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Building the phagocytic cup on an actin scaffold

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Abstract

Cells ingest large particles, such as bacteria, viruses, or apoptotic cells, via the process of phagocytosis, which involves formation of an actin-rich structure known as the phagocytic cup. Phagocytic cup assembly and closure results from a concerted action of phagocytic receptors, regulators of actin polymerization, and myosin motors. Recent studies using advanced imaging approaches and biophysical techniques have revealed new information regarding phagocytic cup architecture, regulation of actin assembly, and the distribution, direction, and magnitude of the forces produced by the cytoskeletal elements that form the cup. These findings provide insights into the mechanisms leading to the assembly, expansion, and closure of phagocytic cups. The new data show that engulfment and internalization of phagocytic targets rely on several distinct yet complementary mechanisms that support the robust uptake of foreign objects and may be precisely tailored to the demands of specific phagocytic pathways.

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Introduction

Phagocytosis is the process of engulfment of large objects that involves binding of receptors on the cell surface to the surface of the phagocytic target, followed by a dramatic cytoskeletal rearrangement to form an actin-supported phagocytic cup. Phagocytosis can be initiated by signals intrinsically present on the surface of the target or via coating of the target (opsonization) with serum components, such as antibodies or the complement (C3). Phagocytic receptors on the cell surface range from those that recognize microbial components (such as scavenger receptors and Dectin-1) to complement receptors (integrin αMβ2/CR3) and antibody-binding Fc receptors (FcR) [1].

Initial binding of phagocytic targets relies not only on random collisions between cells and foreign particles but also on the active target capture via actin-dependent formation of ruffles (broad and flat actin-membrane sheets supported by networks composed of branched actin) or filopodia (needle-like projections containing an actin core composed of parallel actin filament arrays surrounded by the plasma membrane) [2], with both types of protrusions used to sweep and probe the phagocyte's environment in search of foreign objects. Ruffling and filopodia formation may be constitutive or stimulated by cytokines, and their assembly relies on the activity of actin nucleators such as the Arp2/3 complex (ruffles and filopodia) or formins (primarily filopodia) [2,3].

Engagement and clustering of phagocytic receptors upon binding to a target induces a cascade of signaling events that leads to the assembly of phagocytic cups [4,5]. Prior studies have indicated that the shape of the phagocytic cups and their dynamic behavior (extension around the target surface vs. formation of a "sinkhole" that the target is gradually pulled into) may be dependent on the type of receptor being engaged, with IgG-FcR-mediated phagocytosis using pseudopodia-like cup extension around the target vs. complement-mediated phagocytosis involving primarily the sinking mechanism [6,7]. This apparent difference in the phagocytic cup morphology has led to proposals that specific phagocytosis pathways may rely on distinct actin-regulatory mechanisms. However, recent studies have shown that phagocytosis of both IgG-opsonized and complement-opsonized targets by macrophages involves formation of pseudopodia-based phagocytic cups [8,9], suggesting that the distinctions between different types of phagocytosis are not as clear-cut as once thought. Furthermore, integrins may also contribute to IgG-FcR-mediated phagocytosis, especially under the conditions of low IgG density [9,10]. Thus, different types of phagocytosis may involve overlapping or redundant cytoskeletal mechanisms, and the ability of cells to use multiple types of phagocytic actin assemblies, multiple actin nucleators, and a variety of phagocytic receptors is likely to result in an efficient and resilient immune response.

Spatial organization of actin and adhesion receptors in the phagocytic cups

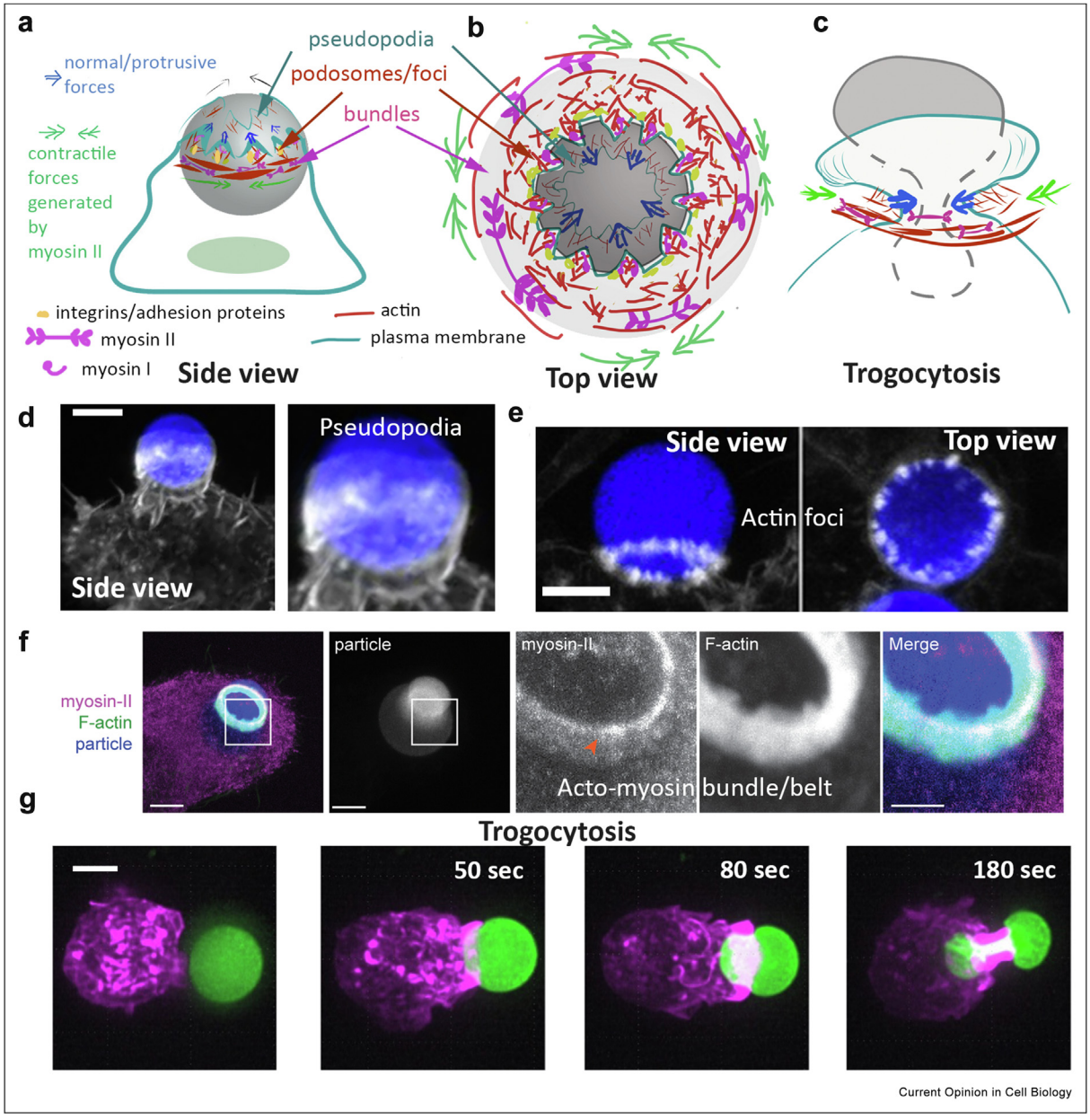
Recent studies using both IgG-opsonized and C3-opsonized phagocytic targets have identified actin puncta/clusters within phagocytic cups formed by macrophages, dendritic cells, and neutrophil-like HL-60 cells [10–14], indicating that phagocytic actin is not assembled into a uniform network but is organized into discrete spots (Figure 1). These actin puncta have been examined using super-resolution microscopy (STED and SIM) and platinum replica electron microscopy in the setting of frustrated phagocytosis [10,12]. Actin foci are composed of branched actin [12] and colocalized with the Arp2/3 complex and its activators and with clusters of phagocytic receptors [10,12], suggesting that receptor cross-linking/clustering may create hot spots of actin assembly along the rim of the cup. In addition, actin foci are associated with cell adhesion receptors (integrins) and intracellular adhesion proteins, such as talin, vinculin, and paxillin [10,11,14] and are reminiscent of podosomes, specialized sites of cell-substrate adhesion found in macrophages and dendritic cells. Podosome assembly requires the Arp2/3 activator WASp [15], which may work synergistically with the actin-regulatory protein cortactin [16]. Similarly, phagocytic actin foci are enriched in WASp and cortactin [10,11,14] and are disrupted by WASp inhibition [10], suggesting that WASp is the predominant Arp2/3 activator in both podosomes and phagocytic actin clusters. Formation of phagocytic podosome-like structures is accompanied by a transient reduction in the podosome number at the cell-substrate interface [14], indicating that both phagosome-associated podosomes and regular podosomes may compete for a limited pool of constituent proteins.

Comparison of the dynamics of phagocytic podosome-like clusters and regular podosomes in RAW 264.7 macrophages shows that the former are more dynamic and short-lived [10]. Furthermore, rings of podosomes formed during frustrated phagocytosis have the tendency to expand and exhibit outward movement, while regular podosomes remain relatively stationary in unstimulated cells [10]. Similarly, lattice light sheet imaging of actin clusters in live RAW macrophages in 3D shows that most of them exhibit forward movement along the surface of the target, together with the rim of the expanding phagocytic cup [11]. Thus, phagocytic actin foci slide along the surface of the target, often in a synchronous and coordinated manner [11], driving efficient engulfment of the target. Phagocytic cup formation and extension has also been clearly linked to phosphoinositide signaling [17]. PI(4,5)P2 is enriched at the leading front of the pseudopod extension in RAW macrophages [18] and controls actin assembly through the Cdc42-WASp-Arp2/3 axis [19]. When examined in 3D, PI(4,5)P2 concentration is lower at the base of the cup and higher at the rim of the cup where pseudopodia extension occurs [18]. During frustrated phagocytosis, reduction in PI(4,5)P2 in the center of the phagocytic wave is even more pronounced. The phagocytic wave/podosome ring forms a fence-like transition zone organizing lipid phosphatases and kinases into spatially separated pools [20]. This allows phosphoinositide segregation, with an external actin-dense zone rich in PI(4,5)P2 and an inside zone, circumscribed by the ring, depleted in PI(4,5)P2 and cortical actin, but rich in PI(3,4)P2 [20]. These observations highlight an additional functional role of the actin foci as an assembly platform for the lipid-modifying enzymes to form a metabolic barrier controlling phosphoinositide signaling, and it would be important to examine this mechanism in more detail in the 3D configuration.

What could be other functional significance of actin foci or podosome formation? Actin foci/podosome-like structures represent sites of close apposition of plasma membrane to the surfaces of phagocytic targets, as shown by IRM [10] and TIRF microscopy [12]. Thus, formation of phagocytic actin foci/podosomes may play an important role in promoting strong adhesion between the phagocyte and its target and help in efficiently enclosing targets of complex shapes [10]. Compared with focal adhesions, which are integrated into a contractile actomyosin skeleton, podosomes represent a more dynamic and self-sustainable type of adhesion since assembly or disassembly of individual podosomes is not always coupled to a larger structure, such as a stress fiber. Unlike focal adhesion formation, podosome assembly does not require contractility and is associated with lower myosin II activity, and the loss of a single podosome does not result in detachment or snapping back of multiple neighboring podosomes. These properties may be advantageous to allow cells to rapidly bind and engulf a target without the risk of target detachment and without the need to completely change the overall cell shape.

In an interesting development, recent work using microparticle traction force microscopy has revealed significant phagocytic forces in the direction perpendicular (normal) to the surface of the phagocytic target, and the magnitude of normal forces turned out to be higher than that of the in-plane forces [11,21]. These forces result in indentation of deformable phagocytic targets at the sites corresponding to actin foci, indicating that actin assembly at the rim of the phagocytic cup results in pushing or protrusive forces directed toward the surface of the target (Figure 1). This finding is consistent with podosome-like organization of actin foci since podosomes also generate protrusive forces [22]. Protrusive forces could be used by phagocytes to probe the target's mechanical properties (stiffness) and could also help close the phagosome or promote pinching off of small portions of phagocytic targets (trogocytosis) (see below).

Figure 1



Phagocytic cup architecture (a, b) Phagocytic cup organization, shown in a side view **(a)** and a top-down view **(b)**. The picture of phagocytic cup organization that arises from multiple studies includes the presence of **ruffles or pseudopodia** at the leading edge of the cup (nucleated primarily by Arp2/3 complex), a ring of **Arp2/3-nucleated actin foci or podosomes** located immediately behind the ruffling edge, and parallel, **stress-fiber-like circumferential actin bundles** collaboratively nucleated by Arp2/3 and formins. The ruffles, along with formin-nucleated filopodia, provide increased surface area to promote the initial contact between the phagocyte and the target. Podosomes/actin foci ensure the tight adhesion between the phagocyte and the target. Actin polymerization at foci is responsible for protrusive forces (**blue arrows**) that may serve a mechanosensory function and may also be involved in trogocytosis. Additional contractile forces (**green double arrows**) are generated via myosin II-mediated sliding of actin filaments. In complement-mediated phagocytosis, focal adhesion-like complexes containing integrin and talin promote receptor-cytoskeleton coupling needed for successful engulfment while in Fc-dependent phagocytosis, explosive actin polymerization along with the tight adhesion may be sufficient to enclose the phagocytic target **(c)** Protrusive and contractile forces cooperate to promote "nibbling" during trogocytosis. (d, e) Phagocytic cup organization **(d)** and actin foci **(e)** in RAW macrophages expressing mEmerald-Lifeact (grey) and ingesting polyacrylamide microparticles (blue). d and left panel in e – side view, right panel in e – top-down view (adapted from [11]) **(f)** Constricting acto-myosin belt in a RAW macrophage ingesting a polyacrylamide particle (adapted from [11]) **(g)** An example of trogocytosis, with a RAW macrophage expressing mEmerald-Lifeact (magenta) ingesting a polyacrylamide microparticle (green). Scale bars, 5 um; zoom scale bar in F, 1 um.

Regulation of phagocytic actin assembly

Assembly of phagocytic actin cups and actin foci relies on the activity of the Arp2/3 complex, however, other proteins, such as formins (Dia1) [8] and members of the Ena/VASP family [23,24], may also contribute to regulation of actin polymerization and provide alternative mechanisms for phagocytic actin assembly. For example, Arp2/3-null mouse macrophages show only partial reduction in FcR-mediated uptake [25] and in *Drosophila*, the loss of Arp2/3, Dia, or Ena does not prevent phagocytosis of apoptotic cells [24]. The use of alternative actin nucleators in the absence of Arp2/3 activity results in changes in cup organization and phagocytic efficiency, indicating that a switch away from the Arp2/3-mediated actin assembly has consequences for phagocytic uptake. For example, inhibition of the Arp2/3 activity disrupts formation of actin foci and reduces phagocytic forces during FcR-mediated phagocytosis [11] and prevents ruffle formation during C3-mediated phagocytosis [8]. Similarly, in the absence of Arp2/3, *Drosophila* macrophages are unable to form large phagocytic cups initiated by lamellipodia and instead use long filopodia to reach out and grab apoptotic debris [24].

The rate of actin assembly and the organization of actin networks forming the cup likely depend on the type of phagocytic receptors engaged by a target and on the mechanical properties of the target. For example, the density of Arp2/3-nucleated actin networks is increased in response to force [26,27], and, therefore, actin foci encountering stiff phagocytic targets may assemble more densely branched actin. This could help explain why stiff targets are more successfully taken up via phagocytosis. In some types of phagocytosis, such as C3R-mediated phagocytosis, actin cytoskeleton is additionally coupled to the surface of the target via focal-adhesion-like complexes that form a mechanosensitive clutch that can respond to substrate rigidity [1].

Roles of myosin motors

Myosins are actin-associated molecular motors that can power many actin-dependent activities relevant to phagocytosis [28]. These activities include regulation of filopodia and pseudopodia formation, contraction of actin bundles/networks, and transport of organelles or proteins (for example, integrins). Since several myosin isoforms have been detected in phagocytic cups/phagosomes, myosins have long been thought to be important for phagocytosis, and, in particular, for phagocytic cup closure [29]. However, the precise roles of myosins in phagocytosis have remained somewhat controversial. In particular, inhibition of myosin II, perhaps the best-studied myosin class with regard to phagocytosis, has produced mixed results, as reviewed in [28].

Recent studies have returned to the question of how myosins contribute to phagocytosis. Phagocytic actin foci are associated with class I myosins (myo1e and myo1f) [11,12]. These myosins have the ability to bind both actin and plasma membrane and may contribute to organizing or regulating actin assembly near the plasma membrane. Indeed, knockout of myo1e/f slows down phagocytic cup closure in 3D and actin turnover in phagocytic actin waves in 2D [12]. Membrane tension plays an important role in regulating both phagocytosis and actin assembly [30], and, therefore, maintenance or modulation of membrane tension by class I myosins may influence phagocytic efficiency.

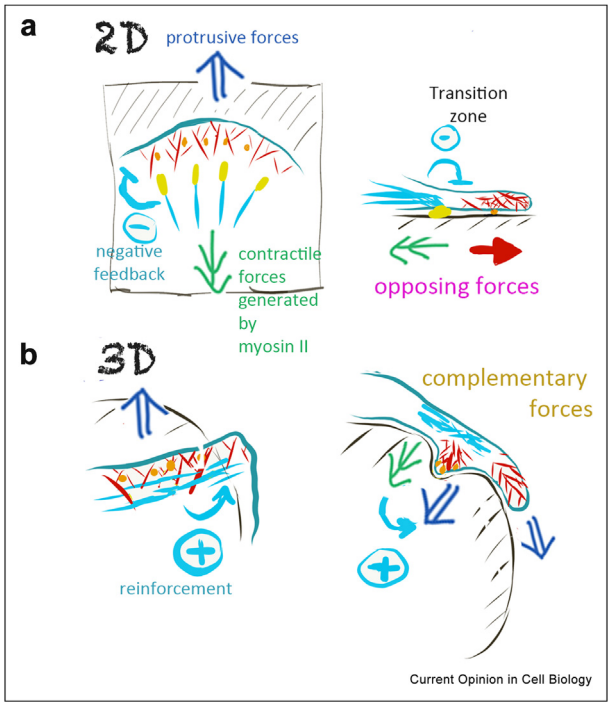
Phagocytic cups/phagosomes have been shown to squeeze deformable targets (red blood cells or apoptotic cells) in a purse-string-like contraction [29,31], while traction force microscopy has revealed the presence of in-plane traction forces during frustrated phagocytosis, with cell contraction being reduced by myosin II inhibition [53]. Thus, myosin II-mediated contractile activity could promote closure of the phagosome (Figure 1). While blebbistatin treatment does not affect phagocytic cup closure in IgG- and C3R-mediated phagocytosis [8,11], it reduces phagocytic forces in IgG-mediated phagocytosis [11] and decreases internalization in Dectin-1-dependent phagocytosis [32].

Effects of Arp2/3-driven polymerization and myosin II activity on actin architecture: what you see in 2D is not necessarily true in 3D

The application of novel force measurement approaches to the studies of phagocytosis allows us to rethink the ways in which different types of actin assemblies work together to control cell architecture and shape. The crosstalk between the Arp2/3-mediated actin polymerization and acto-myosin II contractility is one striking example. In 2D settings, such as cell migration on a planar substrate or frustrated phagocytosis on a flat surface, myosin II creates a pulling force applied to the rear of the lamellipodial actin network (Figure 2a). The force generated by myosin II converts actin networks into actin bundles and stress fibers, and the increase in contractility leads to formation of focal adhesions that apply centripetal/tangential forces in the plane of the substrate. In this case, myosin II-generated pulling forces oppose the Arp2/3-dependent protrusive forces.

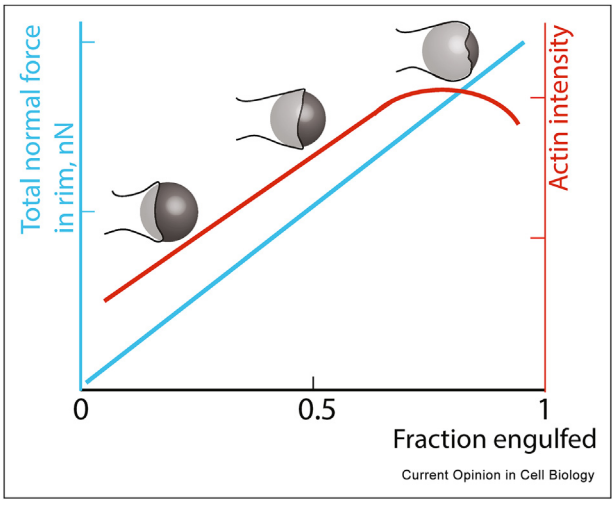
On the contrary, in a three-dimensional phagocytic cup, myosin II activity reinforces the protrusive forces indenting the substrate at the Arp-2/3-rich actin foci [11]. How is this possible? With the Arp2/3-mediated protrusions indenting the surface of the phagocytic target, and myosin-dependent contractile belt squeezing the target, the two types of actin machinery can now cooperate. The actomyosin belt exerts compressive,

Figure 2



Competition and cooperation between protrusive and contractile forces in 2D and 3D (2D) During cell migration on a planar substrate, Arp2/3-nucleated actin polymerization drives leading edge protrusion and leads to assembly of focal complexes (orange), while myosin II-dependent contraction of stress fibers pulls at the lamellipodial actin network and promotes maturation of focal adhesions (yellow). Competition between the pulling and protrusive forces produces negative feedback that slows down forward movement of the leading edge (3D) During phagocytic cup closure in 3D, both protrusive forces (generated by Arp2/3-driven actin assembly) and contractile forces (produced by the acto-myosin belt) are directed towards the center of the phagocytic target, reinforcing and complementing each other. Phagocytic podosomes are shown in orange.

Figure 3



Time course of actin assembly and force production during phagocytic cup closure. Constriction of the phagocytic cup and contraction of actin networks results in an increase in both actin intensity and normal forces.

rather than pulling, forces, reinforcing the pushing action of the Arp2/3-generated actin foci and working with the branched actin network rather than against it (Figure 2b). Furthermore, since branched actin networks undergo self-reinforcement under load leading to increased branch density, as observed *in vitro* [26] and *in cellulo* [27], forces applied by myosin II can facilitate assembly of densely branched Arp2/3-dependent networks. This leads to an increase in both actin intensity and normal forces over time (Figure 3). In addition, as actin filaments buckle under the compressive forces of the membrane tension and myosin contractility [33,34], buckling may also potentiate branching [35]. Previous observations in 3D settings, such as the cytokinetic rings of dividing cells and the contractile cell cortex, suggest that myosin II activity may promote actin filament turnover and disassembly, providing new monomers for actin reorganization and reassembly [36–38]. Whether a similar myosin II-dependent turnover mechanism may operate in the phagocytic cup remains to be determined, although it appears more likely that myosin II plays a distinct, network-reinforcing rather than network-dismantling, role during phagocytosis.

Partial eating or trogocytosis

Not all phagocytosis attempts result in successful engulfment of the entire phagocytic target. When phagocytes are confronted with targets that do not have the mechanical or geometrical properties that favor phagocytosis, they may end up biting off and ingesting small portions of the target (Figure 1c, g). This process, called trogocytosis (from the Greek "trogo" = nibble or chew) plays important physiological roles [39]. Trogocytosis is used by microglia to prune synapses and portions of axons in the developing nervous system [40,41], by embryonic epithelial cells to remove apoptotic cells [31], by neutrophils to nibble on and ultimately destroy antibody-opsonized cancer cells [42], and can even be used by cancer cells to "borrow" immunomodulatory components from immune cells, presumably to evade destruction by the immune system [43]. Partial eating of soft phagocytic targets is associated with intense accumulation of myosin II in the phagocytic cup prior to biting off a portion of the target [11]. Thus, myosin-mediated constriction of the cup may be particularly important in trogocytosis, to allow pinching off of the target cell membrane by the phagocyte. It remains to be determined whether protrusive forces generated by actin foci also help promote trogocytosis via target deformation and indentation.

Box 1. Comparison of phagocytic pathways and associated cell adhesion types

Engulfment mediated by IgGs/Fc receptors proceeds via rapid expansion of the branched actin network, with the phagocyte adhesion mediated primarily by podosomes. Since IgGs specifically recognize foreign objects that have previously been encountered by the immune system and need to be eliminated, IgG-dependent phagocytosis has likely evolved as an extremely fast and efficient process.

Phagocytosis mediated by the signals involved in innate immunity, such as complement, involves formation of integrin-mediated focal adhesions and their coupling to the cytoskeleton. This is a slower, more deliberate process that allows phagocytes multiple opportunities to interact with the target until multiple linkages between the target and the phagocytic cell are formed, leading to eventual internalization.

Podosomes: self-sustainable (do not require attachment to a stress fiber or another larger structure), have high turnover, support strong adhesion that is independent of the long-range actin reorganization, generate localized protrusive/normal forces, do not affect overall cell shape.

Focal adhesions: assemble in a concerted fashion (at least two focal adhesions need to be connected to the ends of a single acto-myosin stress fiber), require myosin contractility, have slow turnover, are associated with large tangential forces and affect the overall cell shape. Focal adhesions are highly mechanosensitive and may allow cells to probe the properties of the target.

Influence of the microenvironment and phenotypic diversity on phagocyte architecture and functions: future directions

A plethora of studies have been done *in vitro* to dissect mechanisms of phagocytosis. These studies have greatly contributed to our understanding of this process since phagocytosis *in vivo* often also involves interactions between a single target and an individual phagocyte, which can be easily mimicked *in vitro*. However, in health and particularly in diseases like cancer, the microenvironment strongly influences the phenotype of phagocytic cells, generating specialized phagocytes such as Tumor Associated Macrophages (TAMs) [44] and tissue macrophages. Specialized tissue macrophages have highly specific molecular signatures and cytokine profiles [45] and differ from circulating monocytes in their responses to foreign objects and tumor cells [46]. Compared to macrophage gene expression profiles, much less is known of their cell architecture/cytoskeletal organization, and how the geometry and physical properties of the microenvironment can fine-tune their properties [47]. Recent studies point toward the shape and size of TAMs as potential prognostic indicators in cancer and as correlates of their cellular origin and expression of phagocytic markers [48,49].

Tissue resident phagocytes also include retinal pigment epithelial cells (RPEs) in the eye, Sertoli cells in the testis, and ovarian granulosa cells [50,51]. These non-professional phagocytes play key roles in maintaining tissue homeostasis by removing older portions of retinal photoreceptors, cell debris (residual bodies) formed during spermatogenesis, and apoptotic oocytes. While phagocytic cups in these cells are also composed of branched actin and exhibit many similarities to the phagosomes of immune cells, some of the signaling and actin remodeling pathways are likely to be different, including reliance on distinct types of receptors, such as the Tryo3-Axl-Mer (TAM) tyrosine kinase receptors and integrin $\alpha V\beta 5$ [50–52].

Future studies will need to address questions related to phagocyte diversity and the effects of tissue microenvironment. Do macrophages residing in various organs and tissues have organ-specific architecture? Can the stiffness or the viscosity of the environment influence and tune phagocytosis? Are there dramatic differences in phagocytic capacity between TAMs in a solid tumor, such as pancreatic cancer, vs. circulating macrophages encountering liquid tumors, for example, leukemia? What are the consequences of tissue aging for the cell architecture of infiltrating macrophages? Intravital imaging approaches for *in vivo* studies and further development of controlled microenvironments for *in vitro* experiments may bring about answers to these questions Box 1.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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