 2008).

2.1.5 Blood endothelial cell markers

The human blood vasculature consists of various vessel types such as arteries, veins and capillaries, each with their own specific phenotype. Despite this many-sided nature, several proteins can be used to identify the blood vasculature. However, most markers considered to be specific for blood or lymphatic vasculature are also expressed on other, non-vascular cell types and should only be used to distinguish between the two types of vasculature (Table 1).

2.1.5.1 Endoglin

Endoglin, also known as CD105, is a homodimeric protein of ~180 kDa in non-reduced form (ten Dijke et al. 2008). It was originally defined by the antibody 44G4, raised against non-T, non-B acute lymphoblastic leukemia (Quackenbush and Letarte 1985). Its expression is highest on blood endothelial cells (Gougos and Letarte 1988; Wang et al. 1993; Fonsatti et al. 2001), with additional, weak expression detected on fibroblasts (Gougos and Letarte 1988), hematopoietic progenitor cells (Cho et al. 2001) and macrophages (Lastres et al. 1992). This high expression on blood vascular endothelium and its absence from lymphatic endothelium (Hirakawa et al. 2003) caused endoglin to be considered as a blood endothelial specific marker.

2.1.5.2 von Willebrand Factor (vWF)

Von Willebrand factor is a large, multimeric glycoprotein, also known as factor VIII-related antigen. The monomeric form has an approximate molecular weight of 250 kDa. However, disulfide linkage can lead to multimers of >20 000 kDa (Sadler 1998; Ordonez 2012).

The protein is synthesized mainly by endothelial cells and megakaryocytes. In megakaryocytes, the production of vWF leads to storage of the final protein in α-granules of the resulting platelets. In endothelial cells on the other hand, it is stored in the so-called Weibel-Palade bodies. These structures are secretory organelles, specific for endothelial cells and store vWF as well as P-Selectin (Ruggeri and Ware 1993; Valentijn et al. 2011). In endothelial cells, vWF is released at the abluminal side of the cell where it enables the adhesion of platelets in case of injury of the endothelium (Turitto et al. 1985), thereby facilitating platelet mediated clotting and closure of wounds.

Immunohistochemically, vWF can be detected on most endothelia with the exception of large blood vessels and lymphatics, which are mostly negative (Turitto et al. 1985). Due to inferior sensitivity, the usage of vWF as vascular marker has declined and been replaced by markers such as PAL-E and CD31.

2.1.6 Lymphatic endothelial cell markers

The study of lymphatic vessels in physiological and pathological conditions has long been hampered by the lack of markers specific for lymphatic vasculature. It was only in 1995, that VEGFR-3 was described as first molecule specific for lymphatic endothelium (Kaipainen et al. 1995). Subsequently, several other molecules have been described with a more or less lymphatic specific expression pattern (Table 1) (Jussila and Alitalo 2002).

2.1.6.1 Prospero-related homeobox 1

Originally, PROX1 was cloned as a homolog to the *Drosophila* gene *prospero* (Oliver et al. 1993). The same publication demonstrated PROX1 expression in tissues as diverse as developing liver and heart, neuronal cells and pancreas. The first clues to the importance of PROX1 came from a study reporting embryonic lethality of PROX1 null mice (Wigle et al. 1999). However, this report concentrated on PROX1 function during murine lens development. Only a follow-up study of the same group revealed the importance of PROX1 during lymphatic development (Wigle and Oliver 1999).

Despite the expression of PROX1 in non-lymphatic cells, the protein has been widely considered as master control gene and has been used as a lymphatic marker (Hong et al. 2002; Wilting et al. 2002; Hirakawa et al. 2003; Baluk and McDonald 2008).

2.1.6.2 Podoplanin

Podoplanin is a mucin-type transmembrane glycoprotein that was first discovered in 1990 in osteoblasts and subsequently shown to be expressed in cells of the osteoblastic lineage as well as in type I alveolar cells of the lung (Nose et al. 1990; Wetterwald et al. 1996). The name podoplanin was coined in 1997 to indicate the fact that the protein could be associated with the flattening of glomerular epithelial podocyte foot processes (Latin: “pes planus”) (Breiteneder-Geleff et al. 1997). Two years later it was shown that podoplanin is expressed in lymphatics and absent from blood vasculature, (Breiteneder-Geleff et al. 1999). Moreover, a follow up study demonstrated mutually exclusive staining of podoplanin and PAL-E and perfect colocalization of podoplanin with VEGFR-3, at that time the only established lymphatic specific marker (Breiteneder-Geleff et al. 1999; Weninger et al. 1999). These findings make podoplanin a marker specific for lymphatic endothelium. Podoplanin has been shown to stimulate LEC adhesion, migration and tubule formation in vitro. Furthermore, knock-out of podoplanin causes mice to die at birth due to respiratory failure and defects in the lymphatic vasculature (Schacht et al. 2003). Over the last years, numerous functions of podoplanin have been found, including development of heart, lung and lymphatic system, platelet aggregation, regulation of the immune system and promotion of tumor progression and metastasis (Astarita et al. 2012).

2.1.6.3 LYVE-1

LYVE-1 is a transmembrane glycoprotein that was identified based on its sequence homology to the hyaluronan receptor CD44 (Banerji et al. 1999). Just like CD44, LYVE-1 binds to extracellular hyaluronan in immobilized and solubilized form (Banerji et al. 1999). During embryonic development LYVE-1 is expressed on all lymphatic endothelial cells. This changes in adults, where LYVE-1 becomes restricted to lymphatic capillaries (Makinen et al. 2005). In addition, it is also expressed on sinusoidal cells of healthy liver and spleen (Mouta Carreira et al. 2001) as well as on various populations of macrophages (Maruyama et al. 2005; Bockle et al. 2008; Schroedl et al. 2008). Despite this non-exclusive expression on lymphatics, LYVE-1 is a widely used lymphatic marker.

The functions of LYVE-1 are not yet fully understood. Several studies suggest a function of LYVE-1 during hyaluronan metabolism (Mandriota et al. 2001; Prevo et al. 2001) as well as a potential association with tumor metastasis (Du et al. 2011). Surprisingly, LYVE-1 null mice develop normally and do not exhibit any alterations in lymphatic development, cell trafficking or levels of hyaluronan in tissue or blood. Similarly, growth of transplanted melanomas and lung carcinomas were unaffected (Gale et al. 2007). On the other hand, LYVE-1 positive lymphatic vessels at the invasive front of early colorectal cancer indicate lymphatic invasion and can serve as a predictive factor for lymph node metastasis in early colorectal cancer (Akishima-Fukasawa et al. 2011).

2.1.7 Pan-endothelial marker CD31

CD31, also known as PECAM-1 (platelet endothelial cell adhesion molecule 1) is a member of the immunoglobulin superfamily. The transmembrane glycoprotein has a molecular weight of 130 kDa, of which ~40% derive from N- and O-linked glycosylation (Newman et al. 1990). CD31 can form homophilic interactions with other CD31 molecules (Sun et al. 1996) as well as heterophilically bind to for example integrin $\alpha v\beta 3$, CD38, CD177 and proteoglycans (Wang et al. 2003; Woodfin et al. 2007).

CD31 is expressed on endothelial cells and a wide variety of hematopoietic and immunological cell types such as platelets, neutrophils, natural killer cells and monocytes (Pusztaszeri et al. 2006; Woodfin et al. 2007). On endothelial cells it is concentrated at the cellular junctions (Simmons et al. 1990) where it is a constituent of the so called lateral border recycling compartment (Mamdouh et al. 2003; Mamdouh et al. 2008). Soon after its discovery, CD31 was shown to be involved in leukocyte transendothelial migration (TEM) (Muller et al. 1993). Subsequently it has been demonstrated that CD31 also plays a role in angiogenesis (DeLisser et al. 1997; Zhou et al. 1999).

Similar to other endothelial markers, CD31 expression is not restricted to vascular endothelial cells. However, while proteins such as endoglin or vWF are able to distinguish blood endothelial cells from lymphatic endothelial cells, CD31 stains endothelia of both vessel types (Pusztaszeri et al. 2006; Baluk et al. 2007). Even though staining of lymphatic endothelium by CD31 is considerably weaker than that of blood vasculature, PECAM-1 has to be considered a pan-endothelial cell marker.

2.1.7.1 Other endothelial markers

In addition to the above listed molecules, a plethora of other markers exist that is more or less specific for endothelia in general or endothelial cells of one type. CD34 is a protein whose function is not yet fully understood. However, its ligand is known to be L-selectin and it has been implicated in cell adhesion as well as erythroid and myeloid differentiation (Lanza et al. 2001). In addition to endothelial cells of blood- and lymphatic vessels CD34 is expressed on hematopoietic progenitor cells, dendritic cells dermal dendrocytes and some fibroblasts (Pusztaszeri et al. 2006; Ordonez 2012).

Ulex europaeus agglutinin I lectin (UEAI) is a plant lectin preferentially binding glycoproteins and –lipids containing α-linked fucose residues. It is a highly specific pan-endothelial marker and has been extensively used for the identification of endothelial derived tumors (Ordonez and Batsakis 1984; Ordonez 2012). However, its expression has also been detected on endothelial progenitor cells (Di Stefano et al. 2009).

In addition to its importance in angiogenesis, VEGFR-2 has been shown to be specifically expressed in endothelial cells as well as mesothelial cells in normal adult tissue (Miettinen et al. 2012). Apart from normal adult tissue, VEGFR-2 is expressed in numerous vascular endothelial tumors such as hemangioma, lymphangioma and angiosarcoma (Miettinen et al. 2012; Ordonez 2012).

Another member of the VEGF-receptor family, VEGFR-3 plays an important role in lymphangiogenesis (see chapter 2.1.2). While it can be detected in most endothelial cells during embryogenesis, its expression was thought to become more specific for lymphatic endothelial cells (Kaipainen et al. 1995). However, subsequent studies demonstrated VEGFR-3 expression also on blood vessels in lung adenocarcinoma (Niki et al. 2001), in colorectal adenocarcinoma (Witte et al. 2002) and a number of other tumors (Valtola et al. 1999; Ordonez 2012).

Vascular endothelial cadherin (VE-cadherin), an adhesion molecule of the endothelial junctions is another noteworthy pan-endothelial marker (Vestweber 2008). It is an important factor for the integrity of vascular endothelium, as blockage with antibodies increased the migration of leukocytes to inflamed peritonea in mice (Gotsch et al. 1997). In addition to blood endothelial junctions, VE-cadherin has been shown to be expressed in button-like junctions of lymphatic vessels (Baluk et al. 2007). As is the case with the other vascular markers, VE-cadherin can also be found on non-vascular cells such as astrocytes and glioma (Boda-Heggemann et al. 2009).

Table 1: Expression of vascular markers.

Marker	Expression in BEC	Expression in LEC	Expression in other cell types	Reference
PAL-E	+	-	Leukocytes	(Keuschnigg et al. 2009)
Endoglin	+	-	Fibroblasts, macrophages, hematopoietic progenitor cells	(Gougos and Letarte 1988; Lastres et al. 1992; Cho et al. 2001)
vWF	+	-	Megakaryocytes, various tumor cells	(Ordonez and Batsakis 1984; Sadler 1998; Ordonez 2012)
PROX1	-	+	Epithelial cells during murine lens development	(Wigle et al. 1999)
Podoplanin	-	+	Epithelial cells and podocytes	(Breiteneder-Geleff et al. 1997)
LYVE-1	(+)	+	Some blood vessels and macrophages	(Mouta Carreira et al. 2001; Maruyama et al. 2005; Cho et al. 2007)
CD34	+	+	Hematopoietic progenitor cells, dendritic cells dermal dendrocytes and some fibroblasts	(Pusztaszeri et al. 2006; Ordonez 2012)
CD31	+	(+)	Platelets, neutrophils, natural killer cells, monocytes	(Pusztaszeri et al. 2006; Woodfin et al. 2007)
UEAI	+	+	Some endothelial progenitor cells	(Di Stefano et al. 2009)
VEGFR-2	+	-	Mesothelial cells	(Miettinen et al. 2012)
VEGFR-3	(+)	+	Corneal epithelium and tumor blood vessels	(Cursiefen et al. 2006; Ordonez 2012)
VE-cadherin	+	+	Astrocytes and glioma	(Boda-Heggemann et al. 2009)

The expression of vessel-type specific and pan-endothelial vascular markers in BEC, LEC and non-vascular cells is summarized here. (+) indicates low and/or sporadic expression.

2.1.8 PV-1, PAL-E, 174/2 and Meca-32

The connection between the terms PV-1, PAL-E and Meca-32 can be confusing as they are sometimes used as synonyms. However, understanding their relationship is important. PAL-E is a mouse monoclonal antibody recognizing human PV-1 (Niemela et al. 2005; Keuschnigg et al. 2012), whereas Meca-32 is the rat equivalent to PAL-E, binding to PV-1 of mouse origin (Ioannidou et al. 2006). In addition to these antibodies, 174/2 is a mouse monoclonal antibody raised against small, isolated

vessels of human lymph nodes in our laboratory (Niemela et al. 2005). Analysis of the antigen bound by 174/2 showed, that it also recognizes PV-1, albeit a different epitope than PAL-E (Niemela et al. 2005; Keuschnigg et al. 2012).

2.1.8.1 Pathologische Anatomie Leiden Endothelium (PAL-E)

In 1985, Schlingemann and colleagues (Schlingemann et al. 1985) published a new monoclonal antibody, designated as PAL-E, which specifically stained the endothelia of capillaries, venules as well as small and medium-sized veins. It only reacted weakly if at all with all types of arteries and large veins. In addition, it was completely absent from the lymphatic vasculature. Despite widespread use for 20 years, the target of the antibody remained elusive, probably due to its functional restriction to frozen section. Hence it took until 2004 before the target of PAL-E was first identified as a secreted from of vimentin (Xu et al. 2004) and then as plasmalemma vesicle associated protein 1 (PV-1) (Niemela et al. 2005). However, this report was subsequently questioned by another group claiming that PAL-E does not recognize PV-1, but instead binds a VEGF binding site within NRP-1 (Jaalouk et al. 2007). In 2012 finally, we unambiguously showed that PAL-E actually does bind to PV-1, which can form complexes with NRP-1 (Keuschnigg et al. 2012).

2.1.8.2 Plasmalemma vesicle associated protein 1

PV-1, previously known as gp68 (Stan et al. 1997), was first identified in rat as the epitope recognized by the endothelial cell specific antibody 21D5 (Ghitescu et al. 1997). In a follow up study, PV-1 was then shown to localize to endothelial structures called caveolae or plasmalemma vesicles (Stan et al. 1997). Caveolae are regularly sized plasmalemmal invaginations of approximately 70 nm (Yamada 1955). They are partially characterized by the expression of the protein caveolin (Rothberg et al. 1992).

Isolation, cloning and characterization of the PV-1 sequence in rat revealed a heavily glycosylated protein of ~50-60 kDa which forms homodimers *in situ*. PV-1 was further classified as an integral type II transmembrane protein with a short intracellular N-terminus and longer extracellular C-terminus containing four N-glycosylation sites as well as a proline-rich region (Figure 5A) (Stan et al. 1999). Further microscopical analyses demonstrated PV-1 expression connected to diaphragms of transcellular channels, fenestrae and stomata of caveolae making it the first common marker of these endothelial structures (Stan et al. 1999). Subsequently, it was also shown that several PV-1 dimers can be found close to each other in the same diaphragm (Stan 2004). SD's and FD's share common structural features: both are formed by radial protein-fibers, connecting in the center of the diaphragm thereby forming a central density or "knob" (Figure 5B) (Palade and Bruns 1968; Clementi and Palade 1969).

Subsequently, it was revealed that not only is PV-1 part of the diaphragms, but it is a key component, the lack of which prevents phorbol myristate acetate-induced

formation of SD's and FD's (Stan et al. 2004). Analysis of the protein sequence of PV-1 in combination with structural data and functional studies lead to a hypothesis of PV-1 integration into stomatal- and fenestral diaphragms: The short intracellular N-terminus would anchor the protein to the plasma membrane and the heavy glycosylation would keep the extracellular part of the protein "afloat". PV-1 homodimers would then form the fibers of the diaphragm with their C-termini interacting to form the central knob (Stan 2005).

After the characterization and analysis of the PV-1 sequence and structure, several studies investigated the regulation of PV-1 expression as well as its functions in various settings. As mentioned already above, PV-1 expression and formation of diaphragms can be induced on human endothelial cells in culture using phorbol myristate acetate (Stan 2004). Furthermore, using human microvascular endothelial cells Carlson-Walter and colleagues demonstrate that PV-1 can be induced using the angiogenic growth factors VEGF or scatter/hepatocyte growth factor (Carson-Walter et al. 2005). Upregulation of PV-1 via VEGF-signaling was also shown by Strickland and colleagues (Strickland et al. 2005). More recently, another report confirms the induction of PV-1 by VEGF in human endothelial cells and additionally shows that angiotensin II also stimulates PV-1 expression (Bodor et al. 2012). Interestingly, two studies investigating PV-1 in mice report a negative regulation of PV-1 by VEGF (Hnasko et al. 2006; Hnasko et al. 2006).

As to the signaling pathways, inhibition of VEGF-signaling via VEGFR-2 by monoclonal antibodies as well as inhibitors against p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase blocked the induction of PV-1 by VEGF and angiotensin II in man (Strickland et al. 2005; Bodor et al. 2012). Again, the opposite was reported for mice where a VEGFR-2 inhibitor increased the amount of PV-1 protein, but not m-RNA (Hnasko et al. 2006).

The exact function of PV-1 has not yet been elucidated. Apart from its requirement for the synthesis of diaphragms, PV-1 might also have additional functional roles. Based on its expression pattern, a sieving and permeability-regulating function was put forward (Stan 2004; Bodor et al. 2012). Several reports connect PV-1 to angiogenesis in various cancer studies. For example, PV-1 was found in membrane vesicles, released from the endocytic compartment (so-called exosomes) of human mesothelioma cells (Hegmans et al. 2004; O'Loughlin et al. 2012). Furthermore, PV-1 was shown to be upregulated in various high-grade human brain tumors and to be associated with blood-brain barrier disruption. In the same study, PV-1 expression in hypoxia related conditions such as ischemia and stroke was demonstrated. The authors then showed decreased tubulogenesis and migration of endothelial cells after PV-1 knock down and hypothesized that PV-1 might play an important role in tumor angiogenesis (Carson-Walter et al. 2005).This upregulation of PV-1 in tumor endothelium was also shown by Strickland and colleagues (Strickland et al. 2005). Similar results were obtained with mice (Shue et al. 2008). In addition to tumors, microvascular leakage connected to increased PV-1 expression was also shown in the retina of patients with diabetes mellitus (Schlingemann et al. 1999) as well as in the glomeruli of patients with renal allografts (Yamamoto et al. 2007).

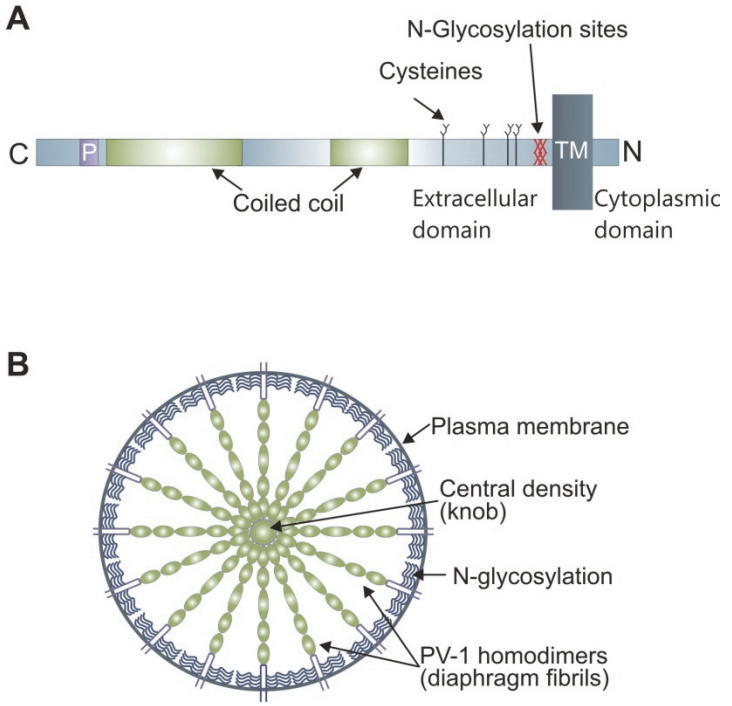

Figure 5: Structure and model of PV-1. A) Schematics of the features and domains of PV-1. **B)** Model of the membrane insertion and diaphragm formation of PV-1 as suggested by (Stan 2007).

In 2012, two groups revealed the importance of PV-1 by demonstrating embryonic lethality in PV-1$^{-/-}$ mice (Herrnberger et al. 2012; Herrnberger et al. 2012; Stan et al. 2012). In a C57BL/6J background, deletion of PV-1 resulted in lethality between E13 and postnatal day 2 (Stan et al. 2012). Similarly, in a C57BL/6N background, embryos survived only until E17.5 (Herrnberger et al. 2012). However, after intercrossing Balb/c, C57BL/6J and 129Sv/J mice, around 20% of the expected number of mice survived until 3-4 month of age (Stan et al. 2012), while mice in a C57BL/6N/FVB-N background were viable up to 4 weeks after birth (Herrnberger et al. 2012). Both studies reported a significantly impaired vascular barrier function due to the absence of diaphragms in fenestrated capillaries. This resulted in hemorrhaging, subcutaneous edema, and protein-losing enteropathy (Herrnberger et al. 2012; Stan et al. 2012). In addition, cardiac defects such as thinner ventricular walls and ventricular septal defects were reported (Herrnberger et al. 2012).

2.1.9 Pathological aspects of the vasculature

The vascular endothelium has long been considered a mere diffusion barrier preventing dispersion of blood components into the surrounding tissue. However, in 1980 it was reported that acetylcholine causes endothelial cells to stimulate

vasodilation (Furchgott and Zawadzki 1980). This study triggered a deeper appreciation for the importance of endothelial cells in vascular homeostasis. Dysfunction of the endothelium has subsequently been implicated in numerous pathological aspects of the vasculature such as hypertension, diabetes, coronary artery disease, chronic renal failure and peripheral artery disease (Endemann and Schiffrin 2004). One major factor in vascular pathologies is angiogenesis. Uncontrolled angiogenesis for example contributes to disorders such as cancer, age-related macular degeneration and inflammatory disorders (Carmeliet 2003; Carmeliet 2005). On the other hand, insufficient angiogenesis can cause vessel malformation and vessel regression, contributing to pathological conditions such as ischemia, preeclampsia, diabetes and Alzheimer's disease (Carmeliet 2003; Carmeliet 2005).

However, this prominent role of angiogenesis in vascular disorders also offers an attractive approach for therapeutic intervention. Anti-angiogenic therapies mostly concentrate on VEGF-signaling as the most potent inducer of angiogenesis. Anti-VEGF- and VEGFR antibodies and small molecule inhibitors of the tyrosine kinase signaling of VEGFR as well as chimeric VEGFR proteins have been developed (Baka et al. 2006; Crawford and Ferrara 2009). By now, several compounds such as bevacizumab, sunitinib, pazopanib and sorafenib have been approved by the US Food and Drug Administration for use in clinics (Potente et al. 2011). Even though these drugs showed great promise, efficiency of VEGF inhibition in cancer treatment was shown to be less beneficial than predicted after preclinical testing (Ferrara 2010; Ebos and Kerbel 2011). Subsequently, several mechanisms conveying resistance against anti-VEGF treatment have been found (Ebos and Kerbel 2011; Potente et al. 2011).

In its intact, healthy form, vascular endothelium promotes the unobstructed flow of blood through the human body and acts as selective barrier for both cells and macromolecules. In order to maintain normal blood circulation, the surface of the endothelium has to be anti-coagulant and anti-thrombotic (Feletou 2011). Endothelial dysfunction can nowadays be understood as a state characterized by impaired vasodilation as well as prothrombotic- and proinflammatory activity. The pathology of endothelia is a complex state with multiple aspects affecting it. However, some of these aspects appear to be present in most pathological conditions.

The main vasodilating factor produced by the vasculature is the molecule nitric oxide (NO). NO is generated through the L-arginine metabolism by the enzyme NO synthase in endothelial cells. In addition to its vasodilating function, NO also exhibits anti-aggregating, anti-inflammatory and growth inhibiting effects (Endemann and Schiffrin 2004). In the early stages of vascular dysfunction the reduced availability of NO is the main contributing factor (Taddei et al. 2003). By itself, NO inhibits adhesion of leukocytes to the vessel wall (Kubes et al. 1991). In addition, decreased bioavailability of NO can upregulate endothelial vascular cell adhesion molecule 1 (VCAM-1) by stimulating the expression of NF-κB (Khan et al. 1996). Furthermore, activated endothelium expresses additional adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), E-selectin and P-selectin. These adhesion molecules offer an adhesive surface to which leukocytes, platelets and erythrocytes can adhere (Gross and Aird 2000). The adhesion of leukocytes to the vessel wall is a prerequisite

for vascular inflammation and migration of leukocytes into the surrounding tissue and subsequent tissue inflammation. In addition, the adhesion of platelets and erythrocytes, while necessary during hemostasis, can lead to atherosclerosis and plaque formation (Gross and Aird 2000; Endemann and Schiffrin 2004; Versari et al. 2009).

In summary, under normal conditions, the activation of vascular endothelium is strictly regulated and occurs only according to environmental requirements. However, vascular dysfunction can cause the delicate balance of physiological, vascular function to tip towards a pathological state (such as thrombosis, hypertension and diabetes).

2.2 Leukocyte trafficking

The main function of leukocytes is to protect the human body from and fight against invading pathogens. However, in order to do that they need to patrol the body in search of foreign antigens. Considering the size-difference of ~125 x 10^{-15} liters vs. 75 liters between a resting lymphocyte and the human body it becomes clear that this process needs to be intricately regulated (von Andrian and Mackay 2000). One of the barriers leukocytes need to cross during their journey is the wall of the blood vessels. The migration of leukocytes across a vessel wall requires several distinct steps summarized under the term “adhesion cascade” (Figure 6) (Butcher 1991). This multistep process includes leukocyte tethering and rolling, activation and firm adhesion, lateral crawling and finally transmigration.

2.2.1 Leukocyte tethering and rolling

The first step of crossing the vascular endothelium has to be the tethering of freely flowing leukocytes to the vessel wall. Shear forces in the larger vessels can rise up to 50 dyn cm^{-2} (von Andrian and Mackay 2000), thereby preventing tethering of leukocytes. However, in postcapillary venules and high endothelial venules (HEV) wall shear stresses are in the range of 1-4 dyn cm^{-2}, hence providing an environment that facilitates leukocyte tethering and subsequent extravasation (Jones et al. 1996).

Tethering and rolling are mediated mostly by selectins (Figure 6A). Selectins are type I membrane glycoproteins with a N-terminal domain homologous to Ca^{2+}-dependent lectins, followed by a single EGF-like domain, two to nine short consensus repeats, a transmembrane region and a short cytoplasmic tail (Bevilacqua 1993). Selectins mediate cell-cell adhesion via interaction of their lectin-like domain with a carbohydrate ligand. In particular, oligosaccharides such as the fucosyl-modified tetrasaccharide sialyl Lewis x and its isomer sialyl Lewis a are bound by all selectins (Springer 1995; Kansas 1996; McEver and Cummings 1997). The three members of this protein family were named after their expression pattern: L-selectin (CD62L),

expressed on leukocytes, P-selectin (CD62P), expressed on platelets and endothelial cells and E-selectin (CD62E), expressed on endothelial cells (Ley and Kansas 2004; Ley et al. 2007).

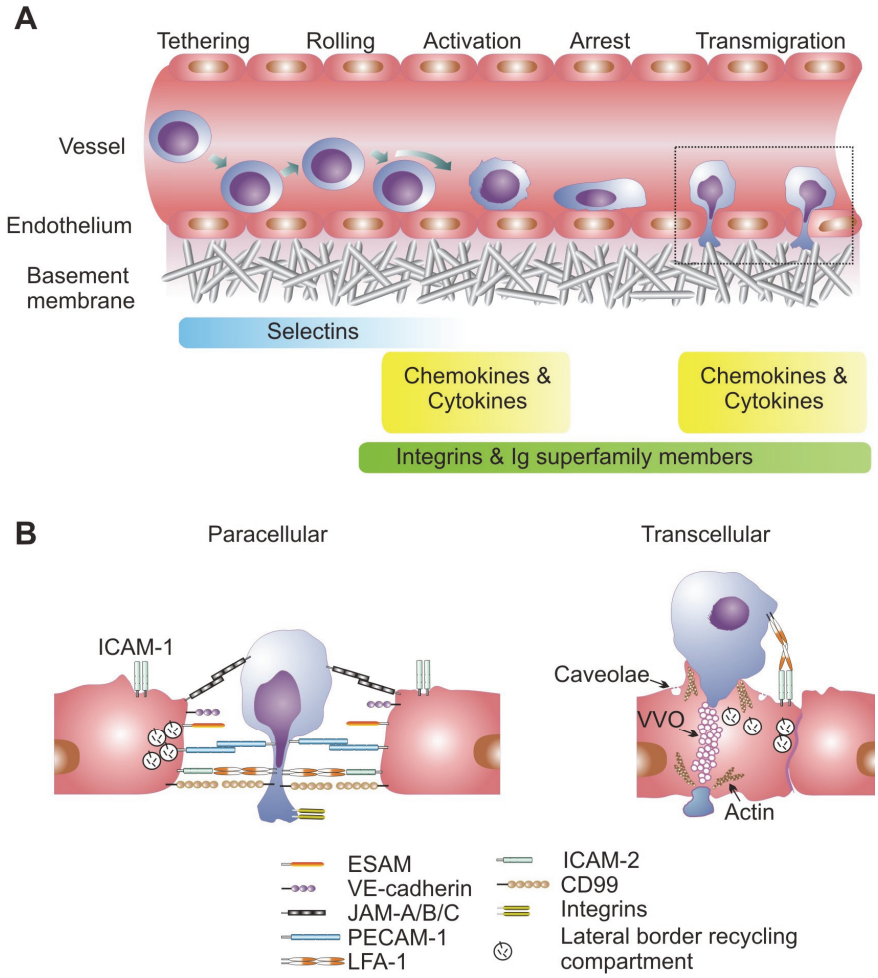

Figure 6: The leukocyte adhesion cascade. A) A schematic representation of the leukocyte adhesion cascade. Freely flowing leukocytes are captured by selectins, which facilitates rolling of leukocytes on the endothelium. Activation of endothelial cells and leukocytes by chemokines and cytokines then triggers integrin mediated arrest. Lateral crawling allows leukocytes to search out sites permissive for transmigration. Crossing of the endothelium occurs then via either the paracellular pathway or the transcellular pathway. **B)** Paracellular and transcellular migration are shown in more detail. Paracellular migration requires the opening of tight junctions and adherens junctions and involves several molecules such as JAMs. Transcellular migration on the other hand seems to involve endothelial structures such as caveolae and VVOs. The later border recycling compartment has been proposed as a common denominator of both pathways. VVO, vesiculo vacuolar organelles

While L-selectin is permanently expressed on leukocytes, P-selectin and E-selectin have to be induced by inflammatory stimuli. P-selectin is stored in α-granules and Weibel-Palade bodies of platelets and endothelial cells and can therefore be rapidly transported to the cell surface. E-selectin on the other hand is regulated at transcriptional level and its expression on the cell surface can hence be seen ~4 hours after activation (Berman et al. 1986; McEver et al. 1989; Kansas 1996; Ley and Kansas 2004; Ley et al. 2007)

Selectins can capture flowing cells and induce rolling in the course of milliseconds. This property is mainly attributed to the fast K_{on} and K_{off} rates of selectin-ligand bonds (Alon et al. 1995), describing the rates with which bonds are formed and released. In addition to the fast on- and off rates, another important property of selectin-ligand bonds is their resistance to mechanical force. This characteristic is referred to as "Catch"-bond, meaning that the binding strength increases once shear stress is applied (Marshall et al. 2003). In fact, both L-selectin and P-selectin actually require flow-induced shear stress to support their bonds, as cells start detaching once the flow is stopped (Finger et al. 1996; Lawrence et al. 1997). The main selectin ligand is the glycoprotein P-selectin glycoprotein ligand 1 (PSGL-1). However, the name is misleading as PSGL-1 can be bound by all three selectins (McEver and Cummings 1997). In addition to PSGL-1, E-selectin can also adhere to glycosylated CD44 and E-selectin ligand 1 (Hidalgo et al. 2007).

After the introduction of the multistep adhesion cascade for leukocyte transmigration, selectins were considered the exclusive molecules capable of initiating contact and mediating rolling under flow. However, after a while this hypothesis was proven incorrect and it was shown that certain integrins such as leukocyte function associated antigen 1 (LFA-1; αLβ2), macrophage antigen 1 (MAC1; αMβ2) and the α4-integrins very late antigen 4 (VLA-4; α4β1) and lymphocyte Peyer's patch adhesion molecule (LPAM-1; α4β7) are also able to support tethering and rolling of leukocytes on endothelial cells (Alon et al. 1995; Berlin et al. 1995; Johnston et al. 1997; Johnston and Butcher 2002).

2.2.2 Leukocyte activation and firm adhesion/arrest

With their fast K_{on} and K_{off} rates selectin-ligand bonds cannot sufficiently slow leukocyte rolling to enable firm adhesion to the vascular endothelium. For this step of the adhesion cascade, additional receptors need to be engaged. The formation of these secondary bonds is triggered by inflammatory chemokines and mediated by integrins on leukocytes binding to their counterparts of the immunoglobulin superfamily on endothelial cells (Harris et al. 2000; Plow et al. 2000; Ley et al. 2007).

In contrast to selectins, which are constitutively active, integrins need to be activated before they are able to form stable bonds. This activation of integrins is mediated by

chemokines, a family of polypeptides comprising more than 50 chemokine ligands and over 20 chemokine receptors (Zlotnik and Yoshie 2012). Historically, both chemokine ligands and their receptors were grouped into families, depending on their arrangement of cysteine residues in the peptide-chain (C, CC, CXC and CX3C, with X referring to any non-cysteine amino acid) (Allen et al. 2007; Zlotnik and Yoshie 2012). Binding of chemokine ligands and receptors is characterized by a large degree of redundancy as most receptors bind several ligands and many ligands bind multiple receptors (Allen et al. 2007).

Integrins are cell surface receptors, mediating cell-cell and cell-extracellular matrix adhesion. An integrin dimer consists of an α- and a β-subunit. At present, in human there are 18 α-subunits and 8 β-subunits known (Takagi and Springer 2002; Hoye et al. 2012). These subunits give rise to 24 distinct integrins. The two subunits of integrins are type I transmembrane glycoproteins, which are noncovalently connected. The C-terminal cytoplasmic domains of integrins are very short, containing only ~50 residues. The overall topology of the extracellular domains include globular N-terminal domains responsible for ligand binding and long stalks connecting the ligand binding site and the transmembrane region (Takagi and Springer 2002; Carman and Springer 2003; Kinashi 2005).

Integrins can be found in distinct confirmations defined by their affinity for their respective ligands. Under most conditions, integrins are found in a basic inactive form, exhibiting low binding affinities. However, upon activation (e.g. through chemokines) integrins switch into states of intermediate and high affinity. This division of integrin activity states into low-, intermediate- and high-affinity states has been the traditional view. However, it has been proposed that these states only represent discrete forms in a continuum of integrin conformations (Ley et al. 2007). This switch from inactive form to active form is termed "inside-out" signaling (Dustin and Springer 1989).

The change of conformation into active, high affinity states occurs very fast, within one second. This fast action is a requirement for integrin function and becomes clear once one considers the conditions under which integrins need to function: Assuming a vessel with a diameter of 100 μm and a physiological shear stress of ~2 dyn cm^{-2} (Jones et al. 1996), leukocytes flow with an average velocity of 2,5 mm sec^{-1}. Even taking the bonds between selectins and their ligands into account, rolling leukocytes still cover distances of ~8-10 x their diameter within one second (Laudanna et al. 2002). Therefore integrin activation needs to be quick to allow formation of bonds. This rapid switch from inactive to active conformations is driven by cytosolic factors such as talin and kindlin 1-3 (Vestweber 2012). However, the required factors for integrin activation vary depending on the integrin in question. For example, kindlin 3 was shown to be important for the activation of β1, β2 and β3 integrins, but less so for the integrin α4β1 (Malinin et al. 2009; Manevich-Mendelson et al. 2009). The integrin ligand also seems to influences the activation, as talin for example is required for firm binding of α4β1 integrin to ICAM-1, but not to VCAM-1 (Hyduk et al. 2011).

Apart from affinity, integrin signaling can also be regulated via clustering of integrin molecules (avidity regulation). During avidity regulation, several integrin

molecules associate driven through a connection with the cytoskeleton. In that way, integrins can modify cellular adhesion irrespective of conformational changes (Takada et al. 1997; Shimaoka et al. 2002). This mode of integrin regulation has for example been shown to play a role in the binding of LFA-1 to ICAM-1 and during formation of immunological synapses (van Kooyk and Figdor 2000; Carrasco et al. 2004).

In addition to the inside-out signaling and subsequent adhesion strengthening, integrins also induce outside-in signaling thereby regulating cellular processes such as apoptosis, motility and proliferation (Shattil 2005; Giagulli et al. 2006). It was hypothesized that outside-in signaling via LFA-1 might affect leukocyte adhesion under flow (Kim et al. 2003). Indeed, it was shown that the lack of two crucial mediators (namely the tyrosine kinases HCK and FGR) of outside-in signaling via LFA-1 and MAC-1 does not impair rapid adhesion of neutrophils under flow. However, the lack of outside-in signaling significantly accelerated the breakage of integrin bonds and detachment of adhering leukocytes (Giagulli et al. 2006).

The most relevant integrins in the context of leukocyte trafficking belong to the β_1, β_2 and β_7 families of integrins with their members $\alpha_L\beta_2$ (LFA-1), $\alpha_M\beta_2$ (MAC-1), $\alpha_4\beta_7$ and $\alpha_4\beta_1$ (very late antigen 4; VLA-4). These integrins and their immunoglobulin superfamily ligands are summarized in table 2. It is noteworthy that in addition to firm adhesion of leukocytes, integrin ligands can also facilitate leukocyte transmigration (Oppenheimer-Marks et al. 1991).

Table 2: Integrins and their ligands.

Integrin	Ligand
$\alpha_L\beta_2$ (LFA-1), $\alpha_M\beta_2$ (MAC-1)	ICAM-1
$\alpha_L\beta_2$	ICAM-2, 3, 4, 5
$\alpha_4\beta_1$ (VLA-4), $\alpha_4\beta_7$	VCAM-1
$\alpha_4\beta_7$	MAdCAM-1

The integrins most important for the leukocyte adhesion cascade and their immunoglobulin superfamily ligands are listed here.

2.2.3 Leukocyte lateral crawling

After firm adhesion, leukocytes have been shown to crawl in a lateral manner before finally crossing the endothelium. This lateral movement seems to be dependent on MAC-1 and ICAM-1 as MAC-$1^{-/-}$ neutrophils exhibit a delayed and impaired extravasation whereas LFA-$1^{-/-}$ cells adhered less efficiently, but crawled similarly to wild type cells (Phillipson et al. 2006). Another study performed on monocytes showed that these cells moved around 20 μm to the nearest junctions in a LFA-1, MAC-1, ICAM-1, ICAM-2 dependent manner. If these molecules were blocked, monocytes were unable to find cellular junctions (Schenkel et al. 2004).

The purpose of this lateral crawling appears to be the search for permissive sites for migration across the endothelium. It has been shown for example *in vivo* that transmigration of neutrophils occurs at regions where the basement membrane components laminin 10, collagen IV and nidogen-2 were reduced by ~60% (Wang et al. 2006). These sites were also associated with gaps between pericytes, thereby offering openings for leukocyte transmigration.

On the other hand, the need for leukocyte crawling is not unambiguous: Taking into account that the architecture of endothelial cells under the influence of flow is elongated, leukocytes have been demonstrated to almost always adhere in the vicinity of an endothelial junction (Dewey et al. 1981; Phillipson et al. 2006). Furthermore, selectins have been shown to capture leukocytes next to endothelial junctions (Burns et al. 1999) and neutrophils have been shown to emigrate from vessels not only through paracellular- but also transcellular pathways (Feng et al. 1998). Therefore the absolute need for lateral crawling remains doubtful. Phillipson *et al*. offered a potential explanation for crawling by showing that MAC-$1^{-/-}$ neutrophils were able to emigrate even without intraluminal crawling. However, the speed of crossing the endothelium was significantly reduced (Phillipson et al. 2006). Therefore, lateral crawling of leukocytes seems to enable them to search for regions more suitable for crossing the endothelial wall.

2.2.4 Transendothelial migration

Once leukocytes have found a permissive site for TEM they need to cross three distinct barriers: the endothelial cells, their basement membrane and finally the surrounding layer of pericytes.

After firm adhesion of leukocytes to the endothelium, they can induce the formation of so-called “docking structures” or “transmigratory cups” (Barreiro et al. 2002; Carman and Springer 2004). These endothelial structures are enriched in ICAM-1, VCAM-1, cytoskeletal components such as actin and vimentin and their connecting proteins ezrin and moesin. (Barreiro et al. 2002; Carman et al. 2003; Carman and Springer 2004; Nieminen et al. 2006; van Buul et al. 2007). Formation of these protrusions was shown to occur in a LFA-1 and VLA-4 dependent manner, triggered by phosphatidylinositol‑4,5-bisphosphate and RHO (Ras homologue) GTPase dependent association with cytoplasmic proteins (Barreiro et al. 2002; Carman and Springer 2004). Due to their parallel orientation to the direction of diapedesis and perpendicular orientation to the direction of flow, it was proposed that these transmigratory cups could serve as guidance structures for correctly oriented paracellular- as well as transcellular migration (Carman and Springer 2004). Furthermore, the formation of docking structures, which finally completely engulfed adherent leukocytes was shown to be dependent on the protein leukocyte-specific protein 1 and aid in the maintenance of endothelial cell barrier function (Phillipson et al. 2008; Petri et al. 2011).

Depending on the location where leukocytes cross the endothelium, leukocyte diapedesis can be subdivided into two types. Paracellular migration refers to leukocytes crossing the endothelium through endothelial junctions between adjacent endothelial cells. In addition, some studies demonstrated a preferential migration of leukocytes at tricellular junctions (Burns et al. 1997; Sumagin and Sarelius 2010). However, this concept has not received much attention. The second type, transcellular migration, describes leukocytes extravasating from the blood vessel through the body of one individual endothelial cell (Carman and Springer 2004; Ley et al. 2007; Sage and Carman 2009). In the following chapter, both pathways of leukocyte diapedesis will be described in more detail.

2.2.4.1 Paracellular migration

Migration of leukocytes through the endothelial cell junctions requires the opening of tight junctions and adherens junctions (Figure 6B). These structures connect neighboring endothelial cells and are crucial for the maintenance of endothelial barrier function. The loosening of endothelial junctions can be facilitated by releasing bonds between adhesion molecules on adjacent endothelial cells. This can be facilitated by the formation of bonds between the adhesion molecules on endothelial cells and leukocytes (Table 3). The intracellular domain of ICAM-1 for example has been shown to be required for RHO GTPase mediated opening of endothelial junctions and subsequent T-cell migration (Greenwood et al. 2003; Millan and Ridley 2005). Moreover, clustering of ICAM-1 and VCAM-1 transmits numerous signals into the cytoplasm and nucleus. Several of these seem to be involved in TEM (van Buul et al. 2007).

Endothelial VE-cadherin is another junctional protein involved in paracellular TEM. Phosphorylation of VE-cadherin is induced after stimulation of ICAM-1 and is required for the loosening of adherens junctions (Turowski et al. 2008). In human umbilical vein endothelial cells (HUVEC), VE-cadherin is phosphorylated by the kinases Src and Pyk2 on the binding sites for p120 and β-catenin, thereby inhibiting their binding to VE-cadherin (Turowski et al. 2008). As this interaction is required for the maintenance of adherens junctions, VE-cadherin phosphorylation ultimately leads to loosened endothelial junctions (Alcaide et al. 2008). This has been confirmed by another study, showing that stabilization of VE-cadherin through the introduction of a VE-cadherin-α-catenin fusion construct strengthens the association of VE-cadherin with actin. Thereby it blocks the induction of vascular permeability in response to VEGF and strongly reduces the extravasation of leukocytes to sites of inflammation (Schulte et al. 2011)

Opening of junctional structures is also connected to the contractile force of myosin light-chain kinase induced through increased levels of Ca^{2+} (Huang et al. 1993; Hixenbaugh et al. 1997). These contractile forces can produce openings *in vitro* large enough to be discernible in light microscopes (i.e. >200 nm). However, *in vivo* these gaps are only in the order of ~ $1x10^{-1}$ nm and by themselves cannot facilitate leukocyte TEM (Majno and Palade 1961; Muller 2011).

Table 3: Endothelial molecules and their ligands involved in paracellular migration.

Endothelial molecule	Endothelial ligand	Leukocyte ligand
JAM-A	JAM-A	CD11a/CD18 (LFA-1)
JAM-B	JAM-B, JAM-C	N/A
JAM-C	JAM-C, JAM-B	CD11b/CD18 (MAC-1)
ICAM-1	N/A	CD11a/CD18, CD11b/CD18
VCAM-1	N/A	CD49d/CD29 (VLA-4)
PECAM-1	PECAM-1	PECAM-1
VE-cadherin	VE-cadherin	N/A
CD99	CD99	CD99
CD99L2	CD99L2	CD99L2
ESAM	ESAM	N/A

This table summarizes the most important endothelial molecules and their counterparts on adjacent endothelial cells and leukocytes that are involved in paracellular migration of leukocytes. For reviews see (Ley et al. 2007; Muller 2011).

The junctional adhesion molecule (JAM)-family with its three members JAM-A, JAM-B and JAM-C is critically involved in processes such as leukocyte TEM and vascular permeability (Weber et al. 2007; Muller 2011). All JAM-molecules share a similar structure composed of a short intracellular domain with a PDZ binding motif, a single transmembrane sequence and an extracellular domain containing two immunoglobulin-like domains (Weber et al. 2007). JAMs can either form homophilic- or heterophilic interactions (Table 3). JAM-A for example interacts usually with other JAM-A molecules. During inflammation however it can also bind to LFA-1 (CD11a/CD18) on leukocytes (Ostermann et al. 2002). Interestingly, blocking of JAM-A *in vitro* does not seem to affect leukocyte TEM (Liu et al. 2000; Schenkel et al. 2004; Shaw et al. 2004) with one exception (Ostermann et al. 2002), whereas blockage *in vivo* leads to decreased TEM and inflammation (Martin-Padura et al. 1998; Woodfin et al. 2009). JAM-C on the other hand can also form homophilic interactions in addition to binding to MAC-1. This binding of MAC-1 is involved both in lymphocyte TEM *in vitro* across HUVECs (Johnson-Leger et al. 2002) as well as neutrophil TEM *in vivo* (Chavakis et al. 2004).

PECAM-1 is another member of the immunoglobulin superfamily and is concentrated at the lateral junctions of endothelial cells. In addition to being an endothelial marker, homophilic interaction between PECAM-1 molecules on leukocytes and endothelium is required for TEM (Muller et al. 1993; Mamdouh et al. 2003). The importance of PECAM-1 for leukocyte TEM both *in vitro* and *in vivo* has been extensively demonstrated using blocking antibodies, soluble PECAM-Fc chimeras, genetic deletion and expression in cells that normally lack it (Muller 2007; Dasgupta et al.

2009). Blockage of PECAM-1 interactions results in tight adhesion of leukocytes to the apical surface of endothelial cells and migration along the junctions, apparently in search for a site permissive for TEM (Liao et al. 1995; Schenkel et al. 2004).

This phenotype has been observed in all settings in human and mouse with one notable exception: The C57BL/6 mouse strain does not respond to any inhibition of PECAM-1 interactions (Duncan et al. 1999; Schenkel et al. 2004; Seidman et al. 2009). However, leukocytes in PECAM-1 deficient C57BL/6 mice do exhibit a functional deficiency related to traversing the basal lamina underneath the endothelium (Wakelin et al. 1996).

The junctional borders of cells are even under non-inflamed conditions active regions within endothelial cells with constitutive membrane recycling. This membrane-recycling has been shown to involve a specialized compartment, designated as "lateral border recycling compartment" (LBRC) (Mamdouh et al. 2008). Closer investigation of this compartment revealed a nature distinct from other vesicular structures such as VVOs, caveolae or recycling endosomes (Mamdouh et al. 2003). Ultrastructurally the LBRC seems to consist of 50 nm large vesicles (Mamdouh et al. 2008) and it has been shown to contain ~30 % of the cell's PECAM-1 pool in addition to JAM-A and CD99 (Mamdouh et al. 2003; Mamdouh et al. 2009). The exact function of the LBRC has not yet been elucidated. However, it has been shown that this vesicular structure is directed towards sites of leukocyte TEM, where it increases the surface of the plasma membrane. This relocation is driven by kinesin motor proteins along microtubules and blocking either the motor proteins or inhibiting association of microtubules inhibits leukocyte TEM (Mamdouh et al. 2003; Mamdouh et al. 2008).

It has been speculated that the purpose of the LBRC could be the removal of ligated surface proteins, the provision of free membrane surface with unligated proteins required for TEM such as PECAM-1, CD99 and JAM-A (Muller 2011).

Unlike PECAM-1 and the JAM family of proteins, CD99 is a unique 32 kDa surface molecule. In the human genome, it is related only to its paralog CD99L2 (Suh et al. 2003). CD99 acts later during TEM than does PECAM-1, but in a similar fashion, that is homophilic interaction of CD99 on endothelial cells and monocytes and neutrophils is required for successful TEM. Monocytes blocked at the CD99-dependent step had partly crossed the endothelium, showing most of their cell bodies at the basal membrane, but retaining their uropod at the apical surface (Schenkel et al. 2002; Lou et al. 2007).

ESAM is a molecule related to the family of JAM-proteins even though it differs significantly in structure, expression profile and binding partners. In contrast to JAM-proteins, expression of ESAM is limited to platelets and junctional areas of endothelial cells. It was shown that homing of neutrophils to inflamed peritoneum was markedly delayed in $ESAM^{-/-}$ mice. Moreover, it was demonstrated that small interfering RNAs, limiting the expression level of ESAM to 5%, reduced the levels of activated Rho GTPases by over 75%. As ESAM is implicated in Rho-activation and

active Rho affects the opening of endothelial junctions, this might be the mechanism by which ESAM facilitates extravasation of neutrophils (Wegmann et al. 2006).

Several other molecules have been implicated in the paracellular migration of leukocytes such as ICAM-2 (Xie et al. 1995), CD99L2 (Bixel et al. 2007) vascular adhesion protein 1 (Koskinen et al. 2004; Stolen et al. 2005; Jalkanen and Salmi 2008), vWF (Petri et al. 2010) and leukocyte specific protein 1 (Petri et al. 2011).

2.2.4.2 Transcellular migration

The importance of paracellular and transcellular types of migration has been extensively debated. Even though some of the earliest *in vivo* studies analyzing leukocyte TEM demonstrated transcellular migration (Marchesi and Florey 1960; Williamson and Grisham 1961; Marchesi and Gowans 1964), the concept was not generally accepted. This might have been due to the fact that early *in vitro* experiments failed to provide clear evidence for transcellular migration (Beesley et al. 1979; Furie et al. 1987; Pawlowski et al. 1988; Muller 2003). Criticism of the concept of transcellular migration finally subsided when a study in 1998 for the first time demonstrated clear, unambiguous evidence for neutrophil transcellular migration *in vivo* (Feng et al. 1998).

Even though the basic concept of leukocyte transcellular TEM is now generally accepted, its quantitative contribution to leukocyte trafficking is not yet clear. Percentages range from 5% of neutrophils migrating transcellularly across HUVECs *in vitro* (Carman and Springer 2004) to 100% of mononuclear leukocytes crossing the murine blood-brain barrier in a transcellular fashion in experimental autoimmune encephalomyelitis (an animal model for human multiple sclerosis) *in vivo* (Wolburg et al. 2005).

Most *in vitro* studies employed either human umbilical vein endothelial cells (HUVECs) or human microvascular endothelial cells isolated from skin (HDMECs) or lung. In general, studies analyzing leukocyte diapedesis using HUVECs reported a low prevalence of transcellular migration of ~5-10% (Carman and Springer 2004; Yang et al. 2005; Millan et al. 2006). However, when migration across microvascular endothelial cells was analyzed, transcellular migration constituted ~30% of total leukocyte TEM (Ferreira et al. 2005; Millan et al. 2006; Carman et al. 2007). A comprehensive list of experimental settings and the quantitative importance of transcellular migration can be found elsewhere (Sage and Carman 2009).

Two additional considerations should be mentioned here: Unambiguously demonstrated transcellular migratory events often occurred only 100-200 nm from endothelial cell borders (Chamberlain and Lichtman 1978; Cho and De Bruyn 1981; Cho and De Bruyn 1986; Feng et al. 1998; Wolburg et al. 2005; Azzali et al. 2008). Since endothelial cells are thinnest near to the junctions, this makes sense as it offers the “path of least resistance” (Chamberlain and Lichtman 1978). However, light microscopy which has been used in most of the recent *in vitro* studies offers only resolutions of maximally 200 nm. As many studies scored transmigration in the

vicinity of endothelial junctions by default as paracellular, the prevalence of transcellular migration might be underestimated (Sage and Carman 2009). In addition, *in vitro* settings usually do not promote the same degree of junctional complexity as *in vivo* settings (Ballermann and Ott 1995; Lee et al. 1999; Noria et al. 1999). As a consequence, junctions in *in vitro* settings could be leakier and therefore promote more paracellular migration than physiological settings do (Sage and Carman 2009).

Numerous in vitro studies have started to shed light on the mechanisms through which leukocytes cross the endothelium in a transcellular manner (Figure 6B). Whereas most groups found transcellular migration to occur via formation of transendothelial pores, some studies also reported the presence of leukocytes within the cytoplasm of endothelial cells, so called "emperiopolesis" (for a review see (Sage and Carman 2009)). However, the migration via transendothelial pores seems to be predominant.

During lateral crawling, leukocytes have been shown to form protrusions on the ventral surface (Wolosewick 1984; Carman et al. 2007). These actin-dependent protrusions (Linder and Aepfelbacher 2003) were found to range in size from 100 nm to 2 μm and have been termed "podosomes" for short forms and "invasive podosomes or invadopodia" for long protrusions (Carman et al. 2007). Podosomes formed rapidly (within 30 sec) under the laterally migrating leukocytes and typically covered an area of ~100 μm^2. Interestingly, formation of podosomes almost always occurred before a transendothelial pore could be established (Carman et al. 2007). Podosomes were shown to be dynamic structures that were formed and retracted in rapid succession, thereby probing the endothelial cell. As it has been suggested that lateral crawling could represent a search for sites permissive for transmigration (Phillipson et al. 2006), formation of podosomes seems to be involved in the same process. Leukocyte protrusions were also found on top of the endothelial nucleus, a location not allowing transcellular migration (Carman et al. 2007). This indicates that leukocytes need to empirically search for permissive sites. Once such places had been identified, leukocytes extended their podosomes to lengths up to 2 μm, thereby bringing endothelial apical and basal membranes into close contact (Carman et al. 2007). Hence, podosomes have been suggested to be required for leukocyte transcellular TEM (Wolosewick 1984; Allavena et al. 1991; Carman et al. 2007). Although not termed podosomes, similar types of protrusions "probing" the endothelium have been described in numerous *in vivo* studies and have been suggested to induce transcellular pore formation (Lossinsky et al. 1989; Lossinsky et al. 1991; Greenwood et al. 1994; Wolburg et al. 2005).

One unifying concept of transcellular migration is the requirement for membrane fusion to open a pore and subsequent membrane fission to reseal the opening. The formation of and probing with podosomes is one contributing and maybe initializing factor (Carman et al. 2007). However, endothelial cells actively contribute to this process. Earlier studies showed that the endothelium concentrates membrane containing structures (e.g. vesicles and VVOs) as well as fusogenic proteins such as vesicle-associated membrane protein 2 and 3 around podosomes (Migliorisi et al.

1987; De Bruyn et al. 1989; Greenwood et al. 1994; Millan et al. 2006; Carman et al. 2007). Disturbance of the SNARE fusion system or knock-down of caveolin-1 both result in impaired transcellular migration (Millan et al. 2006; Carman et al. 2007), suggesting an important role for membrane provision and fusion in this process.

Interestingly, a recent study showed that PECAM-1, originally thought to be involved in paracellular migration, also plays a role during leukocyte transcellular TEM (Mamdouh et al. 2009). The authors showed that monocytes and neutrophils transcellularly migrating across HUVECs in response to N-formyl-methionyl-leucyl-phenylalanine or monocyte chemotactic protein-1 were surrounded by PECAM-1 (Mamdouh et al. 2009). Furthermore, the junctional molecules CD99 and JAM-A were also detected around migrating leukocytes. PECAM-1, CD99 and JAM-A have been shown to locate to the LBRC (Mamdouh et al. 2003; Mamdouh et al. 2008; Mamdouh et al. 2009), which the authors found to be recruited to the site of transcellular pore formation (Mamdouh et al. 2009). These findings could provide a mechanism connecting transcellular- and paracellular migration. However, more work needs to be done to better understand the underlying processes and requirements.

2.2.4.3 Crossing the basement membrane and pericyte-layer

Once leukocytes have crossed the endothelial monolayer, they need to penetrate the underlying basement membrane and, where present, the surrounding pericyte-sheat. (Nourshargh et al. 2010). The basement membrane consists of a network formed by laminins and collagen IV, connected through interactions with nidogen-2 and perlecan (Hallmann et al. 2005). It is formed by endothelial cells in collaboration with pericytes (Ley et al. 2007; Stratman et al. 2009; Nourshargh et al. 2010) and it has been shown that incomplete pericyte coverage results in basement membrane areas with low expression of laminins and collagen IV (Wang et al. 2006; Voisin et al. 2009; Voisin et al. 2010). These low expression regions can be found in many tissues. Moreover, it has been shown that neutrophils and monocytes identify these sites using ventral membrane protrusions and subsequently cross the basement membrane through these openings (Voisin et al. 2009; Voisin et al. 2010). Interestingly, similar permissive sites were found to be involved in the intravasation of dendritic cells into the lymphatic vessels (Pflicke and Sixt 2009).

For polymorphonuclear leukocytes to pass through a physical barrier, they need pores of at least 2 µm diameter (Kuntz and Saltzman 1997). However, openings in the basement membrane are ~40 fold smaller (Abrams et al. 2000). Therefore, the basement membrane has to be remodeled to accommodate leukocyte TEM. Indeed it has been shown that the regions of low laminin- and collagen IV expression are transiently enlarged in response to transmigrating neutrophils (Wang et al. 2006; Voisin et al. 2009; Voisin et al. 2010). However, the underlying mechanism is still unresolved. Proteases such as matrix metalloproteinase 8 and 9 and neutrophil elastase have been suggested to be involved in neutrophil TEM (Yadav et al. 2003; Reichel et al. 2008). Interestingly, monocytes manage to cross the low expression regions

without need for remodeling (Voisin et al. 2009), suggesting that the enlargement of pores in the basement membrane is not absolutely required.

2.2.5 Interstitial migration

After extravasation from the blood vasculature, leukocytes need to travel through the 3-dimensional (3D) matrix in the interstitium before returning via lymphatics and lymph nodes back to the blood vasculature.

The interstitium consist of a mesh-like accumulation of proteins such as elastin, different types of collagens and glycosaminoglycans. The individual components are entwined and cross-linked, thereby forming the interstitial 3D matrix (Comper and Laurent 1978; Scallan et al. 2010). The main producers of this extracellular matrix (ECM) are fibroblasts, who also release matrix-degrading enzymes causing an interstitial turnover time of approximately 50 days (Granger et al. 1984). Leukocyte migration in the ECM has been shown to involve continuous shape changes reminiscent of the amoeboid migration of primitive single-celled eukaryotes (De Bruyn 1946). Cells such as fibroblasts are strongly adhesive and therefore align along ECM fibers with which they form strong interactions (Cukierman et al. 2001; Renkawitz and Sixt 2010). Migration while maintaining firm bonds with the ECM is termed haptokinesis and is mainly dependent on integrins (Renkawitz and Sixt 2010). These bonds are crucial as loss of attachment in this situation results in a special form of apoptosis termed anoikis (Chiarugi and Giannoni 2008). Leukocytes on the other hand can survive in the ECM without anchoring bonds and exhibit shape changes mostly independent of the surrounding matrix (Lammermann and Sixt 2009; Renkawitz and Sixt 2010). It appears as if leukocyte migration in the interstitium can be influenced by guidance cues from the environment, but is not strictly determined by them (Nourshargh et al. 2010). However, guidance cues such as chemokines and lipids are well established factors controlling leukocyte migration by activating both integrins and the actomyosin cytoskeleton (Rot and von Andrian 2004).

This activation of the actomyosin cytoskeleton is the main protrusive force behind amoeboid migration (Krummel and Macara 2006; Lammermann and Sixt 2009). Forward movement is achieved by protruding lamellipodia and filopodia via actin polymerization at the leading edge mediated by Rac (Chhabra and Higgs 2007; Nourshargh et al. 2010). Contraction of actin fibers mediated by type II myosins at the trailing edge then causes its detachment and pushes the cytoplasm forward (Smith et al. 2003). However, these actions need the traction created by connecting the actin cortex to the matrix. This is usually achieved by integrin binding of the ECM (Mitchison and Cramer 1996). Nevertheless, it has been shown that leukocytes such as dendritic cells and $CD4^+$ T-cells can use integrin independent migratory mechanisms for movement in 3D environments (Friedl et al. 1998; Lammermann et al. 2008; Lammermann et al. 2009). This raises the question how dendritic cells move forward without attaching their body to the surrounding matrix.

Analysis of integrin-dependent migration in fibroblasts revealed a tight connection between the protrusion of the leading edge, integrin-mediated adhesion

and myosin II induced contraction (Vanni et al. 2003; Meshel et al. 2005; Lammermann et al. 2008). Interestingly, in dendritic cells this link was missing as leading edge protrusion and trailing edge contraction were found to be "spatio-temporally dissociated" (Lammermann et al. 2008). Blockage of myosin II caused the immobilization of the trailing edge, while the leading edge protruded normally. This immobilization of the trailing edge resulted in strongly impaired migration speeds. Additional experiments then showed that dendritic cells in the ECM migrate by traction independent protrusion and that contraction at the trailing edge is required to propel rigid structures such as the nucleus against external forces such as small pore sizes in the ECM (Lammermann et al. 2008).

Subsequently it has been shown that dendritic cells can switch between an integrin dependent- and an integrin independent mode of interstitial migration depending on the substrate (Renkawitz et al. 2009). This allows dendritic cells to migrate without following ECM fibers but rather following a gradient of guidance molecules such as chemokines (Renkawitz et al. 2009). Interestingly, chemokines have also been shown to influence the way how dendritic cells migrate. Depending on their form of presentation, immobilized CCL21 triggered integrin activation and thus adhesion of migrating dendritic cells. These cells then moved randomly on the surface presenting the chemokines. A soluble gradient of chemokines on the other hand induced chemotaxis without integrin dependent anchoring of the cells (Schumann et al. 2010). Hence both the immune cells as well as the environment can influence the pathways involved in interstitial migration and thereby shape the immune response.

3. AIMS OF THE STUDY

The distinction between the blood vasculature and lymphatic vasculature is a crucial factor in many studies in the fields of immunology, vascular biology and cancer research. To be able to separate the two types of vessels, marker proteins are commonly used. However, the specificity and suitability of these vessel-specific proteins is sometimes questionable and their function not always known. In addition, endothelial cells from one vessel type are often required for *in vitro* experiments. As the isolation and culture of primary endothelial cells is cumbersome, immortalized endothelial cell lines are frequently employed as model systems. Two such cell lines are the human microvascular endothelial cell line 1 (HMEC-1) and the telomerase immortalized microvascular endothelial cell line (TIME).

The aim of this study was to evaluate the lymphatic- and blood vessel specific gene expression, suitability of marker genes and vascular model systems. Furthermore, we wanted to investigate the identity and function of one specific blood vascular marker.

The specific aims of the study were:

I To analyze the gene expression profiles of the immortalized endothelial cell lines HMEC-1 and TIME and primary blood- and lymphatic endothelial cells as well as identify new, stable vessel-type specific markers.

II To investigate the identity of the blood vessel marker PAL-E and analyze a potential relationship with neuropilin-1

III To examine a potential function of PAL-E/PV-1 during the process of leukocyte transmigration.

4. MATERIALS AND METHODS

A more detailed description of the materials and methods used during these studies can be found in the original publications and their respective supplements.

4.1 Antibodies

Table 4: Summary of the primary antibodies used during these studies

Antigen	Clone/ Catalog	Isotype	Label	Source/ Reference	Used in
h Caveolin-1	2234	mouse IgG2a		BD Biosciences	III
h CD31	2C8	mouse IgG1		(Airas et al. 1993)	I, II
h CD31	M-20	goat IgG		Santa Cruz Biotechnology	II
h CLEVER-1	3-372	mouse IgG1		(Irjala et al. 2003)	I
h CLEVER-1	3-372	mouse IgG1	Alexa 488	(Irjala et al. 2003)	I
h COLEC12	AF2690	goat IgG		R&D Systems	I
h HLA-DR5	HB151	rat IgG2b		ATCC	III
h LYVE-1	102-PA50AG	rabbit IgG		Reliatech	
h MCAM	AF932	goat IgG		R&D Systems	I
h NRP-1	446921	mouse IgG2a		R&D Systems	II
h NRP-1	AF3870	sheep IgG		R&D Systems	II
h Podoplanin	18H5	mouse IgG1		Acris Antibodies	I
h PV-1	174/2	mouse IgG1		(Niemela et al. 2005)	I, II, III
h PV-1	174/2	mouse IgG1	FITC	(Niemela et al. 2005)	I, III
h PV-1	PAL-E	mouse IgG2a		(Schlingemann et al. 1985), Abcam and AbD Serotec	I, II
h Vimentin		chicken IgG		gift from J. Eriksson	III
h Vimentin	V9	mouse IgG1		Sigma Aldrich	III
m Endoglin	MJ7/18	rat IgG2a		gift from E. Butcher	III
m PV-1	Meca-32	rat IgG2a		gift from E. Butcher	III
neg. control	20102	mouse IgG2a		R&D Systems	I, II, III
neg. control	3G6	mouse IgG1		(Salmi and Jalkanen 1992)	III
neg. control	AK-1	mouse IgG1		In Vivo Biotech Services	I, II
neg. control	NS-1	mouse IgG1		ATCC	III
neg. control	serum	sheep		AbD Serotec	II
neg. control	serum	goat		Vector Laboratories	I, II
neg. control	serum	rabbit		Harlan-Sera-Lab LTD	I

h, human; m, mouse; ATCC, American Type Culture Collection

Table 5: Secondary antibodies used during these studies.

Antigen	Isotype	Conjugate	Source	Used in
chicken IgG	goat IgG	Alexa 546	Molecular Probes	III
FITC	goat IgG	Alexa 488	Molecular Probes	III
goat IgG	donkey IgG	Alexa 546	Molecular Probes	I, II
goat IgG	donkey IgG	HRP	Santa Cruz Biotechnology	II
mouse IgG	goat IgG	Alexa 488	Molecular Probes	I, III
mouse IgG	goat IgG	PE	Southern Biotechnology	I
mouse IgG	rabbit IgG	HRP	Dako	II, III
mouse IgG	sheep F(ab')2	FITC	Sigma Aldrich	II, III
mouse IgG1	goat IgG	Alexa 488	Molecular Probes	III
mouse IgG1	goat IgG	Alexa 546	Molecular Probes	II
mouse IgG2a	goat IgG	Alexa 546	Molecular Probes	III
mouse IgG2a	goat IgG	Alexa 488	Molecular Probes	II
rabbit IgG	goat IgG	Alexa 488	Molecular Probes	I
rat IgG	goat IgG	Alexa 488	Molecular Probes	III
sheep IgG	donkey IgG	Alexa 546	Molecular Probes	II
sheep IgG	donkey IgG	FITC	Sigma Aldrich	II
sheep IgG	rabbit IgG	HRP	Dako	II

HRP, horseradish peroxidase

4.2 Cells, cell lines and transfectants

Table 6: Primary cells and cell lines used for experiments.

Cell/ Cell line	Description	Source/Reference	Used in
HUVEC	Human umbilical vein endothelial cells	(Jaffe et al. 1973)	I, III
HDMEC	Human dermal microvascular endothelial cells	PromoCell	III
PBMC	Peripheral blood mononuclear cells		I,III
PMN	Polymorphonuclear leukocytes		I, III
HMEC-1	Human microvascular endothelial cell line 1	(Ades et al. 1992)	I
TIME	Telomerase immortalized micro-vascular endothelial cells	(Venetsanakos et al. 2002), ATCC	I
HEK-EBNA PV-1	Human embryonic kidney cell line expressing PV-1	(Graham et al. 1977), ATCC	II
HEK-EBNA NRP-1	Human embryonic kidney cell line expressing NRP-1	(Graham et al. 1977), ATCC	II
HEK-EBNA mock	Human embryonic kidney cell line transfected with empty vector	(Graham et al. 1977), ATCC	II
HEK-EBNA PV-1+NRP-1	Human embryonic kidney cell line expressing PV-1 and NRP-1	(Graham et al. 1977), ATCC	II

HEK EBNA is the designation of the HEK-293 cell line, which expresses the Epstein Barr Virus gene "EBNA-1" under the control of the CMV promotor.

4.3 Methods

Table 7: Methods used for the publications.

Method	Used in
Cell isolation and culture	I, III, III
Co-Immunoprecipitation	II, III
End-point transmigration	III
Flow cytometry	I, II, III
Immunofluorescence	I, III
Immunohistochemistry	I, II, II
In vitro flow assay	I, III
In vivo inflammation model, air pouch	III
In vivo inflammation model, peritonitis	III
Live cell imaging	III
Microarray analysis	I
Transfections	II, III
Western blot	II, III

Selected methods are described in more detail below.

4.3.1 Immunohistochemistry

Frozen sections of 5-7 µm were cut on a Leica CM3050S cryostat, fixed for 3 min in ice-cold acetone and stored at -70 ºC until used. Stainings were performed by incubating the sections for 30-60 minutes with primary antibodies diluted in PBS. After 2x 5 min washes in PBS, sections were stained with species/isotype specific secondary antibodies diluted in PBS, 10% serum. Where it was required, free binding sites were blocked with 5% species specific serum in PBS. This procedure was repeated for the second epitope to be stained. After 2x 5 min washes in PBS the sections were mounted in mounting medium containing DAPI. Stainings were analyzed on a Zeiss LSM510 Meta confocal laser scanning microscope.

4.3.2 End point transmigration and immunofluorescence

Endothelial cells (HUVEC or HDMEC) were seeded on fibronectin treated glass coverslips and grown until they formed an intact endothelial monolayer. For end point transmigration, cells were activated for 4 hours with 100 U TNF-α ml^{-1} medium prior to addition of $1x10^6$ lymphocytes. Cells were allowed to transmigrate for 30-60 minutes at 37°C. After 3 washes with PBS, the cells were fixed with 4% paraformaldehyde either at room temperature or on ice. Cells were subsequently

permeabilized using PBS, 0,2% saponin, 0,2% gelatin. Staining was then performed with primary and secondary antibodies diluted in the same permeabilizing solution.

4.3.3 Co-Immunoprecipitation (Co-IP)

Co-immunoprecipitation was performed using magnetic M-270 Epoxy Dynabeads. Coating of beads with precipitating antibodies was performed as was previously described (Cristea et al. 2005). Protein complexes were subsequently precipitated from cell lysate or lymphocyte depleted tonsil lysate, washed and dried over-night in a speed vac. Dried samples were then resuspended in Laemmli sample buffer with or without mercaptoethanol and analyzed by SDS-PAGE. Proteins were then transferred to nitrocellulose membranes and detected using primary antibodies diluted in PBS-Tween-20 and HRP-coupled secondary antibodies. Signals were visualized using chemiluminescent HRP substrates.

4.3.4 In vitro flow assay

HUVECs, HMECs or TIME were plated into glass capillaries or ibidi μ-slide VI 0,4 chambers and cultured until confluence. Endothelial monolayers were then activated with TNF-α. Capillaries were stabilized by perfusion with binding buffer for one minute at a physiological laminar shear stress of 0,75-1 dyn cm^{-2}. Either PBMCs or PMNs (at 1 x 10^6 cells ml^{-1} in binding buffer) were then perfused through the capillary. Rolling and adhesion of cells were analyzed after 2 or 3 minutes, whereas firm adhesion and transmigration were analyzed at later time points. Analysis was performed by recording 10 fields using an inverted Olympus IX70 microscope. Finally, recorded cells were manually counted offline and effects of antibody treatment or cell line specific differences analyzed using two-tailed student's t-test with unequal variance.

5. RESULTS

5.1 HMEC-1 and TIME are hybrid vascular endothelial cells (I)

For the purpose of high-throughput screening experiments we were searching for suitable endothelial cell lines. The original publications reporting the two cell lines HMEC-1 and TIME have both together been cited over 800 times. Therefore we intended to use these cell lines as model systems for blood vascular endothelial cells.

However, flow cytometric analysis of HMEC-1 and TIME revealed that these cells simultaneously express markers for both blood and lymphatic endothelial cells. Interestingly, no separate subpopulations could be detected, but only one single homogenous one expressing both types of markers, such as PV-1, CD31, Podoplanin, LYVE-1 and CLEVER-1. This was confirmed by immunofluorescence stainings showing double-positive cells. However, flow cytometry only analyzes the expression of a limited number of genes. To get a more complete picture of the nature of HMEC-1 and TIME, we performed microarray analysis of their gene expressions and compared them to those of normal blood- and lymphatic endothelial cells.

Using a multidimensional scaling plot (MDS-plot) we showed that the genome wide expression pattern of HMEC-1 and TIME places them near to, but not among normal BEC- and LEC samples. The inclusion of prostate tissue allowed us to objectively assess the distances. In addition, several genes were differentially expressed between HMEC-1, TIME, BEC and LEC.

As gene expression analysis does not analyze functional properties we then continued to investigate those by analyzing the behavior of HMEC-1 and TIME during leukocyte transmigration. To that end we performed *in vitro* flow assays, which allow the evaluation of leukocyte rolling, adhesion and transmigration. Confirming the findings from our flow cytometric- and gene expression analyses, HMEC-1 and TIME did not behave like our normal blood vascular endothelial control (HUVEC).

Both rolling and adhesion of freshly isolated peripheral blood mononuclear cells (PBMC) were reduced by 60-80% when compared to HUVECs. Polymorphonuclear leukocytes (PMN) on the other hand exhibited impaired rolling and adhesion only on HMEC-1 but appeared to function normally on TIME. Similarly, total numbers of interacting PBMC (firmly adhering and transmigrating) were significantly reduced on both HMEC-1 and TIME. Interaction of PMN with endothelial cells was only reduced on HMEC-1, but not on TIME. Despite those differences, once firmly adhered, PBMC and PMN migrated with similar efficiencies across HMEC-1 and TIME as across HUVECs.

5.2 Analysis of BEC- and LEC specific gene expression using publicly available microarrays (I)

Previous studies searching for vessel specific markers often relied on microarray data to compare blood vascular and lymphatic vascular gene expression. However, these analyses were commonly done with freshly isolated cells from only a few individuals. The limited biological variation is an inherent problem of these analyses. As we already had compiled a list of BEC- and LEC datasets from the public domain we wanted to use these microarrays to identify new, stable markers for blood- and lymphatic endothelial cells.

To that end we compiled two different sets of data: Dataset A contained 14 BEC and 10 LEC samples, all run on the same Affymetrix platform (GPL570). The second set, Dataset B, included 33 BEC and 14 LEC samples. These 47 samples of Dataset B also included the 24 samples from Dataset A. The samples forming Dataset B were derived from 12 separate experiments run on three different Affymetrix platforms (GPL570, GPL571 and GPL5188). For the first comparison we used Dataset A as it does not suffer from inter-platform variation. In the second step we then compared Datasets A and B. Finally we relate our analysis of Dataset B to two published microarray studies investigating BEC- and LEC-specific gene expression (Petrova et al. 2002; Hirakawa et al. 2003).

Our initial comparison of Dataset A to Dataset B revealed only a limited overlap when choosing a threshold of ≥2-fold change in gene expression with a multiple hypotheses corrected p-value of ≤0,05 to define a significantly different expressed gene. In Dataset A we identified 27 BEC-specific genes, while Dataset B contained 28 genes that were significantly changed. 15 of these genes were common to both Datasets. When analyzing LEC-specific genes, we identified 13 in Dataset A and 28 in Dataset B. 10 of these genes appeared in both analyses.

The subsequent comparison of our results from Dataset B to two published studies reporting BEC- and LEC-specific genes resulted in surprising findings. Only a minor overlap of the results could be seen. Only two BEC-specific and three LEC-specific genes were identified in all three studies. Also the overlap between each two individual studies never reached 40 %. The three LEC genes found in all three analyses were the prototype markers PROX1 and podoplanin with the third gene identified as reelin. The BEC specific genes however did not contain any of the commonly used blood vascular markers. Instead the two genes identified in each of the three analyses were neuronal cell adhesion molecule (NRCAM) (Lane et al. 1996) and CXCL1 (Richmond et al. 1988).

Further analysis of our results using IPA software (Ingenuity Systems, www.ingenuity.com) revealed that most of the BEC- and LEC-specific genes identified in our study were mainly involved in the development of cardiovascular structures, tissues and organs (p-values from ≤0,05 - ≤0,0001). These findings support our approach and validate the results.

Building on the previous analyses of Dataset B we wanted to use the vessel type specific gene expression profiles to identify new, stable markers for BEC and LEC. To that end we selected genes that were uniquely identified in our analysis and not reported earlier as vascular markers. Finally we chose the protein melanoma cell adhesion molecule (MCAM) as blood vascular marker and collectin placenta 12 (COLEC12) as lymphatic marker. To test whether our *in silico* findings could be confirmed by independent *in vitro* experiments, we compared the staining pattern of our new markers with those of established BEC- and LEC-specific genes.

Indeed, MCAM positive staining correlated very well with the signal from the BEC-marker PV-1 (Niemela et al. 2005), whereas no colocalization could be detected with the LEC-markers CLEVER-1 (Irjala et al. 2003), LYVE-1 and podoplanin. Similarly, COLEC12 staining showed a high degree of colocalization with CLEVER-1, LYVE-1 and podoplanin stainings and mutually exclusive staining with PV-1. These independent experiments confirmed our *in silico* identification of new BEC- and LEC- specific markers.

5.3 Identification of the epitope recognized by the vascular marker PAL-E (II, III)

PAL-E has been used for over 25 years to identify endothelial cells of blood vascular origin. Due to its absence from lymphatic endothelial cells and strong, specific staining of endothelium of medium-sized veins, venules and capillaries (Schlingemann et al. 1985) PAL-E was considered a good marker. However, probably due to the limited range of applications that PAL-E can be used in, the identity of the epitope recognized by this antibody remained elusive for a long time. 20 years after its first description the antigen recognized by PAL-E was sequentially identified as a secreted form of vimentin (Xu et al. 2004), as PV-1 (Niemela et al. 2005) and finally as NRP-1 (Jaalouk et al. 2007). This study was carried out to clear the confusion surrounding the identity of the PAL-E epitope.

Considering the widespread expression of NRP-1 on non-vascular cells such as thymic epithelial cells, neuronal structures, T- cells and dendritic cells (Gu et al. 2003; Lepelletier et al. 2007) and longtime use of PAL-E as BEC marker we first wanted to compare the staining pattern of PAL-E and an antibody against NRP-1. We stained numerous tissues including peripheral lymph node, liver, thymus, brain (choroid plexus), tonsil and heart. As PAL-E staining is absent from brain tissue with a functional blood-brain barrier (Carson-Walter et al. 2005), we chose choroid plexus as a fenestrated brain tissue constitutively expressing the PAL-E antigen (Schulz and Engelhardt 2005).

No differences between the staining patterns of PAL-E and a PV-1 antibody could be detected in any of the analyzed tissues. Interestingly, also PV-1 and NRP-1

as well as PAL-E and NRP-1 co-stainings revealed a high degree of colocalization. However, in heart and liver significant differences could be detected. To confirm the vascular specificity of the stainings, we performed double stainings with an antibody against CD31 and PAL-E, anti-PV-1 and anti-NRP-1 antibodies. Indeed, all stained vessels were also positive for CD31. Only the anti-NRP-1 antibody bound to some non-vascular structures in the liver.

We then continued to use cells transfected with either a mock plasmid, PV-1 or NRP-1. Confirming the results from the earlier experiments, both PAL-E and the anti-PV-1 antibody bound to PV-1 transfected cells, whereas two different anti-NRP-1 antibodies exclusively recognized the NRP-1 transfected cells. Similarly, analysis of lysates from PV-1 transfected cells and tonsils by immunoblotting showed similar results. PAL-E and the anti-PV-1 antibody both recognized a band in the PV-1 transfected cell lysate and tonsil lysate. The anti-NRP-1 antibody however only bound to tonsil lysate. These experiments indicated that the target of PAL-E is PV-1 and not NRP-1.

A potential physical interaction between PV-1 and NRP-1 would offer an explanation for the identification of NRP-1 as target of PAL-E. To test this hypothesis we performed Co-IP. Using lysates from transfected cells and tonsil we could precipitate NRP-1 with an anti-PV-1 antibody and thereby confirm a physical interaction of PV-1 with NRP-1.

The second study, claiming that PAL-E recognizes a secreted form of vimentin actually reported their PAL-E antibody to be a mIgG2b, whereas the original clone is a mIgG2a. In addition to that, the expression of vimentin a wide variety of cell types made it an unlikely epitope recognized by the BEC marker PAL-E. This theory was confirmed in 2005 (Niemela et al. 2005). However, the expression pattern and localization of PV-1 would fit to a protein involved in transcellular migration. As vimentin has been shown to play a role in transcellular migration (Nieminen et al. 2006) we wanted to look at a potential connection between PV-1 and vimentin.

We first investigated the expression of PV-1 in vimentin deficient (*vim*$^{-/-}$) mice. While the expression pattern of PV-1 was not changed in *vim*$^{-/-}$ mice when compared to wild-type littermates, the expression levels of PV-1 in the vasculature of heart and spleen in *vim*$^{-/-}$ mice were reduced by ~20%. Similarly to tissue stainings, a colocalization of PV-1 with vimentin could also be detected on a cellular level in HUVECs. In resting cells, both PV-1 and vimentin were distributed evenly over the entire cell. However, after activation with TNF-α, PV-1 was redistributed to the peripheral areas where it partially colocalized with vimentin.

Considering the partial colocalization between PV-1 and vimentin and the effect of *vim*$^{-/-}$ on the expression levels of PV-1, this cytoskeletal protein could be physically linked to PV-1. Hence we performed Co-IPs to test this hypothesis. Indeed we managed to precipitate PV-1 from resting and activated HUVECs using an antibody against vimentin, thereby confirming a physical association of PV-1 with vimentin intermediate filaments.

These experiments established the identity of the antigen recognized by the prototype BEC marker PAL-E as PV-1. At the same time they revealed physical interactions between PV-1 and the intermediate filament protein vimentin as well as the angiogenic molecule NRP-1.

5.4 Function of the prototype BEC marker PAL-E (III)

The expression pattern, cellular localization, physical properties and interactions make an involvement of the PAL-E antigen PV-1 in leukocyte trafficking feasible. Therefore we investigated a potential function of PV-1 during transcellular migration of leukocytes.

5.4.1 *In vitro* analyses of PV-1 function (III)

We first performed end point transmigration assays in which endothelial cells are cultured and activated on glass coverslips. After the addition of leukocytes to the endothelial monolayer the cells are fixed, stained and the migration of leukocytes analyzed by microscopy.

Indeed we could detect lymphocytes crossing the endothelium via a transcellular pathway. Transendothelial pores/channels were formed with a diameter of ~7 μm in HUVECs and ~13-15 μm in HDMECs. These channels were outlined by rings of PV-1 and caveolin-1. In addition, vimentin fibers could be seen forming a mesh around extravasating lymphocytes. These results revealed a dynamic redistribution of PV-1 to the site of lymphocyte transmigration and partial colocalization with caveolin-1 and vimentin during this process. However, end point transmigration does not represent a physiological model of leukocyte TEM including shear stress. Therefore we continued to analyze PV-1 function during leukocyte diapedesis using *in vitro* flow assays.

In these experiments we wanted to analyze the effect of a function blocking PV-1 antibody on leukocyte extravasation under the influence of physiological shear stress. Our antibody against PV-1 did not affect the number of rolling or adhering PBMCs. However, transmigration of PBMCs was reduced by ~40%. As PMNs have been reported to use the paracellular pathway under these conditions (Yang et al. 2005), we tested, whether our antibody would also inhibit PMN TEM. Supporting our hypothesis of a role of PV-1 in transcellular migration, no effect of an anti-PV-1 antibody on PMN transmigration could be detected under these settings.

5.4.2 PV-1 function during leukocyte trafficking *in vivo* (III)

Finally, we wanted to investigate the effect of PV-1 blockage on leukocyte trafficking *in vivo*. To that end we performed two different in vivo inflammation models, namely peritonitis and air pouch model. During peritonitis, acute inflammation of the peritoneal cavity was induced by injection of proteose peptone with interleukin-1β. Subsequently, antibodies were injected into the tail-vein and 18 hours later, peritoneal lavages were collected and analyzed. Meca-32, an antibody against mouse PV-1 (Ioannidou et al. 2006) reduced the migration of leukocytes to the site of inflammation by ~85%. Detailed analysis of leukocyte subsets in the peritoneal lavage showed that the migration of neutrophils was reduced by ~65%, whereas the numbers of macrophages and lymphocytes were comparable to those in non-inflamed peritonea.

However, our negative control antibody in the peritonitis experiment was a non-binding one. Hence we performed a second *in vivo* experiment using a different model and including an endothelial-binding negative control antibody. This second model was induced by repeated subcutaneous injections of air and administration of CCL-21 and bovine serum albumin into the resulting air pouch. Here, PV-1 treatment reduced the numbers of leukocytes in the air pouch by ~38% when compared to endoglin antibody treated mice. Analysis of leukocyte subsets revealed a reduction in the numbers of granulocytes by ~44% and those of lymphocytes by ~56%, while numbers of macrophages were not affected. These experiments unambiguously demonstrated a crucial function of PV-1 during leukocyte trafficking to sites of inflammation.

6. DISCUSSION

6.1 Plasticity in the gene expression of primary- and immortalized endothelial cells (I)

The analysis of blood- or lymphatic vasculature specific processes often requires the use of cells from either vessel type. However, even though primary endothelial cells would represent the most physiological model system, immortalized cell lines are often used as replacements. Primary endothelial cells are difficult to isolate and cannot be grown for extended periods. Immortalized cell lines on the other hand are usually well characterized, easy to obtain and culture and usable for up to 40 passages or more.

However, when we analyzed the widely used immortalized endothelial cell lines HMEC-1 and TIME we noticed the expression of lymphatic markers on these cells. The original publications reporting these cell lines have been cited more than 800x. Even though aberrant protein expression and functional defects have been reported already in 2004 (Nisato et al. 2004) and 2007 (Oostingh et al. 2007), these cells remain a widely used model system for blood vascular endothelial cells (Cunha et al. 2010; DiMaio et al. 2011; Lu et al. 2012; Willer et al. 2012). It is well known that the human microvasculature consists of both blood- and lymphatic vessels. Therefore, isolated microvascular endothelial cells consist of a mixture of LEC and BEC. Considering that the original articles describing the generation of HMEC-1 and TIME do not mention any selection for BECs, it could be expected that the immortalized cell lines contain a mixture of both cell types. However, here we clearly show that HMEC-1 and TIME do not consist of a mixture of BEC and LEC, but rather one homogenous population of cells, expressing markers for blood- and lymphatic endothelium.

In order to comprehensively analyze the nature of the cell lines HMEC-1 and TIME we performed a microarray analysis of their gene expression and subsequently compared it to those of 14 BEC and 10 LEC. Indeed, the genome wide expression analyses confirmed the results from our flow cytometric analyses by showing that HMEC-1 and TIME cluster near to but separately from primary BEC- and LEC samples.

It has been previously established that the immortalization of cells can cause severe alteration in their gene expression profile (Boess et al. 2003; Murray et al. 2004; Boerma et al. 2006). In addition, differences in the response to cytokines and interaction with leukocytes have been reported (Lidington et al. 1999; Oostingh et al. 2007). However, not only immortalized endothelial cells can change their phenotype. Primary HUVECs for example have been shown to adapt a lymphatic like phenotype in *in vitro* angiogenesis assays depending on their interaction with the surrounding extracellular matrix environment (Cooley et al. 2010). This importance of the extracellular matrix for the maintenance of cell identity has also been reported elsewhere (Butcher et al. 2009). Even cell culture *per se* has been shown to have an

effect on the expression of cell-lineage specific genes (Amatschek et al. 2007; Wick et al. 2007).

However, changes in the lineage specific gene expression are not restricted to *in vitro* conditions. The expression of BEC- and LEC markers in the same cells can also be observed *in vivo*. During development, the lymphatic lineage marker PROX1 is expressed in a small number of embryonic vein endothelial cells. The initial expression of PROX1 in these cells triggers the upregulation of lymphatic specific genes and slow downregulation of blood specific genes, leading to the formation of the first lymphatic vessels (Adams and Alitalo 2007). A recent study found that LECs also express the cell fate regulating genes for arteries (NOTCH) and veins (COUP-TFII) in addition to the lymphatic marker PROX1 (Kang et al. 2010).

These facts might explain the differences between HMEC-1 and TIME on one hand and primary BEC and LEC on the other hand. In addition it should be mentioned, that our genome wide expression analysis probably overestimates the relatedness between HMEC-1, TIME, BEC and LEC. The MDS plot presents the clustering of the cells according to the expression of over 19 000 genes. However, previous reports found less than 400 genes to be differentially expressed between BEC and LEC (Petrova et al. 2002; Hirakawa et al. 2003). We could support this theory by demonstrating the expression of several genes such as rophilin 2, gremlin 1 and GRAM domain containing 3 in HMEC-1 and TIME, which are not usually found in BECs.

Previous studies of BEC- and LEC specific gene expression often relied on microarray analyses. A common problem of these studies is the limited biological variability. As pointed out above, the cell type specific expression profile can significantly vary. Therefore small sample numbers might cause the results to be skewed. Expanding our initial set of 14 BEC and 10 LEC microarray datasets, we finally assembled a set of 33 BEC and 14 LEC datasets, representing 12 independent experiments run on three different Affymetrix platforms. We argued that this approach would result in the identification of more stable markers. As our samples included cells from different origins, culture conditions and isolation protocols, genes would have to be consistently up- or downregulated to be identified as BEC- or LEC specific in our analysis.

Comparison of our restricted set of samples (Dataset A) with our extensive set (Dataset B) revealed only a limited overlap of the BEC- and LEC specific genes. For example, the increase in the number of LEC samples by 40% in Dataset B more than doubled the number of LEC-specific genes. However, most of the LEC markers identified in Dataset A were also present in Dataset B. This was not the case with BEC specific genes. A possible explanation for this might be the more homogenous nature of LECs when compared to BECs. When we compared our results from Dataset B with two earlier studies (Petrova et al. 2002; Hirakawa et al. 2003) analyzing BEC- and LEC specific gene expression by microarrays, we noticed several interesting points: Firstly, both the isolation of cells as well as the analysis of results differed significantly. While Hirakawa et al isolated LECs using $CD34^-$, $CD31^+$ cells, Petrova et al considered $CD31^-$, $PDPN^+$ cells as LECs. Furthermore, in the first study genes were considered as BEC- or LEC specific if they fulfilled the criteria of increased

expression with a p-value of ≤0,002. In the second report, genes whose expression level was changed 2-fold were classified as BEC- or LEC specific. Interestingly, Hirakawa et al used $CD34^-$, $CD31^+$ staining to isolate LECs even though it has been shown that both of these markers are found on both BEC and LEC (Pusztaszeri et al. 2006; Woodfin et al. 2007; Ordonez 2012). Moreover, in their results CD31 was found to be BEC specific (Hirakawa et al. 2003).

The identification of PDPN and PROX1 as LEC markers supports the validity of our approach. The third identified LEC specific gene, RELN, was shown to be involved in NOTCH-signaling (Forster et al. 2010). The crucial role of NOTCH-signaling during the specialization of BECs and LECs could explain this result. The most surprising finding was the lack of established BEC markers in the results. Neither of the two genes identified in all three studies as BEC specific is commonly used to identify blood vessel endothelium. NRCAM has been shown to interact with laminin (Grumet and Sakurai 1996), a protein found to be BEC-specific (Hirakawa et al. 2003). However, various laminin-isoforms have been shown to be expressed on a wide variety of tissues (Hallmann et al. 2005) including lymphatics (Pflicke and Sixt 2009). CXCL1 on the other hand can be induced by prostaglandin E2 and plays a role during angiogenesis by inducing microvascular cell migration and formation of tubules (Wang et al. 2006). As is the case with all BEC- and LEC markers, none of these genes is endothelium specific. However, they can be used to distinguish blood endothelial cells from lymphatic ones.

To validate our analysis we chose two genes not previously reported as BEC-or LEC markers and analyzed their vessel specific staining in human lymph nodes, chronically inflamed tonsils and malignant tissues. Indeed, staining with the new LEC marker COLEC12 as well as with the BEC marker MCAM correlated very well with the established markers PV-1, LYVE-1, podoplanin and CLEVER-1.

6.2. Identification of the antigen recognized by the prototype endothelial marker PAL-E (II, III)

One of the most widely used blood vascular marker has been the antibody PAL-E (Schlingemann et al. 1985). However, despite its widespread use, the antigen recognized by this antibody remained elusive for many years until the target was finally reported to be a secreted form of vimentin (Xu et al. 2004), PV-1 (Niemela et al. 2005) and a VEGFR-2 binding site within NRP-1 (Jaalouk et al. 2007).

The report of Xu and colleagues actually reported a mIgG2b isotype for their PAL-E clone (Xu et al. 2004), even though the original clone is a mIgG2a (Schlingemann et al. 1985). Therefore their results cannot be considered as valid. Jaalouk et al. on the other hand claimed that the identification of PV-1 as target of PAL-E is wrong, as PAL-E and an antibody against PV-1 failed to inhibit each other in competitive staining experiment (Jaalouk et al. 2007). However, this argument ignores the basic immunological fact that glycoproteins in general can have multiple epitopes.

Our histochemical stainings show that PV-1 colocalizes with NRP-1 and that the absence of vimentin reduces the concentration of PV-1 in endothelial cells. Even though NRP-1 has been shown to be expressed in numerous non-endothelial cell types (Gu et al. 2003; Lepelletier et al. 2007), there is evidence linking PV-1 and NRP-1 in endothelial cells. It is well known that NRP-1 is the coreceptor of VEGFR-2 and that their expressions as well as functions are closely connected (Giraudo et al. 1998; Soker et al. 1998; Petrova et al. 1999). Furthermore, VEGFR-2 has been shown to localize to luminal and abluminal surfaces as well as to the membranes of VVOs. The authors of this study also suggested that these structures could be involved in VEGF-A_{165}-induced vascular leakage (Teesalu et al. 2009). VEGFR-2 has also been found on caveolae and the stomatal diaphragms of VVOs (Dvorak and Feng 2001). Taking into account that PV-1 forms the stomatal diaphragms of VVOs and those of caveolae (Stan 2007), this brings NRP-1 in indirect contact with PV-1 with VEGFR-2 as mediator. Furthermore, VEGF-A_{165} has also been shown to directly regulate PV-1 expression via VEGFR-2 (Strickland et al. 2005; Hnasko et al. 2006). However, one study reported an upregulation of PV-1 expression (Strickland et al. 2005), whereas the second study showed a negative effect of VEGF-A on PV-1 (Hnasko et al. 2006). Nevertheless, our results together with these previously published studies suggest a functional link between PV-1 and NRP-1.

PV-1 forms the diaphragms of endothelial structures such as caveolae, fenestrae and TECs (Stan et al. 2004). In addition, PV-1 is a transmembrane protein and it has been suggested that it could be stabilized by scaffold proteins and a connection to the cytoskeleton (Stan 2007). Considering the reduced amounts of PV-1 in our *vim*$^{-/-}$ mice, it might be feasible that vimentin is the cytoskeletal component stabilizing PV-1 dimers in the plasma membrane. We tested a potential physical association of PV-1 with vimentin and NRP-1 using Co-IP. And indeed we managed to show that PV-1 can bind to both vimentin and NRP-1.

Put together, these findings demonstrate that the PAL-E antigen PV-1 can form complexes with NRP-1 and vimentin and thereby connect for the first time these proteins involved in leukocyte trafficking and angiogenesis.

6.3 Functions of the PAL-E antigen PV-1 (III)

Considering the facts known about PV-1, such as its association with vimentin and caveolin-1 as well as its expression in endothelial cells and function in diaphragm formation, PV-1 could be involved in leukocyte transcellular migration. This was the hypothesis we wanted to investigate.

Immunofluorescence stainings demonstrated an interesting redistribution of PV-1 and caveolin-1 towards the cell borders in TNF-α activated endothelial cells, where PV-1 also partially colocalized with vimentin. This behavior might be a preparation for the adhesion and migration of leukocytes in inflammation. Endothelial cells under flow exhibit an elongated form, leading to attachment of leukocytes near to cell borders (Dewey et al. 1981). Furthermore, transcellular migration has been shown to

occur preferably near endothelial junctions. Near their borders, endothelial cells are often only 200 nm thick, thereby acting as a path of least resistance for migrating leukocytes. Hence the finding that 60% of transcellular pores formed within 1 µm of cellular junctions (Chamberlain and Lichtman 1978) is not surprising. The redistribution of PV-1 and caveolin-1 also indicates an accumulation of "free" plasma membrane in the cell periphery. Thereby, endothelial cells could actively facilitate the probing of leukocytes with podosomes and finally the formation of a transendothelial pore (Feng et al. 2002; Carman and Springer 2004). This preparation for regulated membrane fusion is also supported by additional reports demonstrating the concentration of caveolar proteins in addition to fusogenic proteins (VAMP-2 and 3, SNARE) (Millan et al. 2006; Carman et al. 2007) around migrating leukocytes. These findings also fit to the results of our end point transmigration studies, showing an accumulation of PV-1, caveolin-1 and vimentin around transmigrating leukocytes.

However, so far our results were only indicating a potential involvement of PV-1 in leukocyte TEM. Hence we performed *in vitro* flow assays to determine the actual effect of a blockage of PV-1 by antibodies. At the same time, these experiments would pinpoint the actual step during which PV-1 exerts its function. The results clearly indicated that PV-1 did not have any effect during leukocyte tethering, rolling or firm adhesion. However, the transmigration of lymphocytes was significantly reduced after administration of a function blocking anti-PV-1 antibody. Neutrophil TEM was not affected. As neutrophils are known to cross TNF-α activated HUVECs after 4 hours preferably via a paracellular pathway (Yang et al. 2005), this suggests an involvement of PV-1 during transcellular migration of leukocytes.

The final missing step was the analysis of PV-1 function *in vivo*. To that end we performed two independent experiments. Peritonitis is a model of acute peritoneal inflammation. Under these settings, our anti-PV-1 antibody reduced the influx of leukocytes to the site of inflammation by ~85%. Surprisingly, migration of neutrophils to the peritoneal cavity was reduced by ~65%. Neutrophils are among the first cells to arrive at the inflamed tissue and are thought to reach this location via paracellular migration. However, analysis of neutrophil TEM across TNF-α activated HUVECs showed that migration after 4 hours of activation occurs predominantly via a paracellular pathway, whereas this shifts after 24 hours of activation to a mostly transcellular TEM (Yang et al. 2005). In our model of peritonitis, inflammation is induced 18 hours before analysis of leukocytes in the peritoneal lavage. This might explain the effect of our anti-PV-treatment on neutrophil intravasation *in vivo*. The 18 hour time point also allowed slower moving leukocytes such as monocytes and lymphocytes to reach the site of inflammation. The numbers of these cells in the peritoneal lavage were comparable to those of non-inflamed peritonea, indicating almost complete blockage of migration of monocytes and lymphocytes to the site of inflammation.

To confirm these results in an independent *in vivo* model, we performed an air pouch model to simulate a peripheral inflammation. A weakness of the peritonitis model was the fact that the negative control antibody did not bind to the mouse endothelium. Hence we wanted to use a binding negative control antibody for our air

pouch model. However, identifying an antibody that binds specifically to mouse endothelium but has no potential effect on leukocyte trafficking is challenging. Finally we chose the endoglin antibody MJ7/18 as our negative control. Blocking of PV-1 in these settings reduced the number of leukocytes migrating to the air pouch by ~38% when compared to the endoglin antibody. Indeed, our negative control antibody MJ7/18 also reduced the number of leukocytes in the air pouch by ~28% when compared to our non-binding control antibody. Even though this effect of the endoglin antibody did not reach statistical significance, it indicates that the efficiency of our PV-1 antibody treatment might be underestimated. The effect or our endoglin antibody might be explained by previous reports showing endoglin to be upregulated during inflammation and preferably so in cells associated with infiltration of inflammatory cells (Torsney et al. 2002). Furthermore, endoglin has been shown to be involved in TGF-ß signaling (Lee et al. 2008). TGF-ß signaling in turn is known to cause impaired leukocyte adhesion and transmigration due to inhibition of E-selectin and IL-8 expression (Gamble and Vadas 1988; Cai et al. 1991; Gamble et al. 1993; Pintavorn and Ballermann 1997).

Two recent publications demonstrated embryonic lethality in PV-1$^{-/-}$ mice due to lack of diaphragms and impaired vascular barrier function resulting in vascular leakage (Herrnberger et al. 2012; Stan et al. 2012). These results also support our findings. The location and structure of PV-1 would suggest a “gatekeeper”-role and administration of anti-PV-1 antibodies could interfere with this function by blocking required interactions of endothelial PV-1 with potential ligands. As a result, endothelial structures such as VVOs and caveolae, which are implicated in the transcellular exchange of liquids, macromolecules and cells would remain closed (Feng et al. 1999; Dvorak and Feng 2001; Millan et al. 2006; Carman and Springer 2008).

6.4 Implications and future perspectives (I-III)

In the following paragraphs I would like to speculate about potential implications and future applications of my work.

The two cell lines HMEC-1 and TIME are widely used (Ades et al. 1992; Venetsanakos et al. 2002). The original publications describing the creation of these cell lines have been cited over 800 times (March 2013). Surprisingly, they continue to be used as vascular model systems, even though it has been shown already in 2007 that these cells exhibit an impaired induction of adhesion molecules (Oostingh et al. 2007). Combining our comprehensive analysis with this earlier report clearly indicates that these cells are not suitable for use as blood vascular models. As a consequence, results relying exclusively on experiments carried out using these cell lines should be carefully re-evaluated. In the future it would be interesting to elucidate the mechanisms underlying the conversion of differentiated endothelial cells into a developmentally earlier stage.

To that end it might be useful to establish immortalized endothelial cell lines from mixed and pure populations of blood and lymphatic endothelial cells using telomerase- and large T antigen immortalization procedures. Subsequently, the phenotype and gene expression profile of these cell lines could be followed at regular time points. Alternatively, the roles of well-known cell-fate transcription factors in HMEC-1 and TIME could be studied using siRNA.

In addition, our analysis of microarray studies identifying BEC- or LEC specific genes revealed surprisingly large differences in the results of previous studies (Petrova et al. 2002; Hirakawa et al. 2003). This finding could have implications on many microarray analyses relying on a very limited amount of biological samples. At the same time our list of new, stable BEC- and LEC-specific markers could prove useful for clinicians and pathologists who need to distinguish between vessels of blood- and lymphatic origin on a daily basis.

Even though our approach of combining numerous endothelial gene expression profiles might be a step in the right direction, the selection and number of samples in our dataset is still limited. Considering the plethora of vascular beds such as arteries, capillaries, veins, fenestrated, continuous, discontinuous as well as afferent- and efferent lymphatics etc., there is still an obvious need for isolation and analysis of different endothelial cells from human and mouse to gain a truly comprehensive understanding of the characteristics and differences of the various vascular beds.

My studies investigating the identity of the antigen recognized by the prototype endothelial marker PAL-E might have implications for future studies in the fields of angiogenesis, tumor biology and leukocyte trafficking as they demonstrate a direct interaction between NRP-1 and PV-1.

This connection between PV-1 and NRP-1 could be further investigated in vivo using conditional PV-1- or NRP-1 deficient mice. Alternatively, knock-down of either one or both molecules by siRNA in cultured endothelial cells might enable the analysis of potential effects of PV-1 deficiency on angiogenesis or the effects a lack of NRP-1 might have on the formation of endothelial diaphragms and leukocyte trafficking.

In combination with our results showing a role for PV-1 during leukocyte trafficking, these studies suggest a potential use for an anti-PV-1 antibody in the treatment of cancer and chronic inflammation.

It has been shown that cancer cells can use the same molecules as leukocytes to extravasate from blood vessels (Konstantopoulos and Thomas 2009). For example, selectins have the capacity to bind sialyl Lewis x and sialyl Lewis a tetrasaccharides. While sialyl Lewis x is present on most leukocytes and some endothelial cells, its isomer sialyl Lewis a can be found on tumor cells but not on leukocytes (Stone and Wagner 1993; Mannori et al. 1995; Laubli and Borsig 2010). Further studies revealed that the presence of sialyl Lewis x/a tetrasaccharides on the surface of tumor cells

generally correlates with a poor prognosis due to increased formation of metastases (Nakayama et al. 1995; Sato et al. 1997; Kannagi et al. 2004). In addition to selectins, integrins, another family of molecules involved in leukocyte trafficking, have been shown to have an important role for cancer metastasis (Felding-Habermann et al. 2001; Bakewell et al. 2003).

By showing that PV-1 can physically interact with NRP-1, we connected our leukocyte trafficking molecule to the process of angiogenesis. As the VEGF-VEGFR axis is a major target for current cancer therapies (Ferrara 2009; Rapisarda and Melillo 2012), an antibody targeting PV-1 might be able to simultaneously affect cancer cell extravasation from the blood vessels and tumor angiogenesis.

However, before an anti-PV-1 antibody could be considered for therapeutic purposes, the role of PV-1 during leukocyte trafficking and its potential effect on angiogenesis need to be carefully studied. In addition, potential side-effects of anti-PV-1 antibodies on processes such as vascular permeability or angiogenesis in healthy organs need to be investigated.

To answer some of the above mentioned questions and to study the various functions of PV-1 we have generated full and conditional PV-1 knock-out mice in the lab. Together with sophisticated microscopy approaches such as intravital microscopy, laser scanning confocal microscopy and stimulated emission depletion microscopy we hope to elucidate the roles PV-1 might play in leukocyte homing, tumor growth and angiogenesis.

7. SUMMARY

The blood vasculature and lymphatic vasculature are two fundamental systems on which our body is built upon. Both derive from the same precursor cells and share many characteristics. Despite these structural and developmental similarities, both vascular trees exhibit significant differences in their gene expression and consequently their phenotypic- as well as functional properties. However, it is surprising that the differences between lymphatic- and blood vascular endothelial cells can be traced to the differential expression of less than 400 genes (Petrova et al. 2002; Hirakawa et al. 2003; Wick et al. 2007). This number represents only ~1,2 - 1,5% of the genes in the human genome (Consortium 2004). Despite these minor differences in the gene expression between BEC and LEC, the distinction between vessels of lymphatic origin and blood origin is crucial in vascular biology, cancer research, pathology and angiogenesis.

In these studies we investigate the difference in the gene expression between BEC and LEC for the first time on a large scale. At the same time we comprehensively show that the two widely used endothelial cell lines HMEC-1 and TIME do not represent physiological models of primary blood endothelial cells. These results demonstrate a high degree of plasticity in the cell type specific gene expression. Building on our large scale analysis we identify new, arguably more stable BEC- and LEC specific genes and show that they can be used to distinguish between the two vessel types *in vitro*.

We then continue to investigate the mystery surrounding the identity of the epitope recognized by the prototype blood vascular marker PAL-E. Despite its widespread use for over 20 years and more than 240 studies citing the original publication describing this marker (Schlingemann et al. 1985), its identity remained elusive for decades and was then claimed to be vimentin, PV-1 and NRP-1. Here we finally resolve this confusion by unambiguously demonstrating the target of PAL-E to be PV-1. At the same time we could show a connection between PV-1, vimentin and NRP-1, thereby providing proof for a connection between PV-1 and the cytoskeleton. As our results clearly associate PV-1 and NRP-1, this also offers evidence of a connection between the processes of angiogenesis and leukocyte trafficking (Figure 7).

Finally we identify a role for the antigen recognized by the prototype BEC marker PAL-E by showing its importance in the process of leukocyte trafficking. Several lines of indirect evidence also allowed us to pinpoint the function of PV-1 more specifically to the transcellular pathway of leukocyte diapedesis.

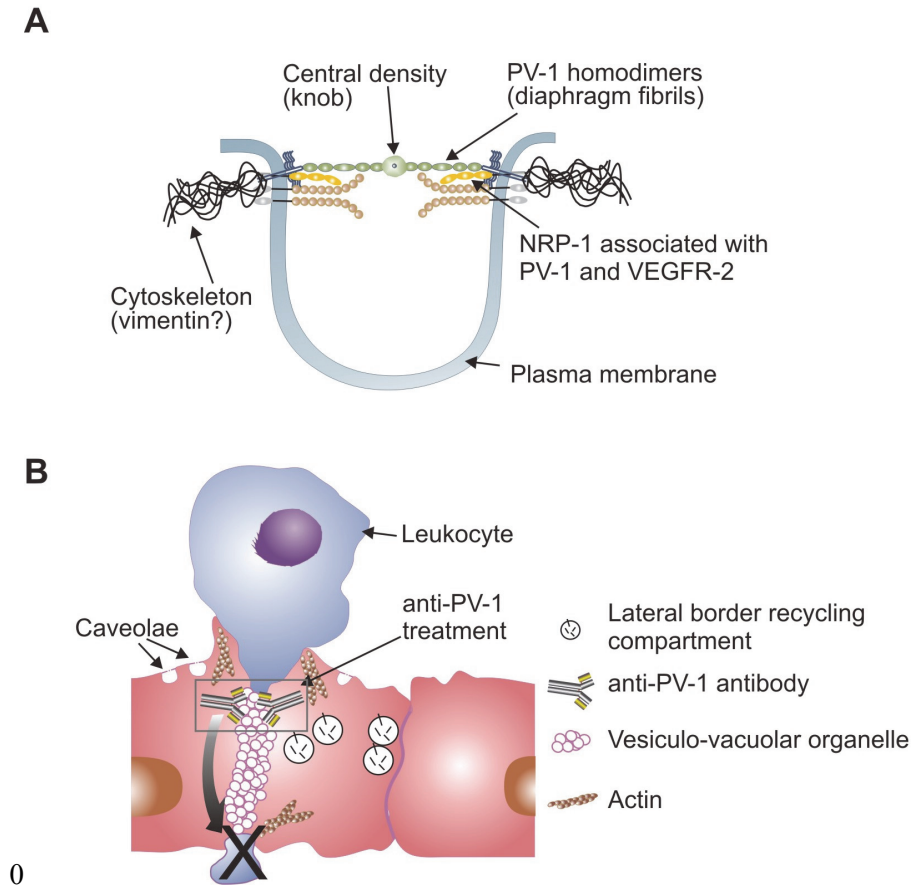

Figure 7: Results at a glance. A) A modified model of PV-1 insertion into the plasma membrane, connection to the cytoskeleton and formation of diaphragms by PV-1 homodimers. Original model suggested by Stan RV. (Stan 2007). **B)** Administration of antibodies against PV-1 prevents leukocyte transendothelial migration through a yet unknown mechanism.

In conclusion, these studies characterize the gene expression profiles of the widely used (>800 citations) endothelial cell lines HMEC-1 and TIME as well as those of primary BEC and LEC. Building on these results we identified a catalog of new, stable markers that can be used for the distinction between vessels of lymphatic- and blood origin. We also solve the confusion surrounding the identity of the antigen recognized by the BEC marker PAL-E and finally managed to identify a physiological function for the protein PV-1 during leukocyte trafficking. These results suggest that PV-1 could be a valid target for anti-inflammatory therapy or maybe even treatment of cancer metastasis.

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