

Phagocytosis and the microtubule cytoskeleton

Rene E. Harrison and Sergio Grinstein

Abstract: Phagocytosis is a critical host defense mechanism used by macrophages and neutrophils to clear invading pathogens. The complex sequence of events resulting in internalization and degradation of the pathogens is a coordinated process involving lipids, signaling proteins, and the cytoskeleton. Here, we examine the role of the microtubule cytoskeleton in supporting both the engulfment of pathogens and their elimination within phagolysosomes.

Key words: macrophage, microtubule, phagocytosis, maturation, Fc receptor.

Résumé : La phagocytose est un mécanisme de défense crucial utilisé par les macrophages et les neutrophiles pour éliminer les agents pathogènes qui envahissent l'hôte. La séquence complexe des événements entraînant l'internalisation et la dégradation des agents pathogènes est un processus coordonné faisant intervenir des lipides, des protéines de signalisation et le cytosquelette. Dans cet article, nous étudions le rôle du cytosquelette de microtubules (MT) dans la phagocytose des agents pathogènes et leur destruction dans les phagolysosomes.

Mots clés : macrophage, microtubule, phagocytose, maturation, récepteur Fc.

[Traduit par la Rédaction]

Phagocytosis

Macrophages are key players in the innate defense response whereby organisms protect themselves against pathogens. Macrophages are immune cells with specialized capacity for cell migration and phagocytosis of complement- and immunoglobulin-opsonized pathogens and are also instrumental in antigen presentation to T cells. The multiple roles of macrophages make this cell type an attractive model for the study of leukocyte biology. This review will concentrate on the potential roles of the microtubule (MT) cytoskeleton in mediating macrophage function, with special emphasis on phagocytosis mediated by Fc γ receptors (Fc γ R).

Phagocytosis of immunoglobulin G (IgG) opsonized particles via Fc γ R is a highly polarized and dynamic event involving clustering of receptors by multimeric ligands, recruitment of a signaling complex to the cross-linked receptors, actin assembly, pseudopod extension, and phagosomal closure (reviewed in Greenberg and Grinstein 2002). Engagement of Fc γ R by IgG-opsonized particles or by immune complexes leads to their clustering and tyrosine phosphorylation by Src-family kinases. This event in turn recruits the tyrosine kinase Syk (Greenberg et al. 1994) as

well as phosphatidylinositol 3'-kinase (PI3K). Lipid products of the type I PI3K accumulate markedly at the phagosomal cup (Marshall et al. 2001). Active PI3K phosphorylates PI-4-P and PI-4,5-P₂ to generate PI-3,4-P₂ and PI-3,4,5-P₃, respectively, which are potent second messengers that engage a variety of signaling cascades, likely by recruiting proteins containing PH (pleckstrin homology) domains (Greenberg 1999; Kapeller and Cantley 1994). The PI3K inhibitor wortmannin blocks pseudopod extension and (or) sealing, which correlates with a decline in local exocytic insertion of intracellular membranes at the plasma membrane (Cox et al. 1999). The formation of pseudopods is coincident with local remodeling of the underlying actin cytoskeleton to form a dense heavy mesh termed the actin cup. Actin cup formation requires activation of the Rho-family GTPases, Rac, and cdc42, although the precise mechanism(s) leading to their activation are not clear.

Macrophages have evolved a variety of killing strategies to defend against invading pathogens including proteolytic degradation, oxidation, and acidification of the pathogens. These are not apparent in the nascent phagosomes but are acquired during the process of maturation. Phagosome maturation is believed to occur by recruitment of new proteins from the cytosol, fusion events with subcompartments of the endolysosomal pathway, and removal and recycling of unnecessary components. These events are mediated by vesicular fusion and fission and are thought to be driven at least in part by the Rab-family of small GTPases. Phagosomes first fuse with early endosomes and acquire markers such as Rab5 and EEA1 (early endosomal antigen 1) (Desjardins et al. 1994; Vieira et al. 2001). Early fusion events involve the activity of the type III PI3K, which generates phosphatidylinositol 3-phosphate (PI3P) on the phagosomal

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membrane (Vieira et al. 2001). During the course of maturation, the phagosomes lose these early markers and acquire late endosome markers, including the mannose-6-phosphate receptor and Rab7, and eventually fuse with lysosomes to form a hybrid organelle: the phagolysosome (Desjardins et al. 1994). During maturation, the phagosomes acquire vacuolar proton pumps and become progressively more acidic and, in parallel, become loaded with acid proteases, like cathepsin D, and acid phosphatases (Geisow et al. 1981). During the course of phagosomal killing, remnants of the internalized pathogens are recycled back to the plasma membrane and some are presented to T cells to mount the cell-mediated immune response.

Microtubule cytoskeleton

MTs are ubiquitous cytoskeletal elements composed of tubulin subunits. Soluble tubulin bound to GTP polymerizes into 25-nm-diameter MT filaments, a process that involves the formation of 13 protofilaments arranged around a hollow core (Walczak 2000). MTs are asymmetric, or so-called "polar" structures, based on the inherent polarity of the tubulin heterodimers, each of which is composed of an α subunit and a β subunit that are only 50% identical in structure (Joshi 1998). This inherent polarity underlies the differential polymerization dynamics observed at the plus, or fast-growing end, versus the minus, or slow-growing end, of the MT, which is often embedded in the juxtannuclear MT organizing center (MTOC) (Joshi 1998). Within the cell, MT organization and function are controlled at a number of levels. The dynamics of MT assembly and disassembly are variable, as is the nucleation process that generates tubules de novo. These variables are controlled, in part, by the association between MTs and three main classes of accessory proteins including MT-associated proteins (MAPs), motors, and signaling molecules.

Two subpopulations of MTs often exist within the same cell, defined by differences in their stability. MTs are classified as dynamic or stable depending on the rate at which tubulin dimers are added or removed (Schulze and Kirschner 1987). Dynamic MTs are effectively disassembled by MT-disrupting agents, including cold or drugs such as colchicine (Bulinski and Gundersen 1991). The dynamic instability of MTs is modulated by the binding of MAPs (Maccioni and Cambiasso 1995). These proteins are generally called "structural" MAPs because they bind to, stabilize, and promote the assembly of MTs (Hirokawa 1994). Stable MTs formed upon binding of structural MAPs accumulate post-translational modifications such as acetylation of α -tubulin subunits within the MT polymer (Schulze and Kirschner 1987). Stable MTs show a half-life on the order of hours, compared with the minutes observed in highly dynamic MTs (Bulinski and Gundersen 1991; Schulze and Kirschner 1987). MAPs are in turn believed to be regulated by phosphorylation/dephosphorylation by protein kinases and phosphatases, respectively (Gundersen and Cook 1999). Other classes of proteins that bind MTs include motors (Amos and Cross 1997) that use MT polarity to sort and displace cargo towards or away from the MTOC (Amos and Cross 1997). MT motors include the retrograde or minus end directed motors, including cytoplasmic dynein, and the

anterograde or plus end directed family of motors, termed the kinesins (Amos and Cross 1997). These proteins use the energy derived from ATP hydrolysis to propel the motor and associated "cargo", including organelles and signaling complexes, along the MTs (Amos and Cross 1997).

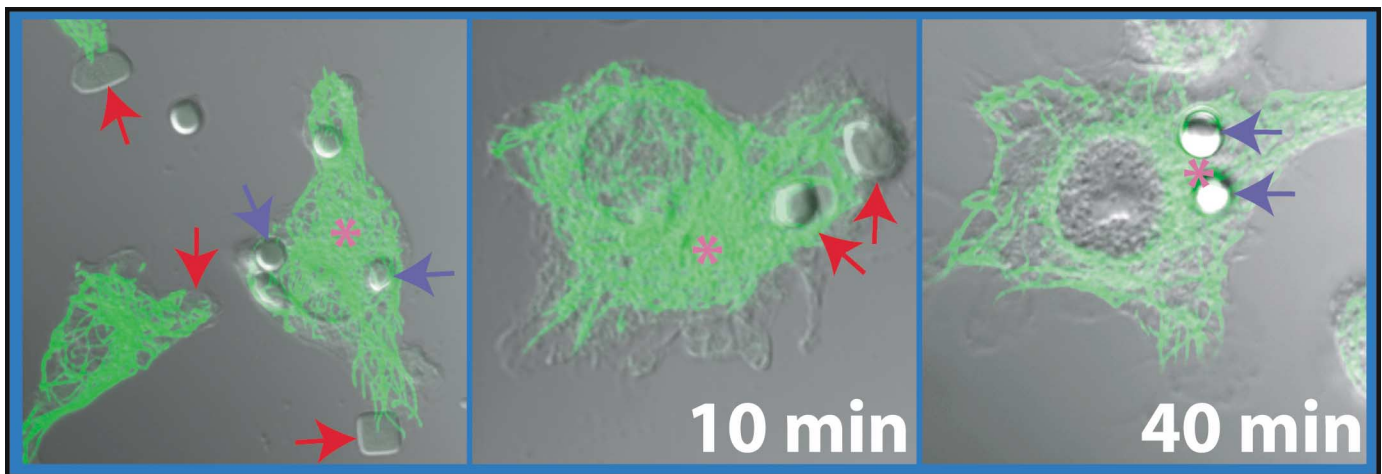
Roles of microtubules

Besides acting as a structural component of cells, it is now known that MTs have significant dynamic roles in the cell, such as mediating chromosome movements during mitosis, organelle positioning, intracellular transport of membranous vesicles, and signal transduction (for reviews, see Gundersen and Cook 1999; Valiron et al. 2001). Importantly, MT turnover plays a key role in cellular processes requiring changes in cell shape in events such as pseudopod formation and cell migration (Nabi 1999).

The movement and positioning of organelles within the cell are largely dependent on their association with MT motors. For instance, the perinuclear clustering of the Golgi complex is dependent on dynein, which holds the stack of cisterns adjacent to the MTOC (Wehland et al. 1983). Material internalized by endocytosis is transported from a peripheral to a juxtannuclear location as it progresses from early to late endosomes; this process similarly requires involvement of MTs (Gruenberg 2001; Gruenberg et al. 1989). Cytoplasmic dynein and its activator dynactin (Gill et al. 1991) are known to maintain late endosomes and lysosomes in the normal perinuclear location (Burkhardt et al. 1997; Harada et al. 1998). Recent evidence has linked Rab-family members directly to MT motor recruitment and activation. Thus, early endocytic events require a functional interplay between Rab5, PI3P production, and an unidentified minus end directed motor (Nielsen et al. 1999). Further, a Rab7-binding protein, Rab7-interacting lysosomal protein (RILP), recruits dynactin to late endocytic organelles, leading to the formation of perinuclear lysosomal aggregates (Cantalupo et al. 2001; Jordens et al. 2001). Rab7 is a key regulator of fusion between late endosomes and lysosomes and is also required for lysosome positioning within the cell (Bucci et al. 2000; Cantalupo et al. 2001; Feng et al. 1995; Jordens et al. 2001; Meresse et al. 1995). RILP is likely to mediate at least some of these functions.

In addition to transporting and maintaining organelles near the center of the cell, MTs also provide tracks for the directional movement of some cell components towards the cell periphery. As expected, MTs carrying out these functions tend to be stable (Bulinski and Gundersen 1991). One of the proposed functions of the MT system is to provide a scaffold at the leading lamellae of migrating cells for the attachment of signaling and (or) motile components that promote the development of polarity and directed cell migration (Rodionov et al. 1993). Cell polarization, migration, and directional extension of pseudopodia in neutrophils (Keller and Niggli 1995) and T cells (Ratner et al. 1997) require the MT cytoskeleton. The interactions between T helper cells and antigen-presenting cells, as well as orientation of cytotoxic T cells towards their target cell, require MTOC re-orientation and directed secretion guided by MTs (Kupfer and Singer 1989; Kupfer et al. 1987). MTs are also required for antibody secretion by plasma cells (Antoine et al. 1980),

Fig. 1. Tight spatial interaction between phagosomes and MTs. RAW264.7 macrophages were allowed to ingest IgG-opsonized red cells and, at the times indicated, the cells were fixed and immunostained for tubulin. Immunofluorescence images are superimposed on the corresponding differential interference contrast images. Red arrows indicate red cells being internalized (phagocytic cups). Blue arrows indicate internalized red cells (formed phagosomes). Purple asterisks denote the MTOC.



secretion of lytic compounds by natural killer cells (Katz et al. 1982), and targeted neutrophil degranulation (Mollinedo et al. 1989). Disrupting the function of the centrifugal motor kinesin alters pseudopod activity and cell polarity, suggesting that polarized membrane insertion guided along MTs is central to these processes (reviewed in Nabi 1999).

As mentioned above, signaling molecules form another class of MAPs. With a surface area nearly comparable with that of the plasma membrane, MTs often serve as scaffolds to bring components of signaling pathways together, possibly to increase the efficiency and specificity of their activation (Gundersen and Cook 1999). By serving as a docking platform, MTs promote the interaction of two or more signaling molecules that might not otherwise interact (Gundersen and Cook 1999). Signaling molecules that associate with MTs in immune cells include protein kinase C isoenzymes β and γ , which translocate to MTs following activation of $\beta 2$ integrins during T cell migration (Volkov et al. 2001). In monocyte-derived osteoclasts, attachment to bone coincides with MT-dependent translocation of PI3K, which is necessary for bone resorption (Lakkakorpi et al. 1997).

Microtubules in phagocytosis

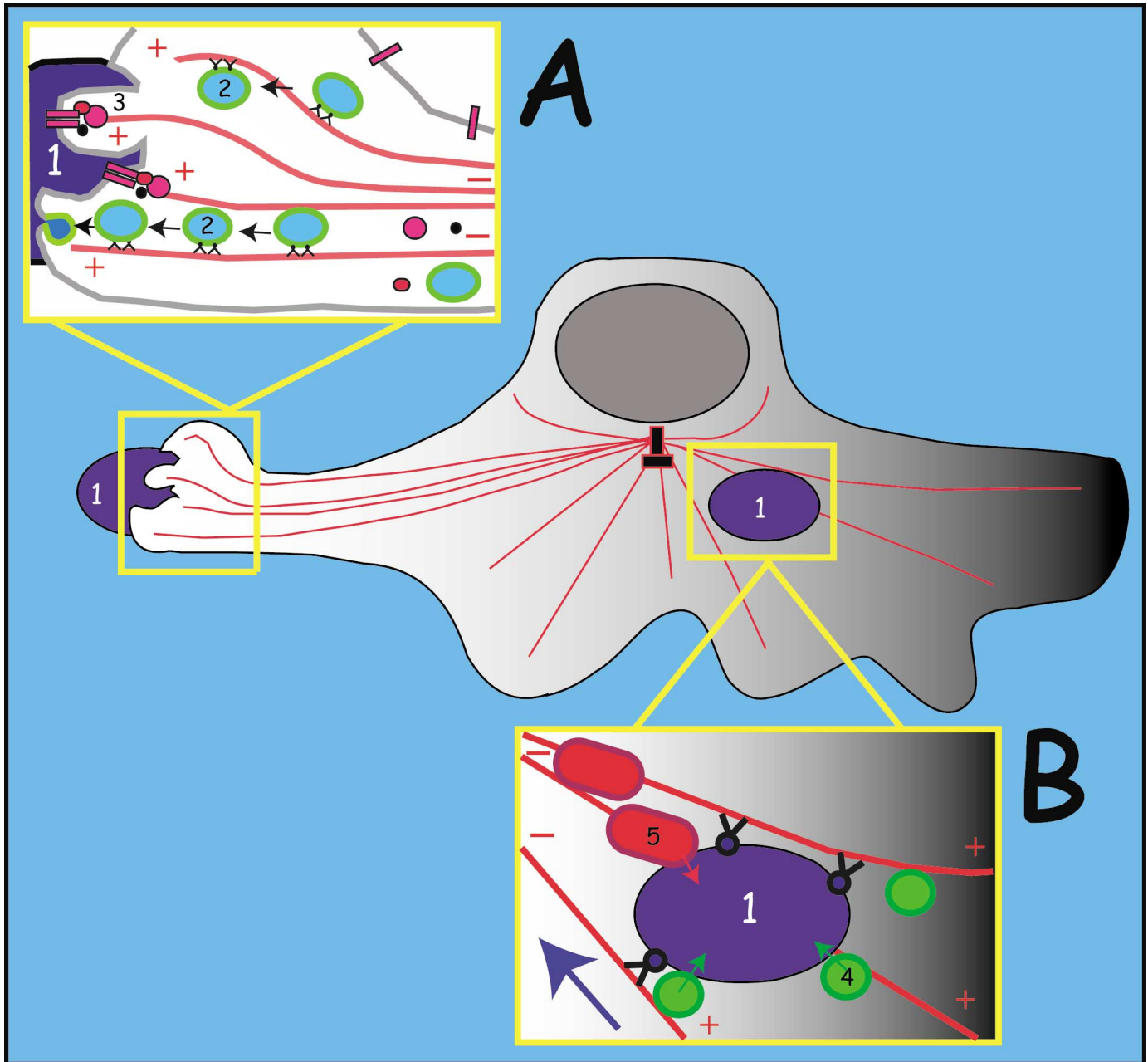
Given the multiple roles of the MT cytoskeleton, it is not surprising that it is also critical for many functions in macrophages. Studies on macrophage MTs have revealed that this cytoskeletal component is highly dynamic: ~80% of cytoplasmic MTs show a rapid turnover rate with a half-life of 7 min or less (Robinson and Vandre 1995). Interestingly, activation of macrophages seems to augment the stable MT component in these cells. Besides cytokines such as IFN- γ , macrophages are activated by bacterial products such as lipopolysaccharide (LPS) and phorbol esters like PMA (phorbol 12-myristate 13-acetate). PMA stimulation causes a rapid increase in MT number in mouse macrophages that peaks at 15 min following stimulation (Robinson and Vandre 1995). LPS increases MT stability by increasing total tubulin and MAP levels in human monocytes and macrophages (Allen et al. 1997). Altered vesicular traffic has been observed

in macrophages primed by thioglycollate (Edelson et al. 1975), IFN- γ , or LPS (Tsang et al. 2000), which may reflect enhanced MT stability in these cells.

MTs appear to play multiple roles in macrophages. Reorientation of the MTOC occurs in macrophages during chemotaxis (Nemere et al. 1985). The formation of actin adhesion structures termed podosomes requires MTs (Linder et al. 2000). Of particular importance for pathogen clearance is the observation that MTs also appear to be key for phagocytosis. Early studies of phagocytosis in mouse macrophages using transmission electron microscopy showed numerous MTs in the region of actin accumulation surrounding the ingested particles (Reaven and Axline 1973) (also see Fig. 1). In human leukocytes, contact of the opsonized particle with its receptors initiates a rapid MT assembly that is maximal by 3 min (Burchill et al. 1978). Numerous studies have since shown that MTs are not only remodeled during phagocytosis but are also essential for optimal Fc γ R-mediated ingestion in primary human monocytes and macrophage cell lines (Athlin et al. 1985, 1986; Bjermer et al. 1988). On the other hand, comparative studies of Fc γ R- versus complement-mediated phagocytosis revealed that only the latter process stringently requires MTs (Allen and Aderem 1996; Newman et al. 1991). The controversy regarding the requirement for MTs during Fc γ R-mediated phagocytosis may be explained by the activation state of the macrophages during these assays. As priming of macrophages has been shown to cause a selective stabilization of a subpopulation of MTs during activation, this subpopulation would be resistant to the effects of MT-depolymerizing agents. It is therefore safe to assume, in the interim, that MTs contribute to both Fc γ R- and complement receptor mediated phagocytosis, although perhaps to different extents. MTs have recently been shown to be important for integrin mobility in macrophages, which is consistent with the requirement for MT integrity during complement-mediated phagocytosis, as the CR3 complement receptor is a $\beta 2$ integrin (Zhou et al. 2001).

What might be the role of the MT subset found at the phagocytic cup during Fc γ R-mediated phagocytosis? MT

Fig. 2. Proposed functions of MTs during Fc γ R-mediated phagocytosis. Main panel: schematic overview of phagosome formation and maturation. The boxed regions in the main panel are magnified in the insets. Inset A: putative roles of MTs at the phagocytic cup. During engulfment of an IgG-opsonized particle (1), MTs may serve as tracks for kinesin-driven vesicle (2) traffic towards the plus ends to allow focal exocytic insertion of endomembranes required for pseudopod extension. MTs may also serve as a scaffold for signaling complexes (3) that are recruited to the vicinity of clustered Fc γ R and are required for local lipid modifications and actin assembly. Inset B: putative role of MTs in phagosomal maturation. Degradation of the internalized particle (1) occurs by dynein-mediated transport of the phagosome towards the minus ends of MTs and fusion with endosomes (4) and lysosomes (5).



disruption reduces pseudopod dynamics during frustrated phagocytosis assays, showing a role in localized membrane protrusive activity during spreading (Rosania and Swanson 1996). As colchicine affects pseudopod formation in macrophages and other cell types, it is plausible that MTs are required for pseudopod formation during Fc γ R-mediated phagocytosis. MTs may contribute to pseudopod extension by guiding localized insertion of endomembrane vesicles to the phagocytic cup, likely via a kinesin motor (see Fig. 2A).

Since disrupting kinesin function alters pseudopod activity and cell polarity in other cells (reviewed in Nabi 1999), polarized membrane insertion may similarly cause protrusive activity during phagocytosis. Recently, we showed that phagocytosis is indeed accompanied by localized exocytosis of recycling endosomal vesicles, which may be a critical source of membrane required for engulfment of large or multiple particles (Bajno et al. 2000). Alternatively, MTs may be assisting in the signaling events that generate

pseudopods. PI3K activity is believed to be regulated, at least in part, by translocation of the kinase from the cytosol to the plasma membrane (Susa et al. 1992). Recently, several subunits of PI3K were shown to associate with MTs in other cell types (Inukai et al. 2000; Kapeller et al. 1995). By tethering PI3K near its plasma membrane substrates, MTs may serve to increase the efficiency of kinase activity. Interestingly, while LPS activation of macrophages does not increase total PI3K activity, it causes a significant increase in plasma membrane associated PI3K activity (Weinstein et al. 2000). We speculate that activation of macrophages and a resultant enhanced MT network may serve to enhance and localize lipid-mediated signal transduction at the plasma membrane in a manner that favors phagocytosis. Therefore, in addition to functioning as tracks for the delivery of endomembrane vesicles to active membrane regions such as the nascent phagosome, MTs may also provide a scaffold for signaling complexes (Fig. 2A).

Microtubules in phagosome maturation

Once internalized, the phagosome matures by sequential fusion events with vesicles of the endocytic pathway. During internalization, early phagosomes briefly contain an actin coat and are believed to be translocated out of the cortical actin network by a mechanism that involves myosin Va (Al-Haddad et al. 2001). This initial translocation is then followed by association of phagosomes with MTs and ensuing maturation. Early phagosomes have been shown to have a preference for MT plus ends (Blocker et al. 1998) and this initial binding requires a unique phagosomal MAP recently characterized (Blocker et al. 1996).

Phagosomes are known to move centripetally along MTs, and immunogold analysis has revealed association of MT motors, including dynein and dynactin subunits, with phagosomal membranes (Habermann et al. 2001). Moreover, phagosomes have been documented to reach speeds of 0.2–1.5 $\mu\text{m/s}$, indicative of MT motor based transport (Blocker et al. 1998). Also, contact and fusion of phagosomes with late endocytic organelles is significantly reduced following MT depolymerization (Desjardins et al. 1994; Blocker et al. 1997). This cytoskeletal interaction is critical for exchange of material between the compartments.

The observed association of phagosomes with Rab5 and Rab7 and the known interaction of these GTPases with MTs and motors suggest a potential mechanism for phagosome maturation along this cytoskeletal network (Fig. 2B). We propose that Rab7 recruits to phagosomes the effector protein RILP, which in turn mediates the association of the vacuole with dynein/dynactin and MTs. In accordance with this model, late phagosomes accumulate near the MTOC (Fig. 1, 40 min). It is also possible that lysosomes migrate to meet the phagosome. Macrophages contain unique tubular lysosomes that form by a kinesin-dependent mechanism and often extend out from the MTOC to the cell periphery (Hollenbeck and Swanson 1990; Knapp and Swanson 1990; Swanson et al. 1992). Thus, phagosomes and lysosomes may meet and “kiss” halfway in their journey along MTs.

Future prospects

Further work should clarify the role of the MT cyto-

skeleton in the diverse aspects of macrophage function. The importance of vesicle traffic along MTs to pathogen killing remains to be explored in detail. Further, the apparent discrepancies regarding the role of MTs in Fc γ R-mediated phagocytosis need to be resolved. Another aspect that needs investigation is the involvement of MTs in antigen processing and presentation.

Understanding MT-based aspects of phagocyte function is not only important in improving our knowledge of the basic cell biology of leukocytes but also has potential usefulness in generating therapeutic targets for pathological immune conditions. MT-disrupting agents, like *Vinca* alkaloids, have already achieved some success as anti-inflammatory agents in human disease models, including type II–III autoimmune diseases, idiopathic thrombocytopenic purpura, and autoimmune hemolytic anemia, which engage phagocytes of the reticuloendothelial system (Tsubakio et al. 1983). In addition, the MT-depolymerizing drug colchicine has widespread use in diseases ranging from acute gouty arthritis to familial Mediterranean fever. Pyrin, the gene product responsible for familial Mediterranean fever, is regulated by IFN- γ and has recently been shown to be a MAP expressed in cells of monocyte lineage (Mansfield et al. 2001).

There is also promise for MT-directed drugs in the treatment of pathogen-mediated diseases. Both *Mycobacterium tuberculosis* and *Salmonella typhimurium* evade lysosomes and successfully survive in incompletely mature phagosomes within macrophages. Interestingly, *Mycobacterium* is successfully cleared following activation of macrophages with IFN- γ , and it will be of interest to determine if MT stabilization by this priming agent aids in lysosomal fusion with the vacuole containing bacteria. *Salmonella* exploits the MT cytoskeleton to generate long *Salmonella*-induced filaments (SIFs), elongated membrane compartments that promote bacteria multiplication in macrophages (Brumell et al. 2002; Garcia-del Portillo et al. 1993). Since MT depolymerization reduces both *Salmonella*-induced filament formation and bacterial proliferation, MTs may provide a potential target to counter infection by this typhoid fever inducing agent.

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