Microtubules regulate PI-3K activity and recruitment to the phagocytic cup during Fcγ receptor-mediated phagocytosis in nonelicited macrophages

Arian Khandani,* Edward Eng,* Jenny Jongstra-Bilen,† Alan D. Schreiber,‡ David Douday,§ Payman Samavarchi-Tehrani,¹ and Rene E. Harrison*,1

*Division of Life Sciences, University of Toronto at Scarborough, Toronto, Ontario, Canada; † Toronto General Research Institute, University Health Network, Department of Immunology, University of Toronto, Toronto, Ontario, Canada; ‡ Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA; and § Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada

Abstract: Phagocytosis is a complex sequence of events involving coordinated remodeling of the plasma membrane with the underlying cytoskeleton. Although the role of the actin cytoskeleton is becoming increasingly elucidated, the role of microtubules (MTs) remains poorly understood. Here, we examine the role of MTs during Fcγ receptor-mediated phagocytosis in RAW264.7 mouse macrophages. We observe that MTs extend into the phagosomal cups. The MT-depolymerizing agents, colchicine and nocodazole, cause a sizeable reduction in phagocytosis of large particles in RAW264.7 cells. Phagocytosis in primed macrophages is unaffected by MT-depolymerizing agents. However, activation of macrophages coincides with an increased population of drug-stable MTs, which persist in functional phagocytic cups. Scanning electron microscopy analysis of unprimed macrophages reveals that pseudopod formation is reduced markedly following colchicine treatment, which is not a consequence of cell rounding. MT depolymerization in these cells does not affect particle binding, Syk, or Grb2-associated binder 2 recruitment or phosphotyrosine accumulation at the site of phagocytosis. Ras activation also proceeds normally in macrophages treated with colchicine. However, MT disruption causes a decrease in accumulation of AKT-pleckstrin homology-green fluorescent protein, a probe that binds to PI-3K products at the sites of particle binding. A corresponding decline in activated AKT is observed in colchicine-treated cells using immunoblotting with a phospho-specific-AKT (ser473) antibody. Furthermore, the translocation of the p85α regulatory subunit of PI-3K is reduced at the phagocytic cup in colchicine-treated cells. These findings suggest that MTs regulate the recruitment and localized activity of PI-3K during pseudopod formation. J. Leukoc. Biol. 82: 000–000; 2007.

Key Words: phagocytosis · pseudopod · phosphoinositides

INTRODUCTION

Phagocytosis via FcγRs is a highly polarized and dynamic event involving signaling complex recruitment, actin assembly, pseudopod extension, and phagosomal closure (reviewed in ref. [1]). Engagement of FcγRs by IgG-opsonized particles leads to their clustering and tyrosine phosphorylation by Src family kinases. Phosphorylation of the immunoreceptor tyrosine activation motif, in turn, provides docking sites for Src homology-2 (SH2)-containing molecules, including the tyrosine kinase Syk [2]. The downstream pathways stimulated by Syk are incompletely understood but include activation of PI-3K, which is composed of a catalytic p110 subunit and one of several possible regulatory subunits [3]. The p110 and p85α subunits of PI-3K have been shown to interact with Syk and FcγRs [4, 5], and lipid products of the kinase markedly accumulate at the phagosomal cup [6]. Active PI-3K phosphorylates phosphatidylinositol (PI) 4-phosphate and PI 4,5-bisphosphate to generate PI 3,4-bisphosphate and PI 3,4,5-trisphosphate (PI(3,4,5)P3), respectively, which are potent, second messengers that engage a variety of signaling cascades, likely by recruiting pleckstrin homology (PH) domain-containing proteins [3, 7, 8]. The PI-3K inhibitor wortmannin blocks pseudopod formation during phagocytosis [9]. The formation of pseudopods is coincident with local remodeling of the cortical actin cytoskeleton to form the actin cup. This event requires activation of the Rho GTPases, Rac1 and cdc42, although the precise mechanism leading to their activation is not clear [10–12]. This has been proposed to be via binding of PH domain-containing, activating factors for these GTPases, such as the Rac exchange factor Vav. Although the contribution of microfilaments to this dynamic, polarized event is largely undisputed, the role of the microtubule (MT) cytoskeleton remains debated.

MTs are composed of tubulin heterodimers, which polymerize out of a centrally located MT organizing center (MTOC; reviewed in ref. [13]). MTs play many roles in regulating

1 Correspondence: Division of Life Sciences, University of Toronto at Scarborough, Toronto, Ontario, M1C 1A4, Canada. E-mail: harrison@utsc.utoronto.ca

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cellular morphogenetic processes, including assembly and positioning of the cleavage furrow [14], intracellular transport [14], and establishment of cell shape and polarity in multiple cell types [15]. MTs also play a significant role in signal transduction, by virtue of multiple kinase and phosphatase interactions with MTs [16]. An involvement of MTs has been observed in major effector functions of immune cells including neutrophil and T cell migration [17, 18], secretion of lytic compounds by cytotoxic T cells [19] and NK cells [20], neutrophil degranulation [21], antibody secretion by plasma cells [22], and podosome formation in macrophages [23].

Early transmission electron microscopy studies of phagocytosis in mouse macrophages showed numerous MTs in the region of actin accumulation surrounding large beads [24]. Several studies since have shown a requirement for MTs in FcγR-mediated phagocytosis in primary human monocytes [25–27] and the J774.2 macrophage cell line [28]. However, these studies did not address the precise role of MTs in phagocytosis. It is interesting that direct, comparative studies of FcγR- versus C3b-mediated phagocytosis, in which macrophages were treated with PMA to express activated complement receptors, showed a requirement for MTs, only for complement-mediated phagocytosis [29]. We re-examined the roles of MTs in FcγR-mediated phagocytosis in resting versus primed macrophages. Our experiments revealed a novel role for MTs in regulating PI-3K during FcγR-mediated phagocytosis. We chose the murine macrophage cell line RAW264.7 for detailed signaling analysis, as these cells are amenable to transfection. We present evidence for a requirement of MTs during FcγR-mediated phagocytosis in mediating PI-3K action at the phagocytic cup.

MATERIALS AND METHODS

Reagents

DMEM, PBS, and penicillin/streptomycin antibiotics were from Wisent Inc. (St-Bruno, Quebec, Canada). Sheep RBCs (sRBCs) and rabbit anti-sRBC IgG were obtained from ICN Biomedicals (Irvine, CA, USA). Polystyrene beads (0.8, 3.3, and 8 μm diameter) were obtained from Bangs Laboratories (Fishers, IN, USA). Fucose 6 was from Roche Diagnostics Corp. (Switzerland), Rhodamine-phalloidin was from Molecular Probes (Eugene, OR, USA). Anti-phosphotyrosine (PY20) and anti-p85 (Z-8) polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cy3-conjugated, donkey anti-human IgG, anti-mouse IgG, anti-rabbit IgG, and FITC-conjugated donkey, anti-human IgG, anti-mouse IgG, and anti-rabbit IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). HRP-conjugated, goat anti-rabbit IgG and donkey anti-mouse IgG were from Pierce Chemical Co. (Rockford, IL, USA). The Ras antibody was obtained from ViroMed Biosafety Laboratories (Minneapolis, MN, USA), National Cancer Institute (Bethesda, MD, USA), and Biological Carcinogenesis Branch Repository (Cumberland, NC, USA). The GST-Raf1 Ras-binding domain (RBD) construct was from Upstate Biotechnology (Lake Placid, NY, USA). Phospho-AKT (p-AKT; ser 473) antibodies were from Cell Signaling (Beverly, MA, USA). Anti-α-tubulin (B-5-1-2) and antiacetylated α-tubulin (6-11B-1) mAbs and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Mouse macrophage isolation, cell culture, and transfection

Primary macrophages were isolated from the peritoneal cavities of BALB/c mice, directly or 2 days after i.p. injection (0.7 ml/20 g mouse) of thioglycollate (TG; sterile 4% solution, aged 3 months), as described previously [30]. Briefly, resident and TG-elicited macrophages were harvested from killed mice by peritoneal lavage by injecting 5 ml ice-cold PBS (without Ca2+/Mg2+) containing 5% heat-inactivated FBS. To prevent activation by glass adherence [31], mouse macrophages from each mice were plated separately on poly-L-lysine or fibrinogen-coated coverslips (coated 0.2 mg/ml or 1 mg/ml, respectively, overnight, prior to blocking with 0.1% BSA) and grown in DMEM with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) for 2 h prior to washing away nonadherent cells. The mouse macrophage RAW264.7 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). The cells were freshly thawed for experiments and used within several passages to prevent activation. RAW264.7 macrophages were cultured in DMEM with 10% heat-inactivated FBS as described [6].

RAW264.7 cells were plated on 25 mm glass coverslips and transiently transfected with Fugene 6, according to the manufacturer’s instructions, and used within 24 h of transfection. To depolymerize MTs thoroughly, cells were treated with 10 μM colchicine for 20 min or 30 μM nocodazole for 30 min before addition of the IgG-opsonized sRBC or bead targets. Macrophage priming was performed by pretreating the cells overnight with IFN-γ (500 U/ml) or for 20 min with PMA (100 nM) in serum-free media, prior to experiments. RAW suspension cells were generated by growing cells in RPMI with 10% FCS in upright flasks in 5% CO2 for 2–3 weeks. Suspension cells were placed on a rotator during colchicine treatment and phagocytosis assays. After 30 min of phagocytosis, external sRBCs were lysed, and cells were spun onto poly-L-lysine-coated coverslips for immunostaining. An equivalent number of suspension cells were plated on coverslips for 6 h to allow spreading prior to phagocytosis assays and were used as controls.

Phagocytosis assays

sRBCs and polystyrene beads (0.8, 3.3, and 8 μm) were opsonized with subagglutinating concentrations of rabbit anti-RBC IgG or human IgG, respectively. Opsonization was for 1 h at room temperature, followed by three washes with PBS. For phagocytosis, macrophages were exposed to opsonized particles in DMEM/FBS at 37°C. To examine early phagocytosis events, sRBCs were added at a concentration of five to nine sRBCs/macrophage into 1 ml media for 4–7 min, followed by vigorous washing to remove unbound particles. The binding index was calculated by counting the number of bound particles per macrophages. To determine the phagocytic index, macrophages were incubated with sRBCs for 5 min in 1 ml media to synchronize phagocytosis. Beads were spun onto the cells by low-speed centrifugation. Unbound particles were then washed off, and cells were incubated at 37°C for 25 min. Externally bound sRBCs were then lysed by a 20-s hypotonic shock, and the cells were fixed with 4% paraformaldehyde in PHEM for 30 min at 4°C. The number of phagosomes per 100 macrophages was determined using differential interference contrast (DIC) microscopy. To identify opsonized polystyrene beads, which were not internalized, the samples were incubated at 4°C with Cy3-labeled, donkey anti-human IgG (1:1000) for 10 min prior to fixation. The phagocytic index was defined as the number of ingested particles per 100 macrophages. A minimum of three replicate wells per condition was studied in each experiment, and the number of individual experiments is indicated in the figure legends. Data are expressed as the mean ± SE. For phagocytic indexes, mean values for the raw data-points (typically over 100 cells) for each n value were compared between treatments. The significance of differences between means was assessed using the Student’s t-test. We chose Student’s t-tests for their high stringency of analysis; two-tailed analyses, as we could not predict if values would increase or decrease; and an unpaired analysis, as we could not assume cells within and between each replicate were identical to one another.

Immunofluorescence and confocal microscopy

Following phagocytosis assays, RAW264.7 cells were washed with MT-preparing 80 mM PIPES (pH 6.9), 20 mM HEPES, 4 mM EGTA, 1 mM MgCl2 (PHEM) buffer and fixed with 4% paraformaldehyde in PHEM for 30 min at room temperature. The fixed cells were permeabilized with 0.1% Triton X-100 in PBS containing 100 mM glycine for 20 min before blocking for 1 h with 5% FBS in PBS. Cells were stained with primary antibodies for 1 h at room temperature in PBS containing 1% FBS. All cells were stained with an anti-α-tubulin antibody (1:2000) to monitor MT integrity. To visualize phosphotyrosine-containing proteins, the PY20 antibody (1:2000) was used. The p85α antibody was diluted 1:200. Following washing, samples were incubated with appropriate FITC- or Cy3-conjugated secondary antibodies (1:1000). To
Scanning electron microscopy (SEM)

For SEM, RAW264.7 cells were grown on plastic coverslips. Following 7 min phagocytosis of IgG-sRBCs, cells were rinsed with ice-cold 0.1 M phosphate buffer (pH 7.4), followed by fixation with 2% gluteraldehyde. After washing with 0.1 M sodium cacodylate buffer, cells were postfixed with buffered 1% OsO4 for 1 h and dehydrated in ethanol. The samples were then critical point-dried and sputter-coated with gold. Samples were examined, and images were acquired in a JEOL JSM 620 SEM.

Ras activation assays

RAW264.7 cells were serum-starved for 1 h and then subjected to various treatments, as described in the text. Cells were lysed with modified radiomunoprecipitation assay buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1% glycerol, 25 mM NaF, 10 mM MgCl2, 1 mM EDTA, 1 mM NaVO4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 250 μM PMSE), and the level of Ras-GTP was determined using an activation-specific probe as described [32]. Briefly, GST-Raf1 RBD fusion protein was induced and purified from Escherichia coli and bound to glutathione beads. This was used to precipitate active Ras (Ras-GTP) from total cell lysates, and the amount of Ras-GTP was then determined by immunoblotting with an anti-Ras antibody and densitometry scanning.

Immunoblotting

Whole cell lysates and Ras-GTP bound on beads were solubilized in Laemmli’s sample buffer. Equal protein amounts were loaded and analyzed by SDS-PAGE and Western blotting as described [33]. Equal loading was determined using the DC protein assay (Bio-Rad, Hercules, CA, USA) and confirmed further by Ponceau red staining of nitrocellulose (NC) membranes. Protein detection was performed by exposing NC blots to the primary antibodies: anti-α-tubulin (1:2000), anti-PY20 (1:1000), anti-Ras (1:25,000), and anti-p-AKT (ser473; 1:1000). Immunodetection was performed using corresponding secondary antibodies conjugated to HRP (diluted 1:5000; 1 μg/ml). Blotting was visualized by the ECL Western blotting detection system (Amersham Pharmacia Biotech, Little Chalfont, UK), according to the manufacturer’s instructions. Protein bands were measured by densitometry scanning of independent experiments using NIH/Scion Image software, and relative pixel intensities were normalized between experiments. Pooled values between experiments were expressed as mean ± SEM-fold activation/induction or reduction of association of proteins compared with control experiments.

RESULTS

Macrophages elicited by TG or primed with IFN-γ are protected from colchicine inhibition of phagocytosis

We investigated whether macrophage activation impacted the effect of MT-depolymerizing drugs on FcyR-mediated phagocytosis. Previous studies of primary cultures using human monocyte-derived macrophages and Bio-gel-activated macrophages showed that FcyR-mediated phagocytosis was unresponsive to anti-MT agents [29, 34]. We compared colchicine sensitivities toward phagocytosis of resident macrophages versus IFN-γ and TG-elicited macrophages. Injection of aged, sterile TG broth was used to induce an acute inflammatory response, which has been shown to enrich for macrophages, vastly different from resident, peritoneal macrophages in terms of size and metabolic and bactericidal activity [35, 36]. We chose mouse peritoneal macrophages, as we could investigate phagocytosis within 2 h of isolation, compared with 1 week, which is required for differentiation of human monocyte-derived macrophages. These macrophages have been shown to up-regulate complement receptors spontaneously, indicative of macrophage activation, during long-term culturing on coated glass coverslips [34].

Phagocytosis of IgG-opsonized sRBCs in nonelicited, resident, peritoneal macrophages was depressed significantly in the presence of colchicine (Fig. 1, B and E) compared with control cells (Fig. 1, A and E). In contrast, TG-elicited macrophages showed no statistically significant difference in phagocytosis in the presence (Fig. 1, D and E) or absence (Fig. 1, C and E) of colchicine. We next investigated whether more classical activators such as IFN-γ affected colchicine susceptibility during phagocytosis. Macrophage activation occurs primarily by exposure to T cell-derived IFN-γ but also by bacterial products including LPS [37, 38] and by phorbol esters [39]. We treated resident macrophages overnight with IFN-γ and compared the phagocytic index to unstimulated, overnight cultures. We observed a significant decrease in phagocytosis in unprimed macrophages, which were exposed to colchicine prior to phagocytosis, compared with control cells (Fig. 1E). This decrease was not as significant as seen with colchicine-treated, resident cells, tested immediately after isolation (Fig. 1, B and E), which may reflect a slight activation as a result of time in culture. IFN-γ-treated macrophages showed no difference in phagocytosis in the presence or absence of colchicine (Fig. 1E), confirming that the activation state of primary macrophages modulates colchicine effects on phagocytosis. Colchicine pretreatment did not affect binding of sRBCs to resident or TG-elicited peritoneal macrophages (not shown).

Macrophage activation appeared to confer a resistance to MT-depolymerizing agents during phagocytosis of IgG-opsonized particles. We examined whether this phenomena occurred in RAW264.7 cells, an established murine macrophage cell line (Fig. 2). Phagocytosis was depressed significantly in colchicine (Fig. 2, B and E) and nocardazole (a reversible, MT-depolymerizing agent; Fig. 2E)-treated cells, compared with control (untreated; Fig. 2, A and E) cells. In contrast, similar amounts of IgG-sRBCs were ingested in IFN-γ-activated RAW264.7 cells pretreated with colchicine (Fig. 2, D and E), as compared with IFN-γ-treated cells alone (Fig. 2, C and E). Cells were treated additionally with γ-lumicolchicine to control for nonspecific effects of colchicine on nucleoside transport at the cell membrane [40]. γ-Lumicolchicine-treated cells showed a
similar phagocytic index to control, untreated RAW264.7 cells (Fig. 2E).

**Macrophage activation is accompanied by enhanced MT stability in RAW264.7 cells**

The above results indicated a differential requirement for MTs during phagocytosis, depending on the activation status of macrophages. We decided to examine more closely the MT stability and distribution in macrophages before and after priming. Previous work has shown that the protein kinase C activator PMA causes MT assembly in neutrophils [34] and macrophages [39], and IFN-γ stimulates MT assembly in vitro [41]. Immunofluorescence using an anti-α-tubulin antibody revealed MTs penetrating into the site of early sRBC attachment in control cells (Fig. 3A). Diffuse tubulin staining was often observed surrounding the cup, which may represent membrane-bound tubulin (Fig. 3A). Following a 20-min colchicine pretreatment, MTs were largely abolished (Fig. 3B) compared with control cells (Fig. 3A). In colchicine-treated cells, MT staining was confined to regions corresponding to the MTOC, and short MTs, often disrupted, extended from it (Fig. 3B). Our findings in unprimed RAW264.7 cells indicate that these macrophages have a high percentage of drug-labile, cytoplasmic MTs, reflective of rapid turnover. In addition, these results reveal a close spatial relationship between MTs and nascent phagosomes. An extensive MT network was observed in IFN-γ-treated cells, and MTs also extended into the sites of phagocytic cup formation, highlighted by phalloidin staining (Fig. 3C). It is important that when IFN-γ-activated macrophages were treated with colchicine, many long MTs persisted, the majority of which extended into processes where actin cups were forming (Fig. 3D).

These observations strongly suggested that activated macrophages had a high content of drug-resistant MTs. We examined MT stability in control and activated RAW264.7 cells. MT stability was quantified by the assessment of levels of acetylated α-tubulin, a post-translational modification, which is used widely as a marker of drug- and cold-stable MT subsets [42, 43]. Stable MT subsets are acetylated on the ε-lysine 40 residue of α-tubulin following tubulin polymerization into MTs [42]. Although these modifications are not thought to stabilize MTs directly, they do correlate with slower MT turnover [43]. Immunofluorescence with an antiacetylated α-tubulin antibody showed an increase in this modified tubulin in IFN-γ-activated (Fig. 3F) and PMA-activated (Fig. 3G) RAW264.7 cells compared with untreated cells (Fig. 3E). To quantify the stable MT subpopulation in RAW264.7 cells, Western blotting for total α-tubulin and acetylated α-tubulin of whole cell lysates was performed. The extent of tubulin modification, as expressed by the relative ratio, modified:total tubulin, was on average two- to threefold higher in PMA- or IFN-γ-activated cells compared with unprimed RAW264.7 cells (Fig. 3H). These findings indicate that macrophage activation changes the composition of cytoplasmic MTs to create a less dynamic and more stable MT network. The increased amount of acetylated α-tubulin in activated RAW264.7 cells correlated with the increase population of colchicine-resistant MTs (Fig. 3D).
Colchicine does not impair sRBC attachment, Src activity, Syk, and Grb2-associated binder 2 (Gab2) recruitment to forming phagosomes

We next turned to determining the role of MTs in FcγR-mediated phagocytosis using unprimed RAW264.7 cells, which contain MTs that are almost completely abolished in the presence of MT-depolymerizing agents (Fig. 3F). To investigate the mechanisms underlying engulfment, we examined membrane events at the site of particle attachment in control and colchicine-treated RAW264.7 cells. To determine whether treatment with colchicine affected the availability of FcRs at the plasma membrane or their ability to bind ligand, RAW264.7 cells were tested for particle-binding ability. IgG-opsonized sRBCs were combined with macrophages for 5 min, following which unbound sRBCs were washed off. Cells were fixed, and bound sRBCs were counted for control and colchicine-treated cells. Colchicine pretreatment did not affect particle binding as compared with control or γ-lumicolchicine-treated cells (Fig. 4, A–C). As binding of IgG-opsonized particles is via FcγR clustering, cell surface expression and lateral movement of the receptor do not appear to require the MT cytoskeleton.

Activation of the FcγR requires enzymatic activity of Src and Syk, which associate with the FcγR (reviewed in ref. [1]). We examined the importance of MTs in tyrosine phosphorylation events at the site of particle attachment, by immunostaining with an antiphosphotyrosine antibody. Following 3D reconstruction of confocal slices, phosphotyrosine accumulation was detectable shortly after the plasmalemma established contact with the opsonized particles in control and colchicine-treated cells (Fig. 4, D and E). Cell lysates, following identical treatments, were also analyzed by Western blotting with the phosphotyrosine antibody. sRBC addition caused the expected, dramatic tyrosine phosphorylation of multiple proteins (Fig. 4F, Lane 2) [44–46]. Colchicine pretreatment of RAW264.7 cells did not block tyrosine phosphorylation of these proteins (Fig. 4F, Lane 3). Accordingly, Syk-GFP accumulated similarly in colchicine-pretreated or control RAW264.7 cells undergoing phagocytosis (Fig. 4, G and H). Similar results were obtained with nocodazole-pretreated RAW264.7 cells (not shown). In addition, transfection of RAW264.7 cells with the adaptor protein Gab2-GFP showed normal recruitment to the phagocytic cup in colchicine-treated cells (not shown). These experiments revealed that Syk and Gab2 recruitment and overall tyrosine phosphorylation in the developing phagosome occurred normally in drug-treated cells, indicating that they are MT-independent events.

MT depolymerization reduces PI-3K activity at the phagosomal cup

As part of our analysis of potential signaling defects caused by colchicine treatment during FcγR-mediated phagocytosis, we examined PI-3K activity by transient transfection of RAW264.7 cells with a fusion protein consisting of the PH domain of AKT, linked to GFP (AKT-PH-GFP). The PH domain of AKT is known to have a high affinity for PI3,4,5P3, and inhibition of PI-3K with wortmannin decreases AKT-PH-GFP accumulation at the phagocytic cup [6, 9]. Exposure of transfected RAW264.7 cells to IgG-opsonized sRBCs induced a marked and rapid redistribution of AKT-PH-GFP to the phagocytic cup in colchicine-treated RAW264.7 cells (Fig. 5, A, C, and E), similar to what has been observed previously [6]. In colchicine-treated RAW264.7 cells, AKT-PH-GFP remained within the cytoplasm and nucleus with little accumulation at sRBC attachment sites (Fig. 5, B, D, and E). This distribution has been described in resting macrophages, which are not undergoing phagocytosis [6]. Accumulation of phosphotyrosine-containing proteins still occurred at the sites of particle contact, which were devoid of AKT-PH-GFP in colchicine-treated cells (Fig.
Although prominent actin cups were reduced in colchicine-treated cells, F-actin accumulation occurred at sites of bound particles, which were lacking detectable AKT-PH-GFP accumulation (Fig. 5G).

To verify further whether MT depolymerization was affecting PI-3K activity at the phagocytic cup, we measured the levels of phosphorylated AKT in these cells. AKT is phosphorylated and activated when it binds to PI3,4,5P3 [47]. We used immunoblotting with a serine 473 p-AKT. A prominent band for p-AKT was observed in cells exposed to IgG beads for 7 min or heat-aggregated IgG for 5 min (Fig. 6A). Immunoblotting for p-AKT was reduced markedly in colchicine-treated cells undergoing phagocytosis (Fig. 6A).

These results implicated a defect in PI-3K activity during phagocytosis in the absence of MTs. The Ras GTPase has been shown to cause PI-3K recruitment and activation following growth factor stimulation [48, 49]. We examined Ras activation during FcγR-mediated phagocytosis to determine whether this upstream event occurred in RAW264.7 cells and was affected by colchicine treatment. The levels of Ras-GTP were measured using coprecipitation assays with the Ras-GTP-binding domain of Raf (Raf-RBD), immobilized to glutathione beads via a GST tag. Ras-GTP levels were increased consistently three- to fourfold following sRBC addition to RAW264.7 cells (Fig. 6B). Coprecipitated, active Ras levels were invariably similar in control and colchicine-treated cells during phagocytosis (Fig. 6B). A 20-min treatment with 10 nM PMA was used as a positive control (Fig. 6B). GST-bound, control beads did not bind any Ras, and total Ras protein was not affected by these acute treatments (not shown). These findings suggest that intact MTs are not required for Ras activation in this system.

MT depolymerization effects on particle internalization are a function of particle size

PI-3K has been implicated in pseudopod extension and closure [50]. As we observed a defect in PI-3K activity at the phagosomal cup in the absence of MTs, we next investigated whether colchicine mimicked effects of PI-3K inhibitors on pseudopod dynamics. Wortmannin studies have shown that uptake of large particles is inhibited more significantly than smaller particles [9], indicating a role for PI-3K in pseudopod extension to

Fig. 3. Activation of RAW264.7 cells increases colchicine-resistant and acetylated α-tubulin levels. (A–D) Serial reconstruction of confocal slices of cells after tubulin immunofluorescence. Cells were exposed to IgG-opsonized sRBCs for 7 min, and bound sRBCs are shown in DIC insets (A and B) or represented by phalloidin staining for actin cups (C and D, insets). Acetylated (acet) α-tubulin immunofluorescence of unprimed (E), IFN-γ-activated (F), or PMA-activated (G) RAW264.7 cells. Original bars, 10 μm. (H) Quantification of the relative amounts of acetylated α-tubulin in unstimulated and activated cultures of macrophages. Equal protein levels of cell lysates of unprimed or activated cells were separated on a SDS-PAGE gel and probed with an anti-acetylated α-tubulin antibody. The mean-fold difference of staining intensities relative to total α-tubulin was determined by densitometry, and numbers represent mean and SE from four independent experiments.
complete large particle internalization [9]. Similar to wortman-
in results, we observed that colchicine pretreatment did not
affect small particle uptake, as no difference in internalized,
IgG-coated, 0.8 μm beads was observed in colchicine-treated
cells versus control (Fig. 7, A and D). In contrast, phagocy-
tosis of larger beads of 3.3 or 8 μm in diameter was potently
inhibited following colchicine treatment as compared with
control cells (Fig. 7, B–D), analogous to the wortmannin effects
on FcγR-mediated phagocytosis [9]. We conclude that MTs are
required for optimal pseudopod extension around large par-
ticles.

MT depolymerization reduces pseudopod
formation during FcγR-mediated phagocytosis

As analysis of membrane surface topology is limited by con-
ventional light microscopy, we evaluated pseudopod formation
following MT disruption using SEM. In control cells, sRBCs
were observed at various stages of ingestion, and membrane
pseudopods were often observed to extend around the attached
sRBCs (Fig. 8A). Colchicine-treated cells were often rounded
up with thin projections, remaining attached to the plastic
coverslips. Analysis of surface membrane architecture at the
site of sRBC attachment showed minimal pseudopod develop-
ment surrounding sRBCs (Fig. 8B). Quantification of these
observations is depicted in Figure 5C, which shows significant
reduction of pseudopod formation in colchicine-treated cells
versus control cells. Membrane retraction has been reported
previously in cells treated with MT-depolymerizing agents [28].
To address whether cell-rounding affected the ability of the
cells to complete phagocytosis, suspension cells were gener-
ated. RAW264.7 cells were grown in suspension for several
weeks and challenged with sRBCs. There was only a slight
decrease in the phagocytic index of suspension cells compared
with cells, which were allowed to attach and spread for 6 h
prior to phagocytosis (Fig. 8D). By comparison, phagocytosis in
colchicine-pretreated, suspension cells was impaired mark-
edly, an inhibition comparable with spread cells (Fig. 8D). We
therefore conclude that defects in pseudopod development
following MT disruption are not a consequence of a reduction
in cell spreading.

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Our analysis of primed versus nonelicited macrophages revealed an important difference in MT stability in these cells. Nonelicited macrophages have a greater percentage of drug-labile MTs than macrophages primed with IFN-γ or TG. Although colchicine almost completely depolymerizes MTs in nonelicited macrophages, a large population of acetylated MTs persists in activated macrophages. These findings may provide insight into the discrepancy in the literature regarding the role of MTs in FcγR-mediated phagocytosis. As we see a direct correlation between macrophage priming and MT stability, it is probable that macrophage activation via phorbol esters, macrophage-eliciting compounds, and extended glass coverslip culturing [51] enhanced the MT stability in previous reports, where MTs were shown not to be required for phagocytosis through FcγR [29, 34]. These studies allowed direct comparisons of FcγR- versus complement-mediated uptake, where macrophage priming is required. Furthermore, as complement-mediated phagocytosis is sensitive to MT-depolymerizing agents, these studies implicated a role for dynamic, drug-labile MTs during phagocytosis of complement-opsonized particles in activated macrophages [29, 34].

The lack of colchicine sensitivity during FcγR-mediated phagocytosis in activated macrophages could possibly be a result of the initiation of a MT-independent signaling cascade for phagocytosis in these cells. However, the correlation between drug-stable MTs in primed macrophages and successful phagocytosis in the presence of colchicine suggests that the MTs may play an important, similar role in phagocytosis in...
activated cells. Previous studies have shown that colchicine inhibits binding and phagocytosis of tumor cells by mouse macrophages; however, pretreatment with PMA diminishes this colchicine sensitivity [52]. Priming appears to cause a selective stabilization of a subpopulation of MTs during the activation process. Another study has shown that macrophage activators, including PMA and the bacterial LPS, increase MT-associated protein (MAP) synthesis in the macrophage-like cell line THP-1 [53]. MAP association with MTs is known to increase MT stability and prevent them from drug-induced disassembly [54]. The intracellular signaling elements that lead to MT stabilization during macrophage activation still remain to be identified.

To decipher the role of MTs in FcγR-mediated phagocytosis, we used nonelicited macrophages. We observed MT ends in the cell periphery at the location of nascent phagosome formation in RAW264.7 cells (Fig. 3). Particle binding may signal to the MT cytoskeleton to cause local MT polymerization into the site of early phagocytosis, as has been observed in human leukocytes [55]. The mechanism of MT targeting to the phagocytic cup is currently unknown. Based on tubulin associations with phosphoinositides [56], we speculate the involvement of phospholipids.

MT depolymerization agents reduced the level of particle ingestion significantly without affecting particle binding. We showed here for the first time a need for intact MTs for maximal pseudopod extension during phagocytic cup formation. A role for MTs in phagocytic cup formation does not reflect an overall effect on FcγR-induced signaling, as colchicine treatment did not affect the recruitment of other, early signaling intermediates such as tyrosine kinase Syk or other tyrosine-phosphorylated products. Recruitment of Gab2 to the phagocytic cup is thought to be mediated by its PH domain [57]; however, we observed Gab2-GFP recruitment in the absence of detectable PI-3K activity. It has been shown that Gab2-GFP may also be recruited to the cup by its Grb2-binding site [57], which may represent the population of Gab2-GFP we observed at the cup in colchicine-treated cells. We also did not observe any effect of MT destabilization on Ras activity, which has been implicated in PI-3K regulation.

Localized PI-3K activity at the phagocytic cup has been documented in detail, and we showed here that it is dependent on intact MT cytoskeleton [6]. Recently, Xu and colleagues [58] reported that PI-3K activity, detected using AKT-PH-GFP at the leading edge of migrating neutrophils, is dependent on MTs. In monocyte-derived osteoclasts, attachment to bone coincides with a translocation of PI-3K to the cytoskeletal fraction, a movement that requires intact MTs [59]. Based on our immunofluorescence results, we speculate that MTs may function as a platform to tether PI-3K near its substrates/effectors. Our data support this as colchicine treatment-disrupted PI-3K activity at the cup, detected by the accumulation of AKT-PH-GFP, which binds to PI-3K products [6, 9]. Although we have no evidence to support direct binding of PI-3K to MTs, the binding of p85α to tubulin via its inter-SH2 domain has been reported [60]. In addition, the p55α and γ-regulatory proteins of PI-3K are capable of binding directly to tubulin in response to insulin in Chinese hamster ovary cells [61]. Our immunofluorescence results suggest that PI-3K is enriched in vesicles, which accumulate at the base and along pseudopods of the phagocytic cup (Fig. 9). It is feasible that MT-mediated transport delivers vesicles containing PI-3K to the sites of phagocytosis. This delivery may likely be driven by the MT (+)-end-directed motor, kinesin. Disrupting kinesin function has been shown to alter pseudopod activity in other cell types (reviewed in ref. [15]).

Here, we show an important role for MTs in membrane dynamics as an integral component of the elaborate signaling cascade involved in FcγR-mediated phagocytosis. Analogous to wortmannin effects on phagocytosis, we observed a particle size-dependent sensitivity of phagocytosis in the presence of MT-disrupting agents (Fig. 4). MTs have been implicated previously in localized membrane pseudopodia activity during cell spreading, although the precise function has not been deter-
mined to date. During frustrated phagocytosis, MT dynamics are required for pseudopod activity in spreading macrophages [62]. Similarly, cell polarization and directional pseudopodia activity in neutrophils [17] and T cells [63] requires the MT cytoskeleton. Our experiments with suspended RAW264.7 cells showed that the role of MT cytoskeleton in FcγR-mediated phagocytosis is independent of its well-established role in cell spreading.

Fig. 7. Colchicine reduces the phagocytic capacity of RAW264.7 cells exposed to large beads. RAW264.7 cells, treated with or without colchicines, were incubated with human, IgG-opsonized, polystyrene beads of 0.8 (A), 3.3 (B), or 8 (C) μm in diameter for 5 min to synchronize phagocytosis, followed by washing to remove unbound beads. Cells were incubated for another 25 min at 37°C to allow phagocytosis to occur. Internalized beads were visualized by DIC and counted to determine the phagocytic index. Beads, which were not internalized successfully, were labeled fluorescently with Cy3-anti-human IgG prior to fixation (A–C, insets) and not included in the phagocytic index. Arrowheads indicate external beads. Original bars, 10 μm. (D) Illustration of phagocytic indexes from three separate experiments showing mean and SEM from three independent experiments. *, Phagocytic indexes of these cells are significantly lower than control cells (P<0.05).

Fig. 8. Colchicine decreases pseudopod formation and extension in macrophages during FcγR-mediated phagocytosis. SEM analysis of surface membrane architecture of untreated (A) and colchicine-pretreated (B) RAW264.7 cells after 7 min of phagocytosis of IgG-sRBCs. Open arrowheads show bound sRBCs with no visible pseudopod membrane extension; closed arrowheads indicate the presence of membrane extension around the sRBCs. *, Bumps, which represent recently internalized sRBCs (A). Original bars, 10 μm. The percentage of bound sRBCs with pseudopods in 100 cells for each treatment is depicted (C). Data represent mean and SEM from three separate experiments. *, Percentage of pseudopod formation in these cells is significantly lower (P<0.05). (D) Phagocytic index of RAW264.7 cells grown in suspension or plated on coverslips for 6 h (spread). Control cells and cells pretreated with colchicine were challenged with sRBCs for 30 min. Data represent mean and SEM from three independent experiments. *, Phagocytic index in these cells are significantly lower (P<0.05).
There is important, clinical relevance to these findings. Colchicine has received widespread, acceptable use in diseases ranging from acute gouty arthritis and Familial Mediterranean Fever [64]. It has not been clear to date how colchicine exerts its anti-inflammatory actions. In acute gouty arthritis, it is speculated that administration of colchicine may decrease the inflammatory response of synovial phagocytes, which ingest the urate crystals, largely coated with IgG [65]. Similarly, the anti-MT agents, Vinca alkaloids, have achieved some success in treatments of Types II and III autoimmune diseases [66]. Therapeutic concentrations of these drugs have been shown experimentally to inhibit FcγR phagocytosis [26], suggesting that their applications may be extended to other phagocyte disorders. Our findings support this notion and provide a mechanism for MT function in phagocytosis.

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**Fig. 9.** Distribution of the PI-3K regulatory subunit, p85α, during phagocytosis depends on intact MTs. Immunofluorescent staining for p85α in untreated cells (A and B) and colchicine-treated cells (C and D) undergoing phagocytosis of 8 μm IgG beads for 7 min. Cells were stained for p85α (A and C), F-actin (B and D), and IgG beads (B and D, insets). Open arrowheads point to enrichment, large, vesicle-like staining of p85α at the base of the phagocytic cup, and closed arrowheads indicate p85α and F-actin staining along the pseudopod extensions. *Sites of bead attachment. Original bars, 10 μm. α-tub, α-Tubulin.**


