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The role of EphB/ephrinB in inflammation

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Abbreviations

ABTS	a substrate of enzyme horseradish peroxidase
ADAM	a disintegrin and metalloprotease
bFGF	basic fibroblast growth factor
BBB	blood brain barrier
BRB	blood retina barrier
COUP-TFII	COUP transcription factor 2
Ca^{2+}	calcium
CD	cluster of differentiation
CD99L2	CD99 antigen-like protein 2
Dll4	delta-like 4
EC	endothelial cells
ECM	extracellular matrix
ERK	extracellular signal-regulated kinases
Eph	named after *e*rythropoietin-*p*roducing human *h*epatocellular carcinoma cell line
Ephrin	*Eph* family *r*eceptor *in*teracting proteins
ESAM	endothelial cell-selective adhesion molecule
ESL1	E-selectin ligand 1
FAK	focal adhesion kinase
FGFR	fibroblast growth factor receptor
FITC	fluorescein isothiocyanate
FoxO1	forkhead box O1
GRIP	glutamate receptor-interacting protein 1
GPI	glycosylphosphatidylinositol
Grb	growth factor receptor-bound protein
GM-CSF	granulocyte/macrophage colony-stimulating factor
HUVECs	human umbilical vein endothelial cells
IL	interleukin
IFNγ	interferon-γ
ICAM-1	intercellular adhesion molecule-1
ICAM-2	intercellular adhesion molecule-2
JAM	junctional adhesion molecule

Jak	Janus kinase
JNK	c-Jun N-terminal kinases
LPS	lipopolysaccharides
LFA-1	lymphocyte function-associated antigen-1 (αLβ2-integrin)
LBRC	lateral border recycling compartment
MAC-1	macrophage antigen-1
MAC-3	macrophage antigen (CD107b)
MCP-1	monocyte chemoattractant protein-1
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
Nck	non-catalytic region of tyrosine kinase adaptor protein 1
NO	nitric oxide
PSGL1	P-selectin glycoprotein ligand 1
PDGFR	platelet-derived growth factor receptor
PTP-BL	tyrosine-protein phosphatase non-receptor type 13 (PTPN13)
PI3K	phosphoinositol 3-kinase
PECAM-1	platelet/endothelial cell adhesion molecule-1
Par	partitioning defective
PDZ	PSD95/Discs-large/ZO1
PEI	polyethylenimine
RGS3	regulator of G-protein signaling 3
ROCK	rho-associated protein kinase
Rac	Ras-related C3 botulinum toxin substrate
SHP2	SH2 domain-containing phosphatase 2
SH2	Src homology 2
SDF-1	stromal cell-derived factor 1
Shh	sonic hedgehog
STAT	signal transducer and activator of transcription
SMC	smooth muscle cells
Tie2	tyrosine kinases that contain immunoglobulin-like loops and epidermal growth factor-similar domains 2
TNFα	tumor necrosis factor α
VEGF	vascular endothelial growth factor

VEGFR	vascular endothelial growth factor receptor
VE-PTP	vascular endothelial protein tyrosine phosphatase
VCAM-1	vascular cell adhesion molecule-1
vWF	von Willebrand factor
VLA-4	very late antigen-4 (α4β1-integrin)
ZO	zonula occludens

ZUSAMMENFASSUNG

Ein zentraler Punkt in der stark regulierten Entwicklung des Gefäßsystems ist die Bindung der membran-assoziierten EphrinB-Liganden an ihren korrespondierenden Rezeptor EphB in Endothelzellen (EZ). Bisher ist jedoch nur wenig über die Funktion des EphrinB/EphB-Systems in adulten Blutgefäßen bekannt. Wir haben in diesem Zusammenhang beobachtet, dass die Liganden EphrinB1 und EphrinB2 auf der Oberfläche von EZ lokalisiert sind und ihre Expression im Verlauf inflammatorischer Prozesse zunimmt, während EphB2-Rezeptoren konstitutiv auf Monozyten exprimiert werden. Insofern stellten wir die Hypothese auf, dass diese Liganden unter pro-inflammatorischen Bedingungen an EphB-Rezeptoren auf Monozyten binden. Um die funktionellen Konsequenzen der Rezeptor-Liganden-Interaktion zu untersuchen, wurden kultivierte EZ gegenüber EphB2-Rezeptorkörpern oder EphB2-Rezeptor überexprimierenden Maus-Myelom-Zellen exponiert und die Expression von EphrinB1 bzw. EphrinB2 in den EZ mittels siRNA herunterreguliert.

Ausgehend von diesem experimentellen Aufbau wurde die pro-inflammatorische Differenzierung der EZ und die Migration von Monozyten durch einen EZ-Monolayer analysiert. Die vorwärtsgerichtete, nachgeschaltete Signalgebung von EphB-Rezeptoren förderte die Aktivierung von Monozyten. Die rückwärtsgerichtete EphrinB-Signalgebung hingegen, induzierte den Abbau des von Willebrand-Faktors auf der Oberfläche der EZ, verstärkte die Expression von E-Selektin, VCAM-1 und des *Granulozyten*-Makrophagen-Kolonie-stimulierenden Faktors (GM-CSF) und stimulierte dadurch die Adhäsion von Monozyten. Dabei zeigte sich, dass die Expression beider Liganden für die Migration der Monozyten durch den EZ-Monolayer essentiell ist. Die Aktivierung der Monozyten durch EphB2 wurde von einem Rückgang der Integrität der Zell-Zell-Verbindungen zwischen den EZ begleitet. Dies wurde nicht nur durch Src-abhängige Phosphorylierung von PECAM-1 und eine Verminderung der SHP-2 Aktivität vermittelt, sondern auch durch die Verdrängung von VE-Cadherin aus den interendothelialen Zellkontakten. Zudem erhöhte die Stimulation mit EphB2 die Permeabilität der EZ. Die endothelzellspezifische Unterdrückung der EphrinB2 Expression *in vivo* wurde durch die Injektion von Tamoxifen in Tie2-CreER^{T2}xephrinB2$^{flox/flox}$ Mäusen induziert. Es wurden vier verschiedene Entzündungsmodelle verwendet, um die

Rolle von EphrinB2 bei der Monozyten-Diapedese und die pro-inflammatorische EZ-Antwort *in vivo* zu analysieren: das TPA induzierte Ohr-Ödem, die Thioglykolat vermittelte Peritonitis, die ApoE-Defizienz-vermittelte Arteriosklerose, sowie das Hinterlauf-Ischmämie Modell. Allerdings deuteten die Ergebnisse aller vier Mausmodelle daraufhin, dass der Verlust der mikrovaskulären endothelialen EphrinB2-Expression keine signifikanten Auswirkungen auf die die Monozyten-Infiltration, den Entzündungsverlauf bzw. Schweregrad oder die Progression der Arteriogenese hat. Diese Befunde könnten auf bestimmte Limitationen der verwendeten Mausmodelle hinweisen oder auf einen kompensatorischen Effekt von EphrinB1 bei EphrinB2-Verlust zurückzuführen sein.Insgesamt deuten unsere Daten zum ersten Mal daraufhin, dass das EphrinB/EphB-System zu den inflammatorischen Reaktionen der EZ beiträgt, wobei die rückwärtsgerichtete EphrinB-Signalgebung die Adhäsion und Transmigration von EphB2-exprimierenden Monozyten unterstützt und die Permeabilität der EZ erhöht.

SUMMARY

The binding of membrane-associated ephrinB ligands to corresponding EphB receptors in endothelial cells (ECs) is pivotal for orchestrating the development of the circulatory system. However, only little is known about their function in adult blood vessels. In this context, we observed that the ligands ephrinB1 and ephrinB2 are localized on the surface of ECs where their expression is upregulated during inflammation, while EphB2 receptors are located on the surface of monocytes. Consequently, we hypothesized that these ligands bind to EphB receptors expressed in monocytes under pro-inflammatory conditions. To study functional consequences of their ligand-receptor interactions, ECs were exposed to EphB2 receptor bodies or EphB2-overexpressing mouse myeloma cells upon siRNA-mediated knockdown of ephrinB1 or ephrinB2.

Based on this experimental setup, we analyzed the pro-inflammatory differentiation of the ECs and the transmigration of monocytes through the EC monolayer. Whereas forward signaling downstream of the EphB receptors promotes activation of monocytes, ephrinB reverse signaling induces the deposition of von Willebrand factor on the ECs surface, increases the expression of E-selectin, VCAM-1 and granulocyte-macrophage colony-stimulating factor in ECs, and consequently stimulates monocyte attachment. Moreover, ephrinB1 and ephrinB2 are crucial for the transmigration of monocytes through the endothelial cell monolayer. Their activation by EphB2 not only promotes PECAM-1 phosphorylation through a Src-dependent mechanism while diminishing SHP-2 activity, but also triggers displacement of VE-cadherin from interendothelial cell junctions. In addition, EphB2 stimulation increases the permeability of endothelial cells. *In vivo*, endothelial cell specific ephrinB2 ablation was induced by injecting tamoxifen in Tie2-CreER^{T2}/ephrinB2$^{flox/flox}$ mice. Three distinct types of inflammatory models-TPA-induced ear edema, thioglycollate-elicited peritonitis, atherosclerosis, as well as an arteriogenesis model were employed to analyze the impact of ephrinB2 on monocyte extravasation and progression of pathological processes. However, all four models indicated that loss of endothelial ephrinB2 has no impact on monocyte infiltration, inflammatory pathology, and progression of arteriogenesis. This might be due to the limitations of the utilized models and compensatory effects of ephrinB1. Collectively, our data indicates for the first time

that the EphB/ephrinB system contributes to inflammatory responses of ECs whereby ephrinB reverse signaling supports adhesion and transmigration of EphB2-expressing monocytes, and increases endothelial cell permeability.

1 INTRODUCTION

1.1 General organization of the vessel wall

The vascular system is a complex network of blood vessels (arteries, arterioles, veins, venules, capillaries) to maintain the circulation of blood flow, which is critical for cell homeostasis by providing nutrients and disposing cellular waste. The wall of both veins and arteries can be divided into three layers: the intima (endothelial cells attached to the basement membrane), the media (smooth muscle cells and elastin fibers), and the adventitia (fibro-elastic connective tissue). This general structure can vary widely. For instance, the capillaries consist of a monolayer of endothelial cells, basement membrane, and some pericytes, whereas arterioles and large venules contain at least one layer of smooth muscle cells and some connective tissues [1].

1.1.1 Characteristics of endothelial cells in the vascular system

The endothelium, lining the inner surface of blood and lymphatic vessels, has diverse functions, including maintenance of endothelial barrier, leukocyte trafficking, angiogenesis, as well as regulation of vasodilation and thrombolysis [2, 3]. Depending on the epigenetic determination and various microenvironmental cues, including biomechanical forces (shear stress and cyclic strain) and biochemical stimulators (e.g. growth factors, cytokines, chemokines, hormones, etc.), endothelial cell phenotypes vary widely. This is referred to as endothelial cell heterogeneity [4]. For instance, endothelial cell thickness is less than 0.1 µm in capillaries and veins, and up to 1 µm in the aorta [5]. Moreover, the composition of intercellular junctions is different as well. Tight junctions between adjacent endothelial cells are well-organized in precapillary arterioles, whereas in postcapillary venules, tight junctions are discontinuous and loose, which is suitable for leukocyte extravasation [6].

1.1.2 Ways of blood vessel formation and remodeling

The formation of blood vessels starts at the early phase of embryonic development through two distinct processes: vasculogenesis and angiogenesis. Additionally, arteriogenesis is an adaptive process of transforming pre-existing arterioles into functional arteries under the condition of ischemia [7].

1.1.2.1 Angiogenesis

Angiogenesis is defined as the formation of new capillaries from pre-existing capillary networks both in embryos and adults, which could be accomplished by endothelial sprouting and non-sprouting angiogenesis. Sprouting angiogenesis involves the specialization of endothelial cells (tip cells, stalk cells and phalanx cells), the interplay of various signaling pathways (e.g. Notch and Notch ligands, VEGF and VEGFRs), and processes of vascular maturation and stabilization (e.g. lumen formation and perfusion, network formation, remodeling, pruning, association with mural cells) [8, 9]. Moreover, arteriovenous differentiation is a crucial process to form arteries and veins in early embryonic development. Activation of the Shh/VEGFA/Notch pathway stimulates the expression of ephrinB2 to function as artery identity. Whereas, COUP-TFII suppresses the Notch signaling to induce the expression of EphB4 in the venous endothelium [9, 10]. Besides sprouting, non-sprouting angiogenesis or intussusception is an alternative way to expand vascular networks. In this process, existing blood vessels are divided by invagination and formation of transluminal pillars [11].

1.1.2.2 Arteriogenesis

Arteriogenesis is characterized by the remodeling of pre-existing small arterioles into much larger conductance arteries [12]. It is triggered mainly by an elevation of shear stress, which is induced by increased blood flow following the occlusion of a conduit artery [13]. Several mechanical sensors on endothelial cells (e.g. integrin, ion channels etc.) are responsible for transferring mechanical stimuli into the intracellular signaling. This leads to the activation of endothelial cells, such as the upregulation of adhesion molecule expression, chemokine and NO release, as well as increased permeability [14]. Meanwhile, smooth muscle cells proliferate, migrate, and change from a contractile to a synthetic phenotype [13]. Furthermore, perivascular macrophages secrete various growth factors (e.g. bFGF), cytokines (e.g. TNFα), and proteases, and thus contribute to vascular wall remodeling [15]. Eventually, these molecular and cellular events result in the remodeling of collateral arterioles, including the enlargement of vessel lumen and an increase in wall thickness. This process generates functional arteries which are capable of restoring blood flow in ischemic tissue where a conduit artery is occluded [16].

1.2 Inflammation and immune cells

Inflammation is an initial reaction of the immune system in response to infection, irritation or injury. The classical signs of acute inflammation are pain, heat, redness, swelling, and loss of function. Persistent pro-inflammatory stimuli lead to chronic inflammation which is related to progression of diverse diseases such as atherosclerosis, arthritis and Alzheimer's disease [17]. Alterations in microcirculation during inflammation contain vasodilation, vascular leakage and increased blood flow [18]. Another hallmark of this process is the recruitment of leukocytes to the inflamed site, which mainly occurs in postcapillary venules [19]. Leukocytes are classified as polymorphonuclear (granulocytes) and mononuclear (agranulocytes) cells according to the appearance of nuclei and the presence of granules in their cytoplasm. Granulocytes consist of neutrophils, eosinophils and basophils. Agranulocytes include monocytes and lymphocytes [20]. Apart from immune cells, cell- or plasma-derived soluble inflammatory mediators, including histamine, platelet activating factor, cytokines, chemokines, complement and ROS, play a role in diverse pathological responses during inflammation, such as vasodilation, chemotaxis, tissue damage and pain [21].

1.3 Leukocyte extravasation

Leukocyte extravasation is a critical process in inflammation and immune surveillance. The cascade of leukocyte extravasation has been subdivided into sequential steps: tethering and rolling, activation, firm adhesion, para- and trans-cellular transmigration, and migration through the basal membrane (Figure 1) [19]. In fact, most leukocytes which attach to inflamed postcapillary venules return to circulation since their adhesion is reversible. Penetration of the endothelial barrier is an irreversible step in leukocyte extravasation [22].

1.3.1 Leukocyte adhesion

The initial procedure for leukocyte recruitment is the adhesion of these cells to the endothelium. This is accomplished by sequential and overlapping steps, including selectin-mediated rolling, arrest chemokines-mediated activation of leukocyte integrins and integrin-mediated firm adhesion [22].

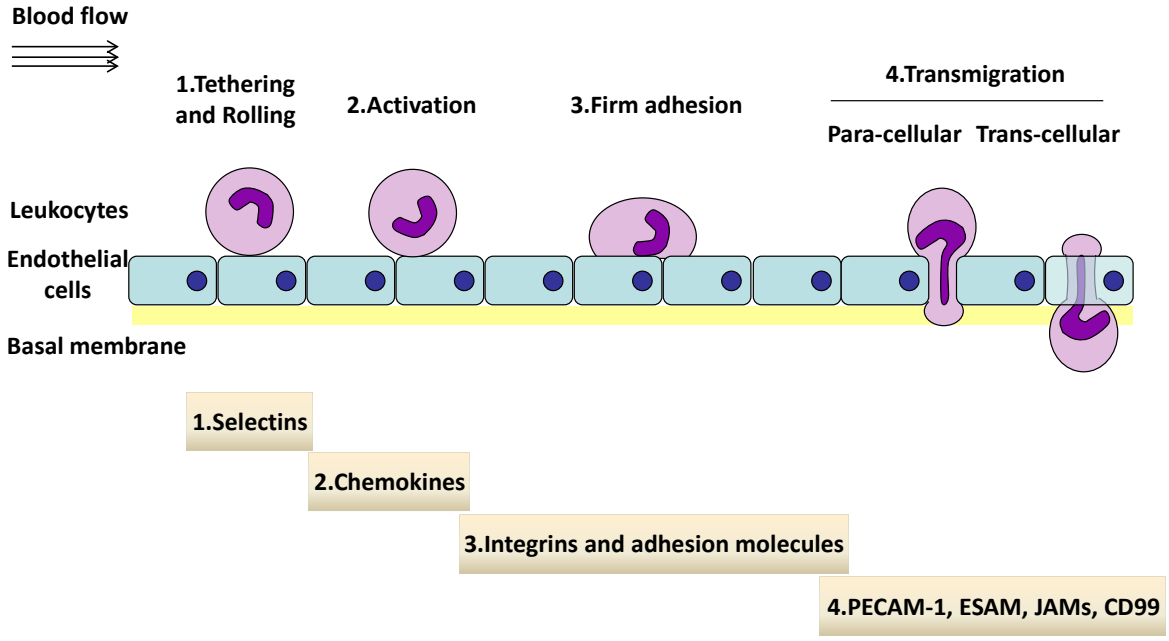

Figure 1: The cascade of leukocyte extravasation.

Multiple steps and key molecules involved in leukocyte extravasation are displayed. Circulating leukocytes are captured by selectins, which initiate leukocyte rolling on endothelium. Exposure to chemokines on endothelial cells activates leukocyte integrins. Firm adhesion is mediated by the interactions of fully activated integrins and adhesion molecules. Leukocytes transmigrate through the endothelial cell monolayer via either a paracellular or transcellular route (adapted from Ley K 2007).

Selectins, comprising P-selectin, L-selectin and E-selectin, interact with P-selectin glycoprotein ligand 1 (PSGL1), glycosylated CD44 and E-selectin ligand 1 (ESL1) expressed in most leukocytes [22]. This binding mediates leukocyte capture (tethering) and sequent rolling along the endothelium to slow down their velocity [23]. Furthermore, rolling of leukocytes allows their binding to arrest chemokines that are deposited on the luminal surface of inflamed endothelial cells, including CXCL12, CCL21, CXCL1, CCL2 and CCL25 [24]. These immobilized chemokines interact and activate G-protein coupled chemokine receptors (GPCR) on the leukocyte surface, which induces intracellular signaling and thereby promoting rapid integrin activation [25].

Firm adhesion of leukocytes is achieved by the binding of their fully activated integrins with the corresponding receptors-Immunoglobulin (Ig) superfamily of

adhesion molecules in endothelial cells. Pro-inflammatory mediators (e.g. TNF-α, IL-1β) induce an increased expression of adhesion molecules in endothelial cells [19, 26]. VCAM-1 interacts with the integrin VLA4. ICAM-1 binds to the integrins LFA-1 and Mac-1 [27]. Moreover, LFA-1 and Mac-1 have distinct roles in leukocyte arrest. While LFA-1 initiates leukocyte adhesion, Mac-1 mediates intraluminal crawling which allows leukocytes to search for the optimal sites of emigration [28, 29].

1.3.2 Leukocyte diapedesis

Once leukocytes crawl to the preferred sites, transendothelial migration or diapedesis occurs, which is accomplished by either a trans- or para-cellular route [19]. The trans-cellular migration is a process that allows leukocytes to pass through the endothelial cytoplasm via vesicular/canalicular systems [30]. The usage of this route is particularly predominant in leukocyte transmigration through blood brain barrier (BBB) and blood retinal barrier (BRB), because of the extremely tight junctions between adjacent endothelial cells [31]. Most diapedesis takes place via the para-cellular route, in which leukocytes extend pseudopods and squeeze through the endothelial cell borders. A bunch of molecules enriched at intercellular junctions, including PECAM-1, ICAM-2, ESAM, CD99, CD99L2 and JAMs, synergetically cooperate to control leukocyte para-cellular transmigration. In addition, ICAM-1 and VCAM-1 play a role in this process as well [32].

The main processes in leukocyte para-cullular transmigration are indicated in the following three aspects. First, leukocyte adhesion evokes clustering of ICAM-1 and VCAM-1 on the endothelial cell surface, which is referred to as “docking structures” or “transmigratory cups” [33, 34]. Second, the loosening of intercellular junctions is a prerequisite for leukocyte transmigration. The reversible formation of gaps is driven by VE-cadherin displacement [35-37]. Phosphorylation of specific tyrosine sites of VE-cadherin is required for this process, as indicated in chapter 1.4.1 [38, 39]. Moreover, downstream signaling pathways of clustered ICAM-1 and VCAM-1 result in endothelial cell contraction and thus weaken intercellular junctions [40-42]. Third, leukocyte transmigration requires targeted membrane recycling from the lateral border recycling compartment (LBRC) [43]. The LBRC is composed of 50 nm vesicle-like structures connected to each other, which

localizes just beneath the lateral plasma membrane. The compartment contains PECAM-1, JAM-A, and CD99 (Figure 2) [44]. In resting endothelial cells, the membrane traffics constitutively between the lateral plasma membrane and the LBRC. Once a leukocyte crosses the endothelium, LBRC membrane is trafficked to the site of diapedesis and surrounds the leukocyte, which is required for the leukocyte transmigration [44, 45].

1.4 Permeability

Increased microvascular permeability, a hallmark of inflammation, occurs in a variety of human pathological conditions, such as ischemia-reperfusion injury, stroke, sepsis and acute respiratory distress syndrome [46]. Diverse mediators, including permeability-increasing factors (e.g. thrombin, histamine, bradykinin, VEGF), oxidants, prostanoids, ischemia and pro-inflammatory stimuli (e.g. TNFα), play a role in regulating endothelial permeability [46]. Indeed, endothelial permeability is achieved by either a trans- or para-cellular pathway. During the trans-cellular route, the substance passes through cell bodies by channels, carriers, pumps or vesicles; in the para-cellular route, it crosses cell-cell contacts via loose intercellular junctions [47]. Impairment of the endothelial barrier not only allows the transport of plasma proteins, solutes, and fluid, resulting in oedematous tissue injury, but also favors leukocyte transmigration [48].

Endothelial cell-cell contacts are composed of two types of adhesive structures, namely adherens junctions (AJs) and tight junctions (TJs), which maintain the endothelial barrier (Figure 2). Adherens junctions contain VE-cadherin and its binding partners [49]. Tight junctions consist of membrane proteins (claudins, occludin, JAM family, ESAM, CD99) and other cytoplasmic components (ZO proteins and others), which are particularly enriched in stringent barriers such as BBB and BRB [50]. Of note, AJs and TJs are regulated mutually. Clustering of VE-cadherin upregulates the expression of claudin-5 through limiting nuclear translocation of the forkhead transcriptional repressor FoxO1–β-catenin complex [51]. Vice versa, JAM-C modulates VE-cadherin-mediated adhesion, as loss of JAM-C increases homophilic adhesive interactions of VE-cadherin dependent on Rap1 [52].

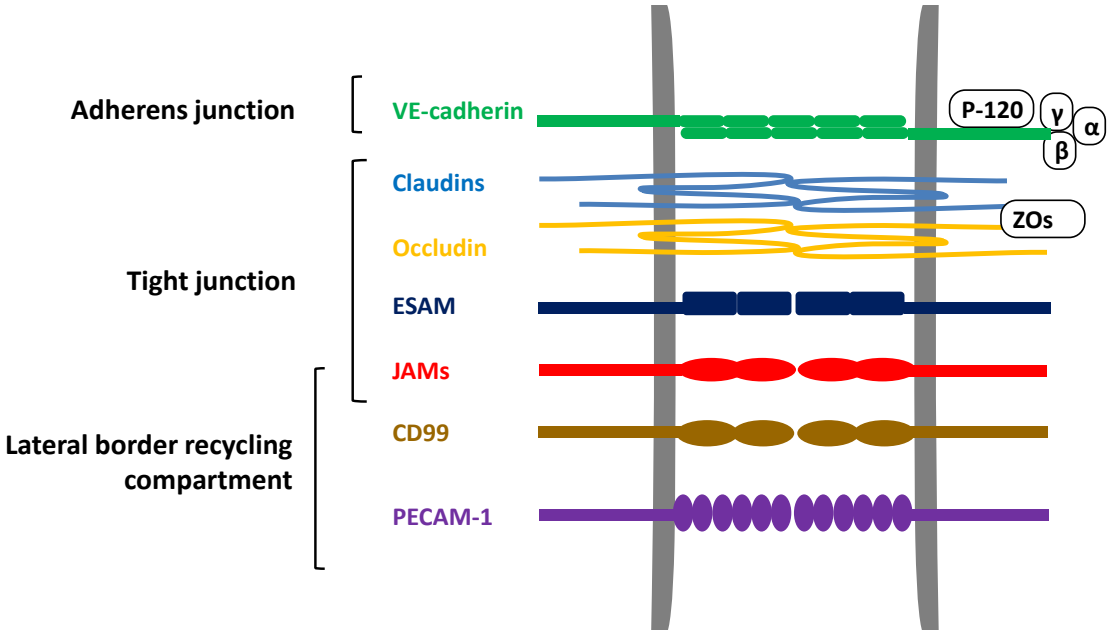

Figure 2: Key molecules in the endothelial cell-cell contacts.
Adherens junctions are composed of VE-cadherin and its binding partner-p120-catenin, β-catenin, and γ-catenin. Tight junctions include claudins (3, 5, 12), occludin, members of the JAM family, ESAM, and cytoplasmic proteins (e.g. ZO proteins). CD99, PECAM-1 and JAM-A are components of the lateral border recycling compartment (adapted from Daniel AE 2013).

1.4.1 Vascular endothelial (VE)-cadherin

VE-cadherin belongs to the cadherin family. It is a transmembrane protein whose cytoplasmic domain interacts with p120-catenin, β-catenin and γ-catenin. These adherens junction components are associated with actin-binding proteins (e.g. α-catenin, vinculin), phosphatases (e.g. VE-PTP), kinases (e.g. src, csk) and growth factor receptors (e.g. VEGFR2 and TGFβ receptor) [48]. VE-cadherin is required for maintaining vascular integrity, and thus controls vascular permeability and represents a barrier for leukocyte extravasation [53]. Functional blockade of VE-cadherin induces an increase in blood vessel permeability, accompanied by the migration of distinct leukocyte subsets [54, 55]. Dismantling of VE-cadherin on the cell surface is accomplished by phosphorylation, internalization and cleavage [56].

Diverse permeability-increasing mediators induce VE-cadherin phosphorylation at specific residues, Y658, Y685, Y731 and S665, which is mediated by Src family kinases [49, 53, 57-60]. This leads to VE-cadherin internalization and the

disassembly of the adherens junction complex [49, 60]. It has recently been reported that veins but not arteries display constitutive and prolonged phosphorylation of Src, Y658 and Y685 of VE-cadherin. This may sensitize veins to permeability-inducing agents, resulting in rapidly reversible openings of endothelial cell junctions [49]. Moreover, internalization of VE-cadherin occurs in a clathrin-dependent manner [60, 61]. VE-cadherin cleavage is accomplished by leukocytes- or tumor cells-released enzymes digesting the extracellular part of VE-cadherin [56, 62]. These processes decrease the abundance of VE-cadherin on the endothelial cell surface, leading to increased vascular leakage.

1.4.2 Tight junctions

Tight junctions play an essential role in regulating paracellular permeability and maintaining cell polarity. Downregulation of claudins, occludin and ZO-1 has been correlated with BBB and BRB dysfunction in various pathophysiological conditions such as stroke, diabetes, inflammation and brain tumors [63-65]. Phosphorylation and redistribution of tight junction proteins away from the cell border are induced by inflammatory or angiogenic mediators (e.g. hypoxia, MCP-1, VEGF or histamine), leading to the impairment of endothelial barrier function [65]. Furthermore, inflammation-induced apical redistribution of JAM-A and upregulation of JAM-C expression exert opposing effects on the modulation of endothelial permeability [66, 67]. JAM-A, which is highly expressed in the brain vascular system, acts as a gatekeeper to decrease permeability in endothelium [68]. On the contrary, JAM-C, highly expressed in lymph nodes, enhances permeability by regulating endothelial contraction and VE-cadherin-mediated adhesion [52, 67].

1.4.3 Endothelial cell contraction

Endothelial cell contraction is a critical mechanism for the disruption of cell-cell adhesion, leading to the gap formation and increased endothelial permeability [69]. It is well-known that myosin light chain (MLC) phosphorylation contributes to actomyosin contractility and endothelial cell contraction [70, 71]. Phosphorylation of MLC is induced by Ca^{2+}/calmodulin-dependent activation of MLC kinase (MLCK), as well as the inhibition of MLC phosphatase (MLCP) activity mediated by RhoA and Rho kinase (ROCK) activity [72-74]. For instance, thrombin-induced

endothelial permeability partially attributes to endothelial cell contraction elicited by an elevation of intracellular Ca^{2+} and activation of Rho/ROCK pathway [75].

1.5 Eph/ephrin system

Eph receptors are named after a cell line in which the first member was discovered: *e*rythropoietin-*p*roducing human *h*epatocellular carcinoma cell line [76]. Eph and the corresponding ligands ephrin (*Eph* family *r*eceptor *in*teracting proteins) have versatile functions in embryos and adults. In the central nervous system, Eph/ephrins have profound effects on the development of neural crest cell, cerebellar granule cell migration, segmentation, topographic mapping, axon guidance and dendritic spine formation in embryonic development [77]. They also play a role in synapse plasticity and the repair of injured nervous system in adults [77, 78]. Moreover, Eph/ephrins are involved in the regulation of epithelium self-renewal, insulin secretion, bone development and remodeling, platelet aggregation and glomerular filtration in the kidney [79-84]. In addition, Eph/ephrins have been widely studied in the pathology of cancer related to tumor growth, invasiveness, angiogenesis and metastasis [85].

1.5.1 Eph/ephrin structure

Eph receptors represent the largest family of receptor tyrosine kinases (RTKs), comprising nine EphA (EphA1-8; EphA10) and five EphB (EphB1-4, EphB6). The subdivision of group A and B depends on the similarities of the extracellular sequences and the binding affinity for corresponding ligands. Ephrin ligands consist of six ephrinA (ephrinA1-6) and three ephrinB (ephrinB1-3). Furthermore, the binding of receptor and ligand is confined in the same subclass. EphA receptors bind to ephrinA ligands and EphB receptors preferentially interact with ephrinB ligands. As an exception, ephrinA5 binds to EphB2 at high concentrations, and ephrinB ligands bind to EphA4 [86].

Eph receptors are transmembrane proteins. The extracellular part of Eph receptors includes an ephrin-binding domain, a cysteine-rich region, and two fibronectin type-III repeats. The cytoplasmic region consists of a juxtamembrane region, the tyrosine kinase domain, a sterile α-motif (SAM) domain and a PDZ-binding motif. EphrinA ligands are linked to the cell membrane via a

glycosylphosphatidylinositol (GPI) anchor. Whereas, ephrinB ligands display an extracellular Eph-binding domain, a transmembrane region, a cytoplasmic tail, and a PDZ-binding domain [87] (Figure 3).

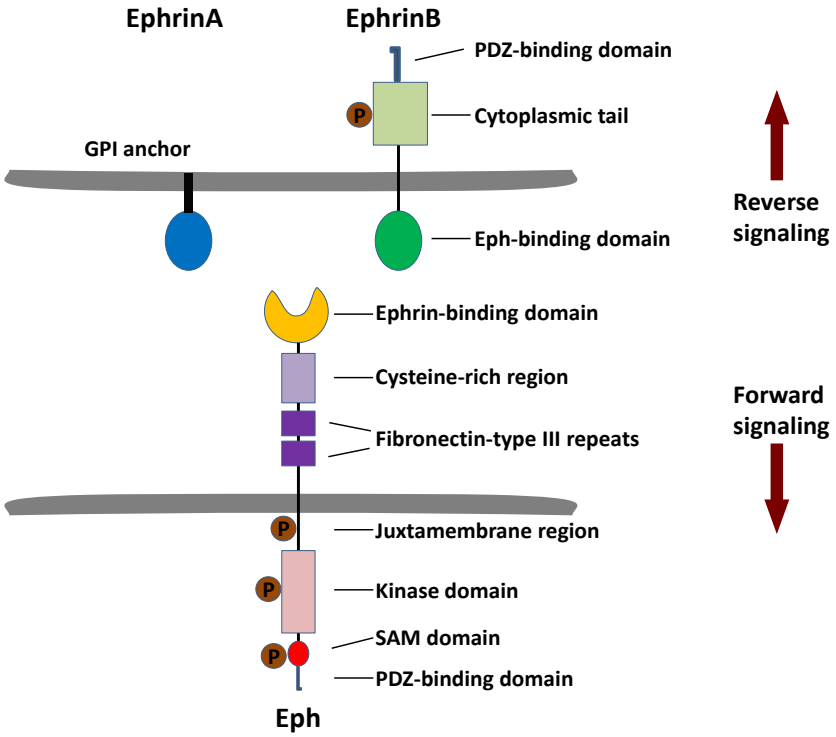

Figure 3: Structure and bidirectional signaling of Eph/ephrin.

The structures of Eph and ephrin are illustrated (refer to the text for detailed description). The binding of Eph and ephrin induces forward signaling through Eph and reverse signaling via ephrin, namely bidirectional signaling (adapted from Salvucci O 2012).

Since receptors and ligands are membrane bound proteins, cell-cell contact is required for inducing bidirectional signaling into the Eph-expressing cells (termed forward signaling) and the ephrin-expressing cells (termed reverse signaling) [88] (Figure 3). Eph and ephrin expressed in opposing cells interact in trans, whereas both expressed in the same cell result in cis interaction [89]. Moreover, once cells contact, clustering of the Eph/ephrin complex occurs progressively, and the degree of clustering determines the signal intensity and final effects. Tetramerization is necessary for evoking downstream signaling [90].

To terminate this interaction, which is required for the detachment of contacting cells, the Eph/ephrin complex is removed from cell surface by endocytosis into

Eph- or ephrin-expressing cells [89]. Intriguingly, this process is accompanied by the internalization of surrounding membrane compartments with other proteins. For instance, EphB4 stimulation results in the internalization of ephrinB2-colocalized VEGFR2 and VEGFR3 into endothelial cells, which is essential for VEGF signaling [91, 92]. An alternative way to eliminate the Eph/ephrin complex is through proteolytic cleavage. This is accomplished by ADAM family metalloprotease and γ-secretase [89, 93]. This process may generate Eph/ephrin fragments that exert additive effects [88, 94-97].

1.5.2 Eph/ephrin signaling

The interaction of Eph and ephrin induces forward and reverse signaling to modulate diverse cell behaviors such as proliferation, differentiation, adhesion and migration [86]. To transduce signals into cells, Eph receptors become tyrosine-phosphorylated and bind to PDZ domain proteins. Subsequently, several signal tranducers are activated: SH2 domain-containing adaptor protein (Grb2/7/10, Nck), Rho and Ras, focal adhesion kinase (FAK), the Jak/Stat pathway and the PI3K pathway [93, 98]. Although ephrinA lacks a cytoplasmic tail, these ligands are capable of triggering downstream signaling through several effectors, such as Src family kinases, ERK, Rac1, and AKT [81, 99-102].

Downstream signals of ephrinB are transmitted through it interacting with a variety of proteins which mediate SH2/PDZ-dependent and -independent signaling pathways [102]. Some of these signals require ephrinB phosphorylation. Phosphorylation of ephrinB is caused by EphB receptor engagement, activation of growth factor receptors (e.g. FGFR, PDGFR, Tie2) or cell membrane proteins (e.g. claudins) [102]. EphB-induced Src family kinases activity is responsible for ephrinB phosphorylation, and delayed recruitment of tyrosine phosphatase PTP-BL results in the dephosphorylation of ephrinB [103]. Phosphorylated ephrinB1 binds to SH2/SH3 domain adaptor proteins such as Grb4 [104] and STAT3 [105, 106]. Moreover, ephrinB interacts with PDZ motif-containing proteins, including PDZ-RGS3 [107], GRIP [108] and syntenin-1 [109]. In addition, ephrinB interacts with some proteins independent of PDZ and SH2 domains, such as Par-6 [110], Connexin 43 [111], Dishevelled (Dsh) [112, 113], as well as Zinc-finger and homeodomain protein 2 (ZHX2) [114]. Furthermore, ephrinB reverse signaling has

been reported to activate Src-, PI3K-, JNK- and ROCK-dependent signaling pathways [115-117].

1.5.3 EphB/ephrinB in the vascular system

EphB4 and ephinB2 play a prominent role in embryonic development of the cardiovascular system. Endothelial ephrinB2 and EphB4 are identified as markers for arteries and veins, respectively. EphrinB2 deficiency in mice leads to prenatal death caused by defects in angiogenesis and capillary network formation, as well as impairments in myocardial trabeculation [118]. EphB4-null mice display the similar phenotype, indicating that EphB4 is the prominent receptor for ephrinB2, and also both forward and reverse signaling are required for vascular development [119]. Moreover, endothelial cell specific ephrinB2 knockout mice exhibit similar defects as ephrinB2-deficient mice [120]. Interestingly, overexpression of ephrinB2 in the endothelium leads to embryonic death caused by severe vascular impairments, such as reduced blood vessel network with thin endothelial connections [91]. Therefore, it reveals that the optimal ephrinB2 expression level in the endothelium is crucial for vascular development. Not only does endothelial ephrinB2 play a predominant role, ephrinB2 in mural cells is critical for their migration, spreading, and adhesion during vessel maturity. Targeted ablation of ephrinB2 in mural cells leads to perinatal lethality caused by abnormal migration of SMCs to lymphatic capillaries, defects of microvascular architecture and tissue edema [121]. Moreover, EphB2 is expressed in mesenchymal cells and binds to endothelial ephrinBs, while EphB3 is expressed in veins and some arteries and binds to mesenchymal ephrinB2. Double mutants of EphB2 and EphB3 exhibit various abnormalities of vascular development. This suggests that EphB and ephrinB interactions between endothelial cells and mesenchymal cells are critical for vascular network formation [122].

In adults, EphB and ephrinB are involved in physiological and pathological angiogenesis, such as wound repair, tumor angiogenesis and intraocular neovascularization [87, 123]. The phosphorylation of ephrinB acts as a marker for angiogenic vessels in neovascularization [105]. Moreover, endothelial ephrinB2 expression is increased in remodeling arterioles undergoing arteriogenesis, which functions in limiting SMC migration to stabilize the vasculature [124]. Of note,

ephrinB reverse signaling negatively regulates blood pressure and SMC contractility via reduced RhoA activity, supported by the evidence that EphB6 knockout mice and SMC specific ephrinB1 knockout mice exhibit higher blood pressure [125, 126].

1.5.4 EphB/ephrinB in immunity and inflammation

The EphB/ephrinB system has been reported to regulate immune cell development and function. This has been widely studied in T cells. EphB6 appears to be essential to modulate T cell function, supported by the evidence that EphB6 knockout mice exhibit impaired T cell responses such as defective proliferation and reduced cytokine release [127]. EphrinBs have been identified as dose-dependent co-regulators together with CD3 on T cell activation. Low concentrations of ephrinB1 and ephrinB2 act as co-stimulators on T cell proliferation and downstream signaling, but switch to inhibitors on high concentrations [128, 129]. Moreover, double null mutant mice of ephrinB1 and ephrinB2 in T cells not only display reduced spleen and thymus size, weight and cellularity, but also show impaired T cell functions such as defective differentiation into Th1 and Th17 cells, reduced immune responses to infection and diminished IL-6 signaling [130]. Whereas, single deletion of ephrinB1 or ephrinB2 in T cells elicits minor effects [131, 132]. This suggests a heavy redundancy among ephrin family members to compensate for the deficiency.

There is accumulating evidence showing that EphB/ephrinB expression is upregulated in various cell types in inflammatory diseases. For instance, the expression of ephrinB1 and EphB2 is strongly increased in leukocytes and endothelial cells in human abdominal aortic aneurysm and carotid atherosclerotic plaques [133, 134]. EphrinB1 expression is significantly increased in synovial fibroblasts and lymphocytes in rheumatoid arthritis [135]. EphrinB2 is most abundantly expressed in endothelial cells at atherosclerosis predilection sites [136] Furthermore, EphB/ephrinB contributes to the progression of inflammation through inducing pro-inflammatory cytokine release and regulating leukocyte chemotaxis. EphrinB1 stimulation enhances the secretion of TNFα in leukocytes and IL-6 in synovial cells [135]. EphB1 elicits the expression of MCP-1 in epithelial cells [137, 138]. Additionally, ephrinB2 stimulates the expression of MCP-1 and IL-8 in

monocytes [136]. With respect to EphB/ephrinB-mediated regulation of leukocyte chemotaxis, the effects are still in controversy. EphrinB2 expressed in endothelial cells facilitates MCP-1-stimulated monocyte transmigration [124, 139]. Pretreatment of leukocytes with ephrinB1 effectively promotes their SDF-1α-dependent migration [135]. On the contrary, precoating ephrinB1, ephrinB2 or EphB2 on the membrane of transwell inhibits basal and chemokine-induced leukocyte migration [133, 134, 140].

1.6 Aims

Although the EphB/ephrinB system has been extensively studied during early embryonic development, its function in the adult vasculature is largely unknown. EphrinB2 is apically expressed on quiescent endothelial cells, and the level of its expression depends on local microenvironmental cues such as hypoxia, contact with SMCs and pro-angiogenic stimulation by VEGF [141]. On the other hand, EphB2 is expressed on the surface of monocytes. [139] The pattern of EphB2/ephrinB2 expression led to the hypothesis that their interaction might be involved in circulating monocyte adhesion to and transmigration through the endothelial cell monolayer, which are pivotal processes during inflammation.

In Part 1, the aim was to study ephrinB2 expression in endothelial cells during inflammation, followed by clarifying the role of endothelial cell ephrinB2 in monocyte diapedesis and the underlying mechanisms.

Considering the unique feature of bidirectional signaling in the Eph/ephrin system, as well as the evidence that EphB2 forward signaling activates monocytes [136], it intrigued us to investigate the impact of ephrinB reverse signaling on the pro-inflammatory differentiation of endothelial cells and monocyte adhesion in Part 2.

In Part 3, to further explore the role of endothelial ephrinB2 in inflammation *in vivo*, tamoxifen inducible endothelial cell specific ephrinB2 knockout mice were generated. Three distinct inflammatory models (ear edema, peritonitis and atherosclerosis) and an arteriogenesis model were used to investigate monocyte extravasation and pathological progression.

Based on the fact of heavy redundancy of ephrinB family members, the aim in Part 4 was to distinguish which ligand (ephrinB1 and/or ephrinB2) is responsible for the observed pro-inflammatory differentiation of endothelial cells induced by EphB2 stimulation.

In Part 5, the aim was to assess the impact of endothelial ephrinB1 during inflammation. The expression of endothelial cell ephrinB1 under inflammatory conditions and its effect on monocyte diapedesis were analyzed.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Chemicals were purchased from the following manufacturers: Roth, Merck and Sigma Aldrich.

2.1.2 Reagents and kits

2.1.2.1 Cell culture Media and supplements

Name of the product	Company
Endothelial cell growth medium	Promocell (Karlsruhe, Germany)
Endothelial cell growth supplement (ECGS)	Promocell
RPMI1640 medium	Invitrogen (Karlsruhe, Germany)
Fetal Bovine Serum (FBS)	Invitrogen
Gelatine	Sigma Aldrich (Heidelberg, Germany)
Fibronectin	BD Bioscience
Hank's BSS	PAA (Cölbe, Germany)
Opti-MEM® reduced serum medium	Invitrogen
Streptomycin	Invitrogen
Penicillin	Invitrogen
Fungizone® antimycotic	Invitrogen
Trypsin	Invitrogen
Trypan blue	Sigma Aldrich
MCDB-131 medium	Invitrogen
Endothelial cell growth supplement (ECGS)	Sigma Aldrich
Heparin salt	Sigma Aldrich
Sodium bicarbonate	Sigma Aldrich
L-glutamine solution	Invitrogen

2.1.2.2 Reagents

Name of the product	Company
MATra-A Reagent	IBA (Göttingen, Germany)
ABTS tablets	Roche (Mannheim, Germany)
SP600125	Biomol (Hamburg, Germany).
PP2	Sigma Aldrich
Sudan III	Merk (Darmstadt, Germany)
Expand Long Template PCR System	Roche (Mannheim, Germany)
Protein A/G PLUS-agarose	Santa Cruz (Heidelberg, Germany)
Protein A magnetic bead	New England Biolabs (Frankfurt, Germany)
Nitrocellulose membrane	GE Healthcare (Hamburg, Germany)
ECL Plus Western Blotting Detection reagent	Millipore (Schwalbach, Germany)
Precision Plus Protein™ standards	Biorad (Munich, Germany)
RNAlater RNA stabilization reagent	Qiagen (Düsseldorf, Germany)
Taq Polymerase	Bioron (Ludwigshafen, Germany)
DNA standards-O'GeneRulerTM DNA Ladder	Fermentas (St.Leon-Rot, Germany)
DAPI	Invitrogen
TSA™-Cy3 system	Perkin Elmer (Rodgau Jügesheim, Germany)
Envision™ labeled polymer-HRP anti-rabbit	DAKO (Hamburg, Germany)
ProLong	Invitrogen
Paraffin	Sigma Aldrich
TPA (12-O-Tetradecanoylphorbol-13-Acetate)	Sigma Aldrich
Thioglycollate broth	Sigma Aldrich
Tamoxifen	Sigma Aldrich
Fluorescence-labeled dextrans	Invitrogen & Sigma Aldrich

2.1.2.3 Kits

Name of the product	Company
Active SHP2 DuoSet IC kit	R&D
Strata Prep PCR purification kit	Agilent Technologies (Waldbronn, Germany)
QIAprep spin miniprep kit	Qiagen
Maxi prep plasmid kit	Macherey-Nagel (Düren, Germany)
pcDNA TM 3.1 Directional TOPO kit	Invitrogen
Competent E.coli	Invitrogen
QuantiTect SYBR Green® kit	Qiagen
Rneasy Mini kit	Qiagen
peqGOLD RNA kit	PEQlab (Erlangen, Germany)
Omniscript RT kit	Qiagen

2.1.3 Small interfering RNAs

siRNA	Target sequence	Source
si RNA ephrinB2	ephrinB2siRNA2GGACUGGUACUAUACCCAC[dT][dT] ephrinB2siRNA2_asGUGGGUAUAGUACCAGUCC[dT][dT] ephrinB2siRNA1GGAAUAAAGAUCCAACAAG[dT][dT] ephrinB2siRNA1_asCUUGUUGGAUCUUUAUUCC[dT][dT]	Sigma Aldrich
si RNA ephrinB1	N/A	Qiagen
negative control	N/A	Qiagen

2.1.4 Primers

RT-PCR

human RPL32 forward	5´-GTT-CATCCGGCACCAGTCAG-3´
human RPL32 reverse	5´-ACGTGCACATGAGCTGCCTAC-3´
human ephrinB1 forward	5´-GGAGGCAGACAACACTGTCA-3´
human ephrinB1 reverse	5´-GAACAATGCCACCTTGGAGT-3´
human ephrinB2 forward	5´-GAAAATACCCCTCTCCTCAACT-3´
human ephrinB2 reverse	5´-CTTCGGAACCGAGGATGTTGTTC-3´
human E-selectin forward	5´-TTCGCCTGTCCTGAAGGATG-3´
human E-selectin reverse	5´-TCAGTTGAAGGCCGTCCTTG-3´
human GM-CSF forward	5´-TCCTGAACCTGAGTAGAGAC-3´
human GM-CSF reverse	5´-CAAAGGGGATGACAAGCAGA-3´

Real time PCR

mouse RPL32 forward	5´-GGGAGCAACAAGAAAACCAA-3´
mouse RPL32 reverse	5´-ATTGTGGACCAGGAACTTGC-3´
mpuse ephrinB1 forward	5´-GTAGGCCAGGGCTATTTCTG-3´
mouse ephrinB1 reverse	5´-TGTCTCATGAGGGTCCAAAA-3´
mouse ephrinB2 forward	5´-CTTTGGAGGGCCTGGATAAC-3´
mouse ephrinB2 reverse	5´-TCTTCATGGCTCTTGTCTGG-3´
human RPL32 forward	5´-AGGCATTGACAACAGGGTTC-3´
human RPL32 reverse	5´-GTTGCACATCAGCAGCACTT-3´
human E-selectin forward	5´-AGGTGAACCCAACAATAGGC-3´
human E-selectin reverse	5´-GCTGCACCTCTCATCATTCC-3´
human VCAM-1 forward	5´-CATGGAATTCGAACCCAAACA-3´
human VCAM-1 reverse	5´-GACCAAGACGGTTGTATCTCTGG-3´

2.1.5 Buffers and solutions

Luria-Bertani (LB) Medium:

1.0 % (w/v) Bacto-tryptone

0.5 % (w/v) Yeast extracts

1.0 % (w/v) NaCl

LB-Agar Plates:

1.5 % (w/v) Agar in LB medium with 50 µg/ml ampicillin

Ringer's solution

154 mM NaCl

5.6 mM KCl

2.4 mM $CaCl_2$

6 mM $NaHCO_3$

5.6 mM Dextrose

30 mg/l Nitroplessid

27 mg/l Adenosin

Zinc fixation

0.1 M Tris HCl, pH 7.4

3.2 mM $Ca(CH_3COO)_2(H_2O)$

22.8 mM $Zn(O_2CCH_3)_2(H_2O)_2$

35.9 mM $ZnCl_2$

Casein blocking serum

0.25 % Casein

0.1 % BSA

15 mM NaN_3

50 mM Tris, pH 7.6

Phosphate-Buffered Saline (PBS) per L

8.0 g NaCl

0.2 g KCl

1.44 g Na_2HPO_4

0.2 g KH_2PO_4

Tris-Buffered Saline (TBS) per L

6.1 g Tris (0.5 M)

8.75 g NaCl (1.5 M)

0.05% PBST or TBST

0.5 ml Tween were add to PBS or TBS

Lysis Buffer for Western blot

150 mM NaCl,

10 mM Tris, PH 7.4

1 % Triton

0.1 M DTT

1 mg/ml Pepstatin A

1 mg/ml Leupeptin

40 mg/ml Pefa-block

Tris-glycine-SDS Running Buffer

25.0 mM Tris

192.0 mM Glycine

0.1 % SDS

Transfer Buffer

25.0 mM Tris

192.0 mM Glycine

20 % Methanol

Blocking Buffer

5 % milk powder in PBST

Stripping Buffer

0.2 M NaOH

5X (Tris-borate-EDTA) TBE buffer

450 mM Tris

450 mM Boric acid

20 mM EDTA, pH 8.0

6X Glycerol loading buffer

10 mM Tris/HCl, pH 7.5

10 mM EDTA, pH 8.0

30 % Glycerol

0.01 % Bromophenol blue

0.01 % Xylene green

Isotonic GA/PFA

1 % Glutaraldehyde

0.3 % Paraformaldehyde

0.07 M Sodium cacodylate buffer

2.1.6 Antibodies

2.1.6.1 Secondary antibodies

Secondary antibody	Use	Source
cy2 conjugated donkey anti-mouse IgG (H+L)	ICC	Jackson laboratories via Dianova (Hamburg,Germany)
cy2 conjugated donkey anti-goat IgG (H+L)	ICC	Jackson laboratories via Dianova
cy2 conjugated donkey anti-rat IgG (H+L)	IHC/ICC	Jackson laboratories via Dianova
Rabbit anti-goat Immunoglobulins	IHC/ICC	Z0454, DAKO
Goat anti-rabbit IgG peroxidase	WB	A6154, Sigma Aldrich
Goat anti-mouse IgG peroxidase	WB	A4416, Sigma Aldrich
Rabbit anti-goat IgG peroxidase	WB	A5420, Sigma Aldrich

2.1.6.2 Primary antibodies

WB: Western blot, ICC: Immunocytochemistry, IHC: Immunohistochemistry

Primary antibody	Use	Source
Rabbit polyclonal anti-phospho-Src (Y419)	WB	AF2685, R&D (Wiesbaden, Germany)
Rabbit polyclonal anti-phospho-PECAM-1 (tyr713)	WB	A0547, Assaybiotech (Herford, Germany)
Rabbit monoclonal anti-Src (36D10)	WB	2109, Cell Signaling (Frankfurt, Germany)
Goat polyclonal anti-ephrinB1	WB/IHC	AF473, R&D
Goat polyclonal anti-ephrinB2	WB/IHC	AF496, R&D
Goat polyclonal anti-VE-cadherin (C19)	WB	sc6458, Santa Cruz
Mouse monoclonal anti-E-selectin	IHC	sc71017, Santa Cruz
Mouse monoclonal anti-VE-cadherin	WB	MAB1989, Millipore (Schwalbach, Germany)
Goat polyclonal anti-EphB2	WB/IHC	AF467, R&D
Mouse monoclonal anti-β-actin (AC-15)	WB	6276, Abcam (Cambridge, UK)
Goat polyclonal anti-CD31 (M20)	WB	sc1506, Santa Cruz
Rat monoclonal anti-CD31 (MEC 13.3)	IHC	sc18916, Santa Cruz
Mouse monoclonal anti-VE cadherin (BV6)	ICC	MAB1987, Millipore
Rat monoclonal anti-F4/80	IHC	T2006, Dianova (Hamburg, Germany)
Rat anti-Mac-3	IHC	550292, BD pharmingen (Heidelberg, Germany)
Mouse monoclonal anti-human PECAM-1 (JC70A)	ICC	M0832, DAKO
HRP-Rabbit anti-human vWF	ELISA	P0026, DAKO

2.1.7 Recombinant proteins

Recombinant protein	Dosage	Source
Human IgG1 Fc MAb (Clone 97924)	1 µg/ml	MAB110, R&D
Recombinant human monocyte chemoattractant protein-1 (MCP-1)	30 ng/ml	279-MC-010, R&D
Recombinant mouse monocyte chemoattractant protein-1 (MCP-1)	2 ng/ml	479-JE-010, R&D
Recombinant human TNFα	1000 U/ml	210-TA-010, R&D
Recombinant mouse TNFα	2 ng/ml	GWB-47E270, Geneway Biotech (San Diego, USA)
Recombinant mouse EphB2/Fc	2 µg/ml	467-B2-200, R&D

2.1.8 Consumables

Tissue culture plates were purchased from Greiner or TPP. Test tubes were obtained from Sarstedt. Transmigration transwells and compatible plates were obtained from BD or Greiner Bio One. Cell scrapers were purchased from Sarstedt Inc**.**

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Isolation and culture of human umbilical vein endothelial cells (HUVECs)

Human umbilical vein endothelial cells were isolated from umbilical cords according to the approval #336/2005 by the local ethics committee. Blood in the umbilical veins was flushed out with 20 ml Hank's buffer. The veins were filled with dispase solution (3.1 g/l) and incubated for 30 minutes at 37°C. The solution together with flushed Hank's solution was centrifuged at 1000 rpm for 5 min, and the pellet was re-suspended in endothelial cell growth medium containing supplements, 5 % FBS, 50 U/ml penicillin, 50 µg/ml streptomycin and 0.25 µg/ml Fungizone® antimycotic. The cells were cultured on standard plates pre-coated with 2 % (w/v) gelatine.

2.2.1.2 siRNA transfection in HUVECs

To transfect one well of a 6-well plate, the transfection complex was made with 3 µg of siRNA and 3 µl of MATra-si reagent in Opti-MEM® medium to a final volume of 200 µl, then mixed thoroughly and incubated for 20 minutes at room temperature. The siRNA complex was added dropwise to the cells. Then the plate was placed on a magnetic plate (Universal Magnet Plate, IBA) for 20 minutes in the incubator allowing the beads to penetrate into the cells. For ephrinB1, efficient knockdown was achieved after 48 hours. However, for sufficient deletion of ephrinB2, cells were subjected to a second transfection after 24 hours (Figure 4).

2.2.1.3 Stimulation of endothelial cells

Endothelial cells were treated with either clustered EphB2 recombinant protein or EphB2-overexpressing mouse myeloma cells. Clustered EphB2 recombinant protein was produced by incubating 2 µg/ml EphB2/Fc and 1 µg/ml anti-IgG Fc antibody for 1 hour at room temperature. 7×10^6/ml mock-transfected and EphB2-overexpressing mouse myeloma cells were administered to the HUVECs on one well of a 6-well plate. In addition, JNK inhibitor, SP600125 (20 µM), and inhibitor of Src family kinases, PP2 (10 µM), were applied 1 hour before exposing HUVECs either EphB2/Fc or EphB2-overexpressing mouse myeloma cells.

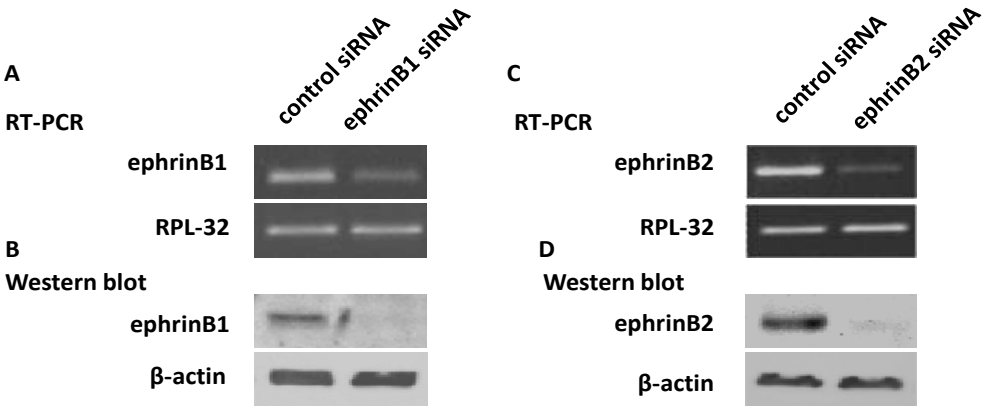

Figure 4: Silencing of ephrinB1 and ephrinB2 mRNA expression in HUVECs.
HUVECs were treated with control siRNA or ephrinB2-specific siRNA or ephrinB1-specific siRNA by magnet-induced transfection. Knockdown efficiency was assessed by PCR (A, C) and Western blot (B, D).

2.2.1.4 Culture of mouse myeloma cells

The mouse myeloma cells P3XTB.A7 were cultured in suspension in RPMI1640 medium, supplemented with 10 % FBS, 50 U/ml penicillin, 50 µg/ml streptomycin and 0.25 µg/ml Fungizone® antimycotic.

2.2.1.5 Generation of EphB2-overexpressing mouse myeloma cells

To get human EphB2 sequence (length 2961bp, Pubmed: 017449), monocyte cDNA template, a pair of designed primer and expand long template PCR system were applied for PCR as follows:

Primer	Amplification	Number of Cycles
Forward: CACCATGGCTCTGCGGAGGCTGGGG GC Reverse: TCAAACCTCCACAGACTGAATCTGGTT CATCTGCG	95°C 30 seconds 58°C 30 seconds 72°C 3 minutes	32

DNA product was purified with the StrataPrep PCR purification kit. Subsequently, the concentration and purity of the EphB2 product were determined by using the NanoDrop ND-1000 spectrophotometer and the absorption of sample A260/280 would be 1.8-2.0. TOPO cloning reaction was carried out using the pcDNA ^{TM} 3.1 Directional TOPO kit. DNA was transformed into competent E. coli. Eight clones were selected and cultured in LB medium containing 50 µg/ml ampicillin overnight individually. Bacterial stock (100 µl H_2O, 100 µl glycerol, 800 µl bacteria) was made. Meanwhile, the construct was extracted by the QIAprep Spin Miniprep kit and analyzed by sequencing. The bacterial stock with correct EphB2 sequence was re-cultured. The plasmid was then extracted and purified by the Maxi Plasmid Purification kit, and eventually eluted with sterile RNase-free water. For the mock, the TOPO cloning reaction without DNA product was performed as described above.

Mouse myeloma cells were transiently transfected with mock or EphB2 plasmid by polyethylenimine (PEI). 1.5×10^6/ml cells were seeded in one well of a 6-well plate in RPMI 1640 medium containing 10 % FBS without antibiotics. 2.5 µg plasmid was incubated with 10 µg PEI in 50 µl Opti-MEM® medium for 15 minutes at room temperature. The mixture was added dropwise to the myeloma cells followed by 6 hours incubation. The transfection process was terminated by adding 2.5 ml complete culture medium. After 48 hours, transfected myeloma cells were ready for stimulation of HUVECs.

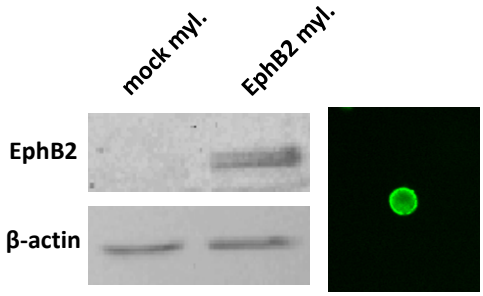

Figure 5: Generation of mock-transfected and EphB2-overexpressing mouse myeloma cells. Western blot and immunofluorescence were performed to analyze the expression of EphB2 in mock-transfected and EphB2-overexpressing mouse myeloma cells. EphB2 was abundantly expressed in EphB2-overexpressing cells but not in mock-transfected cells (left panel). EphB2 is located on the surface of these cells (green, right panel).

2.2.1.6 Culture of brain endothelial cells

bEnd5 cells were cultured in MCDB-131 medium supplemented with 20 % FBS, 2 mM L-glutamine solution, 2 mM penicillin-streptomycin solution, 0.05 mg/ml ECGS, 0.1 mg/ml heparin salt and 1 g/L sodium bicarbonate. This was done by collaborator Dr. Kavi Devraj.

2.2.2 PCR

2.2.2.1 RNA isolation from cells and tissues

Cells were rinsed with ice-cold PBS, and the total RNA was extracted with the PeqGOLD total RNA kit according to the manufacturer's instructions. Tissues were homogenized by sonification in the lysis buffer and RNA was extracted using the RNeasy kit. Finally, RNA was eluted with 30-50 µl RNase-free H_2O and the concentration was measured by using the NanoDrop ND-1000 spectrophotometer.

2.2.2.2 Reverse transcription

Reverse transcription of 500 ng RNA to cDNA was performed by using the Omniscript RT kit according to the manufacturer´s instructions, and the mixture was incubated at 37°C for 1 hour.

2.2.2.3 Polymerase chain reaction (PCR)

The semi-quantification PCR was performed with 5 µl cDNA (5 µg/µl), 1 µl forward primer (20 µM), 1 µl reverse primer (20 µM), 0.75 µl $MgCl_2$, 1.5 µl dNTP, 5 µl 10× buffer and 35.5 µl H_2O to a total volume of 50 µl. PCR amplification was made in an automatic thermocycler (Biometra) using the following programs.

step	temperature	time
Pre-denaturation	95°C	5 minutes
Denaturation Annealing Synthesis	95°C 55-60°C 72°C	30 seconds 30 seconds 1 minutes
Extension	72°C	5-10 minutes

2.2.2.4 Agarose gel electrophoresis

Agarose gel was made by dissolving 1.5 g agarose in 100 ml TEB buffer, and added 4 µl ethidium bromide additionally. The PCR products were mixed with 6x loading buffer. Electrophoresis was performed at 130 V for 45 min. The DNA standard was used to estimate the molecular size of DNA band. The relative band intensity was quantified by Image J.

2.2.2.5 Quantitative real time PCR

Real time PCR was performed with LightCycler instrument (Roche Diagnostics, Penzberg, Germany) by using the QuantiTect SYBR Green® kit. In brief, 5 µl cDNA (100 ng/µl), 1 µl forward primer (20 µM), 1 µl reverse primer (20 µM), 3 µl RNase free H_2O, and 10 µl SYBR Green were added together to a final volume of 20 µl.

2.2.3 Immunohistochemistry and immunocytochemistry

2.2.3.1 Tissue preparation, embedding in paraffin and sectioning

Mice were sacrificed by CO_2 inhalation and cervical dislocation. The chest was opened to expose the heart. A hole was cut in the right ventricle to allow the blood to come out. The left ventricle was cannulated and perfused with Ringer's solution and zinc fixative.

Tissues were isolated and fixed in zinc fixative to retain their antigen and morphology overnight. Then they were dehydrated by passing through 70 %, 85 %, 96 % ethanol and isopropanol. The tissues were incubated in melted paraffin at 60°C overnight. The next day, the tissues were embedded in paraffin blocks and stored at room temperature. The sections of 5 µm thickness were cut by using a microtome and dried for 18 hours at 40°C. Then they were processed for immunofluorescence staining.

2.2.3.2 Cell fixation

Endothelial cells were fixed in methanol for 15 minutes at 4°C. Thereafter, sucking away methanol allows cells to dry. Afterwards, they were further processed for immunofluorescence staining.

2.2.3.3 Immunofluorescence staining

Paraffin sections were rehydrated by passing through Xylol, Xylol, isopropanol, 96 % ethanol, 85 % ethanol, 70 % ethanol and ddH_2O.

The fixed cells or rehydrated sections were blocked by using casein blocking buffer for 30 minutes. Then the cells or sections were incubated with primary antibodies diluted in blocking buffer for 18 hours at 4°C. After completing the washing steps, the cells or sections were incubated with appropriate secondary antibodies for 1 hour at room temperature. For ephrinB1 and ephrinB2 staining, fluorescence amplification system was used after the secondary antibody of rabbit anti-goat. The cells or sections were incubated with DAKO Envsion™ Labeled polymer-HRP anti-rabbit for 30 minutes, followed by TSA™-Cy3 system for 20 seconds. Afterwards, the nuclei were counterstained by DAPI (2 µg/mL, diluted in PBS) for 10 minutes, and the cells or sections were mounted with Mowiol.

2.2.3.4 Confocal microscopy

Confocal microscopy, IX81 microscope equipped with IX-DSU disk unit and the MT20 multiwavelength illumination system, was applied to analyze immunostained tissue sections and cells. Quantitative image analyses were made by using the Olympus CellR-software (Olympus, Hamburg, Germany) or Image J (NIH, USA).

2.2.4 Protein biochemistry

2.2.4.1 Isolation of cellular protein

The cells were rinsed once by ice-cold PBS. Lysis buffer was added onto the cells and incubated for 25 minutes on ice. Afterwards, the lysate was centrifuged at 15000 rpm for 3 minutes at 4°C, and the supernatant was collected in a fresh tube.

2.2.4.2 Co-immunoprecipitation

To precipitate ephrinB2, EphB4-Fc (4 µg/ml) was added to the cell lysate and incubated for 2 hours at room temperature in the rolling machine. Protein A magnetic beads were washed by PBS (pH 7.4) and combined with the mixture to incubate for 2 hours. EphrinB1 was precipitated by utilizing an anti-ephrinB1 antibody (A20) and protein A/G agarose beads using the same procedures described above. Thereafter, precipitates were washed by PBS, mixed with 2×

loading buffer and denatured for 10 minutes at 95°C. Then the samples were further analyzed by SDS-PAGE and western blot.

2.2.4.3 Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were denatured by adding 4× loading buffer and heating them up for 5 minutes at 95°C. 10 % separating gel was prepared and overlaid after polymerization with 4 % stacking gel according to the following recipes. The protein standard was used to check the running progress and to estimate the molecular mass of protein bands. The denatured protein samples were loaded on the gels. Proteins were separated by electrophoresis in running buffer at 80 V for the stacking gel, and 120 V for the separating gel.

	10 % separating gel 10 ml	4% stacking gel 4 ml
ddH_2O	4 ml	2.7 ml
1.5 M Tris-Cl pH8.8	2.5 ml	-
1 M Tris-Cl pH6.8	-	0.5 ml
10 % SDS	0.1 ml	40 µl
10 % APS	0.1 ml	40 µl
30 % Acrylamide	3.3 ml	0.67 ml
TEMED	4 µl	4 µl

2.2.4.4 Western Blotting

The proteins separated by SDS polyacrylamide gel were blotted onto nitrocellulose membrane at 350 mA for 30 to 90 minutes, depending on the molecular weight of the protein of interest. Then, the membrane was immersed into blocking buffer for 30 minutes to prevent the non-specific binding. The membrane was incubated with the first antibody, which was diluted in PBST, for 18 hours at 4°C. Subsequently, the membrane was incubated with appropriate horseradish peroxidase conjugated secondary antibody (1:5000 diluted in blocking buffer) for 1 hour at room temperature. Afterwards, the membrane was developed with Luminata™ Forte

western HRP substrate according to the manufacturer´s instruction. The protein band of interest was detected using Image Quant ^{TM} LAS 4000 mini machine (GE Healthcare) and final analyzed by image J software. If necessary, the blot was incubated in stripping buffer to remove the antibodies, thereby allowing other proteins to be examined in the same blot by re-probing.

2.2.5 Leukocyte adhesion assay

HUVECs were pre-treated either with anti-IgG Fc antibody as control or with pre-clustered EphB2/Fc for 6 hours at 37°C, 5 % CO_2. Afterwards, HUVECs were rinsed by Hank's buffer to get rid of these stimuli, which bind to Fc receptors on THP-1 cells. Calcein AM labeled THP-1 cells were then added to the HUVEC monolayer for 2 hours. The non-adherent THP-1 cells were removed by rinsing with PBS. The number of adherent THP-1 cells was determined by analyzing images with the Image J software.

2.2.6 Transendothelial migration assay

EphrinB1 or ephrinB2 expression was silenced by magnetic transfection of siRNA as described before. HUVECs were then cultured in transwell inserts (8 µm pore size, 24-well format) for 18 hours to form a confluent monolayer. 2×10^5 THP-1 cells were added in the upper chamber. The medium in the lower chamber was supplemented with 30 ng/ml MCP-1. After 18 hours, transmigrated THP-1 cells were collected from the lower chamber and counted using the hemocytometer.

2.2.7 Permeability assay

200.000/cm^2 bEnd5 cells were seeded onto transwell inserts (1.0 µm pore size, 24-well format) coated with 5 µg/cm^2 fibronectin and cultured for 8 days until they formed a monolayer. 5 µM 3 kD TXR-,10 µM 20 kD TMR- and 10 µM 70 kD FITC-labeled dextrans were applied to the upper chamber upon treatment of bEnd5 cells with anti-IgG Fc antibody or pre-clustered EphB2/Fc in serum-free MCDB-131 medium for 8 hours. Aliquots from the lower chamber and the upper chamber were collected after 1 hour and the amount of dextrans was measured in a fluorescence plate reader. Permeability was calculated as lower/upper chamber

fluorescence ratio. The ratio for control conditions was set to 100 %. This assay was done by collaborator Dr. Kavi Devraj.

2.2.8 vWF on cell ELISA

vWF deposition on endothelial cell surface was measured by an *in situ* cell ELISA. HUVECs were exposed to mock-transfected and EphB2-overexpressing mouse myeloma cells for 1 hour, and fixed by isotonic GA/PFA. The fixed cells were blocked with 0.4 % BSA in PBS for 1 hour, and incubated with HRP-rabbit anti-human vWF antibody for 1 hour. The abundance of vWF on the endothelial cell surface was quantified at 405 nm through an ABTS substrate reaction. This assay was done by collaborator Dr. Kerstin Möller.

2.2.9 SHP2 activity assay

HUVECs were exposed to mock-transfected and EphB2-overexpressing mouse myeloma cells for 30 minutes. SHP2 activity was measured using active SHP2 DuoSet IC kit according to the manufacture's instruction.

2.2.10 Animal experiments

All animal experiments were performed with permission of the Regional Council Karlsruhe and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.2.10.1 Culture of murine femoral artery segments

Mice were euthanized by CO_2 inhalation and cervical dislocation. Femoral artery segments were harvested and cultured in DMEM medium supplemented with 15 % FBS and antibiotics. 2 ng/ml mouse TNFα was applied to the artery for 6 hours at 37°C, 5 % CO_2. To explore ephrinB1 expression, the tissue samples were further processed for immunofluorescence staining.

2.2.10.2 Atopic dermatitis model

Atopic dermatitis was performed with VAFNC/NgaTnd Crj mice. The mice were sensitized by cutaneous topical application of 150 ml of 5 % 2,4,6-Trinitrochloro-benzene (picryl chloride, PCl) in acetone and olive oil (95:5) to their bellies and

footpads. Afterwards, the mice were further challenged by cutaneous topical application of 150 ml 0.5 % PCl in olive oil to the back skin once a week for up to 5 weeks. Inflamed skin samples were harvested and processed for immunofluorescence analyses.

2.2.10.3 Generation of ephrinB2$^{EC\text{-}iKO}$ mice

ephrinB2$^{flox/flox}$ mice were crossbred with Tie2-CreER^{T2} transgenic mice to generate Tie2-CreER^{T2}/ephrinB2$^{flox/flox}$ mice. These mice were received intraperitoneal injection of tamoxifen in Miglyol at a dose of 1 mg per day for consecutive 5 days and then waited for 2 weeks. This induces endothelial-specific ablation of *ephrinB2,* which was termed as ephrinB2$^{EC\text{-}iKO}$ mice (Figure 6). For control, Tie2-CreER^{T2}/ephrinB2$^{flox/flox}$ mice were received the same amount of Miglyol. Knockdown efficiency was assessed by ephrinB2 immunostaining in distinct arteries. To investigate Tie2 promoter distribution, R26R/Tie2-CreER^{T2} transgenic mice were subjected to tamoxifen to induce the activity of Cre recombinase. Report gene LacZ expression was indicated by X-gal staining in the different vessels (aorta, femoral artery, auricle vessel) according to protocol. This staining was done by Dr. Evelyn Ernst.

2.2.10.4 Generation of ephrinB2$^{EC\text{-}iKO}$/ApoE$^{-/-}$ mice

Tie2-CreER^{T2}/ephrinB2$^{flox/flox}$ mice were crossbred with ApoE$^{-/-}$ mice to produce Tie2-CreER^{T2}/ephrinB2$^{flox/flox}$/ApoE$^{-/-}$ mice. Tamoxifen injection was administrated in 2 month-old mice as described above, namely ephrinB2$^{EC\text{-}iKO}$/ApoE$^{-/-}$ (Figure 6). In addition, Miglyol was injected as control. When the mice grew up to 6 months of age, they were sacrificed by CO_2 inhalation and then perfused with Ringer's solution and zinc fixative. The aortic arch, including its main branchpoints (brachiocephalic trunk, left common carotid artery and left subclavian artery) was harvested and fixed. Atherosclerotic lesions were analyzed by Sudan III staining using the following procedure: H_2O 1 minute: 70 % ethanol 5 minutes; Sudan III diluted in 70 % ethanol 30 minutes; 80 % ethanol 3 minutes; H_2O 3 times. Moreover, these aortas were dehydrated, embedded in paraffin, and further processed for Mac-3 immunostaining to determine the macrophage contents in the plaques.

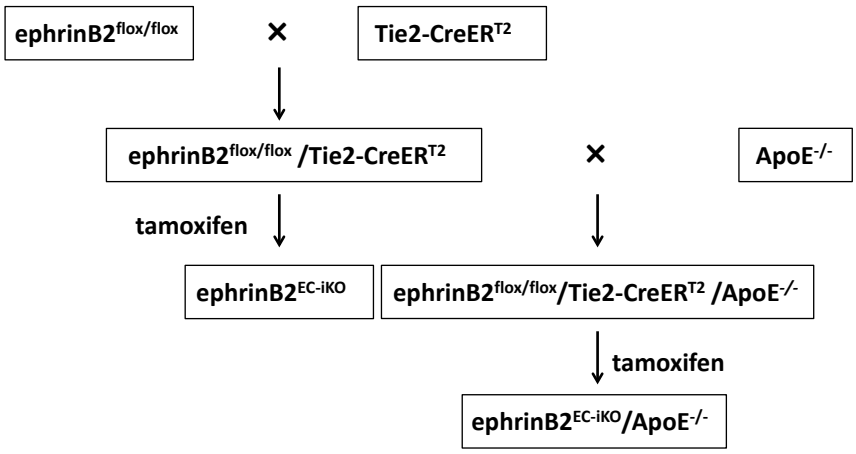

Figure 6: Breeding scheme for producing inducible endothelial cell specific ephrinB2 knockout mice.

2.2.10.5 TPA-induced ear edema

Edema was induced by topically applying 1 µg TPA (12-Otetradecanoylphorbol-13-acetate) dissolved in 20 µl of acetone on the outer surface of one ear. The other ear was applied with acetone as control. After 48 hours, mice were sacrificed by CO_2 inhalation and cervical dislocation, and then perfused with Ringer's solution and zinc fixative. The ears were harvested and processed for histological examination.

2.2.10.6 Arteriogenesis model

Mouse hindlimb ischemia model was generated as described before [124]. Mice were anesthetized with isoflurane, and the femoral artery was ligated distal to the origin of the deep femoral artery. After 7 days, the mice were euthanized by CO_2 inhalation and cervical dislocation, and then perfused with Ringer's solution and zinc fixative with colored pigment, which could not pass through the capillary system allowing for the morphological assessment of growing collateral arterioles. The muscles containing the ligated femoral artery and corresponding collateral arterioles were harvested and further processed for histological examination.

Colored pigment:

8 g HKS® Designers' Gouache (Schmincke) dissolved in 50 ml zinc fixative.

2.2.10.7 Thioglycollate peritonitis model

Mice were injected intraperitoneally with 1 ml of 4 % thioglycollate broth, and euthanized by CO_2 inhalation and cervical dislocation after 6 hours. Peritoneal exudate cells were collected by performing lavage with 5 ml of PBS. The total cell number was estimated by cell counter (Millipore). Macrophages were identified by using Mac-3 staining. Peritoneal tissues were harvested to determine the number of accumulated macrophages by histological examinationd.

2.2.11 Statistical analysis

All results are expressed as means ± SD. Differences between 2 matched experimental groups were analyzed by unpaired Student's t-test (InStat 3.0; GraphPad, USA), with a probability value of $p<0.05$ considered statistically significant. Differences among 3 or more experimental groups were analyzed by one-way ANOVA followed by a Bonferroni post hoc test for selected pairs of groups (InStat 3.0; GraphPad, USA), with a probability value of $p<0.05$ considered statistically significant.

3 RESULTS

3.1 Analysis of the role of endothelial ephrinB2 in inflammation

3.1.1 Expression of ephrinB2 and EphB2 in inflamed skin

To investigate the impact of endothelial ephrinB2 on inflammation, the localization and expression of ephrinB2 and its receptor EphB2 were assessed in a mouse model of atopic dermatitis. Angiogenesis, as a hallmark of inflammation, leads to expanding blood flow in inflamed tissue and further leukocyte transmigration [142]. Immunofluorescence analyses of samples from inflamed mouse skin revealed an increase in the number of PECAM-1-positive capillaries indicating the occurrence of angiogenesis (Figure 7A, C and D). The percentage of ephrinB2/PECAM-1 double-positive capillaries increased significantly during inflammation (Figure 7B, E and F). Additionally, subpopulations of F4/80-positive macrophages in the inflamed tissue expressed the receptor EphB2 on the cell surface (Figure 7G-L).

3.1.2 Effect of endothelial ephrinB2 on monocyte diapedesis and underlying mechanisms

3.1.2.1 Impact of endothelial ephrinB2 on THP-1 cell diapedesis

To analyze the impact of endothelial ephrinB2 on monocyte diapedesis, HUVECs which had been pre-treated with siRNA to knockdown baseline expression of ephrinB2, were seeded on a transwell insert to form a monolayer. THP-1 cells, a monocytic cell line abundantly expressing EphB2 [139], were seeded on top of the HUVEC monolayer. MCP-1 was added to the lower chamber to induce transmigration of the THP-1 cells. The number of transmigrated THP-1 cells in the lower chamber was counted. Silencing of ephrinB2 expression attenuated basal as well as MCP-1-induced THP-1 cell transmigration (Figure. 8).

3.1.2.2 Partial colocalization of ephrinB2 and PECAM-1 at endothelial cell-cell contacts

To elucidate the distribution of ephrinB2 in the HUVEC monolayer, immunofluorescence staining was performed and revealed that ephrinB2 was distributed at interendothelial cell contacts, and partially colocalized with PECAM-1, which is a crucial component of endothelial junctions and required for leukocyte transmigration across the endothelial cell monolayer [143].

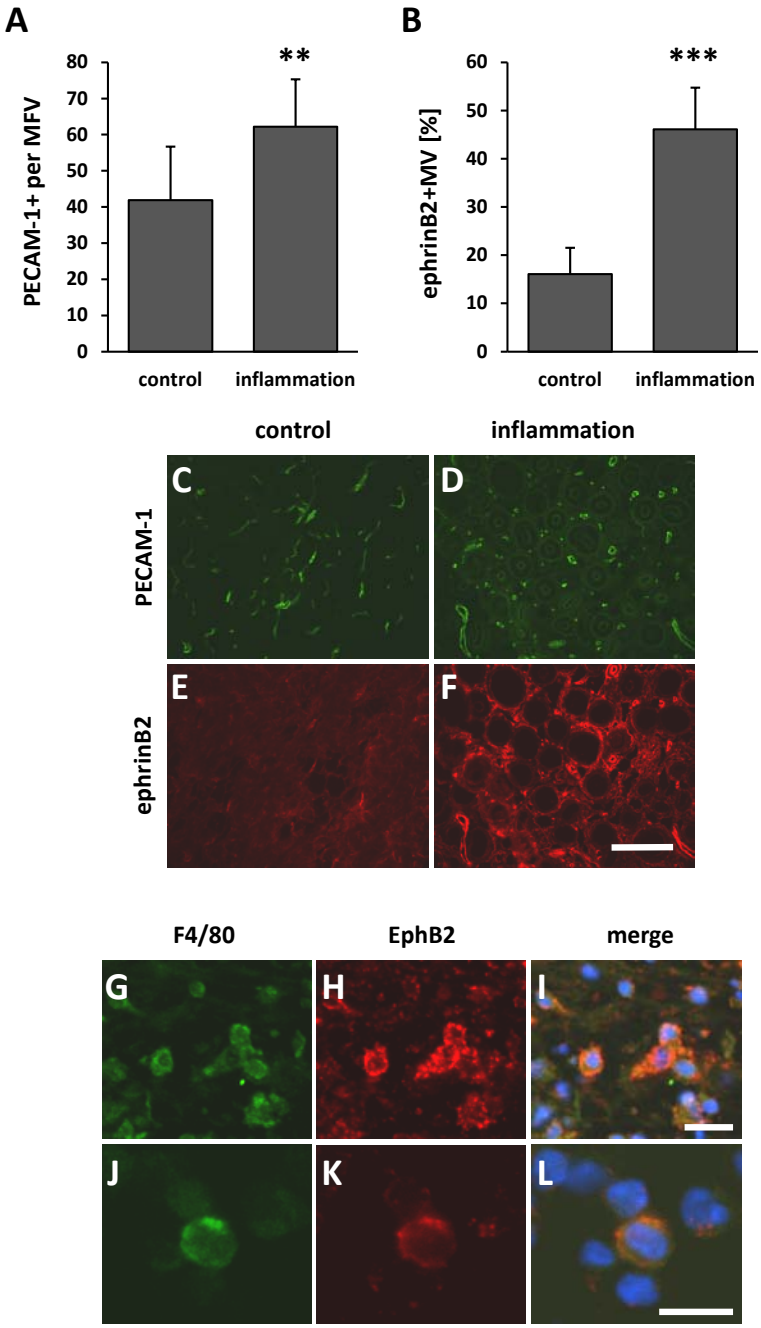

Figure 7: EphrinB2 expression is upregulated during inflammation-associated angiogenesis.

EphrinB2 (red), PECAM-1 (green), EphB2 (red) and F4/80 (green) were detected by immunofluorescence techniques in inflamed mouse skin. Inflammation was associated with an increase in the number of PECAM-1-positive capillaries as indicated by the mean fluorescence value (MFV) of PECAM-1 (A, **p<0.01 vs. control, n=10; compare C and D). The percentage of ephrinB2/PECAM-1 double-positive capillaries increased during inflammation, as PECAM-1 staining was used to normalize the number of capillaries (B, ***p<0.001 vs. control, n=5; compare E and F; scale bar: 100 µm). Moreover, EphB2 receptor was detected on the surface of F4/80-positive macrophages (G-I; scale bar: 20 µm; J-L; scale bar: 10 µm).

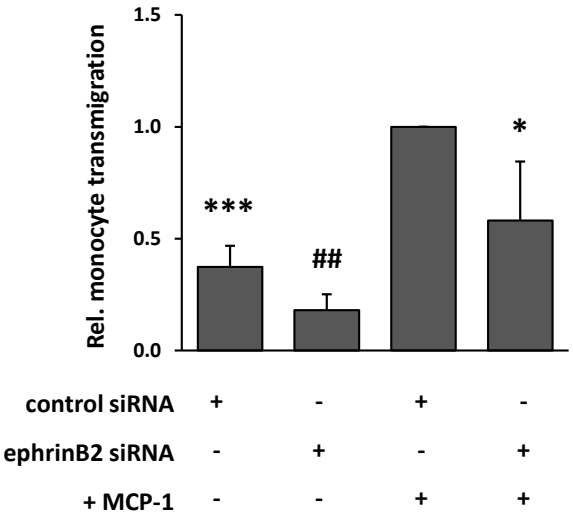

Figure 8: Loss of ephrinB2 attenuates the transmigration of THP-1 cells across an endothelial cell monolayer.

The relative fold of THP-1 cells transmigrating through a HUVEC monolayer in response to MCP-1 was decreased upon siRNA-mediated ephrinB2 knockdown in endothelial cells (***p<0.001, *p<0.05 vs. control siRNA + MCP-1 (set to 1), ##p<0.01 vs. control siRNA, n=6)

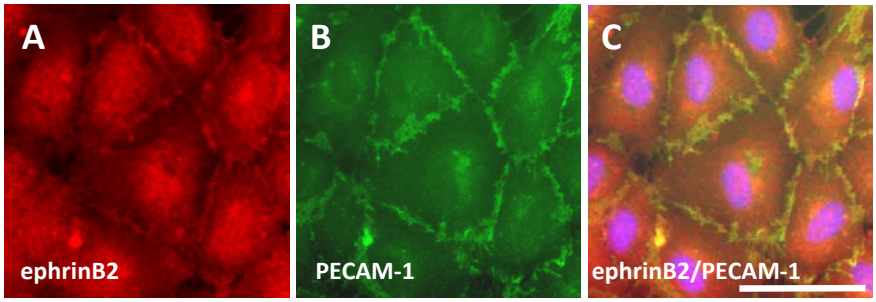

Figure 9: EphrinB2 colocalizes with PECAM-1 in the endothelial cell junctions.

Immunofluorescence staining showed partial colocalization of ephrinB2 (red) and PECAM-1 (green) at intercellular contact sites of a confluent HUVEC monolayer (A-C; scale bar: 50 µm).

3.1.2.3 Analysis of the association of ephrinB2 with PECAM-1 and the phosphorylation of PECAM-1 upon EphB2 stimulation

To further analyze how the association of PECAM-1 and ephrinB2 changes in endothelial cells upon interaction with monocytes, human EphB2-overexpressing mouse myeloma cells were utilized to mimic a characteristic of monocytes along with mock-transfected myeloma cells as negative control. HUVECs were exposed to these cells and subsequent pull-down experiments with cell lysates were carried out. This result revealed that there was little co-precipitation of ephrinB2 and PECAM-1 upon exposure of HUVECs to mock-transfected myeloma cells (Figure 10A). This interaction between ephrinB2 and PECAM-1 was significantly enhanced when HUVECs were exposed to EphB2-overexpressing mouse myeloma cells (Figure 10A). Treatment of HUVECs with the same stimuli elicited PECAM-1 phosphorylation (Figure 10B) which is frequently associated with leukocyte transmigration [144, 145].

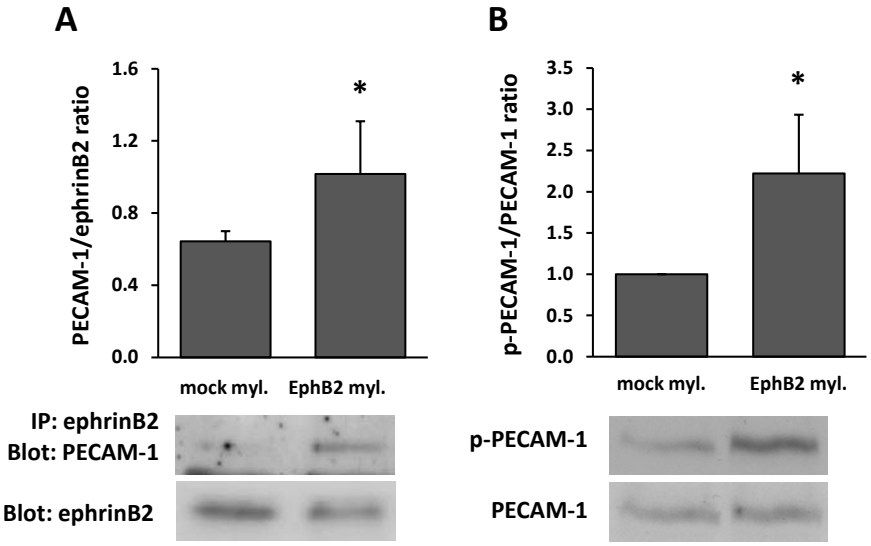

Figure 10: EphB2 stimulation of HUVECs enhances the association of ephrinB2 with PECAM-1 and also induces PECAM-1 phosphorylation.

Co-immunoprecipitation revealed that PECAM-1 associated with ephrinB2 in HUVECs upon exposure to EphB2-overexpressing mouse myeloma cells for 3 hours as compared to control (A, *p<0.05 vs. mock myeloma, n=5). Likewise, PECAM-1 phosphorylation was triggered by the same stimuli within 30 minutes (B, *p<0.05 vs. mock myeloma (set to 1), n=5).

Since the phosphorylation of PECAM-1 largely depends on Src family kinases (SFKs) [146], its inhibitor PP2 was used to clarify whether SFKs are involved in mediating EphB2-induced phosphorylation of PECAM-1. In fact, PP2 abrogated both basal and EphB2-triggered PECAM-1 phosphorylation in HUVECs (Figure 11A). Moreover, Src activity was increased in HUVECs upon interaction with EphB2-overexpressing mouse myeloma cells, as evidenced by phosphorylation at tyrosine residue 419 (Figure 11B). In addition, a reduction of SHP2 activity was observed (Figure 11C).

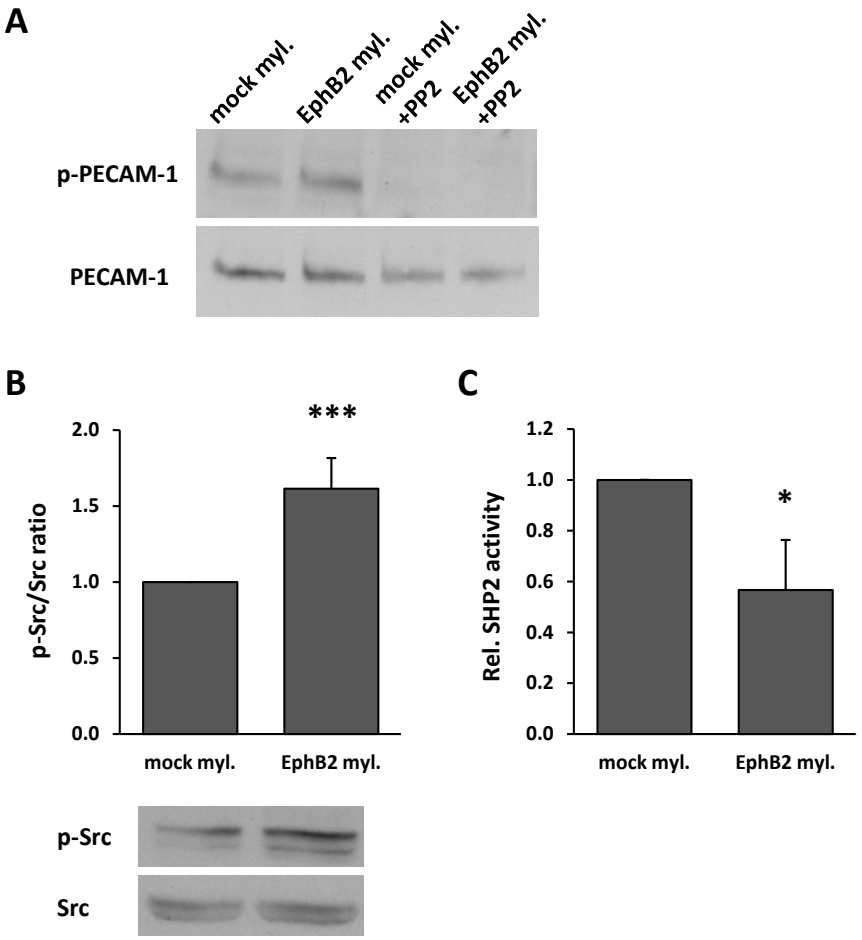

Figure 11: EphB2-induced phosphorylation of PECAM-1 depends on Src family kinases.
HUVECs were pre-treated with DMSO or PP2 (10 µM) for 1 hour before they were exposed to mock-transfected or EphB2-overexpressing mouse myeloma cells for 30 min. PP2 blocked both basal and EphB2-triggered phosphorylation of PECAM-1 as confirmed by Western blot analyses (A). Phosphorylation of Src Y419 was enhanced (B, ***$p<0.001$ vs. mock myeloma (set to 1), n=4), while SHP2 activity was diminished under these conditions (C, *$p<0.05$ vs. mock myeloma (set to 1), n=4).

3.1.2.4 Analysis of the association of ephrinB2 with adherens and tight junction proteins upon EphB2 stimulation

The aforementioned results reveal that ephrinB2 is located at the intercellular junction and associates with PECAM-1 upon EphB2 stimulation. Thus, we further investigated whether ephrinB2 is associated with endothelial cells adherens junction or tight junction proteins. Co-immunoprecipitation revealed that the interaction of VE-cadherin with ephrinB2 was enhanced when HUVECs were exposed to EphB2-overexpressing mouse myeloma cells (Figure 12A). Likewise, the association of ZO-1, a cytoplasmic tight junction protein, with ephrinB2 was strengthened as well (Figure 12B).

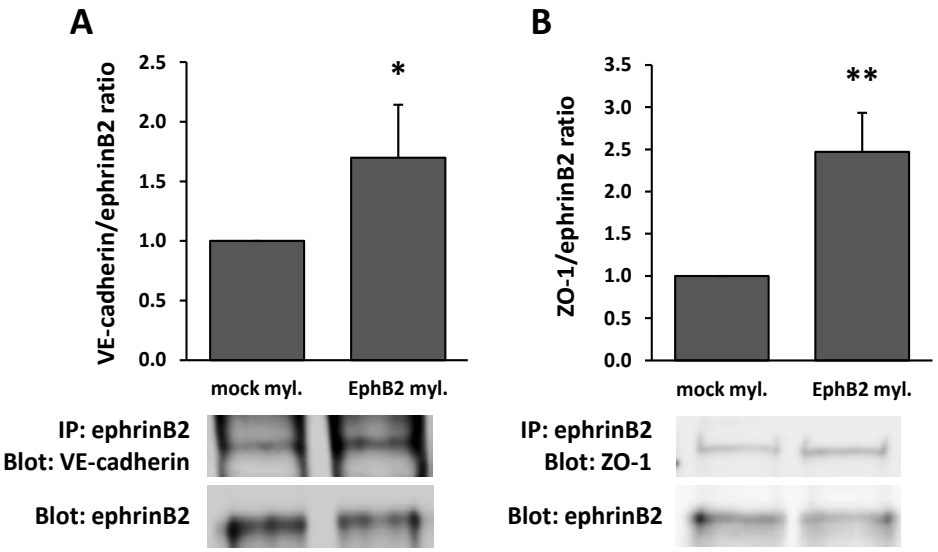

Figure 12: EphrinB2 physically interacts with adherens junction and tight junction proteins upon EphB2 stimulation.

As revealed by co-immunoprecipitation techniques, physical interaction of VE-cadherin (A) or ZO-1 (B) with ephrinB2 was enhanced upon exposure of HUVECs to EphB2-overexpressing mouse myeloma cells (*p<0.05, **p<0.01 vs. mock myeloma (set to 1), n=5).

3.1.2.5 Assessment of the distribution of VE-cadherin upon EphB2 stimulation

VE-cadherin, which is required for maintaining vascular integrity, represents a barrier for leukocyte extravasation [53]. EphB2 stimulation induced partial displacement of VE-cadherin from HUVEC junctions (Figure 13, arrow), which is critical for loosening cell contacts and promoting leukocyte diapedesis [36].

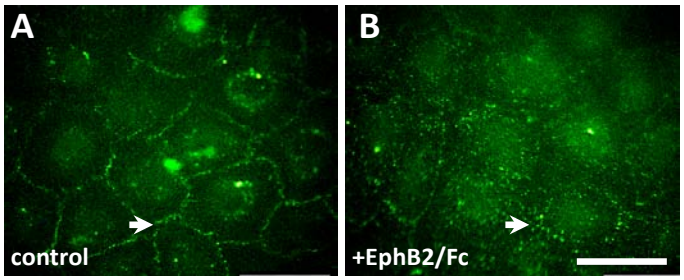

Figure 13: Internalization of VE-cadherin is induced by EphB2 stimulation of HUVECs. Immunofluorescence staining of a HUVEC monolayer was performed. VE-cadherin (green) was displaced from interendothelial cell junctions upon EphB2/Fc stimulation for 30 minutes (B), compared to anti-IgG Fc treatment as control (A, arrow; scale bar: 50 µm).

Taken together, the aforementioned results suggest that the expression of endothelial ephrinB2 increases in the capillaries of the inflamed tissue. EphrinB2 promotes monocyte diapedesis, probably due to EphB2-induced Src activity, PECAM-1 phosphorylation and VE-cadherin internalization in endothelial cells.

3.2 Examination of the impact of ephrinB reverse signaling on the pro-inflammatory responses of endothelial cells

Leukocyte adhesion is an initial process for leukocyte extravasation, including leukocyte rolling, activation and firm adhesion on the inflamed endothelial cells. Pro-inflammatory activation of endothelial cells, such as upregulated expression of adhesion molecules, plays a profound role in this process [19]. EphrinB2 has been reported to induce EphB2 forward signaling on monocytes and to promote the release of pro-inflammatory cytokines (IL-8 and MCP-1) [136]. Considering that the EphB/ephrinB system transduces signals in a bidirectional manner, we hypothesized that EphB2-stimulated reverse signaling via ephrinB ligands might affect the activation of endothelial cells.

3.2.1 Assessment of the expression of pro-inflammatory molecules in endothelial cells upon EphB2 stimulation

Exposure of HUVECs to EphB2-overexpressing mouse myeloma cells (Figure 14A) or soluble EphB2 receptor bodies (Figure 14B-D) elicited the upregulation of

E-selectin expression, both on mRNA and protein level. Since ephrinB reverse signaling has been linked to the activation of c-Jun N-terminal kinases (JNK), which is a well-known determinant of E-selectin expression [115, 147], it was assessed whether JNK is involved in EphB2-stimulated E-selectin expression. To this end, JNK activity was inhibited by SP600125 prior to EphB2 stimulation and this blocked E-selectin expression (Figure 14E).

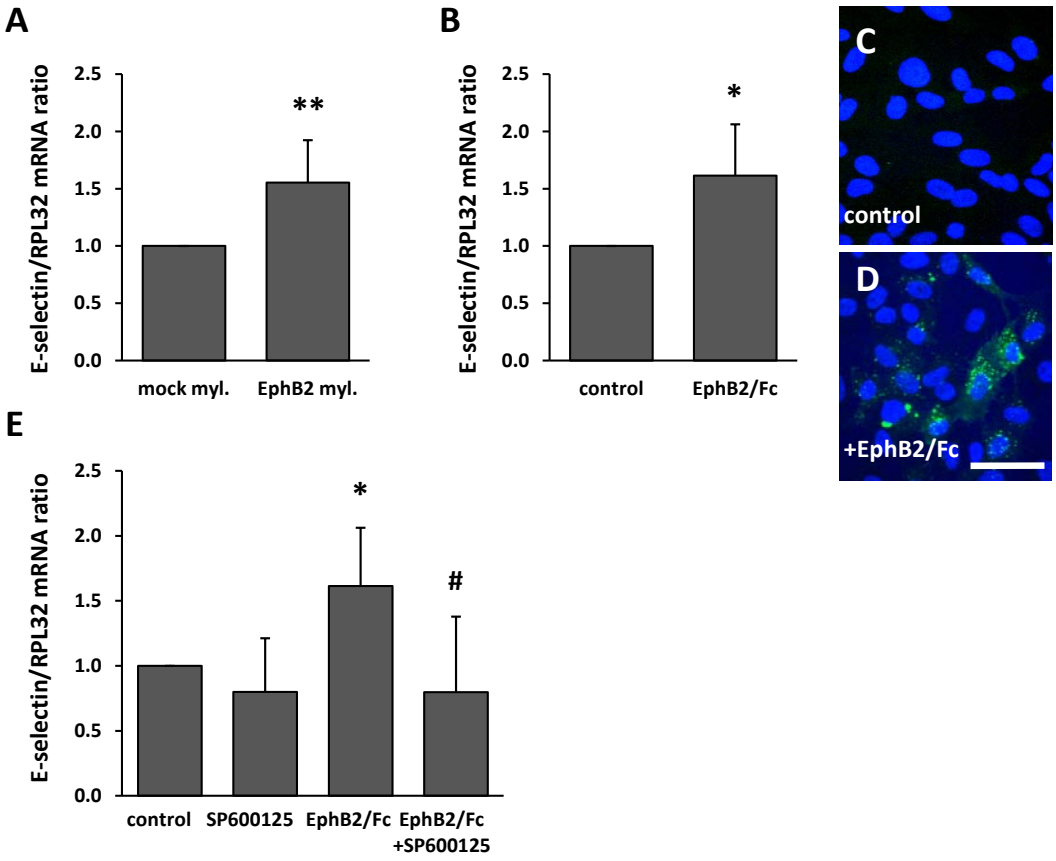

Figure 14: EphB2 stimulation triggers JNK-mediated upregulation of E-selectin expression in endothelial cells.

PCR analyses revealed an increased E-selectin mRNA expression in HUVECs when exposed to EphB2-overexpressing mouse myeloma cells (A, *p<0.05 vs. mock myeloma (set to 1), n=8) or treated with pre-clustered EphB2/Fc (B, *p<0.05 vs. anti-IgG Fc control (set to 1), n=6). Consistently, the upregulation of E-selectin expression was detected in EphB2/Fc-stimulated HUVECs by immunofluorescence techniques (C and D, green; scale bar: 50 µm). Moreover, treatment of HUVECs with the JNK inhibitor SP600125 inhibited EphB2/Fc-induced upregulation of E-selectin expression (E, *p<0.05 vs. anti-IgG Fc control (set to 1), #p<0.05 vs. EphB2/Fc, n=6).

Furthermore, treatment with EphB2-overexpressing mouse myeloma cells elicited the deposition of von Willebrand factor (vWF) on the surface of endothelial cells (Figure 15A), which is a critical determinant of platelet adhesion and leukocyte extravasation [148]. Moreover, treatment with soluble EphB2 receptor bodies resulted in an increased expression of granulocyte/macrophage colony-stimulating factor (GM-CSF) (Figure 15B), which promotes monocytes differentiation into macrophages and subsequently activates leukocytes [149, 150]. The same stimuli induced the upregulation of vascular cell adhesion molecule 1 (VCAM-1) expression (Figure 15C), which mediates leukocyte firm adhesion. In addition, intercellular adhesion molecule-1 (ICAM-1) expression was not changed by EphB2/Fc stimulation (not shown).

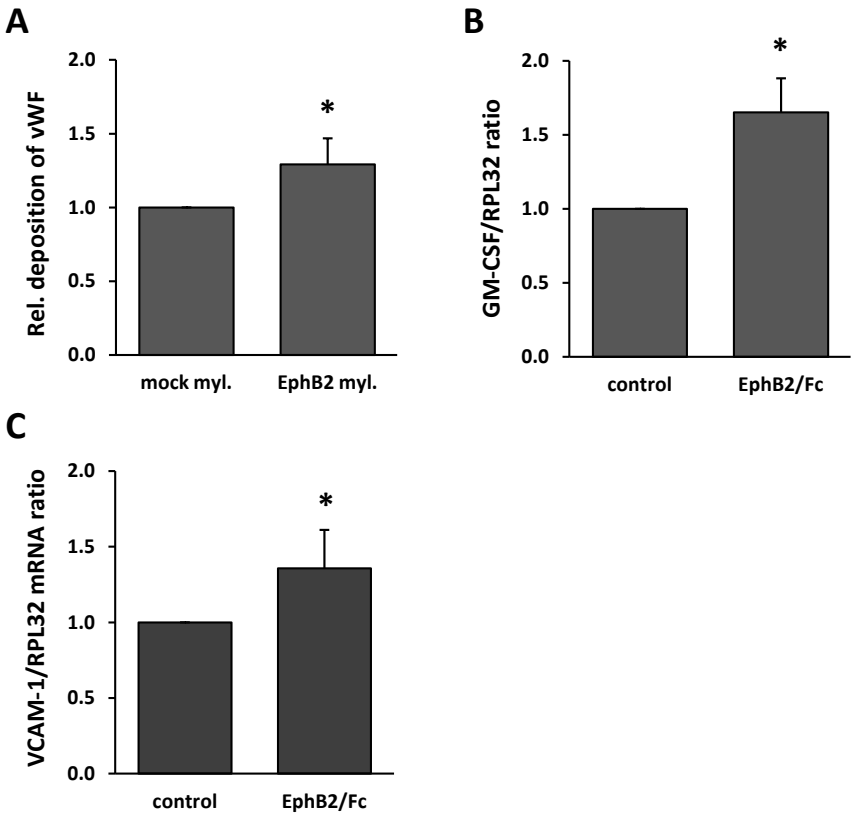

Figure 15: EphB2 elicits GM-CSF and VCAM-1 expression and vWF release in endothelial cells.

ELISA analyses were performed to determine the release of vWF on the surface of endothelial cells, which had been treated with EphB2-overexpressing mouse myeloma cells for 1 hour. This stimulation enhanced vWF release as compared with mock myeloma cells (A, *$p<0.05$ vs. mock myeloma (set to 1), n=5). As evidenced by PCR analyses, the expression of GM-CSF mRNA and VCAM-1 mRNA in endothelial cells was increased upon exposure to clustered EphB2/Fc for 3 hours compared to anti-IgG Fc control (B, C, *$p<0.05$ vs. anti-IgG Fc control (set to 1), n=4-6).

3.2.2 Analysis of THP-1 cell adhesion upon EphB2 stimulation

To further investigate the functional aspect of EphB2-activated endothelial cells, adhesion assays were performed. HUVECs were treated with clustered EphB2/Fc or anti-IgG Fc control for 6 hours, followed by the removal of these stimuli through rinsing HUVECs with PBS. THP-1 cells were then added on top of endothelial cells and allowed to adhere for 2 hours. The number of adherent THP-1 cells to EphB2/Fc pre-treated HUVECs was significantly enhanced (Figure 16).

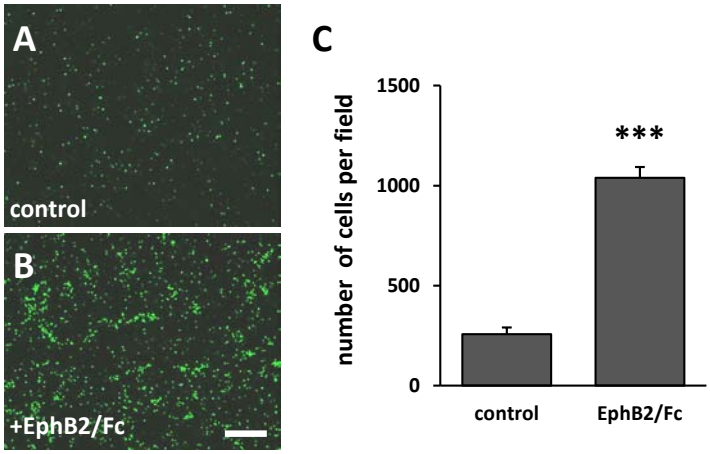

Figure 16: EphB2 stimulation of endothelial cells increases the attachment of THP-1 cells. The number of fluorescent THP-1 cells (green) adhering to EphB2/Fc-prestimulated HUVECs (B; scale bar: 500 µm) was significantly increased as compared to anti-IgG Fc control-treated HUVECs (A). The number of adherent cells was counted per field using image J (C, ***p<0.001 vs. anti-IgG Fc control, n=3).

3.2.3 Examination of the endothelial barrier function upon EphB2 stimulation

A hallmark of inflammation is an increase in endothelial permeability, which leads to edema formation in inflamed tissue [48]. The aforementioned results indicate that EphB2 stimulation increases Src activation (Figure 11B) which is a prominent regulator of vascular permeability [151]. Moreover, EphB2 triggers VE-cadherin internalization, which leads to the disassembly of adherens junctions (Figure 13). Based on these findings, we next hypothesized that EphB2 stimulation may contribute to the disruption of the endothelial barrier function. The bEnd5 mouse brain endothelioma cell line, as an *in vitro* model for blood brain barrier (BBB) [152], was utilized in the permeability assay. bEnd5 cells were seeded on the inserts to form a monolayer and pre-treated with clustered EphB2/Fc. 3 kD TXR,

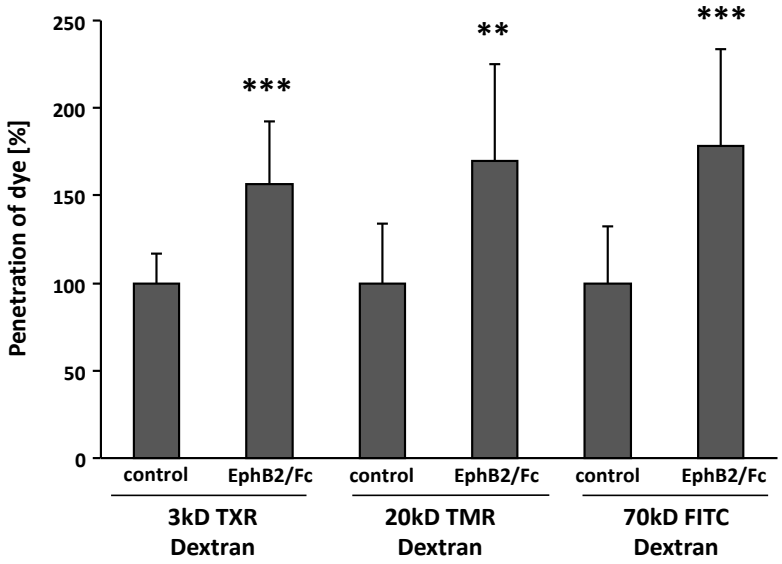

Figure 17: EphB2 impairs the barrier function of brain endothelial cells.
Prolonged EphB2/Fc stimulation of bEnd5 cells for 8 hours increased endothelial permeability as evidenced by the higher amount of penetrating fluorescence-labeled dextrans with different molecular weights (3kD, 20kD and 70kD), as compared to anti-IgG Fc-treated cells (***p<0.001, **p<0.01 vs. anti-IgG Fc control, n=4).

20 kD TMR and 70 kD FITC-labeled dextrans were administrated in the upper chamber. The flux of fluorescence-labeled dextrans across endothelial monolayer into the lower chamber was increased in EphB2/Fc-stimulated bEnd5 cells when compared with anti-IgG Fc antibody-treated cells (Figure 17).

Collectively, these results suggest that EphB2-stimulated reverse signaling in endothelial cells not only upregulates the expression of pro-inflammatory molecules and thereby enhancing THP-1 cell attachment, but also increases permeability of the endothelial cell monolayer.

3.3 Exploration of the effect of endothelial ephrinB2 on inflammation *in vivo*

The above findings and earlier publications suggest that expression of ephrinB2 in endothelial cells is upregulated during inflammation and plays a vital role in monocyte extravasation by interacting with monocyte EphB2. To clarify the role of endothelial ephrinB2 in inflammatory processes, inducible endothelial cell specific ephrinB2 knockout mice (ephrinB2$^{EC\text{-}iKO}$) were utilized to analyze monocyte extravasation under conditions of TPA-induced ear edema and thioglycollate-

elicited peritonitis. Additionally, ephrinB2$^{EC\text{-}iKO}$/ApoE$^{-/-}$ mice were generated to directly explore the impact of endothelial ephrinB2 on the atherosclerotic plaque formation. Finally, an arteriogenesis model was set up to examine the role of ephrinB2 in the collateral remodeling process.

3.3.1 Generation of ephrinB2$^{EC\text{-}iKO}$ mice

We crossbred ephrinB2$^{flox/flox}$ mice with Tie2-CreER^{T2} transgenic mice to generate Tie2-CreER^{T2} /ephrinB2$^{flox/flox}$ mice. Tamoxifen was injected to selectively delete *ephrinB2* expression in endothelial cells, referred to as ephrinB2$^{EC\text{-}iKO}$ mice. For a negative control, the same amount of Miglyol, a solvent of tamoxifen, was injected. To explore the deletion of floxed ephrinB2 in distinct arteries, the distribution of Tie2 promoter was examined first. Reporter gene LacZ expression is only activated by Cre recombinase, whose expression is driven by the Tie2 promoter. Therefore, X-gal staining, which is used to indicate LacZ expression, was performed to determine the distribution of Tie2 promoter in distinct arteries of tamoxifen-treated R26R/Tie2-CreER^{T2} mice. It revealed that the Tie2 promoter was abundantly expressed in endothelial cells of the auricle vessels (Figure 18A), whereas it was sparsely expressed in large arteries, such as the aorta (Figure 18B) and the femoral artery (Figure 18C). Furthermore, to assess the efficiency of ephrinB2 ablation, we performed immunofluorescence staining of ephrinB2 in both auricle and femoral arteries from control and ephrinB2$^{EC\text{-}iKO}$ mice. Deletion of ephrinB2 expression in endothelial cells was displayed in the auricle artery (Figure 18F, D and E). However, only little deletion was detected in the femoral artery (Figure 18G and H). It indicated that the deletion of endothelial ephrinB2 was more efficient in small arteries than in conduit arteries. Therefore, knockdown efficiency of endothelial ephrinB2 is uneven in distinct arteries due to the asymmetrical distribution of Tie2 promoter.

3.3.2 Analysis of TPA-induced ear edema in ephrinB2$^{EC\text{-}iKO}$ mice

TPA-induced ear edema has been widely applied as a model for inflammation [153]. Topical application of TPA for 48 hours resulted in a remarkable increase in ear thickness and the number of accumulated macrophages in the epidermis and dermis, which were assessed by Mac-3 immunostaining (Figure 19A, B, C and E). However, no difference in ear edema formation and macrophage accumulation

could be observed when comparing TPA-treated control and ephrinB2$^{EC\text{-}iKO}$ mice (Figure 19A, B, E and F).

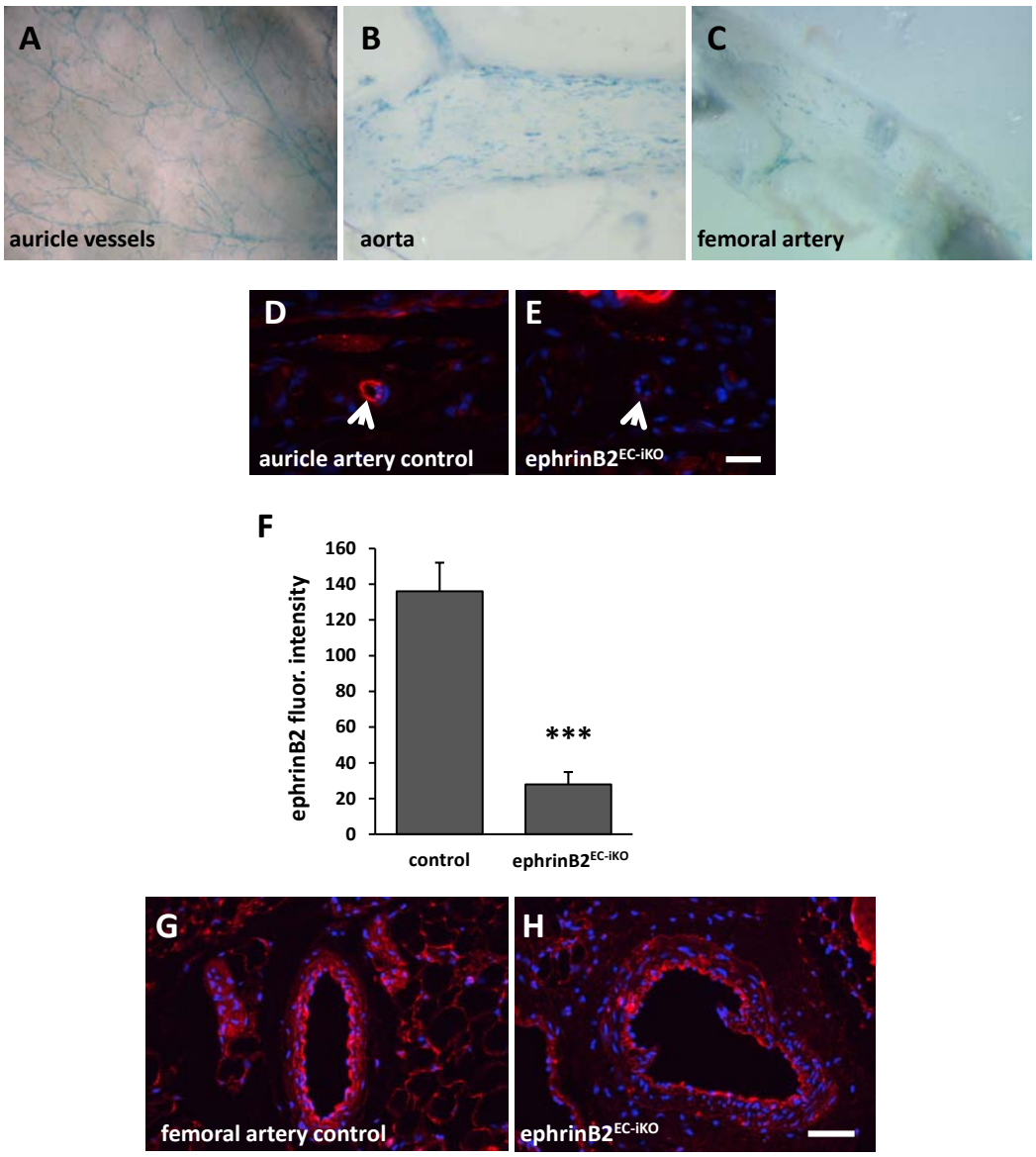

Figure 18: Examination of endothelial ephrinB2 knockdown efficiency in ephrinB2$^{EC\text{-}iKO}$ mice.

X-gal staining was performed to examine the expression of LacZ (blue) and to indicate Tie2 promoter expression in the sections of auricle vessels (A), the aorta (B), and the femoral artery (C) in tamoxifen-treated R26R/Tie2-CreER^{T2} mice. Immunofluorescence analysis was performed to detect ephrinB2 (red) in the auricle and femoral arteries from control (D, G) and ephrinB2$^{EC\text{-}iKO}$ (E, H). In the auricle artery of ephrinB2$^{EC\text{-}iKO}$ mice (E; scale bar: 20 µm), the ephrinB2-specific fluorescence intensity in endothelial cells was reduced by 80% (F, ***$p<0.001$ vs. control, n=5). However, endothelial ephrinB2 expression was not effectively ablated in the femoral artery of ephrinB2$^{EC\text{-}iKO}$ mice (H; scale bar: 50 µm), as compared to control mice (G).

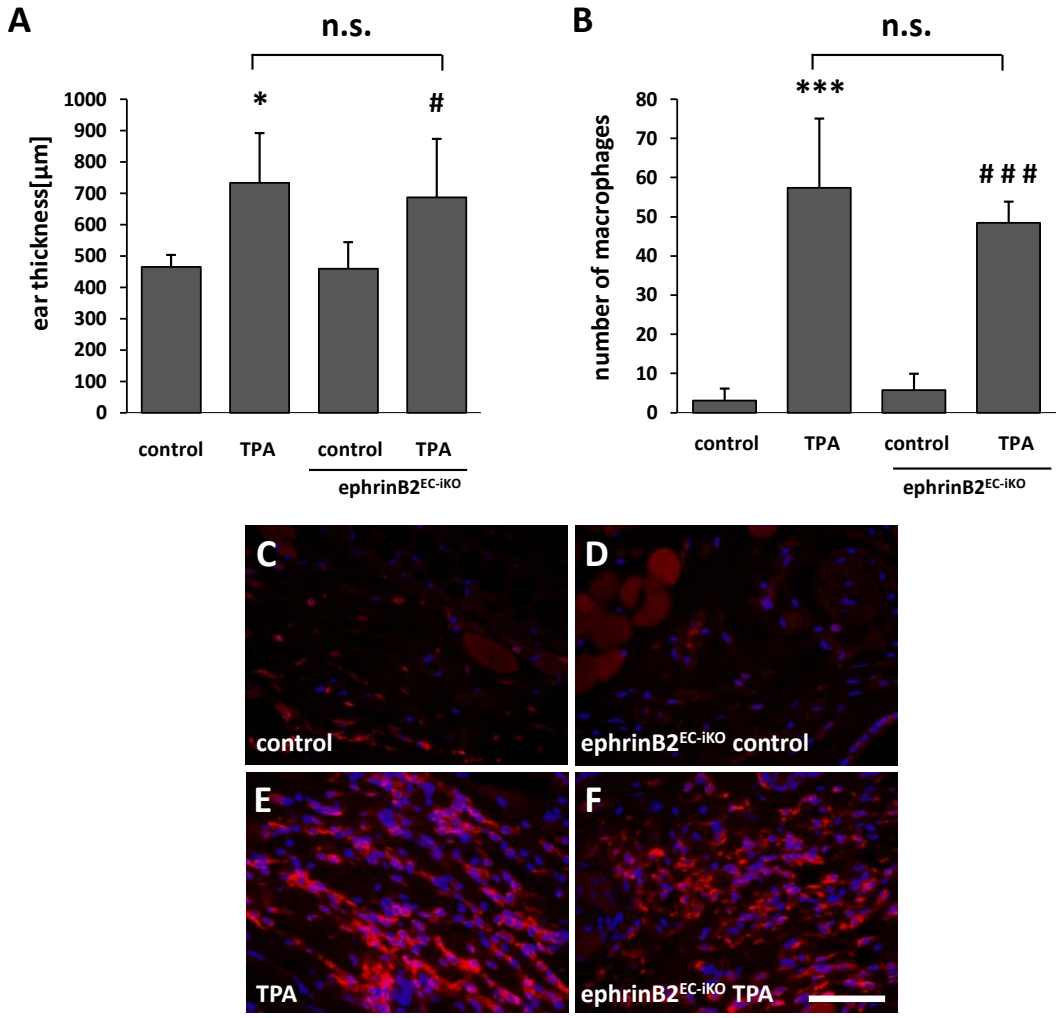

Figure 19: Analysis of ear edema formation and macrophage accumulation induced by TPA in ephrinB2$^{EC\text{-}iKO}$ mice.

Ear thickness was determined in cross-sections of auricles (3 to 4 independent regions per sample) and accumulated macrophages were indicated by Mac-3 immunostaining. Upon TPA treatment, ear thickness was markedly increased (A, *p<0.05 vs. control, #p<0.05 vs. ephrinB2$^{EC\text{-}iKO}$ control, n=5-6). Similarly, the number of accumulated macrophages was significantly increased as well (B, ***p<0.001 vs. control; compare C and E; ###p<0.001 vs. ephrinB2$^{EC\text{-}iKO}$ control, n=5-6; compare D and F; scale bar: 50 µm). However, endothelial ephrinB2 deficiency did not make any difference in ear thickness (A) and the amount of macrophages (B, compare E and F) upon TPA-induced inflammation.

3.3.3 Analysis of thioglycollate-elicited peritonitis in ephrinB2$^{EC\text{-}iKO}$ mice

Peritonitis induced by thioglycollate is utilized to evaluate a sequential recruitment of leukocytes into the peritoneum, starting with an influx of neutrophils and then followed by monocytes [154]. Peritoneal exudate cells and inflamed tissues were collected 6 hours after induction of peritonitis. However, in comparison to control,

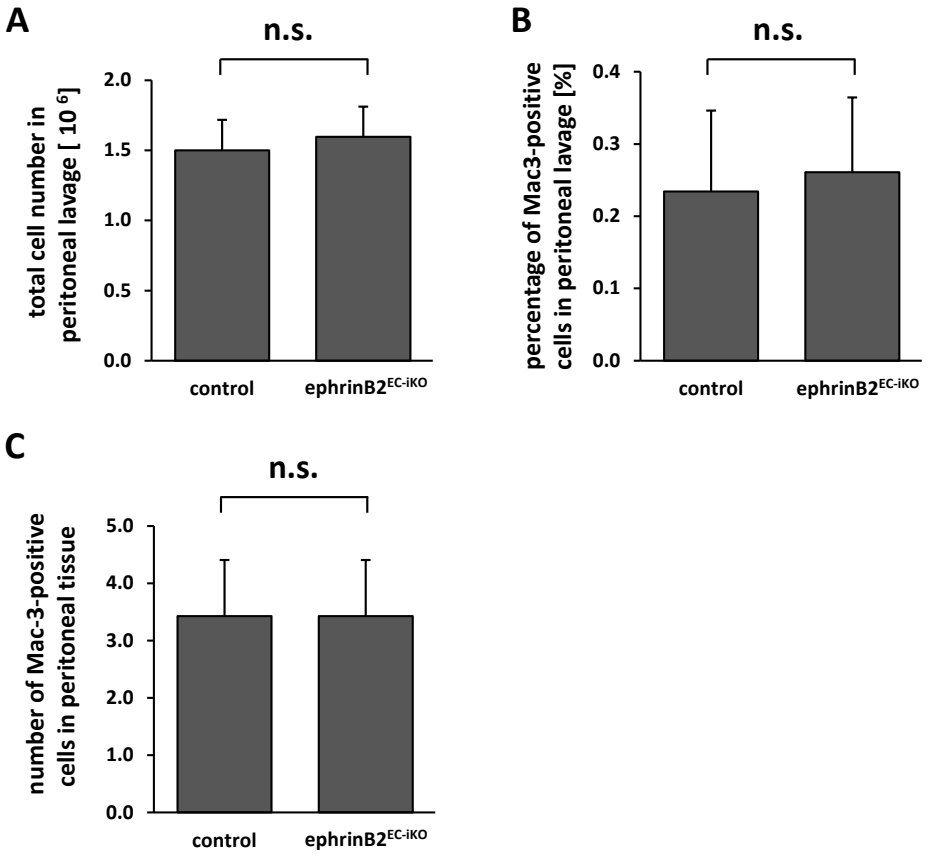

Figure 20: Examination of the influx of cells in lavage and macrophage accumulation in peritoneal tissue in the model of thioglycollate-elicited peritonitis in ephrinB2^{EC-iKO} mice.
Upon induction of peritonitis by intraperitoneally injecting thioglycollate, the total cell number in the lavage was enumerated (A, n=4-6). The percentage of Mac-3-positive cells in lavage (B, n=4-6) and the number Mac-3-positive cells in peritoneal tissue (C, n=7) were determined by immunofluorescence staining. No difference of these parameters was observed when comparing control and ephrinB2^{EC-iKO}mice.

no difference in the number of infiltrated cells or the percentage of Mac-3-positive cells in the lavage was observed in ephrinB2^{EC-iKO} mice. Likewise, the number of macrophages present in peritoneal tissue was also similar in the two groups (Figure 20, A-C).

3.3.4 Analysis of atherosclerosis in ephrinB2^{EC-iKO} mice

Atherosclerosis is a chronic inflammation of the arterial wall [155]. Previous studies indicate that ephrinB2 is most abundantly expressed in endothelial cells at atherosclerosis predilection sites of the mouse aorta. EphrinB2 promotes the adhesion and the pro-inflammatory activation of EphB receptor-expressing

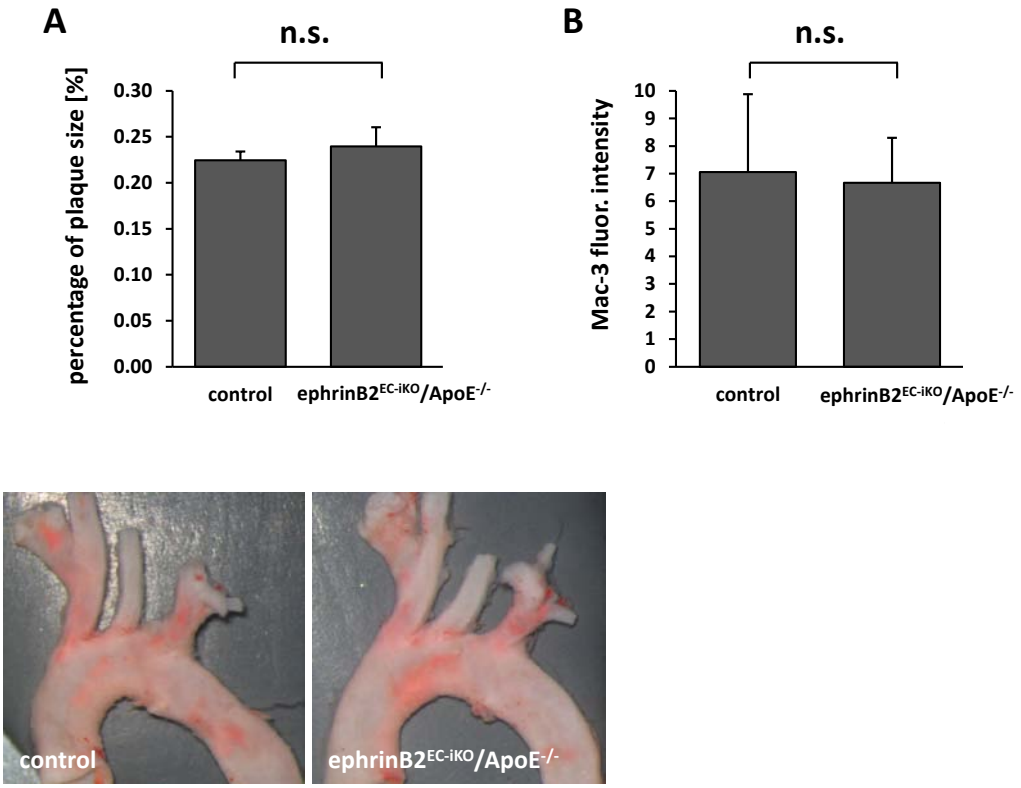

Figure 21: Analysis of plaque formation and plaque-associated macrophage accumulation in ephrinB2$^{EC\text{-}iKO}$/ApoE$^{-/-}$ mice.

The atherosclerotic plaque was visualized by Sudan III staining, and its size was expressed as a percentage of the area stained with dye in the total area of aorta. Quantification of the infiltrated macrophages was assessed by the intensity of Mac-3 staining. There was no difference in plaque size (A) and the number of macrophages (B, n=6) when comparing control and ephrinB2$^{EC\text{-}iKO}$/ApoE$^{-/-}$ mice.

monocytes [136]. Endothelial ephrinB2 may thus contribute to the pathogenesis of atherosclerosis. In this context, Tie2-CreER^{T2}/ephrinB2$^{flox/flox}$/ApoE$^{-/-}$ mice were generated. In 2 month-old mice, while Miglyol was injected as a control, tamoxifen was administrated to delete endothelial ephrinB2 expression, referred to as ephrinB2$^{EC\text{-}iKO}$/ApoE$^{-/-}$ mice. When comparing control and ephrinB2$^{EC\text{-}iKO}$/ApoE$^{-/-}$ mice at 6 months of age, there was no significant difference in plaque size, determined by Sudan III staining. Moreover, the number of macrophages present in the plaques, indicated by Mac-3 staining, was also similar.

3.3.5 Analysis of arteriogenesis in ephrinB2$^{EC\text{-}iKO}$ mice

In case of major arterial occlusion, small arterioles transform into conductance arteries to compensate for the lack of blood flow, a process termed arteriogenesis. [156]. A previous publication shows that endothelial ephrinB2 expression is increased in arterioles undergoing arteriogenesis, which effectively limits SMC migration and facilitates monocyte accumulation [124]. In this regard, we hypothesized that the deficiency of endothelial ephrinB2 may have an inhibitory impact on the progression of arteriogenesis. To verify this, arteriolar remodeling was induced by ligating the femoral artery for 7 days. Compared to sham, the diameter of collateral arteries was increased after ligation (Figure 22E, A and C). However, this parameter was no difference when comparing control and ephrinB2$^{EC\text{-}iKO}$ mice (Figure 22E, C and D). Moreover, macrophage accumulation in the perivascular space of the remodeling collateral arteries was similar in control and ephrinB2$^{EC\text{-}iKO}$ mice (Figure 22J, H and I).

Taken together, four *in vivo* mouse models related to monocyte extravasation and differentiation into macrophages fail to show that endothelial ephrinB2 has an impact on monocyte infiltration, inflammatory pathology, and the progression of arteriogenesis.

3.4 Assessment of the role of ephrinB1 reverse signaling in EphB2-stimulated pro-inflammatory differentiation of endothelial cells

Since ephrinB1 and ephrinB2 in endothelial cells bind to EphB2, we next elucidated which ligand is responsible for EphB2-induced pro-inflammatory activation of the endothelial cells. The expression of both ligands in HUVECs was individually silenced by siRNA pretreatment. While loss of ephrinB1 abolished EphB2-triggered upregulation of E-selectin expression, knockdown of ephrinB2 had no significant effect (Figure 23A). This finding was corroborated by the fact that an increase of E-selectin expression induced by the stimulation with soluble EphB4 receptor bodies, which specifically interact with ephrinB2, was less effective than EphB2 stimulation (Figure 23B).

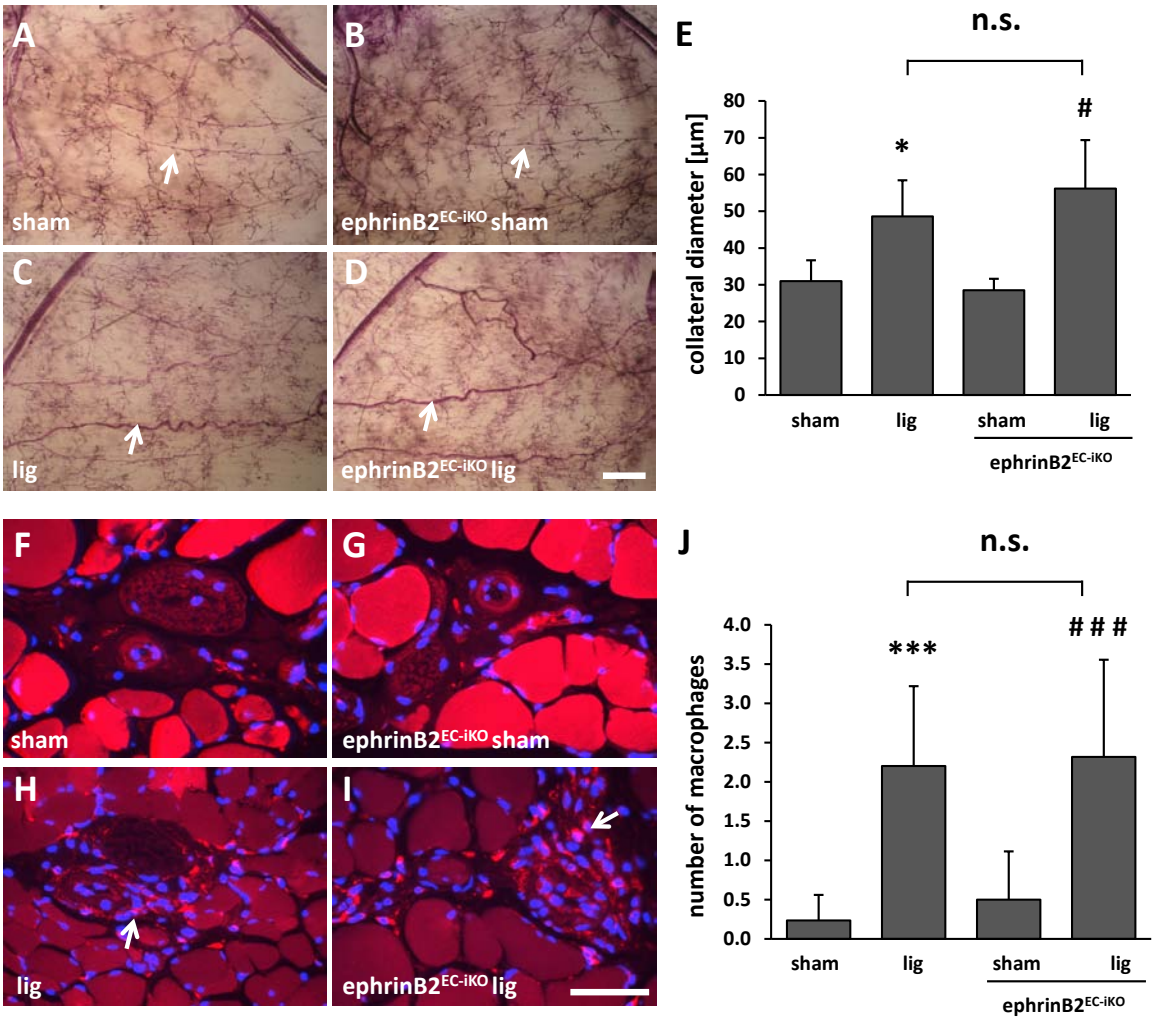

Figure 22: Analysis of the formation of collateral arteries in arteriogenesis in ephrinB2$^{EC\text{-}iKO}$ mice.

Collateral arteries were visualized by pigment perfusion (A-D) and immunostaining (G-J). Diameter of the collateral artery was significantly increased upon arteriogenesis (E, *p<0.05 vs. sham; compare A and C; #p<0.05 vs. ephrinB2$^{EC\text{-}iKO}$ sham, n=5-6; compare B and D; arrow; scale bar: 80 µm). However, the growth of collateral arteries was not blocked in ephrinB2$^{EC\text{-}iKO}$mice as compared to control mice (E, compare C and D; arrow). Perivascular macrophages were determined by Mac-3 staining. No difference in the number of macrophages was detected (J, ***p<0.001 vs. sham; compare F and H; ###p<0.001 vs. ephrinB2$^{EC\text{-}iKO}$ sham, n=5-6; compare G and I; scale bar: 50 µm).

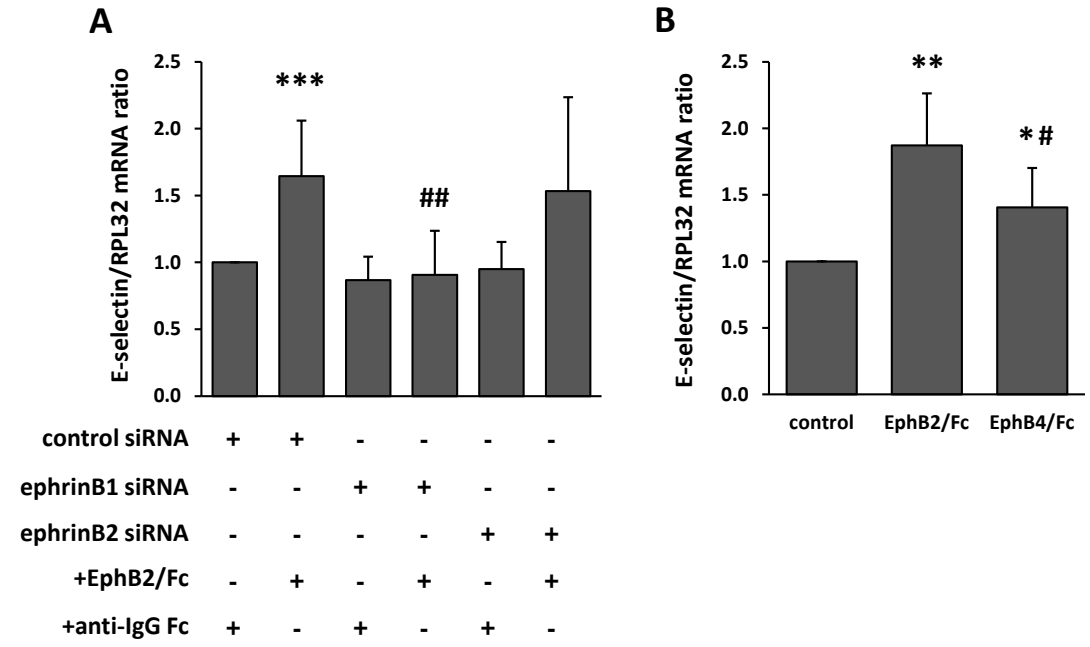

Figure 23: EphB2-induced E-selectin upregulation is dependent on ephrinB1 reverse signaling.

Knockdown of ephrinB1 expression, rather than ephrinB2, attenuated EphB2/Fc-induced upregulation of E-selectin mRNA expression in HUVECs (A, ***p<0.001 vs. control siRNA + anti-IgG Fc control (set to 1), ##p<0.01 vs. control siRNA + EphB2/Fc, n=6-12). Likewise, EphB4/Fc, was not capable of eliciting a significant upregulation of E-selectin mRNA expression in HUVECs as compared to EphB2/Fc treatment (B, **p<0.01, *p<0.05 vs. anti-IgG Fc control (set to 1), #p<0.05 vs. EphB2/Fc, n=7).

3.5 Analysis of the impact of endothelial ephrinB1 on inflammation

There is accumulating evidence indicating that ephrinB1 is upregulated and might be associated with inflammatory pathogenesis by modulating cytokine release and leukocyte chemotaxis [133-135, 140]. Considering the aforementioned results suggesting that ephrinB1 reverse signaling is responsible for EphB2-induced pro-inflammatory change of endothelial cells, it intrigued us to investigate the role of endothelial ephrinB1 in inflammation.

3.5.1 Expression of ephrinB1 under inflammatory conditions

We first examined the expression of endothelial ephrinB1 in inflammation using three different models: inflamed mouse skin model, TNFα-stimulated HUVECs and femoral artery segments.

3.5.1.1 Expression of ephrinB1 in endothelial cells of inflamed mouse skin

Immunofluorescence analyses were performed on the inflamed mouse skin samples from atopic dermatitis model as described before (Figure 7). Inflammation-associated angiogenesis was indicated by the upregulation of PECAM-1-positive capillaries (Figure 24A, C and E). The percentage of ephrinB1/PECAM-1 double-positive capillaries markedly increased during inflammation (Figure 24B, D and F).

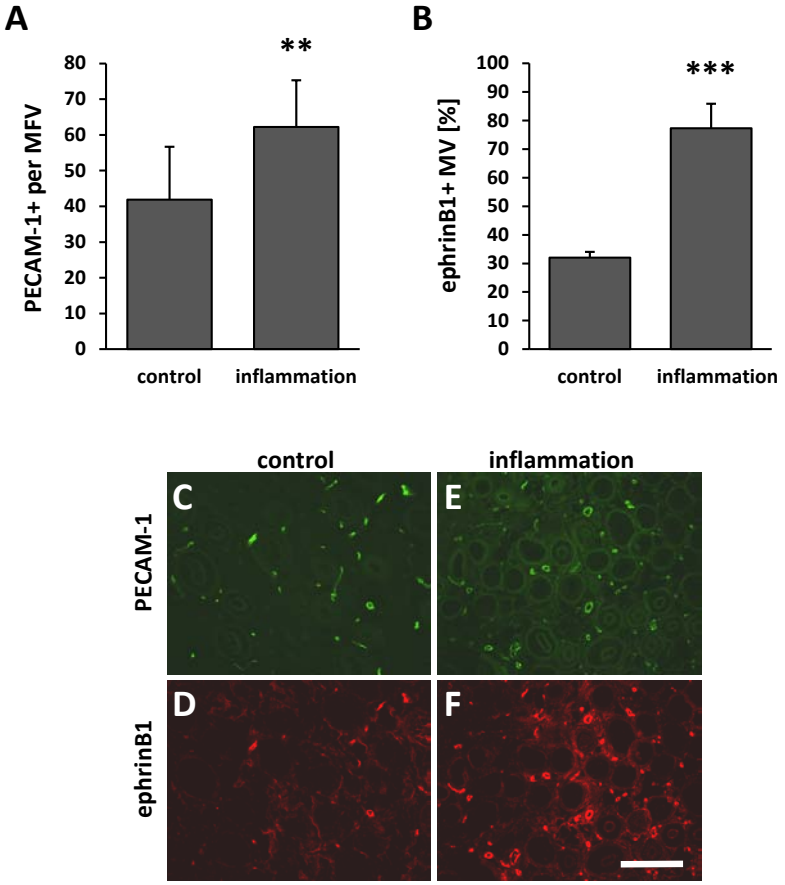

Figure 24: EphrinB1 expression is enhanced in angiogenic capillaries in inflamed mouse skin.

PECAM-1 (green) and ephrinB1 (red) were detected by immunofluorescence staining in murine atopic dermatitis model. Mean fluorescence value (MFV) of PECAM-1 was increased, indicating that inflammation leads to angiogenesis (A, ***p<0.001 vs. control, n=10; compare C and E). The percentage of ephrinB1/PECAM-1 double-positive capillaries was significantly increased (B, **p<0.01 vs. control, n=5; compare D and F; scale bar: 100 µm).

3.5.1.2 Expression of ephrinB1 and ephrinB2 in endothelial cells upon pro-inflammatory stimulation

To analyze the abundance of ephrinB1 and ephrinB2 upon inflammatory stimuli *in vitro*, HUVECs were subjected to the pro-inflammatory cytokine, TNFα. RT-PCR and Western blot analyses were performed and revealed that TNFα triggered the upregulation of ephrinB1 expression (Figure 25A and B), but not ephrinB2 (Figure 25C and D), in endothelial cells.

Next, an *ex vivo* experiment was performed by incubating murine femoral artery segments with TNFα. Immunofluorescence staining was carried out and revealed that endothelial ephrinB1 abundance was increased upon TNFα treatment (Figure 26A, B and C).

3.5.1.3 Expression of endothelial ephrinB1 in venules in the arteriogenesis model

In contrast to ephrinB2, ephrinB1 is also expressed in venous endothelial cells, which allows ephrinB1 to participate in monocyte extravasation. To investigate whether ephrinB1 expression is affected by inflammation-associated vascular remodeling processes, this was examined in the hindlimb ischemia model. Immunofluorescence staining revealed that ephrinB1 expression was significantly upregulated in endothelial cells of venules running in parallel to the remodeling collateral arterioles (Figure 27C, A and B).

3.5.2 Effect of endothelial ephrinB1 on monocyte diapedesis

Since the expression of endothelial ephrinB1 is upregulated under inflammatory conditions, we hypothesized that it might play a role in monocyte extravasation.

3.5.2.1 Role of endothelial ephrinB1 in THP-1 cell transmigration

To examine the impact of ephrinB1 on monocyte diapedesis, transendothelial cell migration assay was performed. HUVECs were pre-treated with specific siRNA to silence ephrinB1. While basal transmigration of THP-1 cells across the endothelial cell monolayer was not affected by loss of ephrinB1, MCP-1-induced transmigration was significantly reduced upon knockdown of ephrinB1 in endothelial cells (Figure 28).

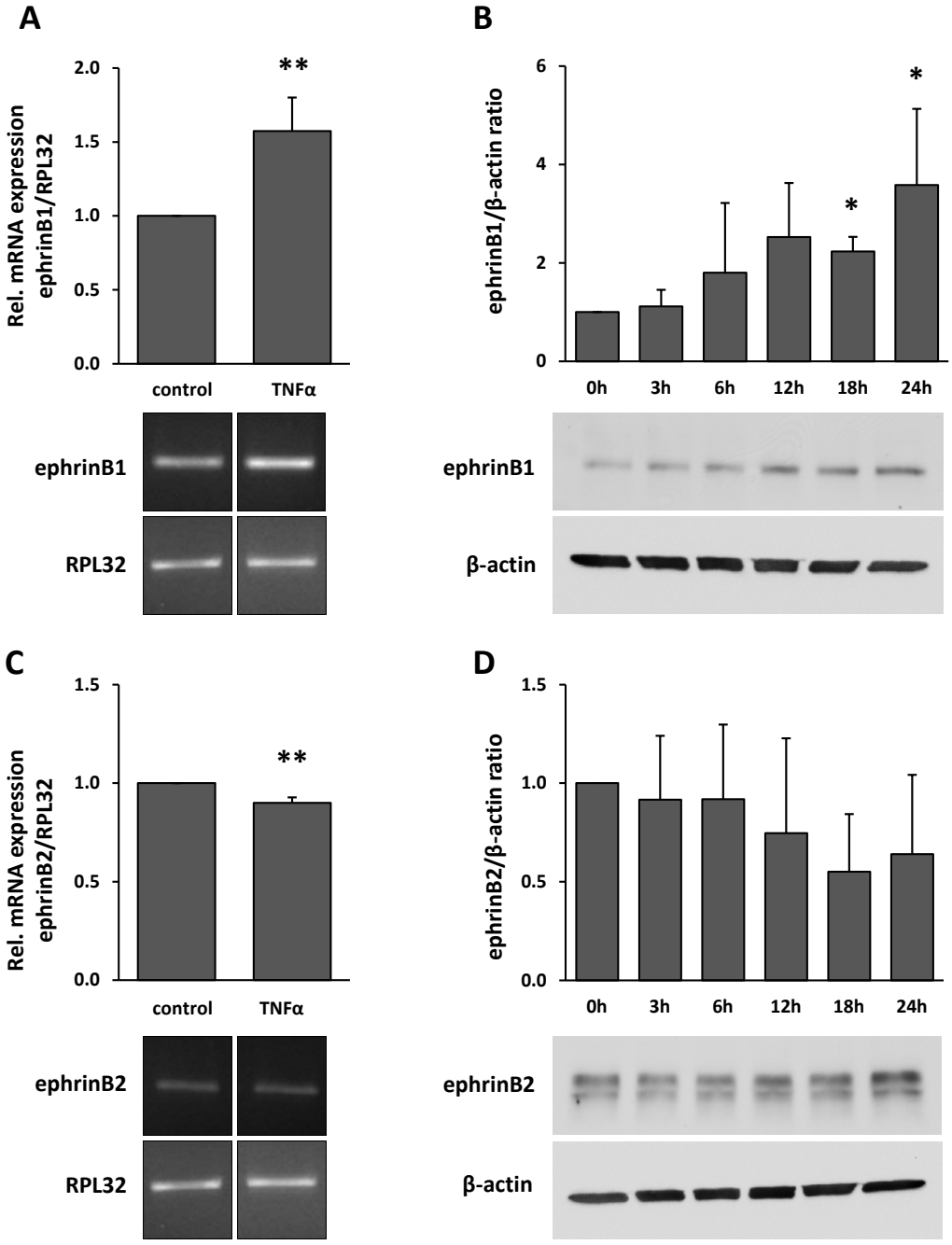

Figure 25: TNFα elevates the expression of ephrinB1, but not ephrinB2, in HUVECs.
Exposure of HUVECs to TNFα (1000 U/ml) increased ephrinB1 expression, assessed by RT-PCR (3 hours treatment; A, **p<0.01 vs. control, n=5) and Western blot analyses (B, *p<0.05 vs. 0 hours, n=4). The abundance of ephrinB2 upon TNFα stimulation was slightly attenuated on mRNA level (3 hours treatment; C, **p<0.01 vs. control, n=5). No significant difference was detected on protein level (D, n=4).

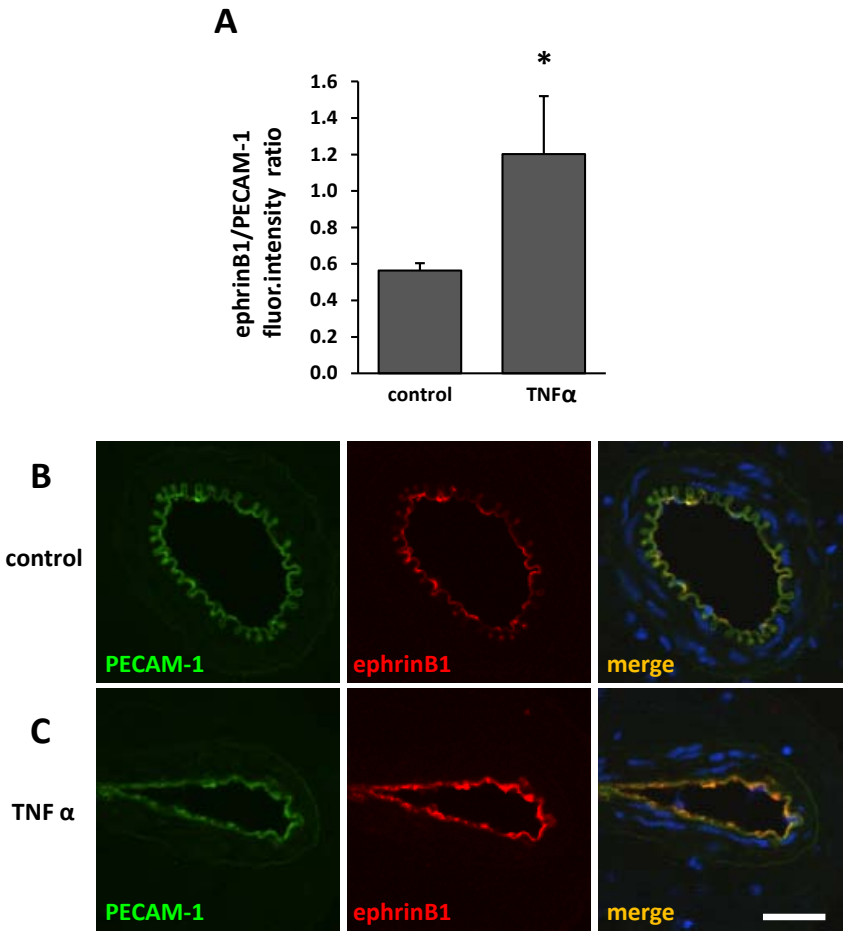

Figure 26: TNFα induces upregulation of ephrinB1 expression in murine endothelial cells.
Freshly isolated murine femoral arteries were incubated with mouse TNFα (2 ng/ml) for 6 hours. EphrinB1 (red) and PECAM-1 (green) were detected by immunofluorescence staining. TNFα treatment increased the abundance of ephrinB1 in endothelial cells (A, *p<0.05 vs. control, n=3; compare B and C; scale bar: 50 µm).

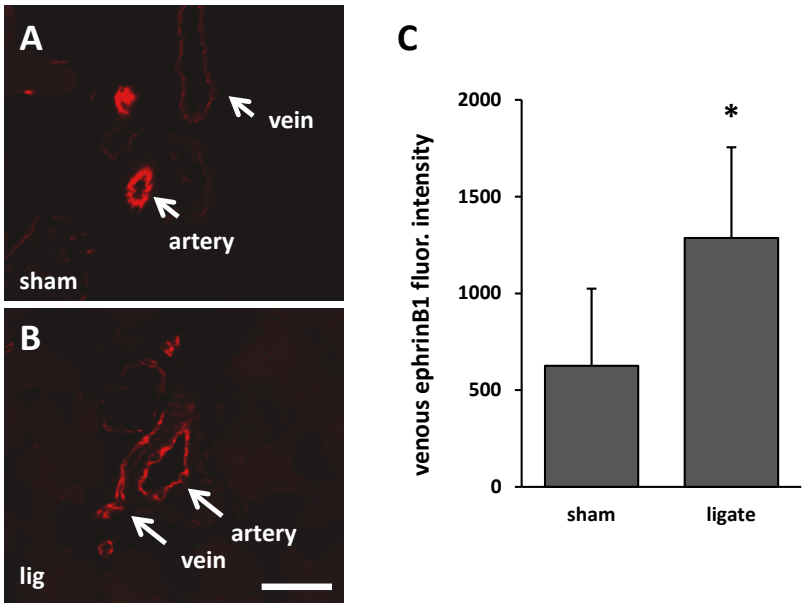

Figure 27: Endothelial ephrinB1 expression is upregulated in venules near to the remodeling arterioles in the arteriogenesis model.

EphrinB1 expression (red) was assessed by immunofluorescence staining. As compared with sham-operated control (A), fluorescence intensity of endothelial ephrinB1 was significantly increased in venules, adjacent to collateral arterioles undergoing remodeling (C, *p<0.05 vs. sham, n=5; compare A and B; scale bar: 50 µm).

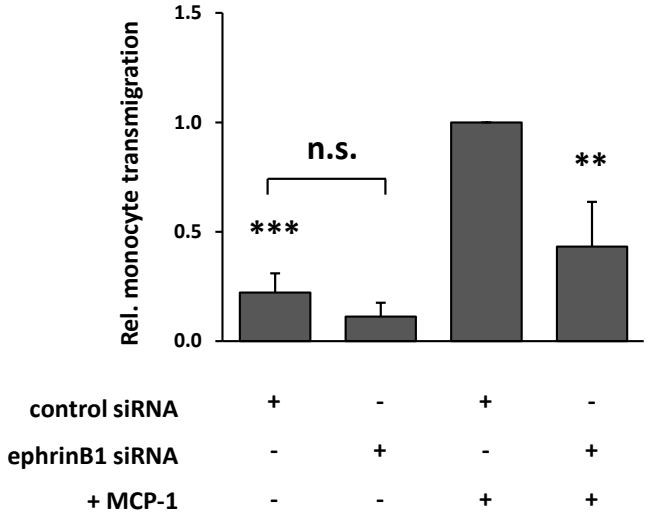

Figure 28: EphrinB1 supports THP-1 cell transmigration through an endothelial cell monolayer.

EphrinB1 expression in endothelial cells was effectively silenced by siRNA transfection. MCP-1-induced transmigration of THP-1 cells was blunted in ephrinB1-deficient endothelial cells (***p<0.001, **p<0.01 vs. control siRNA+MCP-1 (set to 1), n=6).

3.5.2.2 Examination of the association of PECAM-1 and ephrinB1 upon EphB2 stimulation

To elucidate the mechanism through which ephrinB1 assists monocyte transmigration, we hypothesized that ephrinB1 might directly interact with PECAM-1 in the same manner as ephrinB2. Co-immunoprecipitation was performed in HUVECs subjected to mock-transfected and EphB2-overexpressing mouse myeloma cells. However, PECAM-1 did neither co-precipitate upon exposure to mock nor EphB2-overexpressing mouse myeloma cells.

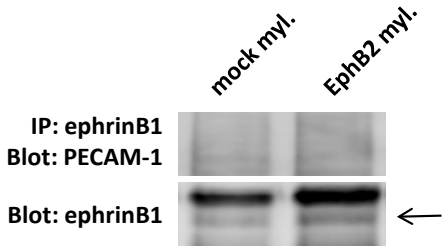

Figure 29: EphrinB1 does not physically interact with PECAM-1.

Analysis of the interaction between ephrinB1 and PECAM-1 was carried out by pulling down ephrinB1 (IP) with its antibody and subsequently detected PECAM-1 and ephrinB1 by western blot analysis. PECAM-1 did not co-precipitate with ephrinB1 even upon EphB2 stimulation.

Taken together, these results indicate that endothelial ephrinB1 expression is upregulated during inflammation and plays a role in monocytes extravasation. EphrinB1 reverse signaling not only mediates EphB2 stimulated-pro-inflammatory molecule expression in endothelial cells, but also facilitates monocyte diapedesis.

4 DISCUSSION

4.1 The role of endothelial ephrinB2 in inflammtion

4.1.1 Endothelial ephrinB2 expression is upregulated during inflammation

Eph receptors represent the largest family of receptor tyrosine kinases (RTKs). Binding to their corresponding ligand ephrins induces bidirectional signaling, by which Ephs and ephrins control numerous physiological and pathological functions [88]. In endothelial cells, an array of Eph receptors (EphA2, EphB1-B4) and ephrins (A1, B1, B2) are expressed [157]. Among them, ephrinB2 has been intensively studied in the embryonic vasculature as a marker of arterial endothelial cells. In the adult organism, the effect of ephrinB2 is largely unknown. In our study, we noted that ephrinB2 was abundantly expressed in endothelial cells during inflammation-related angiogenesis in the atopic dermatitis model. This is in line with previous findings showing that ephrinB2 expression is enhanced under inflammatory conditions. Highly expressed EphrinB2 has been detected in intestinal epithelial cells during inflammatory bowel diseases and also in endothelial cells at arteriosclerosis predilection sites [136, 137]. This finding intrigued us to further investigate the role of endothelial ephrinB2 during inflammation.

EphB receptors are extensively expressed in leukocytes. For instance, lymphocytes express EphB1-4, B6; monocytes express EphB1-3, EphB6; and granualocytes express EphB1-B4 [157]. EphBs have profound effects on modulating immune cell development, activation and chemotaxis [130, 133, 135, 157]. In this study, EphB2 was located on the surface of macrophages. This is in line with previous reports demonstrating that EphB2 expression on the surface of monocytes is upregulated during their differentiation into macrophages and also upon stimulation with PMA [134, 136].

4.1.2 Endothelial ephrinB2 facilitates monocyte transmigration

Previous publications have indicated that ephrinB2 is located on the luminal surface of quiescent endothelial cells, which allows it to interact with receptors expressed on circulating leukocytes [141]. In this context, ephrinB2 interacts with EphB2-expressing monocytes and induces EphB2 forward signaling, leading to the release of IL-8 and MCP-1 from monocytes [136]. Thus, we hypothesized that

binding of endothelial cell ephrinB2 and monocyte EphB2 may affect monocyte extravasation during inflammation. We initially focused on understanding the role of ephrinB2 in monocyte transmigration through the endothelial cell monolayer since this process is a critical and irreversible step. Primary HUVECs were utilized in *in vitro* studies as endothelial cells in both the umbilical artery and vein abundantly express ephrinB2 [141]. THP-1 cells, a monocytic cell line with EphB2 expression on the cell surface, were applied in the transendothelial cell migration assay [139]. The results indicated that MCP-1-induced THP-1 cell transmigration through the endothelial cell monolayer was blocked by loss of endothelial ephrinB2, providing evidence to suggest that endothelial ephrinB2 facilitates monocyte transmigration. This is in agreement with previous reports indicating that MCP-1-stimulated transmigration of monocytes or murine J774 macrophage cells is disturbed when ephrinB2 reverse signaling is blocked by monomeric ephrinB2 or by endothelial cells overexpressing the truncated cytoplasmic domain of ephrinB2 [124, 139].

4.1.3 EphB2 induces ephrinB2 association with intercellular junction proteins and VE-cadherin internalization in endothelial cells

In addressing the mechanisms through which ephrinB2 supports monocyte transmigration, we investigated the subcellular distribution of ephrinB2 in a confluent HUVEC monolayer. Consistent with the previous study, the present results revealed that ephrinB2 was restricted in the cell borders and partially colocalized with PECAM-1 [139, 141]. However, in quiescent endothelial cells, such as EC/SMC coculture spheroids *in vitro* or umbilical cords *in vivo*, ephrinB2 is expressed on the luminal surface of endothelial cells [141].

In order to explore the impact of EphB2 on the activation of endothelial cells, HUVECs were exposed to EphB2-overexpressing mouse myeloma cells that mimic the characteristic of monocytes. This stimulus enhanced the association of endothelial cell ephrinB2 with intercellular junction proteins, such as PECAM-1, VE-cadherin, and ZO-1. This is partly compatible with previous findings indicating that EphB4, which specifically binds to ephrinB2, triggers the translocation of ephrinB2 from cell surface to the junctions where it associates with PECAM-1 [139, 141]. Moreover, displacement of VE-cadherin from cell junctions was observed in response to EphB2/Fc treatment. This is a critical process for loosening adherens

junctions and thereby favoring leukocyte transmigration [35]. However, the mechanism by which EphB2 induces VE-cadherin displacement is not clear yet, but it might result from endocytosis of the EphB/ephrinB complex and together with its surrounding plasma membranes into ephrinB- or EphB-expressing cells in case of cell contact [89]. For instance, EphB4 stimulation leads to the EphB4/ephrinB2 complex endocytosis, accompanied by the internalization of VEGFR2 and VEGFR3 which is colocalized with ephrinB2, and this process is crucial for VEGF signaling [91, 92]. Moreover, it is well-known that VEGFR2 codistributes with VE-cadherin at cell contacts upon VEGF stimulation [158]. In addition, it has been suggested that VEGFR2 might form a protein complex with ZO-1, VE-cadherin and β-catenin in HUVECs [159]. Therefore, the results obtained in our study and previous findings support the idea of a protein complex comprising ephrinB2, VEGFR2 and cell junction proteins. Its assembly/disassembly and endocytosis might be controlled by EphB and/or VEGF treatment. This highlights the need for a more detailed analysis of EphB2-induced translocation of ephrinB2 to the cell border, leading to the association of ephrinB2 with junction proteins and ultimately the internalization of this complex.

4.1.4 EphB2 elicits Src-dependent PECAM-1 phosphorylation

In this study, endothelial cell exposure to EphB2-overexpressing mouse myeloma cells increased Src activity, as the phosphorylation of Src at tyrosine residue 419 was detected. This is in accordance with previous findings indicating that ephrinB reverse signaling is mediated by Src family kinases, which promotes ephrinB phosphorylation and initiates downstream signaling [103]. It is well-known that Src family kinases play a profound role in leukocyte transmigration, supported by the fact that their inhibitor PP2 is capable of blocking the transmigration [160]. This is partly due to Src being able to elicit the phosphorylation of molecules pivotal for leukocyte transmigration such as cortactin which regulates actin remodeling, and VE-cadherin whose phosphorylation leads to disassembly of adherens junctions [38, 39, 160]. Moreover, Src is critical for targeted recycling of membrane from the lateral border recycling compartment (LBRC) to the site of transendothelial migration, which is a prerequisite for leukocyte transmigration [161].

Furthermore, the present results demonstrated that EphB2 stimulation induced Src family kinases-dependent PECAM-1 phosphorylation, as PP2 diminished the basal

and EphB2-stimulated PECAM-1 phosphorylation. Leukocyte transmigration induced by a variety of mediators, such as hypoxia, glucose, LPS or crosslinking of ICAM-1, is associated with the phosphorylation of PECAM-1 in endothelial cells [144, 145, 162, 163]. However, phosphorylated PECAM-1 is not detectable during monocyte transmigration. This is probably due to the limited sensitivity of methods, rapid phosphorylation and dephosphorylation of PECAM-1 within a few seconds, and the low percentage of endothelial PECAM-1 (5-10%) that is involved in the interaction with leukocytes. Nevertheless, PECAM-1 phosphorylated on tyrosine residues appears to be restricted to the LBRC [161]. Specifically, tyrosine 663 is verified to be essential for leukocyte transmigration by regulating the target recycling of LBCR in endothelial cells [164].

In addition, the results from this study revealed that SHP2 phosphatase activity was reduced following EphB2 stimulation. It has been well-established that SHP2 is physically associated with phosphorylated PECAM-1. Moreover, even though it is not well defined, some studies indicate that PECAM-1 is a substrate of SHP2. If this is the case, decreased SHP2 activity may protect the phosphorylation of PECAM-1 [165-168]. Inhibition of SHP2 has been linked to the disassembly of adherens junctions by enhancing tyrosine phosphorylation of VE-cadherin and by increasing RhoA activity, which eventually results in edema formation and endothelial cell dysfunction [169, 170].

Consequently, EphB2-stimulated VE-cadherin internalization, Src kinase activity and PECAM-1 phosphorylation in endothelial cells might be the mechanisms for supporting monocyte transmigration. However, it remains to be investigated whether ephrinB reverse signaling increases the phosphorylation of other Src kinase substrates, such as VE-cadherin and ZO-1, which may disrupt cell junctions and promote leukocyte transmigration.

4.2 EphB2 evokes pro-inflammatory responses of endothelial cells

4.2.1 EphB2 increases pro-inflammatory molecule expression in endothelial cells

Next, it was hypothesized that ephrinB reverse signaling might modulate the pro-inflammatory activation of endothelial cells and affect monocyte adhesion. Indeed, the results indicated that EphB2 stimulation elicited JNK-dependent upregulation of E-selectin expression. E-selectin promotes leukocyte rolling on the endothelial

cell surface by interacting with its ligands on leukocytes, such as PSGL1, CD44 and ESL1 [171]. Furthermore, the results revealed that EphB2-induced increase of E-selectin expression was inhibited by the JNK inhibitor SP600125, suggesting that JNK acts in the downstream of ephrinB reverse signaling, which is consistent with earlier findings [115, 116]. In addition, our study indicated that VCAM-1 expression was increased upon EphB2 stimulation, which mediates leukocyte firm adhesion by binding to integrin VLA-4 [26]. It also revealed that vWF was deposited on the surface of EphB2-treated endothelial cells, which acts as a key regulator of hemostasis and inflammation. Various pro-inflammatory mediators (e.g. histamine and endotoxin) elicit vWF secretion from endothelial cell Weibel-Palade bodies to form string-like structure on their surface [172]. The deposited vWF interacts with PSGL-1 and β2-integrins on leukocytes to mediate their rolling and firm adhesion [173, 174]. Also, vWF binding to platelet GPIb promotes stable interactions of platelets with endothelial cells and thus provides a sticky surface to allow the adherence of leukocytes and to subsequently activate them [148, 175]. Moreover, our results showed that GM-CSF expression was increased in EphB2-stimulated endothelial cells. It is well-known that pro-inflammatory mediators trigger GM-CSF expression, and it plays a role in activating leukocytes, including regulation of their adhesion and enhancement of cytokine and superoxide release [150, 176-180].

4.2.2 EphB2 promotes monocyte adhesion

Furthermore, the present results showed that monocytic cells preferentially adhered to EphB2-prestimulated endothelial cells. This is partially in line with previous reports that ephrinB2 reverse signaling in endothelial cells and EphB4 forward signaling in monocytes are both required for monocyte adhesion [139]. Monocytes preferentially stick to ephrinB2-rich endothelial cells of the aorta, rather than to ephrinB2-poor endothelial cells in the mesenteric artery, and this adhesion is inhibited by monomeric ephrinB2 or EphB4 [136]. Collectively, EphB2-stimulated reverse signaling promotes pro-inflammatory differentiation of endothelial cells, and subsequently enhances leukocyte adhesion.

4.2.3 EphB2 increases endothelial cell permeability

Increased vascular permeability is associated with tissue edema and leukocyte extravasation [48]. In our study, EphB2 stimulation disrupted brain endothelial cell barrier. Similar effects of ephrinB1 reverse signaling have been reported in epithelial cells. In these cells, ephrinB1 physically interacts with claudin1/4 on the same cell membrane. When cell-cell contact occurs, claudin-induced ephrinB1 reverse signaling increases paracellular permeability [181]. Exactly how EphB2 modulates the endothelial barrier function is unclear but may involve three possible mechanisms. One mechanism is that EphB2 stimulates Src activity. Activation of Src disrupts endothelial cell monolayer integrity by the phosphorylation of VE-cadherin, MLCK and FAK, which results in cell junction disassembly and endothelial cell contraction [182-184]. Therefore, deletion of Src, either by inhibitors or genetic ablation, blocks mediator-driven increases in endothelial permeability [184]. Another possible mechanism is that ephrinB reverse signaling has been reported to increase RhoA/ROCK activity, which promotes endothelial cell contraction and loosens their intercellular contacts [113, 117]. Finally, EphB2 induces the association of ephrinB2 with endothelial junction proteins, and this may affect the localization and function of these junction proteins. However, these possible mechanisms need to be investigated in detail in further studies.

4.3 The impact of endothelial ephrinB2 on inflammatory models

On the one hand, the binding of endothelial ephrinB2 with monocyte EphB2 promotes monocyte diapedesis and induces forward signalling to activate monocytes [136]. On the other hand, this interaction elicits ephrinB reverse signaling, which is capable of promoting pro-inflammatory differentiation of endothelial cells and increasing permeability of the endothelial monolayer. To further investigate the functional role of endothelial ephrinB2 in inflammation *in vivo*, we utilized four different inflammation-associated models on inducible endothelial cell specific ephrinB2 knockout mice, and thus analyzed monocyte recruitment and inflammatory pathology.

In TPA-induced ear edema and thioglycollate-elicited peritonitis, it was observed that endothelial ephrinB2 had no impact on monocyte infiltration and inflammatory response, which might be due to the localization of endothelial ephrinB2 and the

harsh pro-inflammatory condition. EphrinB2, as a determinant of the arterial endothelial cell phenotype, is not expressed in postcapillary venules where leukocyte diapedesis occurs during inflammation [22]. Moreover, both TPA and thioglycollate are extremely harsh stimuli to evoke inflammation. As a consequence, both models are suboptimal to study the impact of ephrinB2 which appears to have supportive rather than rate-limiting effects on the inflammatory process. Moreover, loss of endothelial ephrinB2 had no impact on plaque size and macrophage accumulation in the atherosclerosis model. This might be due to the insufficient deletion of ephrinB2 in the aorta, as a result of the limited expression of Tie2 promoter in endothelial cells of large conduit arteries.

With respect to the hindlimb ischemia model, it has been reported that ephrinB2 is abundantly expressed in endothelial cells of remodeling collateral arterioles, which might play a role in arteriogenesis by limiting SMC migration and supporting monocyte recruitment. [124]. However, the present results indicated that deletion of ephrinB2 in endothelial cells had no impact on the growth of collaterals as well as perivascular macrophage accumulation which is a hallmark of arteriogenesis [15]. This may be due to the fact that monocytes originate from parallel venules but not collateral arterioles. A recent study has revealed that monocytes emigrate from neighbouring venules, and then migrate towards the remodeling collateral arterioles to support the progression of arteriogenesis. When the paired venule is ligated along with the feeder arteriole, monocyte recruitment is abolished [185].

Besides the limitations of the utilized models, the redundancy of ephrinB family members also has to be considered. EphB2 receptor is capable of binding to ephrinB1, ephrinB2 and ephrinB3 [113]. While ephrinB3 is not expressed on the endothelial cells, ephrinB1 might compensate for loss of ephrinB2 in endothelial cells of ephrinB2$^{EC\text{-}iKO}$ mice [157]. The redundancy among ephrinB family members has already been indicated in previous publications. T cells specific double null ephrinB1 and ephrinB2 mutant mice display impairment of immune cell development and functions. However, the single knockout of ephrinB1 or ephrinB2 in T cells has minor effects [130-132]. Therefore, considering the limitations of the current models and a possible ephrinB1-dependent compensatory mechanism, the results cannot fully exclude a role of endothelial ephrinB2 in monocyte extravastion and inflammatory processes.

4.4 The role of endothelial ephrinB1 in inflammation

4.4.1 EphrinB1 reverse signaling mediates EphB2-induced E-selection upregulation in endothelial cells

As discussed before, EphB2 binds with ephrinB1 and ephrinB2 to induce reverse signaling in endothelial cells. Next, our study delineated that ephrinB1 reverse signaling was mainly responsible for EphB2-induced pro-inflammatory differentiation of endothelial cells. Loss of ephrinB1, but not ephrinB2, decreased EphB2-induced upregulation of E-selectin expression. This is further supported by applying EphB4, which specifically binds to ephrinB2 to induce ephrinB2 reverse signaling in endothelial cells. It indicated that an increase of E-selectin expression elicited by EphB4 was much less when comparing with EphB2 stimulation. These findings intrigued us to focus on the role of endothelial ephrinB1 in inflammation.

4.4.2 Endothelial ephrinB1 expression is increased in inflammation

EphrinB1 is required for embryonic development, as ablation of ephrinB1 results in perinatal lethality associated with defects in neural crest cell-derived tissues, incomplete body wall closure, and abnormal skeletal patterning [186]. In adults, it has been observed that ephrinB1 expression is upregulated in various cell types during different models of inflammation, and it also plays a role in promoting the release of inflammatory cytokines and regulating leukocyte migration [133-135, 140]. Moreover, ephrinB1, expressed by arterial and venous endothelial cells, transduces reverse signals to promote capillary sprout formation and corneal angiogenesis [122, 187, 188]. To date, the effect of endothelial cell ephrinB1 during inflammation is not known. In this study, endothelial ephrinB1 expression was enhanced under several inflammatory conditions. In the atopic dermatitis model, ephrinB1 expression was increased in endothelial cells during inflammation-associated angiogenesis. In line with this, TNFα, as a pro-inflammatory cytokine, elicited upregulation of ephrinB1 expression in HUVECs. This is further supported by an *ex vivo* experiment, indicating that ephrinB1 expression was elevated in endothelial cells of TNFα-stimulated murine femoral artery. With respect to ephrinB2 expression, it was unaffected in HUVECs in response to TNFα, which is consistent with previous findings using iris endothelial cells [189, 190]. Additionally, the results also showed that endothelial ephrinB1 was strongly expressed in venules running in parallel to collateral arterioles

undergoing arteriogenesis. The venules are the zones where monocytes originally emigrate from [185]. Hence, these findings suggest that the upregulation of endothelial ephrinB1 may play a role in monocyte extravasation through its interaction with EphB2 in monocytes.

4.4.3 Endothelial ephrinB1 promotes monocyte transmigration

This study revealed that endothelial ephrinB1 facilitated monocyte transmigration through an endothelial cell monolayer. This is supported by the finding that MCP-1-induced THP-1 cell transmigration was inhibited by knockdown of endothelial ephrinB1 expression. Exactly how ephrinB1 assists monocyte transmigration is at present unclear, but may depend on the association of ephrinB1 and endothelial junction proteins. Our results indicated that unlike ephrinB2, ephrinB1 did not physically interact with PECAM-1 in endothelial cells. Nevertheless, some previous publications have revealed that ephrinB1 is associated with tight junction proteins in different cell types to modulate their functions. For instance, ephrinB1 interacts with the claudin-5/ZO-1 complex at the lateral membrane in cardiomyocytes, which is essential for stabilizing cell morphology and cardiac tissue architecture [191]. EphrinB1 creates a complex with adjacent claudin1/4 in epithelial cells to regulate cell adhesion and paracellular permeability [181]. In addition to direct association with tight junction proteins, ephrinB1 is capable of regulating the distribution of ZO-1 and Cingulin by interacting with scaffold protein Par-6. This process eventually affects tight junction formation [110]. These findings provide us with a hint that ephrinB1 may be related to adherens and/or tight junction proteins in endothelial cells. Its interaction with EphB2 may modulate the activation of these junction proteins (phosphorylation and/or internalization) and thereby controlling endothelial cell-cell contacts. However, this possibility needs to be further investigated.

4.5 Summary of the effects of EphB/ephrinB during inflammation

Taken together, inflamed tissues display an increased expression of ephrinB1 and ephrinB2 in endothelial cells of angiogenic capillaries, which bind to EphB2 receptors expressed in monocytes. This ligand-receptor interaction plays a role in monocyte extravasation mediated by ephrinB reverse signaling in endothelial cells. EphrinB1 and ephrinB2 facilitate monocyte transmigration through the endothelial

cell monolayer. This might be due to EphB2 stimulated Src-dependent PECAM-1 phosphorylation, VE-cadherin internalization and ephrinB2 association with junctional proteins (PECAM-1, VE-cadherin, ZO-1). Coevally, EphB2-stimulated reverse signaling upregulates E-selectin expression mediated by ephrinB1/JNK, enhances VCAM-1 and GM-CSF expression as well as vWF deposition, and thus reinforces monocyte adhesion. Furthermore, EphB2 stimulation disrupts the endothelial cell barrier function. Collectively, considering previous findings showing that EphB forward signaling stimulates the release of pro-inflammatory cytokines from monocytes [136], it is tempting to speculate that the EphB/ephrinB system may amplify pro-inflammatory responses by their bidirectional signaling in both endothelial cells and leukocytes.

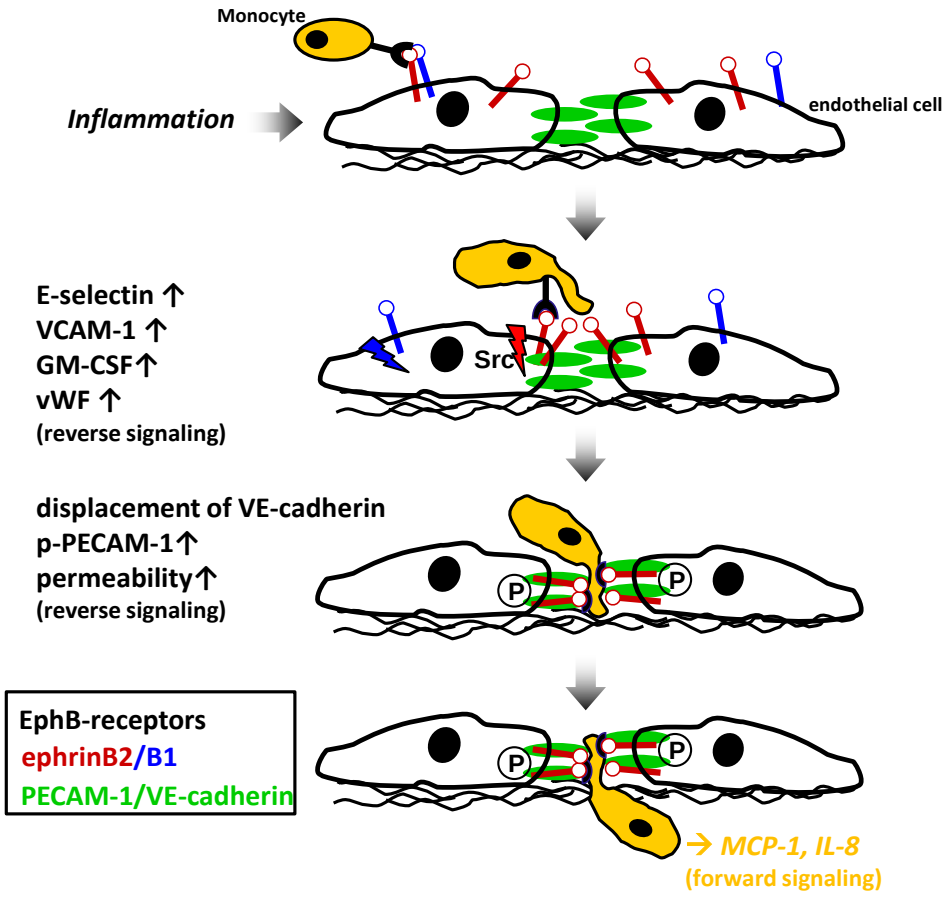

Figure 30: Summary of EphB/ephrinB-mediated effects during inflammation

Binding of monocytic EphB2-receptors to endothelial ephrinB-ligands triggers reverse signaling in endothelial cells, leading to monocyte adhesion and transmigration, as well as increased permeability of an endothelial cell monolayer. EphB2 forward signaling in monocytes promotes their activation.

4.6 Perspective

Based on the aforementioned findings in this study, some questions need to be addressed in the future.

1. What is the role of endothelial ephrinB reverse signaling in inflammation *in vivo*? This question could be addressed by using inflammatory models on inducible endothelial cell specific ephrinB1 and ephrinB2 double knockout mice to assess the progression of inflammation and leukocyte extravasation.
2. Does EphB2 induce translocation of ephrinB1 and ephrinB2 in endothelial cells? Does EphB2 promote formation of a complex containing ephrinB and endothelial cell junction proteins? Does EphB2 regulate activation of junction proteins such as phosphorylation and internalization, resulting in loosening cell-cell contacts? Furthermore, These questions intrigue us to investigate the distribution and interaction of ephrinB and intercellular junction proteins upon EphB2 stimulation in more detail.
3. What are the downstream effectors of ephrinB reverse signaling in endothelial cells, that are responsible for EphB2-induced endothelial permeability and leukocyte diapedesis? Several candidates remain to be analyzed, for instance, MLCK, FAK, RhoA and ROCK activity.

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