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Variations on the theme of podosomes: a matter of context

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Summary

Extensive *in vitro* studies have described podosomes as actin-based structures at the plasma membrane, connecting the cell with its extracellular matrix and endowed with multiple capabilities. Contractile actin-myosin cables assemble them into a network that constitutes a multifaceted cellular superstructure taking different forms – with common characteristics – but manifesting different properties depending on the context of study. Their morphology and their role in cell functioning and behavior are therefore now apprehended in *in vivo* or *in vitro* situations relevant to physiological processes. We focus here on three of them, namely: macrophage migration, antigen presentation by dendritic cells and endothelial cell sprouting during angiogenesis to highlight the characteristics of podosomes and their functioning shaped by the microenvironment.

1 Podosomes

1.1 Introduction

Cells communicate constantly with their environment. The interactions are of many types and involve

soluble, matrix and mechanical factors. For instance, cells probe their surroundings with filopodia, adhere to the extracellular matrix (ECM) through focal or fibrillar adhesion sites and deploy lamellipodia in order to migrate. Podosomes form another class of structures restricted to certain cells and/or certain contexts but endowed with multiple capabilities. Unlike the structures mentioned above, they are endowed with proteolytic activities that allow them to degrade matrix proteins. Podosomes are therefore intimately associated with the process of cellular invasion, where cells breach anatomical barriers and penetrate tissues. Recent advances in the field have established that these dynamic structures also exhibit mechanosensory attributes allowing the exploration of the pericellular environment. This property must now be integrated in understanding how they participate in physiological processes. This review does not deal with related structures described in tumor cells and named invadopodia, which are associated with cancer pathology.

One essential component of the microenvironment is the ECM, a collection of extracellular molecules secreted by cells that provides them with structural and biochemical support. The interstitial matrix is predominant, consisting in structural components such as collagen-I (fibrillar) and elastin, whose main role is to resist tension, and components that facilitate contacts with cells such as fibronectin and laminins. Other key components are proteoglycans such as aggrecan and perlecan that, through their long glycosaminoglycan chains, capture water and make the matrix resistant to compression. Basement membranes (BMs) are specialized ECM that delimit all epithelia and endothelia but also envelop muscle, fat and nerve cells, and serve as structural barriers [1]. They consist primarily of laminins (various isoforms depending on tissue type) and collagen-IV, crosslinked by non-covalent interactions with nidogens and perlecan [2], thus forming a compact and resistant mesh that plays an essential role in maintaining tissue integrity while delivering homeostasis signals. Tissue maintenance, repair and expansion as well as tissue surveillance by immune-competent cells, are processes that require matrix remodeling [1]. These are the physiological processes in which podosomes play a key role.

Structurally, podosomes are actin-based membrane structures in direct contact with the ECM. First characterized as autonomous entities, it is now recognized that they are building blocks of a network, interconnected by contractile actin filaments and linking the actin cytoskeleton to the plasma membrane [3-5]. As plasma membrane domains, podosomes are privileged sites of interactions with the pericellular environment that regulates their formation, lifespan, subcellular distribution and architecture. As cytoskeleton domains, podosomes derive their morphological and functional characteristics from the dynamics of actin polymerization and adopt a dynamic collective behavior [5, 6].

Podosomes are particularly abundant in cells of the myelomonocytic lineage, such as macrophages [7],

immature dendritic cells [8] and osteoclasts [9]. They are also prominent in megakaryocytes [10, 11]. In these cell types, they constitute a major part of the actin cytoskeleton and are formed constitutively upon cell adhesion. They are also found in non-hematopoietic cells. In these cell types, their formation involves ECM-derived signals but also requires specific extracellular induction. These can be mechanical cues and/or specific soluble factors that depend on the cell tissue origin. Podosomes are often arranged in clusters or in ring-shaped superstructures called “rosettes”.

Podosomes have been extensively described for over three decades and in a wide variety of cell types: *in vitro*, in the bidimensional context of the culture dish, under hyperoxic atmosphere and often using a gelatin film to mimic ECM. These explorations have revealed their large repertoire of functions [12]. More recently, podosome presentation and functions have been investigated *ex vivo* or in conditions that better mimic physiological processes *in vivo*. These studies have highlighted the predominant role of the microenvironment in the acquisition of podosome characteristics and functioning. Thus, it turns out that although the different types of podosomes share many components, fine details of their composition, characteristics and subcellular arrangement are determined by the context. Below we describe the main features of podosomes updated with the latest advances in the field, including the characterization of the elements that have led to better understanding of their organization and dynamics and their functional employment in the context of the various functions associated with them. We then describe three physiological processes in which podosomes described *in vitro* or *ex vivo* could occur *in vivo*: macrophage migration across tissues, antigen presentation and uptake by immature dendritic cells and endothelial cell sprouting during angiogenesis. This review does not deal with intracellular signaling pathways, which are known to involve Src and RhoGTPases as pivotal actors of podosome assembly. They have been reviewed elsewhere [13].

1.2 The podosome is an actin structure that connects the cytoskeleton with the plasma membrane

Typically, a podosome is a punctate (0.5-1.0 μm in diameter) structure located at the plasma membrane and is principally made of filamentous actin. It is a highly dynamic organelle undergoing constant disassembly and reformation on a minute time-scale, and characterized by a rapid turnover of actin within the structure [9] (Figure 1A-D). Actin polymerization begins at the plasma membrane and is mediated by the Arp2/3 complex activated by Wiskott–Aldrich syndrome protein (WASp or N-WASp) downstream of the small G protein Cdc42. While the podosome enlarges through the generation of branched actin on a rigid matrix, the core structure grows perpendicular to the substratum over a height of about 0.6 μm (Figure 1C, D). Cortactin (CTTN) and the multi-domain scaffold protein Tks5 (SH3PXD2A) are often used as podosome markers. Cortactin consolidates the Arp2/3-generated actin filaments, whereas Tks5 plays an essential, but yet poorly understood, role in

the subcellular orientation of the actin nucleation sites [14]. Integrins and CD44 are the dominant transmembrane adhesion molecules of podosomes and constitute the bridge that connects the inside with the outside. The transmembrane metalloprotease MT1-MMP (MMP-14) is selectively delivered in transport vesicles to the basal side of the podosome through a microtubule/motor-protein network [15]. The enzyme remains spatially confined to the site of podosome formation through interactions with actin filaments [16]. Surrounding the columnar actin core, numerous structural proteins associated with integrins have been identified, including paxillin, vinculin and talin (Figure 1 C, D) [12, 17]. The actin core and adhesion ring give the podosome the characteristic bipartite appearance in immunofluorescent microscopy (Figure 1A, B) which is also useful for recognizing the structures. High-resolution microscopy has now shown that the adhesion ring of integrin-associated proteins is not really continuous but instead forms a polygon of discrete complexes [5, 18-20]. Characterization of podosomes in the orthogonal plane (perpendicular to the basal membrane) has revealed the existence of a third element, the podosome cap. It covers the column of actin filaments like an umbrella and contains proteins such as formin-like protein, FMNL1 [21], inverted formin, INF2 [22], supervillin, SVIL [23], and the sphingolipid long-chain base-responsive protein LSP1 [24]. This structure may serve as a hub for incoming transport vesicles, regulate podosome growth or channel the myosin contractile forces associated with podosomes [23, 24] (Figure 1C, D). Detailed analysis of the actin network has allowed a better understanding of the architecture of the organelle and brought to light the mechanics of their sensory properties. At the base of the columnar structure, the detection of Arp2/3 serves as a signature for the formation of a branched actin network [25]. This branched actin core is surrounded by unbranched antiparallel contractile filaments that radiate from the top of the podosome near the cap to the ring of adhesion sites [4, 9, 26] (Figure 1D). Pioneering work using atomic force microscopy has shown that podosomes exert forces on the matrix in an oscillating movement by using the myosin IIA motor of these contractile filaments [27]. The core of actin filaments grows upwards from the basal side. In turn, the acto-myosin contractile cables tethered onto the matrix at the level of the ring stretch, inducing the recruitment of mechano-sensitive protein vinculin at these sites. The stored forces are restored in protruding forces at the core and are in turn relayed onto the ECM [3, 5, 27]. Talin, vinculin and paxillin are required for actin polymerization and protrusion force generation [28]. Myosin IIA contracts the actin network filaments and applies tension to the bound vinculin molecules, counterbalancing core growth and eventually reducing podosome size and protrusion. Depending on the rigidity of the matrix, the magnitude of the forces leads to distinct levels of recruitment of the mechano-sensitive protein vinculin at sites where the actin filaments connect to the cell membrane [5]. In this way, the exerted forces translate the physical property of the substrate into biochemical signals to the podosomes, which in turn are transmitted to the cell and may lead to differential podosome composition. The formin INF2 was recently shown to regulate contracting events at individual podosomes from the cap structure [22]. The adhesive ring also undergoes pressure oscillations resulting from the movements of the core [5, 6]. Thus, adhesion

proteins sustain tension and feedback on actin-driven protrusion and the integrity and dynamics of protrusive cores and adhesive rings are coordinated by the actomyosin apparatus [28].

Another set of contractile actin/myosin-filaments has been revealed by the use of super-resolution microscopy and these connect podosomes with each other [3, 5, 23]. These connections ensure their regular distribution [4, 23, 29] and enable their collective and synchronized functioning [6, 23] (Figure 1B, D). In fact, connectivity extends well beyond the nearest neighbors of podosomes, as shown by the continuous redistribution of podosome components throughout podosome clusters [30]. The formin FHOD1 was recently shown to regulate the actomyosin-based contractility of these connecting filaments within podosome clusters in primary macrophages [22]. These interconnecting filaments appear to be associated with the radiating filaments described above. Super-resolution correlative light and electron microscopy (SR-CLEM) has allowed the detection of another network of filaments that has no apparent connection with podosome cores. These short filaments are restricted to the ventral side of the plasma membrane and form a thick layer of cortical actin at the bottom of the entire podosome cluster [19].

The dynamic behavior of podosomes comprises *de novo* formation, dissolution and reformation. Podosomes also undergo continuous fission and fusion events, where neighboring structures are in close spatial vicinity. In between these podosomes, SR-CLEM detects a thick bundle of unbranched filaments that may originate from the actin core. The filaments close to and on top of the core are decorated with zyxin but not with vinculin, suggesting that they do not associate with the plasma membrane. In contrast to the radiating fibers and interconnecting cables, these interpodosomal connections are insensitive to inhibition of actin polymerization, suggesting that these pools of actin are not dynamically coupled [19].

Podosomes are thus endowed with mechano-sensory qualities and podosome clusters function as a coordinated mechanosensory area [31]. These mechano-sensory properties may either combine or, more likely, alternate with their matrix-degrading activity (Figure 1C, D).

1.3. The various signals regulating podosome formation

1.3.1. Matrix signals transmitted at the level of the plasma membrane

Under conventional cell culture conditions, podosomes appear in their characteristic form in cells seeded on a smooth and rigid plastic dish, as shown in Figure 1. For myelomonocytic cells, integrin-mediated adhesion to the ECM-coated substratum triggers the assembly of podosomes, which form the predominant adhesive structures for these cells. Different matrices engage different integrins and thus trigger distinct signaling cascades: integrin $\beta 2$ bound to fibrinogen drives podosome formation in macrophages [27], integrin $\beta 3$ bound to osteopontin is involved in osteoclasts [32], and integrin $\beta 1$

bound to collagen-I is a potent triggering signal for megakaryocytes [10, 11]. The matrix also regulates the lifespan of podosomes: when megakaryocytes are seeded on collagen-I, podosomes triple their longevity as compared to cells seeded on fibrinogen [11]. The geometry of the substrate dictates podosome subcellular localization, arrangement and morphology, while also impacting their lifetime. On a surface lined with reliefs, dendritic cells accumulate podosomes along ridges and these podosomes are more stable than those formed in non-rugged areas [33]. These properties are well illustrated by cells seeded on a collagen-I matrix: podosomes tend to line up on the ridges and follow the meanders of the deposited collagen-I fibers [11] and may even change their architecture and composition [34]. Here, podosome accumulation and presentation dictated by collagen-I topography could be mediated by the newly discovered interpodosomal network [19]. As another example to illustrate the prominent role of the ECM, podosomes barely form when endothelial cells derived from liver sinusoids are seeded on a soft matrix. In contrast, on a stiff substratum corresponding to a matrix associated with liver fibrosis, they form abundantly and with a highly amplified proteolytic power [35]. Matrix signals also play a decisive role in podosome formation induced by cytokines. Thus, collagen-IV is essential for podosome induction by the vascular endothelial growth factor VEGF in microvascular endothelial cells [36], while fibronectin is the preferred ECM for TGF- β -induced podosome formation in endothelial cells derived from large arterial vessels [37].

Podosomes may also be induced by suppressing the tractive forces exerted by cells on a matrix. Fibroblasts do not normally present podosomes on a rigid matrix but classical focal adhesion contacts. When the cells are spread on a fluid matrix composed of lipid-attached Arg-Gly-Asp (RGD) peptides, they assemble podosome-like structures that convert into focal adhesion contacts when a force is applied to the fluid lipid-RGD bilayer [38]. These findings highlight the role of mechano-signals in the regulation of podosome formation. A constricted environment also promotes podosome formation. Arterial endothelial cells confined into a micro-channel scaffold device spontaneously assemble podosomes [39]. Within the channel, the podosomes are mainly arranged in clusters, while they mainly form rosettes when entering or exiting the confined space. Therefore, physical constraints influence podosome organization and disturbance of cell polarity may be involved in their formation.

Although adhesion is a pre-requisite for podosome formation and podosomes gather information about matrix composition by the engagement of specific integrins, kinetic studies performed in osteoclasts suggest that this interaction is not the prime event that leads to their assembly as cortactin and paxillin are detected first at the site of podosome formation and precede integrin recruitment [40]. Podosome formation is stimulated by a local loss of contractility and the recruitment of negative regulators of RhoA such as p190RhoGAP [38, 41, 42]. As F-actin depolymerization promotes cortactin phosphorylation [43], the nucleation of the filaments could be triggered by Arp2/3 and/or a cortactin-enriched primer complex, on which ECM signals converge [44, 45]. As mentioned above, the dynamic

behavior of podosomes includes podosome dissolution and reformation. Beside its proteolytic activity, MT1-MMP also serves to imprint spatial memory for podosome reformation after dissolution, and therefore contributes to the construction of the structure [16].

1.3.2. Alteration of gene expression and other signals

MicroRNAs are small noncoding RNAs that play a critical role in regulating several cellular functions through transcriptional silencing. Either a loss or a gain of micro-RNA expression can regulate podosome formation. In vascular smooth muscle cells (VSMCs), miR-143 and -145 regulate a switch between differentiated (contractile/non-proliferative) and dedifferentiated (synthetic/migratory/proliferative) phenotypes. These micro-RNAs prevent the formation of podosomes in VSMCs by repressing the transcription of PKC α (PRKCA) and PDGFR α (PDGFRA), and of fascin (FSCN1), respectively [46]. Expression of miR-143 and -145 is repressed by the proto-oncogene Src, an event that may promote podosome formation. In contrast, miR-155 induces podosome assembly in arterial endothelial cells [47]. MiR-155 represses the transcription of the BMP-regulated transcription factor Smad5. As Smad5 signals to cells in the basal state, it is likely that miR-155 alters cell homeostasis by suppressing a regulatory signal. Consistent with this, miR-155 is known to influence endothelial to mesenchymal transition by counteracting RhoA function [48].

More recently, cell fate specification pathways were also shown to regulate podosome formation. Blocking Notch signalling frees the way for the formation of podosomes in endothelial tip cells, one of the first steps in the process of angiogenesis. As Notch mainly interferes with gene transcription, podosome induction is very likely the immediate consequence of altered gene expression [49]. Podosomes are also involved in embryonic development where cell migration is instrumental for generating tissues in the appropriate position, as shown in the zebrafish model [50]. In the nematode *C. elegans*, podosomes are used by the anchor cell, a specialized somatic gonadal cell, to migrate across the basement membrane in order to connect the developing uterine with vulval tissues [51].

Another means to control podosome induction is oxygen or nutrient deficiency [52], or the production of reactive oxygen species (ROS) [53]. However, the how and why of these observations have not yet been explored. There are thus several ways in which podosome formation may be induced or modulated. Table 1 summarizes the various signals that have been shown to regulate podosome assembly in endothelial cells. These regulations are dynamic as podosomes in turn act on the microenvironment.

2. Macrophage podosomes and trans-tissue migration

Macrophages originate predominantly from blood-borne monocytes that migrate into tissues. They differentiate in a tissue-dependent manner, giving rise to tissue-specific cells (such as Kupffer cells in the liver or glial cells in the brain). Besides tissue-specific characteristics, two broad distinctions are made in the macrophage population, namely M1 and M2. The two subpopulations distinguish themselves by expression of specific cell surface markers and the secretion of specific sets of cytokines which lead to two opposite functional characteristics. The M1 signature is associated with elimination of (damaged) tissue and of foreign organisms (initiation phase of inflammation) whereas the M2 signature is associated with tissue repair (resolution phase of inflammation) [54]. In fact, M1 and M2 are the two extreme of a continuum of phenotypes *in vivo* and subpopulations exist that do not fit these two categories [55]. Macrophages are confronted by matrices whose rigidity and topography must be decoded for an “all-terrain” migration. In porous collagen-I based matrices, cellular deformability is sufficient for their progression and they move in an amoeboid fashion. In more dense matrices, macrophages form podosomes and use their proteolytic properties to forge ahead through the stroma in a mesenchymal migration mode [56]. Both M1 and M2 populations produce podosomes but those associated with the M2 signature tend to be more dynamic and were shown to contribute to mesenchymal mobility [57].

On a two-dimensional surface under *in vitro* conditions, podosomes are evenly distributed over the whole ventral (attached side) of macrophages. As mentioned above, podosomes predominantly serve as adhesion sites because they lack other matrix contacts. The even distribution is lost when macrophages are set to migrate in response to chemokines such as CSF-1. A new population of large podosomes with a rapid turnover (short half-life) accumulates at the leading edge. The enhanced dynamics of M2 macrophage podosomes is essential for the migration process, in particular when cells migrate across the endothelium (process of diapedesis) [8]. When analyzed in more detail, the chemokine-exposed macrophages may be seen to possess two distinct populations of podosomes: i) large dynamic “precursors” located at the periphery and at the leading edge; and ii) smaller and more stable “successors”, located more centrally and often derived from splitting precursors [23]. The differences between the two podosome populations may be due to the differential regulation of myosin-II by the cap components, LSP1 dominating in precursors and SVIL in successors [24]. Interestingly, LSP1 regulates podosome oscillations, protrusive force generation and podosome lifespan [24].

An example of an *in vitro* two-dimensional environment is where cells are crawling on an endothelium or certain epithelia attached to their basement membrane such as in lung, bladder, intestine or certain glands. However, most environments are three-dimensional. In a collagen-I gel mimicking the environment of macrophages in connective tissues *in vivo*, podosomes take on a totally different appearance and are consequently named “3D podosomes” [58]. Instead of having the typical

core/ring/cap moieties, they present as globular structures at the end of long protrusions with less distinct boundaries between the substructures. Nevertheless, they remain recognizable as podosomes thanks to their specific markers, their proteolytic capacity and their dynamics. They are larger, less numerous, and their composition slightly differs from their 2D counterparts.

In vitro, macrophages carrying 3D podosomes and exposing MT1-MMP at the plasma membrane dig tunnels through which they migrate (Figure 2A) [59], a phenomenon that reflects trans-tissular migration. Indeed, the podosomes in HIV-1 infected macrophages are more stable than those of uninfected cells and their size and degradation potential are increased as a result of Hck (hematopoietic cell kinase, the predominant member of the Src-family of tyrosine kinases in these cells) stimulation by the viral protein Nef. Such changes are reflected by the accelerated migration of these cells towards inflammatory sites [42]. Conversely, diminishing podosome size and degradation potential through Hck inhibition reduces cell migration [60-62]. It is likely that 3D podosomes also possess the adhesion properties of “2D” podosomes because integrins and CD44 localize at these structures [58]. However, the function of mechanosensory and matrix signal transduction remains difficult to explore in three-dimensional contexts.

3. Dendritic cell podosomes, antigen presentation and uptake

Dendritic cells patrol the blood and tissues with the purpose of capturing and presenting foreign elements to naïve T cells to allow them to mount a defensive response. Dendritic cells are genuine professional antigen-presenting cells and they exist in several sub-populations: interstitial, dermal, plasmacytoid, inflammatory and Langerhans cells. In the absence of foreign bodies, they are said to be immature (iDC). They constantly probe their environment with pattern recognition receptors (PRR) that recognize molecules (mainly sugars, lipids, proteins and nucleic acids) shed by infectious agents such as bacteria, fungi and viruses [63]. The signaling events that ensue “pattern” binding to their receptors lead to the internalization of the antigen by endocytosis or phagocytosis, to the redistribution of the proteins involved in antigen processing, to enhanced expression of the major histocompatibility complex and to the release of cytokines (interferon β , interleukins, TNF α and RANTES) that facilitate T-cell differentiation. In short, a set of reactions occur that lead to priming of the adaptive immune system [64].

In vitro, iDCs podosomes are arranged in clusters (Figure 1A). In dense matrices of gelled collagen-I or Matrigel, iDCs form 3D podosomes at the tip of cell protrusions and adopt the mesenchymal migration mode similar to macrophages [65]. As a consequence of antigen recognition, iDC turn into mature dendritic cells (mDC). Concomitantly, podosomes disappear and cells form long dendrites instead (hence the name of the cells). By reducing adhesion to the ECM, this transformation also

marks a transition to a faster migration mode, like DCs migrating towards lymph nodes to present antigens to naïve T cells *in vivo* [66].

Podosomes participate specifically in the antigen seeking, capture and uptake phases of T cell immune response triggering. First, the topographical cues seem to act as a triggering signal for podosome construction. When iDCs are seeded on a porous membrane, podosomes form precisely on the top of the pores [67, 68]. How extracellular topographical cues are signaled into the cells is not clear. It may occur through the recruitment of curvature-sensing proteins, such as the BAR proteins CIP4, FBP17, ASAP1 or IRSp53 into the plasma membrane [69]. The topography also influences podosome morphology: the ring of adhesive proteins containing vinculin, paxillin, talin and other components remains positioned at the entry of the membrane pore, whereas the actin core descends into the pore at the same time as the protrusion (Figure 2B). The extension of the protruding actin core is dependent on myosin-II activity and therefore involves contractility. The protruding membrane exposes MT1-MMP and PRRs and becomes a privileged site for matrix proteolysis, antigen capture and endocytosis [67, 68]. This podosome architecture seems ideally suited for iDCs to seek “pathological patterns” across epithelia, which may occur in the intestine, lung or nasal cavities [67].

4. Podosomes of endothelial cells in sprouting angiogenesis

Endothelial cells line the inner surface of all blood vessels. Besides their multiple functions at the blood/tissue interface, they also play a key role in the process of vascular bed formation during angiogenesis, *i.e.*, the process whereby new vessels arise from existing ones and lead to the extension of the vascular network. This mainly concerns the micro-vasculature that buds to establish vascularization during development or restores irrigation of injured or ischemic tissues. The developing retina of a newborn mouse, in which vascularization is initiated in the first week after birth, has been a great source for studying the process of angiogenesis. The main angiogenic factor involved is VEGF and endothelial cells respond to it in different ways. Cells with the highest cell surface receptor (VEGFR2) density turn into “tip cells”, whereas the adjacent cells become “stalk cells”. Tip cells develop filopodia equipped with VEGFR2 that capture surrounding VEGF, and with integrins allowing them to dock into newly explored territories. They are the leading migratory cells. The adjacent cells proliferate and extend a stalk that pushes tip cells further into the surrounding tissue. In this way, an initial vascular sprout gradually turns into a growing capillary. The fate of the stalk cells depends on a process referred to as lateral inhibition [64]. VEGF enhances the membrane expression of Delta-like 4 (Dll4) in the tip cell and, by signaling to Notch receptors, imposes the “stalk phenotype” on adjacent endothelial cells (a juxtacrine signaling mode). The process described above suggests that tip cells must somehow suppress even low levels of Notch signaling. This may occur through a mechanism of cis-inhibition, where Notch and Notch-ligands are expressed on the

same membrane and create non-productive signaling complexes that prevent trans-activation [70]. Thus, stalk cells have high Notch activity whereas tip cells have low Notch activity.

In vitro, microvascular endothelial cells exposed to VEGF form podosomes [36, 49, 71-73]. These podosomes are interconnected by contractile actin-myosin filaments in an annular superstructure called a rosette. They have a strong proteolytic activity especially against collagen-IV [36]. Pharmacological inhibition of Notch signaling produces the same effects [49]. These observations led us to investigate podosome formation in tip cells displaying low Notch activity and exposed to VEGF *in situ* using the model of the mouse retina.

Immunofluorescence staining of podosome markers detected podosomes in tip cells of the developing retinal vasculature. In their native microenvironment, podosomes are globular in shape, interact with the BM, and their occurrence coincides with reduced collagen-IV vessel coverage [49] (Figure 2C). The cables connecting the podosomes are clearly visible without recourse to super-resolution microscopy. Based on *in vitro* data, we postulate that podosomes intervene in tip cells *in vivo* at two levels: initially in the process of sprouting to breach the BM and invade the ECM; and at a later stage in the process of anastomosis, where tip cells must first remove the basement membrane sheet of an existing capillary in order to adhere to the endothelial cells to allow the sprouts to interconnect.

5. Conclusions and perspectives

Our knowledge of podosomes has increased tremendously in recent years, thanks in particular to technological developments in microscopy [31]. Accumulating evidence of the role of podosomes in the degradation of collagen-IV and in the breaching of anatomical barriers during angiogenesis makes them potential therapeutic targets in aberrant neo-vascularization processes. The intervention of podosomes in macrophages and iDCs requires further exploration *in vivo* but their predicted role in transtissular invasion offers new avenues for intervention in poorly controlled innate and adaptive immune responses. Processes involving podosomes are not limited to the examples that we have presented in this review. For instance, they take center stage in the formation of the sealing zone in osteoclasts and engage them in the process of bone resorption. The maturation of the postsynaptic plate in the neuromuscular junction [74] and the fusion of myoblasts [75] are also physiological phenomena in which podosomes are thought to play a decisive role. The investigation of podosomes in *in vitro* conditions has revealed their multiple potentialities but it is likely that the different functions of podosomes do not intervene simultaneously. Like iDCs and macrophages, osteoclasts and endothelial cells can also present antigens [76, 77], and the latter can form elongated podosomes when cells are seeded on top of a porous membrane [37]. Thus, the presentation and properties of podosomes concern the microenvironment rather than cells. It is the microenvironment that determines the precise role that podosomes play *in vivo* by regulating their assembly, arrangement, architecture,

lifespan, composition and functions.

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Competing interest

The authors declare no competing interests

Figure legends

Figure 1. Structure and organization of podosomes. (A) In an immature dendritic cell, the immunofluorescent staining of actin (red) and vinculin (green), visualized in the basal plane, reveals the core and ring of podosomes and their group arrangement. (B) Schematic representation of (A). (C) Diagram of the cell with its podosomes and surrounding F-actin networks in sagittal view (along the dotted line shown in (B)), showing the actomyosin cables that interconnect the podosomes and the podosome cap. (D) Detail of podosomes: the core, seat of actin polymerization, the ring, consisting in plaque proteins allowing matrix adhesion, the cap, where the radiating actin filament bundles terminate, the cables of actomyosin that assemble the individual podosomes into a network and the recently discovered actin bundles associating neighbor podosomes in close vicinity. For the sake of clarity, the set of short filaments forming a thick layer of cortical actin at the bottom of the podosome cluster [19] is not shown. Podosomes are endowed with a mechanosensory activity orchestrated by forces generated at several points (arrows), which combines or alternates with their proteolytic activity depending on the matrix characteristics (not described in the figure). N.B., The schematic representation uses information gathered from data obtained in different cell types.

Figure 2. Podosome presentation in different *in vitro* or *ex vivo* contexts. (A) Podosomes allow macrophages to tunnel into the matrix to infiltrate the tissues. In a three-dimensional, compact collagen-I matrix (shown as a block), macrophages form protrusions with globular-shaped podosomes at their ends. These so-called 3D podosomes do not present the core/ring organization that characterizes podosomes in a 2D situation (Figure 1A). Matrix proteolysis by podosomes combined with the dynamic of protrusions drills a tunnel which, *in vivo*, may allow trans-tissular migration of macrophages and other cells. (B) iDC podosomes participate in the recognition and treatment of antigens. Side view of a Boyden chamber showing iDCs seeded on a gelatin-coated polycarbonate filter. Podosomes form on top of the pores, as the topographical changes initiate podosome construction and shape its morphology: the adhesive ring remains positioned at the level of the filter but the actin core extends into the pore. Extension of the actin core is dependent on myosin-II activity. The protrusion that now exposes MT1-MMP (not shown in the figure) and PRR becomes a prime site for matrix proteolysis, antigen capture and endocytosis. This podosome architecture could allow iDCs to detect distant pathogens. (C) Endothelial cell podosomes locally digest the BM during angiogenesis. Endothelial cells acquire invasive properties to allow the emergence of blood vessel branches. (1) Developing vasculature at day 6 in the mouse retina stained with the isolectin B4 endothelial marker. (2) Immunofluorescent staining of the boxed area in (1) showing the angiogenic front at higher magnification F-actin (red), cortactin (green) and BM collagen-IV (white). (3) Immunofluorescent staining of the boxed area in (1) showing a region of the distal and mature vasculature at higher magnification: in the absence of pro-angiogenic stimulus, the basement membrane wraps the tube composed of endothelial cells and provides them with survival and quiescence signals. (4) Zoom of the boxed area in (2); in response to VEGF, some cells become tip cells and assemble podosomes that locally degrade the underlying BM. (5) Zoom of the boxed area in (2); filopodia emerge through the gaps thus created. (6) Zoom of the boxed area in (2); the cell buds and the filopodia grip the ECM by engaging integrins, allowing this cell to extrude from the parent vessel to give rise to vascular branching. A sketch depicting each situation is presented in the bottom panel.

Table I: Different types of signals regulate podosome formation in endothelial cells. Many factors are able to modulate podosome formation, especially in cells where the structures are inducible such as endothelial cells. The signals are often of extracellular origin such as cytokines, nature and properties of the matrix, but may also result from internal changes concerning the metabolism and microRNAs. These regulations are dynamic as podosomes in turn act upon the microenvironment. Up and down arrows indicate if the assembly/formation of podosomes is enhanced (green) or reduced (red), respectively.

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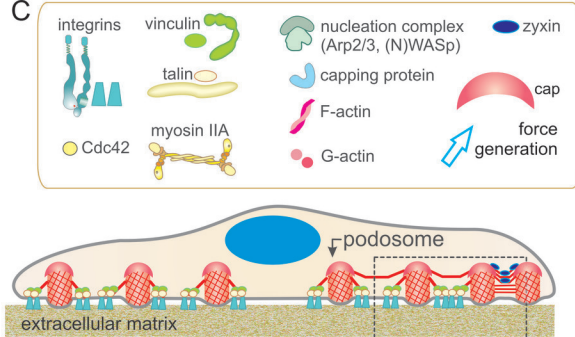
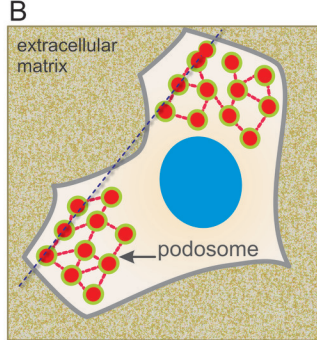
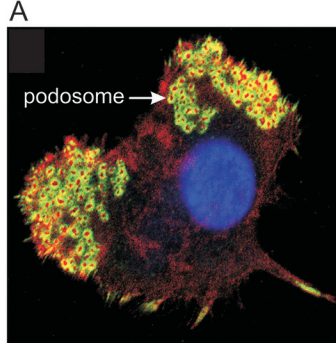
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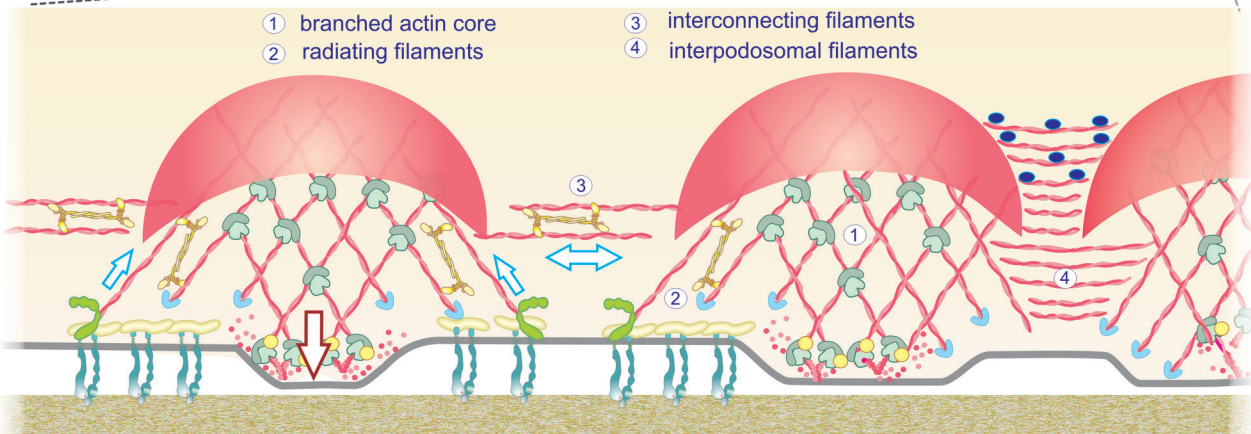
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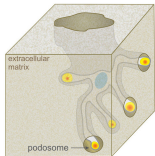
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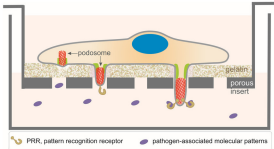
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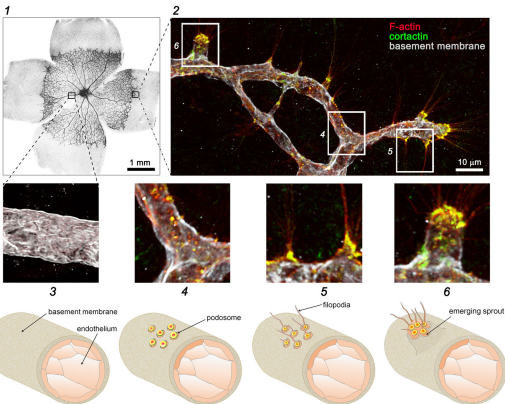
A Macrophage podosomes



B Dendritic cell podosomes



C Endothelial cell podosomes



<i>Nature of signal</i>	<i>Mediator</i>	<i>Podosomes</i>	<i>Vascular bed</i>	<i>References</i>
<i>Cytokines</i>	VEGF-A	↑	capillary vein	[36, 71-73]
	TGF-β	↑	aorta	[37, 47]
	TNF-α	↑	vein	[72]
<i>Extracellular matrix</i>	Collagen-IV Laminin 8/10	↑	capillary	[36]
	Fibronectin, Collagen-I	↓		
	Collagen-I fibers	↑	aorta vein capillary liver sinusoids	[34]
<i>Juxtacrine signaling</i>	Notch pathway	↓	capillary	[49]
	exosomes	↑	capillary	[53]
<i>Metabolism</i>	reactive oxygen species, nutriments	↑	capillary	[53]
<i>Micro-RNAs</i>	miR-155	↑	aorta	[47]
<i>Mechanical</i>	confinement	↑	aorta	[39]
	stiffness	↑	liver sinusoids	[35]