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The physiological and pathophysiological roles of platelet CLEC-2

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Summary

CLEC-2 is a C-type lectin receptor which is highly expressed on platelets but also found at low levels on different immune cells. CLEC-2 elicits powerful platelet activation upon engagement by its endogenous ligand, the mucin-type glycoprotein podoplanin. Podoplanin is expressed in a variety of tissues including lymphatic endothelial cells, kidney podocytes, type I lung epithelial cells, lymph node stromal cells and the choroid plexus epithelium. Animal models have shown that the correct separation of the lymphatic and blood vasculatures during embryonic development is dependent on CLEC-2-mediated platelet activation. Additionally, podoplanin deficient mice show abnormalities in heart, lungs, and lymphoid tissues, whereas absence of CLEC-2 affects brain development. This review summarizes the current understanding of the molecular pathways regulating CLEC-2 and podoplanin function and suggests other physiological and pathological processes where this molecular interaction might exert crucial roles.

Keywords

Platelets; CLEC-2; podoplanin; lymphangiogenesis

The role of platelets beyond haemostasis

Platelets are anucleated circulating cells highly specialized to promptly react upon vessel injury, adhere to the exposed subendothelial matrix and trigger a series of complex intracellular signalling pathways leading to the formation of a shear-resistant haemostatic plug, which seals the wound limiting excessive bleeding. This haemostatic function plays a fundamental role in vertebrates, which employ a closed, high-pressure circulatory system to efficiently deliver oxygen and nutrients to tissues. The evolutionary origin of mammalian platelets has been traced through nucleated thrombocytes in other vertebrates to the primitive circulating haemocytes present in many invertebrates, which are involved in multiple defence mechanisms including phagocytic and haemostatic, wound-sealing functions. Despite the presence of a well-developed immune system in superior vertebrates, some non-haemostatic rudimentary functions persist in mammalian platelets, including bactericidal, phagocytic and inflammatory activities. Given that the haemostatic function is

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only affected when platelet counts fall quite severely (e.g. the physiologic human platelet count range is $150\text{--}400 \cdot 10^3/\mu\text{L}$ but function is adversely affected below $50 \cdot 10^3/\mu\text{L}$), these additional non-haemostatic functions might partly explain the high platelet counts found in mammals (1, 2).

Platelets play important pro-inflammatory roles linked to several common diseases such as atherosclerosis. Under vascular inflammatory conditions, platelets are recruited to sites of endothelial cell activation, where they firmly adhere and release pro-inflammatory compounds. Subsequently, adherent platelets recruit circulating leukocytes and promote their infiltration, contributing to the development of atherosclerotic plaques. When disrupted, these lesions often lead to life-threatening thrombosis (3). Platelets also stimulate the formation of neutrophil extracellular traps (NETs) that entrap microorganisms but which can also cause significant damage to adjacent tissues. Another well-known non-haemostatic function of platelets is the preservation of vascular integrity of mature blood vessels, as very low platelet counts lead to the appearance of petechiae in the skin, a sign of broken capillary blood vessels (4). Additionally, platelets intervene in tissue regeneration after vascular injury through the release of angiogenesis modulators. Autologous platelet administration in the form of fibrin clots provides an adhesive support that effectively reduces the time required for wound healing after dental, orthopaedic and muscle/tendon surgery, although controlled clinical studies are still required to confirm the effectiveness of this novel approach in clinical settings (5, 6).

Role of platelets in embryonic development

A recent study has revealed an unexpected developmental role of platelets triggering the remodelling and closure of the ductus arteriosus (DA) in preterm newborns. Impairment of this process was present in mouse models with defective platelet biogenesis or adhesion. The authors also showed that thrombocytopenia is an independent predictor for failure of DA closure in preterm human infants (7), although this association needs to be confirmed in larger prospective studies. On the other hand, the antiplatelet drugs indomethacin and ibuprofen are the standard medical treatment to drive DA closure in neonates, and there are no reports of increased incidence of patent DA in patients with Glanzmann thrombastenia, suggesting that the role of platelets in DA closure might be mediated by novel, aggregation-independent mechanisms.

The field of platelet biology has been further boosted with the discovery of a novel role for platelets in regulating embryonic lymphatic development (8-11). The lymphatic system plays important roles in homeostatic maintenance such as in facilitating the return of exuded tissue fluid back to the blood circulation at the thoracic duct, the transport of surveying immune cells to secondary lymphoid tissues, and the absorption of lipids in the intestine villi through lacteal vessels. The lymphatic system develops once the blood vascular system is formed and circulation established. Soon after arterial-venous separation takes place, around embryonic day (E) 9.0 in mice and gestation week 6 in humans, a subpopulation of endothelial cells in the cardinal vein commits to the lymphatic lineage and sprouts out to form primordial lymph sacs that later expand and mature into lymphatic plexuses running in parallel but separately from blood vessels. Although many important questions remain unsolved in the lymphangiogenesis field, contributions from multiple groups allowed the recognition of the essential factors required for normal lymphatic development, including the homeobox transcription factor Prox1, which controls the acquisition of a lymphatic cell profile by venous endothelial cells. These Prox1-positive cells then respond to vascular endothelial growth factor-C (VEGF-C) released by neighbouring mesenchymal cells through activation of VEGFR-3/Neuropilin-2 receptor signalling leading to lymphatic sprouting and polarized migration into the surrounding tissue (12).

Systemic oedema and abnormal lymphatic vessels during mid-gestation due to blood-lymphatic misconnections were found in mice embryos deficient in the mucin glycoprotein podoplanin (13, 14), expressed in lymphatic endothelium but absent from blood vessels, or lacking the glycosyltransferase T-synthase critical for the correct biosynthesis of podoplanin (15). Similar blood-lymphatic phenotypes were also unexpectedly observed in mice embryos deficient in the haematopoietic signalling proteins Syk, SLP-76 or PLC γ 2 (16-18), despite none of these proteins being expressed in the developing lymphatic vessels. These surprising observations were clarified after the discovery of the C-type lectin receptor CLEC-2 in platelets as the endogenous ligand for podoplanin, which upon ligand binding induces a signalling cascade involving tyrosine phosphorylation by the kinase Syk and the downstream adapter SLP-76 and activation of PLC γ 2 (19, 20). These data led to the hypothesis that CLEC-2 dependent activation upon podoplanin binding is a fundamental requirement for successful blood-lymphatic separation during embryogenesis. Since CLEC-2 is highly expressed in platelets but also present at low levels in other hematopoietic cells (21), confirmation of the fundamental role of platelets in lymphatic development was only obtained after the description of blood-filled lymphatics in mouse models deficient in the megakaryocyte-platelet lineage or after platelet-specific deletion of CLEC-2, Syk or SLP-76 (8-11).

CLEC-2: a platelet-activating C-type lectin receptor

The CLEC-2 gene *CLEC1B* is located on chromosome 12 in the Dectin-1 gene cluster with six other C-type lectin receptors known as important pattern recognition receptors for the activation of innate immunity (22). CLEC-2 is a type II transmembrane protein containing a C-terminal extracellular domain that lacks the conserved amino acids required for carbohydrate binding. CLEC-2 was first identified in a bioinformatic screen (23) and later recognized as a platelet activating receptor for the snake venom toxin rhodocytin (19). The subsequent identification of podoplanin as the endogenous ligand for CLEC-2 came from the observation that podoplanin-expressing tumour cells induce platelet aggregation in a remarkably similar manner to that of rhodocytin with a long lag phase preceding aggregation and a complete dependency on Src family kinases and PLC γ 2 activities (20). CLEC-2 is a hemITAM bearing receptor present as a homodimer on the platelet surface with a single YxxL motif in its cytoplasmic tail downstream of a tri-acidic amino acid region (DEDG). Pharmacological inhibition of Syk or mutations of the conserved tyrosine on CLEC-2 or the conserved arginines in the phosphotyrosine binding motifs in the Syk SH2 domains result in complete abrogation of CLEC-2 signalling, supporting a model in which Syk crosslinks two phosphorylated receptors (19, 20, 24-26). Binding of CLEC-2 ligands induces a powerful activation cascade (Figure 1) that shares many of the signalling features of the ITAM platelet collagen receptor GPVI FcR γ -chain, including sequential activation of Src and Syk family kinases, adapter proteins such as LAT and SLP-76, and activation of PLC γ 2 (19). There are however notable differences between these two pathways. First, the proximal phosphorylation events upon CLEC-2 ligation are mediated by both Src and Syk tyrosine kinases, whereas phosphorylation of the ITAM is believed to be mediated solely by Src kinases. Additionally, phosphorylation of CLEC-2 in human platelets is strongly dependent on actin polymerisation, release of the secondary mediators ADP and TxA₂, and activation of Rac, demonstrating a critical role for feedback events in CLEC-2 signalling (27, 28), whereas these are not required for optimal phosphorylation of the GPVI-FcR γ -chain complex in response to GPVI-specific agonists. It is noteworthy, however, that this dependence on secondary mediators does not apply in the same way to all proteins in the CLEC-2 signalling cascade (27). Finally, the absence of SLP-76 completely abolishes GPVI downstream signalling, whereas there is a residual level of CLEC-2 dependent activation in the absence of this adapter protein (24). In this context, a role for the adapter proteins Dok-2 and ADAP complementing SLP-76 function in CLEC-2 mediated signalling has been

suggested by a recent proteomic study that detected tyrosine phosphorylation of these proteins following rhodocytin-induced platelet activation (27). Dok-2 tyrosine phosphorylation upon CLEC-2 activation also leads to an increased interaction with the phosphatase SHIP-1, indicating that this lipid phosphatase might play a negative regulatory role in CLEC-2 signalling (27).

Role of CLEC-2 in haemostasis and thrombosis

Although podoplanin is not expressed in blood endothelial cells, platelets, or the exposed subendothelial matrix, a role for CLEC-2 in haemostasis has been reported by demonstration of an increase in tail bleeding using a filter blotting assay in mice in which the C-type lectin receptor has been selectively depleted from the platelet surface using a specific antibody (29). Interestingly, there was no increase in tail bleeding in mice depleted of CLEC-2 using the same antibody or in CLEC-2-deficient mice when measured with a non-filter based assay (11, 30, 31). Consistent with this, there was no detectable increase in tyrosine phosphorylation of CLEC-2 on a collagen surface under shear conditions (31). On the other hand, two groups have reported a role for CLEC-2 in two mouse thrombosis models using the antibody-depleted and CLEC-2-deficient mouse models (11, 29). Additionally, antibody-mediated combined depletion of GPVI and CLEC-2, or genetic deficiency of both platelet proteins, leads to a dramatic haemostatic defect and profound impairment of arterial thrombus formation in mice (30). Interestingly, prolongation of tail bleeding time is not observed in chimeric Syk-deficient mice (32, 33) or mice treated with the selective Syk inhibitor PRT060318 (34), suggesting that defects observed upon concurrent GPVI/CLEC-2 deficiency are mediated by a combination of loss of adhesion and signalling. Further studies are required to investigate the mechanism of the functional redundancy between these two receptors in haemostasis and thrombosis including the possible identification of an additional CLEC-2 ligand within the vasculature.

Podoplanin: a widely expressed glycoprotein with poorly understood roles

Podoplanin is a highly O-glycosylated type-1 transmembrane sialomucin glycoprotein which does not share homology with any other characterized protein. Podoplanin is expressed by a wide variety of different cell types, including kidney podocytes, type-1 lung alveolar cells, the choroid plexus epithelium, lymphatic endothelial cells (LEC) and fibroblastic reticular cells within secondary lymphoid organs. It is also present on a subpopulation of macrophages, osteocytes and osteoblasts, thymus type 1 epithelial cells and Sertoli cells (35, 36).

Podoplanin contains a very short cytoplasmic tail of only nine amino acids that interacts with ezrin-radixin-moesin (ERM) proteins (Figure 2), which provide a fundamental, regulated link between integral membrane proteins and the actin cytoskeleton and are involved in co-ordinating cell shape, motility and adhesion (37). ERM proteins also influence molecular clustering at the immune synapse, with perturbations of ERM function having an inhibitory effect on T-cell receptor clustering (38). Podoplanin expression correlates with the redistribution of ezrin to membrane projections, increased ezrin phosphorylation and the formation of actin-rich filopodia, suggesting that this and other ERM proteins may play a major role in the regulation of the cytoskeleton and cell motility by podoplanin (39, 40). Activation of ERM proteins is regulated by RhoA-dependent kinase (ROCK) phosphorylation. The Rho family GTPases promote ERM protein recruitment to the plasma membrane while ERM proteins themselves are thought to positively regulate Rho activity (37). siRNA mediated knockdown of podoplanin in microvascular LECs leads to abrogated RhoA activation and reduced capillary tube formation and cell migration (41, 42), while in cancer cell lines podoplanin upregulation induces RhoA activation. This

upregulation correlates with the acquisition of a migratory phenotype and the onset of epithelial to mesenchymal transition (EMT) (43), which plays a key role in cancer metastasis. Reactivated EMT is also the cause of organ fibrosis, which leads to the loss of functionality of epithelial structures. However, EMT is also a normal developmental event in which epithelial cells are converted into migratory mesenchymal cells (44). In this context, podoplanin deficient mice show impaired embryonic myocardial formation due to abnormal EMT of the coelomic epithelium that correlates with down-regulation of RhoA (45). These data confirm a complex role of podoplanin in the modulation of RhoA activity and cell migration in both embryonic development and in pathological conditions.

Proposed molecular mechanisms of lymphangiogenesis modulation by platelets

The burning question that has yet to be answered is why CLEC-2 mediated platelet signalling through Syk, SLP-76 and PLC γ 2 is required for normal lymphatic development. It has been proposed that CLEC-2 induced secretion of angiogenic modulators from α -granules could modulate lymphatic endothelium (46). Reports that mice deficient in the integrin-interacting protein kindlin-3 develop blood filled lymphatics led to the suggestion that integrin α IIb β 3-mediated platelet aggregation would be required to seal lymphatic-venous misconnections allowing correct blood lymphatic separation (14). However, both of these hypotheses seem unlikely considering the absence of reported lymphatic abnormalities in patients lacking platelet dense or α -granules, in patients with Glanzmann thrombasthenia (47), and in mice deficient in α IIb- or β 3-integrin subunits (48, 49), or in the global regulator of integrins, talin (50). The observed phenotype in kindlin-3 constitutive knockout mice could be explained by its functional role in β 1, β 2 and β 3 integrin activation in endothelial cells (51, 52) through binding by their FERM domain. In this context, integrin α 9 β 1 deficient mice demonstrate fatal bilateral chylothorax and defective lymphatic valve morphogenesis and function (53, 54), while inhibition of integrin α 4 β 1 function blocks LEC migration and lymphangiogenesis (55).

A potential alternative theory has emerged from recent studies reporting that the CLEC-2 ligand podoplanin promotes cell motility. As mentioned above, podoplanin interacts with the actin cytoskeleton through its binding to the ERM protein family, modulating the activity of Rho family GTPases and altering cell migration capacity in the absence of a ligand (40, 42, 43, 56). We therefore proposed that engagement of CLEC-2 and podoplanin might abrogate podoplanin constitutive signalling and thus lymphatic endothelial cell migration capacity. Since CLEC-2 receptor clustering is an important component of CLEC-2 dependent platelet activation (28), we speculated that CLEC-2 mediated intracellular signalling and receptor clustering induced upon podoplanin binding would reinforce podoplanin clustering and abrogate podoplanin-mediated signalling and subsequent cell migratory potential.

In a series of *in vitro* studies, using primary human lymphatic endothelial cells able to induce both human and mouse platelet activation through CLEC-2, we demonstrated that platelets abrogate the migration and tube formation capacity of human lymphatic endothelial cells (10) through a mechanism involving CLEC-2 and Syk, as CLEC-2 or Syk deficient platelets demonstrate a significantly weaker effect on LEC behaviour. A similar inhibitory effect on LEC migration was observed after antibody-mediated podoplanin clustering, while releasates from CLEC-2 activated platelets did not reproduce the effect, further suggesting that platelets influence LEC behaviour through direct cell-cell contacts. Our results are consistent with previous reports showing the inhibitory effect on migration of anti-podoplanin antibodies *in vitro* (57) and podoplanin-Fc *in vivo* (58).

Osada et al. confirmed the direct inhibitory effect of platelets on lymphatic endothelial cell migration through CLEC-2 mediated podoplanin crosslinking, and additionally suggested a role for released bone morphogenetic protein-9 (BMP-9) in platelet modulation of lymphangiogenesis using a GPVI agonist to obtain platelet releasates (59). Previous data have indicated that different platelet agonists evoke different releasate profiles (60), which Osada et al. propose may explain the discrepant results obtained with CLEC-2 and GPVI induced platelet releasates. However, it should be noted that no difference in the composition of the releasates by these two agonists has been reported and that this seems unlikely given that they signal through similar pathways. The authors also showed that pre-treatment of platelets with a $\alpha\text{IIb}\beta_3$ inhibitor did not attenuate platelet-induced inhibition of LEC migration, proliferation and tube formation, further suggesting that platelet aggregation is not required for lymph/blood vessel separation (59).

Other potential roles of CLEC-2 in physiological processes

There is currently considerable speculation on whether platelet CLEC-2 is involved in other important biological pathways given that podoplanin is expressed in a wide variety of other cells including kidney podocytes, type I lung epithelial cells, lymph node stromal cells and choroid plexus epithelium.

Brain development

One of the striking phenotypes of CLEC-2 deficient mice is the haemorrhaging foci found in the brain ventricles during embryogenesis at day E12.5 onwards (10, 61). The lymphatic system is not present in the brain, but podoplanin is highly expressed on the choroid plexus epithelium suggesting that the interaction between CLEC-2 and podoplanin is required for the correct formation of this structure. The choroid plexus, part of the blood-cerebrospinal fluid barrier, plays an active role in the development, homeostasis, and repair of the central nervous system by controlling the entry of amino acids and hormones from the periphery into the brain, clearing toxins and drugs, and synthesizing trophic and angiogenic factors (62). The choroid plexus has also been suggested as one of the entry points for immune cells into the central nervous system in inflammatory conditions or traumatic brain injury (63). The specific role of CLEC-2 in the biology of the choroid plexus is not yet understood and currently represents an area of active investigation in our group.

Kidney physiology

The kidney glomeruli works as a filtration barrier that allows the passage of water and small molecules into the urinary space while keeping cells and proteins in the capillary blood. The glomerular capillary wall is composed of a fenestrated endothelium, the glomerular basement membrane, and specialized epithelial cells called podocytes due to their characteristic interdigitating foot processes that cover the basement membrane. Podocytes processes are considered the most important determinant of capillary wall selective permeability, as disturbances of their architecture are usually associated with proteinuria and represent a common early symptom of many renal diseases. The podocytes react to injury or damage in many diseases by losing their characteristic morphology. This so called foot process effacement is characterized by cell flattening due to RhoA-dependent rearrangements in the podocyte actin cytoskeleton that normally supports their delicate architecture and opposes the high hydrostatic pressure necessary for glomerular filtration (64). For that reason, proteins regulating the actin cytoskeleton are of paramount importance for podocyte shape maintenance and function. Podoplanin and podocalyxin are negatively charged proteins expressed in the apical membrane of podocytes that faces the luminal urinary side. Although their role in kidney physiology is as yet incompletely understood, evidence suggests that they prevent the passage of negatively charged proteins into the

urinary space and keep adjacent podocytes processes separated. There are several reports of direct interactions of both molecules with ezrin and the actin cytoskeleton that are disrupted in nephrotic syndromes. Furthermore, their absence or antibody-mediated blockade precludes the maintenance of normal podocyte shape, leading to foot process effacement, dysfunctional glomerular filtration barrier and proteinuria (64-67).

Lung physiology

Podoplanin is highly expressed at the apical membrane of type I alveolar epithelial cells lining most of the gas exchange surface of the lung, especially in the microvillus-type protrusions present between type I and neighbouring type II epithelial cells. Mice lacking podoplanin show abnormal lung development during late gestation with narrower airspaces and thicker mesenchyme. These mice fail to expand their alveolar sacs at birth, resulting in perinatal respiratory failure and mortality (68, 69). Mice deficient in CLEC-2 also show reduced airspace in the lung alveoli and fluid present in the larger lung airways, and fail to inflate their lungs normally at birth. During development, the airway epithelium secretes fluid into the lumen of the lung which influences its branching dynamics and morphology. This liquid must be cleared at birth and a large portion (~40%) of the clearance is due to flow through the lymphatics. The disruption of lymphatic function is therefore a potential precursor of the lung inflation failure observed in podoplanin and CLEC-2 deficient mice (10).

While it is tempting to suggest that the presence of podoplanin in lung and kidney vascular beds might represent an additional haemostatic mechanism to ensure prompt blood loss arrest upon endothelial damage in these highly vascularized tissues, currently there are no direct evidences to support such tissue-specific regulation of haemostasis.

Immune response

The recent demonstration of the presence of CLEC-2 in immune cells, including neutrophils, monocytes, dendritic cells, natural killer and B cells indicates that it may also play a role in the regulation of immunity (21, 70). Activation of CLEC-2 signalling in myeloid cells in the presence of LPS leads to an increased production of the anti-inflammatory cytokine IL-10, suggesting that CLEC-2 could play a role in the resolution of inflammation (21). In this context, it has also been shown that a subpopulation of macrophages upregulates the CLEC-2 ligand podoplanin after LPS stimulation *in vitro* (71) or during peritoneal inflammation *in vivo*. These so called fibroblastic macrophages are highly phagocytic cells that have the potential to promote the resolution of acute inflammation by supporting the clearance of pro-inflammatory leucocytes by phagocytosis (35). A role for podoplanin in the intravasation of dendritic cells into afferent lymphatic vessels towards draining lymph nodes for subsequent antigen presentation has been proposed due to its capacity to form complexes and immobilize CCL21, a chemokine involved in dendritic cell docking to the lymphatic endothelium (72). Additionally, CLEC-2 deficiency in dendritic cells has been shown to impair their entry into lymphatics and trafficking to lymph nodes (73).

Development of secondary and tertiary lymphoid structures

The development of secondary lymphoid organs such as lymph nodes during embryogenesis results from the crosstalk between lymphoid tissue inducer cells and mesenchymal cells expressing podoplanin. In mature secondary lymphoid organs, podoplanin is highly expressed on fibroblastic reticular cells, which support their sophisticated architecture and provide a conduit regulating the transport of small molecules including antigens and chemokines (74). Podoplanin deficient mice lack peripheral lymph nodes and show defective follicles and germinal centers in the spleen and Peyer's patches, possibly due to impaired development or function of fibroblastic reticular cells and lymphatic endothelial

cells (75). Noteworthy, podoplanin-expressing interleukin 17-producing helper T (Th17) cells have been shown to play an important pathogenic role in the development of tertiary lymphoid structures during autoimmune inflammation in a model of experimental autoimmune encephalomyelitis (75) and to infiltrate the joints of arthritic mice (76). The potential role of podoplanin in the development of rheumatoid arthritis (RA) is also supported by its discovery in the fibroblast-like synoviocytes of RA patients known to mediate inflammation and tissue destruction in this pathology, whereas it is absent from the synovium of healthy subjects (77). Given the emerging roles of podoplanin in lymphoid structure formation and autoimmunity, it is tempting to hypothesize a modulating role of yet unidentified CLEC-2 positive cells in these processes. This will be certainly a critical area for investigation in the coming years.

Vascular integrity

A recent report has revealed a crucial role of platelet ITAM signalling through CLEC-2 and GPVI in vascular integrity in models of immune complex-mediated skin inflammation (reverse passive Arthus reaction) and LPS-induced lung inflammation (78). This exciting observation raises new questions regarding the day-to-day role of CLEC-2 and GPVI in vessel preservation and during angiogenesis in processes such as wound repair, post-ischemic neovascularization and carcinogenesis. It will also be important to establish if the same or a related mechanism underlies the role of CLEC-2 in prevention of blood-lymphatic mixing and brain haemorrhaging during development. Clearly, however, neither of these two developmental processes involves GPVI, as a similar phenotype is not present in GPVI-deficient mice (79, 80).

Lymphatics maintenance

In adult tissues, lymphangiogenesis occurs through the expansion of existing lymphatic networks or by the recruitment of macrophage-like lymphatic progenitors during normal physiological processes such as wound healing, in pathological conditions such as inflammation or tumour metastasis, or as a consequence of ionizing radiation therapies that induce extensive damage to existing lymphatic vessels (12, 81). In addition to its role in lymphatic development during embryogenesis, CLEC-2 also plays an uncharacterized role in lymphatic vessel maintenance during adulthood, as irradiated wild type mice reconstituted with CLEC-2-deficient foetal liver cells show blood-filled mesenteric lymphatic vessels and Peyer's patches seven weeks after reconstitution, while radiation chimeras reconstituted with wild-type cells develop no abnormalities during the same time frame (10). Furthermore, postnatal inactivation of the endothelial T-synthase causes blood/lymphatic vessel misconnections and fatty liver disease due to direct chylomicron deposition via misconnected portal vein and intestinal lymphatic systems (15). Future work is required to address whether CLEC-2 also plays a role during the expansion of adult lymphatics associated with inflammation, wound repair or cancer metastasis.

Cancer metastasis

Metastasis is the main cause of cancer-associated mortality but still remains poorly understood. Metastatic dissemination requires that cancer cells from a primary tumour acquire a migratory and invasive phenotype, degrading the surrounding extracellular matrix and moving to the lymph and blood vessels. Entry into the bloodstream allows their colonization of anatomically distant organs, where they develop secondary metastases. During their transit within the circulation, tumour cells come into contact with platelets, which can further define their metastatic potential through multiple mechanisms (82). Experimental blockade of key platelet adhesion receptors inhibits tumour cell-induced platelet aggregation and diminishes metastasis (83). Podoplanin is upregulated in different cancer cell types, especially on the leading edge of tumours, and contributes to cancer

pathogenesis by promoting tumour-mediated lymphangiogenesis, increased cell invasion and lymph node metastatic potential (40, 84–86). Beyond its role in increasing the intrinsic metastatic potential of tumours, the capacity of podoplanin to induce platelet activation and aggregation could facilitate the arrest and extravasation of circulating cancer cells (87), increasing metastasis. Since podoplanin expression levels have an important prognostic value, it has been suggested that podoplanin might be an attractive therapeutic target. In this context, an anti-human podoplanin antibody (NZ-1) that inhibits podoplanin-induced platelet activation abrogated experimental metastasis due to neutralization of the interaction between podoplanin and CLEC-2 (88). Recently, a chimeric humanized anti-human podoplanin antibody inhibiting podoplanin-induced platelet aggregation and experimental metastasis was developed as a potential novel anticancer agent (89). It is uncertain however whether this approach will interfere with the role of endogenous podoplanin on other cell types, leading to deleterious side effects.

Cardiovascular disease

A direct link between lymphatic dysfunction and the pathogenesis of obesity, atherosclerosis and cardiovascular disease, which are leading causes of mortality and disability in developed countries, has been proposed (12). Mice with heterozygous mutations in VEGFR3 or Prox1 develop chronic tissue inflammation in association with increased fat accumulation. Arterial inflammation is a significant component of atherosclerotic disease. The *vasa vasorum*, a microvasculature network found in the adventitial layer of large arteries that supplies oxygen and nutrients and removes waste products from the arterial wall, plays an important role in plaque development by providing an entry pathway for monocytes to migrate to sites of early atherogenesis. The adventitial lymphatic vessels found in the proximities of the *vasa vasorum* might play an important protective role by draining lipids, inflammatory cells and cytokines from the arterial wall reducing its local concentration and therefore constraining atherosclerotic development (12). The interaction between podoplanin-expressing activated macrophages and platelet CLEC-2 leads to platelet aggregation and degranulation (71), which might contribute to the thrombogenicity of atherosclerotic plaques containing foam cells (90), although its relative importance compared to other thrombogenic components of the plaque such as tissue factor, cholesterol or collagen remains to be elucidated.

Conclusions

After the discovery of the fundamental roles of podoplanin in physiological processes and the pathogenesis of various diseases and the identification of CLEC-2 as its endogenous ligand, there is great expectation that understanding the mechanisms that govern this interaction will provide important clues for the therapeutic targeting of various human conditions. This exciting but still intriguing area of research will certainly unveil new surprising aspects of platelet biology beyond haemostasis, with potential implications for the use of antiplatelet agents.

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Figure 1. CLEC-2 mediated platelet signalling

CLEC-2 dimers are cross-linked by podoplanin expressing cells causing clustering of the receptor on the platelet surface. One Syk molecule binds to a pair of CLEC-2 molecules. CLEC-2 phosphorylation and Syk phosphorylation is mediated only partially by Src family kinases (SFK) but largely by Syk itself. Once activated, Syk phosphorylates the downstream LAT signalosome.

Figure 2. Podoplanin-mediated constitutive signalling

Podoplanin interacts with the ezrin/radixin/moesin (ERM) protein family, which provides a structural link to the actin cytoskeleton, and promotes RhoA activation and cell migration in the absence of a ligand. Podoplanin expression also induces epithelial to mesenchymal transition in different cell types.