Control of vascular integrity via endothelial growth factor and integrin cell adhesion receptor pathways

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To my family

"Nothing has such power to broaden the mind as the ability to investigate systematically and truly all that comes under thy observation in life"
-Marcus Aurelius

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred throughout the text by their roman numerals (I, II). Original publications have been reproduced at the end of the thesis with the permission of the copyright holders.

I Endothelial destabilization by angiopoietin-2 via integrin β1 activation

Laura Hakanpää, Tuomas Sipilä, Veli-Matti Leppänen, Prson Gautam, Harri Nurmi, Guillaume Jacquemet, Lauri Eklund, Johanna Ivaska, Kari Alitalo and Pipsa Saharinen.
Nat. Commun. 6: 5962, 2015.

II Targeting β1-integrin inhibits vascular leakage in endotoxemia

Laura Hakanpää, Elina A. Kiss, Guillaume Jacquemet, Ilkka Miinalainen, Lauri Eklund, Johanna Ivaska and Pipsa Saharinen.
Proc. Natl. Acad. Sci. U.S.A. 115: E6467-E6476, 2018.

ABBREVIATIONS

ARDS	acute respiratory distress syndrome
ANGPT	angiopoietin
ALI	Acute lung injury
Akt	PKB, protein kinase B
BEC	blood microvascular endothelial cell
BM	basement membrane
cAMP	cyclic adenosine monophosphate
CARS	compensatory anti-inflammatory response syndrome
Cdc24	cell division control protein 24
CHO	chinese hamster ovary
CLP	cecal ligation and puncture
CNV	choroidal neovascularization
DAMP	danger associated molecular pattern
DLC1	Deleted in liver cancer 1
DME	diabetic macular edema
E	embryonic day
EC	endothelial cell
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase 3
ERK	extracellular signal-regulated kinase
FAK	Focal adhesion kinase
F-actin	filamentous actin
FGD5	FYVE, RhoGEF and PH domain containing 5
FLD	fibrinogen like domain
FN	fibronectin
FOXO1	forkhead box protein O1
GEF	Guanine nucleotide exchange factor
GPCR	G-protein coupled receptor
GTPase	guanosine triphosphatase
HeLa	Henrietta Lack's epithelial cell line
HIF	hypoxia inducible factor
HUVEC	human umbilical vein endothelial cell
HPMEC	human pulmonary microvascular endothelial cells
ICAM	intercellular adhesion molecule
IL-1β	interleukin-1β
IL-6	interleukin-6
LDL	low-density lipoprotein
LDV	leucine-aspartate-valine
LLC	Lewis lung carcinoma cell line
LNM-35	NCI-H460-LNM35 carcinoma cell line
LPS	lipopolysaccharide
MLC	myosin light-chain
MLCK	myosin light-chain kinase
MLCP	myosin light-chain phosphatase
PAMP	pathogen associated molecular pattern
PDGF	platelet derived growth factor
PECAM1	platelet and endothelial cell adhesion molecule 1, also CD31
PI3K	phosphoinositide 3 kinase

P	postnatal day
PDR	proliferative diabetic retinopathy
PROX1	prospero homeobox protein 1
PRR	pathogen recognition receptor
Rab5	Ras-related protein
Rac1	Rac family small GTPase 1
Rap1	Ras-related protein 1
RGD	arginine-glycine-aspartic acid
RhoA	Ras homolog gene family, member A
ROCK	Rho-associated protein kinase
ROS	reactive oxygen species
SHARPIN	SHANK associated RH domain interacting protein
S1P	sphingosine 1 phosphate
SIRS	systemic inflammatory response syndrome
Src	proto-oncogene tyrosine-protein kinase
TGFβ	transforming growth factor β
TEM	transmission electron microscopy
TIE1	tyrosine kinase with Ig and epidermal growth factor homology domains
TIE2	TEK receptor tyrosine kinase
TNF-α	tumor necrosis factor alpha
VCAM -1	vascular cell adhesion molecule 1
VE-cadherin	vascular endothelial cadherin (official name Cadherin-5)
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VE-PTP	vascular endothelial cell specific phosphotyrosine phosphatase (official name protein tyrosine phosphatase receptor type B)
nAMD	neovascular age-related macular degeneration
ZO-1	zonula occludens 1

ABSTRACT

Vascular integrity is essential for proper vessel function, and for the maintenance of tissue and organ homeostasis. Endothelial cells (ECs) in the inner lining of the blood vessels form a barrier that dynamically regulates permeability across the vessel wall. Permeability via EC-EC junctions is transiently increased during inflammation, whereas abnormally or persistently elevated EC permeability promotes disease pathogenesis. For example, in sepsis, systemic capillary leakage compromises blood perfusion, and may lead to hypovolemic shock and multiorgan failure. Despite the significant amount of research on the mechanisms that control the EC barrier, no targeted therapies currently exist to seal the leaky vessels and maintain tissue perfusion.

The aim of this study was to investigate how vascular permeability is controlled via an EC-derived growth factor angiopoietin-2 (ANGPT2), which is upregulated in various human diseases, including sepsis. ANGPT2 was found to signal via β1-integrin, and therefore the function of endothelial β1-integrin in vascular permeability was investigated. The results identified a novel signaling pathway, where ANGPT2–β1-integrin signaling promotes EC permeability. β1-integrin was found to play a previously uncharacterized role in inflammation-induced vascular permeability, and an antibody against β1-integrin inhibited vascular leakage, improved EC junction integrity and protected from cardiac failure in LPS-induced murine endotoxemia.

Earlier studies have shown that ANGPT2 destabilizes blood vessel integrity in a context-dependent manner via its classical receptor TEK receptor tyrosine kinase (TIE2) on ECs. These studies have raised interest on ANGPT2 as a potential target in various diseases, including cancer and ocular neovascular diseases. This study revealed that ANGPT2 can promote EC destabilization independently of TIE2, which is downregulated during inflammation. These results suggest that a better understanding of the signaling function of ANGPT2 is necessary, in order to optimally target ANGPT2 in disease.

This study also highlights the crucial role of endothelial β1-integrin in controlling inflammation-induced EC permeability. The results showed that various inflammatory agents induced EC monolayer destabilization via β1-integrin, manifested by the loss of junctional VE-cadherin, the formation of actin stress fibers, and altered EC-extracellular matrix (ECM) adhesions. The EC-ECM adhesions that formed in inflammation were elongated fibrillar adhesions that can be distinguished from focal adhesions by the presence of the adapter protein tensin-1. Furthermore, β1-integrin promoted inflammation-induced EC contractility and reduced the EC barrier function. Importantly, targeting β1-integrin using a monoclonal antibody, or via a heterozygous genetic deletion in the endothelium of gene-targeted mice decreased vascular leakage in LPS-induced murine endotoxemia. Notably, the β1-integrin antibody was effective both as a prophylactic and as an intervention therapy, administered after the onset of systemic inflammation and vascular leakage, and its mechanism of action was independent of attenuating systemic inflammation, and of the vascular stabilizing function of TIE receptors.

In summary, this thesis provides new knowledge on the mechanisms that lead to vascular leakage via ANGPT2 and β1-integrin. β1-integrin was identified as a potentially universal regulator of EC permeability. A major finding was that targeting the EC β1-integrin in a preclinical model of sepsis decreased vascular leakage, thereby improving cardiac function. The results of this thesis call for further studies in evaluating the translational potential of β1-integrin mediated vascular permeability.

TIIVISTELMÄ

Verisuoniston oikeanlainen toiminta on välttämätöntä kudosten ja elinten toiminnalle. Verisuonten sisäpinnan endoteelisolukerros muodostaa verisuonten sisäseinämän ylläpitäen kudosten tasapainoa ja immuunivastetta. Verisuonten endoteelisolukerroksen läpäisevyys lisääntyy tulehdusreaktiossa sekä monissa sairauksissa. Esimerkiksi sepsiksessä kapillaariverisuonten vuoto aiheuttaa nesteen kertymistä kudoksiin ja samalla heikentää veren virtausta ja kudosten hapen saantia, mikä voi johtaa septiseen sokkiin ja monielinvaurioon. Mittavasta tutkimustiedosta huolimatta kapillaarivuotoon ei toistaiseksi ole lääkettä, ja uusia keinoja verisuonivuodon estoon sairauksissa tarvitaan kipeästi. Tässä väitöskirjatutkimuksessa löydettiin uusi signaalinvälitysreitti, joka lisää verisuonten läpäisevyyttä tulehduksen yhteydessä. Prekliinisissä kokeissa verisuonten vuotoa pystyttiin estämään hiiren sepsis-mallissa.

Tutkimuksen tarkoituksena oli selvittää niitä solutason mekanismeja, jotka säätelevät endoteelisolujen läpäisevyyttä ja miten verisuonivuotoa voidaan estää prekliinisessä sepsis-mallissa. Tutkimus keskittyi erityisesti selvittämään endoteelisolujen angiopoietiini-2 (ANGPT2)-kasvutekijän sekä soluadheesioproteiinien, integriinien, merkitystä endoteelisolujen liitosten purkautumisessa ja verisuonivuodossa tulehduksessa.

Tutkimuksessa löysimme uuden signaalinvälitysreitin, joka lisää endoteelisolukerroksen läpäisevyyttä. Endoteelisolujen väliset liitokset heikentyivät sekä ANGPT2- että β1-integriini-välitteisen viestinnän seurauksena. Havaitsimme, että tulehduksen välittäjäaineet aktivoivat β1-integriinin toimintaa, mikä johti soluliitosten heikkenemiseen. Lisäksi osoitimme, että tulehduksen aiheuttama verisuonivuoto oli vähäisempää hiiren sepsismallissa, jos toinen β1-integriiniä koodaava alleeli oli poistettu hiiren endoteelisoluista tai jos β1-integriinin toimintaa estettiin vasta-aineella.

ANGPT2-kasvutekijän ennestään tunnetut tehtävät välittyvät endoteeliperäisen TIE2-reseptorin kautta. ANGPT2:n määrä lisääntyy monissa sairauksissa, ja ANGPT2 on lääkekehityksen kohteena erityisesti silmän verisuonisairauksien hoitoon. Tämä tutkimus osoitti, että ANGPT2 lisää endoteelisoluviljelmän läpäisevyyttä myös ilman TIE2-reseptoria, jonka määrä laskee tyypillisesti tulehduksessa.

Työssä kuvasimme miten endoteelin läpäisevyys nousee ANGPT2-välitteisesti muuttuneen β1-integriinin toiminnan seurauksena. Huomasimme, että ANGPT2–β1-integriini-signaalinvälitys johti endoteelisoluliitosten heikkenemiseen, aktiinitukirangan muutokseen stressisäikeiseksi, sekä uudentyyppisten soluadheesioiden muodostumiseen lisäten endoteelisolukerroksen läpäisevyyttä tulehduksessa. Pre-kliinisessä sepsis mallissa β1-integriiniä estävä vasta-aine vähensi merkittävästi verisuonivuotoa sekä estohoitona että interventiohoitona, kun vasta-aine annosteltiin systeemisen tulehduksen ja verisuonivuodon jo alettua. Vasta-ainehoito myös kohensi endoteelisoluliitoksia ja ehkäisi sepsiksen aiheuttamaa sydämen vajaatoimintaa. Mekanistisesti, β1-integriini-vasta-aineen verisuonia parantavat vaikutukset eivät johtuneet yleisestä tulehduksen laskusta.

Yhteenvetona voidaan todeta, että tässä väitöskirjassa esitetään uutta tietoa endoteelisolujen läpäisevyyteen ja verisuonivuotoon johtavista mekanismeista. Tässä työssä kuvataan β1-integriini endoteelisolujen läpäisevyyden säätelijäksi. ANGPT2 aktivoi β1-integriinin johtaen adheesiomuutoksiin ja endoteelin läpäisevyyden nousuun. Lyhytaikainen β1-integriini-vasta-ainekäsittely sekä yhden β1-integriini-alleelin poisto vähensivät sepsiksen aiheuttamaa verisuonivuotoa ja suojasivat sepsiksen aiheuttamalta sydämen vajaatoiminnalta hiirimallissa. Väitöskirjatyön tutkimustuloksilla voi olla merkitystä kehitettäessä verisuonia vakauttavia hoitoja.

INTRODUCTION

Blood and lymphatic vasculatures span throughout the body maintaining tissue oxygenation and fluid homeostasis. The blood vessels consist of endothelial cells (EC) that form the inner layer of the blood vessels, mural cells that cover the endothelium, and an extracellular matrix (ECM) surrounding the vessels. The ECs form a semi permeable barrier with vascular bed specific sieving activity (Monahan-Earley et al., 2013). The EC barrier is regulated by the cellular actin cytoskeleton and adherens and tight junctions that connect ECs together, as well as EC adhesion to the underlying basement membrane (BM). Integrin cell adhesion receptors serve to transport signals from cells to the ECM and *vice versa*. They also anchor ECs to the BM and mediate connections of junctional proteins to the actin cytoskeleton (Cerutti and Ridley, 2017).

Acute and chronic inflammation increase fluid leakage and inflammatory cell infiltration into the tissues through the EC barrier. When acute inflammation turns chronic, vessel undergo profound changes in their structure and function (Claesson-Welsh, 2015). In systemic inflammation such as sepsis, the overwhelming inflammatory response causes capillary leakage leading to decreased blood volume and shock. Tissue edema and hypoxia contribute to subsequent organ failure. Mortality of patients with sepsis remains high. 30 million patients are estimated to develop sepsis annually, with an estimate of 6 million deaths from septic shock (Gyawali et al., 2019). In case of microbial sepsis, rapid initiation of antibiotic use is crucial. However, when septic shock develops, current treatments can be ineffective, and as of now, no targeted therapies exist to correct the capillary leakage (Gyawali et al., 2019).

Two major growth factor receptor signaling systems regulate vascular morphogenesis and EC functions: Vascular endothelial growth factor (VEGF)–VEGF receptor (VEGFR) and the angiopoietin (ANGPT)–TIE growth factor receptor systems. An important function of the ANGPT1–TIE2 signaling system is the maintenance of vascular stability, whereas ANGPT2, which is produced by activated ECs in vascular diseases, functions as a context dependent agonist/antagonist for TIE2 (Saharinen et al., 2017a).

Circulating ANGPT2 levels are increased in various vascular diseases, including sepsis, where high ANGPT2 levels correlate with poor patient prognosis (Leligdowicz et al., 2018). In addition, preclinical results have demonstrated an essential role of ANGPT2–TIE2 signaling in vascular destabilization and leakage (Eklund et al., 2017). The current work aimed to discover molecular mechanisms that lead to EC destabilization. We sought to elucidate how ANGPT2 and integrins are involved in EC destabilization, and how this leads to vascular leakage.

We found that ANGPT2 and β1-integrin decreased EC stability in inflammation and in ECs where TIE2 levels were decreased. In inflammation, ANGPT2 supported the formation of β1-integrin and tensin-1-positive fibrillar matrix adhesions and actin stress fibers, and decreased VE-cadherin in EC junctions. ANGPT2–β1-integrin signaling promoted stress fibers via intracellular ERK and RhoA-ROCK signaling pathways. Moreover, β1-integrin mediated inflammation-induced EC contractility and permeability. Notably, both β1-integrin antibodies or a heterozygous EC-specific deletion of β1-integrin decreased vascular leakage in a preclinical murine sepsis (endotoxemia) model. In addition, β1-integrin antibodies protected from endotoxemia-induced cardiac failure and improved EC junction integrity. Discoveries in this work shed light onto why elevated ANGPT2 levels are harmful in vascular leakage syndromes, and suggest that endothelial β1-integrin acts as a mediator of EC permeability. These findings may have translational impact for regulation of vascular stability and leakage.

REVIEW OF THE LITERATURE

1. Blood and lymphatic vascular systems

The vasculature comprises of blood vascular and lymphatic vascular systems, which carry blood and lymph throughout the body sustaining physiological functions, respectively. However, the blood and lymphatic vessels are also involved in disease (Monahan-Earley et al., 2013).

1.1 Blood vascular system

The cardiovascular system in vertebrates is a closed system where blood leaves and enters the circulation via the heart. The cardiovascular system is the first organ system to develop during embryogenesis, and its correct function ensures the functioning of the gas-exchange system of the lungs (Monahan-Earley et al., 2013).

1.1.1 Structure and development of the blood vasculature

The inner layer of the blood vessel wall consists of a single EC layer. The ECs are flat in shape, however, depending on the vessel size, EC thickness can vary 100-fold in the human body (Florey, 1966). One example are the cubic ECs of the post-capillary high endothelial venules that aid lymphocyte circulation from the blood stream to lymph nodes (Miyasaka and Tanaka, 2004).

The blood vasculature is a hierarchic network of vessels with different functions. Arteries and arterioles transport oxygen rich blood from the heart to tissues, and veins and venules return carbon dioxide rich blood to the heart, destined to small circulation of the lungs followed by gas-exchange in the alveolar capillaries. The capillaries also connect peripheral arterioles and venules in the tissues (Monahan-Earley et al., 2013). The endothelium of arteries and veins is covered by a smooth muscle cell layer. The endothelial and smooth muscle cells are embedded in the extracellular matrix (ECM). The ECM plays a major role in vessel development, growth and maturation. Approximately 300 genes code for ECM proteins, consisting of 200 different glycoproteins, over 30 proteoglycans and over 40 collagens that assemble into various ECMs. The ECM provides important signaling cues during vascular development and supports functions of the mature vessels by interacting with EC surface receptors (Hynes and Naba, 2012). The inner EC layer of arteries and veins is called *tunica intima*, the smooth muscle cell layer is called *tunica media*, and the outermost ECM layer is called *tunica adventitia*. *Tunica adventitia* connects the vessels to the surrounding tissues and organs (Mazurek et al., 2017). Especially in arteries, the *tunica media* and *tunica adventitia* contain fibers that provide elasticity and support the vessel structure under high pressure. Veins have thinner wall structures than arteries and valves to prevent back flow of the blood (Udan et al., 2013).

Capillaries serve to release oxygen, hormones and nutrients into tissues, and respond to angiogenic signals (Augustin and Koh, 2017). Only one single red blood cell can pass through the capillaries at a time. The capillaries are covered by pericytes in a vascular bed and tissue specific manner (Mazurek et al., 2017). The capillaries are embedded in a specialized ECM, the basement membrane (BM), which offers mechanical support to the vessels (Marchand et al., 2019). The BM mainly consists of collagen IV and laminin, forming a network supported by nidogen. The major proteoglycan in the vascular BM is perlecan (Thomsen et al., 2017). In angiogenic vessels, in disease and during injury, the BM composition is altered and a provisional matrix, which is rich in plasma fibronectin (FN), is formed. The early provisional matrix matures into late provisional matrix, enriched with FN produced by the ECs (Barker and Engler, 2017).

The luminal side of the endothelium is lined with a glycocalyx that consists of proteoglycans, glycoproteins, glycosaminoglycans, and plasma proteins. The thickness of the glycocalyx varies depending on the vascular bed, and is important for maintenance of the vascular barrier and other vascular functions (Uchimido et al., 2019).

During embryogenesis, the vascular system initially forms via vasculogenesis. Angioblasts, or the precursors of ECs that originate from extraembryonic and embryonic mesoderm, migrate to the sites of blood islands to form the first vessels, a primary capillary plexus (Adams and Alitalo, 2007; Swift and Weinstein, 2009). According to mouse embryo studies, vasculogenesis takes place during embryonic days (E) 6.5–9.5 of the 21 days long murine pregnancy (Drake and Fleming, 2000).

The vascular tree continues to grow mainly via angiogenesis and vasculogenesis. Angiogenesis, or the formation of new blood vessels from preexisting ones, is driven by growth factor gradients and signals from the surrounding matrix. The primary capillary plexus branches out into a complex network where capillary, venous and arterial vessel specification and vessel hierarchy are distinguished. Vessels can also expand by a progress called intussusception, where one vessel divides into two (Swift and Weinstein, 2009). Maturing ECs secrete growth factors to attract mural cells that cover the maturing vessels and further differentiate for organ specific functions (Jain, 2003; von Tell et al., 2006). Hypoxia guides vessel growth to meet the oxygen need in a growing embryo, but also during pathological vessel growth in various diseases (Koch et al., 2011).

The endothelial cells and the vessels they form show remarkable tissue and vessel-type specific heterogeneity, reflecting their specialized functions (Monahan-Earley et al., 2013). Thus, even though ECs share many functions, there is no single protein known that would be entirely specific to ECs, or expressed to the same extent in all ECs (Aird, 2007b). Due to this heterogeneity, genetic mouse models that have been designed to target EC-specific genes via endothelial specific gene promoters seldom work with the same efficiency in all EC types or in all tissues (Minami and Aird, 2005). Recent advancements in e.g. single cell sequencing technologies will elucidate the knowledge of the molecular heterogeneity of the vasculature.

1.1.2 Functions of the blood vascular system

In addition to its function as a transport system, the blood vessels control blood flow and pressure, which are crucially regulated by vascular integrity (see 1.3), as well as mechanisms of vasodilation and vasosuppression. The blood vasculature also has essential functions during wound healing and in control of local tissue inflammation (Aird, 2007a; Schwartz et al., 2010). Moreover, tight control of the blood clotting cascade is crucial for homeostatic regulation of the vasculature. Upon injury, platelets are attracted to the site of injury by von Willebrand factor produced by ECs. Tissue injury triggers the clotting cascade via tissue factor, which becomes exposed due to retraction or apoptosis of ECs, leading to thrombin production that further enhances coagulation and fibrin clot formation to seal the vessel. Proteinase activated receptors on ECs sense the pro-coagulogenic factors and are involved in the initiation of clotting (Yau et al., 2015), whereas ECs also inhibit unnecessary clotting. The blood clot is eventually dissolved, via release of pro-fibrinolytic molecules and metalloproteinases by ECs (Yau et al., 2015). In certain diseases, such as sepsis, aggravated coagulation processes may occur, leading to disseminated intravascular coagulation, highlighted by shortage of coagulation factors and increased bleeding (Gando et al., 2016).

1.2 Lymphatic vascular system

The lymphatic vascular system is important for the control of tissue fluid homeostasis, lipid adsorption in the gut, and for the function of the adaptive immune system. The lymphatic ECs share properties with blood vascular ECs but have evolved into a different purpose. The lymphatic capillaries lack mural cell coverage, whereas larger collecting lymphatic vessels are covered by smooth muscle cells. The lymphatic network is an open-ended system. Lymphatic capillaries collect the lymph from the interstitial space in the peripheral tissues. The lymphatic vessels gather at the thoracic duct, where the lymph is returned to the blood stream (Vaahtomeri et al., 2017). The lymph nodes are specialized structures in this system, and are essential for the adaptive immunity (Schwager and Detmar, 2019). A specialized hybrid vessel, termed the Schlemm's canal, can be found in the eye. It shares features of both blood and lymphatic vessels, but its formation relies on the lymphatic growth factor receptor signaling (Aspelund et al., 2014).

The first lymphatic structures, the jugular lymph sacs, arise in the developing embryo, after commitment of the lymphatic ECs at E9.5, via lymphatic EC migration from the cardinal vein (Vaahtomeri et al., 2017). Moreover, lymphatic progenitors from non-venous origin contribute to the development of the lymphatic vasculature in an organ-specific manner (Martinez-Corral et al., 2015). Lymphangiogenesis can be further induced in many pathological conditions, like inflammation, tissue repair and cancer (Vaahtomeri et al., 2017).

1.3 Endothelial cells and the vascular barrier

The integrity of the EC layer is pivotal for the proper function of the vascular barrier, which is maintained via EC-EC and EC-ECM adhesions and contributes to the permeability of the vessels in a vascular bed -specific manner.

In general, arterial ECs form tighter EC-EC connections than ECs in the veins. In certain tissues such as in the skin, lungs and the heart, as well as in the central nervous system, capillary ECs form a continuous endothelium that allows the passage of only small molecules, like water, and prevents the passage of plasma proteins and circulating cells. The lowest permeability is in the blood-brain and the blood-retinal-barriers. Fenestrated endothelium can be found in the kidney glomeruli, intestine and endocrine glands. Fenestrated EC-EC junctions have pores, but the cell layer appears organized and continuous. Pores in the fenestrae permit the passage of small peptides, and also allow fast water and solute transport, which is important for the function of these vascular beds. Sinusoidal endothelium is discontinuous and unorganized and the most permeable, allowing passage of large plasma proteins. Sinusoids can be found for example in the liver and the bone marrow (Augustin and Koh, 2017).

Two distinct routes have been reported to mediate permeability across the vascular endothelium. In the paracellular route protein and cell passage occurs via the EC junctions, whereas in the transcellular route transport occurs via transcytosis through ECs, especially in the sinusoidal vascular beds (Augustin and Koh, 2017). The vesiculo-vacuolar organelles (VVO) are vacuolar structures of ECs that have been shown to carry out extravasation of macromolecules. VVOs span the whole EC width from luminal to abluminal side, forming a structure through which plasma proteins can leak efficiently (Cheng and Nichols, 2016). In certain tissues the paracellular route has been found to be the major mechanism of inflammation induced permeability and leukocyte extravasation (Schulte et al., 2011). The regulation of paracellular permeability via EC junctions and the actin cytoskeleton is discussed below.

1.4 Endothelial cell junctions

EC integrity and paracellular permeability are regulated via adherens and tight junctions that can be seen as an electron dense area in the transmission electron microscope and encompass most of the EC longitude (Wallez and Huber, 2008). ECs undergo adhesional changes as the junctions form. Initial focal contacts turn into focal adhesions, which are in contact with the adherens junctions, tight junctions and actin cytoskeleton in the matured endothelium (Kasa et al., 2015). Adherens and tight junctions are intertwined with each other and with the actin cytoskeleton.

1.4.1 Adherens junctions

Adherens junctions are dynamic structures that allow regulated passage of molecules and immune cells across the ECs. The composition of adherens junctions varies across the vasculature, but the most abundant proteins are vascular endothelial cadherin (VE-cadherin) and catenins, of which ECs express α-, β-, and γ-catenins and p120-catenin (Campbell et al., 2017). Adherens junction proteins are linked to actin cytoskeleton, and changes in actin filaments affect adherens junctions, and *vice versa*. β- and γ-catenin (or plakoglobin) link VE-cadherin to the actin cytoskeleton by binding to the cadherin tail via their arm region, and by forming a complex with α-catenin via their amino terminal region. α-catenin further links this cadherin-catenin complex to the actin cytoskeleton (Figure 1). VE-cadherin is also in contact with p120-catenin. In addition to forming a link between VE-cadherin and actin cytoskeleton, p120-catenin regulates VE-cadherin expression and trafficking (Campbell et al., 2017; Gavard, 2014).

1.4.2 VE-cadherin

VE-cadherin is a transmembrane glycoprotein present in adherens junctions in virtually all vascular beds. VE-cadherin is a member of the classical cadherin family that in blood vessels forms homotypic (zipper-like) interactions across the EC junctions in a calcium-dependent manner (Lampugnani et al., 2018).

VE-cadherin is needed for the proper formation of the vasculature. Murine derived embryonic bodies fail to develop vessels *in vitro*, if they are mutated to lack the *Cdh5* gene coding for VE-cadherin. VE-cadherin inactivation, however, does not influence the proliferative capacity of stem cells (Vittet et al., 1997). VE-cadherin knock-out mice die at E9.5 during embryonic development, due to impaired ECs survival and angiogenesis (Carmeliet et al., 1999).

VE-cadherin is considered as a key regulator of vascular stability, and its conditional deletion in adult mice or inhibition using blocking antibodies resulted in increased vascular permeability in the lungs and the heart (Corada et al., 1999; Frye et al., 2015). No alterations in tight junction protein claudin-5 were found in mice lacking the *Chd5* gene (Frye et al., 2015). However, in cultured ECs VE-cadherin is known to regulate the composition of tight junctions (Taddei et al., 2008).

VE-cadherin associates with the platelet and endothelial cell adhesion molecule 1 (PECAM1, termed CD31 from hereon) and the vascular endothelial growth factor receptor (VEGFR) 2 through its intracellular domain. These interactions, together with integrin cell adhesion receptors, also comprise the EC mechanosensory complex, which mediates the EC responses to fluid shear stress (Tzima et al., 2005).

The appearance of VE-cadherin in the lymphatic vasculature differs from that of the blood vasculature. VE-cadherin is organized into button-like structures in lymphatic capillaries, but adopts the zipper-like morphology in lymphatic capillaries undergoing lymphangiogenesis and in collecting lymphatic vessels. The button-like junctions contain gaps that are thought to facilitate fluid uptake from the interstitium. The zipper-like lymphatic capillary junctions of embryonic lymphatic vessels mature into button-like junctions after birth. Lymphatic VE-cadherin junctions retain plasticity and undergo remodeling from buttons to zippers during inflammation (Baluk et al., 2007; Yao et al., 2012), and in lacteal lymphatic capillaries when neuropilin-1 and VEGFR1 are deleted, promoting VEGFR2 signaling and resisting chylomicron uptake (Zhang et al., 2018).

The junctional assembly of VE-cadherin is under complex regulation of various protein kinases, phosphatases and small guanosine triphosphatases (GTPases), that determine VE-cadherin phosphorylation status and downstream signaling (Dejana and Lampugnani, 2018). Nine amino acid residues can be phosphorylated in the tail region of VE-cadherin by various kinases. Five different phosphorylation sites have been characterized to regulate permeability (Orsenigo et al., 2012; Potter et al., 2005; Turowski et al., 2008; Wallez et al., 2007). Physiological differences in basal VE-cadherin phosphorylation have been found, including variation between arteries and veins. The phosphorylation of VE-cadherin is increased upon inflammatory or angiogenic stimuli, and can be mediated via the proto-oncogene tyrosine protein kinase (Src), and other kinases, like the p21-activated kinase PAK (Gavard and Gutkind, 2006), stimulating VE-cadherin internalization. However, increased phosphorylation of VE-cadherin is insufficient to lead to its internalization, and additional stimulus is needed (Lambeng et al., 2005; Orsenigo et al., 2012).

1.4.3 Tight junctions

The abundance of tight junction proteins varies in different vascular beds due to differences in vessel structure. For example, in post-capillary venules, where leukocyte trafficking occurs, tight junctions are less prominent and receptors required for leukocyte adhesion abundant, especially during inflammation. On the contrary, the blood-brain-barrier, which is a specialized vascular bed to protect the brain, has a high content of tight junction proteins to fortify the endothelial structure and prevent leakiness. Large arteries, like the aorta, have also well-organized tight junctions that mediate resistance to high rates of pulsatile blood flow (Aird, 2007a).

Claudins and occludins represent the most important tight junction components in ECs. They participate in permeability regulation and interact with adherens junctions. Claudin-5 has been shown to be important in maintaining blood-brain-barrier integrity, and its deletion in the mouse genome results in death after birth due to hemorrhaging (Tsukita et al., 2019). Another important component is the intracellular zonula occludens-1 (ZO-1) to which the claudins and occludins are linked (Figure 1). ZO-1 also associates with proteins involved in cellular tension sensing, mediating interaction of tight junction proteins with VE-cadherin. ZO-1 has been shown to regulate angiogenesis and support EC integrity via both VE-cadherin and the actin cytoskeleton (Tornavaca et al., 2015).

1.5 Endothelial actin cytoskeleton

Most of the data concerning EC actin cytoskeleton is derived from *in vitro* studies. A cortical actin structure is essential for endothelial monolayer integrity. It is formed during EC adhesion to ECM, after the maturation of initial focal complexes into stable focal contacts (Figure 1). The cortical actin contains filamentous actin (F-actin) that is formed via polymerization of globular β- and γ-actins (G-actin) through nucleation and elongation steps. F-actin also forms the membrane skeleton that adjusts EC membranes accordingly, whereas the cortical actin rim is a separate structure that interacts with

membrane skeleton. Membrane skeleton is composed of short F-actin fibrils, whereas cortical actin is composed of longer fibrils, which are in continuous contact with several actin binding proteins that link them to other cellular structures (Prasain and Stevens, 2009). Filamin links actin to cell membrane proteins and links F-actin fibrils for the construction of cell-cell contacts and cell-matrix-contacts where cortical actin is formed (Kumar et al., 2019).

The third type of F-actin, actin stress fibers, are composed of short F-actin fibrils. Typically, when stress fibers form, the cortical actin rim is dismantled. Stress fibers may stretch through the cell cytoplasm, and generate centripetal tension by acquiring contractile forces via the actomyosin contractility machinery (Figure 1). Stress fiber contractility promotes retraction of EC membranes, pulling contacting ECs away from each other and leading to gap formation in the junctions of EC monolayers. On the contrary, cortical actin supports membrane stability (Prasain and Stevens, 2009).

Contractility of F-actin is generated via binding of myosin motor proteins along with cross-linking proteins such as alpha-actinin to stress fibers (Figure 1). Phosphorylation of the myosin light chain (MLC) of myosin II regulates its activity. At least two distinct signaling pathways lead to contractile stress fiber formation. The Ca^{2+}/Calmodulin pathway activates MLC kinase (MLCK) which phosphorylates MLC. Additionally, the Ras homolog gene family member A (RhoA) – Rho-associated protein kinase (ROCK) signaling can induce MLC activity via direct phosphorylation, or indirectly, via phosphorylation-mediated inhibition of the MLC phosphatase (Huveneers et al., 2015; Kassianidou et al., 2017). Formation of the F-actin fibril is regulated by several actin binding proteins of the GTPase protein family. Whereas RhoA can promote stress fiber formation, Ras-related C3 botulinum toxin substrate 1 (Rac1) and Ras-related protein 1 (Rap1) support cortical actin (Huveneers et al., 2015). In ECs, the FYVE, RhoGEF and PH domain containing 5 (FGD5), which is a Guanine nucleotide exchange factor (GEF), inhibits stress fiber formation via Rap1 and the cell division control protein 42 (Cdc42) mediated signaling promotes cortical actin (Braun et al., 2019).

2. Growth factor regulation of endothelial cells

Extracellular signals regulate multiple EC functions via two major endothelial growth factor receptor systems: the angiopoietin (ANGPT)–TIE and the vascular endothelial growth factor (VEGF)–VEGFR system. Both systems are vital in the development of the blood and lymphatic vasculatures, and in homeostasis (Lohela et al., 2009; Saharinen et al., 2017a).

2.1 VEGF–VEGFR system

VEGF–VEGFR system comprises of five ligands: VEGF-A (termed VEGF from hereon), - B, -C and -D, the placental growth factor (PlGF), and three receptors: VEGFR1, VEGFR2 and VEGFR3. VEGF has four splice variants that differ in their matrix binding properties, with different signaling outcomes. VEGF is a ligand for VEGFR1 and VEGFR2, and can also bind neuropilins 1 and 2. VEGF-B and PlGF bind VEGFR1, and VEGF-C and VEGF-D bind VEGFR3, but also VEGFR2 in their fully processed forms (Jha et al., 2017). In adult mice, VEGFR3 is enriched in lymphatic ECs, although the receptor is also found in blood vessels. VEGF is produced by virtually all cells of the body, and its expression is highly upregulated in hypoxia (Apte et al., 2019). VEGFRs are major regulators of EC functions, such as EC proliferation, cell survival and migration, but the receptors are also found on other cell types (Greenwald et al., 2019; Licht and Keshet, 2013).

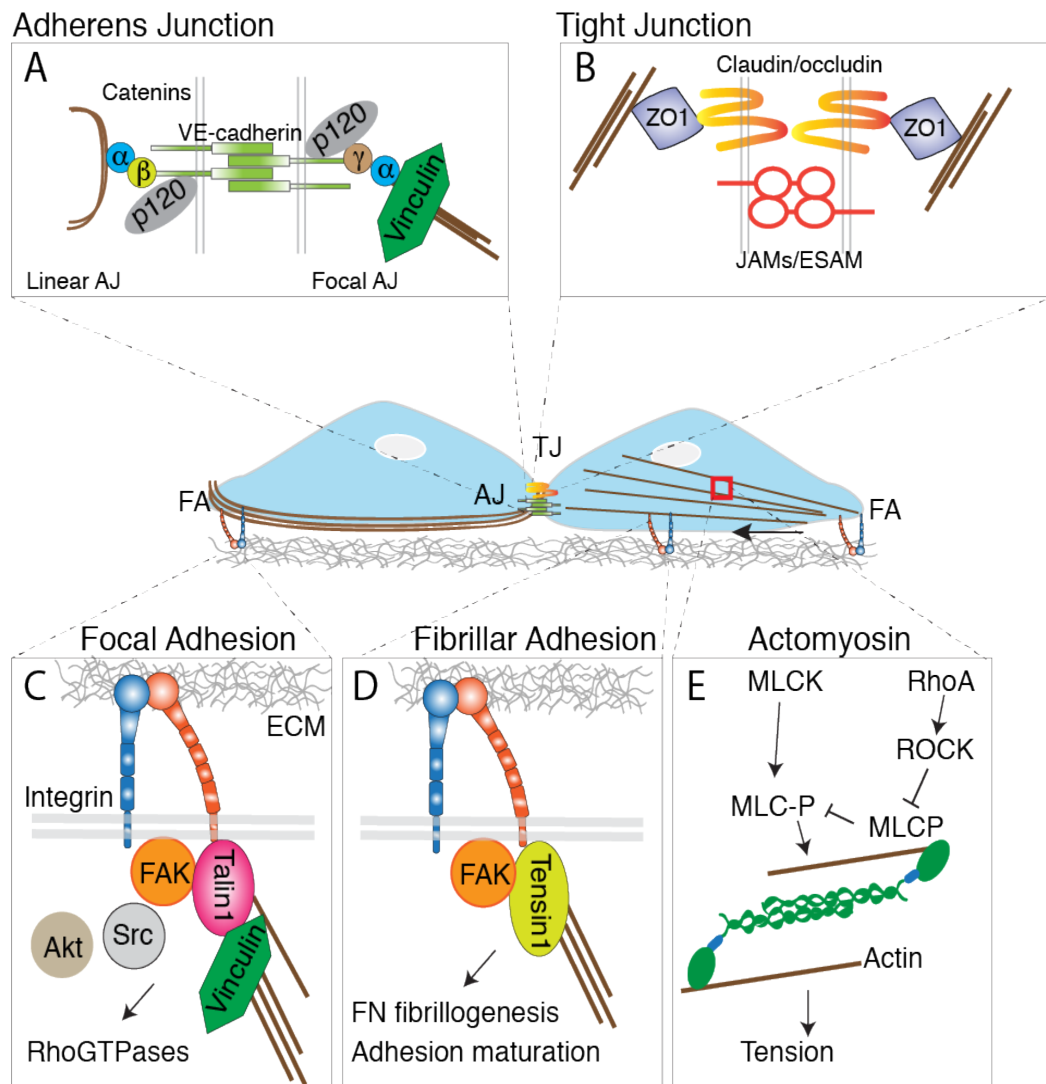


Figure 1. Endothelial cell-cell and cell-matrix adhesions. A) Adherens junctions (AJ) connect to actin cytoskeleton via catenins and B) tight junctions (TJ) connect to actin cytoskeleton via ZO1 binding to claudins and occludins. Focal AJs may further contain vinculin that enforces the actin coupling and mediates tension, whereas linear AJs associate with cortical actin in cells with less tension. C) Focal adhesions (FA) and D) fibrillar adhesions connect to actin cytoskeleton via integrin adaptors. FA signaling is complex, and only as subset of the signaling mediators are illustrated. Fibrillar adhesions were originally characterized in fibroblasts and are enriched with tensin1 instead of talin1. E) Actin stress fiber tension is elicited by myosin binding to actin fibers. Activation of the actomyosin contractility is under dynamic regulation. Information derived from multiple sources (Georgiadou and Ivaska, 2017; Huveneers et al., 2015; Lo, 2017).

VEGF was originally described as the Vascular Permeability Factor, and it is a strong inducer of vascular permeability via VEGFR2 (Li et al., 2016; Senger et al., 1983). VEGF signaling is crucial during development, and heterozygous deletion of even a single allele of *Vegf* results in death of the mouse embryos at around E11 due to impaired vascular formation resulting in various developmental anomalies (Carmeliet et al., 1996; Ferrara et al., 1996), and homozygous deletion of *Vegfr2* in mice result in a similar phenotype (Shalaby et al., 1995).

In adult mice, autocrine VEGF plays a role in EC survival, and conditional EC specific deletion of VEGF leads to death of the mice over 25 weeks (Lee et al., 2007). In addition, inhibition of VEGF

for 2-3 weeks using a VEGF trap, induces the regression of capillaries e.g. in the thyroid and small intestine within, and loss of fenestrae in the capillaries of the kidney (Kamba et al., 2006).

VEGF is induced by the hypoxia inducible factor (HIF) signaling in response to low oxygen pressure, resulting in neoangiogenesis (Majmundar et al., 2010). This mechanism is at play in solid tumors, where hypoxia drives VEGF expression and angiogenesis. Similarly, in neovascular eye diseases, VEGF is the main driver of pathological angiogenesis. Drugs that target VEGF have therefore been used in cancer and neovascular eye diseases, with higher efficacy in the latter (Ferrara and Adamis, 2016).

2.2 Angiopoietin (ANGPT)–TIE system

The ANGPT–TIE system is composed of two major ligands, ANGPT1 and ANGPT2, and two receptors, the Tyrosine kinase with Ig and epidermal growth factor homology domains 1 (TIE1) and the TEK receptor tyrosine kinase (TIE2). ANGPT growth factors modulate the phosphorylation status of the TIE receptors in this system. ANGPT1 is an agonistic ligand for TIE2, whereas ANGPT2 is a context dependent agonist/antagonist. A third ligand, ANGPT4, has also been characterized, but not studied extensively (Eklund et al., 2017).

2.2.1 TIE receptors

TIE2 and TIE1 receptors are type I transmembrane protein receptor tyrosine kinases (Dumont et al., 1994; Partanen et al., 1992). Both receptors are enriched in the ECs of both vascular and lymphatic vessels (Partanen et al., 1992), but there is some expression also in haematopoietic cells, and e.g. TIE2 is expressed in a subtype of macrophages (TIE2 expressing macrophages) and hematopoietic stem cells (Arai et al., 2004; Batard et al., 1996; De Palma et al., 2005).

TIE2 and TIE1 are closely related. Their ectodomains consist of three fibronectin type III domains, three epidermal growth factor repeats and three Ig-like domains (Figure 2) (Barton et al., 2006). Both receptors have an intracellular, carboxyterminal kinase domain that mediates downstream signaling (Figure 2). TIE2 ligand binding domain resides in the second Ig repeat, whereas TIE1 does not directly bind ANGPTs (Barton et al., 2006; Davis et al., 2003), and therefore its signaling mechanisms are not completely understood.

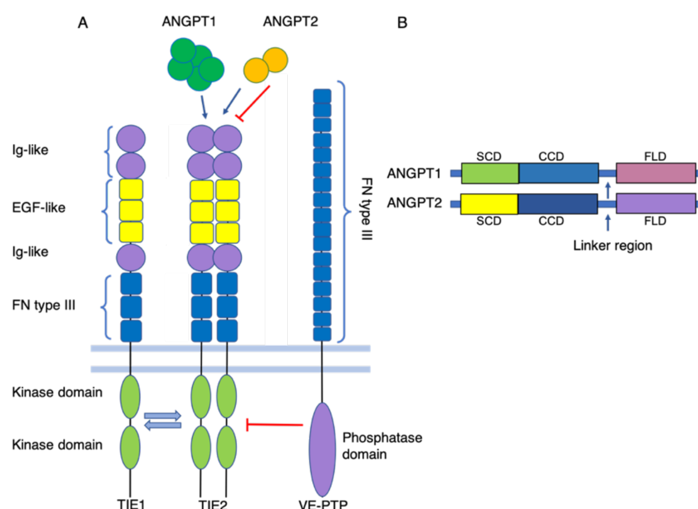


Figure 2. Schematic presentation of the ANGPT–TIE system. A) TIE extracellular domains consist of Ig-like domains, FN type III domains, and EGF-like domains. Intracellular domains of TIEs and of VE-PTP consist of kinase and phosphatase domains, respectively. Blue arrows indicate receptor activation, red arrow indicates receptor inhibition. B) Structure of ANGPTs. SCD = superclustering domain, CCD = coiled-coil domain, FLD = fibrinogen-like domain. Linker regions (blue arrows). Panel A) adapted from Thurston & Daly (Thurston & Daly, 2012) Panel B) adapted from study I.

TIE1 interacts with TIE2 in EC-EC junctions upon ANGPT stimulation of ECs, regulating TIE2 activity and internalization (Korhonen et al., 2016). The interacting interfaces of the receptors were found to depend on charged regions within the receptor ectodomains (Seegar et al., 2010). *TIE1* gene silencing in cultured ECs, or deletion in the mouse genome, also impaired ANGPT1 induced TIE2 phosphorylation further indicating the importance of TIE1 in TIE2 biology (D'Amico et al., 2014; Korhonen et al., 2016; Savant et al., 2015). Moreover, TIE1 was needed for ANGPT2 agonistic activity, but the detailed mechanisms are yet to be clarified (D'Amico et al., 2014; Korhonen et al., 2016).

TIE2 phosphorylation is negatively regulated by the vascular endothelial protein tyrosine phosphatase (VE-PTP, official name PTPRB). VE-PTP is a membrane bound tyrosine phosphatase with 17 fibronectin type III repeats in its extracellular domain, and an intracellular phosphatase domain (Figure 2) (Eklund et al., 2017). VE-PTP regulates ANGPT–TIE signaling by dephosphorylating TIE2 at the kinase domain thus inhibiting downstream signaling (Li et al., 2009). Antibodies targeting VE-PTP extracellular domain induce the formation of similar enlarged vessels as does the genetic inactivation of VE-PTP (Winderlich et al., 2009).

2.2.2 Angiopoietin-1

ANGPT1 is a secreted glycoprotein that consists of an N-terminal superclustering domain, a coiled-coil domain and a C-terminal receptor binding domain (Figure 2) (Leppanen et al., 2017; Thurston and Daly, 2012). ANGPT1 can form dimers or trimers via the coiled-coil domain that further cluster into oligomers such as tetramers, pentamers and higher order multimers, via the superclustering domain (Kim et al., 2005; Saharinen et al., 2017b). ANGPT1 is produced in mesenchymal cells surrounding vessels (Davis et al., 1996). Using a fluorescent reporter mouse, ANGPT1 expression has been identified in the pericytes of choriocapillaries and in neuronal cells of the ganglion and inner nuclear layers of the retina (Park et al., 2017). In addition, pericytes in the lung express ANGPT1 (Kato et al., 2018). ANGPT1 is also produced and stored in the granules of platelets, from where it can be rapidly released (Brindle et al., 2006; Li et al., 2001).

ANGPT1 binding to TIE2 on cell membranes induces TIE2 translocation to EC junctions. Here, TIE2 receptors interact *in trans*, from one cell to another, resulting in TIE2 phosphorylation and downstream activation of the phosphoinositide 3 kinase (PI3K)– protein kinase B (from hereon called Akt) pathway (Fukuhara et al., 2008; Saharinen et al., 2008). In general, ANGPT1 binding to TIE2 induces downstream signaling leading to EC stabilization via the actin cytoskeleton, EC-EC junction enforcement and anti-inflammatory signaling, however, in certain vascular beds ANGPT1 can also induce non-leaky vascular remodeling. Akt phosphorylates the transcription factor forkhead box O1 (FOXO1). This leads to FOXO1 cytoplasmic localization preventing its nuclear translocation and transcription of its target genes, including *ANGPT2* (Figure 5) (Daly et al., 2006; Wilhelm et al., 2016). Another result from PIK3 signaling is phosphorylation of the endothelial nitric oxide synthase (eNOS), which signals for endothelial stability (Fukuhara et al., 2008; Kim et al., 2000a; Saharinen et al., 2008). ANGPT1 can also promote the migration of sub-confluent ECs via extracellular signal-regulated kinase (ERK) signaling and TIE2 localized in EC-ECM contact sites (Fukuhara et al., 2008; Saharinen et al., 2008). Although ANGPT1 does not bind to TIE1, it stimulates TIE1 receptor phosphorylation, likely via its interaction with TIE2 (Korhonen et al., 2016; Saharinen et al., 2005).

2.2.3 Angiopoietin-2

ANGPT2 is structurally homologous to ANGPT1 (Figure 2), but forms mostly dimers via the coiled-coil domain, resulting in weak TIE2 agonist activity, despite of similar TIE2 binding affinity of the

ANGPT1 and ANGPT2 fibrinogen-like domains (FLDs) (Davis et al., 2003; Fiedler et al., 2003; Maisonpierre et al., 1997; Saharinen et al., 2017b). ANGPT2 has been found to activate TIE2 to some extent in the lymphatic ECs and in non-inflammatory conditions, whereas ANGPT2 acts an antagonist, inhibiting TIE2 phosphorylation and downstream signaling in inflammation (Figure 5) (Gale et al., 2002; Kim et al., 2016; Korhonen et al., 2016; Reiss et al., 2007; Souma et al., 2018).

ANGPT2 expression is increased in numerous diseases, such as sepsis, cancer, neovascular eye diseases and many others (Saharinen et al., 2017a). ANGPT2 produced by activated ECs is stored and secreted via Weibel-Palade bodies, which are storage granules that harbor, in addition to ANGPT2, von Willebrand factor and P-selectin, released upon a regulatory stimulus. Although considered an EC-specific growth factor, ANGPT2 is also expressed by retinal horizontal cells in adult mice (Hackett et al., 2002). *In vitro*, ANGPT2 is released from Weibel-Palade bodies upon various stimuli, such as phorbol 12-myristate 13-acetate (PMA) and the inflammatory agents thrombin and histamine (Fiedler et al., 2004). The expression of ANGPT2 is controlled by the Akt–FOXO1 pathway (Daly et al., 2004; Potente et al., 2005), and is elevated by tumor necrosis factor-α (TNFα), VEGF, hypoxia, hyperglycemia, and during angiogenesis (Hackett et al., 2002; Kim et al., 2000b; Mandriota and Pepper, 1998; Rasul et al., 2011).

ANGPT2 induces TIE2 translocation to the junctions of cultured ECs, where it is the only endogenously expressed ligand. In comparison to exogenous ANGPT1, ANGPT2 induces weak TIE2 phosphorylation, which is increased if TIE2 is ectopically expressed (Saharinen et al., 2008). In stressed HUVEC cultures, where Akt signaling is low, ANGPT2 can act as a TIE2 agonist and, similar to ANGPT1, phosphorylate TIE2 and induce Akt activation (Daly et al., 2006). When added to ECs together with ANGPT1, ANGPT2 acts as an antagonist, and inhibits ANGPT1–TIE2 signaling dose-dependently (Yuan et al., 2009).

2.3 ANGPT–TIE system in vascular development

ANGPT–TIE signaling is essential for proper vascular development. Genetic deletion of either *Tie2* or *Angpt1* in mice results in embryonic death at E10.5–E12.5 because of defects in the cardiovascular development (Dumont et al., 1994; Jeansson et al., 2011; Sato et al., 1995; Suri et al., 1996). In these mice, the embryos have less ECs, and the developing vasculature fails to mature normally. Ectopic expression of *Angpt2* during embryonic development results in vascular defects and a phenotype comparable to that of *Tie2* or *Angpt1* deleted mouse embryos, and lethality at around E10 (Maisonpierre et al., 1997). *Angpt1* can be deleted after E12.5 without causing major defects or lethality, indicating a specific timeframe where its activity is needed (Jeansson et al., 2011).

Genetic mouse models have further revealed that ANGPT2 is not as crucially needed for embryonic blood vascular development as ANGPT1. *Angpt2* deficient pups are born, but die within two weeks after birth due to generalized lymphatic dysfunction. Specifically, *Angpt2* deletion leads to impaired zipper-to-button transformation of the junctions of initial lymphatic vessels, and to altered pericyte coverage of the maturing lymphatic vessels (Gale et al., 2002; Zheng et al., 2014). Interestingly, the lymphatic defects are corrected in an *Angpt1* knock-in into the *Angpt2* locus (Gale et al., 2002).

Universal deletion of *Tie2* leads to impaired vascular development and embryonic edema (Thomson et al., 2014). Not surprisingly, a similar phenotype was found in *Angpt1* and *Angpt2* double knock-out mice. If universal deletion is induced at E12.5, the *Angpt1-Angpt2* double knock-out mice survive, but develop embryonic edema and have defective lymphatic vascular development (Thomson et al., 2014). Deletion of *Angpt1* and *Angpt2* at E16.5 allows the mice to develop postnatally. At the age of three weeks the mice develop glaucoma that arise due to defective function of the ocular lymphatics

and the Schlemm's canal, similar to *Tie2* deleted mice (Thomson et al., 2014). Interestingly, heterozygous mutations in *Tie2* and *Angpt1* have also been found in human patients with primary congenital glaucoma (Souma et al., 2018). The less studied ANGPT4 (originally ANGPT3 in the mouse) was recently found to be important for venous remodeling in the mouse retina as well as retinal fluid clearance and neuronal function (Elamaa et al., 2018).

Embryonic *Tie1* deletion is lethal at E13.5. TIE1 null mice have compromised capillary remodeling, and loss of ECs in the microvasculature (Puri et al., 1995). *Tie1* can be deleted in adult mice without harmful effects under homeostasis. Interestingly, when syngeneic tumors are implanted in the knockout mice, tumor growth is decreased, but the mechanisms are not yet understood (D'Amico et al., 2014). TIE1 is also an important factor in lymphatic development and in the development of the postnatal retinal vasculature (D'Amico et al., 2014; Qu et al., 2010). Lymphatic capillaries and the collecting lymphatics fail to develop if only the TIE1 ectodomain is expressed, and the TIE1 intracellular domain is required both during embryonic development and after birth (Shen et al., 2014). As mentioned above, TIE1 has been found to be required for certain ANGPT-mediated vascular responses, however, the mechanism explaining the various biological phenotypes of *Tie1* gene targeted mice have remained somewhat elusive until now. Further highlighting the importance and complexity of TIE receptor signaling, the universal deletion of *Ve-ptp* leads to embryonic lethality, and causes severe defects in angiogenesis, but not vasculogenesis, and in heart function (Baumer et al., 2006; Dominguez et al., 2007).

3. Integrins

Integrins are cell adhesion receptors that mediate EC adhesion to the ECM, or to other cells. Integrins are found in all cell types and are essential for many cellular functions, including cell movement, cell division and sensing the environment. Integrins also serve as pathogen receptors for several bacteria and viruses (Bachmann et al., 2019; Stewart and Nemerow, 2007). Integrins form αβ heterodimers that differ in their specificity for ECM components as their ligands. 18 alpha subunits and 8 beta subunits have been found in vertebrates, making up 24 different integrin heterodimers (Figure 3). By binding to the ECM, integrins connect the ECM to the intracellular actin cytoskeleton. Uniquely, integrin signaling within a single cell is bidirectional (Sun et al., 2016b).

3.1 Structure and function of integrins

3.1.1 Integrin structure and activation

The integrin subunits are 90-160 kD in size and are comprised of several subdomains that form the complex structure. In brief, integrins contain a large ectodomain, flexible linker region, a transmembrane helix spanning the cell membrane, and, in most cases, a short cytoplasmic domain

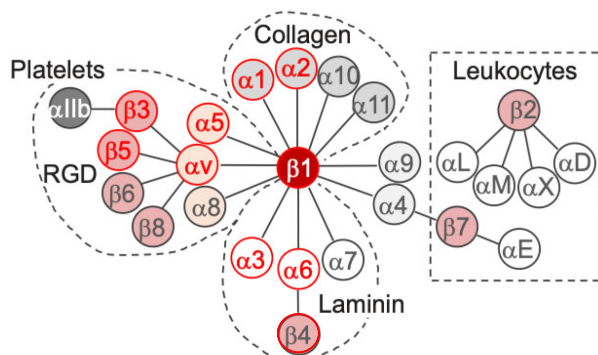


Figure 3. **Integrin heterodimers.** Schematic presentation of integrin heterodimers and major ligands. Integrins reported to be expressed in the ECs are marked with red circles. Information for the schematic has been combined from several articles (Hodivala-Dilke et al., 2003; Humphries et al., 2006; Welser et al., 2017).

(Figure 4) (Bachmann et al., 2019). The extracellular portion of alpha subunits consist of the β-propeller, a thigh domain, and two calf domains (Figure 3). Some alpha chains additionally have an alpha-I domain (also called alpha-A) inside the β-propeller. The β-propeller has several subdomains, including the N-terminal Ca^{2+} binding domains, which affect ligand binding. The extracellular domain of the beta subunit consists of a beta-I domain, a plexin-semaphorin-integrin domain, four EGF modules, and a beta-tail domain (Figure 4) (Bachmann et al., 2019).

Integrins do not have any enzymatic activity, and are activated via a conformational change into an open-extended conformation (Figure 4). Integrins can be activated outside-in via ligand binding to integrin ectodomain, or inside-out via adaptor protein binding to integrin intracellular tail. Inside-out activation of the integrins leads to a conformational change as the intracellular and membrane spanning portions of alpha and beta subunits separate from each other allowing the extracellular head domain to open into an extended form from a bent closed conformation (Figure 4). This opening enhances ECM binding of integrins. Talin is one of the best characterized integrin adapter proteins that binds to integrin beta cytoplasmic tails mediating integrin inside-out activation (Kechagia et al., 2019). Outside-in signaling is supported by ligand binding to the extracellular region, and similarly promotes the open extended conformation (Bachmann et al., 2019). As of recently, inside-in signaling of integrins has also been reported to take place, regulating anoikis (Alanko et al., 2015).

Ligand binding of integrins is dependent on Ca^{2+}, Mn^{2+}, and Mg^{+} ions. Integrins bind to various ECM proteins, such as collagens, laminins and fibronectins, as well as cell adhesion molecules such as vascular cell adhesion molecule (VCAM) and intercellular cell adhesion molecule (ICAM). Typically, a given integrin can bind many ligands, which may, to some extent, be explained by shared consensus sequences recognized by the integrin ligand binding domain. Four major types of ligand-integrin interactions have been reported: 1) binding of the RGD (arginine-glycine-aspartic acid) peptide motif, present e.g. in FN and vitronectin, 2) binding of the LDV (leucine-aspartate-valine) motif, found in eg. FN and in cell adhesion molecules, 3) binding of alpha-I domain containing integrins to laminin and to collagen via the GFOGER motif and 4) highly specialized laminin binding (Bachmann et al., 2019; Humphries et al., 2006).

3.1.2 Integrin mediated cell adhesions

The characterization of the integrin adhesome has led to the identification of approximately 200 proteins that regulate integrin mediated cell adhesion (Horton et al., 2016; Winograd-Katz et al., 2014). Collectively, integrin mediated adhesions are dynamic structures that transduce mechanical forces in stable and migratory cells and in homeostasis and disease (Sun et al., 2016b). Actin

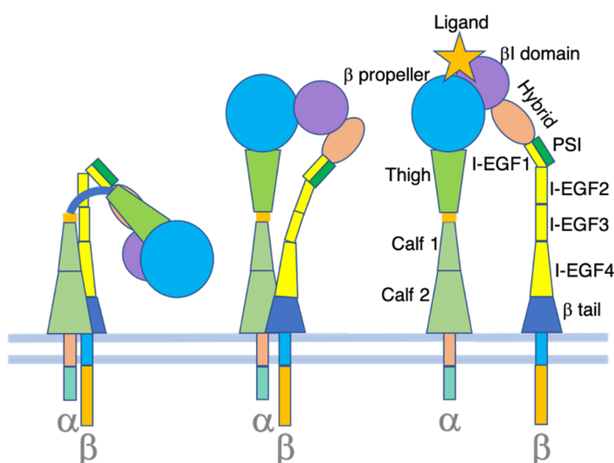


Figure 4. A schematic of the three integrin conformational states. From left to right: bent-closed, extended closed, and extended open conformations. Ligand or adaptor binding induces conformational activation of integrins. Inhibitory adaptor protein binding confines integrins into bent-closed conformation. The characteristic protein domains of alpha- and beta- subunits are indicated for the open extended conformation. Schematic adapted from Su et al. (Su et al., 2016).

cytoskeleton rearrangements are orchestrated via integrin activation and clustering, facilitating adhesional changes and cell membrane movement. Various types of integrin-mediated adhesions have been observed that differ in their function, subcellular location and molecular composition. An important factor affecting the adhesion formation is the composition of the ECM, which stimulates integrin outside-in signaling. The adhesions include nascent adhesion, focal contacts, focal adhesions, fibrillar adhesions, podosomes and invadopodia (Geiger and Yamada, 2011). Nascent adhesions and focal contacts can transform rapidly into focal adhesions during cell migration and further mature into fibrillar adhesions. Fibrillar adhesions are elongated adhesions, which contain FN, α5β1-integrin and the adapter protein tensin promoting mechanotransduction and adhesive signaling (Figure 1) (Georgiadou and Ivaska, 2017; Pankov et al., 2000).

Podosomes are specialized actin structures that mediate mechanosensing and matrix degradation, most importantly in immune cells like monocytes (Linder and Wiesner, 2016). Invadosomes, including podosomes, are called invadopodia. Leukocytes and cancer cells use invadopodia for matrix degradation during BM invasion (Seano and Primo, 2015). Integrins and integrin adaptors link to the actin cytoskeleton core of the invadopodia, along with several actin binding proteins, like cortactin (Seano and Primo, 2015). Interestingly, another podosome-like adhesion structure has been identified, which does not connect to the actin cytoskeleton, but rather to clathrin-containing structures via αvβ5-integrin mediating cell adhesion (Lock et al., 2019).

An essential feature in integrin adhesions is integrin trafficking that facilitates the dynamic nature of cell adhesions. In *in vitro* cancer cell models active β1-integrin is trafficked frequently to intracellular vesicles, whereas inactive β1-integrin is endocytosed but rapidly returned to the cell membrane (Arjonen et al., 2012; Moreno-Layseca et al., 2019). The integrins are endocytosed in Ras-related protein (Rab5) positive early endosomes that mature into late endosomes, and can be targeted for degradation by lysosomes, or alternatively recycled back to the cell membrane in Rab11 positive recycling endosomes (Moreno-Layseca et al., 2019).

3.2 Integrin adaptor proteins

Integrin adaptors are involved in both activation and inhibition of integrins. 88 adaptor proteins that directly bind integrins, have been reported so far, but only a small subset of these has been well characterized. Some adaptors bind many integrin tails and some have a more limited binding specificity. For example, β1-integrin has been reported to bind 32 adaptor proteins, of which five adaptors are unique to β1-integrin, and many of these have not been studied in detail (Bachmann et al., 2019). Adaptors regulate integrin signaling, of which the best characterized is the mechanosensory signaling, but additional functions affecting e.g. metabolism are emerging (Bachmann et al., 2019). Activating adaptors switch the integrin heterodimer from a low affinity ligand binding conformation to a high affinity one. The best characterized activating adaptors are talins (Figure 4) and kindlins, which are essential for integrin clustering at cell adhesions.

Integrin inhibiting adaptors bind either the beta or the alpha subunit. Integrin cytoplasmic domain associated protein 1 (ICAP1), filamin and Docking protein 1 (Dok1) bind beta subunits, whereas SHANK associated RH domain interacting protein (SHARPIN), nischarin and the mammary-derived growth inhibitor bind alpha subunits (Morse et al., 2014).

Some adaptor proteins link integrins to actin cytoskeleton. The most studied are talins, tensins (Figure 4) and filamin, which bind universally to several integrin beta tails. Talin-1–integrin–actin complex is the first to assemble in the formation of focal contacts that mature to focal adhesions (Figure 1).

Talin-2, in contrast, is found in fibrillar adhesions, but its function is unclear (Gough and Goult, 2018). Tensin-1 and tensin-3 promote fibrillar adhesions in fibroblasts, whereas tensin-2 can be found in focal adhesions. Tensin-1 and -3 promote the activated integrin conformation, in analogy to talin-1. Tensin-1 can replace talin in the β1-integrin tail, and promote fibrillar adhesion formation in the cell center (Figure 1) (Georgiadou and Ivaska, 2017).

Regulation of the integrin activation state is crucial for proper cellular function. Activating and inhibiting adaptors compete in binding to the beta tail determining the conformational state. For example, talin and kindlin mediated integrin activation is inhibited by filamin binding. Additionally, talin binding is inhibited by Dok1, and ICAP1 inhibits binding of kindlin. Inhibitors that bind to the integrin alpha tail, like SHARPIN, bind to the same conserved site, but can still inhibit binding of integrin activators to the beta tail (Morse et al., 2014).

Some of the integrin binding proteins have catalytic activity, like kinases and phosphatases, and they mediate signaling at cell adhesion sites, including the Focal adhesion kinase (FAK) (Tanjore et al., 2008). In addition, several signaling pathways are induced downstream of activated integrins including Src, ERK, and Akt-mediated signaling (Figure 1) (Georgiadou and Ivaska, 2017; Huveneers et al., 2015; Lo, 2017). Integrins can also be phosphorylated by several kinases, which may regulate the adaptor binding (Chatterjee et al., 2018; Gahmberg et al., 2019). In platelets, the role of phosphorylation has been better characterized. Tyrosine phosphorylation of the β3 integrin tail by Src regulates αIIbβ3-integrin signaling, with effects for example in platelet aggregation (Durrant et al., 2017; Phillips et al., 2001). In summary, the integrin signaling is complex, and regulates in a context-dependent manner various cellular functions including cell adhesion, migration, cell division, organelle compartmentalization, chromosome segregation, proliferation and metabolism (Bachmann et al., 2019).

3.3 Endothelial integrins

ECs express a subset of integrins. The integrin expression pattern may vary depending on the vascular bed studied, and in quiescent and angiogenic ECs (Stupack and Cheresh, 2002). ECs have been mainly reported to express five different β1-integrin heterodimers, two different αv-integrin containing heterodimers, and the α6β4-integrin (Figure 3) (Hodivala-Dilke et al., 2003; Welser et al., 2017). Further, it seems that αvβ8-integrin is needed in a non-EC-specific manner for the development of the vasculatures in the brain, spinal cord and eye (Table 1). Endothelial RGD binding integrins can bind to several ligands containing the consensus sequence, but α5β1-integrin is considered as the major FN receptor, αvβ5-integrin is primarily a vitronectin receptor, and αvβ3-integrin binds FN and vitronectin. All of them can bind other RGD containing ligands, e.g. osteopontin (Humphries et al., 2006). The other EC integrins have binding specificity towards collagens and laminins (Figure 2) (Hodivala-Dilke et al., 2003). Endothelial β1-integrin can heterodimerize with several alpha subunits to form integrins with varying specificity to ECM proteins (Figure 2) (Hodivala-Dilke et al., 2003). In addition, lymphatic ECs express α9β1-integrin (Bazigou et al., 2009).

The functions of EC integrin heterodimers have been studies during vascular development (Table 1), whereas the roles of EC integrins in the mature vasculature are less well understood. In addition, various EC integrins have been studied during postnatal angiogenesis or in the tumor vasculature, however, less is known about the function of EC integrins in other types of diseases. Below, the most insightful studies have been referred to, and focus has been taken on the known roles of β1-integrin during development.

β1-integrin is a major integrin expressed by ECs, and known to be critical for embryonic development. Ubiquitous deletion of *Itgb1* leads to retarded development and death of the embryos by E5.5 (Stephens et al., 1995). Embryos, where *Itgb1* has been deleted from the endothelium, die between E9.5–10.5 (Table 1) (Lei et al., 2008; Tanjore et al., 2008). β1-integrin is dispensable for the formation of the vascular plexus via vasculogenesis, but is necessary during the angiogenic expansion of the vasculature (Table 1) (Lei et al., 2008; Tanjore et al., 2008). Deletion of endothelial β1-integrin at different developmental time points using various Cre lines has elucidated the function of β1-integrin during vascular development. Deletion of β1-integrin using the *Tie2*-Cre driver mice is lethal by E10.5, whereas its deletion using the *Tie1*-Cre delays the death of the embryos by one day, until E11.5 (Table 1) (Carlson et al., 2008), and using the constitutive *Cdh5-Cre* until E13.5–E17.5 (Yamamoto et al., 2015). Deletion using the constitutive *Cdh5-Cre* revealed that β1-integrin is essential in arterial lumen formation (Table 1) (Zovein et al., 2010). Deletion of β1-integrin postnatally using a tamoxifen-inducible *Cdh5-iCre* compromised the development of the retinal vasculature (Table 1) (Yamamoto et al., 2015).

In contrast to β1-integrin, other endothelial integrins appear to play less critical functions during vascular development (summarized in Table 1), and even the endothelial deletion of *Itga5* did not result in a major vascular phenotype (Li et al., 2012; van der Flier et al., 2010). However, although the *Itga9-/-* mice are born normally, the lymphatic vascular development is defective, due to abnormal formation of lymphatic valves (Table 1) (Bazigou et al., 2009; Huang et al., 2000).

Studies in adult mice have revealed functions of various EC integrins in disease processes (summarized in Table 1). A recent report proposed that α5β1-integrin promotes angiogenesis in the context of the brain and heart ischemia (Table 1) (Lee et al., 2018; Li et al., 2012; Pang et al., 2018). In addition, α5β1-integrin has been found to contribute to chronic arterial inflammation (Table 1) (Al-Yafeai et al., 2018). VEGF has been reported to induce vascular growth via α1β1- and α2β1-integrins, and this signaling seems to promote both VEGFR2 mediated lymphangiogenesis in healing wounds and tumor angiogenesis (Hong et al., 2004; Senger et al., 1997; Senger et al., 2002). αvβ5-integrin in turn, has been shown to mediate VEGF-induced vascular permeability *in vivo*, via FAK recruitment by Src kinase (Table 1) (Eliceiri et al., 2002).

The function of EC integrins in tumor angiogenesis has been puzzling. *Itgb3* and *Itgb5* deletions in mice resulted in enhanced angiogenesis in implanted tumors (Table 1) (Reynolds et al., 2002), whereas *Itga1* deletion decreased tumor angiogenesis (Pozzi et al., 2000). Furthermore, it has been suggested that the FN binding α5β1- and αvβ3-integrins might play essential roles in tumor angiogenesis; however, contrasting results have also been published (Raab-Westphal et al., 2017). Despite of the many studies indicating that β1-integrin is essential in developmental angiogenesis (Table 1), *Itga5* deletion using *Tie2-Cre* in mice revealed that α5β1-integrin is not essential for tumor angiogenesis, and neither is *Itgav* (Murphy et al., 2015). In general, it is now considered that some of the poorly understood functions of integrins can be compensated by other integrin heterodimers in the tumor vasculature, thus complicating attempts to inhibit integrin mediated tumor angiogenesis, and it further seems that the partial benefit of small molecular targeting of FN binding integrins in the tumor vasculature has been a result of secondary effects (Murphy et al., 2015; Raab-Westphal et al., 2017).

Integrins have also been found to interact with growth factor receptor systems in ECs, including the VEGF and TIE receptor systems. While integrin – growth factor ligand or receptor interactions have been mostly reported to be activating, also negative regulation of the growth factor receptors by

integrins have been reported (Ivaska and Heino, 2011). In the blood vascular ECs, VEGFR2 has been reported to interact with αvβ3-integrin during angiogenesis, and similarly, in the lymphatic ECs, VEGF-A, -B and -C have been shown to bind to α9β1-integrin during lymphangiogenesis (Vlahakis et al., 2007; Vlahakis et al., 2005). α5β1-integrin also interacts with VEGFR3 *in vitro* in lymphatic ECs, promoting survival signals (Zhang et al., 2005).

Similarly, interactions of TIE receptors with integrins have been reported. TIE2 was found to co-immunoprecipitate with both α5β1- and αvβ3-integrin from ECs, and both TIE2 and TIE1 were found to interact with integrins using recombinant proteins (Cascone et al., 2005; Dalton et al., 2016; Thomas et al., 2010). ANGPTs have been also found to bind various integrins in ECs and non-ECs, like cardiomyocytes (Dallabrida et al., 2004), breast cancer cells (Imanishi et al., 2007) and in glioma, where the ANGPT2-αvβ1-integrin signaling promoted glioma metastasis (Hu et al., 2006). One report also describes endothelial interactions where ANGPT2 co-immunoprecipitated from TIE2-low EC tip cells with αvβ5- and α5β1-integrins (Felcht et al., 2012). However, the *in vivo* significance of integrin interactions is in many cases not thoroughly understood (see the discussion for further details on TIE-ANGPT-integrin signaling).

Table 1. Genetically modified integrin mouse lines with vascular phenotypes or vascular integrin deletion.

	Deletion	Vascular phenotype	Reference
α1	*Itga1*-/-	Reduced tumor angiogenesis	(Pozzi et al., 2000)
α4	*Itga4*flox/flox;*Tie2*-Cre	No vascular phenotype, followed up to 1 year	(Priestley et al., 2007)
	*Itga4*loxp/loxp;*Tie2*-Cre	Increased lymphangiogenesis and lymph node metastasis	(Garmy-Susini et al., 2013)
α5	*Itga5*-/-	Embryonic death at E9.5, defects in vasculogenesis and angiogenesis, reduced FN deposition	(Francis et al., 2002)
	*Itga5*flox/flox;*Tie2*-Cre	No obvious vascular defects in vascular beds studied up to 18 months, attenuated hypoxia-induced cerebral angiogenesis, no compensatory increase in αvβ3 levels	(van der Flier et al., 2010) (Li et al., 2012)
	*Itga5*flox/flox;*Pdgfrb*-Cre	35% lethality before E17.5; edema, defective formation of lymphovenous valves	(Turner et al., 2014)
	Itga5+/-;*Ldlr*-/-	Reduction of atherosclerotic lesions in atheroprone regions	(Sun et al., 2016a)
	*Itga5*flox/flox;*Cdh5*-iCre;*Apoe*-/-	Deletion induced in adulthood, mice on high fat diet had less oxidized low-density lipoprotein (oxLDL) induced atheromas, and matrix deposition of FN was reduced	(Al-Yafeai et al., 2018)
	*Itga5*flox/flox;*Tie2*-Cre	Smaller infarction zones and increased BBB integrity in a brain ischemia model	(Roberts et al., 2017)
		Aggravated progression of experimental autoimmune encephalomyelitis (EAE) in adult mice, elevated permeability of spinal cord blood vessels	(Kant et al., 2019)
αv/α5	*Itga5flox/flox:Itgav*flox/flox;*Tie2*-Cre	Most of the embryos die by E14.5, defects in the major vessels, minor differences according to genetic background	(van der Flier et al., 2010)
	*Itga5flox/flox:Itgav*flox/flox;*Cdh5*-Cre	Normal tumor growth and tumor angiogenesis.	(Murphy et al., 2015)
α6	*Itga*6flox/flox;*Tie1*-Cre	Increased tumor growth and tumor vessel angiogenesis mediated by elevated VEGF signaling	(Germain et al., 2010)
	*Itga*6flox/flox;*Tie2*-Cre	Decreased ischemia-induced angiogenesis	(Bouvard et al., 2012)
		Reduced tumor growth and reduced tumor vessel number, reduced TIE2-expressing macrophage infiltration	(Bouvard et al., 2014)
	*Itga*6flox/flox;*Pdgfrb*-Cre, embryonic deletion	No effect on physiological angiogenesis, retinal vasculature; in tumor vasculature less pericytes, reduced stability, increased diameter, abnormal BM	(Reynolds et al., 2017)
α9	*Itga9*-/-	P6-P12 pups smaller, respiratory distress due to chylothorax, edema and lymphocyte accumulation in the chest cavity	(Huang et al., 2000)
α9	*Itga9*-/-;*Tie2*-Cre or *Cdh5*-Cre	Deficient lymphatic valve formation and FN deposition to matrix	(Bazigou et al., 2009)
αv	*Itgav*-/-	80% death at E9.5-11.5 but not due to angiogenic defects, embryos show extensive vasculogenesis and angiogenesis, 20% die at P0 from hemorrhage	(Bader et al., 1998)
	Itgav flox/αv null;*Tie2*-Cre or *Nestin*-Cre	No phenotype in cerebral vasculature with *Tie2*-Cre, with *Nestin*-Cre cerebral vascular defects.	(McCarty et al., 2005)
β1	*Itgb1*-/-	Embryonic lethality after implantation	(Fassler and Meyer, 1995) (Stephens et al., 1995)
	*Itgb1*flox/flox;*Tie2*-Cre or *Tie1*-Cre	Embryonic lethality at E10.5-11.5	(Carlson et al., 2008)
	*Itgb1*flox/flox;*Tie2*-Cre	Embryonic lethality at E9.5-10, abnormal vascular patterning	(Lei et al., 2008; Tanjore et al., 2008)
	*Itgb1*flox/flox;*Cdh5*-Cre-ERT or *Itgb1e3*/e3;*Cdh5*-Cre	Embryonic lethality at E13.5-17.5, disrupted vasculature, hemorrhage, abnormal arterial lumen formation	(Zovein et al., 2010)
	*Itgb1*flox/flox;*sma22α*-Cre *Itgb1*e3/e3;*sma22α*-Cre *Itgb1*flox/flox;*Wnt1*-Cre *Itgb1*e3/e3;*Wnt1*-Cre	Defects in vascular wall development in the aortic arch and branching vessels, lethality embryonic or postnatal depending on deletion and Cre-line (smooth muscle cell or neural crest silenced)	(Turlo et al., 2012)

	*Itgb1*lox/lox, *Pdgfb*-iCre or *Cdh5*-iCre	Deletion induced postnatally, deficiency in retinal vessel sprouting and VE-cadherin localization during vascular development and maturation, hyperproliferation of vascular front	(Yamamoto et al., 2015)
	*Itgb1*lflox/flox;*EC-SLC-Cre*	Deletion in adult mice, disturbed alignment of aortic ECs in the outer curvature	(Xanthis et al., 2019)
β3/β5	*Itgb3*-/-;*Itgb5*-/- or *Itgb5*-/-	Enhanced tumor neoangiogenesis	(Reynolds et al., 2002)
β3	*Itgb3*-/-	Increased VEGF stimulated permeability	(Reynolds et al., 2004)
		Coronary capillaries fail to form in male mice	(Weis and Cheresh, 2011)
		Enhanced vascular leakage after endotracheal LPS, increased mortality after LPS or CLP induced sepsis, increased mesenteric vascular leakage after LPS	(Su et al., 2012)
	Itgb3mut+/+	Phosphorylation sites Tyr747 and Tyr 759 mutated to alanines, normal vascular development, reduced tumor vasculature	(Mahabeleshwar et al., 2006)
β4	*Itgb4*-1355T+/+	Reduced tumor vascularization, reduced retinal neovascularization	(Nikolopoulos et al., 2004)
β5	*Itgb5*-/-	Reduced vascular leakage in models of acute lung injury and (ALI) and ventilation induced lung injury (VILI)	(Su et al., 2007)
		Reduced vascular leakage after LPS or cecal ligation and puncture (CLP)	(Su et al., 2013)
		Less VEGF induced vascular leakage. No reduction in *Itgb5*+/- or *Itgb3*-/- mice.	(Eliceiri et al., 2002)
β8	*Itgb8*-/-	65% lethality during midgestation due to improper vascularization of the yolk sac and the placenta. 35% lethality perinatally, intracerebral haemorrhage, abnormal capillaries in the brain, brain ECs are hyperplastic.	(Zhu et al., 2002)
	*Itgb*8flox/flox-*Nestin*-Cre	Brain haemorrhage at P0, abnormal vascular morphogenesis, disorganizes glia. Mice followed to adulthood appeared normal due to an unknown repair mechanism.	(Proctor et al., 2005)
		Hemorrhage in the brain, abnormal vascular morphogenesis, leaky vasculature	(Proctor et al., 2005)
		Retinal hemorrhage, under-developed retinal vasculature, elevated branch point density and vascular coverage	(Arnold et al., 2012)

4. Regulation of endothelial barrier function in inflammation and neovascular disease

Transient increase in vascular permeability is an essential part of a normal inflammatory response, when increasing plasma transport and immune cell infiltration across the vascular wall occur. Acute vascular leakage is induced by the release of inflammatory agents, such as histamine produced by mast cells, and released upon acute infection or allergy. In addition to increase in EC permeability, inflammation induces EC activation and changes in pericyte coverage and ECM composition, including the deposition of a provisional matrix, rich in plasma FN (Rahimi, 2017). The inflammatory ECM is considered to play a role in angiogenic destabilization of ECs via guiding EC adhesions (Arroyo and Iruela-Arispe, 2010). Moreover, alterations of the ECM occur during chronic inflammatory responses, which may further promote immune cell activation (Sorokin, 2010). In acute inflammation, vascular permeability affecting mostly post-capillary venules is reversible and decreased when inflammation resolves. However, prolonged inflammation or chronic inflammatory diseases that develop over time are characterized by chronic vascular permeability, which leads to changes in the capillary and venule structure, endothelial dysfunction and vascular remodeling, driven by e.g. VEGF (Claesson-Welsh, 2015; Rahimi, 2017).

4.1 Vascular destabilizing factors

Various growth factors, pro-inflammatory cytokines and other agents produced during inflammation promote EC activation and permeability. Well known mediators of the fast type I EC activation are thrombin, bradykinin and histamine, which can increase EC permeability in minutes via binding to cell surface heterotrimeric G-protein coupled receptors (GPCRs) (Beckers et al., 2010; Birukov and Karki, 2018). Desensitization of the GPCR signaling, which occurs within 10-20 minutes, results in reversible EC permeability increase. In contrast, type II EC activation is induced by inflammatory cytokines such as TNFα or interleukin (IL)-1β, which produce a slower response (Pober and Sessa, 2007). During microbial infection, lipopolysaccharide (LPS), which is a cell wall constituent of Gram-negative bacteria, and a strong inflammatory mediator, induces EC activation and permeability via the Toll like receptor 4, both *in vitro* and *in vivo*, affecting both capillaries and larger vessels (Chatterjee et al., 2008; Gong et al., 2008). LPS is also a strong activator of the innate immune system, leading to immune cell secretion of numerous proinflammatory cytokines, including TNFα and IL-1β.

The intracellular signaling cascades induced by type I and type II mediators are conveyed via partially overlapping downstream signaling molecules. Notably, GPCRs, in contrast to type II receptor signaling, induce a strong Ca^{2+} signal via phospholipase C, resulting in prostaglandin I_2 and nitric oxide (NO) synthesis by ECs. However, both type I and II mediators induce rearrangements in the actin cytoskeleton that are associated with increased contractility. Thrombin signals via protease activated receptor 1 to activate RhoA, which inhibits the activity of the MLCP, and via calmodulin, which activates MLCK, leading to MLC phosphorylation (Beckers et al., 2010; Birukov and Karki, 2018). Thrombin also increases the expression of ANGPT2, and ANGPT2 has been found to mediate thrombin induced Ca^{2+} signaling and EC permeability (Benest et al., 2013; Huang et al., 2002) (Rathnakumar et al., 2016). TNFα and IL-1β stimulation of ECs also leads to actin stress fiber formation. TNFα suppresses cyclic adenosine monophosphate (cAMP) and Rac1 signaling, thus promoting stress fiber formation (Schlegel and Waschke, 2009), and IL-1β increases contractility via RhoA (Ganter et al., 2008). TNFα also induces expression of ANGPT2, and the effects of TNFα are further enhanced by ANGPT2 (Fiedler et al., 2006; Kim et al., 2000b). *In vitro*, LPS activates several tyrosine kinases, such as the Src family kinases, Protein kinase C, and Rho kinase that rearrange the

actin cytoskeleton into stress fibers and promote adherens junction disruption (Bannerman and Goldblum, 1997; Bannerman et al., 1998; Barabutis et al., 2013; Chatterjee et al., 2008; Gong et al., 2008; Joshi et al., 2014; Zhao and Davis, 2000). *In vivo*, LPS induced leakage of post capillary venules involves decreased endothelial Rac1 activity (Schlegel and Waschke, 2009).

Contractile actin stress fibers contribute to decreased EC monolayer integrity, but various inflammatory agents, such as histamine and bradykinin, also directly regulate VE-cadherin phosphorylation and internalization, leading to dismantling of EC junctions (Dejana et al., 2008; Orsenigo et al., 2012). TNFα also induces the phosphorylation of VE-cadherin, β- and γ-catenin, leading to VE-cadherin internalization (Dejana et al., 2008). IL-1β decreases junctional VE-cadherin independently of its classical myeloid differentiation factor 88 (MyD88)-NFκB pathway, via MyD88-ARF nucleotide binding site opener (ARNO)-ADP ribosylation factor 6 (ARF6) pathway (Zhu et al., 2012).

VEGF, which is produced by many cell types in acute inflammation, in allergy, and in hypoxia, is a potent inducer of vascular permeability, contributing to vascular leakage in tumors and in neovascular eye diseases (Ferrara et al., 2003). VEGF promotes paracellular permeability by inducing the phosphorylation of VE-cadherin Y658 through the adapter protein Tsad leading to Src activation, and VE-cadherin internalization via β-arrestin-mediated endocytosis (Dejana et al., 2008; Fukumura et al., 2001; Gavard and Gutkind, 2006; Sun et al., 2012). *In vivo*, basal VE-cadherin phosphorylation has been observed in veins, which is suggested to contribute to the higher permeability of veins than arteries. VEGF also contributes to the permeability of capillaries by inducing fenestrae formation in small venules and capillaries (Roberts and Palade, 1995). Thus, various permeability inducing agents use both distinct and overlapping signaling pathways to regulate EC permeability.

4.1 Diapedesis

In addition to increased fluid leakage, the paracellular route is also used by inflammatory cells during diapedesis, i.e. the controlled passage of white blood cells from the vasculature to tissues. Upon encountering an invading pathogen, macrophages and dendritic cells secrete cytokines that activate the ECs locally. Activated ECs express various cell adhesion proteins such as E- and P-selectin, ICAM1 &2 and VCAM1 that mediate sequential binding of leukocyte integrins to ECs during leukocyte rolling, adhesion and arrest (Vestweber, 2015). Differential integrin adaptor binding determines the rolling and arrest phases (Lefort et al., 2012). Transient opening of VE-cadherin junctions is necessary for leukocyte diapedesis. The leukocytes do not pass the EC barrier in mutated mice where VE-cadherin cannot dissociate from α-catenin (Schulte et al., 2011). The EC junctions loosen as VE-cadherin is dephosphorylated at Y731 upon contact with leukocytes, and VE-PTP receptor dissociates from VE-cadherin. Additionally, the platelet F11 receptor (also known as JAMA) either on the surface of the ECs or leukocytes, as well as CD31, may participate (Vestweber, 2015). EC actomyosin forms a tight actin cytoskeleton structure around the diapedesis site, ensuring that there is no plasma leakage to tissues (Filippi, 2016). Although inflammation normally resolves, persistent inflammatory infiltrates are present during pathological or chronic inflammation (Phillipson and Kubes, 2011; Vestweber, 2015). In summary, diapedesis does not induce fluid leakage, and the mechanism is therefore different from vascular permeability leading to fluid accumulation and tissue edema during inflammation.

4.3 Sepsis

Sepsis is a dramatic example of increased capillary permeability that can lead to hypovolemic shock due fluid leakage into the tissues. Clinically, sepsis is most often caused by Gram positive- and Gram negative bacteria, but also by viral or fungal infections. In addition, a sterile sepsis-like syndrome called systemic inflammatory release syndrome (SIRS) can initiate from e.g. mechanical trauma or burns. Similarly, strong hypersensitivity reactions like anaphylactic shock may lead to hypovolemia due to SIRS. Microbial antigens, or danger-signals from damaged cells are recognized by pathogen- or danger associated molecular patter (PAMPs and DAMPs, respectively) receptors (PRR) on immune cells (Wentowski et al., 2019).

In sepsis, PRR stimulation leads to an overwhelmed inflammatory response via neutrophils and monocytes. The innate immune complement system and the coagulation cascade are hyperactivated, leading to thrombin production. In some cases, the dysfunctional coagulation cascade activation leads to disseminated intravascular coagulation (DIC), where fibrin clots are formed in the capillaries causing ischemia. The complement and coagulation cascade further reinforce each other (Wentowski et al., 2019). The hyperinflammatory phase can lead to compensatory immunosuppression, highlighted by apoptosis of immune cells like dendritic cells and lymphocytes. Further, anti-inflammatory Th2 signaling overrides proinflammatory Th1 signaling, which may lead to immunoparalysis; a condition where the body cannot sustain innate or adaptive immune responses (Rittirsch et al., 2008).

The incidence of sepsis is high among infants and elderly people, and also higher among men that women. Specific genetic burden towards sepsis is inconsistently studied. In addition, no large-scale studies have been conducted to study the risk factors of sepsis (Rello et al., 2017; Wentowski et al., 2019). The symptoms of sepsis vary according to the initial cause, affected organs, and the patient history (Rello et al., 2017). In the clinics, sepsis can be suspected if respiratory rates are elevated, systolic blood pressure has dropped, and if the patient has a mental change. The initial treatment involves infusion with antibiotics and controlled fluid resuscitation. Increased lactate levels predict capillary hypoperfusion leading to organ failure. Several other circulating factors, like procalsitonin, have been investigated as biomarkers of sepsis (Wentowski et al., 2019). Notably, ANGPT2 levels, measured upon arrival to an emergency unit, have in numerous studies been found to predict poor prognosis in sepsis patients (Leligdowicz et al., 2018).

In the clinics, the different phases of sepsis progression are difficult to determine. Preclinically, sepsis can be divided into two major phases. In the SIRS phase, where proinflammatory actions are elicited by the body, the ECs start to respond to inflammatory stimuli in an uncontrolled manner, and become dysfunctional. In the compensatory anti-inflammatory response syndrome (CARS) phase organs suffer from hypoperfusion as the contents of the capillaries leak heavily into the tissues (Bauer et al., 2006).

For the large part, EC dysfunction and leakage are thought to drive organ failure. In sepsis, the endothelial hemostatic balance is compromised as the ECs turn prothrombic and antifibrinolytic. From preclinical models of sepsis, it is understood that leukocyte diapedesis accelerates, and the cytokines produced by leukocytes enhance EC inflammation. In particular, inflammatory cytokines like TNFα, IL-1β, and IL-6 drive EC permeability and vascular leakage (Matsumoto et al., 2018). Organ function is largely compromised in septic shock. Heart function decreases in septic shock due to insufficient myocardial function (Merx and Weber, 2007). The cardiac depression can be measured using echocardiography, and is caused by a combination of activated ECs and monocytes, inflammatory cytokines and complement activation (Merx and Weber, 2007). The glomerular and

peritubular capillaries of the kidney are especially challenged in sepsis and thus acute kidney failure is a common consequence of sepsis. The liver, recieving 25 % from all blood, suffers from hypoperfusion due to a collapse of the sinusoidal vascular bed, and in the lungs, permeability increase in the pulmonary vasculature disrupts fluid clearance in the alveoli leading to lung injury and acute respiratory distress syndrome (ARDS) (Ince et al., 2016).

4.3 Vascular permeability in neovascular diseases

Vascular permeability is also increased in many common but difficult to cure diseases, highlighted by hypoxia induced neoangiogenesis or inflammation induced vascular remodeling (Baluk et al., 2014; Fallah et al., 2019; Lyle and Taylor, 2019), including various infectious diseases, cancer, atherosclerosis and several ocular diseases. In the eye, pathogenesis of proliferative diabetic retinopathy (PDR), diabetic macular edema (DME) and neovascular age-related macular degeneration (nAMD), is attributed to hypoxia-inducible angiogenic growth factors that also increase EC permeability (Baluk et al., 2014; Fallah et al., 2019; Lyle and Taylor, 2019).

Tumor vasculature grows to support the oxygen demand of the increasing tumor mass. The main angiogenic stimulus in the tumor is VEGF, which is produced by multiple cell types in response to hypoxia-induced activation of HIF. In addition, the tumor microenvironment is rich in various other growth factors, such as ANGPT2, as well as inflammatory cell derived pro-inflammatory cytokines that together with VEGF induce a leaky tumor vasculature. The tumor endothelium is characterized by poorly developed EC junctions, incomplete BM and a low pericyte coverage. The leaky vessels are poorly perfused, leading to hypoxia and reinforcement of the angiogenesis cascade. Leakage also results in increased tumor interstitial pressure, which hinders delivery of chemotherapies, but increases metastasis (Chiang et al., 2016; Martin et al., 2019).

Atherosclerosis is a chronic inflammatory disease where large and medium sized arteries accumulate cholesterol in regions of turbulent flow, developing atheromas. The atheromas and subsequent plaques consists of inflammatory monocytes producing proinflammatory cytokines, like TNFα, smooth muscle cells and lipids, including low-density lipoprotein (LDL) (Back et al., 2019). Inflammatory cytokines activate the ECs resulting in elevated reactive oxygen species (ROS) levels and decreased NO signaling. Increased HIF and VEGF signaling further promote endothelial activation, leakage, monocyte infiltration and inflammation. Furthermore, the plaque alters and hinders blood flow, and unstable plaques can rupture and travel to smaller sized arteries causing ischemia (Gimbrone and Garcia-Cardena, 2013).

4.4 ANGPT–TIE pathway and vascular stability

The ANGPT–TIE pathway regulates both vascular stability and leakage in homeostasis and disease, respectively. Various diseases associated with endothelial activation and vascular leakage, such as sepsis, cancer, neovascular eye diseases, atherosclerosis and diabetes, are reported to have imbalances in the ANGPT–TIE system. In general, ANGPT2 levels increase in activated ECs in various diseases, whereas ANGPT1 levels may decrease. Simultaneously, TIE receptor expression can decrease. These changes in the endothelium lead to decreased ANGPT1/ANGPT2 ratio, impaired vascular stability due to decreased TIE2 signaling, and hence facilitate plasma leakage into tissues (Saharinen et al., 2017a).

In human sepsis, elevated ANGPT2 levels have been associated with poor patient prognosis correlating with high mortality (Bhandari et al., 2006; Davis et al., 2010; Ebihara et al., 2009; Gallagher et al., 2008; Giamarellos-Bourboulis et al., 2008; Giuliano et al., 2007; Kumpers et al.,

2010; Kumpers et al., 2008; Mankhambo et al., 2010; Orfanos et al., 2007; Page et al., 2011; Parikh et al., 2006; Ricciuto et al., 2011; Siner et al., 2009; Statz et al., 2018; van der Heijden et al., 2009; van der Heijden et al., 2008). Decrease in ANGPT1 levels has also been reported in sepsis patients (Ebihara et al., 2009; Giuliano et al., 2007; Mankhambo et al., 2010; Ricciuto et al., 2011). Interestingly, TIE2 levels have been found to correlate with the outcome of ARDS. Certain genetic variations in humans were associated with high TIE2 expression; such individuals had a 28% lower risk of developing ARDS after microbial infection. In contrast, patients harboring a more common variation associated with lower TIE2 levels were 31% more likely to develop ARDS (Ghosh et al., 2016).

Several lines of evidence from mouse sepsis models suggest a functional role for ANGPT2 in sepsis. LPS elevates ANGPT2 expression and thus attenuates ANGPT1–TIE2 EC stabilizing signaling *in vivo* (Korhonen et al., 2016; Zeng et al., 2016). In line with this, heterozygous deletion of ANGPT2, or *in vivo* ANGPT2 targeting with siRNA, protected from LPS-induced acute lung injury (ALI) (David et al., 2012). In the lung, ANGPT2 attenuates TIE2 signaling and induces vascular remodeling during *Mycoplasma pulmonis* infection (Tabruyn et al., 2010). *Tie2* heterozygous mice, that express 50% less TIE2, were more susceptible to both LPS and cecal ligation and puncture (CLP) -induced sepsis than wild type mice (Ghosh et al., 2016). TIE2 signaling was reported to inhibit tissue factor activation, which can lead to thrombin production hence potentially contributing to DIC (Higgins et al., 2018). Highlighting the counteracting role of the ANGPT1, ANGPT1 inhibits vascular leakage induced by histamine, thrombin and VEGF *in vivo* (Baffert et al., 2006; Gamble et al., 2000; Thurston et al., 2000).

Numerous reports have found a correlation between increased circulating ANGPT2 levels and poor patient prognosis in cancer (Chen et al., 2018; Goede et al., 2010; Hacker et al., 2016; Helfrich et al., 2009; Park et al., 2007). Murine tumor models have shown that ANGPT2 is upregulated in the blood vessels co-opted by the tumor. This induces pericyte detachment from the capillary endothelium and vessel regression generating hypoxic areas that further induce the expression of ANGPT2 and VEGF, leading to enhanced angiogenesis in the tumor (Holash et al., 1999; Scholz et al., 2016). Pericyte detachment and ANGPT2-mediated activation of the endothelium promote metastasis and impair tumor vessel integrity leading to leakiness (Falcon et al., 2009; Imanishi et al., 2007; Keskin et al., 2015; Minami et al., 2013; Schulz et al., 2011).

Antibodies blocking ANGPT2–TIE2 interaction inhibit solid tumor growth in mice (Oliner et al., 2004), reducing metastasis (De Palma and Lewis, 2013), and improving blood vessel endothelial integrity at metastatic sites (Holopainen et al., 2012). Further benefit has been reported when both ANGPT2 and VEGF were blocked at the same time (Daly et al., 2013; Hashizume et al., 2010; Kienast et al., 2013; Scheuer et al., 2016). Alas, clinical trials testing ANGPT2 targeting compounds did not result in improvement of patient outcome in ovarian cancer. However, ANGPT2 targeting in combination with VEGF inhibitors is still under investigation, and currently in clinical trials in solid tumors (Fukumura et al., 2018).

In the eye, ANGPT2 contributes to the pathological neovascularization of both retinal and choroidal vessels, whereas ANGPT1 suppressed pathological neovascularization and vascular leakage (Lee et al., 2014b). Currently, combinatorial targeting of ANGPT2 and VEGF is being tested in clinical trials for the treatment of DME and nAMD (Saharinen et al., 2017a). In addition to DR and DME, serum ANGPT2 levels are further elevated in type II diabetic patients, which correlates with diabetic pathologies, like diabetic angiopathy (Li et al., 2015; Rasul et al., 2011).

ANGPT2 is expressed in response to turbulent flow in atheroprone regions of the arteries, where it strengthens inflammation and promotes endothelial activation (Le Dall et al., 2010; Post et al., 2008). Antibodies against ANGPT2 reduced the formation of fatty streaks in hypercholesterolemic (low-density lipoprotein receptor−/−; apolipoprotein B100/100) mice, indicating that ANGPT2 contributes to early development of atherosclerotic plaques (Theelen et al., 2015).

Recently, an inducible endothelial specific deletion of VE-PTP was reported. Deletion of VE-PTP postnatally protected in *Ins2^{Akita}129/SvEv* mice from diabetes. VE-PTP inhibition induced the activation of TIE2, leading to activation of eNOS and inhibition of FOXO1 nuclear translocation attenuating inflammatory signals (Carota et al., 2019). In venous malformation, the endothelial TIE2 receptor has a gain of function -mutation that leads to TIE2 hyperactivation, causing over grown, distorted vessels with excessive lumens and poor smooth muscle cell coverage. Also, the matrix beneath the endothelium is altered. This condition is painful and highlights the fact that a balance in TIE2 activity is essential for proper vessel development and homeostasis (Kangas et al., 2018).

The above-mentioned pathological conditions are a result of complex signaling. *In vitro* studies have revealed potential mechanisms of action for the ANGPT-TIE system, and ANGPT1–TIE2 signaling stabilizes the endothelium by several means. Importantly, ANGPT1 promotes VE-cadherin stability in the junctions and supports the cortical actin cytoskeleton, whereas ANGPT2 counteracts these effects especially when inflammatory stimuli are present (David et al., 2012; Gamble et al., 2000) (see Figure 5 for details).

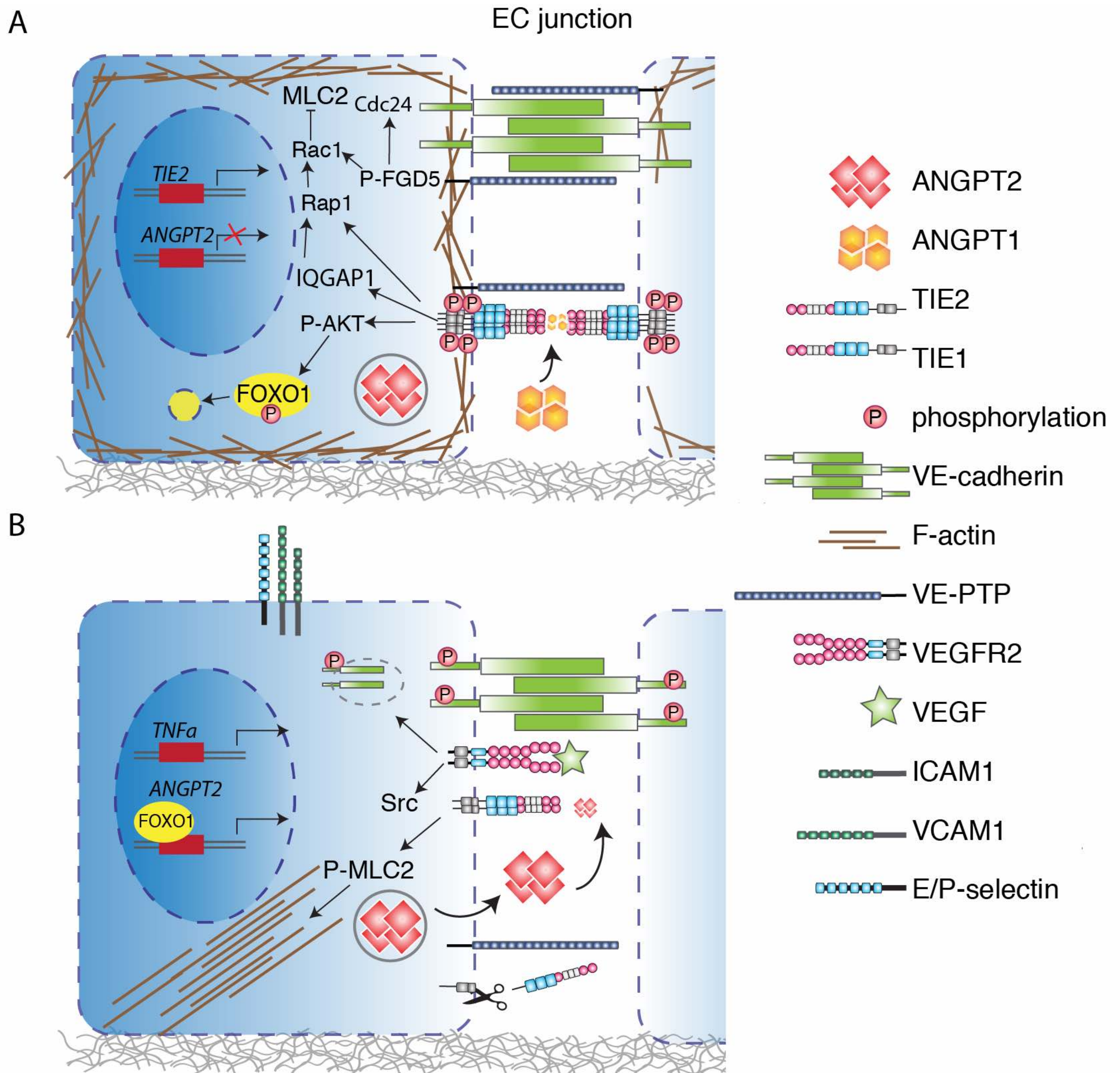


Figure 5. ANGPT–TIE signaling in EC quiescence and destabilization. A) ANGPT1-TIE2 stabilizing signaling in ECs. ANGPT1 induces the phosphorylation of TIE2, and TIE1, leading to Akt activation, FOXO1 phosphorylation and its nuclear exclusion and degradation, attenuating ANGPT2 expression. ANGPT1–TIE2 signaling further promotes cortical actin cytoskeleton via Rap1-Rac1-mediated signaling, attenuating MLC phosphorylation. VE-PTP stabilizes VE-cadherin at EC-EC junctions and further sustains EC integrity. If VE-PTP is inhibited, phosphorylated FGD5 inhibits stress fiber formation via Rap1 and supports cortical actin via Cdc24. ANGPT1 inhibits inflammatory gene expression via inhibition of NFκB signaling. B) ANGPT1–TIE signaling is attenuated in inflammation *in vivo*. ANGPT2 is released from the Weibel-Palade bodies in response to inflammatory stimuli both *in vitro* and *in vivo*. Excess of ANGPT2 inhibits ANGPT1-TIE2 signaling. *In vivo*, inflammatory signals induce the proteolytic cleavage of TIE1, supporting ANGPT2 antagonistic function. FOXO1 gene expression, including ANGPT2, is induced, leading to a positive feedback loop, fortifying EC destabilization. In synergy with inflammatory agents and VEGF, ANGPT2 dismantles VE-cadherin from cell junctions. VEGF-VEGFR2 signaling that activates Src kinase induces VE-cadherin internalization. ICAM1, VCAM1, and selectins are upregulated enabling diapedesis (not illustrated). Information illustrated here is drawn from multiple sources (Frye et al., 2015; Saharinen et al., 2017b; Salomao et al., 2008).

AIM OF THE STUDY

This thesis aimed to elucidate how ANGPT2 regulates EC monolayer destabilization and vascular permeability. Specifically, the aim was to delineate the interplay between ANGPT2, TIE receptors and endothelial integrins in EC destabilization. The study was undertaken to understand the mechanisms underlying the loss of the EC integrity in inflammation, which is expected to facilitate the design of vascular stabilizing therapies for diseases associated with vascular leakage. The specific questions were:

I How does ANGPT2 signal to destabilize the endothelium, and do endothelial integrins play a role in this?

II Do ANGPT2 and β1-integrin signaling promote endothelial destabilization in inflammation and vascular leakage in sepsis? What are the mechanisms that promote EC permeability in inflammation?

MATERIALS AND METHODS

5. Cell lines and treatments (I&II)

Altogether, this thesis presents similar results from three different primary human ECs derived from different vascular beds (Table 2). *In vitro* EC studies were conducted on glass coverslips or plastic wells, coated with FN. *In vivo*, however, ECs experience various ECM molecules and matrix stiffnesses, which were not considered in our cellular assays. Another limitation is that blood flow, which regulates many EC functions *in vivo*, was not examined.

Primary human ECs (Table 2) were used between passages 1-5, except HUVECs between passages 1-7. EC culture and experiments, unless otherwise mentioned, were performed on FN coated culture plates and Transwell inserts. All other cell lines (Table 2) were cultured, and used for experiments, without coating.

Table 2. Cell lines.

Cell line	Source/Reference	Description	Growth medium*	Used in:
HMVEC (referred to BEC)	Lonza	Primary human Neonatal skin microvascular EC	EBM2	I, II
HMVEC	Promocell	Primary human Neonatal skin microvascular EC	EBM2	I, II
HPMEC	Promocell	Primary human pulmonary microvascular EC	EBM2	I, II
HUVEC,	Promocell, Cell Applications	Primary human umbilical vein EC	EBM2	II
b.END3	ATCC	Immortalized, murine cerebral EC	EBM2	II
CHO	ATCC	Immortalized, Chinese hamster ovary cells	DMEM	I
HeLa	ATCC	Immortalized, Epithelial cell from cervical cancer	DMEM	I
LLC	ATCC	Immortalized, murine lung cancer cell line	DMEM	I
NCI-H430-LNM-35 (LNM-35 for short)	ATCC	Immortalized, human lung cancer cell line	RPMI	I
293-GPG-VSV-G	(Ory et al., 1996)	Immortalized, human derived packaging cell line for retroviral production	DMEM (4.5 g/l glucose)	I
293FT	(Ory et al., 1996)	Immortalized, human derived packaging cell line for lentiviral production	DMEM (4.5 g/l glucose)	I, II

*) All media were from Lonza. EBM2 medium was supplemented with Lonza endothelial supplement kit; DMEM and RPMI with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin; DMEM 4.5 glucose/l for the culture of 293-GPG-VSV-G with 10% FBS, 1% glutamine, 0.2% penicillin, 0.2% streptamycin, 0.2% puromycin, 0.6% neomycin and 0.00008% tetracycline; and DMEM 4.5 glucose/l for 293FT with 10% FBS, 1% L-glutamine, 0.2% penicillin and 0.2% streptomycin.

5.1 Genetic manipulation of cell lines (I&II)

5.1.1 shRNA and siRNA mediated gene silencing

shRNA mediated gene silencing could result in off-target effects, and activation of compensatory mechanism during the 48 h silencing period. To rule out off-target effects, all major results were verified with another shRNA target sequence (Table 3) or with siRNA for TIE2 silencing in Study I.

siRNA approach was not suitable to validate the effects of β1-integrin silencing in confluent monolayers, since siRNA transfection requires the use of subconfluent EC cultures, whereas β1-integrin silenced cells are impaired in cell proliferation and do not grow to confluency. Further confirmation of the results was obtained using the inhibitory β1-integrin antibody Mab13, in addition to shRNA. Direct plasmid transfection can be inefficient in ECs, and was not considered an option for efficient gene targeting. The re-expression of silenced genes had to be ruled out in verifying the specificity of integrin silencing, since the efficiency of integrin overexpression was found poor in the ECs. Overexpression of the mouse TIE2 ectodomain in TIE2 silenced ECs was successful and TIE2 re-expression rescued the silenced EC phenotype.

Different shRNA vectors (Table 3) from the TRC1 library (Broad Institute, www.broadinstitute.org) were used together with packaging plasmids pCMVg and pCMVdelta8.9 to produce lentiviral particles in 293FT cells (Ory et al., 1996). Cells were transduced with shRNA lentivirus particles in the presence of 0.1% Polybrene (Sigma-Aldrich) for 48h. For siRNA silencing of *TIE2*, BECs were starved overnight in 1% FBS EBM2, without other supplements, and transduced twice for 24 h on subsequent days, with siRNA against *TIE2* or scramble sequences (Sigma-Aldrich, SC-3667 siTIE2) using Oligofectamin (Invitrogen). The main results were repeated with two different shRNA expressing lentiviruses (Table 3).

Table 3. Lentiviral shRNA target sequences

Target	shRNA sequence	Used in
TIE2	GCTTCTATACAAACCCGTTAA	I
TIE2	CGCTACCTACTAATGAAGAAA	I
TIE1	GACTGGAGCAACACAGTAGAA	I
TIE1	CTCTGACTTAAGCTGCCTCAA	I
ITGB1	GCCTTGCATTACTGCTGATAT	I, II
ITGB1	GCCCTCCAGATGACATAGAAA	I, II
ITGAV	GTGAGGTCGAAACAGGATAAA	I
ITGB5	GCTGTGCTATGTTTCTACAAA	I
TENSIN1	CCGACTACTCACTTCAGCATT	II
TENSIN1	GCCTTTATCCACTCGAATTAT	II
ANGPT2	GATGATAGAAATAGGGACAAA	I, II
ANGPT2	CCCTAATTCTACAGAAGAGAT	I, II
ITGA5	CTCCTATATGTGACCAGAGTT	II
ITGA5	CCTCAGGAACGAGTCAGAATT	II
ITGB3	GATGCAGTGAATTGTACCTAT	II
ITGB3	CCTTAGCCTTTGTCCCAGAAT	I, II

5.1.2 Expression vector cloning and retroviral overexpression (I)

To study weather ANGPT1 and ANGPT2 fibrinogen like domain (FLD) and N-terminal linker region have differential functions in integrin binding and activation, the respective domains were interchanged via cloning, and chimeric angiopoietins were produced in CHO cells. Angiopoietin chimeras ANGPT1-ANGPT2-Flag and ANGPT2-ANGPT1-Flag were constructed as follows: the ANGPT fibrinogen like domain (FLD) and the N-terminal linker region (amino acids R262-F498 in ANGPT1; K249-F496 in ANGPT2) were changed between ANGPT1 and ANGPT2 using two-step PCR and cloned into the pMXS vector. The constructs were tagged with a Flag-peptide coding sequence (DYKDDDDK), which was attached to the chimeric angiopoietin C-terminus, followed by a stop codon. ANGPT2 and ANGPT2-ANGPT1 contained the native ANGPT2 signal sequences, whereas ANGPT1 and ANGPT1-ANGPT2 were expressed under the Igκ light chain signal sequence.

Membrane bound murine TIE2 ectodomain (mTIE2-ECD) was cloned by PCR into the pMXs vector (a kind gift from Dr. Kitamura University of Tokyo, Japan) resulting in a deletion of the intracellular I824-A1124 amino acids in mTIE2. A membrane bound form of eGFP was created by attaching a myristoylation and palmitoylation sequence from the lyn kinase (MGCIKSKRKDNLNDDGVD) (Zacharias et al., 2002) to the eGFP N-terminus by PCR, and inserting the PCR fragment in to pMXs. Retroviruses were produced in 293-GPG-VSV-G cells, and transduced into cells in the presence of 0.1% Polybrene for 48 h.

5.2. Stimulation of cells in culture (I&II)

HeLa cells were starved for 2 h in 2% FBS-DMEM, and stimulated in the starvation medium (I). ECs were starved for 1 h in starvation media consisting of overnight-conditioned medium diluted (1:5) with serum free medium (final FBS concentration 1%) (I&II), and stimulated with various agents as listed in Table 4. Experimental details are found in the original articles I&II.

Table 4. Reagents used in cell stimulations.

Stimulus	Concentration	Cell type	Provider, cat. Nro.	Used in:
E. Coli LPS O111:B4	2.5 μg/ml	BEC	Sigma-Aldrich, L4391	II
E. Coli LPS O55:B5	10 μg/ml	BEC	Sigma-Aldrich, L2880	II
rhIL-1β	10 ng/ml	BEC, HMVEC	R&D, 201-LB	II
Thrombin	0.1, 0.2 or 1 U/ml	BEC, HMVEC, HUVEC	Sigma-Aldrich, 10602400001	II
S-Blebbistatin	10 mol/L	BEC	Santa Cruz, SC-204253	II
rhANGPT1	60 nM	HeLa	R&D, 923-AN	I
rhANGPT2	60 nM or 10 μg/ml	HeLa/BEC	R&D, 623-AN	I, II
Rat anti-integrin β1 mAb13 (Table 5)	0.1, 4 or 10 μg/ml	BEC, HUVEC	BD Biosciences, 552828	I, II
Hamster anti-integrin β1 HMβ1 (Table 5)	10 μg/ml	b.END3	Biolegend, 102210	II
Cilengitide	10 μM	b.END3/BEC	Sigma-Aldrich	II

6. Immunological and RNA-based methods (I&II)

Table 5. Antibodies used; antibody clone indicated for functional antibodies.

PRIMARY ANTIBODIES			
Antigen	**Antibody (functionality, clone)**	**Provider, cat. Nro.**	**Used in:**
Human TIE1	Goat polyclonal	R&D, AF619	I, II
Human TIE2	Goat polyclonal	R&D, AF313	I
Mouse Tie2	Goat polyclonal	R&D, AF762	I, II
Human ANGPT2	Goat polyclonal	R&D, AF623	I, II
Human VE-cadherin	Mouse monoclonal	BD Biosciences, 555661	I, II
Human VE-cadherin	Rabbit monoclonal	Cell Signaling, 2500	I, II
Mouse VE-cadherin	Rat monoclonal	BD Biosciences, 555289	I, II
Mouse VE-cadherin	Rat monoclonal	eBioscience, 14-1441	I, II
His-tag	Rabbit polyclonal	Cell Signaling, 2365	I
Flag-tag	Rabbit polyclonal	Sigma Aldrich, F7425	I
Human fibronectin	Rabbit polyclonal	Sigma Aldrich, F3648	I
Human β-catenin	Mouse monoclonal	BD Biosciences, 610153	I
Human ZO-1	Rabbit polyclonal	Invitrogen, 61-7300	I, II
Human p-ERK1 Thr202/Tyr204	Rabbit polyclonal	Cell Signaling, 9101	I

Rat ERK	Rabbit polyclonal	Cell Signaling, 9102	I
Mouse p-Akt Ser473	Rabbit polyclonal	Cell Signaling, 9271	I
Mouse Akt	Rabbit polyclonal	Cell Signaling 9272	I
Human β3-integrin	Rabbit monoclonal	Abcam, ab75872	I, II
Human β5-integrin	Rabbit polyclonal	Abcam, ab15459	I
Mouse β1-integrin	Rat monoclonal (activating, 9EG7)	BD Biosciences, 553715	I
Human β1-integrin	Mouse monoclonal (activating, 12G10)	Abcam, ab30394	I, II
Human β1-integrin	Mouse monoclonal	Millipore, Mab2252	I
Human β1-integrin	Mouse monoclonal (blocking, mAb13)	BD Biosciences, 552828	I, II
Mouse β1-integrin	Armenian hamster monoclonal (blocking, HMβ1)	BioLegend, 102210	II
Hamster α5-integrin	Mouse monoclonal	Hybridoma bank, PB1	I
Mouse CD31	Armenian hamster monoclonal	Millipore, Mab1398Z	I, II
Human αvβ3-integrin	Mouse monoclonal	Millipore, Mab1976	I
Human αvβ5-integrin	Mouse monoclonal	Millipore, Mab20197	I
Mouse α4-integrin	Rat monoclonal (blocking, PS/2)	BioXCell, BE0071	II
Human α5-integrin	Mouse monoclonal	Novus, NBP2-50146	II
Human α5-integrin	Goat polyclonal (activating, SNAKA51)	R&D, AF1864	I, II
Human talin-1	Mouse monoclonal	Sigma Aldrich, T3287	II
Human tensin-1	Rabbit polyclonal	Sigma Aldrich, SAB4200283	II
Mouse S100A8	Goat polyclonal	R&D, AF3059	II
Mouse VCAM1	Goat polyclonal	R&D, AF643	II
Mouse desmin	Rabbit monoclonal	Millipore, 04-585	II
Mouse Ly6G/C	Rat monoclonal	Biolegend 108402	II
SECONDARY ANTIBODIES			
Antigen	**Antibody, conjugate**	**Provider, cat. Nro.**	**Used in:**
Rabbit IgG	Goat polyclonal, biotinylated	GE Healthcare, E0432	I, II
Goat IgG	Rabbit polyclonal, biotinylated	GE Healthcare, E0466	I, II
Mouse IgG	Goat polyclonal, biotinylated	GE Healthcare, E0433	I, II
Rabbit IgG	Swine polyclonal, HRP-conjugated	GE Healthcare, P0217	I, II
Goat IgG	Rabbit polyclonal, HRP-conjugated	GE Healthcare, P0493	I, II
Mouse IgG	Goat polyclonal, IRDye 800CW	LI-COR, 926-32210	II
Goat IgG	Donkey polyclonal, IRDye 680RD	LI-COR, 925-68074	II
Goat IgG	Donkey polyclonal, Alexa fluor488	Thermo Scientific, A11055	I, II
Rabbit IgG	Donkey polyclonal, Alexa fluor488	Thermo Scientific, A21206	I, II
Mouse IgG	Donkey polyclonal, Alexa fluor488	Thermo Scientific, A21202	I, II
Goat IgG	Donkey polyclonal, Alexa fluor594	Thermo Scientific, A11058	I, II
Rabbit IgG	Donkey polyclonal, Alexa fluor594	Thermo Scientific, A21207	I, II
Mouse IgG	Donkey polyclonal, Alexa fluor594	Thermo Scientific, A21203	I, II
Rat IgG	Donkey polyclonal, Alexa fluor488	Thermo Scientific, A21208	I, II
Rat IgG	Donkey polyclonal, Alexa fluor594	Thermo Scientific, A21209	I, II
Goat IgG	Donkey polyclonal, Alexa fluor647	Thermo Scientific, A21447	I, II
Armenian hamster IgG	Goat polyclonal, Alexa fluor568	Thermo Scientific, A21112	II
Rat IgG	Chicken polyclonal, Alexa647	Thermo Scientific, A21472	
Armenian hamster IgG	Goat polyclonal, FITC conjugated	Jackson Immunoresearch, 127-095-160	I, II

6.1 Immunofluorescence staining of cultured cells on coverslips and for TIRF

Cells on coverslips were fixed in 4% PFA-PBS, washed with PBS and permeabilized for 5 min with 0.1% Triton X-100. For talin-1 or tensin-1 staining, the cells were fixed with 4% PFA containing 0.2% Triton X-100 for 10 min and permeabilized with 0.2% Triton X-100 in PBS. Samples were blocked in 1% bovine serum albumin (BSA) -PBS, incubated for 30 min with primary antibodies (Table 5) RT in 1% BSA-PBS, washed with PBS, blocked, incubated for 30 min with secondary antibodies (Table 5) at RT, washed, and mounted using semisolid mounting medium with DAPI (Abcam). TIRF (total internal resonance fluorescence) samples were similarly stained in TIRF glass bottom wells (Ibidi) without BSA and post-fixed with 4% PFA.

6.2 Whole-mount staining (I&II)

For whole mount tissue and *en face* aorta preparation, mice were anaesthetized and perfused with 1% PFA-PBS. Aortas and tracheas were further fixed for 1 h in 1% PFA-PBS, and ear skin overnight at 4 °C in 4% PFA. Tissues were blocked with donkey immunomix (5% donkey serum, 0.2% BSA, 0.3% TritonX-100, 0.05% sodium azide in Dulbecco's PBS) for 1 h RT. Whole mount staining was performed using indicated primary antibodies for 24–48 h at 4°C, followed by extensive washing using 0.3%-TritonX-100-PBS RT, incubation with secondary antibodies for 16 h at RT, washing and post-fixing of the samples. Tissues were mounted in DAPI-Vectashield.

6.3 Immunohistochemistry of frozen mouse tissue sections (II)

After sacrificing the mice, lungs were fixed in 4% PFA-PBS o/n, washed in PBS and treated with 25% sucrose o/n. Tissues were immersed in OCT compound (Tissue Tek) and frozen in dry ice. Frontal frozen sections of the lungs were washed with 0.3% Triton X-100-PBS, blocked with BSA or donkey immunomix, treated with primary antibodies o/n at +4 °C in blocking buffer, washed with 0.3% Triton X-100-PBS, treated with secondary antibodies for 2 h RT, washed with 0.3% Triton X-100-PBS, rinsed with PBS, and mounted in DAPI-Vectashield.

6.4 Immunoprecipitation and Western blot

Cell lysates were prepared in (I) MPER (ThermoFisher Scientific) or (II) PLCLB or RIPA buffers, and tissue lysates were prepared in RIPA buffer, containing protease and phosphatase inhibitors (Sigma-Aldrich). Soluble TIE1 (II) ectodomain was analyzed using Western blot from EC supernatants, and from mouse serum as described previously (Korhonen et al., 2016). For analysis of the phosphorylated TIE2, equal amounts of total protein from mouse lung lysates were used for immunoprecipitation with anti-mTIE2 (R&D Systems) and the protein G-Dynabeads (ThermoFisher Scientific) at +4 °C. The immunocomplexes or total cell lysates, supernatant and serum, were boiled in Laemmli sample buffer and separated on a polyacrylamide gel (Bio-Rad and ThermoFisher Scientific) and transferred to a methanol-activated PVDF membrane (Merck Millipore). The membrane was blocked in 0.05% Tween-20–TBS containing 5% BSA or BBLicor (Bio-Rad), and probed with primary antibodies (Table 5) at +4 °C overnight, followed by secondary antibodies (Table 5) 30 min RT. SuperSignal West Pico and Femto Luminol reagents (ThermoFisher Scientific) or (II) the Odyssey CLx near-infrared fluorescence imaging system (LI-COR) were used for detection. (I&II) Fluorescent Western blots were converted to grayscale images in Adobe Photoshop CS6. Quantifications of band intensities were performed using LI-COR software or ImageJ software.

6.5 ELISA (I&II)

Standard sandwich enzyme linked immunosorbent assay was used to measure human ANGPT2 from EC supernatants using the Quantikine kit according to the manufacturer's instructions (R&D Systems) (I). Blood was collected through the heart of anaesthetized mice and serum was separated by centrifugation. Standard sandwich enzyme linked immunosorbent assays were used to measure mANGPT2, mIL-1β, mTNFα and mIL-6 serum levels with the Quantikine kits (R&D Systems) according to the manufacturer's instructions (II).

6.6 Quantitative real time PCR

For Q-RT-PCR, total RNA from cell cultures was isolated using RNA NucleoSpin II Kit (Macherey-Nagel), or from murine tissues with Trisure reagent (Bioline) according to the manufacturer's protocol. RNA was reverse transcribed to cDNA using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen), RT2 First Strand Kit (Qiagen), or iScript cDNA Synthesis Kit (Bio-Rad). RT-PCR was carried out using the DyNAmo HS SYBR Green master mix (Thermo Scientific) and run on a BIO-RAD C1000 Thermal cycler (Bio-Rad). Primers used are listed in Table 6. Estimate of *Angpt2* mRNA copy number was calculated based on control samples of known *Angpt2* cDNA concentrations. Integrin expression was analyzed using the RT2 profiler PCR Array Wound Healing (Qiagen). Mouse samples were normalized to *Gapdh* and human samples to *GAPDH* or *CD31*. Fold changes were calculated using the comparative CT (threshold cycle) method.

Table 6. Q-RT-PCR primers*.

Target:	Forward (5'-3')	Reverse (5'-3')	Used in:
ANGPT1	AACATGGGCAATGTGCCTACACTT	CATTCTGCTGTATCTGGGCCATCT	I
ANGPT1	ACGTGGAACCGGATTTCTCT	TTTAGTACCTGGGTCTCAACATCT	I
ANGPT2	CAGATTTTGGACCAGACCAGTGA	TCAATGATGGAATTTTGCTTGGA	I
ANGPT4	ATCCAGCGCCGTGAGAATG	AAATGTTCGTACTGGGCATAGG	I
ANGPT4	CAGGACTGTGCAGAGATCCA	TCTCCGAAGCCCTGTTTGTA	I
Angpt1	CATTCTTCGCTGCCATTCTG	GCACATTGCCCATGTTGAATC	II
Angpt2	TTAGCACAAAGGATTCGGACAAT	TTTTGTGGGTAGTACTGTCCATTCA	II
Tnfa	CATCTTCTCAAAATTCGAGTGACAA	CATTGAGGTGGAGAGCTTTC	II
Il1b	GTGTGACGTTCCCATTAGAC	CATTGAGGTGGAGAGCTTTC	II
Il6	AGTTGCCTTCTTGGGACTG	AGGTCTGTTGGGAGTGGTATC	II
Gapdh	CCACTAGGCGCTCACTGTTC	CCCCATACGACTGCAAAGAC	II
ITGB1	CCGCGCGGAAAAGATGA	GAATTTGTGCACCACCCACAA	II
PECAM1	CTGCTGACCCTTCTGCTCTGTTC	GGCAGGCTCTTCATGTCAACACT	II
TNS1	TCTTGAAGGGAGACATCTTG	TTGAAAGCATCATCAAGGTC	II

*) Primers from Sigma-Aldrich

7. *In vitro* assays

7.1 Microscopy and image analysis (I&II)

This thesis entails a large body of image analyses, which is the method of choice to observe cellular morphology and subcellular localization of proteins, and may not be replaced by other methods. Majority of the *in vitro* analyses were focused to monitor the presence of VE-cadherin or stress fibers (Table 7), which were used to reveal the EC monolayer integrity. Image quantitation was carried out using manual thresholding (VE-cadherin and other analyses, Table 7) or visual evaluation (stress fibers), whereas a more automated approach would offer an investigator-blinded approach. In study I, VE-cadherin cell surface expression was used to complement immunofluorescence studies, but this, nor VE-cadherin internalization, were not investigated in study II (Orsenigo et al., 2012). Additionally, traction force microscopy, utilized in study II, could have been an elegant means to report functional effects of TIE2 and integrin silencing also in study I. In addition, imaging was used to observe vascular structures and inflammatory cells in tissues using whole mount preparations, complemented with protein and RNA analysis.

Table 7. Image analyses in short*

Analysis	Material	Image type	Quantification	Used in:
VE-cadherin	EC coverslips	40x epifluorescence/63x confocal	Total area of pixels per image ** normalized to the total cell number ***	I: Fig 1; II: Fig 1, Fig 2, Fig 7, Fig S3, Fig S4, Fig S6
Stress fibers	EC coverslips	40x epifluorescence	Presence of stress fibers extending across the longitudinal cell axis normalized to the total cell number per image ***	I: Fig 1, Fig 3, Fig S1, Fig S2, Fig S3, Fig S5, Fig S6; II: Fig S5
EC elongation	EC coverslips	40x epifluorescence	EC length/width ratio	I: Fig S1
ZO-1	EC coverslips	63x confocal	Total length of ZO-1 positive junctions per image normalized to the total cell number **	II: Fig 2, Fig S3
α5-integrin adhesions	EC coverslips	63x confocal	Total area of pixels per image ** normalized to the total cell number ***	II: Fig 7
β5-integrin adhesions	HeLa	63x confocal	total pixels/area **	II: Fig 4
ANGPT2 binding	HeLa	63x confocal	Number of cells that stain positive for ANGPT2 normalized to total cell number ***	II: Fig 4
Tensin-1	TIRF wells	63x TIRF /100x TIRF	Total area of pixels per image ** normalized to the total cell number ***	II: Fig 7
TIE2 complementation	EC coverslips	40x epifluorescence	Ratio of SF positive+mTIE2-ECD+ or SF positive+GFP+ cells normalized to the total cell number ***	I: Fig 5
EC spreading, β1-integrin adhesions	EC coverslips	63x confocal	Total pixels from the central 50% of cell area **	I: Fig2, Fig S4
EC spreading, cell area	EC coverslips	63x confocal	Total actin pixels per area**	II: Fig S1, Fig S7
α5-integrin recycling	EC coverslips	63x confocal	Total pixels per area of α5-integrin positive vesicles quantified from cropped images: 1 cell/image	II: Fig 7
Cancer cell transmigration	Transwell inserts	40x epifluorescence	Total count of GFP positive cancer cells in the insert or total GPF signal	I: Fig 1, Fig S2
Aortic EC junctions	*En face* whole mount	63x confocal maximum intensity projections including the complete VE-cadherin signal from the total thickness	total pixels/area **	I: Fig 6
EC junctions	Fixed 80nm sections of trachea and lungs	4800x TEM images	Relation of electron dense area to the total length of EC-EC contact	II: Fig 5
Vascular leakage	Whole mounts of trachea and ear skin	5x epifluorescent	total pixels/area of fluorescent tracer **	II: Fig 3, Fig 6, Fig S10, Fig S12
VCAM-1	Frozen O.C.T. sections of lungs	20x epifluorescent	total pixels/area of fluorescent tracer **	II: Fig 4
S100A8	Frozen O.C.T. sections of lungs	Slide scanner whole tissue images	total pixels/area of fluorescent tracer **	II: Fig S11
Ly6/G	Frozen O.C.T. sections of lungs	20x epifluorescent	total pixels/area of fluorescent tracer **	II: Fig 4

*) All image analyses were performed with ImageJ software (version 1.46, http://imagej.nih.gov/ij/), from at least three independent experiments, and results are expressed as the mean with standard deviation. **) Using a similar threshold value for each image within one experiment. ***) DAPI positive cell nuclei.

Images of cells on coverslips, whole mounts and frozen tissue sections were captured using an Axio Imager Z2 microscope (Zeiss) connected to a Hamamatsu Orca Flash 4.0 LT camera, or using a laser scanning confocal microscope (Zeiss LSM 780 or Zeiss LSM 880). Three-dimensional maximum intensity projections were digitally reconstructed from confocal z-stacks. TIRF images of EC tensin-1 and talin-1 were acquired using a Laser-TIRF 3 Imaging System (Zeiss) with an EMCCD camera (Hamamatsu ImageEM C9100-13; Chip size 512x512; Hamamatsu Photonics K:K:) controlled by the Zen software (Zen 2012 Blue Edition Systems; Zeiss), or using the Nikon Eclipse Ti-E N-STORM inverted microscope, with an EMCCD camera (512x512) controlled by the NIS-Elements advanced research ver.4.2 (Nikon) (II). Whole tissue sections were imaged using a 3DHistech Pannoramic 250 FLASH II digital slide scanner. Transwell inserts were imaged with Zeiss Axiovert microscope.

7.2 Analysis of cell surface expressed TIE2 and VE-cadherin (I)

shRNA lentivirus transduced BECs were treated with sulfo-NHS-SS-biotin (0.2mg/ml) in Dulbecco's PBS (DPBS) for 30min at +4°C. The reaction was stopped using 20 mM sodium-2-mercapto-ethane-sulphonate (MesNa) in 50mM Tris-HCl pH 8.6, 100mM NaCl in DPBS for 15min at +4°C, and subsequently replaced with 20 mM iodoacetamide in DPBS for 10min in +4°C. After washing with DPBS, the cells were lysed (25 mM Tris pH 7.4, 100 mM NaCl, 2mM MgCl2, 0,5 mM EGTA, 5% glycerol and 1% Triton-X100 supplemented with the protease inhibitor cocktail). The lysates were incubated with Streptavidin-coupled Dynabeads (Life Technologies) for 60 min and boiled in 1xLaemmli loading buffer. Surface-expressed, biotinylated VE-cadherin and TIE2 were analyzed using Western blot.

7.3 TIE2 complementation assay (I)

BECs grown to 50% confluence were transduced for 24 h with lentiviral shRNA against TIE2 or scramble sequences, followed by transduction with retroviruses coding for membrane-anchored mouse TIE2 ectodomain (mTIE2-ECD) or membrane-anchored GFP for another 24 h. Cells were fixed and mTIE2-ECD transduced samples were stained using specified antibodies against mTIE2.

7.4 Tumor cell-EC transmigration assay (I)

The transmigration assay was used to investigate the integrity of the EC monolayer, and served as a functional *in vitro* evidence on the roles of ANGPT2 and TIE2 in the ECs. However, as other options, *in vitro* permeability conducted with fluorescent tracers, or impedance measurements (Bischoff et al., 2016), of which the latter was carried out in study II, could offer knowledge on EC monolayer permeability without the apparent interactions between cancer cells and ECs.

Tumor cell transmigration across an EC monolayer was performed using Transwell chambers (6.5 mm insert diameter, 8 µm pore size, Corning Life Sciences, NY, USA). 100 000 BECs, transduced with TIE2 or scramble shRNA lentiviruses, were seeded on the upper compartment overnight. 100 000 LLC-GFP cells were applied on top of the confluent EC monolayer for 9 hours, with complete growth media in both chambers. Inserts were fixed in 4% PFA-PBS and mounted onto glass slides. Alternatively, 100 000 LNM-35-GFP cells were applied on top of the confluent BEC monolayer for 5 or 9 hours and treated with control or anti-ANGPT2 antibody.

7.5 Spreading assay (I&II)

EC spreading assays were used to monitor the development of EC adhesions on FN. Since collagens represent ligands for β1-integrins, the studies could have been complemented using additionally collagen coated coverslips (Humphries et al., 2006).

BECs or b.END3s transduced with scramble or *TIE2* shRNA lentiviruses for 48 h, were detached, and let to recover in complete medium for 30 min. 15 000 cells were seeded on coverslips, pre-coated with 10 µg/ml of FN for 1 h, in the presence antibodies against human β1-integrin mAb13, mouse β1 integrin HMβ1, control IgG (Table 5), and/or cilengitide (Table 4), and let adhere for indicated times. Coverslips were fixed and stained.

7.6 Integrin activation assay (I)

The integrin activation assay provided a quantitative means to evaluate ANGPT2 and ANGPT1-induced α5β1-integrin activation, measured by fluorescent FN binding. The assay was performed in CHO cells, which express α5β1-integrin, but not other FN binding integrins, enabling the assessment of α5β1-integrin only. This assay is complementary to ELISA, which was used to measure ANGPT2 binding to integrin without information on integrin activation status.

To determine the effect of ANGPTs on integrin activation, the binding of cells to a fluorescently labelled FN7-10 fragment of FN was measured using fluorescence activated cell sorting (FACS). In short, CHO cells, or CHO cells transduced with retroviral vector for mTIE2-ECD, were collected and preincubated 30 min with various concentrations of rhANGPT2, rhANGPT1 or EDTA, and subsequently with fluorescent FN, anti-hamster α5β1-integrin antibody (recognizing total α5β1-integrin, PB1, Table 5), and Alexa-488 conjugated anti-mouse secondary antibody and analyzed using FACS. The results were expressed as the α5β1-integrin activation index, calculated as a ratio of mean fluorescent intensity of activated (Alexa-647-FN binding measured against background signal in the presence of EDTA) relative to total α5β1-integrin (PB1 staining). Alternatively, CHO cells transduced with plasmid constructs for ANGPT1-ANGPT2-flag, ANGPT2-Flag or ANGPT2-ANGPT1-flag were used similarly as above to measure integrin activation.

7.7 Fibronectin matrix remodeling (I)

BECs were plated on vitronectin (Invitrogen) coated coverslips and transduced with scramble or *shTIE2* lentiviruses. Cells were maintained in EBM2, supplemented with FN-depleted serum which was prepared by rotating the serum with gelatine sepharose (GE Healthcare, Fairfield, CT, USA) two times for 1 h, and spinning the sepharose containing serum with Micro Bio-Spin chromatography columns (Bio-Rad, Hercules, CA, USA). Depletion of FN was confirmed using Western blotting. Cells were stained for FN and analyzed using confocal microscopy.

7.8 α5-integrin internalization assay (II)

The α5-integrin internalization assay was used to follow the recycling of α5β1-integrin in the ECs, after inflammatory stimuli, which we found to be dependent on ANGPT2. However, this assay did not include an acid wash step to remove unbound antibody before internalizing the antibody at +37°C, but instead the cells were rinsed before the antibody was let to internalize. Thus, some of the antibody-labeled α5-integrin could recycle from the cell surface directly into EC-ECM adhesions, and not via

the internalized vesicles. However, the analysis was based on the intensity of α5-integrin positive intracellular vesicles, and thus, the results were not affected by this possibility.

Confluent BEC coverslips transfected with shScr or shANGPT2 lentiviral vectors (Table 3) were labeled on ice for 30 min with non-function blocking monoclonal antibody against the α5-integrin ectodomain or with control antibodies (goat IgG isotype, Sigma-Aldrich, both at 2 μg/ml in Dulbecco's PBS containing 1% BSA) (Table 5). Cells were subsequently rinsed with ice-cold Dulbecco's PBS, incubated at +37 °C for 1 h, and stimulated with LPS O111:B4, IL-1β or thrombin. Cells were fixed with 4% PFA-PBS, and the internalized α5-integrin antibody and control Ig were detected after permeabilization using anti-goat-Alexa488 secondary antibody. The internalized α5-integrin antibody was localized and quantified in intracellular vesicles (confirmed using confocal z-stack projections) around the nucleus.

7.9 Measurement of EC barrier function (II)

Impedance measurements using the XCelligence system were used to measure the integrity of the EC monolayer. The advantage of this method is the continuous (one-minute interval) recording of the EC monolayer. This method was accompanied by evaluation of the EC morphology and VE-cadherin quantitation at selected time points. IL-1β could not induce a detectable decrease in EC monolayer impedance using this system, which applies a high frequency electrical current (50 000 Hz) (Robilliard et al., 2018), whereas the system was sensitive enough for the robust impedance drop elicited by thrombin. Alternative equipment, like the ECIS system, would allow the use of a selection of lower frequency electrical currents, detecting more sensitive paracellular changes in the EC monolayer (Robilliard et al., 2018).

For non-invasive electrical cell impedance measurements, ECs were grown to confluency on E-plates with gilded electrodes. Continuous impedance measurements were performed (XCelligence, Acea Biociences) before and after stimulation of cells with thrombin in the presence of function-blocking human β1 integrin Ab mAb13 or control antibody. Alternatively, BECs silenced for 48 h using shScr or shITGβ1 lentiviral vectors were used in impedance measurements. The start of the stimulation was set as time point zero. Results were expressed as Cell Index (normalized impedance).

7.10 Traction force microscopy (II)

Traction force microscopy (TFM) was performed to measure EC contractility after thrombin stimulation in the presence or absence of inhibitory mAb13 antibody against β1-integrin. In short, Hydrogels were prepared on glass coverslips to achieve a Young's modulus of 3 kPa, as determined in Georgiadou et al. (Georgiadou et al., 2017). HUVECs were plated onto TFM gels O/N and imaged live at +37 °C with a 3i Marianas Spinning disk confocal microscope (Intelligent Imaging Innovations, 3i Inc.). Z stacks of the beads and a phase-contrast image of the colonies were recorded for 6-10 replicates per sample (each 12-16 images). Imaging was repeated after mAb13 antibody and thrombin addition. Reference images for every monolayer without cells (after trypsin detachment) were recorded in a similar manner. To extract the bead displacement fields, a MATLAB software package (MathWorks), utilizing a correlation algorithm was used (provided by T. Betz University of Münster, Münster, Germany). Traction forces were determined as described in Peuhu et al. using the same MATLAB software package after the Fourier transform traction force algorithm (Peuhu et al., 2017).

7.11 Time-lapse microscopy (II)

BECs expressing Paxillin-TagGFP2 were generated using the LentiBrite™ Paxillin-GFP Lentiviral Biosensor (Merck Millipore). Paxillin-TagGFP2 BECs were plated on μ-Slide 8-Well slides (Ibidi) and 48 h later stimulation media, supplemented with 50 mM HEPES, was added. Cells were stimulated with thrombin or IL-1β and imaged using a Marianas spinning disk confocal microscope controlled by SlideBook 6 (Intelligent Imaging Innovations, Inc.), equipped with a Yokogawa CSU-W1 scanning unit, an inverted Zeiss Axio Observer Z1 body and a 100× (NA 1.4 oil, Plan-Apochromat, M27) objective. Images were acquired every two minutes using an Orca Flash4 sCMOS camera (chip size 2,048 × 2,048; 2 x 2 camera binning enabled; Hamamatsu Photonics), at 37°C and in the presence of 5% CO_2. Images were processed using ImageJ to remove background (rolling ball radius: 10 pixels), and to compensate bleaching (exponential fit method) and drifting (StackReg, Rigid body).

8. *In vivo* mouse experiments (I&II)

In study I, we performed *in vivo* experiments using ANGPT2 overexpression in the murine vascular endothelium. Overexpression of murine ANGPT2 did not induce any severe phenotype in otherwise healthy transgenic mice. Inflammatory challenge and/or a functional approach could have been taken to further assess harmful ANGPT2 signaling *in vivo*. In study II, we performed functional leakage assays (see below) using a murine endotoxemia model. Endotoxemia was induced using a high dose of LPS, resulting in the development of systemic inflammation within hours. This method is widely used, and thus comparison of our results with those from other laboratories is an advantage. However, sepsis does not always progress fast, and here another preclinical model, the CLP model, could have been used to complement the endotoxemia model. Intervention studies, where HMβ1 β1-integrin functional antibody would be administered after the onset of CLP-induced sepsis, would further strengthen our findings. A potential limitation of our study is that the mechanism of the injected HMβ1 antibody may involve multiple cell types. However, we observed vascular localization of HMβ1, and confirmed our studies using a heterozygous endothelial deletion of β1-integrin, indicating that inhibiting endothelial β1-integrin protects from vascular leakage in endotoxemia.

The mice (Table 8) were maintained in the Laboratory Animal Centre of the University of Helsinki according to IACUC and IRB guidelines. The National Animal Experiment Board in Finland approved animal experiments used in this study. Transgenic female and male mice (Table 8) at the age of 8–18 weeks were genotyped and used for experiments. Single transgenic or wild type littermates were used as controls for double transgenic mice. Wild type mice (C57Bl/6J) were housed shortly before experiments, and male mice were used in the experiments.

8.1 Vascular leakage

β1- (HMβ1) or α4-integrin (PS/2) blocking antibodies (Table 6), or control (hamster IgG) (all 2.5 mg/kg) were administered into 2–4-month-old wild type male mice using intraperitoneal (i.p.) injection. Endotoxemia was induced 24 h later, with intraperitoneal administration (i.p.) of LPS (O55:B5, 11 mg/kg) for 2 h or 16 h. Throughout this work, the mice were sacrificed after maximum of 16 h after LPS administration. Alternatively, mice were first given LPS 2 h followed by i.p. injection of HMβ1 or control antibody for further 14 h. Mice were anaesthetized with i.p. injections of xylazine (10 mg/kg) and ketamine (80 mg/kg). 100 nm Alexa-594 labelled fluorospheres (0.6 % solution in 1% BSA-PBS) or 70 kD Texas red labeled Dextran (1% solution in 1% BSA-PBS) (Invitrogen, ThermoFisher Scientific) were injected intravenously through the tail vein, and let to

circulate for 4 min. The animals were perfused with PBS and 1% PFA in PBS via the left ventricle, followed by tissue collection, staining, imaging, and image analysis (for analysis, see Table 7).

8.2 Echocardiography

β1-integrin blocking antibodies (HMβ1), or control (hamster IgG) (all 2.5 mg/kg) (Table 5) were administered into male mice using intraperitoneal (i.p.) injection for 24 h. Cardiac function was monitored 24 h later, and again 16 h after LPS (O55:B5, 11 mg/kg) administration under isoflurane anaesthesia using Vevo 2100 ultrasound system (Fujifilm, VisualSonics) with a linear array 40-MHz transducer. After obtaining parasternal short-axis view at the papillary muscle level, two-dimensional B-Mode and M-mode recordings at sweep speed of 1200 Hz were done. M-mode images were used to measure left ventricular end diastolic diameter (LVEDD) and left ventricular end systolic diameter (LVESD). Ejection fraction (EF) and fraction shortening (FS) were calculated as EF (%) = ((LVEDD – LVESD)2/LVEDD2) x 100 and FS (%) = ((LVEDD – LVESD)/LVEDD) x 100.

8.3 Analysis of EC-EC junctions using transmission electron microscopy (TEM)

β1-blocking antibodies (HMβ1), or control (hamster IgG) (all 2.5 mg/kg) were administered into male mice using i.p. injection. 24 h later LPS (O55:B5, 11 mg/kg) was administered i.p. for 16 h. Tissue samples from lung and trachea were first fixed in 1% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4, and then post fixed in 1% osmium tetroxide, dehydrated in acetone and embedded in Epon LX 112 (Ladd Research Industries). Semi-thin sections were stained with toluidine blue to locate vascular structures for ultrastructural analysis. For TEM, 80 nm sections were cut with a Leica Ultracut UCT microtome and examined in Tecnai G2 Spirit transmission electron microscope (Fei Europe). Images were captured by Quemesa CCD camera (Olympus Soft Imaging Solutions GMBH).

Table 8. Genetic mouse lines and wild type mice (I&II)

Mouse	Description	Injection/ treatment	Experimental procedure	Source/reference	Used in:
C57Bl/6J	Wild type mice	Ab or LPS i.p. injection, anaesthesia i.p.	Murine endotoxemia, vascular leakage	Charles River or Janvier	II
VEC-tTA/$^{\text{Tet-OS-}Ang2}$	Inducible mANGPT2 overexpression in EC W/O tetracycline	Tetracycline (Sigma) 2.0 mg/mL in 5 % sucrose in drinking water (Tet off), anaesthesia i.p.	Aortic *en face* preparations	(Holopainen et al., 2012)	I
Itgb1$^{fl/fl}$	Floxed *Itgb1* allele	Tamoxifen (Sigma) i.p. injection (30 µl of 1 mg/ml) x3 P1-P7, LPS i.p. injection, anaesthesia i.p.	Murine endotoxemia, vascular leakage	The Jackson Laboratory; (Raghavan et al., 2000)	II
Pdgfb-iCre^{ERT2}	Tamoxifen inducible expression of the Cre recombinase under the *Pdgfb* promoter	Tamoxifen (Sigma) i.p. injection (30 µl of 1 mg/ml) x3 P1-P7, Ab or LPS i.p. injection, anaesthesia i.p.	Murine endotoxemia, vascular leakage	The Jackson Laboratory; (Madisen et al., 2010)	II
B6.Cg.-Gt(ROSA)26Sort$^{m14(CAG-tdTomato)Hze/J}$	tdTomato fluorescent protein expression upon Cre expression	Tamoxifen (Sigma) oral gavage (2 mg/mouse/day) 5x adult mice, anaesthesia i.p.	Tracheal whole mounts	The Jackson Laboratory; (Madisen et al., 2010)	II

Statistical analysis (I&II)

For pairwise comparisons of two treatment groups, Student's t-tests (two-tailed, unequal variance) were used (I&II). For comparisons of multiple groups, Dunnet's (I) or Tukey's (II) multiple comparison tests were used in conjunction with ANOVA post-hoc pairwise testing. A P-value of less than 0.05 was considered statistically significant. Experimental group sizes are indicated in the figure legends of the original publications.

Collectively, our results demonstrated that ANGPT2 and β1-integrin mediated EC monolayer destabilization in inflammation, and when the TIE2 levels in ECs were decreased via shRNA silencing. Moreover, the results suggested that ANGPT2 and β1-integrin may act in the same signaling pathway that involves integrin recycling and integrin activation by the N-terminal domain of ANGPT2. Finally, the results suggested that targeting β1-integrin signaling may have translational relevance via inhibition of vascular leakage in inflammation and sepsis.

9. ANGPT2–β1-integrin signaling pathway destabilizes the endothelium (I)

In study I, we discovered that ANGPT2 destabilized EC monolayers via β1-integrin-dependent signaling, when the levels of the cognate ANGPT2 receptor TIE2 were decreased, and that ANGPT2 activated β1-integrin via the ANGPT2 N-terminal domain.

TIE2 silencing destabilizes endothelial monolayers

In order to investigate the function of EC-secreted ANGPT2 on EC monolayer integrity, we silenced ANGPT2, TIE2 or TIE1 in primary human ECs. TIE2 silencing led to the loss of cortical actin cytoskeleton and to the formation of actin stress fibers (Figure 6 A, B), leading to elongated EC morphology. In contrast, ANGPT2 or TIE1 silenced ECs retained a cortical actin phenotype similar to scrambled control silenced ECs (I: Fig 1, Fig S1).

Actin stress fibers can generate centripetal tension that weakens EC-EC contacts in adherens junctions. In line with this, immunofluorescence analysis showed that VE-cadherin and β-catenin, both located in the adherens junctions of control cells, were reduced in the cell–cell contacts of TIE2 silenced ECs (I: Fig 1, Fig S2). Supporting these observations, cell surface VE-cadherin, but not total VE-cadherin, was decreased in TIE2 silenced cells. In contrast, tight junction protein ZO-1 was retained in the EC-EC contacts of TIE2 silenced cells (I: Fig S2).

Functionally, LNM-35 cancer cell transmigration across the EC monolayer was inhibited in samples treated with anti-ANGPT2 antibodies (I: Fig 1, Fig S2). This result complements earlier studies where ANGPT2 blocking antibodies improved EC-EC junctions and inhibited lung metastasis (Holopainen et al., 2012). In line with the decreased integrity, TIE2 silenced monolayers were more permissive for tumor cell transmigration than control cells (I: Fig 1).

ANGPT2 is required for destabilization of TIE2-silenced EC monolayers

We considered that ANGPT2 signaling may be deregulated upon loss of its classical TIE2 receptor. Indeed, when ANGPT2 was co-silenced with TIE2, ECs retained their cortical actin shape, indicating that ANGPT2 was necessary for stress fiber formation in TIE2 silenced cells, via a TIE2-independent mechanism (Figure 6 C; I: Fig 1, Fig S2).

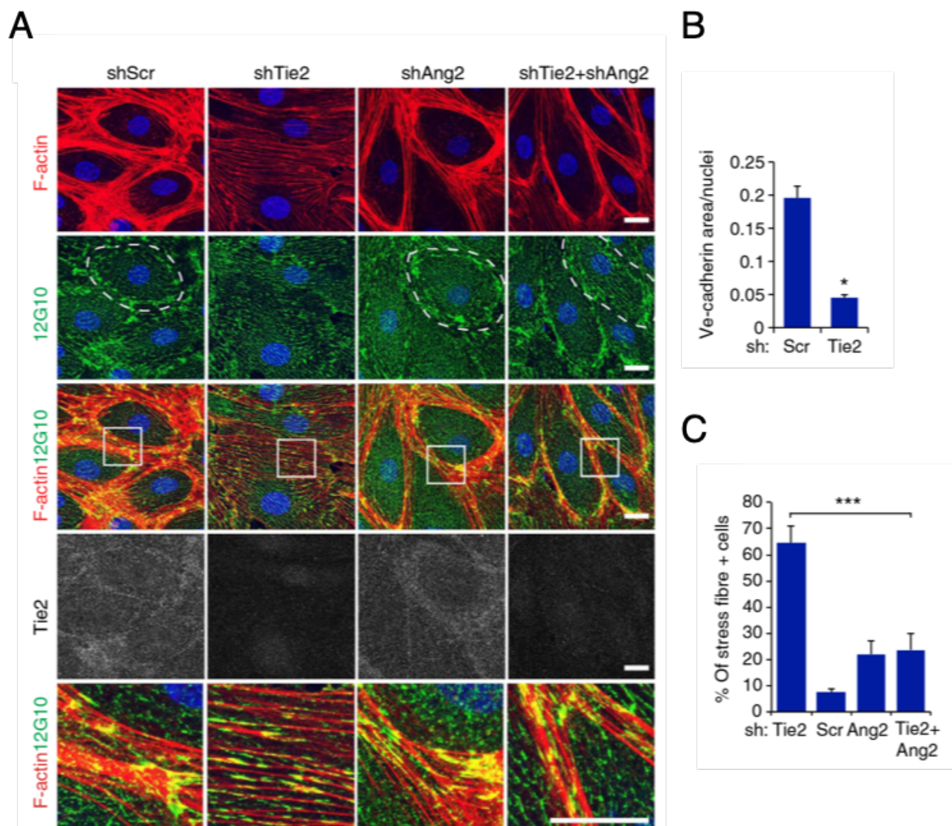


Figure 6. ANGPT2 induces β1-integrin translocation and destabilizes the TIE2 silenced monolayer. A-C: ECs were silenced for control (shScr), TIE2 (shTie2), ANGPT2 (shAngpt2) or both TIE2 and ANGPT2. A) ECs were stained with β1-integrin (12G10) and TIE2 antibodies. Actin was visualized with Phalloidin staining. Representative images were obtained with confocal microscopy. B) Quantification of VE-cadherin from microscopic images (Table 7). C) Actin stress fiber positive ECs were quantified from microscopic images. Scale bars: 20 µm. The figures have been modified from Hakanpää et al. 2015 (study I) with the permission from Macmillan Publishers Ltd.

TIE2 silencing alters EC-ECM adhesions

Integrins have been reported to function as alternative receptors for ANGPT2 both in ECs and non-ECs (Carlson et al., 2001; Felcht et al., 2012; Scholz et al., 2011). Since β1-integrin was the most prominent integrin subunit expressed in BECs (I: Fig 3), we investigated whether β1-integrin activity was altered in the TIE2-silenced cells using activation-state-specific antibodies against β1-integrin (12G10 and 9EG7, Table 5). Active β1-integrin containing focal adhesions were located in the periphery of control ECs, whereas β1-integrin was located along the actin stress fibers extending across the longitudinal axis of TIE2 silenced ECs (Figure 6 A; I: Fig 2, Fig S3). In contrast to focal adhesions present in control cells, the β1-integrin positive adhesions in TIE2 silenced ECs resembled fibrillar adhesions. Moreover, FN remodeling was altered in the TIE2 silenced ECs (I: Fig 2). During short term spreading on FN, the TIE2 silenced ECs also harbored more centrally located β1-integrin positive adhesions than control ECs, indicating increased β1-integrin activity upon TIE2 silencing (I: Fig 2, Fig S4). Thus, TIE2 silencing altered EC-ECM adhesions and increased active β1-integrin. Notably, when both TIE2 and ANGPT2 were silenced, the β1-integrin-positive elongated adhesions were decreased, indicating that ANGPT2 was required for the increased β1-integrin activation in TIE2 silenced ECs (Figure 6 A; I: Fig 2).

Co-silencing of β1-integrin protects from destabilization of TIE2-silenced EC monolayers

To investigate whether β1-integrin was required for the alteration of the actin cytoskeleton in the ECs, β1-integrin was co-silenced together with TIE2. Although β1-integrin expression was significantly reduced in the silenced ECs, some β1-integrin remained in adhesions localizing at the cell periphery, and the silencing did not affect the EC cortical actin cytoskeleton. However, β1-integrin silencing prevented the formation of the actin stress fibers in the TIE2-co-silenced ECs (I: Fig 3). In contrast,

high concentration of β1-integrin-blocking antibodies (mAb13, Table 5) disrupted both actin stress fibers and the cortical actin rim of TIE2 silenced and control cells, respectively (I: Fig 3, Fig S3). Thus, we contemplated that central β1-integrin adhesions promoted actin stress fibers, and were more sensitive to decreased β1-integrin levels when compare to peripheral focal adhesions that stabilized the cortical actin in control cells.

In addition to β1-integrin, BECs expressed β3- and β5-integrins, which together with the αv-integrin, form RGD-binding heterodimers (I: Fig 3). However, silencing of αv-, β3- or β5-integrins did not rescue the cortical actin structure in the TIE2 silenced cells. αvβ3-integrin signal was detected at the junctional area in EC monolayers, and in focal adhesions. In line with previous reports, silencing αv- or β3-integrin in ECs resulted in disorganized cortical actin and increased stress fiber phenotype, implicating that αvβ3-integrin activity stabilizes the EC cortical actin cytoskeleton (I: Fig 3, Fig S3). Taken together, EC monolayer destabilization induced by TIE2 silencing was specifically dependent on β1-integrins, but not αv-integrins.

To understand more about the intracellular signaling pathways that were activated in TIE2 silenced ECs, we utilized phospho-protein signaling blots. Akt and ERK were strongly phosphorylated in TIE2 silenced cells, however, co-silencing of TIE2 and ANGPT2 or TIE2 and β1-integrin, decreased the phosphorylation of both signaling mediators to the level of control cells (I: Fig S5). Increased Rho kinase activity induces the formation of actin stress fibers. Functional inhibition of the PI3K or the Rho kinase pathways using kinase specific inhibitors, decreased stress fibers and supported the formation of cortical actin structures in the TIE2 silenced cells (I: Fig S5).

Effect of ANGPT2 on EC junctions *in vivo*

It has been previously shown that overexpression of mANGPT2 in the endothelium (*VE-cadherin-tTA* (*VEC-tTA*)/*Tet-OS-Angpt2* mice) resulted in increased lung metastasis (Holopainen et al., 2012). In those studies, it was found that EC integrity of pulmonary capillaries adjacent to extravasated tumor cells was decreased: disturbed association of ECs to the underlying BM, reduced EC-EC junctions and gaps between the ECs were described. However, the changes were significantly less in wild type tumor-bearing littermates suggesting that the increased ANGPT2 levels aggravated EC destabilization by tumor cells (Holopainen et al., 2012).

In order to study the effects of ANGPT2 on vascular stability, we used the transgenic *VEC-tTA/Angpt2* mice that secrete ANGPT2 into the circulation (I: Fig S8). Interestingly, whole-mount staining of *en face* prepared mouse aortas for VE-cadherin, CD31, active β1-integrin, filamentous actin and TIE2 revealed marked differences between *VEC-tTA/Angpt2* and control mice. The *VEC-tTA/Angpt2* mice showed more irregular VE-cadherin and CD31 staining in all aortic regions analyzed, when compared to control littermates, with an interdigitating pattern at EC–EC junctions. Active β1-integrin was detected in the junctional area of aortic ECs of control mice, whereas it was detected in central elongated adhesions in the aortic ECs in *VEC-tTA/Angpt2* mice (I: Fig 6). In addition, in the ascending aorta, filamentous actin was resembling a stress fiber phenotype in *VEC-tTA/Angpt2* mice, but not in control mice, where actin signal was more restricted to junctional area of aortic ECs (I: Fig S8). Furthermore, TIE2 co-localized with VE-cadherin staining in the aortic ECs of control mice but less in *VEC-tTA/Angpt2* mice (I: Fig 6). Altogether, the altered VE-cadherin and CD31 positive EC junctions in *VEC-tTA/Angpt2* mice were associated with altered immunohistochemical localization of actin, TIE2 and β1-integrin.

ANGPT2 can activate β1-integrin via the ANGPT2 N-terminal domain

The results suggested that ANGPT2 can signal via β1-integrin in ECs expressing low TIE2 levels. Thus, we investigated ANGPT2 signaling in cell systems that do not express TIE2 (I: Fig S6). First, we studied HeLa adenocarcinoma cells ectopically expressing ANGPT2 or HeLa stimulated with recombinant ANGPT2. Based on immunofluorescence stainings, recombinant or ectopically expressed ANGPT2 was found to be deposited in cell–matrix adhesions of Hela cells, adjacent to active β1-integrin staining, and ANGPT2 also stimulated the formation of central β-integrin containing adhesions (I: Fig 4, Fig S6). ANGPT2 deposition was significantly inhibited by the inhibitory β1-integrin antibody, mAb13, but not by cilengitide, which mainly inhibits αv-integrins (I: Fig 4, Fig S6). ANGPT1 localization was diffuse, and independent of β1-integrin after stimulation of HeLa cells (I: Fig S6).

Secondly, we examined whether ANGPT2 is able to activate β1-integrin. Since integrin activation results in increased ability of integrins to bind ECM proteins, we measured the binding of a recombinant FN fragment (encompassing the FN domains 7–10) to the cell surface of CHO cells, which express α5β1-integrin, but not TIE2. Interestingly, incubation of CHO cells with ANGPT2 enhanced the binding of FN to CHO cells dose-dependently, whereas ANGPT1 did not (I: Fig 4). Notably, when mTIE2 ectodomain was expressed in CHO cells, ANGPT2 mediated integrin activation was decreased, suggesting that mTIE2 (murine) ectodomain captured ANGPT2, inhibiting ANGPT2 signaling via β1-integrin. Similarly, complementing TIE2 silenced EC by the mTIE2 ectodomain stabilized the cortical actin cytoskeleton (I: Fig 5). Further, in HeLa overexpressing full length human TIE2, ANGPT2 bound to TIE2 receptor, as did ANGPT1, inducing the typical receptor internalization, whereas overexpression of TIE1, that does not bind ANGPTs, had no effect (I: Fig S7).

The mechanism of ANGPT2-mediated β1-integrin activation was investigated more closely using chimeric angiopoietin growth factors. Expression vectors were generated for angiopoietins where the N- and C-terminal domains of ANGPT1 and ANGPT2 were changed *vice versa* (I: Fig 4). In summary, results from both HeLa and CHO cells pointed towards a crucial function of the N-terminal, but not the C-terminal domain, of ANGPT2 and none of the ANGPT1 domains in β1-integrin signaling, whereas overexpression of TIE2 interfered with ANGPT2–β1-integrin signaling.

10. ANGPT2 and β1-integrin in endothelial destabilization in inflammation (II)

In this study we discovered that β1-integrin was required for inflammation-induced EC destabilization and permeability, and provided evidence on how ANGPT2 may regulate β1-integrin in inflammation. Notably, in a preclinical murine model of endotoxemia, we demonstrated that inhibitory β1-integrin antibody decreased vascular leakage and protected from cardiac failure.

Inflammatory agents mediate EC monolayer destabilization via β1-integrin

Our previous results identified that ANGPT2 can signal via β1-integrin when the levels of its cognate receptor, TIE2, were decreased. Inflammation is known to decrease the expression of both TIE1 and TIE2 (Ghosh et al., 2016; Kim et al., 2016; Korhonen et al., 2016; Kurniati et al., 2013; Li et al., 2014). Thus, we hypothesized that ANGPT2 might signal via β1-integrin in inflammation, and asked whether β1-integrin is involved in inflammation induced EC monolayer destabilization that affects the integrity of EC-EC junctions. We utilized several inflammatory agents: thrombin, IL-1β and LPS that are known to cause EC monolayer destabilization.

To investigate the function of β1-integrin in inflammation-induced EC monolayer permeability, ECs were stimulated with the inflammatory agents, and β1-integrin was simultaneously inhibited. For inhibition of β1-integrin, we used shRNA silencing or a β1-integrin antibody (mAb13), which stabilizes the β1-integrin in an inactive, closed conformation, via binding to the beta-I-domain in the headpiece of β1-integrin (Byron et al., 2009; Mould et al., 1996). In line with its ability to inhibit β1-integrin binding to the ECM, a high concentration of mAb13 inhibited EC spreading on FN, and thereby, also interfered with EC junction organization (II: Fig S1). Notably, a low mAb13 concentration (0.1 μg/ml) did not affect EC monolayer integrity and was selected for further studies.

All inflammatory agents tested induced the destabilization of the EC monolayer. IL-1β and thrombin induced a significant loss of VE-cadherin (II: Fig 1, Fig 2, Fig S3, Fig S5, Fig S6) although with varying kinetics (II: Fig S4). IL-1β, thrombin and LPS also stimulated the formation of actin stress fibers, quantified for LPS (II: Fig S5), in confluent EC monolayers. shRNA silencing of ANGPT2, β1-integrin or α5-integrin, but not β3-integrin, protected from inflammation-induced monolayer destabilization, i.e. the formation of stress fibers and the loss of VE-cadherin or the tight junction protein ZO-1 that connects catenins to actin cytoskeleton (II: Fig 1, Fig 2, Fig S3, Fig S5, Fig S6). In addition to inflammatory agents, stimulation with a high concentration of recombinant ANGPT2 induced the loss of VE-cadherin in a β1-integrin dependent manner (II: Fig 7).

mAb13 prevented the thrombin induced loss of VE-cadherin, whereas it had no effect on VE-cadherin in unstimulated control ECs (II: Fig 1). mAb13 also significantly decreased thrombin induced EC monolayer permeability which was analyzed using noninvasive electrical cell impedance monitoring (II: Fig 1). Blebbistatin, an inhibitor of nonmuscle myosin II, prevented thrombin-induced loss of VE-cadherin and stress fiber formation (II: Fig 1). mAb13 and blebbistatin had similar effects suggesting that β1-integrin was involved in tension mediated loss of EC junction integrity. This was verified using traction force microscopy, which demonstrated that mAb13 attenuated thrombin induced cellular contractility and traction force generation (II: Fig 1).

Inflammation alters EC-ECM adhesions

We hypothesized that β1-integrin promotes inflammation-induced EC permeability via altered EC-ECM adhesions. Therefore, we investigated the dynamics of EC–ECM adhesions after inflammatory stimulation using spinning disk confocal microscopy of paxillin-GFP expressing ECs. Both control and thrombin-stimulated ECs contained focal adhesions localized in the cell periphery, however, thrombin stimulation additionally induced the formation of centrally located cell adhesions (II: SMovie, Fig. 7). This finding was confirmed in fixed ECs: β1- and α5-integrin were enriched at centrally located, elongated matrix adhesions after IL-1β, thrombin, or LPS stimulation (II: Fig 7).

Using TIRF microscopy, we found that talin-1 localized in focal adhesions in ECs in non-inflammatory conditions (II: Fig S19), whereas tensin-1 that shares a binding site with talin-1, and binds to β1-integrin in fibrillar adhesions, was less visible (II: Fig 7, Fig S19). However, LPS and IL-1β stimulations significantly increased tensin-1 positive adhesions along stress fibers, in a pattern similar to active β1- and α5-integrins. The increase in tensin-1 was ANGPT2-dependent, since LPS induced significantly less tensin-1 positive adhesions in ANGPT2 silenced ECs (II: Fig 7, Fig S19). Supporting a functional role for tensin-1, more VE-cadherin was retained in EC junctions after IL-1β stimulation in tensin-1 silenced ECs compared to control cells (II: Fig 7).

Taken together, inflammatory agents stimulated the formation of fibrillar EC–ECM adhesions, which contained α5β1-integrin and tensin-1. ANGPT2-mediated increase in tensin-1 positive fibrillar adhesions promoted monolayer destabilization in inflammation, most likely via coupling α5β1-integrin to actin stress fibers and contractile forces that led to decreased junctional localization of VE-cadherin and ZO-1.

ANGPT–TIE pathway in the inflamed endothelium

It has been previously shown that inflammation downregulates TIE1 and TIE2 mRNA and protein levels, and results in the cleavage of the TIE1 ectodomain, leading to vessel destabilization *in vivo* (Figure 5) (Korhonen et al., 2016). Therefore, we studied whether β1-integrin signaling was linked to decreased TIE pathway activity in EC inflammation *in vitro*. We found that TIE1 was cleaved within 30 min after IL-1β or thrombin stimulation of ECs, and this was not affected by mAb13 treatment (II: Fig S13). Thus, mAb13 did not improve EC monolayer integrity via enhanced expression of the TIE receptors. Instead the inflammatory agents induced the downregulation of TIE1 also in an *in vitro* EC model system, similarly to *in vivo*.

Since α5-integrin was localized in fibrillar adhesions following LPS, IL-1β, and thrombin stimulations, we studied whether α5β1-integrin trafficking was dependent of ANGPT2. α5-integrin was followed, using an antibody recognizing the α5-integrin ectodomain, from the cell membrane (labeled on ice) to internalized perinuclear vesicles. All inflammatory agents stimulated the recycling of α5-integrin from internalized vesicles to inflammatory EC-ECM adhesions, and interestingly, the recycling from the vesicles was inhibited by ANGPT2 silencing (II: Fig 7, Fig S20). In summary, inflammatory agents induced ANGPT2-dependent recycling of α5-integrin, and the cleavage of TIE1, which was not affected by mAb13 (Figure 8).

Inhibition of β1-integrin decreases vascular leakage in endotoxemia inflammation-independently

From the *in vitro* experiments it was evident that inhibition of β1-integrin supported EC integrity in inflammation. Therefore, we decided to test the concept *in vivo*, in inflammation-induced vascular leakage. Murine endotoxemia is manifested by systemic inflammation, vascular leakage, and organ failure, thus mimicking human sepsis. Whereas the function of β1-integrin had not been investigated in endotoxemia, several studies had demonstrated that ANGPT2 aggravated whereas ANGPT1 and TIE2 protected from endotoxemia (Leligdowicz et al., 2018). Moreover, previous work has shown that mouse ANGPT1, TIE1 and TIE2 were downregulated, whereas ANGPT2 was upregulated in endotoxemia (II: Fig 4, Fig S9) (Korhonen et al., 2016).

We administered sub-lethal doses of LPS, or PBS as a control, in C57Bl/6J mice and measured vascular leakage after two and 16 hours. In addition, mice received injections of control or β1-integrin inhibitory (HMβ1) antibodies 24 hours before LPS (preventive treatment), or 2 hours after LPS (intervention).

Vascular leakage was visualized with fluorescent tracers injected via the tail vein. Two different tracers were used: fluorospheres of a diameter of 100 nm that correlate to the size of a 1000 kD protein, and 70 kD dextran. 100 nm spheres do not leak out in homeostatic conditions, aside from sinusoidal vascular beds, which were not analyzed in this work, whereas 70 kD dextran, which is approximately the size of albumin, may leak out from capillaries. The tracers were analyzed 4 min after injection, followed by perfusion of the vasculature via the left ventricle. The vasculature in the

ear and in the trachea of PBS-administered control mice showed no leakage, whereas leakage was induced in LPS-administered mice (Figure 7; II: Fig 3, Fig. 4, Fig S10, Fig S12). However, vascular leakage was significantly decreased in mice that received the HMβ1 antibody, but not the control antibody, prior to LPS administration (Figure 7; II: Fig 3, Fig S12). HMβ1 did not increase vascular leakage in PBS-treated control mice (Figure 7; II: Fig 3, Fig S12). Importantly, administration of HMβ1 two hours after LPS, when systemic inflammation and vascular leakage were increased, decreased the leakage in an intervention setting (II: Fig. 4, Fig S10).

Inflammatory response after LPS administration, i.e. levels of serum proinflammatory cytokines, VCAM-1 expression, and neutrophil infiltration in the lungs, were similar in mice that received the HMβ1 or control antibodies (II: Fig 4, Fig S9), indicating that attenuated inflammation did not explain for the observed decrease in vascular leakage in HMβ1 administered mice. In addition, α4-integrin function blocking antibody, which binds the leukocyte α4β1-integrin, had no effect on LPS-induced vascular leakage, suggesting that leukocyte integrin was not involved (II: Fig S12). Moreover, using the conditionally targeted *Itgb1flox/flox;Pdgfb-iCRE* mice, we found that heterozygous inducible *Itgb1* deletion in the endothelium of adult mice protected from LPS-induced vascular leakage, indicating an EC autonomous function for β1-integrin in regulation of vascular leakage (II: Fig. 6).

Inhibitory antibody against β1-integrin protects from cardiac failure in murine endotoxemia

In addition to reduced vascular leakage, HMβ1 protected from LPS-induced cardiac failure. Heart function was monitored using echocardiography 24 hours after HMβ1 antibody, and again 16 hours after LPS administration. LPS reduced the cardiac output measured by ejection fraction and fractional shortening. Prophylactive treatment with the HMβ1, but not the control antibody, significantly increased cardiac output after LPS administration (II: Fig 3).

Inhibitory antibody against β1-integrin does not affect ANGPT–TIE signaling

Over time, we observed an increase in *Angpt2* mRNA and ANGPT2 serum protein levels, and a decrease in *Angpt1* mRNA levels in LPS-administered mice, as reported also by Korhonen et al. (Korhonen et al., 2016). The levels were similar in mice that received either HMβ1 or control antibodies (II: Fig 4, Fig S9). Similarly, the HMβ1 antibody had no effect on LPS-induced decrease in TIE2 and TIE1 protein levels in the lung lysates, or soluble TIE1 in serum samples (II: Fig. 4, Fig S13). Consequently, the LPS-induced decrease of TIE2 phosphorylation was unaltered by the HMβ1 treatment (II: Fig 4).

Inhibitory antibody against β1-integrin improves EC junctions in endotoxemia

We asked, if HMβ1 could directly improve the integrity of the blood vessels in endotoxemia. Interestingly, 48 hours after i.p. injection in mice, HMβ1 was detected in the tracheal vasculature (II: Fig S15), overlapping with staining for CD31. We utilized transmission electron microscopy to visualize the ultrastructure of EC junctions in HMβ1 or control antibody treated mice after LPS or PBS administration. In mice that received LPS, the cell junction length was significantly shorter than in PBS treated control mice (II: Fig 5, Fig S14). However, the junction length was preserved in HMβ1 administered LPS-treated mice, when compared to control antibody treated mice. Improvement in the EC-EC integrity was seen both in the pulmonary microvasculature and in capillaries, postcapillary venules, and collecting veins in the tracheal vasculature (II: Fig 5).

Overall, targeting β1-integrin either using HMβ1 or genetic deletion decreased vascular leakage in murine endotoxemia. HMβ1 protected from cardiac failure and improved EC-EC junctions, but did not affect LPS-induced inflammatory response or TIE signaling.

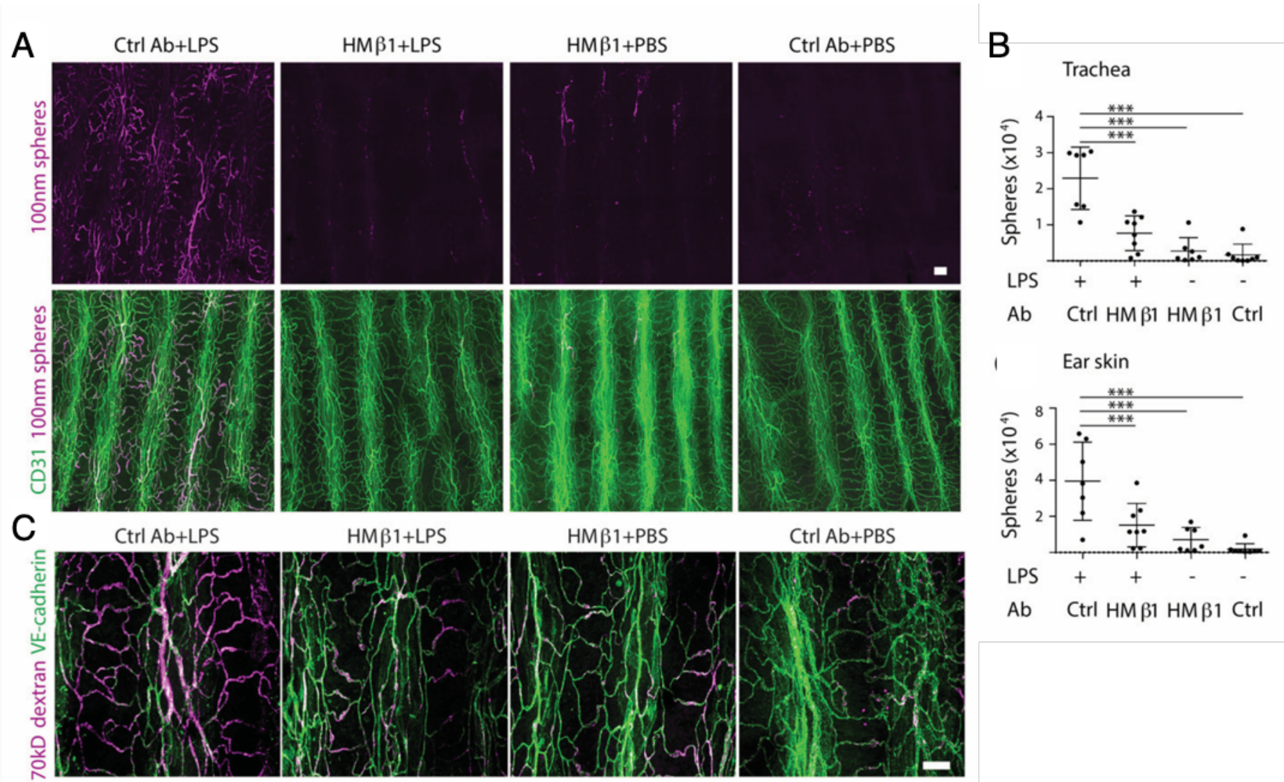


Figure 7. β1-integrin targeting antibody decreases vascular leakage in murine endotoxemia. A, C) Adult wild type mice we administered with HMβ1 or control antibodies for 24 h, followed by 16 h of LPS, and injection of either 100 nm fluorospheres or 70 kD dextran to the circulation for 4 min. B) Quantification of leaked tracer. Scale bars: 100 µm. The figures have been modified from Hakanpää et al. 2018 (study II) with the permission from The National Academy of Sciences.

DISCUSSION

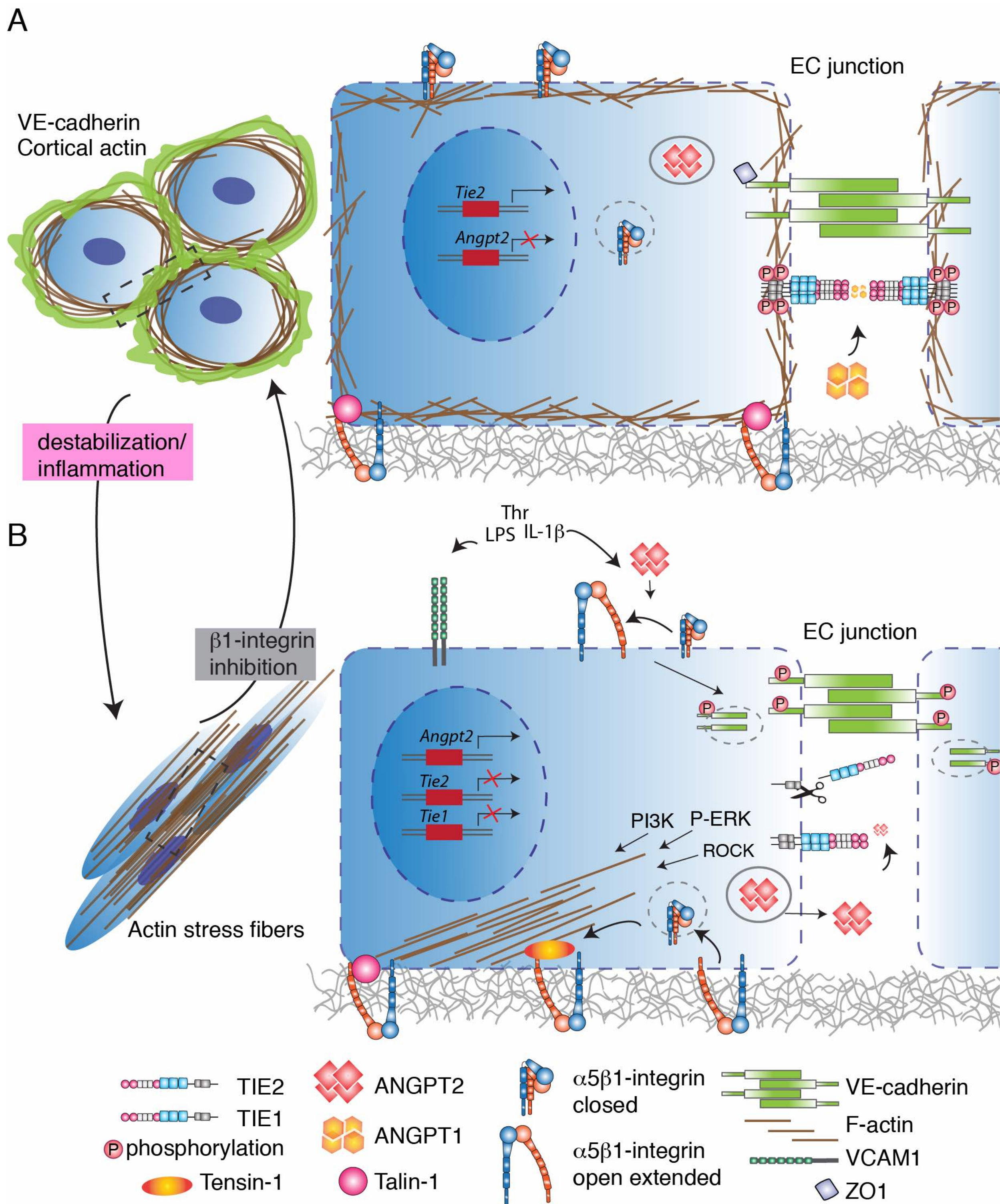
The results of this thesis provided new insight into the functions of ANGPT2 and β1-integrin in EC monolayer destabilization in inflammation and vascular leakage in endotoxemia. The main results are discussed below.

11. ANGPT2–β1-integrin interactions in EC destabilization (I)

We found that TIE2 silencing in ECs led to monolayer destabilization, in line with a previous publication (Parikh et al., 2006), and that unexpectedly, the monolayer destabilization could be prevented by co-silencing of ANGPT2. This was surprising, since ANGPT2 is known to act as a weak context-dependent agonist/antagonist of TIE2. To identify the mechanism by which ANGPT2 could signal in the absence of TIE2, we considered integrins, which have been implicated in ANGPT2 signaling, although with little mechanistic insight. We discovered that expression of β1-, but not αvβ3- or αvβ5-integrins, was necessary for EC monolayer destabilization in TIE2 silenced ECs.

In subsequent studies we aimed to more thoroughly characterize ANGPT2 signaling via β1-integrin, and hypothesized that ANGPT2 and β1-integrin could interact directly. Our results showed that ANGPT2 bound to β1-integrin ectodomain, but the β1-integrin domain mediating the binding was not identified. Using an integrin activation assay we found that ANGPT2 activated α5β1-integrin via the ANGPT2 N-terminus. In these studies, the N- and C-terminal domains of ANGPT1 and ANGPT2 were changed *vice versa*. This approach was possible, since ANGPT1 did not activate integrin. The ANGPT N-terminal domain consists of the coiled-coil and superclustering domains that participate in dimerization/trimerization and further multimerization of the ANGPTs (Davis et al., 2003; Kim et al., 2005; Leppanen et al., 2017). However, previously, the N-terminal domains of ANGPT1 or ANGPT2 have not been linked to any signaling function. Thus, besides the differential TIE2 agonist activity of ANGPT1 and ANGPT2, the ability to activate α5β1-integrin is a major difference between the signaling mechanisms of the homologous ANGPT1 and ANGPT2. More work is required to understand the potential function of the ANGPT2 N-terminus.

Figure 8. Summary of the findings: ANGPT2–β1-integrin pathway induces EC destabilization and leakage in inflammation. A) In endothelial homeostasis, EC s are stabilized by VE-cadherin and TIE2 receptor signaling, and talin-1 positive focal adhesions. ANGPT2 expression is low, whereas TIE2 expression is high. B) In systemic inflammation *in vivo*, the TIE1, TIE2 and ANGPT1 levels are decreased. TIE1 is cleaved and ANGPT2 acts as an antagonistic TIE2 ligand, decreasing the stabilizing phospho-TIE2 and downstream signaling. TIE1 is also cleaved in cultured ECs stimulated with IL-1β or thrombin. Inflammation, or TIE2 silencing, decreases junctional VE-cadherin. Inflammatory agents also decrease ZO-1. Inflammation also induces α5β1-integrin activation and translocation to tensin-1 positive fibrillar adhesions via an ANGPT2-dependent mechanism that may involve integrin recycling. The inflammatory adhesions promote stress fiber formation leading to increase in EC permeability and intracellular tension. TIE2 silencing increases ERK phosphorylation and stress fibers via the PI3K and ROCK pathways. Silencing of ANGPT2, α5- or β1-integrin, but not β3- or β5-integrins in ECs stabilizes the EC monolayer, retains cortical actin and junctional VE-cadherin of TIE2 silenced and inflammatory agent stimulated ECs. Inhibitory β1-integin antibody stabilizes the EC monolayer *in vitro* in inflammation, and decreases vascular leakage protecting from sepsis-induced cardiac failure in an *in vivo* model of murine endotoxemia. The information in this figure is derived from publications of this thesis.



Previous work has suggested various mechanisms by which ANGPT2 can signal via integrins. It was reported that ANGPT2 induced the sprouting of TIE2 low ECs, in an integrin-dependent manner, in an *in vitro* sprouting assay. Although the specific integrins were not identified, sprouting was inhibited by antibodies against α5β1-, αvβ3-, and αvβ5-integrin (Felcht et al., 2012). It was additionally found that ANGPT2 co-immunoprecipitated with αvβ5- and α5β1-integrin from TIE2 low ECs, and in a cell-free system also with αvβ3-integrin. All three integrins also bound to ANGPT2 in an ELISA assay, in an acidic environment, resembling angiogenic microenvironment, whereas

ANGPT2 binding to TIE2 was observed in both acidic and physiological pH. However, ANGPT2 was not found to promote the activation of β1-integrin, as measured using conformation specific antibodies (Felcht et al., 2012).

In another study, ANGPT2 was found to bind to CHO cells in a flow cytometric assay more strongly via α5β1-integrin than via αvβ3-integrin (Lee et al., 2014a). siRNA targeting of either α5- or β1-integrin abolished ANGPT2 binding in this assay. The ANGPT2–α5β1-integrin interaction was mapped to a C-terminal glutamine 362 of ANGPT2, and to the CALF-domains of α5-integrin ectodomain. Mutation of Glut-362 disrupted ANGPT2 binding to α5-integrin, but not to TIE2 (Lee et al., 2014a). Both of the above-mentioned studies concluded that ANGPT2 does not compete with FN in binding to RGD-recognizing integrins. Lee et al. further suggested that the α5-integrin tailpiece is essential for ANGPT2–α5β1-integrin interaction, since ANGPT2 did not bind a chimera where α5-integrin tailpiece was substituted with that of an αv-integrin, but did bind to an α5β1-β3-integrin chimera, where the tail piece of β3-integrin was cloned to that of β1-integrin (Lee et al., 2014a). In summary, both N- and C-termini of angiopoietins have been implicated in interacting with (α5)β1-integrin, and our work suggested that the ANGPT2 N-terminus activates α5β1-integrin. Further work is needed to elucidate this question.

Recently, ANGPT2 was reported to destabilize endothelial integrity in a heart ischemia model, by antagonism of ANGPT1–TIE2 signaling, but it also polarized proinflammatory macrophages through α5β1-integrin signaling (Lee et al., 2018). In HUVECs, α5β1-integrin silencing decreased ANGPT2-induced FAK phosphorylation, indicating that ANGPT2 induced FAK via α5β1-integrin (Lee et al., 2018).

Adding to the complexity, several lines of evidence suggest that ANGPT1 can also signal via β1-integrin. Originally, α5β1-integrin was found to sensitize TIE2 to low levels of ANGPT1 in cells growing on FN (Cascone et al., 2005). These results were more recently supported by another study, which showed that α5β1-integrin was necessary for ANGPT1-mediated TIE2 signaling in cultured ECs, and for TIE1-TIE2 interaction in EC-EC junctions (Korhonen et al., 2016). Thus, matrix composition and the abundance of TIE receptors appear to play a role in ANGPT1–TIE2 signaling. In another study, FN was found to promote α5β1-integrin–TIE2 interaction assessed using co-immunoprecipitation in a cell-free system, whereas interaction of α5β1-integrin with TIE1 did not require FN. In the same study, investigators found using co-immunoprecipitation that the ANGPT1 FLD, but not ANGPT2 FLD, bound to α5β1- and αvβ3 integrins in the absence of TIE receptors (Dalton et al., 2016).

The studies by Cascone et al. and Korhonen et al. have been subsequently supported by studies using brain ECs. It was shown that TIE2 is activated in an α5-integrin dependent manner in an *in vitro* model of oxygen-glucose deprivation/restoration of brain ECs. Upon α5-integrin silencing, TIE2, FAK and Akt phosphorylation were attenuated. Similarly, ANGPT1-induced TIE2 phosphorylation was decreased after oxygen deprivation when α5-integrin was silenced. In an *in vivo* model of mouse brain ischemia, phosphorylated TIE2 colocalized with α5-integrin, as detected using confocal microscopy (Pang et al., 2018). In contrast, another study found that the BBB integrity was increased upon α5-integrin silencing in mouse brain ischemia models *in vivo* (Table 1) (Roberts et al., 2017). Recently, in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis, EC deletion of α5-integrin in mice enhanced the symptoms and vascular leakage of EAE at early stages of the disease, but not at the late stages (Table 1) (Kant et al., 2019; Wang et al.,

2019a). Here, the authors did not, however, report whether this signaling is dependent or independent of TIE2 signaling.

Collectively, our studies together with those from other laboratories suggest that ANGPT2–integrin signaling could occur via multiple mechanisms, in a context-dependent manner. Based on our results it is tempting to speculate that ANGPT2 signals via β1-integrin when TIE2 signaling is suppressed (Figure 8), such as in the inflammatory endothelium, and that some of the vascular destabilizing functions of ANGPT2 might be mediated via its poorly characterized N-terminal domain. It remains to be investigated how ANGPT1 and ANGPT2 differentially signal via α5β1-integrin. One explanation that can be envisioned is that ANGPTs can interact with integrins via multiple mechanism and some of these mechanisms are shared between ANGPT1 and ANGPT2, whereas some interactions occur in a unique ANGPT2-dependent manner, such as the N-terminus dependent β1-integrin activation. Further, ANGPT–integrin signaling could be modified by the presence or absence of the TIE2 receptor, as well as by the ECM composition, but these theories remain to be confirmed.

Thus, our results add to the existing body of evidence that ANGPT2 signals via integrins. The novel aspects of our study include: 1) identification of a specific function for β1-integrin, but not other integrins, in ANGPT2 mediated EC destabilization, and 2) discovery that ANGPT2 can activate α5β1-integrin, whereas previous work mostly measured ANGPT2 binding to integrins. 3) We demonstrated specificity between ANGPT2 and ANGPT1 in their N-terminal domains in α5β1-integrin activation, whereas in many studies comparisons between ANGPT1 and ANGPT2 were not performed. Knowledge on 1) the effects of ANGPT-integrin signaling on ECs and 2) the effects of ANGPT-integrin signaling on vascular functions including vessel integrity, should facilitate attempts to target ANGPT2 in disease.

12.1 Signaling in TIE2 silenced ECs (I)

Using a phosphoprotein screen, we discovered that TIE2 silencing in ECs increased Akt and ERK phosphorylation in an ANGPT2- and β1-integrin-dependent manner. PI3K inhibition decreased stress fiber formation in TIE2 silenced BECs and supported actin rearrangement into a cortical actin rim, indicating that the increase in Akt phosphorylation was functionally relevant (Figure 8). The increased Akt activity in TIE2 silenced cells was surprising, since Akt is a key downstream signaling mediator of the ANGPT1–TIE2 pathway (Figure 8). However, the PI3K–Akt pathway is activated downstream of multiple cell surface receptors, including integrins (Moreno-Layseca and Streuli, 2014). Although not well understood, it has been suggested that Akt can be differentially activated by various cell surface receptors, leading to distinct cellular outcomes. A recent report suggested that Akt could be activated in a specific subcellular location via ectopic expression of a constitutively active R-Ras, but not after VEGF stimulation, explaining barrier promoting properties of R-Ras, but not VEGF (Li et al., 2017).

In addition, ERK is known to be activated downstream of the integrin-FAK pathway, leading to RhoA activation (Burridge and Wittchen, 2013). Indeed, ERK can be activated in response to stress fiber formation, and ERK phosphorylation decreases upon inhibition of stress fiber formation in ECs (Hsu et al., 2010). In fibroblasts, ERK phosphorylation correlates with increased tension and Myosin II activity (Hirata et al., 2015). As expected, an inhibitor of ROCK abolished already formed stress fibers and β1-integrin positive central adhesions, leading to formation of peripheral β1-integrin positive focal adhesions that overlapped with cortical actin (Figure 8).

12. ANGPT2 and β1-integrin signaling in inflammation (II)

We extended our studies in TIE2 silenced ECs to inflammation, since upregulation of ANGPT2 and downregulation of TIE1 and TIE2 are hallmarks of inflamed endothelium. Inflammatory agents induce barrier loss and stress fibers in EC cultures, and thus we expected a role for β1-integrin in these processes.

Our novel finding was that β1-integrin was required in ECs for IL-1β-, thrombin-, and LPS-induced EC destabilization, permeability and contractility, and that β1-integrin silencing similarly protected from inflammation-induced EC barrier loss (Figure 8). Based on our results, it is plausible that β1-integrin functions downstream of multiple inflammatory agents, providing a common downstream target in EC permeability signaling. Indeed, the inflammatory agents IL-1β, thrombin, and LPS signal via different cell surface receptors and induce EC destabilization with different kinetics. However, here all inflammatory agents induced the loss of VE-cadherin in EC junctions, as well as the formation of actin stress fibers (Figure 8). Thus, β1-integrin likely functions upstream of mechanisms that regulate VE-cadherin and actin cytoskeleton in inflammation induced EC permeability pathways. Previously, ANGPT2 has been found to prime ECs for inflammation by e.g. sensitizing ECs for TNFα , in line with our results (Fiedler et al., 2006; Benest et al., 2013; Rathnakumar et al., 2016).

mAb13 is a β1-integrin blocking antibody that binds to the headpiece of β1-integrin (beta-I domain) and prevents β1-integrin heterodimers from binding FN (Mould et al., 1996). mAb13 did not significantly alter VE-cadherin or actin cytoskeleton when used at a low concentration. Instead, mAb13 pretreatment inhibited the IL-1β or thrombin induced loss of VE-cadherin, translocation of β1-integrin into fibrillar adhesions, and stress fiber formation (Figure 8). Mould et al. have reported that mAb13 preferentially binds to ligand-free β1-integrin heterodimers, inhibiting ligand (FN) binding (Mould et al., 1996). Thus, it is likely that in ECs mAb13 binds to a pool of inactive β1-integrin heterodimers and prevents their binding to ECM proteins. This likely explains how mAb13 prevents fibrillar adhesion formation, which is associated with adhesion maturation in fibroblasts. Moreover, whereas mAb13 inhibited inflammatory agent-induced incorporation of β1-integrin into fibrillar adhesions, the small pool of β1-integrin already engaged at focal adhesions would be largely unaffected. This concept is in line with our finding that very high concentrations of mAb13 were required to impair EC stability.

One possibility how mAb13 can affect EC barrier maintenance in inflammation is via FAK, which has been reported to maintain endothelial stability but also act downstream of e.g. VEGF to increase permeability. Conditional deletion of FAK in the vascular endothelium in mice led to increased basal vascular permeability (Schmidt et al., 2013). On the other hand, VEGF-induced permeability was decreased in mice lacking endothelial FAK (Chen et al., 2012). Thus, similarly to β1-integrin, FAK is known to play a dual role in regulation of both vascular stability and stimuli-induced permeability. Since FAK signals downstream of multiple integrins, it remains to be found out if FAK is involved in the regulation of the EC barrier function via β1-integrin. In line with our studies and supporting a role for FAK, a recent publication showed that silencing of α5- or β1-integrin in ECs abolished rhANGPT2 induced increase in FAK phosphorylation (Lee et al., 2018).

Alternatively, mAb13 may affect Src kinase-mediated EC permeability, which is reported to regulate VEGF, bradykinin and TNFα induced EC permeability (Adam et al., 2016; Kim et al., 2009) in part via increased stress fiber formation (Burridge and Wittchen, 2013). Recently, Src has been linked to FAK activation on stiff matrices, and subsequent VE-cadherin phosphorylation and EC barrier disruption (Wang et al., 2019b). Another recent study showed dynamic Src-mediated regulation of

the EC barrier, via expression of activated Src. Initially, active Src induced the phosphorylation of VE-cadherin on Tyr731, which enhanced barrier function. Subsequently, Src induced the phosphorylation of VE-cadherin on Tyr685, promoting disruption of EC barrier function (Klomp et al., 2019). Previously, it has been reported that even though Src promotes initial thrombin mediated BBB break down *in vivo*, it is involved in the long-term improvement of the barrier (Liu et al., 2010). Thus, FAK and Src are capable of both stabilizing and destabilizing the EC barrier in a dynamic, context-dependent manner. The potential contribution of FAK or Src to β1-integrin-mediated EC barrier regulation remains to be found out.

Conclusively, the results by us and others indicate a dual function for β1-integrin in 1) supporting EC monolayer stability, and in 2) actively promoting EC monolayer destabilization following inflammatory stimuli.

13. ECM adhesions in EC destabilization and inflammation (II)

In order to understand the function of β1-integrin, EC adhesion to FN was investigated. A novel finding was that inflammation induced the formation of fibrillar adhesions that contained the α5β1-integrin and the adapter protein tensin-1 that were both required for inflammation-induced loss of junctional VE-cadherin, and further β1-integrin was required for thrombin induced increase of cellular tension. Talin-1 is a well-established mediator of mechanosensitive coupling of active integrins to actin, via an integrin–talin–vinculin complex, whereas tensin-1, a marker of fibrillar adhesions, has been less studied (Bachmann et al., 2019). However, tensin-1 can bridge several integrin heterodimers to actin cytoskeleton, and its coupling to α5β1-integrin in fibroblasts promotes FN fibrillogenesis. Moreover, α5β1-integrin, but not αvβ3-integrin, translocated to fibrillar adhesions during fibroblast adhesion maturation and an inhibitor of tensin-1 prevented fibrillar adhesion formation, but did not affect focal adhesions (Pankov et al., 2000). Supporting our findings, a recent report using fibroblasts demonstrated that tensin-1 and tensin-3 maintain the active state of β1-integrin in fibrillar adhesions, suggesting that the integrin–tensin complex has mechanosensitive properties similar to the integrin–talin complex (Georgiadou and Ivaska, 2017). Although our work did not elucidate the mechanisms leading to increased tensin-1 in inflammation, it is tempting to speculate that this would involve e.g. the activity of Src, which has been found to phosphorylate the cytoplasmic NPxY motif of β1-integrin supporting tensin-1 binding, while inhibiting talin-1 binding (McCleverty et al., 2007).

We further discovered that inflammatory agents enhanced the recycling of α5β1-integrin from internalized vesicles into fibrillar adhesions, in an ANGPT2-dependent manner (Figure 8). Integrin recycling provides a mechanism of dynamic regulation of integrin activation, and active and inactive β1-integrins are known to undergo differential recycling. Active integrins are recycled faster than inactive dimers, and the recycling pathways are different, resulting in distinct pools of active and inactive integrins (Moreno-Layseca et al., 2019). In this respect, it is interesting to note that segregation of β1-integrin into spatially distinct nanoclusters of active and inactive integrins, was recently reported to regulate integrin overall activity (Spiess et al., 2018).

Both talin-1 and tensin-1 have been found to play a role in vascular formation and stability, although their endothelial specific functions are not fully understood. Tensin-1 knock-out mice show decreased angiogenic capacity in a Matrigel plug assay, and isolated ECs from these mice also have defective tube forming capacity *in vitro* (Shih et al., 2015). Interestingly, tensin-1 knock-out mice suffer from renal failure, but other tissues seemed unaffected (Lo et al., 1997). An EC specific deletion of talin-1 led to the death of gene targeted embryos due to early vascular defects by E3 (Monkley et al., 2011).

A recent study investigated the role of talin-1 in the endothelial barrier function in the mature vasculature of adult mice, using the inducible *Cdh5-Cre* (*Tln1-1$^{flox/flox}$;Cdh-5-Cre*^{ERT}). After 16 days of tamoxifen injection, *Tln1-1$^{flox/flox}$;Cdh-5-Cre*^{ERT} mice (*Talin1-ECKO*) started to die due to severe bleeding in the microvasculature of the intestine (Pulous et al., 2019) whereas other organs were not affected. However, in addition to intestinal capillaries, also the junctions of retinal vessels were disorganized with more internalized VE-cadherin in the *Talin1-ECKO* mice (Pulous et al., 2019). It was suggested that the phenotype was mediated via β1-integrin, although *in vivo* evidence was lacking. Indeed, since talin-1 is a universal adaptor of several integrins, it is plausible that talin-1 deletion affected the function of several integrins. However, the high tissue specificity of talin-1 and tensin-1 knock-outs is remarkable, suggesting that perhaps other integrin activators can compensate for these adaptors in other tissues. Overall, the functions and tissue specificities of integrin adaptors in ECs needs to be more thoroughly investigated.

14.1 β1-integrin inhibitory antibody in vascular leakage *in vivo* (II)

We found that a β1-integrin functional antibody, HMβ1, that has been reported to inhibit capillary formation during mouse embryogenesis (Carlson et al., 2008), prevented LPS-induced vascular leakage in mice, both as a pretreatment and as an intervention therapy, administered after the onset of systemic inflammation. Although high levels of HMβ1 disrupted EC adhesion *in vitro*, the HMβ1 dose of 2.5 mg/kg, which was used in our studies *in vivo,* did not affect the CD31 positive junctions in the tracheal vasculature, during the 40 hour-experiment. This suggests that ECs *in vivo* are not highly sensitive to β1-integrin loss, possibly due to vascular maturation including BM and pericyte support, or due to compensation by other integrin heterodimers. It is also possible that the dose of HMβ1 did not reach a sufficiently high concentration *in vivo,* to cause loss of EC integrity and that longer treatments would be needed to fully inhibit β1-integrin. Supporting these possibilities, a high molar concentration of ATN-161, a peptide inhibitor of α5β1-integrin, induced EC apoptosis during retinal neovascularization, and JSM6427, a small molecule inhibitor of α5β1-integrin, inhibited neovascularization in rodent, rabbit, and non-human primate models without any adverse effects (Sui et al., 2018; Zahn et al., 2009). Our results using HMβ1 suggest that short-term β1-integrin inhibition does not lead to aberrant changes in a mature vasculature, suggestive of a potential therapeutic window. Similarly, Zovein et al. have reported that adult retinal vasculature was mostly unaffected by β1-integrin blocking antibodies (Zovein et al., 2010).

14. Destabilizing function of endothelial β1-integrin in the vasculature

EC specific β1-integrin has previously been found important for vascular formation and maintenance of EC polarity in arteries, as summarized in Table 1. In addition, Yamamoto et al. showed that β1-integrin was necessary in the growing vasculature to inhibit EC proliferation, to maintain vascular stability, and VE-cadherin and FN deposition (Yamamoto et al., 2015). We found that heterozygous deletion of β1-integrin in the endothelium of adult mice protected from LPS-induced vascular leakage, but did not lead to any obvious vascular defects, including vascular leakage or capillary loss. The effect of homozygous EC specific deletion of β1-integrin in adult mice has not been published so far, and was also not studied in this work.

Our results highlighting a vascular destabilizing role for β1-integrin in the mature vasculature are supported by previous studies using integrin knock-out mice and an α5β1-integrin targeting peptide that demonstrated a proinflammatory function for α5β1-integrin. Specifically, α5β1-integrin was found to promote atherosclerosis, since the ATN-161 peptide that inhibits α5β1-integrin, reduced

atherosclerosis and endothelial inflammation in *ApoE*$^{-/-}$ mice that develop atherosclerosis on a high fat diet (Yurdagul et al., 2014). In addition, EC specific deletion of α5-integrin protected from atherosclerosis in the *ApoE*$^{-/-}$ mice (Table 1) (Al-Yafeai et al., 2018). Further, knock-in mice, where the α5-integrin intracellular domain was replaced by that of the α2-integrin, did not develop atherosclerosis in *ApoE*$^{-/-}$ background, suggesting that the cytoplasmic domain of α5-integrin was needed for the formation of atherosclerotic plaques (Budatha et al., 2018). Mechanistically, the α5β1-integrin-mediated progression of arterial inflammation and atherosclerosis has been found to depend on the mechanotransduction signaling via actin cytoskeleton and membrane lipid rafts (Albarran-Juarez et al., 2018; Sun et al., 2016a), FN deposition into the ECM (Budatha et al., 2018; Feaver et al., 2010), and downregulation of anti-inflammatory cAMP signaling (Yun et al., 2019). Increased ANGPT2 levels have been reported in atherosclerosis (Le Dall et al., 2010; Post et al., 2008). Theelan et al. found that a neutralizing ANGPT2 antibody reduced serum triglyceride levels and the formation of fatty streaks in arteries of hypercholesterolemic LDL$^{-/-}$;ApoB$^{100/100}$ mice (Theelen et al., 2015). Our studies support the concept that elevated ANGPT2 signaling in the aortic ECs decreases junctional TIE2, promoting inflammatory signaling and predisposing the vasculature to ANGPT2–β1-integrin signaling and EC destabilization.

In addition to atherosclerosis, α5β1-integrin has been also implicated in neovascular diseases of the eye. ATN-161 was found to inhibit neovascularization and vascular leakage in a preclinical model of laser-induced choroidal neovascularization (CNV), resembling choroidal neovascular formation in nAMD (Maier et al., 2007; Umeda et al., 2006; Wang et al., 2011). More recently, another integrin binding peptide, AXT107, was reported to potentiate EC junction integrity *in vitro* by disrupting the α5β1-integrin heterodimer. Interestingly, AXT107 also improved EC barrier function *in vivo* in a CNV model, as efficiently as the VEGF trap that is used to treat wAMD in patients (Mirando et al., 2019).

Thus, work from other laboratories has shown that α5β1-integrin promotes chronic inflammation in atherosclerosis and hypoxia-associated neoangiogenesis and leakage in the eye. Our work, on the other hand, discovered that β1-integrin promotes vascular leakage in acute EC responses such as endotoxemia-induce vascular leakage. We demonstrated that 1) short-term blocking or heterozygous deletion of β1-integrin protected from vascular leakage, and 2) administering the β1-integrin functional antibody decreased vascular leakage, improved EC junctions, and protected from cardiac failure in murine endotoxemia.

Subsequent work should identify, 1) which α-integrins are essential in regulation of vascular leakage and 2) which αβ-integrin heterodimers are essential in homeostasis. Based on our results we suggest that β1-integrin plays a dual role, being required for both homeostatic vascular stability and specifically for inflammation-induced vascular leakage.

15.1 Integrins in regulation of EC stability and vascular permeability (I&II)

Mechanistic insight into the function of β1-integrin signaling has been obtained using cultured ECs. All alpha subunits capable of binding to β1-integrin, namely integrins α1, α2, α3, α5, α6 and αv, were expressed in cultured ECs (I), although α5-integrin was expressed at least four times higher than the other alpha subunits. However, the high expression levels of α5-integrin did not correlate with an EC stabilizing function, since silencing of αv- but not α5-integrin destabilized EC monolayers. Our results are in line with previous findings that αvβ3-integrin stabilizes EC cortical actin (Su et al., 2012), and in addition, we demonstrate that αvβ3-integrin stabilizes EC junctions.

Our results also concur with previous work showing that ANGPT2 can stimulate αvβ3-integrin internalization, which would add to the mechanisms by which ANGPT2 can promote EC destabilization (Thomas et al., 2010).

Instead, our results demonstrated that α5-integrin silencing, similarly to β1-integrin silencing, protected from EC destabilization in TIE2 silenced or inflammatory stimulated ECs (I-II). α5β1-integrin was also activated upon ANGPT2 stimulation in an integrin activation assay (I), and inflammatory agents induced α5-integrin recycling that was ANGPT2-dependent (II). Finally, inflammation stimulated the formation of α5β1-integrin positive fibrillar adhesions (II) (Figure 7). Previously, it has been shown that α5β1-integrin translocates to central fibrillar adhesions creating tension that is essential for FN fibrillogenesis and assembly into the ECM in fibroblasts (Faurobert et al., 2013; Pankov et al., 2000). Thus, the adhesion maturation observed in fibroblasts shows similarity to inflammation-induced adhesion formation in ECs.

The αvβ3-integrin has been found necessary for maintenance of cortical actin in EC monolayers, via sphingosine 1 phosphate (S1P) (Su et al., 2012). S1P is known to promote cortical actin through Rac1 activity, and it is also involved in the formation of adherens junctions (Belvitch and Dudek, 2012). On the contrary, it has been shown that blocking antibody against αvβ5-integrin decreases LPS-, VEGF-, transforming growth factor β (TGFβ)– and thrombin-elicited drop in EC permeability, and further prevents RhoA-mediated stress fiber formation (Su et al., 2013; Su et al., 2007). Based on these reports, it would seem that αvβ5- and αvβ3-integrins have opposing effects on the endothelial barrier, via RhoA or Rac1, respectively.

Yamamoto et al. proposed that β1-integrin is needed for VE-cadherin maintenance *in vitro* in HUVEC junctions and *in vivo* in the postnatal retina (Table 1) (Yamamoto et al., 2015). In line with Yamamoto et al., we conclude that β1-integrin is required for maintenance of EC monolayers, since high levels of β1-integrin antibody disrupted EC adhesion and thereby EC junctions (II). In our studies, silencing of β1-integrin did not however, disrupt the EC monolayer. This can be possibly explained by the incomplete loss of β1-integrin from the focal adhesions during the 72 hour-silencing, although 96% of the β1-integrin mRNA and the majority of the β1-integrin protein was depleted. Moreover, since our silencing experiments were based on lentiviral vectors, all experiments were performed using confluent EC monolayers to avoid any potential effects of β1-integrin loss on EC division and subsequent adhesion (Malan et al., 2010), whereas Yamamoto et al. utilized siRNAs to silence β1-integrin.

αvβ3-integrin was originally proposed an angiogenic role, and the expression of αvβ3-integrin is increased in the activated endothelium (Weis and Cheresh, 2011). However, studies of αv- or β3-integrin knockout mice have revealed that αvβ3-integrin may not be uniquely required for angiogenesis (Bader et al., 1998; Hodivala-Dilke et al., 1999), rendering the role of αvβ3-integrin in angiogenesis controversial. Furthermore, VEGF stimulation enhances angiogenesis in *Itgb3-/-* mice (Table 1) (Reynolds et al., 2004), suggesting compensatory mechanism via other integrins, like α5β1-integrin. Further studies using β3-integrin deleted mice showed increased vascular leakage in a murine model of ALI, and mortality in murine sepsis (Table 1). In this study, the β3-integrin was deleted ubiquitously, but effects through macrophage or platelet β3-integrins were ruled out by bone marrow transplantation (Su et al., 2012). In a cerebral injury model, αvβ3-integrin inhibitory peptide alone, and together with recombinant ANGPT1, improved the cerebral vasculature (Han et al., 2010).

In the tumor vasculature, simultaneous silencing of α5- and αv-integrins had no effect on tumor angiogenesis (Table 1) (Murphy et al., 2015). The authors thus propose that the effects of blocking the function of these integrins with antibodies or small molecular inhibitors relies on dominant negative functions, but offer no conclusive evidence to support this theory (Murphy et al., 2015). In addition, since many of the integrin heterodimers are ubiquitously expressed, the use of blocking reagents *in vivo* is likely to target various cell types simultaneously. In our studies, we confirmed the results using both inhibitory antibodies and endothelial heterozygous gene deletion of β1-integrin.

15. Preclinical approaches of vascular stabilization in sepsis (II)

Decreased endothelial barrier function and increased EC permeability are associated with numerous diseases, including atherosclerosis, ocular neovascular diseases, cancer and sepsis (Jeong et al., 2019). Systemic inflammation results from the aberrant activation of the innate immune system, leading to the production of numerous inflammatory mediators including TNF-α, IL-1β, IL-6, and thrombin, which can act on ECs to cause vascular leakage. Additionally, EC apoptosis may contribute to loss of vascular integrity in sepsis (Saharinen et al., 2017a; Wentowski et al., 2019).

The fundamental problem remains that there are no treatments for pathological vascular leakage in sepsis. Several clinical trials testing various therapeutic approaches have failed to improve patient survival, such as targeting of the IL-1 receptor or activated protein C (Marshall, 2014). Therapies aiming to attenuate inflammation may show complex outcomes, since the inflammatory response also serves the vital purposes of eradication of the invading pathogen, and tissue regeneration and repair (Cooke, 2019). Various preclinical approaches, some of which are discussed below, have targeted the vasculature aiming to improve the hemodynamics, vascular perfusion and integrity.

Repurposing of existing drugs for alternative use is an attractive approach to provide a faster route to novel therapies. Imatinib, a kinase inhibitor of the Abl and Arg kinases, has shown efficacy in decreasing vascular leakage in murine sepsis models (Waller, 2010). Imatinib was reported to decrease VEGF-induced vascular leakage in the skin and to attenuate acute pulmonary edema and vascular leakage in the kidney in a CLP model (Aman et al., 2012). Another study concluded that LPS-induced lung catalase activity, a marker of oxidative EC injury and DNA damage, was decreased using a prophylactive imatinib dosing in mice. Further, LPS-elicited increase in lung caspase3/7 activity, circulating TNFα and IL-6 as well as pulmonary edema were diminished, whereas survival after LPS administration was improved in imatinib-treated mice compared to control group (Stephens et al., 2015). Similarly, in ALI, prophylactic imatinib improved lung morphology and decreased LPS-induced increase of total protein levels and inflammatory cell counts in bronchoalveolar lavage. However, contradicting results have also been reported. Specifically, prophylactic imatinib enhanced the symptoms of ventilation induced lung injury (VILI) (Letsiou et al., 2015).

VEGF inhibitors are in therapeutic use in several cancers. However, VEGF inhibition using e.g. bevacizumab has yielded controversial reports in preclinical models of sepsis. In one preclinical study, bevacizumab decreased vascular leakage in the lung, spleen and kidney, and increased the overall survival (Jeong et al., 2013). However, another study-conducted after the clinical trial for bevacizumab in the treatment of sepsis was canceled-reported no significant benefit on overall survival in a CLP model of murine sepsis (Besnier et al., 2017). It is likely that VEGF plays a less prominent role in vascular permeability in sepsis, compared to hypoxia-driven neovascular diseases.

Preclinically, a promising way of alleviating sepsis and sepsis-like syndromes, has been the manipulation of the ANGPT–TIE2 signaling pathway (David et al., 2011; Kumpers et al., 2011;

Witzenbichler et al., 2005). In murine LPS-induced endotoxemia, adenoviral overexpression of ANGPT1 improved the survival of septic mice, and ameliorated lung injury, in part via restoring eNOS function (Witzenbichler et al., 2005). In a murine VILI model, human ANGPT1 recombinant protein therapy decreased inflammation and VEGF and ANGPT2 expression, but not vascular leakage (Hegeman et al., 2010). A powerful way of promoting TIE2 signaling has been achieved via inhibition of VE-PTP that dephosphorylates TIE2. In mice, administration of a VE-PTP blocking antibody, or genetic deletion of VE-PTP, decreased VEGF and histamine-induced vascular leakage (Frye et al., 2015). VE-PTP antibodies did, however, increase vascular leakage in TIE2 negative mice, potentially via disturbing the VE-PTP–VE-cadherin association. Interestingly, it was recently reported that VE-PTP inhibition can stabilize EC junctions independently of VE-cadherin, and even independently of its phosphatase activity (Frye et al., 2015; Juettner et al., 2019). However, potential adverse effects due to long-term VE-PTP inhibition, namely downregulation of TIE2 in inflammation, and a tendency for aggregation of ANGPT1, may limit the use of these approaches.

Although ANGPT2 enforces inflammation promoting vascular leakage in sepsis, a synthetic antibody ABTAA that clusters ANGPT2 fortified TIE2 signaling, resulting in decreased leakage and prolonged survival in a murine CLP model (Han et al., 2016). Mechanistically, ABTAA was found to diminish inflammation, via decreased expression of ICAM-1, VCAM-1 and E-selectin, neutrophil infiltration to lungs as well as expression of proinflammatory cytokines (Han et al., 2016). The beneficial effects of ABTAA were not limited to sepsis, but instead, ABTAA was found to normalize the leaky tumor vasculature, and decrease neovascularization and leakage in a murine laser-induced choroidal neovascularization model (Kim et al., 2019; Park et al., 2016). ABTAA appeared more efficacious than ANGPT2-TIE2 blocking antibodies that have shown some efficacy in various models (Lomas-Neira et al., 2016; Ziegler et al., 2013).

In contrast to ABTAA, VE-PTP or ANGPT1, which are associated with decreased inflammation, the inhibitory β1-integrin antibody in our studies did not attenuate inflammation or enhance TIE2 activation, suggesting a novel mechanism of action for the β1-integrin antibody, potentially involving direct effects on the EC barrier. Thus, one interesting possibility would be to combine ANGPT2 antibodies, like ABTAA, with an inhibitor or an antibody targeting β1-integrin. Vascular integrins have also been considered as targets for sepsis before. β5-integrin knock-out mice have been reported to survive better in LPS-induced endotoxemia, manifested by decreased albumin leakage to small bowel and colon (Table 1) (Su et al., 2013). Weather αvβ5-integrin has similar endothelial functions as β1-integrin, is yet to be investigated. Since α5β1-integrin targeting has showed promise in other preclinical disease models, it would be of interest to determine the α-integrin subunit that is involved in vascular leakage in systemic inflammation. In summary, our results demonstrated that the inhibitory β1-integrin antibody decreased vascular leakage by enhancing vascular stability in murine endotoxemia as a prophylactic and as an intervention therapy.

CONCLUSIONS AND FUTURE PROSPECTS

This thesis investigated the mechanisms that promote vascular permeability in inflammation, and more specifically, the roles of ANGPT2 and β1-integrin in endothelial destabilization. High ANGPT2/ANGPT1 ratio has been associated with various human diseases characterized by increased capillary leakage, including sepsis, and functional studies in mice have demonstrated a key role for ANGPT2 in many of the diseases. However, β1-integrin has not been previously reported to increased endothelial permeability and pathological leakage.

We discovered 1) a novel signaling pathway of ANGPT2 signaling via β1-integrin that leads to EC destabilization in inflammation and in TIE2 low ECs, 2) that inflammation regulates EC-ECM adhesion dynamics via formation of tensin-1 and α5β1-integrin positive fibrillar adhesions that create tension leading to weakening of the EC barrier function, and 3) inhibiting β1-integrin function in *in vivo* preclinical model of sepsis decreased vascular leakage and protected from endotoxemia-induced cardiac failure.

The results established a novel concept, whereby ANGPT2–β1-integrin signaling plays a role in vascular destabilization in inflammation, especially when the levels of TIE2 are decreased. Our results showing that heterozygous deletion of EC β1-integrin in endotoxemia decreases vascular leakage, strongly supports the concept. However, based on our results, it is not possible to formally prove the existence of the ANGPT2–β1-integrin signaling *in vivo*. The results showing that inhibition of β1-integrin not only improved vascular integrity, but also improved cardiac function in endotoxemia, are intriguing from a translational point of view, since these effects were obtained in the presence of EC and humoral inflammation. Thus, the mechanism of action of β1-integrin inhibition did not involve decreased inflammation, which might be beneficial in diseases where eradication of microbial pathogens is necessary. The results of this thesis encourage further studies to determine whether β1-integrin inhibition, in one way or another, might be utilized in future therapy development to inhibit vascular leakage in e.g. sepsis, or in numerous other diseases involving endothelial permeability.

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REFERENCES

Adam, A.P., Lowery, A.M., Martino, N., Alsaffar, H., and Vincent, P.A. (2016). Src Family Kinases Modulate the Loss of Endothelial Barrier Function in Response to TNF-alpha: Crosstalk with p38 Signaling. PLoS One *11*, e0161975.

Adams, R.H., and Alitalo, K. (2007). Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol *8*, 464-478.

Aird, W.C. (2007a). Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. Circ Res *100*, 158-173.

Aird, W.C. (2007b). Phenotypic heterogeneity of the endothelium: II. Representative vascular beds. Circ Res *100*, 174-190.

Al-Yafeai, Z., Yurdagul, A., Peretik, J.M., Alfaidi, M., Murphy, P.A., and Orr, A.W. (2018). Endothelial FN (Fibronectin) Deposition by 51 Integrins Drives Atherogenic Inflammation. Arterioscl Throm Vas *38*, 2601-2614.

Alanko, J., Mai, A., Jacquemet, G., Schauer, K., Kaukonen, R., Saari, M., Goud, B., and Ivaska, J. (2015). Integrin endosomal signalling suppresses anoikis. Nat Cell Biol *17*, 1412-1421.

Albarran-Juarez, J., Iring, A., Wang, S., Joseph, S., Grimm, M., Strilic, B., Wettschureck, N., Althoff, T.F., and Offermanns, S. (2018). Piezo1 and Gq/G11 promote endothelial inflammation depending on flow pattern and integrin activation. J Exp Med *215*, 2655-2672.

Aman, J., van Bezu, J., Damanafshan, A., Huveneers, S., Eringa, E.C., Vogel, S.M., Groeneveld, A.B., Vonk Noordegraaf, A., van Hinsbergh, V.W., and van Nieuw Amerongen, G.P. (2012). Effective treatment of edema and endothelial barrier dysfunction with imatinib. Circulation *126*, 2728-2738.

Apte, R.S., Chen, D.S., and Ferrara, N. (2019). VEGF in Signaling and Disease: Beyond Discovery and Development. Cell *176*, 1248-1264.

Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G.Y., and Suda, T. (2004). Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell *118*, 149-161.

Arjonen, A., Alanko, J., Veltel, S., and Ivaska, J. (2012). Distinct recycling of active and inactive beta1 integrins. Traffic *13*, 610-625.

Arnold, T.D., Ferrero, G.M., Qiu, H., Phan, I.T., Akhurst, R.J., Huang, E.J., and Reichardt, L.F. (2012). Defective retinal vascular endothelial cell development as a consequence of impaired integrin alphaVbeta8-mediated activation of transforming growth factor-beta. J Neurosci *32*, 1197-1206.

Arroyo, A.G., and Iruela-Arispe, M.L. (2010). Extracellular matrix, inflammation, and the angiogenic response. Cardiovasc Res *86*, 226-235.

Aspelund, A., Tammela, T., Antila, S., Nurmi, H., Leppanen, V.M., Zarkada, G., Stanczuk, L., Francois, M., Makinen, T., Saharinen, P.*, et al.* (2014). The Schlemm's canal is a VEGF-C/VEGFR-3-responsive lymphatic-like vessel. J Clin Invest *124*, 3975-3986.

Augustin, H.G., and Koh, G.Y. (2017). Organotypic vasculature: From descriptive heterogeneity to functional pathophysiology. Science *357*.

Bachmann, M., Kukkurainen, S., Hytonen, V.P., and Wehrle-Haller, B. (2019). Cell Adhesion by Integrins. Physiol Rev *99*, 1655-1699.

Back, M., Yurdagul, A., Jr., Tabas, I., Oorni, K., and Kovanen, P.T. (2019). Inflammation and its resolution in atherosclerosis: mediators and therapeutic opportunities. Nat Rev Cardiol *16*, 389-406.

Bader, B.L., Rayburn, H., Crowley, D., and Hynes, R.O. (1998). Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all alpha v integrins. Cell *95*, 507-519.

Baffert, F., Le, T., Thurston, G., and McDonald, D.M. (2006). Angiopoietin-1 decreases plasma leakage by reducing number and size of endothelial gaps in venules. Am J Physiol Heart Circ Physiol *290*, H107-118.

Baluk, P., Fuxe, J., Hashizume, H., Romano, T., Lashnits, E., Butz, S., Vestweber, D., Corada, M., Molendini, C., Dejana, E.*, et al.* (2007). Functionally specialized junctions between endothelial cells of lymphatic vessels. J Exp Med *204*, 2349-2362.

Baluk, P., Phillips, K., Yao, L.C., Adams, A., Nitschke, M., and McDonald, D.M. (2014). Neutrophil dependence of vascular remodeling after Mycoplasma infection of mouse airways. Am J Pathol *184*, 1877-1889.

Bannerman, D.D., and Goldblum, S.E. (1997). Endotoxin induces endothelial barrier dysfunction through protein tyrosine phosphorylation. Am J Physiol *273*, L217-226.

Bannerman, D.D., Sathyamoorthy, M., and Goldblum, S.E. (1998). Bacterial lipopolysaccharide disrupts endothelial monolayer integrity and survival signaling events through caspase cleavage of adherens junction proteins. J Biol Chem *273*, 35371-35380.

Barabutis, N., Handa, V., Dimitropoulou, C., Rafikov, R., Snead, C., Kumar, S., Joshi, A., Thangjam, G., Fulton, D., Black, S.M.*, et al.* (2013). LPS induces pp60c-src-mediated tyrosine phosphorylation of Hsp90 in lung vascular endothelial cells and mouse lung. Am J Physiol Lung Cell Mol Physiol *304*, L883-893.

Barker, T.H., and Engler, A.J. (2017). The provisional matrix: setting the stage for tissue repair outcomes. Matrix Biol *60-61*, 1-4.

Barton, W.A., Tzvetkova-Robev, D., Miranda, E.P., Kolev, M.V., Rajashankar, K.R., Himanen, J.P., and Nikolov, D.B. (2006). Crystal structures of the Tie2 receptor ectodomain and the angiopoietin-2-Tie2 complex. Nat Struct Mol Biol *13*, 524-532.

Batard, P., Sansilvestri, P., Scheinecker, C., Knapp, W., Debili, N., Vainchenker, W., Buhring, H.J., Monier, M.N., Kukk, E., Partanen, J.*, et al.* (1996). The Tie receptor tyrosine kinase is expressed by human hematopoietic progenitor cells and by a subset of megakaryocytic cells. Blood *87*, 2212-2220.

Bauer, M., Brunkhorst, F., Welte, T., Gerlach, H., and Reinhart, K. (2006). [Sepsis. Update on pathophysiology, diagnostics and therapy]. Anaesthesist *55*, 835-845.

Baumer, S., Keller, L., Holtmann, A., Funke, R., August, B., Gamp, A., Wolburg, H., Wolburg-Buchholz, K., Deutsch, U., and Vestweber, D. (2006). Vascular endothelial cell-specific phosphotyrosine phosphatase (VE-PTP) activity is required for blood vessel development. Blood *107*, 4754-4762.

Bazigou, E., Xie, S., Chen, C., Weston, A., Miura, N., Sorokin, L., Adams, R., Muro, A.F., Sheppard, D., and Makinen, T. (2009). Integrin-alpha9 is required for fibronectin matrix assembly during lymphatic valve morphogenesis. Dev Cell *17*, 175-186.

Beckers, C.M., van Hinsbergh, V.W., and van Nieuw Amerongen, G.P. (2010). Driving Rho GTPase activity in endothelial cells regulates barrier integrity. Thromb Haemost *103*, 40-55.

Belvitch, P., and Dudek, S.M. (2012). Role of FAK in S1P-regulated endothelial permeability. Microvasc Res *83*, 22-30.

Benest, A.V., Kruse, K., Savant, S., Thomas, M., Laib, A.M., Loos, E.K., Fiedler, U., and Augustin, H.G. (2013). Angiopoietin-2 is critical for cytokine-induced vascular leakage. PLoS One *8*, e70459.

Besnier, E., Brakenhielm, E., Richard, V., and Tamion, F. (2017). Does anti-VEGF bevacizumab improve survival in experimental sepsis? Crit Care *21*, 163.

Bhandari, V., Choo-Wing, R., Lee, C.G., Zhu, Z., Nedrelow, J.H., Chupp, G.L., Zhang, X., Matthay, M.A., Ware, L.B., Homer, R.J.*, et al.* (2006). Hyperoxia causes angiopoietin 2-mediated acute lung injury and necrotic cell death. Nat Med *12*, 1286-1293.

Birukov, K.G., and Karki, P. (2018). Injured lung endothelium: mechanisms of self-repair and agonist-assisted recovery (2017 Grover Conference Series). Pulm Circ *8*, 2045893217752660.

Bischoff, I., Hornburger, M.C., Mayer, B.A., Beyerle, A., Wegener, J., and Furst, R. (2016). Pitfalls in assessing microvascular endothelial barrier function: impedance-based devices versus the classic macromolecular tracer assay. Sci Rep *6*, 23671.

Bouvard, C., De Arcangelis, A., Dizier, B., Galy-Fauroux, I., Fischer, A.M., Georges-Labouesse, E., and Helley, D. (2012). Tie2-dependent knockout of alpha6 integrin subunit in mice reduces post-ischaemic angiogenesis. Cardiovasc Res *95*, 39-47.

Bouvard, C., Segaoula, Z., De Arcangelis, A., Galy-Fauroux, I., Mauge, L., Fischer, A.M., Georges-Labouesse, E., and Helley, D. (2014). Tie2-dependent deletion of alpha6 integrin subunit in mice reduces tumor growth and angiogenesis. Int J Oncol *45*, 2058-2064.

Braun, L.J., Zinnhardt, M., Vockel, M., Drexler, H.C., Peters, K., and Vestweber, D. (2019). VE-PTP inhibition stabilizes endothelial junctions by activating FGD5. EMBO Rep *20*, e47046.

Brindle, N.P., Saharinen, P., and Alitalo, K. (2006). Signaling and functions of angiopoietin-1 in vascular protection. Circ Res *98*, 1014-1023.

Budatha, M., Zhang, J., Zhuang, Z.W., Yun, S., Dahlman, J.E., Anderson, D.G., and Schwartz, M.A. (2018). Inhibiting Integrin alpha5 Cytoplasmic Domain Signaling Reduces Atherosclerosis and Promotes Arteriogenesis. J Am Heart Assoc *7*.

Burridge, K., and Wittchen, E.S. (2013). The tension mounts: stress fibers as force-generating mechanotransducers. J Cell Biol *200*, 9-19.

Byron, A., Humphries, J.D., Askari, J.A., Craig, S.E., Mould, A.P., and Humphries, M.J. (2009). Anti-integrin monoclonal antibodies. J Cell Sci *122*, 4009-4011.

Campbell, H.K., Maiers, J.L., and DeMali, K.A. (2017). Interplay between tight junctions & adherens junctions. Exp Cell Res *358*, 39-44.

Carlson, T.R., Feng, Y., Maisonpierre, P.C., Mrksich, M., and Morla, A.O. (2001). Direct cell adhesion to the angiopoietins mediated by integrins. J Biol Chem *276*, 26516-26525.

Carlson, T.R., Hu, H., Braren, R., Kim, Y.H., and Wang, R.A. (2008). Cell-autonomous requirement for beta1 integrin in endothelial cell adhesion, migration and survival during angiogenesis in mice. Development *135*, 2193-2202.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C.*, et al.* (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature *380*, 435-439.

Carmeliet, P., Lampugnani, M.G., Moons, L., Breviario, F., Compernolle, V., Bono, F., Balconi, G., Spagnuolo, R., Oosthuyse, B., Dewerchin, M.*, et al.* (1999). Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. Cell *98*, 147-157.

Carota, I.A., Kenig-Kozlovsky, Y., Onay, T., Scott, R., Thomson, B.R., Souma, T., Bartlett, C.S., Li, Y., Procissi, D., Ramirez, V., *et al.* (2019). Targeting VE-PTP phosphatase protects the kidney from diabetic injury. J Exp Med *216*, 936-949.
Cascone, I., Napione, L., Maniero, F., Serini, G., and Bussolino, F. (2005). Stable interaction between alpha5beta1 integrin and Tie2 tyrosine kinase receptor regulates endothelial cell response to Ang-1. J Cell Biol *170*, 993-1004.
Cerutti, C., and Ridley, A.J. (2017). Endothelial cell-cell adhesion and signaling. Exp Cell Res *358*, 31-38.
Chatterjee, A., Snead, C., Yetik-Anacak, G., Antonova, G., Zeng, J., and Catravas, J.D. (2008). Heat shock protein 90 inhibitors attenuate LPS-induced endothelial hyperpermeability. Am J Physiol Lung Cell Mol Physiol *294*, L755-763.
Chatterjee, D., D'Souza, A., Zhang, Y., Bin, W., Tan, S.M., and Bhattacharjya, S. (2018). Interaction Analyses of 14-3-3zeta, Dok1, and Phosphorylated Integrin beta Cytoplasmic Tails Reveal a Bi-molecular Switch in Integrin Regulation. J Mol Biol *430*, 4419-4430.
Chen, X.L., Nam, J.O., Jean, C., Lawson, C., Walsh, C.T., Goka, E., Lim, S.T., Tomar, A., Tancioni, I., Uryu, S., *et al.* (2012). VEGF-induced vascular permeability is mediated by FAK. Dev Cell *22*, 146-157.
Chen, Y., Wu, Y., Zhang, X., Zeng, H., Liu, Y., Wu, Q., Chen, Y., Zhu, G., Pan, Q., Jin, L., *et al.* (2018). Angiopoietin-2 (Ang-2) is a useful serum tumor marker for liver cancer in the Chinese population. Clin Chim Acta *478*, 18-27.
Cheng, J.P.X., and Nichols, B.J. (2016). Caveolae: One Function or Many? Trends Cell Biol *26*, 177-189.
Chiang, S.P., Cabrera, R.M., and Segall, J.E. (2016). Tumor cell intravasation. Am J Physiol Cell Physiol *311*, C1-C14.
Claesson-Welsh, L. (2015). Vascular permeability--the essentials. Ups J Med Sci *120*, 135-143.
Cooke, J.P. (2019). Inflammation and Its Role in Regeneration and Repair. Circ Res *124*, 1166-1168.
Corada, M., Mariotti, M., Thurston, G., Smith, K., Kunkel, R., Brockhaus, M., Lampugnani, M.G., Martin-Padura, I., Stoppacciaro, A., Ruco, L., *et al.* (1999). Vascular endothelial-cadherin is an important determinant of microvascular integrity in vivo. Proc Natl Acad Sci U S A *96*, 9815-9820.
D'Amico, G., Korhonen, E.A., Anisimov, A., Zarkada, G., Holopainen, T., Hagerling, R., Kiefer, F., Eklund, L., Sormunen, R., Elamaa, H., *et al.* (2014). Tie1 deletion inhibits tumor growth and improves angiopoietin antagonist therapy. J Clin Invest *124*, 824-834.
Dallabrida, S.M., Euloth, M., and Rupnick, M.A. (2004). The role of angiopoietin-1 in physiologic and pathologic cardiac remodeling and in promoting cardiac myocyte survival. J Am Coll Cardiol *43*, 236a-236a.
Dalton, A.C., Shlamkovitch, T., Papo, N., and Barton, W.A. (2016). Constitutive Association of Tie1 and Tie2 with Endothelial Integrins is Functionally Modulated by Angiopoietin-1 and Fibronectin. PLoS One *11*, e0163732.
Daly, C., Eichten, A., Castanaro, C., Pasnikowski, E., Adler, A., Lalani, A.S., Papadopoulos, N., Kyle, A.H., Minchinton, A.I., Yancopoulos, G.D., *et al.* (2013). Angiopoietin-2 Functions as a Tie2 Agonist in Tumor Models, Where It Limits the Effects of VEGF Inhibition. Cancer Research *73*, 108-118.
Daly, C., Pasnikowski, E., Burova, E., Wong, V., Aldrich, T.H., Griffiths, J., Ioffe, E., Daly, T.J., Fandl, J.P., Papadopoulos, N., *et al.* (2006). Angiopoietin-2 functions as an autocrine protective factor in stressed endothelial cells. Proc Natl Acad Sci U S A *103*, 15491-15496.
Daly, C., Wong, V., Burova, E., Wei, Y., Zabski, S., Griffiths, J., Lai, K.M., Lin, H.C., Ioffe, E., Yancopoulos, G.D., *et al.* (2004). Angiopoietin-1 modulates endothelial cell function and gene expression via the transcription factor FKHR (FOXO1). Genes Dev *18*, 1060-1071.
David, S., Ghosh, C.C., Mukherjee, A., and Parikh, S.M. (2011). Angiopoietin-1 requires IQ domain GTPase-activating protein 1 to activate Rac1 and promote endothelial barrier defense. Arterioscler Thromb Vasc Biol *31*, 2643-2652.
David, S., Mukherjee, A., Ghosh, C.C., Yano, M., Khankin, E.V., Wenger, J.B., Karumanchi, S.A., Shapiro, N.I., and Parikh, S.M. (2012). Angiopoietin-2 may contribute to multiple organ dysfunction and death in sepsis*. Crit Care Med *40*, 3034-3041.
Davis, J.S., Yeo, T.W., Piera, K.A., Woodberry, T., Celermajer, D.S., Stephens, D.P., and Anstey, N.M. (2010). Angiopoietin-2 is increased in sepsis and inversely associated with nitric oxide-dependent microvascular reactivity. Crit Care *14*, R89.
Davis, S., Papadopoulos, N., Aldrich, T.H., Maisonpierre, P.C., Huang, T., Kovac, L., Xu, A., Leidich, R., Radziejewska, E., Rafique, A., *et al.* (2003). Angiopoietins have distinct modular domains essential for receptor binding, dimerization and superclustering. Nat Struct Biol *10*, 38-44.
De Palma, M., and Lewis, C.E. (2013). Macrophage regulation of tumor responses to anticancer therapies. Cancer Cell *23*, 277-286.
De Palma, M., Venneri, M.A., Galli, R., Sergi Sergi, L., Politi, L.S., Sampaolesi, M., and Naldini, L. (2005). Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. Cancer Cell *8*, 211-226.
Dejana, E., and Lampugnani, M.G. (2018). Endothelial cell transitions. Science *362*, 746-747.

Dejana, E., Orsenigo, F., and Lampugnani, M.G. (2008). The role of adherens junctions and VE-cadherin in the control of vascular permeability. J Cell Sci *121*, 2115-2122.

Dominguez, M.G., Hughes, V.C., Pan, L., Simmons, M., Daly, C., Anderson, K., Noguera-Troise, I., Murphy, A.J., Valenzuela, D.M., Davis, S.*, et al.* (2007). Vascular endothelial tyrosine phosphatase (VE-PTP)-null mice undergo vasculogenesis but die embryonically because of defects in angiogenesis. Proc Natl Acad Sci U S A *104*, 3243-3248.

Drake, C.J., and Fleming, P.A. (2000). Vasculogenesis in the day 6.5 to 9.5 mouse embryo. Blood *95*, 1671-1679.

Dumont, D.J., Gradwohl, G., Fong, G.H., Puri, M.C., Gertsenstein, M., Auerbach, A., and Breitman, M.L. (1994). Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. Genes Dev *8*, 1897-1909.

Durrant, T.N., van den Bosch, M.T., and Hers, I. (2017). Integrin alphaIIbbeta3 outside-in signaling. Blood *130*, 1607-1619.

Ebihara, I., Hirayama, K., Nagai, K., Kakita, T., Miyamoto, Y., Nagai, M., Ogawa, Y., Fujita, S., Shimohata, H., Kai, H.*, et al.* (2009). Angiopoietin balance in septic shock patients treated by direct hemoperfusion with polymyxin b-immobilized fiber. Ther Apher Dial *13*, 520-527.

Eklund, L., Kangas, J., and Saharinen, P. (2017). Angiopoietin-Tie signalling in the cardiovascular and lymphatic systems. Clin Sci (Lond) *131*, 87-103.

Elamaa, H., Kihlstrom, M., Kapiainen, E., Kaakinen, M., Miinalainen, I., Ragauskas, S., Cerrada-Gimenez, M., Mering, S., Natynki, M., and Eklund, L. (2018). Angiopoietin-4-dependent venous maturation and fluid drainage in the peripheral retina. Elife *7*.

Eliceiri, B.P., Puente, X.S., Hood, J.D., Stupack, D.G., Schlaepfer, D.D., Huang, X.Z., Sheppard, D., and Cheresh, D.A. (2002). Src-mediated coupling of focal adhesion kinase to integrin alpha(v)beta5 in vascular endothelial growth factor signaling. J Cell Biol *157*, 149-160.

Falcon, B.L., Hashizume, H., Koumoutsakos, P., Chou, J., Bready, J.V., Coxon, A., Oliner, J.D., and McDonald, D.M. (2009). Contrasting actions of selective inhibitors of angiopoietin-1 and angiopoietin-2 on the normalization of tumor blood vessels. Am J Pathol *175*, 2159-2170.

Fallah, A., Sadeghinia, A., Kahroba, H., Samadi, A., Heidari, H.R., Bradaran, B., Zeinali, S., and Molavi, O. (2019). Therapeutic targeting of angiogenesis molecular pathways in angiogenesis-dependent diseases. Biomed Pharmacother *110*, 775-785.

Fassler, R., and Meyer, M. (1995). Consequences of lack of beta 1 integrin gene expression in mice. Genes Dev *9*, 1896-1908.

Faurobert, E., Rome, C., Lisowska, J., Manet-Dupe, S., Boulday, G., Malbouyres, M., Balland, M., Bouin, A.P., Keramidas, M., Bouvard, D.*, et al.* (2013). CCM1-ICAP-1 complex controls beta1 integrin-dependent endothelial contractility and fibronectin remodeling. J Cell Biol *202*, 545-561.

Feaver, R.E., Gelfand, B.D., Wang, C., Schwartz, M.A., and Blackman, B.R. (2010). Atheroprone hemodynamics regulate fibronectin deposition to create positive feedback that sustains endothelial inflammation. Circ Res *106*, 1703-1711.

Felcht, M., Luck, R., Schering, A., Seidel, P., Srivastava, K., Hu, J., Bartol, A., Kienast, Y., Vettel, C., Loos, E.K.*, et al.* (2012). Angiopoietin-2 differentially regulates angiogenesis through TIE2 and integrin signaling. J Clin Invest *122*, 1991-2005.

Ferrara, N., and Adamis, A.P. (2016). Ten years of anti-vascular endothelial growth factor therapy. Nat Rev Drug Discov *15*, 385-403.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-Braxton, L., Hillan, K.J., and Moore, M.W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature *380*, 439-442.

Ferrara, N., Gerber, H.P., and LeCouter, J. (2003). The biology of VEGF and its receptors. Nat Med *9*, 669-676.

Fiedler, U., Krissl, T., Koidl, S., Weiss, C., Koblizek, T., Deutsch, U., Martiny-Baron, G., Marme, D., and Augustin, H.G. (2003). Angiopoietin-1 and angiopoietin-2 share the same binding domains in the Tie-2 receptor involving the first Ig-like loop and the epidermal growth factor-like repeats. J Biol Chem *278*, 1721-1727.

Fiedler, U., Reiss, Y., Scharpfenecker, M., Grunow, V., Koidl, S., Thurston, G., Gale, N.W., Witzenrath, M., Rosseau, S., Suttorp, N.*, et al.* (2006). Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. Nat Med *12*, 235-239.

Fiedler, U., Scharpfenecker, M., Koidl, S., Hegen, A., Grunow, V., Schmidt, J.M., Kriz, W., Thurston, G., and Augustin, H.G. (2004). The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. Blood *103*, 4150-4156.

Florey, L. (1966). Endothelial Cell. Brit Med J *2*, 487-&.

Francis, S.E., Goh, K.L., Hodivala-Dilke, K., Bader, B.L., Stark, M., Davidson, D., and Hynes, R.O. (2002). Central roles of alpha5beta1 integrin and fibronectin in vascular development in mouse embryos and embryoid bodies. Arterioscler Thromb Vasc Biol *22*, 927-933.

Frye, M., Dierkes, M., Kuppers, V., Vockel, M., Tomm, J., Zeuschner, D., Rossaint, J., Zarbock, A., Koh, G.Y., Peters, K., *et al.* (2015). Interfering with VE-PTP stabilizes endothelial junctions in vivo via Tie-2 in the absence of VE-cadherin. J Exp Med *212*, 2267-2287.

Fukuhara, S., Sako, K., Minami, T., Noda, K., Kim, H.Z., Kodama, T., Shibuya, M., Takakura, N., Koh, G.Y., and Mochizuki, N. (2008). Differential function of Tie2 at cell-cell contacts and cell-substratum contacts regulated by angiopoietin-1. Nat Cell Biol *10*, 513-526.

Fukumura, D., Gohongi, T., Kadambi, A., Izumi, Y., Ang, J., Yun, C.O., Buerk, D.G., Huang, P.L., and Jain, R.K. (2001). Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. Proc Natl Acad Sci U S A *98*, 2604-2609.

Fukumura, D., Kloepper, J., Amoozgar, Z., Duda, D.G., and Jain, R.K. (2018). Enhancing cancer immunotherapy using antiangiogenics: opportunities and challenges. Nat Rev Clin Oncol *15*, 325-340.

Gahmberg, C.G., Gronholm, M., Madhavan, S., Jahan, F., Mikkola, E., Viazmina, L., and Koivunen, E. (2019). Regulation of cell adhesion: a collaborative effort of integrins, their ligands, cytoplasmic actors, and phosphorylation. Q Rev Biophys *52*, e10.

Gale, N.W., Thurston, G., Hackett, S.F., Renard, R., Wang, Q., McClain, J., Martin, C., Witte, C., Witte, M.H., Jackson, D., *et al.* (2002). Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. Dev Cell *3*, 411-423.

Gallagher, D.C., Parikh, S.M., Balonov, K., Miller, A., Gautam, S., Talmor, D., and Sukhatme, V.P. (2008). Circulating angiopoietin 2 correlates with mortality in a surgical population with acute lung injury/adult respiratory distress syndrome. Shock *29*, 656-661.

Gamble, J.R., Drew, J., Trezise, L., Underwood, A., Parsons, M., Kasminkas, L., Rudge, J., Yancopoulos, G., and Vadas, M.A. (2000). Angiopoietin-1 is an antipermeability and anti-inflammatory agent in vitro and targets cell junctions. Circ Res *87*, 603-607.

Gando, S., Levi, M., and Toh, C.H. (2016). Disseminated intravascular coagulation. Nat Rev Dis Primers *2*, 16037.

Ganter, M.T., Roux, J., Miyazawa, B., Howard, M., Frank, J.A., Su, G., Sheppard, D., Violette, S.M., Weinreb, P.H., Horan, G.S., *et al.* (2008). Interleukin-1beta causes acute lung injury via alphavbeta5 and alphavbeta6 integrin-dependent mechanisms. Circ Res *102*, 804-812.

Garmy-Susini, B., Avraamides, C.J., Desgrosellier, J.S., Schmid, M.C., Foubert, P., Ellies, L.G., Lowy, A.M., Blair, S.L., Vandenberg, S.R., Datnow, B., *et al.* (2013). PI3Kalpha activates integrin alpha4beta1 to establish a metastatic niche in lymph nodes. Proc Natl Acad Sci U S A *110*, 9042-9047.

Gavard, J. (2014). Endothelial permeability and VE-cadherin: a wacky comradeship. Cell Adh Migr *8*, 158-164.

Gavard, J., and Gutkind, J.S. (2006). VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. Nat Cell Biol *8*, 1223-1234.

Geiger, B., and Yamada, K.M. (2011). Molecular Architecture and Function of Matrix Adhesions. Csh Perspect Biol *3*.

Georgiadou, M., and Ivaska, J. (2017). Tensins: Bridging AMP-Activated Protein Kinase with Integrin Activation. Trends Cell Biol *27*, 703-711.

Germain, M., De Arcangelis, A., Robinson, S.D., Baker, M., Tavora, B., D'Amico, G., Silva, R., Kostourou, V., Reynolds, L.E., Watson, A., *et al.* (2010). Genetic ablation of the alpha 6-integrin subunit in Tie1Cre mice enhances tumour angiogenesis. J Pathol *220*, 370-381.

Ghosh, C.C., David, S., Zhang, R., Berghelli, A., Milam, K., Higgins, S.J., Hunter, J., Mukherjee, A., Wei, Y., Tran, M., *et al.* (2016). Gene control of tyrosine kinase TIE2 and vascular manifestations of infections. Proc Natl Acad Sci U S A *113*, 2472-2477.

Giamarellos-Bourboulis, E.J., Kanellakopoulou, K., Pelekanou, A., Tsaganos, T., and Kotzampassi, K. (2008). Kinetics of angiopoietin-2 in serum of multi-trauma patients: correlation with patient severity. Cytokine *44*, 310-313.

Gimbrone, M.A., Jr., and Garcia-Cardena, G. (2013). Vascular endothelium, hemodynamics, and the pathobiology of atherosclerosis. Cardiovasc Pathol *22*, 9-15.

Giuliano, J.S., Jr., Lahni, P.M., Harmon, K., Wong, H.R., Doughty, L.A., Carcillo, J.A., Zingarelli, B., Sukhatme, V.P., Parikh, S.M., and Wheeler, D.S. (2007). Admission angiopoietin levels in children with septic shock. Shock *28*, 650-654.

Goede, V., Coutelle, O., Neuneier, J., Reinacher-Schick, A., Schnell, R., Koslowsky, T.C., Weihrauch, M.R., Cremer, B., Kashkar, H., Odenthal, M., *et al.* (2010). Identification of serum angiopoietin-2 as a biomarker for clinical outcome of colorectal cancer patients treated with bevacizumab-containing therapy. Br J Cancer *103*, 1407-1414.

Gong, P., Angelini, D.J., Yang, S., Xia, G., Cross, A.S., Mann, D., Bannerman, D.D., Vogel, S.N., and Goldblum, S.E. (2008). TLR4 signaling is coupled to SRC family kinase activation, tyrosine phosphorylation of zonula adherens proteins, and opening of the paracellular pathway in human lung microvascular endothelia. J Biol Chem *283*, 13437-13449.

Gough, R.E., and Goult, B.T. (2018). The tale of two talins - two isoforms to fine-tune integrin signalling. FEBS Lett *592*, 2108-2125.

Greenwald, A.C., Licht, T., Kumar, S., Oladipupo, S.S., Iyer, S., Grunewald, M., and Keshet, E. (2019). VEGF expands erythropoiesis via hypoxia-independent induction of erythropoietin in noncanonical perivascular stromal cells. J Exp Med *216*, 215-230.

Gyawali, B., Ramakrishna, K., and Dhamoon, A.S. (2019). Sepsis: The evolution in definition, pathophysiology, and management. SAGE Open Med *7*, 2050312119835043.

Hacker, U.T., Escalona-Espinosa, L., Consalvo, N., Goede, V., Schiffmann, L., Scherer, S.J., Hedge, P., Van Cutsem, E., Coutelle, O., and Buning, H. (2016). Evaluation of Angiopoietin-2 as a biomarker in gastric cancer: results from the randomised phase III AVAGAST trial. Br J Cancer *114*, 855-862.

Hackett, S.F., Wiegand, S., Yancopoulos, G., and Campochiaro, P.A. (2002). Angiopoietin-2 plays an important role in retinal angiogenesis. J Cell Physiol *192*, 182-187.

Han, S., Arnold, S.A., Sithu, S.D., Mahoney, E.T., Geralds, J.T., Tran, P., Benton, R.L., Maddie, M.A., D'Souza, S.E., Whittemore, S.R.*, et al.* (2010). Rescuing vasculature with intravenous angiopoietin-1 and alpha v beta 3 integrin peptide is protective after spinal cord injury. Brain *133*, 1026-1042.

Han, S., Lee, S.J., Kim, K.E., Lee, H.S., Oh, N., Park, I., Ko, E., Oh, S.J., Lee, Y.S., Kim, D.*, et al.* (2016). Amelioration of sepsis by TIE2 activation-induced vascular protection. Sci Transl Med *8*, 335ra355.

Hashizume, H., Falcon, B.L., Kuroda, T., Baluk, P., Coxon, A., Yu, D., Bready, J.V., Oliner, J.D., and McDonald, D.M. (2010). Complementary actions of inhibitors of angiopoietin-2 and VEGF on tumor angiogenesis and growth. Cancer Res *70*, 2213-2223.

Hegeman, M.A., Hennus, M.P., van Meurs, M., Cobelens, P.M., Kavelaars, A., Jansen, N.J., Schultz, M.J., van Vught, A.J., Molema, G., and Heijnen, C.J. (2010). Angiopoietin-1 treatment reduces inflammation but does not prevent ventilator-induced lung injury. PLoS One *5*, e15653.

Helfrich, I., Edler, L., Sucker, A., Thomas, M., Christian, S., Schadendorf, D., and Augustin, H.G. (2009). Angiopoietin-2 levels are associated with disease progression in metastatic malignant melanoma. Clin Cancer Res *15*, 1384-1392.

Higgins, S.J., De Ceunynck, K., Kellum, J.A., Chen, X., Gu, X., Chaudhry, S.A., Schulman, S., Libermann, T.A., Lu, S., Shapiro, N.I.*, et al.* (2018). Tie2 protects the vasculature against thrombus formation in systemic inflammation. J Clin Invest *128*, 1471-1484.

Hirata, H., Gupta, M., Vedula, S.R., Lim, C.T., Ladoux, B., and Sokabe, M. (2015). Actomyosin bundles serve as a tension sensor and a platform for ERK activation. EMBO Rep *16*, 250-257.

Hodivala-Dilke, K.M., McHugh, K.P., Tsakiris, D.A., Rayburn, H., Crowley, D., Ullman-Cullere, M., Ross, F.P., Coller, B.S., Teitelbaum, S., and Hynes, R.O. (1999). Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. J Clin Invest *103*, 229-238.

Hodivala-Dilke, K.M., Reynolds, A.R., and Reynolds, L.E. (2003). Integrins in angiogenesis: multitalented molecules in a balancing act. Cell Tissue Res *314*, 131-144.

Holash, J., Maisonpierre, P.C., Compton, D., Boland, P., Alexander, C.R., Zagzag, D., Yancopoulos, G.D., and Wiegand, S.J. (1999). Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science *284*, 1994-1998.

Holopainen, T., Saharinen, P., D'Amico, G., Lampinen, A., Eklund, L., Sormunen, R., Anisimov, A., Zarkada, G., Lohela, M., Helotera, H.*, et al.* (2012). Effects of angiopoietin-2-blocking antibody on endothelial cell-cell junctions and lung metastasis. J Natl Cancer Inst *104*, 461-475.

Hong, Y.K., Lange-Asschenfeldt, B., Velasco, P., Hirakawa, S., Kunstfeld, R., Brown, L.F., Bohlen, P., Senger, D.R., and Detmar, M. (2004). VEGF-A promotes tissue repair-associated lymphatic vessel formation via VEGFR-2 and the alpha1beta1 and alpha2beta1 integrins. FASEB J *18*, 1111-1113.

Horton, E.R., Humphries, J.D., James, J., Jones, M.C., Askari, J.A., and Humphries, M.J. (2016). The integrin adhesome network at a glance. J Cell Sci *129*, 4159-4163.

Hsu, H.J., Lee, C.F., Locke, A., Vanderzyl, S.Q., and Kaunas, R. (2010). Stretch-induced stress fiber remodeling and the activations of JNK and ERK depend on mechanical strain rate, but not FAK. PLoS One *5*, e12470.

Hu, B., Jarzynka, M.J., Guo, P., Imanishi, Y., Schlaepfer, D.D., and Cheng, S.Y. (2006). Angiopoietin 2 induces glioma cell invasion by stimulating matrix metalloprotease 2 expression through the alphavbeta1 integrin and focal adhesion kinase signaling pathway. Cancer Res *66*, 775-783.

Huang, X.Z., Wu, J.F., Ferrando, R., Lee, J.H., Wang, Y.L., Farese, R.V., Jr., and Sheppard, D. (2000). Fatal bilateral chylothorax in mice lacking the integrin alpha9beta1. Mol Cell Biol *20*, 5208-5215.

Huang, Y.Q., Li, J.J., Hu, L., Lee, M., and Karpatkin, S. (2002). Thrombin induces increased expression and secretion of angiopoietin-2 from human umbilical vein endothelial cells. Blood *99*, 1646-1650.

Humphries, J.D., Byron, A., and Humphries, M.J. (2006). Integrin ligands at a glance. J Cell Sci *119*, 3901-3903.

Huveneers, S., Daemen, M.J., and Hordijk, P.L. (2015). Between Rho(k) and a hard place: the relation between vessel wall stiffness, endothelial contractility, and cardiovascular disease. Circ Res *116*, 895-908.

Hynes, R.O., and Naba, A. (2012). Overview of the matrisome--an inventory of extracellular matrix constituents and functions. Cold Spring Harb Perspect Biol *4*, a004903.

Imanishi, Y., Hu, B., Jarzynka, M.J., Guo, P., Elishaev, E., Bar-Joseph, I., and Cheng, S.Y. (2007). Angiopoietin-2 stimulates breast cancer metastasis through the alpha(5)beta(1) integrin-mediated pathway. Cancer Res *67*, 4254-4263.

Ince, C., Mayeux, P.R., Nguyen, T., Gomez, H., Kellum, J.A., Ospina-Tascon, G.A., Hernandez, G., Murray, P., De Backer, D., and Workgroup, A.X. (2016). The Endothelium in Sepsis. Shock *45*, 259-270.

Ivaska, J., and Heino, J. (2011). Cooperation between integrins and growth factor receptors in signaling and endocytosis. Annu Rev Cell Dev Biol *27*, 291-320.

Jain, R.K. (2003). Molecular regulation of vessel maturation. Nat Med *9*, 685-693.

Jeansson, M., Gawlik, A., Anderson, G., Li, C., Kerjaschki, D., Henkelman, M., and Quaggin, S.E. (2011). Angiopoietin-1 is essential in mouse vasculature during development and in response to injury. J Clin Invest *121*, 2278-2289.

Jeong, G.H., Lee, K.H., Lee, I.R., Oh, J.H., Kim, D.W., Shin, J.W., Kronbichler, A., Eisenhut, M., van der Vliet, H.J., Abdel-Rahman, O.*, et al.* (2019). Incidence of Capillary Leak Syndrome as an Adverse Effect of Drugs in Cancer Patients: A Systematic Review and Meta-Analysis. J Clin Med *8*.

Jeong, S.J., Han, S.H., Kim, C.O., Choi, J.Y., and Kim, J.M. (2013). Anti-vascular endothelial growth factor antibody attenuates inflammation and decreases mortality in an experimental model of severe sepsis. Crit Care *17*, R97.

Jha, S.K., Rauniyar, K., Karpanen, T., Leppanen, V.M., Brouillard, P., Vikkula, M., Alitalo, K., and Jeltsch, M. (2017). Efficient activation of the lymphangiogenic growth factor VEGF-C requires the C-terminal domain of VEGF-C and the N-terminal domain of CCBE1. Sci Rep *7*, 4916.

Joshi, A.D., Dimitropoulou, C., Thangjam, G., Snead, C., Feldman, S., Barabutis, N., Fulton, D., Hou, Y., Kumar, S., Patel, V.*, et al.* (2014). Heat shock protein 90 inhibitors prevent LPS-induced endothelial barrier dysfunction by disrupting RhoA signaling. Am J Respir Cell Mol Biol *50*, 170-179.

Juettner, V.V., Kruse, K., Dan, A., Vu, V.H., Khan, Y., Le, J., Leckband, D., Komarova, Y., and Malik, A.B. (2019). VE-PTP stabilizes VE-cadherin junctions and the endothelial barrier via a phosphatase-independent mechanism. J Cell Biol *218*, 1725-1742.

Kamba, T., Tam, B.Y., Hashizume, H., Haskell, A., Sennino, B., Mancuso, M.R., Norberg, S.M., O'Brien, S.M., Davis, R.B., Gowen, L.C.*, et al.* (2006). VEGF-dependent plasticity of fenestrated capillaries in the normal adult microvasculature. Am J Physiol Heart Circ Physiol *290*, H560-576.

Kangas, J., Natynki, M., and Eklund, L. (2018). Development of Molecular Therapies for Venous Malformations. Basic Clin Pharmacol Toxicol *123 Suppl 5*, 6-19.

Kant, R., Halder, S.K., Bix, G.J., and Milner, R. (2019). Absence of endothelial alpha5beta1 integrin triggers early onset of experimental autoimmune encephalomyelitis due to reduced vascular remodeling and compromised vascular integrity. Acta Neuropathol Commun *7*, 11.

Kasa, A., Csortos, C., and Verin, A.D. (2015). Cytoskeletal mechanisms regulating vascular endothelial barrier function in response to acute lung injury. Tissue Barriers *3*, e974448.

Kassianidou, E., Hughes, J.H., and Kumar, S. (2017). Activation of ROCK and MLCK tunes regional stress fiber formation and mechanics via preferential myosin light chain phosphorylation. Mol Biol Cell *28*, 3832-3843.

Kato, K., Dieguez-Hurtado, R., Park, D.Y., Hong, S.P., Kato-Azuma, S., Adams, S., Stehling, M., Trappmann, B., Wrana, J.L., Koh, G.Y.*, et al.* (2018). Pulmonary pericytes regulate lung morphogenesis. Nat Commun *9*, 2448.

Kechagia, J.Z., Ivaska, J., and Roca-Cusachs, P. (2019). Integrins as biomechanical sensors of the microenvironment. Nat Rev Mol Cell Biol *20*, 457-473.

Keskin, D., Kim, J., Cooke, V.G., Wu, C.C., Sugimoto, H., Gu, C., De Palma, M., Kalluri, R., and LeBleu, V.S. (2015). Targeting vascular pericytes in hypoxic tumors increases lung metastasis via angiopoietin-2. Cell Rep *10*, 1066-1081.

Kienast, Y., Klein, C., Scheuer, W., Raemsch, R., Lorenzon, E., Bernicke, D., Herting, F., Yu, S., The, H.H., Martarello, L.*, et al.* (2013). Ang-2-VEGF-A CrossMab, a Novel Bispecific Human IgG1 Antibody Blocking VEGF-A and Ang-2 Functions Simultaneously, Mediates Potent Antitumor, Antiangiogenic, and Antimetastatic Efficacy. Clinical Cancer Research *19*, 6730-6740.

Kim, I., Kim, H.G., So, J.N., Kim, J.H., Kwak, H.J., and Koh, G.Y. (2000a). Angiopoietin-1 regulates endothelial cell survival through the phosphatidylinositol 3'-Kinase/Akt signal transduction pathway. Circ Res *86*, 24-29.

Kim, I., Kim, J.H., Ryu, Y.S., Liu, M., and Koh, G.Y. (2000b). Tumor necrosis factor-alpha upregulates angiopoietin-2 in human umbilical vein endothelial cells. Biochem Biophys Res Commun *269*, 361-365.

Kim, J., Park, J.R., Choi, J., Park, I., Hwang, Y., Bae, H., Kim, Y., Choi, W., Yang, J.M., Han, S.*, et al.* (2019). Tie2 activation promotes choriocapillary regeneration for alleviating neovascular age-related macular degeneration. Sci Adv *5*, eaau6732.

Kim, K.T., Choi, H.H., Steinmetz, M.O., Maco, B., Kammerer, R.A., Ahn, S.Y., Kim, H.Z., Lee, G.M., and Koh, G.Y. (2005). Oligomerization and multimerization are critical for angiopoietin-1 to bind and phosphorylate Tie2. J Biol Chem *280*, 20126-20131.

Kim, M., Allen, B., Korhonen, E.A., Nitschke, M., Yang, H.W., Baluk, P., Saharinen, P., Alitalo, K., Daly, C., Thurston, G.*, et al.* (2016). Opposing actions of angiopoietin-2 on Tie2 signaling and FOXO1 activation. J Clin Invest *126*, 3511-3525.

Kim, M.P., Park, S.I., Kopetz, S., and Gallick, G.E. (2009). Src family kinases as mediators of endothelial permeability: effects on inflammation and metastasis. Cell Tissue Res *335*, 249-259.

Klomp, J.E., Shaaya, M., Matsche, J., Rebiai, R., Aaron, J.S., Collins, K.B., Huyot, V., Gonzalez, A.M., Muller, W.A., Chew, T.L.*, et al.* (2019). Time-Variant SRC Kinase Activation Determines Endothelial Permeability Response. Cell Chem Biol *26*, 1081-1094 e1086.

Koch, S., Tugues, S., Li, X., Gualandi, L., and Claesson-Welsh, L. (2011). Signal transduction by vascular endothelial growth factor receptors. Biochem J *437*, 169-183.

Korhonen, E.A., Lampinen, A., Giri, H., Anisimov, A., Kim, M., Allen, B., Fang, S., D'Amico, G., Sipila, T.J., Lohela, M.*, et al.* (2016). Tie1 controls angiopoietin function in vascular remodeling and inflammation. J Clin Invest *126*, 3495-3510.

Kumar, A., Shutova, M.S., Tanaka, K., Iwamoto, D.V., Calderwood, D.A., Svitkina, T.M., and Schwartz, M.A. (2019). Filamin A mediates isotropic distribution of applied force across the actin network. J Cell Biol *218*, 2481-2491.

Kumpers, P., Gueler, F., David, S., Slyke, P.V., Dumont, D.J., Park, J.K., Bockmeyer, C.L., Parikh, S.M., Pavenstadt, H., Haller, H.*, et al.* (2011). The synthetic tie2 agonist peptide vasculotide protects against vascular leakage and reduces mortality in murine abdominal sepsis. Crit Care *15*, R261.

Kumpers, P., Hafer, C., David, S., Hecker, H., Lukasz, A., Fliser, D., Haller, H., Kielstein, J.T., and Faulhaber-Walter, R. (2010). Angiopoietin-2 in patients requiring renal replacement therapy in the ICU: relation to acute kidney injury, multiple organ dysfunction syndrome and outcome. Intensive Care Med *36*, 462-470.

Kumpers, P., Lukasz, A., David, S., Horn, R., Hafer, C., Faulhaber-Walter, R., Fliser, D., Haller, H., and Kielstein, J.T. (2008). Excess circulating angiopoietin-2 is a strong predictor of mortality in critically ill medical patients. Crit Care *12*, R147.

Kurniati, N.F., Jongman, R.M., vom Hagen, F., Spokes, K.C., Moser, J., Regan, E.R., Krenning, G., Moonen, J.R., Harmsen, M.C., Struys, M.M.*, et al.* (2013). The flow dependency of Tie2 expression in endotoxemia. Intensive Care Med *39*, 1262-1271.

Lambeng, N., Wallez, Y., Rampon, C., Cand, F., Christe, G., Gulino-Debrac, D., Vilgrain, I., and Huber, P. (2005). Vascular endothelial-cadherin tyrosine phosphorylation in angiogenic and quiescent adult tissues. Circulation Research *96*, 384-391.

Lampugnani, M.G., Dejana, E., and Giampietro, C. (2018). Vascular Endothelial (VE)-Cadherin, Endothelial Adherens Junctions, and Vascular Disease. Cold Spring Harb Perspect Biol *10*.

Le Dall, J., Ho-Tin-Noe, B., Louedec, L., Meilhac, O., Roncal, C., Carmeliet, P., Germain, S., Michel, J.B., and Houard, X. (2010). Immaturity of microvessels in haemorrhagic plaques is associated with proteolytic degradation of angiogenic factors. Cardiovasc Res *85*, 184-193.

Lee, H.S., Oh, S.J., Lee, K.H., Lee, Y.S., Ko, E., Kim, K.E., Kim, H.C., Kim, S., Song, P.H., Kim, Y.I.*, et al.* (2014a). Gln-362 of angiopoietin-2 mediates migration of tumor and endothelial cells through association with alpha5beta1 integrin. J Biol Chem *289*, 31330-31340.

Lee, J., Park, D.Y., Park, D.Y., Park, I., Chang, W., Nakaoka, Y., Komuro, I., Yoo, O.J., and Koh, G.Y. (2014b). Angiopoietin-1 suppresses choroidal neovascularization and vascular leakage. Invest Ophthalmol Vis Sci *55*, 2191-2199.

Lee, S., Chen, T.T., Barber, C.L., Jordan, M.C., Murdock, J., Desai, S., Ferrara, N., Nagy, A., Roos, K.P., and Iruela-Arispe, M.L. (2007). Autocrine VEGF signaling is required for vascular homeostasis. Cell *130*, 691-703.

Lee, S.J., Lee, C.K., Kang, S., Park, I., Kim, Y.H., Kim, S.K., Hong, S.P., Bae, H., He, Y., Kubota, Y.*, et al.* (2018). Angiopoietin-2 exacerbates cardiac hypoxia and inflammation after myocardial infarction. J Clin Invest *128*, 5018-5033.

Lefort, C.T., Rossaint, J., Moser, M., Petrich, B.G., Zarbock, A., Monkley, S.J., Critchley, D.R., Ginsberg, M.H., Fassler, R., and Ley, K. (2012). Distinct roles for talin-1 and kindlin-3 in LFA-1 extension and affinity regulation. Blood *119*, 4275-4282.

Lei, L., Liu, D., Huang, Y., Jovin, I., Shai, S.Y., Kyriakides, T., Ross, R.S., and Giordano, F.J. (2008). Endothelial expression of beta1 integrin is required for embryonic vascular patterning and postnatal vascular remodeling. Mol Cell Biol *28*, 794-802.

Leligdowicz, A., Richard-Greenblatt, M., Wright, J., Crowley, V.M., and Kain, K.C. (2018). Endothelial Activation: The Ang/Tie Axis in Sepsis. Front Immunol *9*, 838.

Leppanen, V.M., Saharinen, P., and Alitalo, K. (2017). Structural basis of Tie2 activation and Tie2/Tie1 heterodimerization. Proc Natl Acad Sci U S A *114*, 4376-4381.

Letsiou, E., Sammani, S., Zhang, W., Zhou, T., Quijada, H., Moreno-Vinasco, L., Dudek, S.M., and Garcia, J.G. (2015). Pathologic mechanical stress and endotoxin exposure increases lung endothelial microparticle shedding. Am J Respir Cell Mol Biol *52*, 193-204.

Li, F., Sawada, J., and Komatsu, M. (2017). R-Ras-Akt axis induces endothelial lumenogenesis and regulates the patency of regenerating vasculature. Nat Commun *8*, 1720.
Li, J.J., Huang, Y.Q., Basch, R., and Karpatkin, S. (2001). Thrombin induces the release of angiopoietin-1 from platelets. Thromb Haemost *85*, 204-206.
Li, L., Qian, L., and Yu, Z.Q. (2015). Serum angiopoietin-2 is associated with angiopathy in type 2 diabetes mellitus. J Diabetes Complicat *29*, 568-571.
Li, L., Welser-Alves, J., van der Flier, A., Boroujerdi, A., Hynes, R.O., and Milner, R. (2012). An angiogenic role for the alpha5beta1 integrin in promoting endothelial cell proliferation during cerebral hypoxia. Exp Neurol *237*, 46-54.
Li, R., Zijlstra, J.G., Kamps, J.A., van Meurs, M., and Molema, G. (2014). Abrupt reflow enhances cytokine-induced proinflammatory activation of endothelial cells during simulated shock and resuscitation. Shock *42*, 356-364.
Li, X., Padhan, N., Sjostrom, E.O., Roche, F.P., Testini, C., Honkura, N., Sainz-Jaspeado, M., Gordon, E., Bentley, K., Philippides, A.*, et al.* (2016). VEGFR2 pY949 signalling regulates adherens junction integrity and metastatic spread. Nat Commun *7*, 11017.
Li, Z., Huang, H., Boland, P., Dominguez, M.G., Burfeind, P., Lai, K.M., Lin, H.C., Gale, N.W., Daly, C., Auerbach, W.*, et al.* (2009). Embryonic stem cell tumor model reveals role of vascular endothelial receptor tyrosine phosphatase in regulating Tie2 pathway in tumor angiogenesis. Proc Natl Acad Sci U S A *106*, 22399-22404.
Licht, T., and Keshet, E. (2013). Delineating multiple functions of VEGF-A in the adult brain. Cell Mol Life Sci *70*, 1727-1737.
Linder, S., and Wiesner, C. (2016). Feel the force: Podosomes in mechanosensing. Exp Cell Res *343*, 67-72.
Lo, S.H. (2017). Tensins. Current Biology *27*, R331-R332.
Lo, S.H., Yu, Q.C., Degenstein, L., Chen, L.B., and Fuchs, E. (1997). Progressive kidney degeneration in mice lacking tensin. J Cell Biol *136*, 1349-1361.
Lock, J.G., Baschieri, F., Jones, M.C., Humphries, J.D., Montagnac, G., Stromblad, S., and Humphries, M.J. (2019). Clathrin-containing adhesion complexes. J Cell Biol *218*, 2086-2095.
Lohela, M., Bry, M., Tammela, T., and Alitalo, K. (2009). VEGFs and receptors involved in angiogenesis versus lymphangiogenesis. Curr Opin Cell Biol *21*, 154-165.
Lomas-Neira, J.L., Heffernan, D.S., Ayala, A., and Monaghan, S.F. (2016). Blockade of Endothelial Growth Factor, Angiopoietin-2, Reduces Indices of Ards and Mortality in Mice Resulting from the Dual-Insults of Hemorrhagic Shock and Sepsis. Shock *45*, 157-165.
Lyle, A.N., and Taylor, W.R. (2019). The pathophysiological basis of vascular disease. Lab Invest *99*, 284-289.
Madisen, L., Zwingman, T.A., Sunkin, S.M., Oh, S.W., Zariwala, H.A., Gu, H., Ng, L.L., Palmiter, R.D., Hawrylycz, M.J., Jones, A.R.*, et al.* (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat Neurosci *13*, 133-140.
Mahabeleshwar, G.H., Feng, W., Phillips, D.R., and Byzova, T.V. (2006). Integrin signaling is critical for pathological angiogenesis. J Exp Med *203*, 2495-2507.
Maier, A.K., Kociok, N., Zahn, G., Vossmeyer, D., Stragies, R., Muether, P.S., and Joussen, A.M. (2007). Modulation of hypoxia-induced neovascularization by JSM6427, an integrin alpha5beta1 inhibiting molecule. Curr Eye Res *32*, 801-812.
Maisonpierre, P.C., Suri, C., Jones, P.F., Bartunkova, S., Wiegand, S.J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T.H., Papadopoulos, N.*, et al.* (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science *277*, 55-60.
Majmundar, A.J., Wong, W.J., and Simon, M.C. (2010). Hypoxia-inducible factors and the response to hypoxic stress. Mol Cell *40*, 294-309.
Malan, D., Wenzel, D., Schmidt, A., Geisen, C., Raible, A., Bolck, B., Fleischmann, B.K., and Bloch, W. (2010). Endothelial beta1 integrins regulate sprouting and network formation during vascular development. Development *137*, 993-1002.
Mandriota, S.J., and Pepper, M.S. (1998). Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia. Circ Res *83*, 852-859.
Mankhambo, L.A., Banda, D.L., Group, I.P.D.S., Jeffers, G., White, S.A., Balmer, P., Nkhoma, S., Phiri, H., Molyneux, E.M., Hart, C.A.*, et al.* (2010). The role of angiogenic factors in predicting clinical outcome in severe bacterial infection in Malawian children. Crit Care *14*, R91.
Marchand, M., Monnot, C., Muller, L., and Germain, S. (2019). Extracellular matrix scaffolding in angiogenesis and capillary homeostasis. Semin Cell Dev Biol *89*, 147-156.
Marshall, J.C. (2014). Why have clinical trials in sepsis failed? Trends Mol Med *20*, 195-203.
Martin, J.D., Seano, G., and Jain, R.K. (2019). Normalizing Function of Tumor Vessels: Progress, Opportunities, and Challenges. Annu Rev Physiol *81*, 505-534.
Martinez-Corral, I., Ulvmar, M.H., Stanczuk, L., Tatin, F., Kizhatil, K., John, S.W., Alitalo, K., Ortega, S., and Makinen, T. (2015). Nonvenous origin of dermal lymphatic vasculature. Circ Res *116*, 1649-1654.

Matsumoto, H., Ogura, H., Shimizu, K., Ikeda, M., Hirose, T., Matsuura, H., Kang, S., Takahashi, K., Tanaka, T., and Shimazu, T. (2018). The clinical importance of a cytokine network in the acute phase of sepsis. Sci Rep *8*, 13995.

Mazurek, R., Dave, J.M., Chandran, R.R., Misra, A., Sheikh, A.Q., and Greif, D.M. (2017). Vascular Cells in Blood Vessel Wall Development and Disease. Adv Pharmacol *78*, 323-350.

McCarty, J.H., Lacy-Hulbert, A., Charest, A., Bronson, R.T., Crowley, D., Housman, D., Savill, J., Roes, J., and Hynes, R.O. (2005). Selective ablation of alphav integrins in the central nervous system leads to cerebral hemorrhage, seizures, axonal degeneration and premature death. Development *132*, 165-176.

McCleverty, C.J., Lin, D.C., and Liddington, R.C. (2007). Structure of the PTB domain of tensin1 and a model for its recruitment to fibrillar adhesions. Protein Sci *16*, 1223-1229.

Merx, M.W., and Weber, C. (2007). Sepsis and the heart. Circulation *116*, 793-802.

Minami, T., and Aird, W.C. (2005). Endothelial cell gene regulation. Trends Cardiovasc Med *15*, 174-184.

Minami, T., Jiang, S., Schadler, K., Suehiro, J., Osawa, T., Oike, Y., Miura, M., Naito, M., Kodama, T., and Ryeom, S. (2013). The calcineurin-NFAT-angiopoietin-2 signaling axis in lung endothelium is critical for the establishment of lung metastases. Cell Rep *4*, 709-723.

Mirando, A.C., Shen, J., Silva, R.L.E., Chu, Z., Sass, N.C., Lorenc, V.E., Green, J.J., Campochiaro, P.A., Popel, A.S., and Pandey, N.B. (2019). A collagen IV-derived peptide disrupts alpha5beta1 integrin and potentiates Ang2/Tie2 signaling. JCI Insight *4*.

Miyasaka, M., and Tanaka, T. (2004). Lymphocyte trafficking across high endothelial venules: dogmas and enigmas. Nat Rev Immunol *4*, 360-370.

Monahan-Earley, R., Dvorak, A.M., and Aird, W.C. (2013). Evolutionary origins of the blood vascular system and endothelium. J Thromb Haemost *11 Suppl 1*, 46-66.

Monkley, S.J., Kostourou, V., Spence, L., Petrich, B., Coleman, S., Ginsberg, M.H., Pritchard, C.A., and Critchley, D.R. (2011). Endothelial cell talin1 is essential for embryonic angiogenesis. Dev Biol *349*, 494-502.

Moreno-Layseca, P., Icha, J., Hamidi, H., and Ivaska, J. (2019). Integrin trafficking in cells and tissues. Nat Cell Biol *21*, 122-132.

Moreno-Layseca, P., and Streuli, C.H. (2014). Signalling pathways linking integrins with cell cycle progression. Matrix Biol *34*, 144-153.

Morse, E.M., Brahme, N.N., and Calderwood, D.A. (2014). Integrin cytoplasmic tail interactions. Biochemistry *53*, 810-820.

Mould, A.P., Akiyama, S.K., and Humphries, M.J. (1996). The inhibitory anti-beta1 integrin monoclonal antibody 13 recognizes an epitope that is attenuated by ligand occupancy. Evidence for allosteric inhibition of integrin function. J Biol Chem *271*, 20365-20374.

Murphy, P.A., Begum, S., and Hynes, R.O. (2015). Tumor angiogenesis in the absence of fibronectin or its cognate integrin receptors. PLoS One *10*, e0120872.

Nikolopoulos, S.N., Blaikie, P., Yoshioka, T., Guo, W., and Giancotti, F.G. (2004). Integrin beta4 signaling promotes tumor angiogenesis. Cancer Cell *6*, 471-483.

Orfanos, S.E., Kotanidou, A., Glynos, C., Athanasiou, C., Tsigkos, S., Dimopoulou, I., Sotiropoulou, C., Zakynthinos, S., Armaganidis, A., Papapetropoulos, A.*, et al.* (2007). Angiopoietin-2 is increased in severe sepsis: correlation with inflammatory mediators. Crit Care Med *35*, 199-206.

Orsenigo, F., Giampietro, C., Ferrari, A., Corada, M., Galaup, A., Sigismund, S., Ristagno, G., Maddaluno, L., Koh, G.Y., Franco, D.*, et al.* (2012). Phosphorylation of VE-cadherin is modulated by haemodynamic forces and contributes to the regulation of vascular permeability in vivo. Nat Commun *3*, 1208.

Ory, D.S., Neugeboren, B.A., and Mulligan, R.C. (1996). A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. P Natl Acad Sci USA *93*, 11400-11406.

Page, A.V., Kotb, M., McGeer, A., Low, D.E., Kain, K.C., and Liles, W.C. (2011). Systemic dysregulation of angiopoietin-1/2 in streptococcal toxic shock syndrome. Clin Infect Dis *52*, e157-161.

Pang, D., Wang, L., Dong, J., Lai, X., Huang, Q., Milner, R., and Li, L. (2018). Integrin alpha5beta1-Ang1/Tie2 receptor cross-talk regulates brain endothelial cell responses following cerebral ischemia. Exp Mol Med *50*, 117.

Pankov, R., Cukierman, E., Katz, B.Z., Matsumoto, K., Lin, D.C., Lin, S., Hahn, C., and Yamada, K.M. (2000). Integrin dynamics and matrix assembly: tensin-dependent translocation of alpha(5)beta(1) integrins promotes early fibronectin fibrillogenesis. J Cell Biol *148*, 1075-1090.

Parikh, S.M., Mammoto, T., Schultz, A., Yuan, H.T., Christiani, D., Karumanchi, S.A., and Sukhatme, V.P. (2006). Excess circulating angiopoietin-2 may contribute to pulmonary vascular leak in sepsis in humans. PLoS Med *3*, e46.

Park, D.Y., Lee, J., Kim, J., Kim, K., Hong, S., Han, S., Kubota, Y., Augustin, H.G., Ding, L., Kim, J.W.*, et al.* (2017). Plastic roles of pericytes in the blood-retinal barrier. Nat Commun *8*, 15296.

Park, J.H., Park, K.J., Kim, Y.S., Sheen, S.S., Lee, K.S., Lee, H.N., Oh, Y.J., and Hwang, S.C. (2007). Serum angiopoietin-2 as a clinical marker for lung cancer. Chest *132*, 200-206.

Park, J.S., Kim, I.K., Han, S., Park, I., Kim, C., Bae, J., Oh, S.J., Lee, S., Kim, J.H., Woo, D.C., *et al.* (2016). Normalization of Tumor Vessels by Tie2 Activation and Ang2 Inhibition Enhances Drug Delivery and Produces a Favorable Tumor Microenvironment. Cancer Cell *30*, 953-967.

Partanen, J., Armstrong, E., Makela, T.P., Korhonen, J., Sandberg, M., Renkonen, R., Knuutila, S., Huebner, K., and Alitalo, K. (1992). A novel endothelial cell surface receptor tyrosine kinase with extracellular epidermal growth factor homology domains. Mol Cell Biol *12*, 1698-1707.

Peuhu, E., Kaukonen, R., Lerche, M., Saari, M., Guzman, C., Rantakari, P., De Franceschi, N., Warri, A., Georgiadou, M., Jacquemet, G., *et al.* (2017). SHARPIN regulates collagen architecture and ductal outgrowth in the developing mouse mammary gland. EMBO J *36*, 165-182.

Phillips, D.R., Prasad, K.S., Manganello, J., Bao, M., and Nannizzi-Alaimo, L. (2001). Integrin tyrosine phosphorylation in platelet signaling. Curr Opin Cell Biol *13*, 546-554.

Phillipson, M., and Kubes, P. (2011). The neutrophil in vascular inflammation. Nat Med *17*, 1381-1390.

Pober, J.S., and Sessa, W.C. (2007). Evolving functions of endothelial cells in inflammation. Nat Rev Immunol *7*, 803-815.

Post, S., Peeters, W., Busser, E., Lamers, D., Sluijter, J.P., Goumans, M.J., de Weger, R.A., Moll, F.L., Doevendans, P.A., Pasterkamp, G., *et al.* (2008). Balance between angiopoietin-1 and angiopoietin-2 is in favor of angiopoietin-2 in atherosclerotic plaques with high microvessel density. J Vasc Res *45*, 244-250.

Potente, M., Urbich, C., Sasaki, K., Hofmann, W.K., Heeschen, C., Aicher, A., Kollipara, R., DePinho, R.A., Zeiher, A.M., and Dimmeler, S. (2005). Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. J Clin Invest *115*, 2382-2392.

Potter, M.D., Barbero, S., and Cheresh, D.A. (2005). Tyrosine phosphorylation of VE-cadherin prevents binding of p120- and beta-catenin and maintains the cellular mesenchymal state. J Biol Chem *280*, 31906-31912.

Pozzi, A., Moberg, P.E., Miles, L.A., Wagner, S., Soloway, P., and Gardner, H.A. (2000). Elevated matrix metalloprotease and angiostatin levels in integrin alpha 1 knockout mice cause reduced tumor vascularization. Proc Natl Acad Sci U S A *97*, 2202-2207.

Prasain, N., and Stevens, T. (2009). The actin cytoskeleton in endothelial cell phenotypes. Microvasc Res *77*, 53-63.

Priestley, G.V., Ulyanova, T., and Papayannopoulou, T. (2007). Sustained alterations in biodistribution of stem/progenitor cells in Tie2Cre+ alpha4(f/f) mice are hematopoietic cell autonomous. Blood *109*, 109-111.

Proctor, J.M., Zang, K., Wang, D., Wang, R., and Reichardt, L.F. (2005). Vascular development of the brain requires beta8 integrin expression in the neuroepithelium. J Neurosci *25*, 9940-9948.

Pulous, F.E., Grimsley-Myers, C.M., Kansal, S., Kowalczyk, A.P., and Petrich, B.G. (2019). Talin-Dependent Integrin Activation Regulates VE-Cadherin Localization and Endothelial Cell Barrier Function. Circ Res *124*, 891-903.

Puri, M.C., Rossant, J., Alitalo, K., Bernstein, A., and Partanen, J. (1995). The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. EMBO J *14*, 5884-5891.

Qu, X., Tompkins, K., Batts, L.E., Puri, M., and Baldwin, H.S. (2010). Abnormal embryonic lymphatic vessel development in Tie1 hypomorphic mice. Development *137*, 1285-1295.

Raab-Westphal, S., Marshall, J.F., and Goodman, S.L. (2017). Integrins as Therapeutic Targets: Successes and Cancers. Cancers (Basel) *9*.

Raghavan, S., Bauer, C., Mundschau, G., Li, Q., and Fuchs, E. (2000). Conditional ablation of beta1 integrin in skin. Severe defects in epidermal proliferation, basement membrane formation, and hair follicle invagination. J Cell Biol *150*, 1149-1160.

Rahimi, N. (2017). Defenders and Challengers of Endothelial Barrier Function. Front Immunol *8*, 1847.

Rasul, S., Reiter, M.H., Ilhan, A., Lampichler, K., Wagner, L., and Kautzky-Willer, A. (2011). Circulating angiopoietin-2 and soluble Tie-2 in type 2 diabetes mellitus: a cross-sectional study. Cardiovasc Diabetol *10*.

Rathnakumar, K., Savant, S., Giri, H., Ghosh, A., Fisslthaler, B., Fleming, I., Ram, U., Bera, A.K., Augustin, H.G., and Dixit, M. (2016). Angiopoietin-2 mediates thrombin-induced monocyte adhesion and endothelial permeability. J Thromb Haemost *14*, 1655-1667.

Reiss, Y., Droste, J., Heil, M., Tribulova, S., Schmidt, M.H.H., Schaper, W., Dumont, D.J., and Plate, K.H. (2007). Angiopoietin-2 impairs revascularization after limb ischemia. Circ Res *101*, 88-96.

Rello, J., Valenzuela-Sanchez, F., Ruiz-Rodriguez, M., and Moyano, S. (2017). Sepsis: A Review of Advances in Management. Adv Ther *34*, 2393-2411.

Reynolds, A.R., Reynolds, L.E., Nagel, T.E., Lively, J.C., Robinson, S.D., Hicklin, D.J., Bodary, S.C., and Hodivala-Dilke, K.M. (2004). Elevated Flk1 (vascular endothelial growth factor receptor 2) signaling mediates enhanced angiogenesis in beta3-integrin-deficient mice. Cancer Res *64*, 8643-8650.

Reynolds, L.E., D'Amico, G., Lechertier, T., Papachristodoulou, A., Munoz-Felix, J.M., De Arcangelis, A., Baker, M., Serrels, B., and Hodivala-Dilke, K.M. (2017). Dual role of pericyte alpha6beta1-integrin in tumour blood vessels. J Cell Sci *130*, 1583-1595.

Reynolds, L.E., Wyder, L., Lively, J.C., Taverna, D., Robinson, S.D., Huang, X., Sheppard, D., Hynes, R.O., and Hodivala-Dilke, K.M. (2002). Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. Nat Med *8*, 27-34.

Ricciuto, D.R., dos Santos, C.C., Hawkes, M., Toltl, L.J., Conroy, A.L., Rajwans, N., Lafferty, E.I., Cook, D.J., Fox-Robichaud, A., Kahnamoui, K., *et al.* (2011). Angiopoietin-1 and angiopoietin-2 as clinically informative prognostic biomarkers of morbidity and mortality in severe sepsis. Crit Care Med *39*, 702-710.

Rittirsch, D., Flierl, M.A., and Ward, P.A. (2008). Harmful molecular mechanisms in sepsis. Nat Rev Immunol *8*, 776-787.

Roberts, J., de Hoog, L., and Bix, G.J. (2017). Mice deficient in endothelial alpha5 integrin are profoundly resistant to experimental ischemic stroke. J Cereb Blood Flow Metab *37*, 85-96.

Roberts, W.G., and Palade, G.E. (1995). Increased Microvascular Permeability and Endothelial Fenestration Induced by Vascular Endothelial Growth-Factor. Journal of Cell Science *108*, 2369-2379.

Robilliard, L.D., Kho, D.T., Johnson, R.H., Anchan, A., O'Carroll, S.J., and Graham, E.S. (2018). The Importance of Multifrequency Impedance Sensing of Endothelial Barrier Formation Using ECIS Technology for the Generation of a Strong and Durable Paracellular Barrier. Biosensors (Basel) *8*.

Saharinen, P., Eklund, L., and Alitalo, K. (2017a). Therapeutic targeting of the angiopoietin-TIE pathway. Nat Rev Drug Discov *16*, 635-661.

Saharinen, P., Eklund, L., Miettinen, J., Wirkkala, R., Anisimov, A., Winderlich, M., Nottebaum, A., Vestweber, D., Deutsch, U., Koh, G.Y., *et al.* (2008). Angiopoietins assemble distinct Tie2 signalling complexes in endothelial cell-cell and cell-matrix contacts. Nat Cell Biol *10*, 527-537.

Saharinen, P., Kerkela, K., Ekman, N., Marron, M., Brindle, N., Lee, G.M., Augustin, H., Koh, G.Y., and Alitalo, K. (2005). Multiple angiopoietin recombinant proteins activate the Tie1 receptor tyrosine kinase and promote its interaction with Tie2. J Cell Biol *169*, 239-243.

Saharinen, P., Leppanen, V.M., and Alitalo, K. (2017b). SnapShot: Angiopoietins and Their Functions. Cell *171*, 724-724 e721.

Salomao, R., Martins, P.S., Brunialti, M.K., Fernandes Mda, L., Martos, L.S., Mendes, M.E., Gomes, N.E., and Rigato, O. (2008). TLR signaling pathway in patients with sepsis. Shock *30 Suppl 1*, 73-77.

Sato, T.N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W., and Qin, Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature *376*, 70-74.

Savant, S., La Porta, S., Budnik, A., Busch, K., Hu, J., Tisch, N., Korn, C., Valls, A.F., Benest, A.V., Terhardt, D., *et al.* (2015). The Orphan Receptor Tie1 Controls Angiogenesis and Vascular Remodeling by Differentially Regulating Tie2 in Tip and Stalk Cells. Cell Rep *12*, 1761-1773.

Scheuer, W., Thomas, M., Hanke, P., Sam, J., Osl, F., Weininger, D., Baehner, M., Seeber, S., Kettenberger, H., Schanzer, J., *et al.* (2016). Anti-tumoral, anti-angiogenic and anti-metastatic efficacy of a tetravalent bispecific antibody (TAvi6) targeting VEGF-A and angiopoietin-2. MAbs *8*, 562-573.

Schlegel, N., and Waschke, J. (2009). Impaired cAMP and Rac 1 signaling contribute to TNF-alpha-induced endothelial barrier breakdown in microvascular endothelium. Microcirculation *16*, 521-533.

Schmidt, T.T., Tauseef, M., Yue, L., Bonini, M.G., Gothert, J., Shen, T.L., Guan, J.L., Predescu, S., Sadikot, R., and Mehta, D. (2013). Conditional deletion of FAK in mice endothelium disrupts lung vascular barrier function due to destabilization of RhoA and Rac1 activities. Am J Physiol Lung Cell Mol Physiol *305*, L291-300.

Scholz, A., Harter, P.N., Cremer, S., Yalcin, B.H., Gurnik, S., Yamaji, M., Di Tacchio, M., Sommer, K., Baumgarten, P., Bahr, O., *et al.* (2016). Endothelial cell-derived angiopoietin-2 is a therapeutic target in treatment-naive and bevacizumab-resistant glioblastoma. EMBO Mol Med *8*, 39-57.

Scholz, A., Lang, V., Henschler, R., Czabanka, M., Vajkoczy, P., Chavakis, E., Drynski, J., Harter, P.N., Mittelbronn, M., Dumont, D.J., *et al.* (2011). Angiopoietin-2 promotes myeloid cell infiltration in a beta(2)-integrin-dependent manner. Blood *118*, 5050-5059.

Schulte, D., Kuppers, V., Dartsch, N., Broermann, A., Li, H., Zarbock, A., Kamenyeva, O., Kiefer, F., Khandoga, A., Massberg, S., *et al.* (2011). Stabilizing the VE-cadherin-catenin complex blocks leukocyte extravasation and vascular permeability. EMBO J *30*, 4157-4170.

Schulz, P., Fischer, C., Detjen, K.M., Rieke, S., Hilfenhaus, G., von Marschall, Z., Bohmig, M., Koch, I., Kehrberger, J., Hauff, P., *et al.* (2011). Angiopoietin-2 drives lymphatic metastasis of pancreatic cancer. FASEB J *25*, 3325-3335.

Schwager, S., and Detmar, M. (2019). Inflammation and Lymphatic Function. Front Immunol *10*, 308.

Schwartz, B.G., Economides, C., Mayeda, G.S., Burstein, S., and Kloner, R.A. (2010). The endothelial cell in health and disease: its function, dysfunction, measurement and therapy. Int J Impot Res *22*, 77-90.

Seano, G., and Primo, L. (2015). Podosomes and invadopodia: tools to breach vascular basement membrane. Cell Cycle *14*, 1370-1374.

Seegar, T.C., Eller, B., Tzvetkova-Robev, D., Kolev, M.V., Henderson, S.C., Nikolov, D.B., and Barton, W.A. (2010). Tie1-Tie2 interactions mediate functional differences between angiopoietin ligands. Mol Cell *37*, 643-655.

Senger, D.R., Claffey, K.P., Benes, J.E., Perruzzi, C.A., Sergiou, A.P., and Detmar, M. (1997). Angiogenesis promoted by vascular endothelial growth factor: regulation through alpha1beta1 and alpha2beta1 integrins. Proc Natl Acad Sci U S A *94*, 13612-13617.

Senger, D.R., Galli, S.J., Dvorak, A.M., Perruzzi, C.A., Harvey, V.S., and Dvorak, H.F. (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science *219*, 983-985.

Senger, D.R., Perruzzi, C.A., Streit, M., Koteliansky, V.E., de Fougerolles, A.R., and Detmar, M. (2002). The alpha(1)beta(1) and alpha(2)beta(1) integrins provide critical support for vascular endothelial growth factor signaling, endothelial cell migration, and tumor angiogenesis. Am J Pathol *160*, 195-204.

Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X.F., Breitman, M.L., and Schuh, A.C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature *376*, 62-66.

Shen, B., Shang, Z., Wang, B., Zhang, L., Zhou, F., Li, T., Chu, M., Jiang, H., Wang, Y., Qiao, T.*, et al.* (2014). Genetic dissection of tie pathway in mouse lymphatic maturation and valve development. Arterioscler Thromb Vasc Biol *34*, 1221-1230.

Shih, Y.P., Sun, P., Wang, A., and Lo, S.H. (2015). Tensin1 positively regulates RhoA activity through its interaction with DLC1. Biochim Biophys Acta *1853*, 3258-3265.

Siner, J.M., Bhandari, V., Engle, K.M., Elias, J.A., and Siegel, M.D. (2009). Elevated serum angiopoietin 2 levels are associated with increased mortality in sepsis. Shock *31*, 348-353.

Sorokin, L. (2010). The impact of the extracellular matrix on inflammation. Nat Rev Immunol *10*, 712-723.

Souma, T., Thomson, B.R., Heinen, S., Carota, I.A., Yamaguchi, S., Onay, T., Liu, P., Ghosh, A.K., Li, C., Eremina, V.*, et al.* (2018). Context-dependent functions of angiopoietin 2 are determined by the endothelial phosphatase VEPTP. Proc Natl Acad Sci U S A *115*, 1298-1303.

Spiess, M., Hernandez-Varas, P., Oddone, A., Olofsson, H., Blom, H., Waithe, D., Lock, J.G., Lakadamyali, M., and Stromblad, S. (2018). Active and inactive beta1 integrins segregate into distinct nanoclusters in focal adhesions. J Cell Biol *217*, 1929-1940.

Statz, S., Sabal, G., Walborn, A., Williams, M., Hoppensteadt, D., Mosier, M., Rondina, M., and Fareed, J. (2018). Angiopoietin 2 Levels in the Risk Stratification and Mortality Outcome Prediction of Sepsis-Associated Coagulopathy. Clin Appl Thromb Hemost *24*, 1223-1233.

Stephens, L.E., Sutherland, A.E., Klimanskaya, I.V., Andrieux, A., Meneses, J., Pedersen, R.A., and Damsky, C.H. (1995). Deletion of beta 1 integrins in mice results in inner cell mass failure and peri-implantation lethality. Genes Dev *9*, 1883-1895.

Stephens, R.S., Johnston, L., Servinsky, L., Kim, B.S., and Damarla, M. (2015). The tyrosine kinase inhibitor imatinib prevents lung injury and death after intravenous LPS in mice. Physiol Rep *3*.

Stewart, P.L., and Nemerow, G.R. (2007). Cell integrins: commonly used receptors for diverse viral pathogens. Trends Microbiol *15*, 500-507.

Stupack, D.G., and Cheresh, D.A. (2002). ECM remodeling regulates angiogenesis: endothelial integrins look for new ligands. Sci STKE *2002*, pe7.

Su, G., Atakilit, A., Li, J.T., Wu, N., Bhattacharya, M., Zhu, J., Shieh, J.E., Li, E., Chen, R., Sun, S.*, et al.* (2012). Absence of integrin alphavbeta3 enhances vascular leak in mice by inhibiting endothelial cortical actin formation. Am J Respir Crit Care Med *185*, 58-66.

Su, G., Atakilit, A., Li, J.T., Wu, N., Luong, J., Chen, R., Bhattacharya, M., and Sheppard, D. (2013). Effective treatment of mouse sepsis with an inhibitory antibody targeting integrin alphavbeta5. Crit Care Med *41*, 546-553.

Su, G., Hodnett, M., Wu, N., Atakilit, A., Kosinski, C., Godzich, M., Huang, X.Z., Kim, J.K., Frank, J.A., Matthay, M.A.*, et al.* (2007). Integrin alphavbeta5 regulates lung vascular permeability and pulmonary endothelial barrier function. Am J Respir Cell Mol Biol *36*, 377-386.

Su, Y., Xia, W., Li, J., Walz, T., Humphries, M.J., Vestweber, D., Cabanas, C., Lu, C.F., and Springer, T.A. (2016). Relating conformation to function in integrin alpha(5)beta(1). P Natl Acad Sci USA *113*, E3872-E3881.

Sui, A., Zhong, Y., Demetriades, A.M., Shen, J., Su, T., Yao, Y., Gao, Y., Zhu, Y., Shen, X., and Xie, B. (2018). ATN-161 as an Integrin alpha5beta1 Antagonist Depresses Ocular Neovascularization by Promoting New Vascular Endothelial Cell Apoptosis. Med Sci Monit *24*, 5860-5873.

Sun, X., Fu, Y., Gu, M., Zhang, L., Li, D., Li, H., Chien, S., Shyy, J.Y., and Zhu, Y. (2016a). Activation of integrin alpha5 mediated by flow requires its translocation to membrane lipid rafts in vascular endothelial cells. Proc Natl Acad Sci U S A *113*, 769-774.

Sun, Z., Guo, S.S., and Fassler, R. (2016b). Integrin-mediated mechanotransduction. J Cell Biol *215*, 445-456.

Sun, Z., Li, X., Massena, S., Kutschera, S., Padhan, N., Gualandi, L., Sundvold-Gjerstad, V., Gustafsson, K., Choy, W.W., Zang, G.*, et al.* (2012). VEGFR2 induces c-Src signaling and vascular permeability in vivo via the adaptor protein TSAd. J Exp Med *209*, 1363-1377.

Suri, C., Jones, P.F., Patan, S., Bartunkova, S., Maisonpierre, P.C., Davis, S., Sato, T.N., and Yancopoulos, G.D. (1996). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell *87*, 1171-1180.

Swift, M.R., and Weinstein, B.M. (2009). Arterial-venous specification during development. Circ Res *104*, 576-588.

Tabruyn, S.P., Colton, K., Morisada, T., Fuxe, J., Wiegand, S.J., Thurston, G., Coyle, A.J., Connor, J., and McDonald, D.M. (2010). Angiopoietin-2-driven vascular remodeling in airway inflammation. Am J Pathol *177*, 3233-3243.

Taddei, A., Giampietro, C., Conti, A., Orsenigo, F., Breviario, F., Pirazzoli, V., Potente, M., Daly, C., Dimmeler, S., and Dejana, E. (2008). Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5. Nat Cell Biol *10*, 923-934.
Tanjore, H., Zeisberg, E.M., Gerami-Naini, B., and Kalluri, R. (2008). Beta1 integrin expression on endothelial cells is required for angiogenesis but not for vasculogenesis. Dev Dyn *237*, 75-82.
Theelen, T.L., Lappalainen, J.P., Sluimer, J.C., Gurzeler, E., Cleutjens, J.P., Gijbels, M.J., Biessen, E.A., Daemen, M.J., Alitalo, K., and Yla-Herttuala, S. (2015). Angiopoietin-2 blocking antibodies reduce early atherosclerotic plaque development in mice. Atherosclerosis *241*, 297-304.
Thomas, M., Felcht, M., Kruse, K., Kretschmer, S., Deppermann, C., Biesdorf, A., Rohr, K., Benest, A.V., Fiedler, U., and Augustin, H.G. (2010). Angiopoietin-2 stimulation of endothelial cells induces alphavbeta3 integrin internalization and degradation. J Biol Chem *285*, 23842-23849.
Thomsen, M.S., Routhe, L.J., and Moos, T. (2017). The vascular basement membrane in the healthy and pathological brain. J Cereb Blood Flow Metab *37*, 3300-3317.
Thomson, B.R., Heinen, S., Jeansson, M., Ghosh, A.K., Fatima, A., Sung, H.K., Onay, T., Chen, H., Yamaguchi, S., Economides, A.N., *et al.* (2014). A lymphatic defect causes ocular hypertension and glaucoma in mice. J Clin Invest *124*, 4320-4324.
Thurston, G., and Daly, C. (2012). The complex role of angiopoietin-2 in the angiopoietin-tie signaling pathway. Cold Spring Harb Perspect Med *2*, a006550.
Thurston, G., Rudge, J.S., Ioffe, E., Zhou, H., Ross, L., Croll, S.D., Glazer, N., Holash, J., McDonald, D.M., and Yancopoulos, G.D. (2000). Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat Med *6*, 460-463.
Tornavaca, O., Chia, M., Dufton, N., Almagro, L.O., Conway, D.E., Randi, A.M., Schwartz, M.A., Matter, K., and Balda, M.S. (2015). ZO-1 controls endothelial adherens junctions, cell-cell tension, angiogenesis, and barrier formation. J Cell Biol *208*, 821-838.
Tsukita, S., Tanaka, H., and Tamura, A. (2019). The Claudins: From Tight Junctions to Biological Systems. Trends Biochem Sci *44*, 141-152.
Turlo, K.A., Noel, O.D., Vora, R., LaRussa, M., Fassler, R., Hall-Glenn, F., and Iruela-Arispe, M.L. (2012). An essential requirement for beta1 integrin in the assembly of extracellular matrix proteins within the vascular wall. Dev Biol *365*, 23-35.
Turner, C.J., Badu-Nkansah, K., Crowley, D., van der Flier, A., and Hynes, R.O. (2014). Integrin-alpha5beta1 is not required for mural cell functions during development of blood vessels but is required for lymphatic-blood vessel separation and lymphovenous valve formation. Dev Biol *392*, 381-392.
Turowski, P., Martinelli, R., Crawford, R., Wateridge, D., Papageorgiou, A.P., Lampugnani, M.G., Gamp, A.C., Vestweber, D., Adamson, P., Dejana, E., *et al.* (2008). Phosphorylation of vascular endothelial cadherin controls lymphocyte emigration. J Cell Sci *121*, 29-37.
Tzima, E., Irani-Tehrani, M., Kiosses, W.B., Dejana, E., Schultz, D.A., Engelhardt, B., Cao, G., DeLisser, H., and Schwartz, M.A. (2005). A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. Nature *437*, 426-431.
Uchimido, R., Schmidt, E.P., and Shapiro, N.I. (2019). The glycocalyx: a novel diagnostic and therapeutic target in sepsis. Crit Care *23*, 16.
Udan, R.S., Culver, J.C., and Dickinson, M.E. (2013). Understanding vascular development. Wiley Interdiscip Rev Dev Biol *2*, 327-346.
Umeda, N., Kachi, S., Akiyama, H., Zahn, G., Vossmeyer, D., Stragies, R., and Campochiaro, P.A. (2006). Suppression and regression of choroidal neovascularization by systemic administration of an alpha5beta1 integrin antagonist. Mol Pharmacol *69*, 1820-1828.
Vaahtomeri, K., Karaman, S., Makinen, T., and Alitalo, K. (2017). Lymphangiogenesis guidance by paracrine and pericellular factors. Genes Dev *31*, 1615-1634.
van der Flier, A., Badu-Nkansah, K., Whittaker, C.A., Crowley, D., Bronson, R.T., Lacy-Hulbert, A., and Hynes, R.O. (2010). Endothelial alpha5 and alphav integrins cooperate in remodeling of the vasculature during development. Development *137*, 2439-2449.
van der Heijden, M., Pickkers, P., van Nieuw Amerongen, G.P., van Hinsbergh, V.W., Bouw, M.P., van der Hoeven, J.G., and Groeneveld, A.B. (2009). Circulating angiopoietin-2 levels in the course of septic shock: relation with fluid balance, pulmonary dysfunction and mortality. Intensive Care Med *35*, 1567-1574.
van der Heijden, M., van Nieuw Amerongen, G.P., Koolwijk, P., van Hinsbergh, V.W., and Groeneveld, A.B. (2008). Angiopoietin-2, permeability oedema, occurrence and severity of ALI/ARDS in septic and non-septic critically ill patients. Thorax *63*, 903-909.
Vestweber, D. (2015). How leukocytes cross the vascular endothelium. Nat Rev Immunol *15*, 692-704.
Vittet, D., Buchou, T., Schweitzer, A., Dejana, E., and Huber, P. (1997). Targeted null-mutation in the vascular endothelial-cadherin gene impairs the organization of vascular-like structures in embryoid bodies. Proc Natl Acad Sci U S A *94*, 6273-6278.

Vlahakis, N.E., Young, B.A., Atakilit, A., Hawkridge, A.E., Issaka, R.B., Boudreau, N., and Sheppard, D. (2007). Integrin alpha9beta1 directly binds to vascular endothelial growth factor (VEGF)-A and contributes to VEGF-A-induced angiogenesis. J Biol Chem *282*, 15187-15196.

Vlahakis, N.E., Young, B.A., Atakilit, A., and Sheppard, D. (2005). The lymphangiogenic vascular endothelial growth factors VEGF-C and -D are ligands for the integrin alpha9beta1. J Biol Chem *280*, 4544-4552.

von Tell, D., Armulik, A., and Betsholtz, C. (2006). Pericytes and vascular stability. Exp Cell Res *312*, 623-629.

Waller, C.F. (2010). Imatinib mesylate. Recent Results Cancer Res *184*, 3-20.

Wallez, Y., Cand, F., Cruzalegui, F., Wernstedt, C., Souchelnytskyi, S., Vilgrain, I., and Huber, P. (2007). Src kinase phosphorylates vascular endothelial-cadherin in response to vascular endothelial growth factor: identification of tyrosine 685 as the unique target site. Oncogene *26*, 1067-1077.

Wallez, Y., and Huber, P. (2008). Endothelial adherens and tight junctions in vascular homeostasis, inflammation and angiogenesis. Biochim Biophys Acta *1778*, 794-809.

Wang, L., Zhang, X., Liu, X., Feng, G., Fu, Y., Milner, R., and Li, L. (2019a). Overexpression of alpha5beta1 integrin and angiopoietin-1 co-operatively promote blood-brain barrier integrity and angiogenesis following ischemic stroke. Exp Neurol *321*, 113042.

Wang, W., Lollis, E.M., Bordeleau, F., and Reinhart-King, C.A. (2019b). Matrix stiffness regulates vascular integrity through focal adhesion kinase activity. FASEB J *33*, 1199-1208.

Wang, W., Wang, F., Lu, F., Xu, S., Hu, W., Huang, J., Gu, Q., and Sun, X. (2011). The antiangiogenic effects of integrin alpha5beta1 inhibitor (ATN-161) in vitro and in vivo. Invest Ophthalmol Vis Sci *52*, 7213-7220.

Weis, S.M., and Cheresh, D.A. (2011). alphaV integrins in angiogenesis and cancer. Cold Spring Harb Perspect Med *1*, a006478.

Welser, J.V., Halder, S.K., Kant, R., Boroujerdi, A., and Milner, R. (2017). Endothelial alpha6beta4 integrin protects during experimental autoimmune encephalomyelitis-induced neuroinflammation by maintaining vascular integrity and tight junction protein expression. J Neuroinflammation *14*, 217.

Wentowski, C., Mewada, N., and Nielsen, N.D. (2019). Sepsis in 2018: a review. Anaest Intens Care M *20*, 6-13.

Wilhelm, K., Happel, K., Eelen, G., Schoors, S., Oellerich, M.F., Lim, R., Zimmermann, B., Aspalter, I.M., Franco, C.A., Boettger, T.*, et al.* (2016). FOXO1 couples metabolic activity and growth state in the vascular endothelium. Nature *529*, 216-220.

Winderlich, M., Keller, L., Cagna, G., Broermann, A., Kamenyeva, O., Kiefer, F., Deutsch, U., Nottebaum, A.F., and Vestweber, D. (2009). VE-PTP controls blood vessel development by balancing Tie-2 activity. J Cell Biol *185*, 657-671.

Winograd-Katz, S.E., Fassler, R., Geiger, B., and Legate, K.R. (2014). The integrin adhesome: from genes and proteins to human disease. Nat Rev Mol Cell Biol *15*, 273-288.

Witzenbichler, B., Westermann, D., Knueppel, S., Schultheiss, H.P., and Tschope, C. (2005). Protective role of angiopoietin-1 in endotoxic shock. Circulation *111*, 97-105.

Xanthis, I., Souilhol, C., Serbanovic-Canic, J., Roddie, H., Kalli, A.C., Fragiadaki, M., Wong, R., Shah, D.R., Askari, J.A., Canham, L.*, et al.* (2019). beta1 integrin is a sensor of blood flow direction. J Cell Sci *132*.

Yamamoto, H., Ehling, M., Kato, K., Kanai, K., van Lessen, M., Frye, M., Zeuschner, D., Nakayama, M., Vestweber, D., and Adams, R.H. (2015). Integrin beta1 controls VE-cadherin localization and blood vessel stability. Nat Commun *6*, 6429.

Yao, L.C., Baluk, P., Srinivasan, R.S., Oliver, G., and McDonald, D.M. (2012). Plasticity of button-like junctions in the endothelium of airway lymphatics in development and inflammation. Am J Pathol *180*, 2561-2575.

Yau, J.W., Teoh, H., and Verma, S. (2015). Endothelial cell control of thrombosis. BMC Cardiovasc Disord *15*, 130.

Yuan, H.T., Khankin, E.V., Karumanchi, S.A., and Parikh, S.M. (2009). Angiopoietin 2 is a partial agonist/antagonist of Tie2 signaling in the endothelium. Mol Cell Biol *29*, 2011-2022.

Yun, S., Hu, R., Schwaemmle, M.E., Scherer, A.N., Zhuang, Z., Koleske, A.J., Pallas, D.C., and Schwartz, M.A. (2019). Integrin alpha5beta1 regulates PP2A complex assembly through PDE4D in atherosclerosis. J Clin Invest *130*.

Yurdagul, A., Jr., Green, J., Albert, P., McInnis, M.C., Mazar, A.P., and Orr, A.W. (2014). alpha5beta1 integrin signaling mediates oxidized low-density lipoprotein-induced inflammation and early atherosclerosis. Arterioscler Thromb Vasc Biol *34*, 1362-1373.

Zacharias, D.A., Violin, J.D., Newton, A.C., and Tsien, R.Y. (2002). Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. Science *296*, 913-916.

Zahn, G., Vossmeyer, D., Stragies, R., Wills, M., Wong, C.G., Loffler, K.U., Adamis, A.P., and Knolle, J. (2009). Preclinical evaluation of the novel small-molecule integrin alpha5beta1 inhibitor JSM6427 in monkey and rabbit models of choroidal neovascularization. Arch Ophthalmol *127*, 1329-1335.

Zeng, H., He, X.C., Tuo, Q.H., Liao, D.F., Zhang, G.Q., and Chen, J.X. (2016). LPS causes pericyte loss and microvascular dysfunction via disruption of Sirt3/angiopoietins/Tie-2 and HIF-2 alpha/Notch3 pathways. Sci Rep-Uk *6*.

Zhang, F., Zarkada, G., Han, J., Li, J., Dubrac, A., Ola, R., Genet, G., Boye, K., Michon, P., Kunzel, S.E.*, et al.* (2018). Lacteal junction zippering protects against diet-induced obesity. Science *361*, 599-603.

Zhang, X., Groopman, J.E., and Wang, J.F. (2005). Extracellular matrix regulates endothelial functions through interaction of VEGFR-3 and integrin alpha5beta1. J Cell Physiol *202*, 205-214.

Zhao, Y., and Davis, H.W. (2000). Endotoxin causes phosphorylation of MARCKS in pulmonary vascular endothelial cells. J Cell Biochem *79*, 496-505.

Zheng, W., Nurmi, H., Appak, S., Sabine, A., Bovay, E., Korhonen, E.A., Orsenigo, F., Lohela, M., D'Amico, G., Holopainen, T.*, et al.* (2014). Angiopoietin 2 regulates the transformation and integrity of lymphatic endothelial cell junctions. Genes Dev *28*, 1592-1603.

Zhu, J.W., Motejlek, K., Wang, D.N., Zang, K.L., Schmidt, A., and Reichardt, L.F. (2002). beta 8 integrins are required for vascular morphogenesis in mouse embryos. Development *129*, 2891-2903.

Zhu, W., London, N.R., Gibson, C.C., Davis, C.T., Tong, Z., Sorensen, L.K., Shi, D.S., Guo, J., Smith, M.C., Grossmann, A.H.*, et al.* (2012). Interleukin receptor activates a MYD88-ARNO-ARF6 cascade to disrupt vascular stability. Nature *492*, 252-255.

Ziegler, T., Horstkotte, J., Schwab, C., Pfetsch, V., Weinmann, K., Dietzel, S., Rohwedder, I., Hinkel, R., Gross, L., Lee, S.*, et al.* (2013). Angiopoietin 2 mediates microvascular and hemodynamic alterations in sepsis. J Clin Invest.

Zovein, A.C., Luque, A., Turlo, K.A., Hofmann, J.J., Yee, K.M., Becker, M.S., Fassler, R., Mellman, I., Lane, T.F., and Iruela-Arispe, M.L. (2010). Beta1 integrin establishes endothelial cell polarity and arteriolar lumen formation via a Par3-dependent mechanism. Dev Cell *18*, 39-51.

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