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Title page

Nectins and Nectin-Like Molecules drive vascular development and barrier function

Hermans Doryssa[1], Rodriguez-Mogeda Carla[2], Kemps Hannelore[3,4], Bronckaers Annelies[3], de Vries Helga E.[2], Broux Bieke[1]

[1]UHasselt, Biomedical Research Institute (BIOMED), Department of Immunology and Infection, Diepenbeek, Belgium
[2]MS Center Amsterdam, Molecular Cell Biology and Immunology, Vrije Universiteit Amsterdam, Amsterdam Neuroscience, Amsterdam UMC location VUmc, Amsterdam, The Netherlands
[3]UHasselt, Biomedical Research Institute (BIOMED), Department of Cardio & Organ Systems, Diepenbeek, Belgium
[4]KU Leuven, Department of Cardiovascular Sciences, Center for Molecular and Vascular Biology, Leuven, Belgium

Abstract

Angiogenesis, barriergenesis and immune cell migration are all key physiological events that dependent on the functional characteristics of the vascular endothelium. The protein family of Nectins and Nectin-like molecules (Necls) is a group of cell adhesion molecules that are widely expressed by different endothelial cell types. The family includes four Nectins (Nectin-1 to -4) and five Necls (Necl-1 to -5) that either interact with each other by forming homo- and heterotypical interactions or bind to ligands expressed within the immune system. Nectin and Necl proteins are mainly described to play a role in cancer immunology and in the development of the nervous system. However, Nectins and Necls are underestimated players in the formation of blood vessels, their barrier properties, and in guiding transendothelial migration of leukocytes. This review summarizes their role in supporting the endothelial barrier through their function in angiogenesis, cell-cell junction formation, and immune cell migration. In addition, this review provides a detailed overview of the expression patterns of Nectins and Necls in the vascular endothelium.

Corresponding author
Bieke Broux, PhD
Bieke.broux@uhasselt.be

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Nectin; Nectin-like molecule; Endothelial cells; Angiogenesis; Adherens junction; Migration

Conflict of interest
The authors declare that they have no conflict of interest.

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Introduction

Blood vessels, lined by endothelial cells (ECs), form one of the most regulated barriers of the body. Continuous vascular remodeling enables blood vessels to adapt to the body's needs and to guide cells of the immune system towards inflammatory sites. Blood vessel formation relies on the dynamic endothelial cytoskeleton and the correct environmental guidance cues. Junctional molecules, including adherens junctions (AJ) and tight junctions (TJ), regulate the barrier's integrity and permeability, while specific cell adhesion molecules (CAMs) support the migration of immune cells to sites of infection. Members of the Nectin and Nectin-like molecule (Necl) protein family (other nomenclature is summarized in Table 1) are mostly described for their roles in the context of cancer immunology and development of the central nervous system. However, Nectins and Necls are also widely expressed across the vascular endothelium (Table 2), suggesting that they are important players in vascular development and/or function. They are anchored to the actin cytoskeleton, expressed at cell-cell contact sites and are part of the Ca^{2+}-independent superfamily of immunoglobulin CAMs (IgCAMs) as immune cell ligands used for extravasation [1-3]. In this way, the Nectin and Necl protein family, consisting of four Nectins (Nectin-1 to -4) and five Necls (Necl-1 to -5), participates in processes essential for physiological homeostasis (Fig. 1). Generally, Nectins and Necls share the same protein structure, *i.e.* a cytoplasmic tail, a single transmembrane region, and an extracellular region with three Ig-like domains (Fig. 2). However, they differ in their amino acid composition at the cytoplasmic tail which affects their intracellular properties, e.g. Nectins exhibit a PDZ binding motif (named after the first three proteins discovered to share the domain, *i.e.* postsynaptic density-95 (PSD-95), Discs-large, Zona occludens 1 (ZO-1) domains) which allows them to interact with the actin anchoring protein, afadin, while Necls do not [4].

Nectin and Necls mediate weak cell-cell adhesion, compared to cadherins, through *cis*-interactions on the same cell, followed by homo- and heterophilic *trans*-interactions on neighboring cells (Fig. 1), either with other members of their family or their immune cell ligands: DNAX accessory molecule 1 (DNAM1/CD226), class I-restricted T-cell-associated molecule (CRTAM/CD355), T-cell-activated increased late expression (Tactile/CD96) and T cell immunoreceptor with Ig and ITIM domains (TIGIT) [5]. For an extensive review on the physical binding properties of Nectins and Necls, see [4]. To date, Nectins and Necls have mainly been studied and reviewed in fibroblasts and epithelial cells [6], in neurodevelopment and disease (*i.e.* axon guidance, synapse formation, myelination) [4, 5], and as malignant cell targets [7, 8]. However, many of the Nectin and Necl functions in epithelial cells can be translated to ECs, which pinpoints them as interesting study targets in vascular diseases. In this review, we will describe the heterogeneous roles of the Nectin and Necl protein family in angiogenesis, the construction of junctional molecules and supporting immune cell migration across inflamed endothelium, with the focus on human cell systems.

Table 1 Gene nomenclature of the Nectin and Necl protein family in *Homo sapiens* used in literature. Nomenclature was extracted from National Center for Biotechnology Information (NCBI) and UniProt. CD, cluster of differentiation; HVE, human herpesvirus entry receptor; PRR/PVRL, poliovirus receptor related gene; HIgR, Herpesvirus Ig-like receptor; CADM, Cell adhesion molecule; SYNCAM, Synaptic cell adhesion molecule; IGSF, Immunoglobulin superfamily gene; TSLL/TSLC, Tumor suppressor gene; TAG, tumor-associated glycoprotein.

	Nomenclature
Nectin-1	CD111, HVEC, PRR1, PVRL1, HIgR
Nectin-2	CD112, HVEB, PRR2, PVRL2, PVRR2
Nectin-3	CD113, PPR3, PRR3, PVRL3, PVRR3
Nectin-4	PVRL4, PRR4
Necl-1	CADM3, SYNCAM3, IGSF4B, TSLL1
Necl-2	CADM1, SYNCAM1, IGSF4, IGSF4A, TSLC1, RA175
Necl-3	CADM2, SYNCAM2, IGSF4D
Necl-4	CADM4, SYNCAM4, IGSF4C, TSLL2
Necl-5	CD155, HVED, PVR, PVS, TAGE4

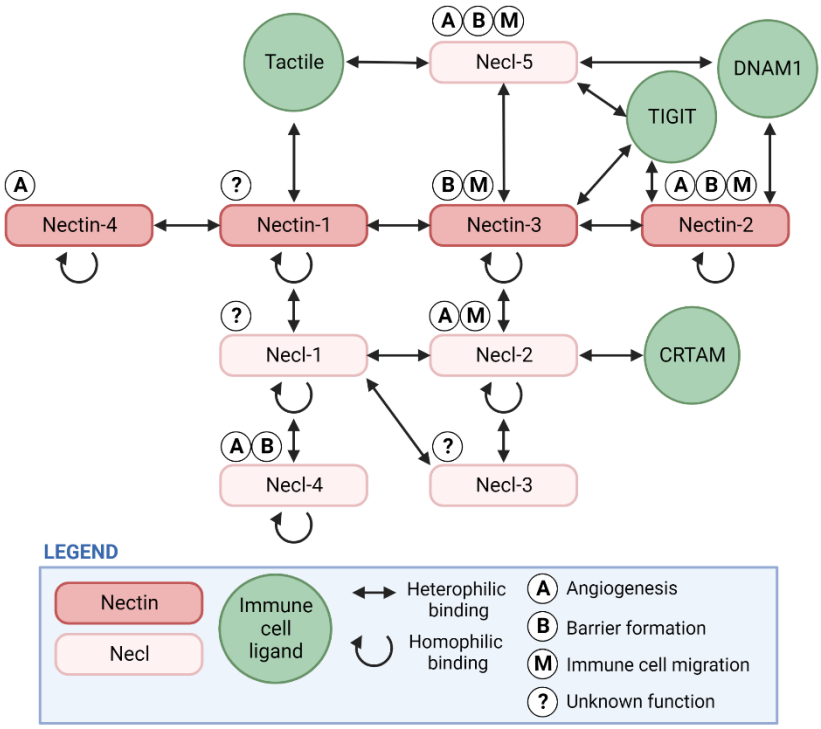

Fig. 1 Extracellular *trans*-interactions of the Nectin and Nectin-like protein family. The family consists of four Nectins (Nectin-1 to -4) and five Necls (Necl-1 to -5). Their involvement in the biological processes: angiogenesis (A), barrier formation (B) and immune cell migration (M) are allocated to each protein. The (?) points out their unknown role in these events. Homophilic and heterophilic interactions are shown in looped arrows and double-headed arrows, respectively. Immune cell ligands, including DNAX accessory molecule 1 (DNAM1/CD226), class I-restricted T-cell-associated molecule (CRTAM/CD355), T-cell-activated increased late expression (Tactile/CD96) and T cell immunoreceptor with Ig and ITIM domains (TIGIT), are shown in green. Figure adapted from [4, 5] and created with BioRender.com.

Angiogenesis

The formation of new blood vessels from pre-existing ones, a phenomenon designated as angiogenesis, is characterized by the dynamic interaction of ECs with their surrounding extracellular environment to establish a new vascular network. In adult tissue, there are two types of angiogenesis: sprouting angiogenesis in which new blood vessels bud from existing capillaries and intussusceptive angiogenesis where a capillary wall protrudes into the lumen of a blood vessel causing the tube to split in two branches [9]. In the following sections, we provide an overview on the general steps of sprouting angiogenesis and we will discuss how Nectins and Necls regulate this process in physiological and pathological conditions (Fig. 2a, b).

Sprouting angiogenesis

Sprouts of ECs emerge from pre-existing capillary endothelium and invade into adjacent tissues in response to angiogenic factors, e.g. vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) [10]. In turn, ECs secrete angiopoietin-2 (ANG-2) which causes pericyte detachment from the vessel through endothelial TIE2 receptor inactivation, while the underlying basement membrane is degraded by local matrix metalloproteinases (MMPs) [11, 12]. Junctions between adjacent ECs loosen and vascular permeability increases, resulting in the extravasation of plasma proteins, providing a temporary scaffold of extracellular matrix (ECM) along which ECs migrate. Angiogenic proteins bound to the ECM are released by proteases which creates a pro-angiogenic milieu and stimulates the motility and invasiveness of ECs [12, 13]. In sprouting angiogenesis, a specialized EC, called the tip cell, creates a leading edge of the outgrowing sprouts and protrudes filopodia expressing receptors for guidance cues such as netrins, ephrins and semaphorins [11, 12, 14]. While the tip cell continuously probes the environment, the adjacent stalk cells proliferate to elongate the emerging vessel sprout. Selection of tip and stalk cells occurs mainly through VEGF-A/VEGFR2 stimulation and DLL4/Notch signaling [11, 12, 15]. As the tip cell migrates under influence of VEGF-A, signaling molecules crucial for EC chemotaxis and motility are activated, including the Rho family of guanine nucleotide (GTP) binding proteins: Rho, Rac and Cdc42 [10, 16].

To form a mature blood vessel, ECs located in the outgrowing sprout undergo morphological differentiation to create tubular structures [17]. This requires pervasive remodeling of the actin cytoskeleton, which is mediated by the adaptor protein afadin [18]. By binding to F-actin and by activating signaling molecules such as Rap1, Rac and Cdc42, afadin coordinates the rearrangement of actin filaments during EC migration and the establishment of intercellular contacts (see 'Barrier formation'). Here, colocalization of Rap1 with afadin is primarily observed in the leading edge of the sprout and at cell-cell contacts and is crucial for the assembly of junctional proteins [17, 18]. In addition, Rap1 and afadin are important regulators in the VEGF-dependent activation of the Akt/eNOS signaling pathway, which is crucial for vascular remodeling and maturation [17]. A vascular lumen is formed by fusion of intracellular vacuoles from adjacent ECs [11]. Induction of blood flow occurs after the fusion of a tip cell with the tip cell of a neighboring vessel branch, a process supported by myeloid cells [11, 12]. Perfusion of the blood vessel induces a quiescent EC phenotype due to reduced VEGF expression in response to oxygen and nutrient delivery [11]. The vessel becomes stable by ECM deposition and coverage by mural cells, *i.e.* pericytes and vascular smooth muscle cells, mediated via paracrine mediators such as platelet-derived growth factor B (PDGF-B), angiopoietin-1 (ANG-1)-TIE2 receptor signaling, and transforming growth factor-β (TGF-β) [10, 11].

Hence, angiogenesis can be considered as a highly regulated set of consecutive events in which signaling pathways, growth factors and their receptors, ECM, proteases and vascular cells all act cooperatively to form a mature blood vessel [10].

Role of Nectins and Necls in angiogenesis

Nectins and Necl proteins are widely expressed by different EC types, including endothelial progenitor cells (EPCs) and micro/macrovascular ECs (summarized in Table 2), and accordingly play a crucial role in regulating angiogenesis. Since Necl-3 expression has not been studied in ECs, it will not be discussed here. EPCs are bone marrow-derived progenitor cells present in the peripheral circulation, vessel walls and the bone marrow, have a greater capacity to proliferate, and promote blood vessel formation by their cytokine production or differentiation into mature vascular ECs in case of tissue repair [1, 19]. Nectin-2 is highly expressed on quiescent circulating EPCs to ensure their progenitor stage. When EPCs participate in blood vessel development, such as tube formation, Nectin-2 is downregulated and ERK signaling is activated [1]. In contrast, Necl-2 is upregulated and essential for EPCs to participate in TNFα/NF-κB-induced angiogenesis *in vitro*, thereby promoting tissue repair. Consequently, Necl-2 silencing or NF-κB inhibition in EPCs abrogates TNFα-induced EPC migration and tube formation [19].

Vascular ECs exhibit differential regulation and localization of Necl-4, Necl-5 and Nectin-2 expression during angiogenesis (Table 2). Upon tube formation or increasing cell density, ECs upregulate Necl-4 at cell-cell contact sites while Necl-5 and Nectin-2 are downregulated, indicating diverse functional properties [1, 2, 20-22]. However, both Necl-4 and -5 are concentrated at the leading edge of the tip cell during EC migration and *cis*-interact with VEGFR2 through their extracellular domain [2, 20, 21]. When ECs are sparsely cultured, Necl-4 and -5 at the leading edge promote cell migration, proliferation and cell survival by supporting the VEGF-induced pro-angiogenic response [2, 20]. Necl-4 inhibits Rho/ROCK signaling through activation of PTPN13 phosphatase, thereby facilitating PI3K/Rac1 pathway activation responsible for cell movement [2, 23]. Moreover, Necl-4 promotes cell proliferation through an augmented activation of the PLC/Raf/MEK/ERK pathway [2, 23]. Consequently, endothelial Necl-4 silencing leads to reduced cell proliferation, tubulogenesis and delayed wound healing in response to VEGF *in vitro* [2, 23]. When confluent, Necl-4 inhibits phosphorylation of VEGFR2 receptor tyrosine residues through PTPN13, resulting in decreased cell movement and proliferation due to suppression of PLC/Raf/MEK/ERK and PI3K/Rac1 pathways [2]. For an extensive review on the intracellular pathways induced by Nectins and Necls, see [6].

Necl-5 mainly functions in the early stages of angiogenesis as it is crucial for tip cell polarization [20, 21]. In absence of Necl-5, ECs are unable to form a leading edge and the VEGFR2-integrin αvβ3 interaction is abrogated [20]. This suppresses VEGF-induced capillary-network formation, EC migration and proliferation, while enhancing apoptosis and cell adhesion to ECM-proteins, due to failing Rap1 and Akt/eNOS activation [20, 21]. Accordingly, Necl-5 deficient mice display an impaired blood flow recovery after hind limb ischemia *in vivo*, which is associated with diminished neovascularization while normal vascular development is not affected [20]. In the mouse fibroblast cell line NIH3T3, Necl-5 also *cis*-interacts with the PDGF receptor (PDGFR) and integrin αvβ3 through

their extracellular domains and promotes PDGF-mediated cell migration by Rac/Cdc42 activation, and PDGF-induced proliferation by Ras/Raf/MEK/ERK signaling [24-28]. One mechanism of action is that Necl-5 recruits cytoplasmic Sprouty2, which alleviates its inhibitory effect on PDGF-induced Ras signaling [27, 28]. The physical and functional PDGFR-Necl-5-integrin αvβ3 interaction enhances their clustering at the leading edge and induces synergistic signaling responses [24, 26]. Although the *cis*-interaction and intracellular responses were investigated in NIH3T3 cells, Necl-5 knockdown in HUVECs also impairs PDGF-induced cell migration and leading edge formation, indicating that these findings might be applicable to ECs and angiogenesis [20]. In addition, the confluency-dependent downregulation of Necl-5 is mediated by *trans*-interaction with Nectin-3 on adjacent cells, which causes endocytosis of Necl-5 in NIH3T3 cells [21]. This mechanism of contact inhibition through Necl-5-Nectin-3 interaction can likely be translated to vascular ECs since Nectin-3 expression shows an opposite cell density-dependent expression in ECs [1, 2]. At cell-cell junctions, Nectin-3 is the only Nectin that *cis*-interacts with the PDGFR in NIH3T3 cells and supports cell survival through PI3K/Akt signaling [29].Although Necl-5 and Nectin-2 show a similar density-dependent expression profile, they exert opposite functions. *In vitro*, Nectin-2 suppresses angiogenic processes including EC proliferation, tubulogenesis and cell-cell contact formation. *In vivo*, Nectin-2 deficiency in mice induces increased blood vessel formation in the retina and spleen. This suggests that Nectin-2 and Necl-5 might outbalance each other's angiogenic effects [1, 22, 30]. On the other hand, Nectin-2 stabilizes DLL4 expression at the cell surface through multiple PDZ domain protein (MPDZ/MUPP1) binding, thereby regulating DLL4 interaction with Notch and supporting tip cell competition [31]. Although MPDZ silencing in ECs reduces DLL4-Nectin-2 colocalization, Nectin-2 deficiency does not impact DLL4 expression [31].

Besides the physiological angiogenic functions of Nectins and Necls, Nectin-4 is highly expressed by vascular ECs in malignant conditions, such as breast cancer, where it promotes tumor angiogenesis and metastasis (extensively reviewed as a potential target for cancer therapy in [8]). More specifically, cancer cells shed Nectin-4 into the microenvironment by which it interacts with endothelial integrin-β4 and induces a pro-angiogenic response through Src, PI3K, Akt, iNOS signaling [32, 33]. This results in enhanced tube formation of vascular ECs *in vitro*, and new blood vessel formation *in ovo* and *in vivo*, thereby promoting pathological angiogenesis [32]. Hence, Nectin-4 silencing reduces the proliferation and tube formation of carcinogenic ECs *in vitro* [33]. In addition, Nectin-2 is implicated to be involved in tumor angiogenesis and metastasis since its downregulation is associated with increased vascular permeability [22].

Altogether, Nectins and Necl molecules function in different stages of angiogenesis, including EC proliferation, migration, leading edge formation and contact inhibition, to maintain tissue integrity and enhance wound healing. Moreover, these molecules show dual functions depending on the stage of blood vessel formation, *i.e.* sparse/migrating or confluent/tube forming ECs. Although they play pivotal roles *in vitro*, Necl-5 or Nectin-2 deficiency *in vivo* somehow results in milder phenotypic changes than expected, *i.e.* normal vascular development or dysregulated angiogenesis in a limited amount of organs, suggesting compensatory mechanisms of other members of the protein family [20, 30]. For example, researchers found that Nectin-2 silencing in HUVECs results in a compensatory change in Nectin-1, Necl-1 and Necl-4 expression [1].

Table 2: Expression profile of Nectin and Necl proteins within the human endothelium. Macrovascular endothelium includes human umbilical vein endothelial cells (HUVEC), human aortic endothelial cells (HAEC), human lymphatic endothelial cells, arteries and veins of different tissues (based on immunohistochemistry). Microvascular endothelium includes human dermal microvascular endothelial cells (HDMEC), human brain microvascular endothelial cells (HBMEC), human lung microvascular endothelial cells and corneal endothelium.

	Endothelial cell expression				**Cell density dependent expression**		Ref.
	Macrovasc. endothelium	Microvasc. endothelium	Endothelial progenitors	Cellular location	Sparse	Confluent	
Nectin-1	+	+	+	A, J	↗	↘	[1, 3, 34]
Nectin-2	+	ND	+	J	↗	↘	[1-3, 30, 35]
Nectin-3	+	ND	+	J	↘	↗	[1-3]
Nectin-4	-	-	ND	ND	ND	ND	[3, 33, 36]
Necl-1	ND	ND	+	ND	ND	ND	[1]
Necl-2	+	+	+	C, LE	ND	ND	[19, 37, 38]
Necl-3	ND	ND	ND	ND	ND	ND	ND
Necl-4	+	+	+	J, LE	↘	↗	[1, 2]
Necl-5	+	ND	ND	J, LE	↗	↘	[2, 3, 20, 35]

ND = not determined; + = present; - = absent; A = apical; C = cytoplasmic; J = junctional; LE = leading edge; ↗ = upregulated; ↘ = downregulated.

Barrier formation

After blood vessel formation, the endothelium is tightly regulated to form a barrier that restricts the movement of molecules and cells between the blood and the interstitial compartments. Moreover, ECs become polarized with an apical site containing CAMs and transporters and a basolateral site attached to the basement membrane [39]. AJ and TJ expression dictates the barrier function of endothelial and epithelial cells. Both types of junctions are connected to the cytoskeleton through their F-actin binding proteins. TJ including junctional adhesion molecules (JAMs), occludin and claudins are connected to F-actin through zonula occludens (ZO) family proteins. AJ are composed of Nectins and cadherins and are connected to the cytoskeleton through afadin and catenin, respectively [40]. Although Necls are not officially recognized as AJ, *trans*-interaction with Nectins is essential for AJ formation [41]. In general, TJ connect areas of the plasma membrane and are expressed at the apical side of AJ, while AJ join actin filaments of consecutive cells at the basolateral level [42-44]. However, this apico-basal alignment is less restricted in the vascular EC cell junction apparatus compared to epithelial cells, since TJs and AJs are often intermingled in ECs [45, 46]. Despite these notable differences between endothelial and epithelial cells, the essential mechanisms of Nectin-mediated cell junction formation are common to both polarized endothelial and epithelial cells. For an extensive review on the intracellular pathways involved in Nectin-mediated AJ and TJ formation, see [6]. Five members of the Nectin/Necl protein family show junctional expression at cell-cell contact sites (Table 2), including Nectin-1 to -3 and Necl-4 and -5, which highlights their function in barrier formation. In this section, we discuss how AJ support the formation of TJ and the crucial role of Nectins/Necls herein (Fig. 2b).

Role of Nectins and Necls in barrier formation

Nectins are Ca^{2+}-independent adhesion molecules that mediate weak cell-cell adhesion, compared to cadherins, through homo- and heterophilic binding with other members of their family, using afadin as actin-anchoring protein [5, 34, 47]. For example, Nectin-1 and Nectin-3 homo- and heterophilic binding strength is weaker than E-cadherin homophilic binding when quantitatively analyzed by separation force measurements or intermolecular force microscopy [48, 49]. Besides mediating cell-cell adhesion as part of AJ, the Nectin-afadin complex also regulates the formation of stronger cadherin-based AJ and TJ. Cadherins are Ca^{2+}-dependent adhesion molecules that only engage in homophilic binding and are linked to the cytoskeleton through α- and β-catenins [34, 40, 50]. When cadherins were first discovered, they were named after their primary localizations: E-cadherin on epithelial cells; N-cadherin on neurons; P-cadherin on placenta cells; and VE-cadherin on vascular ECs [51]. The main endothelial AJ, VE-cadherin, binds to β-catenin or plakoglobin, which interact with α-catenin, thereby connecting cadherins to the actin cytoskeleton of adjacent cells [52].

Nectins form homophilic *cis*-dimers at the cell surface followed by *trans*-interaction with other Nectins/Necls through homo- and heterotypical binding to initiate adhesion between neighboring ECs. Afadin binds to Nectin-*trans*-interactions, through its PDZ domain, and to α-catenin, which recruits a non-interacting cadherin-β-catenin complex. In this way, cadherins are engaged at the Nectin-based AJ which promotes cadherin-*trans*-interactions by adjacent cells, resulting in the formation of mature AJ [40, 47, 53]. In contrast to Nectins, Necl molecules cannot bind afadin since they do not exhibit a PDZ-binding motif, and thus cannot connect with a cadherin-

catenin complex. Consequently, when Nectin-3 and Necl-5 expressing cells *trans*-interact in a heterophilic binding, cadherin is only recruited to the Nectin-3 side. However, when the Nectin-3-Necl-5 interaction is blocked, the formation of cadherin-based AJ is inhibited, indicating that *trans*-interaction is crucial [41]. Although it is generally accepted that Necl proteins do not bind afadin, there are indirect indications showing a Necl-4-afadin connection. Rap1 or afadin silencing in vascular ECs inhibits the confluency-dependent upregulation and junctional expression of Necl-4. Moreover, junctional Necl-4 expression colocalizes with VE-cadherin, suggesting that Necl-4 can act as AJ [2].

The F-actin-binding protein drebrin is identified as an additional and essential partner of the Nectin-afadin complex in ECs. Drebrin connects the actin cytoskeleton to afadin and thereby stabilizes Nectin-based AJ [54, 55]. Consequently, drebrin-deficient vascular ECs show disrupted cell-cell contacts *in vitro*, impaired barrier integrity and loss of Nectin-2 and -3 at intercellular junctions through protein internalization, whereas afadin or VE-cadherin expression are not affected, suggesting an essential function in the maintenance of Nectin-based AJ [54-56].

During and after AJ formation, the Nectin-afadin complex first recruits JAM-A through transient afadin-ZO-1 interaction, followed by occludin and claudins, resulting in the formation of TJ [40, 50]. Rap1- or afadin-deficient endothelial and epithelial cells show impaired AJ and TJ formation and localization *in vitro*, including decreased Nectin-2, VE-cadherin, JAM-A and claudin-5 expression at intercellular junctions, combined with an irregular actin filament architecture, resulting in reduced barrier integrity [17, 47, 50, 54]. Moreover, Nectin-2 and/or Nectin-3 silencing in vascular ECs is associated with downregulation of VE-cadherin, prominent barrier disruption and a reduced transendothelial electrical resistance [1, 54]. Recently, serum-derived lysophosphatidic acid (LPA) was identified as a factor that promotes the formation and organization of AJs and TJs, *i.e.* E-cadherin, nectin-2, and ZO-1, in mouse epithelial cells [57]. This serum LPA-dependency is possibly similar in ECs.

Next to junction formation, the Nectin-afadin complex is profoundly involved in the establishment of apico-basal cell polarity. Generally, AJ are located at the basal side of TJ, e.g. Nectin-1 is expressed at the basal side of ZO-1 in endothelium, while Nectin-3 is at the basal side of JAM-A in epithelium [34, 40, 58]. In afadin-deficient cells or when E-cadherin and JAM-A are expressed in the absence of Nectin-3, cells are unable to generate apico-basal alignment of AJ and TJ. Subsequent co-expression of Nectin-3 in addition to E-cadherin and JAM-A induces structural alignment, indicating Nectin-dependency [58].

In conclusion, Nectins mediate a key function in the formation and maintenance of a functional endothelial and epithelial cell barrier as they participate in the initial steps of AJ and TJ generation and determine cell polarity.

Immune cell migration

Immune cells in the bloodstream reach injury and infection sites by crossing the vessel walls in a process called transendothelial migration (TEM). The initial step of TEM is a weak and transient interaction between ECs and rolling leukocytes in postcapillary venules, close to the inflammation site. This is followed by a firm leukocyte arrest to ECs, adhesion, crawling and subsequent diapedesis through a paracellular or transcellular route [59, 60]. All these steps are sequential but can overlap, which complicates the exact timeline of this process. Different combinations of CAMs and chemokine receptors are required for tissue-specific homing [61]. Four main families of CAMs have been identified: selectins, integrins, IgCAMs, and cadherins [61, 62]. Here, we will summarize the general process of immune cell migration without focusing on a specific tissue. Finally, we highlight the role of Nectin and Necl interactions in supporting immune cell migration (Fig. 2c).

Transendothelial migration

Endothelial cell activation

During inflammation, the adhesive properties of endothelial- and leukocyte-associated CAMs change. Inflammatory stimuli, such as histamine and TNFα, induce rapid or slow EC activation, respectively. Rapid activation results in the expression of pre-formed CAMs while a slow activation drives a transcriptional induction of *de novo* CAMs and chemo-attractants [63]. Ultimately, both lead to an upregulation of CAMs, *i.e.* selectins and IgCAMs. Selectins are transmembrane proteins, mediating heterophilic transient Ca^{2+}-dependent adhesion between ECs and leukocytes [64]. In mammals, L-selectin is expressed on most leukocytes, P-selectin on platelets and ECs, and E-selectin on activated ECs [65]. All three bind to the same ligand P-selectin Glycoprotein Ligand 1 (PSGL-1) present on leukocytes, while each can also bind to selectin-specific ligands [66, 67].

Tethering and rolling

Selectins facilitate the capture of flowing leukocytes [68, 69]. This establishes a low affinity and reversible binding, allowing immune cells to adhere weakly and roll along the surface of the endothelium, following the blood flow direction [70]. Leukocyte microvilli often stabilize rolling by slowing down the cell, promoting the interaction of EC-produced chemo-attractants and chemokine receptors, thereby facilitating *in situ* stimulation of leukocyte integrins [71, 72]. Integrins are transmembrane proteins, consisting of a heterodimer of different α- and β-subunits which participate in cell motility [73]. Using their large extracellular domain, integrins bind to ECM proteins or IgCAMs on ECs, e.g. integrin αLβ2 (Lymphocyte Function-Associated Antigen 1; LFA-1) binds to Intercellular adhesion molecule 1 (ICAM-1), and integrin α4β1 (Late Antigen-4; VLA-4) to vascular cell adhesion molecule 1 (VCAM-1) [74, 75]. Upon ligand binding, integrins form focal adhesions, *i.e.* clusters of integrins in the cell membrane [76]. Leukocytes in the bloodstream regularly maintain inactive, low affinity integrins that require to be activated and undergo conformational changes to modulate high affinity binding to activated ECs [77]. This is triggered by extracellular signals, such as cytokines, chemokines or growth factors [78]. Selectin-mediated rolling therefore triggers transient and weak bonds between leukocyte integrins and endothelial IgCAMs which slowly increases their binding affinity [79, 80].

Arrest, adhesion and crawling

Leukocyte adhesion and arrest are supported by IgCAMs, which mediate Ca^{2+}-independent homophilic and heterophilic cell-cell and cell-ECM interactions [81]. Besides the well-known ICAM-1 and VCAM-1, other members of this family are neural-CAM (NCAM), melanoma-CAM (MCAM), activated leukocyte-CAM (ALCAM), platelet/endothelial-CAM-1 (PECAM-1/CD31) and Nectins [82-85]. On the EC surface, integrin-IgCAM binding recruits adaptor proteins that link the actin cytoskeleton to IgCAMs and protrude the apical membrane, forming docking structures for leukocytes via RhoG GTPase, where VCAM-1 and ICAM-1 will be clustered [86, 87]. On the immune cell surface, integrin-IgCAM binding promotes actin polymerization which forms protrusive lamellipodia and filopodia, structures needed for further migration and sensing the environment [88]. A crawling leukocyte can move in opposite direction of the blood flow, is integrin-dependent and chemokine-stimulated. Integrins in the leading edge of protrusive leukocyte structures contact the apical side of the endothelium. Immune cells crawl on the ECs, scanning for exit cues that provide chemotactic guidance to leave the vessel. This triggers changes in leukocyte morphology and weakening of endothelial junctions and/or formation of intracellular pores depending on paracellular or transcellular migration [89, 90].

Transmigration

Immune cells can transmigrate via paracellular or transcellular routes. Paracellular transmigration is the migration of leukocytes through EC junctions, while transcellular migration is through the EC body [60]. Most leukocytes use the paracellular route to leave the peripheral circulation *in vivo* due to a low TJ expression [91]. Selectin and IgCAM clustering on the EC surface activates signaling pathways that dissemble VE-cadherin and rearrange the actin cytoskeleton. Due to the loss of VE-cadherin and other junctional molecules, leukocytes can migrate in between ECs [92]. Some of these molecules are internalized and recycled in the lateral border recycling compartment (LBRC), a vesicular trafficking system of CAMs and junctional proteins which supports transmigration [93-95]. However, leukocytes might prefer to migrate transcellular in particular locations, such as the blood-brain barrier (BBB) [96]. BBB-ECs support the immune cell privilege of the central nervous system (CNS) as their high TJ expression complicates paracellular migration of leukocytes. In case of transcellular migration, the LBRC, carrying PECAM-1, CD99, JAM-A and other CAMs, is recruited to the chosen site of diapedesis at the apical surface and will surround the leukocyte [93, 97]. LBRC vesicles will fuse with the apical membrane creating a transcellular pore that allows leukocyte protrusions to move across [90]. How the LBRC is targeted to the apical surface is still unknown. Several factors might influence transcellular migration, such as the tightness of EC junctions, their stiffness and whether leukocytes can breach these junctions [96].

Role of Nectins and Necls in immune cell migration

Necl-5 and Nectin-2 are the most investigated family members in the process of (chemokine-mediated) immune cell migration, using HUVECs and HDMECs. These DNAM1 ligands are expressed at the intercellular junctions of vascular ECs, similar to PECAM [3, 35, 98]. The TEM of monocytes and effector memory $CD4^+$ T lymphocytes across resting endothelium is inhibited when blocking either endothelial Necl-5 or DNAM1 on leukocytes *in vitro*, inducing a similar blockade as anti-PECAM or anti-LFA-1 antibodies [35, 98, 99]. Here, monocytes were shown to be arrested at the apical surface of the endothelium, at sites of interendothelial junctions [35, 98]. These findings

support the notion that DNAM1-Necl-5 interaction is involved in immune cell diapedesis, but not in early leukocyte adhesion to ECs. Using a similar mechanism as PECAM, Necl-5 stimulation by DNAM1 induces phosphorylation of immunoreceptor tyrosine-based inhibitory motif (ITIM) by a Src-dependent mechanism, leading to redistribution and binding of Shp-2. Moreover, Necl-5 is the only known Nectin family molecule that partly resides in the LBRC [98]. Besides Necl-5, the DNAM1-Nectin-2 interaction is involved in the migration of effector memory $CD4^+$ T lymphocytes [99]. Tactile (CD96), expressed by T lymphocytes and natural killer (NK) cells, is an additional receptor for Necl-5. Although this interaction is less explored in the migration process, it was shown that Tactile is also involved in effector memory $CD4^+$ T lymphocyte migration across resting endothelium, to a similar extent as DNAM1 [99]. Given that DNAM1 is widely expressed amongst leukocytes and Necl-5 is functionally comparable to PECAM, this interaction may have a more general role in immune cell migration.

Nectin-2 is the main counter-receptor of Nectin-3, expressed by the majority of T lymphocytes and monocytes [3]. Consequently, *in vitro* TEM of T lymphocytes and monocytes is inhibited by either Nectin-2 blockade on ECs or Nectin-3 blockade on the corresponding leukocytes [3, 30]. Herein, Nectin-2 is likely to be involved in diapedesis as endothelial Nectin-2 silencing does not affect the adhesion of monocytes [3, 100]. In addition, Nectin-2 is the only Nectin expressed by high endothelial venules, which are actively involved in lymphocyte egress or homing to secondary lymphoid organs and confirm the relevance of Nectin-2 during lymphocyte extravasation and recirculation (11, 12). *In vivo*, Nectin-2 deficient mice display a 50% reduction in the amount of migrated leukocytes when applying the dorsal air pouch model to mimic a microvascular inflammatory response. The decreased leukocyte migration is due to a declined amount of monocytes and macrophages, whereas T lymphocyte migration was unaffected [100]. In pathological conditions, such as atherosclerosis, Nectin-2 deficient mice exhibit a reduced lesion size and more stable plaques, attributable to an altered immune cell composition in lesions. Again, less macrophages infiltrated, while the T lymphocyte number remained unchanged. This confirms that endothelial Nectin-2 is mainly involved in TEM of monocytes and macrophages during inflammation [100]. Whether this effect on myeloid cells is mainly mediated by DNAM1-Nectin-2 interaction or Nectin-3-Nectin-2 interaction still needs to be explored. The loss of *in vivo* effects on T lymphocytes may indicate a redundant role of Nectin-2 in their migration towards inflammatory sites.

Another unexplored interaction is the CRTAM-Necl-2 interaction. In contrast to DNAM1, CRTAM only binds one ligand within the Nectin family, providing a more specific interaction. Necl-2 is widely expressed by macro- and microvascular ECs throughout the body (Table 2), while CRTAM is mainly expressed by activated cytotoxic lymphocytes, including NK cells, $CD8^+$ and $CD4^+$ cytotoxic T lymphocytes [37, 101, 102]. *In vitro*, the CRTAM-Necl-2 interaction plays an important role in adhesion [101, 103]. More specifically, CRTAM-transfected cells adhere to Necl-2-coating under laminar flow conditions and remain attached when applying increasing shear stress, thereby mimicking the physiological blood flow and demonstrating the strength of the interaction. Consequently, cell adhesion is inhibited when blocking CRTAM [101]. In addition, different *in vivo* models support the involvement of CRTAM-Necl-2 interaction in immune cell infiltration. Herein, $CRTAM^+$ lymphocytes traffic to inflammatory sites and reside in the intestinal mucosa characterized by high Necl-2 expression [104-106]. In naive

mice, CRTAM or Necl-2 deficiency leads to reduced $CD4^+$ and $CD8^+$ T lymphocyte numbers in the intestinal mucosa, without affecting T cell numbers in the spleen [105]. In pathological conditions, elevated numbers of $CRTAM^+$ lymphocytes infiltrate the inflamed gut after T cell-mediated colitis [104], the lungs after influenza virus infection [104], and the skin in an epidermal Necl-2 overexpressing mouse model for autoimmune alopecia [106]. Although the direct interaction of $CRTAM^+$ leukocytes and ECs has not been investigated yet, these findings implicate a potential role of the CRTAM-Necl-2 interaction in immune cell migration since Necl-2 deficiency or overexpression lead to reduced or increased T cell accumulation, respectively. Therefore, this interaction could be more specific than DNAM1 since CRTAM expression is controlled and limited within the leukocyte population.

Altogether, the endothelium is a source of Nectins and Necl molecules while monocytes, T lymphocytes and NK cells are the main immune cells expressing their ligands: DNAM1, CRTAM, Tactile and TIGIT. However, the current knowledge on the involvement of their interactions in TEM is limited to *in vitro* studies on resting endothelium (based on chemotaxis). To date, it is not well understood how Nectin/Necl expression is regulated in activated ECs (e.g. Nectin-2 expression is upregulated on inflamed human lymphatic ECs [30]). Moreover, most *in vitro* studies focus on HUVECs, while the migratory properties of leukocytes also depend on the EC type, such as BBB-ECs. Although, the Nectin/Necl expression pattern on HBMECs is still undetermined, except for Necl-4 [2], a recent proteomic study on mouse brain capillaries shows altered expression profiles during neuroinflammation, i.e. downregulation of Necl-1 to -4 and upregulation of Nectin-2 [107], which highlights their function at the BBB.

Conclusion and Perspectives

Although well-described in the context of cancer immunology and development of the central nervous system, the role of the Nectin and Necl protein family is often neglected in vascular development and function. Therefore, in this review, we described the role of the Nectin and Necl protein family in blood vessel formation, barrier formation and immune cell migration. During sprouting angiogenesis, Necl-4 and -5 are promoting EC proliferation, migration and survival, while Nectin-2 outbalances their functions by suppressing angiogenic cell communication. In malignant conditions, Nectin-4 expression is induced and promotes tumor angiogenesis. Once the blood vessel is formed, Nectin-3 and Necl-4 sense cell-cell contact and will counteract the pro-angiogenic response. Next, the initial steps of AJ and TJ formation and their apico-basal alignment are highly supported by Nectin-1 to -3, Necl-4 and -5, showing junctional expression at cell-cell contact site. In addition, Nectin-2 and Necl-5 support the diapedesis of $DNAM1^+$ or $Nectin\text{-}3^+$ monocytes, macrophages and $CD4^+$ T lymphocytes through vascular ECs, either via the paracellular route since they are expressed at AJ, or via the transcellular route since Necl-5 is present in the LBRC. On the other hand, Necl-2 is involved in the adhesion and homing of $CRTAM^+$ cytotoxic T lymphocytes or NK cells to sites of infection.

However, there are still remaining questions concerning the biological roles of Nectins/Necls, their dysfunction in pathological conditions, as well as their use as therapeutic targets. Although Nectin-1 and Necl-1 are expressed by ECs, their function in angiogenesis, barrier formation and TEM are poorly investigated. In addition, Nectin and Necl proteins are interesting study targets in case of BBB dysfunction, and associated neurological disorders such as multiple sclerosis [107], stroke, or Alzheimer's disease, since an altered EC expression pattern can affect AJ and TJ stability and promote immune cell migration (e.g. Nectin-2 and Necl-5). Herein, the Nectin/Necl expression

profile and function can be studied in post-mortem human brain tissue and in rodent disease models displaying a (EC-specific) Nectin/Necl deficiency or overexpression. These *in situ* and *in vivo* models will enhance our knowledge on the Nectin and Necl protein family, encourage translational research and help identifying therapeutic targets. However, the potential compensatory expression of other Nectins/Necls should be taken into account. Besides the described function of the Nectin and Necl protein family in EC migration and cell-cell adhesion in this review, the interaction of Nectins and Necls with their ligands expressed on leukocytes also triggers immunological effector functions [108]. Thereby this – often neglected – protein family participates in the construction of the body's protective EC barrier as well as in regulating the immune cell response in inflammatory conditions.

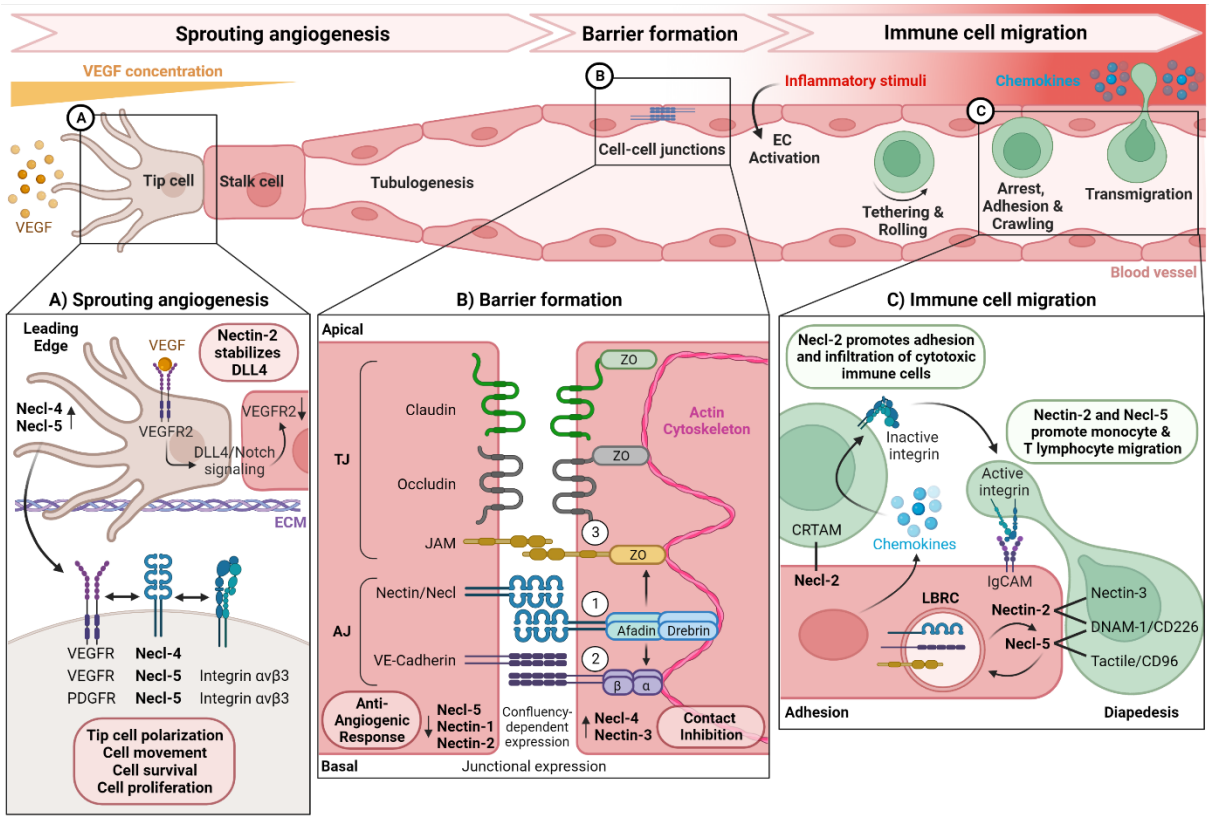

Fig. 2 Overview of Nectin and Necl protein functions in angiogenesis, cell-cell junction formation and immune cell migration. **(a)** Sprouting angiogenesis is triggered by angiogenic factors such as VEGF. The tip cell forms the leading edge of the outgrowing sprout and probes the environment for guidance cues, supported by the ECM, while the adjacent stalk cells proliferate to elongate the emerging vessel. Selection of tip and stalk cells occurs through VEGF/VEGFR2 stimulation and DLL4/Notch signaling. Necl-4 and -5 are concentrated at the leading edge to promote EC migration, survival, proliferation and tip cell polarization. Both *cis*-interact with VEGFR through their extracellular region, while Necl-5 can also interact with PDGFR and integrin αvβ3, having synergistic signaling effects. Nectin-2 stabilizes DLL4 expression and regulates its interaction with Notch, supporting tip cell competition. **(b)** Upon tube formation, ECs upregulate Necl-4 and Nectin-3 at cell-cell contact sites, while Necl-5, Nectin-1 and Nectin-2 are downregulated, which triggers signals of contact inhibition and an anti-angiogenic response. Next, Nectin *trans*-interactions trigger the formation of AJ and TJ. (1) Nectin *cis*-dimers *trans*-interact with other Nectins/Necls through homo- and hetero-typical binding. Afadin binds the interaction and connects Nectins with the actin cytoskeleton, which is stabilized by Drebrin. (2) The Nectin-Afadin complex recruits α-catenin, followed by β-catenin coupled to VE-Cadherin. The clustering of Nectin-Afadin and Cadherin-catenin interactions at cell-cell contact sites forms a mature AJ. (3) The Nectin-Afadin complex next recruits JAM-A through ZO, the actin-scaffolding protein for TJ, followed by occludin and claudins, resulting in the formation of a mature TJ. Moreover, the Nectin-afadin complex supports apico-basal cell polarity, since AJ are generally located at the basal side of TJ. **(c)** Inflammatory stimuli trigger EC activation, leading to an upregulation of CAMs. Weak and transient selectin-mediated leukocyte-EC interactions lead to tethering and rolling of the leukocyte. Inactive and low affinity integrins are slowly activated and undergo conformational changes, triggered by EC-produced chemokines, to ensure high affinity binding to IgCAMs. This leads to firm leukocyte arrest to ECs, adhesion, crawling and subsequent diapedesis through a paracellular or transcellular route. CRTAM-Necl-2 interaction supports leukocyte adhesion, while DNAM1-Necl-5, DNAM1-Nectin-2, Nectin-3-Nectin-2 and Tactile-Necl-5 interactions are involved in monocyte and T lymphocyte diapedesis. Moreover, Necl-5 is the only known Nectin family molecule that partly resides in the LBRC, together with other CAMs, AJ and TJ molecules. α, α-catenin; AJ, adherens junction; β, β-catenin; CAM, cell adhesion molecule; CRTAM, class I-restricted T-cell-associated molecule; DNAM1, DNAX accessory molecule 1; EC, endothelial cell; ECM, extracellular matrix; IgCAM, immunoglobulin CAM; LBRC, lateral border recycling compartment; Necl, nectin-like molecule; Tactile, T-cell-activated increased late expression; TJ, tight junction; VEGFR, vascular endothelial growth factor receptor; ZO zonula occludens. Figure created with BioRender.com.

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