MTOC Reorientation Occurs during Fc γ R-mediated Phagocytosis in Macrophages[□] [▽]

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Cell polarization is essential for targeting signaling elements and organelles to active plasma membrane regions. In a few specialized cell types, cell polarity is enhanced by reorientation of the MTOC and associated organelles toward dynamic membrane sites. Phagocytosis is a highly polarized process whereby particles $>0.5\ \mu\text{m}$ are internalized at stimulated regions on the cell surface of macrophages. Here we provide detailed evidence that the MTOC reorients toward the site of particle internalization during phagocytosis. We visualized MTOC proximity to IgG-sRBCs in fixed RAW264.7 cells, during live cell imaging using fluorescent chimeras to label the MTOC and using frustrated phagocytosis assays. MTOC reorientation in macrophages is initiated by Fc γ R ligation and is complete within 1 h. Polarization of the MTOC toward the phagosome requires the MT cytoskeleton and dynein motor activity. cdc42, PI3K, and mPAR-6 are all important signaling molecules for MTOC reorientation during phagocytosis. MTOC reorientation was not essential for particle internalization or phagolysosome formation. However Golgi reorientation in concert with MTOC reorientation during phagocytosis implicates MTOC reorientation in antigen processing events in macrophages.

INTRODUCTION

Phagocytosis is a specialized mechanism for cells of the innate immune system to clear pathogens and dying cells from the body. Phagocytosis is initiated at the site of particle/pathogen attachment, creating a polarized region of activity within the macrophage. This process is a rapid, highly orchestrated event that recruits a multitude of signaling proteins, mobilizes organelles, and causes dramatic cytoskeletal rearrangements. Phagocytosis is initiated by ligation of Fc γ receptors to IgG-opsonins on the target cell. Engaged Fc γ Rs cluster at the site of particle contact, which in turn recruits Src, Syk, and PI3K (Greenberg and Grinstein, 2002). Local activation of cdc42 and rac1 initiates actin remodeling that coincides with the growth of membrane pseudopods (Greenberg and Grinstein, 2002). Within minutes, the particles are engulfed by the pseudopods into the cytoplasm as a membrane-bound phagosome. Particle contents within the phagosome are then degraded by fusion with the macrophage endocytic machinery. Phagolysosome formation is concomitant with retrograde translocation of the phagosome along the microtubule (MT) cytoskeleton

(Blocker *et al.*, 1997; Harrison and Grinstein, 2002; Harrison *et al.*, 2003). Over the next several hours, particulate antigens from the phagosome will bind to specialized antigen-presentation proteins that will become displayed on the cell surface to initiate the adaptive immune response. Exogenous antigens bind to Major Histocompatibility Complex II (MHCII) proteins, which are largely delivered to phagosomes from Golgi-derived endocytic organelles (Ramachandra and Harding, 2000). Macrophages are also capable of cross-presenting antigens that become complexed with MHC I molecules in the endoplasmic reticulum (ER), before surface presentation (Groothuis and Neefjes, 2005).

Rapid cell polarization also occurs in other immune cells including cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, which reorganize their cytoplasm to face bound antigen-presenting cells (APCs) or target cells for destruction. Cellular reorganization in these cells is characterized by movement of the MT organizing center (MTOC) and the Golgi to a site between the nucleus and the APC/target cell (Kupfer and Dennert, 1984). Movement of the MTOC in T-cells orients the Golgi complex for rapid delivery of cytokines (Kupfer *et al.*, 1991) and lytic granules (Kupfer *et al.*, 1985; Yannelli *et al.*, 1986; Podack and Kupfer, 1991) to the cell surface contact site with the APC/target cell. Recently, it was shown that MTOC contacts the plasma membrane in CTLs and this polarized movement is required to deliver lytic granules to the immunological synapse (Stinchcombe *et al.*, 2006).

MTOC reorientation also occurs during cell migration (Malech *et al.*, 1977; Gotlieb *et al.*, 1981; Kupfer *et al.*, 1982; Rubino *et al.*, 1984; Singer and Kupfer, 1986; Rogers *et al.*, 1992), which can be induced experimentally by wounding confluent cell monolayers (Kupfer *et al.*, 1982; Gotlieb *et al.*, 1983). In these assays, the MTOC, along with the Golgi complex, reorients to a position between the wound edge and the nucleus (Kupfer *et al.*, 1982). MTOC reorientation in wounded fibroblast monolayers is similarly involved in

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Abbreviations used: APC, antigen-presenting cell; DIC, differential interference contrast; GSK-3 β , glycogen synthase kinase 3 β ; MHC, major histocompatibility complex; MT, microtubule; MTOC, microtubule organizing center; PI3K, phosphatidylinositol 3-kinase; sRBC, sheep red blood cell; TIRFM, total internal reflection fluorescence microscopy.

plasma membrane events, because it is thought to optimize focal delivery of intracellular membranes to the leading lamellae (Bergmann *et al.*, 1983). Recently, extensive research has been undertaken to characterize upstream signaling regulation of MTOC reorientation in wound assays in fibroblasts and astrocytes (Etienne-Manneville and Hall, 2001, 2003; Palazzo *et al.*, 2001; Magdalena *et al.*, 2003; Stinchcombe *et al.*, 2006). Cell polarization toward the wound edge in fibroblasts involves both MT stabilization and MTOC reorientation, although these events are mechanistically distinct (Gundersen *et al.*, 2005). It is believed that the movement of the MTOC during migration is mediated by a cdc42-dependent capture and sliding mechanism involving microtubules and plasma membrane-bound dynein at the cell cortex (Palazzo *et al.*, 2001; Gundersen, 2002). In wounded astrocyte monolayers, cdc42 has been proposed to initiate MTOC reorientation by activating another key polarity protein, mammalian partition-defective 6 (mPAR-6) protein, which acts with atypical PKC ζ to regulate adenomatous polyposis coli association with MTs (Etienne-Manneville and Hall, 2001, 2003). In addition, it has been proposed that the rearward movement of the nucleus contributes to MTOC reorientation in migrating fibroblasts, with cdc42, actin, and myosin playing key roles in this mechanism (Gomes *et al.*, 2005).

Although local remodeling of the plasma membrane at the site of phagocytosis has been extensively studied, less is known about the intracellular reorganization of the cytoplasm during phagocytosis. Here we provide the first description of MTOC reorientation during phagocytosis in macrophages. We utilized three complementary techniques to assay MTOC reorientation during Fc γ R-mediated phagocytosis. Transfection of RAW264.7 cells with GFP-chimeric proteins and live fluorescent imaging were performed to visualize reorientation of the MTOC toward the phagosome and its coordinated movement with MT dynamics. Immunostaining of the MTOC during phagocytosis allowed quantification of cytoskeletal and signaling proteins contributions to MTOC reorientation. Finally, we used a "frustrated phagocytosis" assay to uncouple MTOC reorientation from retrograde phagosome transport. A combination of DIC, epifluorescent, and confocal microscopy technologies were used for a detailed analysis of key receptor, cytoskeletal, and signaling regulators involved in MTOC polarization during phagocytosis in macrophages.

MATERIALS AND METHODS

Cell Line and Reagents

RAW264.7 macrophages (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Wisent, St-Bruno, Quebec, Canada) containing 10% heat-inactivated fetal bovine serum (FBS, Wisent). RPMI 1640 containing 25 mM HEPES (HPMI) was also purchased from Wisent. Sheep RBCs (sRBCs) and rabbit anti-sheep sRBC IgG and IgM were obtained from ICN Biomedicals (Costa Mesa, CA). Polystyrene beads (0.8, 3.1, and 8 μ m) were purchased from Bangs Laboratories (Fishers, IN). Monoclonal anti-dynein (IC, clone 70.1) was obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Alexa Fluor 555 dye and Oregon Green 488-phalloidin were from Invitrogen (Burlington, Ontario, Canada). FuGENE-6 was purchased from Roche Diagnostics (Indianapolis, IN). Mouse monoclonal anti- γ -tubulin and anti-FLAG antibodies were from Sigma-Aldrich. Rabbit anti-pericentrin antibodies were from Covance (Madison, WI). Rat anti-LAMP-1 (ID4B) antibody was from the Developmental Studies Hybridoma Bank (Iowa City, IA). Cy2-, Cy3-, and Cy5-conjugated donkey anti-mouse, -rabbit, -rat, or -human IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA). All other reagents were purchased from Sigma-Aldrich.

Cell Transfection, Microinjection, and Inhibitor Treatments

Cells were transfected using FuGENE-6 and used for experimentation the following day. DNA constructs used were γ -tubulin-GFP, CLIP-170-head-

GFP, cdc42 N17-GFP, rac1 N17-GFP, FLAG-tagged mPAR-6, p50 dynamin-GFP, and luminal-GFP. For microinjection, cells were grown to 70–80% confluency on round coverslips and loaded into a microinjection chamber in HPMI. Microinjection was conducted using an Eppendorf FemtoJet injection system (Fremont, CA). A 1:1 mixture of monoclonal anti-dynein antibodies (1.2 mg/ml, clone 70.1, Sigma) and Alexa Fluor 555 (Invitrogen) was microinjected into RAW264.7 cells. Cells were returned to 37°C and incubated for 5–6 h before carrying out phagocytosis assays.

Where specified, 10 μ M colchicine or nocodazole was added to the cells 20 min before phagocytosis to depolymerize MTs, 2 μ M cytochalasin D was added 30 min before phagocytosis to inhibit actin polymerization, and 100 μ M LY294002 was administered to the cells 20 min before phagocytosis to prevent PI3K activity.

Assays for MTOC Reorientation during Phagocytosis

Live Fluorescent Assay. RAW264.7 cells were grown to 70–80% confluency in DMEM in Lab-TekII Chamber no. 1.5 Coverglass System wells (Nalge Nunc International, Naperville, IL), before transfection with γ -tubulin-GFP or CLIP-170-head-GFP. Lab-TekII chambers were loaded into a heat-regulated chamber (37°C) supplied with 5% CO₂. For Fc γ R-mediated phagocytosis, RAW cells were exposed to IgG-sRBCs, which were opsonized with a rabbit anti-sheep RBC IgG antibody as described (Harrison *et al.*, 2003). For Mac-1-mediated phagocytosis, RAW cells were serum starved for 2 h and then incubated with 100 nM PMA for 20 min before addition of C3bi-sRBCs. C3bi-sRBCs were opsonized with rabbit anti-sheep RBC IgM antibody and C5-deficient serum (Jongstra-Bilen *et al.*, 2003). Epifluorescent imaging was performed on an inverted Axiovert 200 microscope (Zeiss). Once sRBC contact was made, fluorescent and DIC images were acquired every 30 s to 2 min, for a period of ~20 min to 2.5 h.

Immunostaining Assay. Control and treated RAW264.7 cells were exposed to IgG-opsonized sRBCs or polystyrene beads (0.8, 3.1, or 8 μ m) opsonized with human IgG (Harrison *et al.*, 2003). Opsonized particles were added at a 1:5 ratio to the macrophages to minimize multiple particle contact with the macrophages. After a 5-min incubation at 37°C, the cells were vigorously washed with PBS to remove unbound particles and to synchronize phagocytosis. Phagocytosis was then allowed to proceed with cells in DMEM/FBS at 37°C, for 30 min, unless otherwise indicated.

Frustrated Phagocytosis Assay. Transfected and control RAW264.7 cells were grown on tissue culture flasks to 80% confluence before detachment by mechanical scraping. Cells were centrifuged at 1000 rpm for 5 min and resuspended in 1 ml HPMI and rotated for 4 h at room temperature to allow receptor recovery. Drug treatments were typically administered within the last 30 min of suspension. Cells, 500 μ l, were added to glass or human IgG-opsonized coverslips (Touret *et al.*, 2005) containing DMEM/FBS. After 5, 10, 15, 30, 45, or 60 min of plating on the coverslips, cells were washed and fixed with 4% paraformaldehyde/PBS. For control experiments, cells were plated onto glass or human IgG coverslips for 30 or 60 min and then incubated with IgG-opsonized beads for 30 min before fixation. For control experiments using suspension cells, RAW264.7 cells grown in suspension for 3 h were incubated with IgG-sRBCs for 30 min while rotating at room temperature. The cells were then plated onto human IgG-coated or glass coverslips and fixed after 30 or 60 min, respectively. Cells were then fixed and processed for immunofluorescence. Z-sections of the cells, 0.25- μ m-thick, were taken on a Zeiss LSM 510 META confocal microscope (Thornwood, NY). Z-sections were combined into a Z-stack, and XZ-sections of the Z-stack were reconstructed to visualize MTOC positioning in the cell.

Immunofluorescence

RAW264.7 cells were fixed with 4% paraformaldehyde/PBS. In instances where phagocytosis was experimentally inhibited, externally bound IgG beads or IgG-sRBCs were first stained with Cy5-conjugated anti-human or anti-rabbit IgG antibodies, respectively, before fixation. Cells were permeabilized using 0.1% Triton X-100/PBS containing 100 mM glycine for 20 min. After permeabilization, cells were washed and blocked with 5% FBS/PBS for 1 h. Cells were then incubated with primary antibodies in PBS and 1% FBS for 1 h. The primary antibody dilutions used were: γ -tubulin (1:5000), pericentrin (1:1000), FLAG (1:1000), LAMP-1 (1:2), and giantin (1:1000). Actin was stained with Oregon Green 488 phalloidin (1:500). All cells were stained with DAPI to visualize the nucleus. Internalized particles were stained with Cy2- or Cy3-conjugated anti-human or anti-rabbit IgG antibodies. Cells were washed and incubated with fluorescent secondary antibodies, before mounting and imaging with confocal or epifluorescence microscopy.

Quantification of Phagocytosis

The amount of phagocytic activity of the macrophages was determined by calculating the number of sRBCs internalized per macrophage. Measurements were done in triplicates ($n > 20$).

Analysis and Quantification of MTOC Reorientation

MTOC reorientation during phagocytosis was determined using a “zonal” classification system as outlined in Figure 2, A and D. For immunostained phagocytosis assays, the MTOC was scored according to its perinuclear location compared with the position of the internalized phagosome/bound particle (Figure 2A). The nucleus was partitioned into three zones that were established with lines (gray) that were perpendicular through the nucleus relative to the plane of the internalized phagosome/bound particle within or on the cell (dashed line; Figure 2A). If the MTOC was found adjacent to the back half of the nucleus, with respect to the phagosome/bound particle, it was considered *not oriented*. MTOCs that were located at the front of the nucleus facing the phagosome/bound particle were considered *fully oriented*, whereas MTOC located between these two zones were scored as *partially oriented* (Figure 2A). Only interphase, mononuclear RAW264.7 cells were analyzed. DAPI staining was utilized to visualize the nucleus. MTOC reorientation was only scored in cells with one phagosome/bound particle that was located within the same focal plane as the MTOC.

A similar zonal classification system was used for frustrated phagocytosis assays, with the human IgG-opsonized coverslip taking the place of the phagosome/bound particle (Figure 2D). The MTOC was determined to be *fully oriented* if it was located between the nucleus and the coverslip. The MTOC was scored as *not oriented* if it was located adjacent to the nucleus on the opposite half of the cell away from the site of frustrated phagocytosis. The MTOC was considered *partially oriented* if it was located between the *fully* and *not oriented* zones (Figure 2D).

For control frustrated phagocytosis experiments using plated or suspension cells, XZ-section images were reconstructed to visualize the MTOC position in the cell. To determine MTOC reorientation, MTOC reorientation was quantified based on the proximity of the MTOC with respect to the opsonized particle (IgG-bead or IgG-sRBC) compared with the proximity of the MTOC to the coverslip. The ratio (or percentage) of the number of cells having the MTOC closer to the opsonized particle than the coverslip compared with the number of cells having the MTOC closer to the coverslip than the opsonized particle was determined.

Statistical Analysis

All fully oriented MTOC data presented was tested for significance using a one-way analysis of variance (ANOVA, $\alpha = 0.05$). All quantification of phagocytosis was tested for significance using a Student's *t* test ($\alpha = 0.05$) against the control. All quantification involving MTOC proximity to the opsonized particle or coverslip was tested for significance using a Student's *t* test ($\alpha = 0.05$) against the IgG coverslip group. All experimental data were repeated in at least triplicates.

RESULTS

MTOC Reorientation Occurs during Phagocytosis in Macrophages

To investigate whether the polarized process of phagocytosis involves reorientation of the MTOC, we transfected RAW264.7 cells with GFP constructs to visualize the MTOC before induction of phagocytosis with IgG-sRBCs and live fluorescent imaging. The MTOC was visualized with γ -tubulin-GFP (Figure 1A and Supplementary Movie 1A). In Figure 1A, phagocytosis was initiated at the opposite side of the nucleus to where the γ -tubulin-GFP was located. The MTOC began reorienting 6 min after initial sRBC contact and internalization (Figure 1A and Supplementary Movie 1A). Full MTOC reorientation toward the phagosome occurred in 80 min. Although retrograde transport of the phagosome was observed, the bulk of the movement mediating phagosome/MTOC proximity came from reorientation of the MTOC (Figure 1A and Supplementary Movie 1A). On average, MTOC reorientation toward the site of the internalized particle occurred within 48 ± 8.6 min ($n = 13$).

To visualize MTs, we also did live fluorescent imaging of MTOC reorientation in RAW264.7 cells transfected with CLIP-170 head-GFP constructs (Figure 1B and Supplementary Movie 1B). Transfection of RAW264.7 cells with β -tubulin-GFP resulted in a high background of soluble β -tubulin-GFP that precluded imaging of cytoplasmic MTs during phagocytosis. CLIP-170-head-GFP consists of the N-terminal MT binding-domain of CLIP-170 (Komarova *et al.*, 2002), and expression of this construct did not inhibit phagocytosis

Figure 1. MTOC reorientation during phagocytosis in macrophages. (A and B) DIC and epifluorescent images of a RAW264.7 cell transfected with γ -tubulin-GFP (A) or CLIP-170-head-GFP (B). (See also Supplementary Movies 1A and 1B.) RAW264.7 cells were exposed to IgG-sRBCs, and time 0 represents time of sRBC attachment. Movement of γ -tubulin-GFP begins 6 min after sRBC contact (A) and directional MTOC movement toward the phagosome in CLIP-170-head-GFP-transfected cells starts at 5 min (B, cell on right) and 10 min (B, cell on left). Asterisk indicates the site of sRBC internalization in epifluorescent stills, arrows indicate MTs at the cell cortex at the site of phagocytosis, and arrowheads indicate the cortical MTs diagonal and posterior to the MTOC and phagosome. Scale bars, 10 μ m.

(Figure 1B and Supplementary Movie 1B). Furthermore, as CLIP-170-head-GFP uniformly labeled the cytoplasmic MTs, MT contact at the phagocytic cup and retrograde transport of the phagosomes along the MTs could be imaged simultaneously with MTOC reorientation. Figure 1B shows two CLIP-170-head-GFP-transfected cells undergoing phagocytosis. In both cells, MTs extend to the cell margin at the site of the phagocytic cup shortly after particle contact (Figure 1B and Supplementary Movie 1B). Movement of the MTOC occurs afterward, with a small tug of the MTOC toward the particle occurring in the cell where phagocytosis happened on the same side of the nucleus as the MTOC. In the other cell, where the MTOC was considered partially oriented at the time of phagocytosis, MTOC reorientation toward the phagocytosis occurs when MTs appear to be tethered at the cortex, at cortical sites in front of and behind the MTOC (Figure 1B and Supplementary Movie 1B).

To determine whether the observed MTOC phenomenon was strictly found in Fc γ receptor-mediated phagocytosis, live fluorescent imaging of MTOC reorientation in RAW264.7 cells transfected with γ -tubulin-GFP was carried out for Mac-1-mediated phagocytosis. No MTOC reorientation toward C3bi-opsonized sRBCs was evident, with only retrograde movement of the phagosome occurring during imaging (Supplementary Figure 3 and Supplementary Movie 3).

As live fluorescent analysis is limited to single cell assays and is prone to photobleaching, we used two other phagocytosis assays to document and quantify MTOC reorientation in macrophages. RAW264.7 cells were fed IgG-sRBCs and fixed at different time intervals, and the MTOCs were

Figure 2. Assays for MTOC reorientation during phagocytosis. (A) Cartoon illustration of MTOC reorientation during phagocytosis “zonal” scoring regime in immunostained cells, as described in *Materials and Methods*. Solid gray bars divide zones used to quantify MTOC reorientation during phagocytosis. (B) RAW264.7 cells were exposed to IgG-sRBCs and fixed at given time intervals. Cells were immunostained for γ -tubulin (red) and sRBC (green). (C) Quantification of MTOC reorientation during phagocytosis at the indicated time intervals. * $p < 0.05$ compared with 5 min. Data are mean $\pm$ SEM from three experiments ($n > 100$). (D) Illustration of MTOC reorientation scoring protocol for cells plated on glass or on IgG-coated coverslips to induce “frustrated phagocytosis” (see *Materials and Methods*). (E) Representative XY confocal images for RAW264.7 cells plated on glass or IgG-coated coverslips at 15 min and stained for γ -tubulin (red) and actin (green). (F and G) XZ confocal reconstructions of RAW264.7 cells plated on glass (F) or IgG-opsonized coverslips (G) for indicated time intervals. Cells were fixed and stained for γ -tubulin (red) and actin (green). (H) Quantification of MTOC reorientation in RAW264.7 cells plated on glass or IgG coverslips for indicated time intervals. * $p < 0.05$ compared with 10 min for glass and 5 min for IgG coverslips. Data are mean $\pm$ SEM from three experiments ($n > 30$). Scale bars, 10 μ m.

immunostained with either γ -tubulin or pericentrin antibodies (Figure 2B). MTOC reorientation in RAW264.7 cells that had ingested a single sRBC was scored according to the diagram in Figure 2A. When cells were challenged with sRBCs for 5 min and fixed and immunostained, 23.3% of cells had MTOC that were fully oriented, 26.1% of cells exhibited partially oriented MTOCs, whereas 50.6% of cells showed nonoriented MTOCs toward the sRBC (Figure 2C). Because sRBCs land randomly on the macrophages, 50% of the MTOCs would be not oriented toward particles, by chance alone. Within 30 min, 67.9% of cells had MTOCs that were fully polarized toward the phagosome (Figure 2, B and C). Interestingly, after 30 min of phagocytosis, γ -tubulin staining adjacent to sRBC was often more diffuse, compared with cells at earlier time points or those not undergoing phagocytosis, which had a more bright, compact γ -tubulin staining (Figure 2B).

Inward movement of the phagosome along MTs has been carefully studied by numerous groups (Blocker *et al.*, 1997; Harrison *et al.*, 2003). Because our fixed MTOC immunostaining assay during phagocytosis cannot tease out the contribution of MTOC reorientation from retrograde MT transport of phagosomes, we also used a frustrated phagocytosis assay for detailed analyses of MTOC reorientation in RAW264.7 cells. Briefly, suspended RAW264.7 cells were

plated on IgG-coated or unopsonized glass coverslips for different times and fixed and stained for the MTOC. Macrophages will bind to IgG-coated coverslips and attempt to ingest the coverslip by extending radial pseudopods, which cause spreading along the coverslip (Rabinovitch *et al.*, 1975; Michl *et al.*, 1979; Cannon and Swanson, 1992; Marshall *et al.*, 2001; Touret *et al.*, 2005). After 15 min of attachment to IgG coverslips, RAW264.7 cells were spread and engaged in frustrated phagocytosis as characterized by broad actin rings (Figure 2E; Jongstra-Bilen *et al.*, 2003). Cells were typically rounder and devoid of actin rings when plated on glass coverslips for 15 min (Figure 2E). MTOCs were scored as fully oriented if γ -tubulin staining was observed at the bottom of the nucleus, facing the coverslip, on XZ-reconstructed confocal images (see Figure 2D, *Materials and Methods*). After 5 min, only 29.1% of cells plated on IgG coverslips had fully oriented MTOCs toward the coverslip (Figure 2, G and H). Very few cells adhered to glass coverslips at this time point, so MTOC reorientation was not quantified in these cells. For cells plated on IgG coverslips for 10 min, 55.1% had MTOCs that were polarized to a basal region of the cell, whereas even after 15 min, only 44.4% of cells plated on glass had MTOCs facing the opsonized coverslip (Figure 2, F–H). An extended time-course analysis revealed that only after 1 h of plating did the majority of cells plated on

Figure 3. MTs are required for MTOC reorientation during phagocytosis and actin is necessary for MTOC reorientation toward smaller particles. (A) Immunostaining of MTOC in RAW264.7 cells transfected with rac1 N17-GFP (blue, inset), or treated with 10 μ M nocodazole, 10 μ M colchicine, or 2 μ M cytochalasin D. After 30 min of exposure to IgG-sRBCs or large 8- μ m IgG-beads, cells were fixed and immunostained for γ -tubulin (red) and IgG-sRBCs (green). Cells transfected with rac1 N17-GFP served as a control for MTOC reorientation in cells where particle internalization is inhibited. Arrow indicates bound sRBC location on RAW264.7 cells. (B) XZ confocal reconstructions of untreated RAW264.7 cells and cells treated with MT and actin inhibitors, before plating on IgG coverslips for 15 min. (C) MTOC reorientation shown in A was quantified with respect to single, bound sRBCs in RAW264.7 cells, in addition to the quantification of MTOC reorientation in cytochalasin D-treated cells exposed to large 8- μ m IgG-beads. * p < 0.05 compared with rac1 N17-GFP cells. Data are mean $\pm$ SEM from three replicate experiments (n > 30). (D) Quantification of MTOC reorientation during frustrated phagocytosis in control (untreated) cells, or nocodazole-, colchicine- or cytochalasin D-treated RAW264.7 cells. * p < 0.05 compared with control. Data are mean $\pm$ SEM from three replicate experiments (n > 30). Scale bars, 10 μ m.

glass coverslips exhibit fully oriented MTOCs (Supplementary Figure 1A). When cells plated on glass coverslips for 1 h were exposed to IgG-opsonized particles, the MTOCs were largely reoriented toward the particle, not the glass coverslip (Supplementary Figure 1, B and C). A similar preference of MTOC reorientation toward the IgG-sRBC versus the coverslip was observed when RAW264.7 cells grown in suspension were fed IgG-sRBCs, before plating on glass for 1 h (Supplementary Figure 1, D and E). These studies indicate that Fc γ R-mediated-signaling is the predominant motivating force behind MTOC reorientation in phagocytosis and frustrated phagocytosis assays. In many XZ images of RAW264.7 cells on IgG coverslips, fully oriented MTOC appeared to be at the very base of the cell, although the MTOC did not approach the plasma membrane within TIRF microscopy resolution, in γ -tubulin-GFP-transfected cells (not shown). The majority of cells (64.3%) plated on IgG-coated coverslips for 15 min showed fully oriented MTOCs toward the coverslip (Figure 2, G and H).

MTOC Reorientation during Phagocytosis Requires MTs but Large Particles Do Not Require F-Actin

Our live fluorescent imaging studies showed that MTs grow into the actin-rich phagocytic cup before MTOC reorientation. To show a definitive role for MTs and actin in mediating MTOC reorientation during phagocytosis we pretreated the cells with 10 μ M nocodazole, 10 μ M colchicine, or 2 μ M cytochalasin D before phagocytosis assays. Depolymerization of MTs and F-actin inhibits particle internalization (Walter *et al.*, 1980; Athlin *et al.*, 1986; Koval *et al.*, 1998; Tse *et al.*, 2003), so for these studies we scored MTOC reorientation with respect to bound particles (see Figure 2A). MTOCs were scored as fully oriented if they were located between the nucleus and bound particles. MTOCs were only tabulated in macrophages bound to a single particle. Because untreated macrophages readily internalize particles, we used cells transfected with dominant negative rac1 N17-GFP constructs as a positive control. rac1 N17 constructs

inhibit particle internalization (Caron and Hall, 1998); however, dominant negative rac1 constructs do not interfere with MTOC reorientation in wounded cell culture monolayer assays (Etienne-Manneville and Hall, 2001; Palazzo *et al.*, 2001). Most of the RAW264.7 cells transfected with rac1 N17-GFP had fully oriented MTOCs toward the bound particle (Figure 3, A and C). IgG-sRBCs were not internalized in nocodazole- or colchicine-treated cells and only 16.6% and 18.8% of cells had MTOCs that were fully oriented toward the site of bound particles, respectively (Figure 3, A and C). The requirement for intact MTs during MTOC reorientation in frustrated phagocytosis was also analyzed. Pretreatment of RAW264.7 cell with nocodazole or colchicine did not affect cell spreading on IgG-coated coverslips (Figure 3B). However, MTOC reorientation toward the opsonized coverslip was reduced in both nocodazole- and colchicine-treated cells, compared with control, untreated cells plated on IgG coverslips for 15 min (Figure 3, B and D).

Actin is not required for MTOC reorientation in endothelial cells, T-cells, fibroblasts, and astrocytes (Gotlieb *et al.*, 1983; Nemere *et al.*, 1985; Etienne-Manneville and Hall, 2001; Palazzo *et al.*, 2001). Disruption of the actin cytoskeleton with cytochalasin D led to a reduction in the amount of MTOC reorientation toward bound sRBC particles (Figure 3, A and C). However, interestingly, although cytochalasin D completely inhibited cell spreading on IgG-coated coverslips, 54.7% of cells had MTOC that were fully oriented toward the opsonized substrate after 15 min of plating (Figure 3, B and D). The discrepancy between the results from the two assays may be due to differences in assay type or the level of Fc γ R signaling. To enhance IgG ligand in the fixed phagocytosis assay, we exposed RAW264.7 cells treated with cytochalasin D to large 8- μ m IgG beads for 30 min. Nearly 63% of cytochalasin D-treated cells showed fully oriented MTOCs toward the bound particles, similar to the results observed in the frustrated phagocytosis assay (Figure 3C).

Figure 4. Dynein is essential for polarization of the MTOC during phagocytosis. (A) Immunofluorescence and DIC images of RAW264.7 cells microinjected with monoclonal anti-dynein (clone 70.1) antibodies or transfected with p50 dynamitin-GFP (insets show microinjected and transfected cell, respectively). Cells were exposed to IgG-beads or IgG-sRBCs for 30 min and fixed and immunostained for pericentrin and γ -tubulin, respectively (top panels). Before fixation, external sRBCs were lysed with water, and external beads were immunostained to differentiate between bound versus internalized particles. Arrows indicate location of internalized IgG-bead/sRBCs. (B) Quantification of MTOC reorientation toward the IgG-bead/sRBC from three replicate experiments. * $p < 0.05$ compared with control. Data are mean $\pm$ SEM from three experiments ($n > 15$). (C and D) XZ confocal reconstructions of nontransfected RAW264.7 cells (C) and cells transfected with p50 dynamitin-GFP (D) after plating on IgG coverslips for 15 min and immunostaining for γ -tubulin (C and D, bottom panel). (E) Quantification of MTOC reorientation during frustrated phagocytosis in control (nontransfected) cells or p50 dynamitin-GFP transfected RAW264.7 cells. * $p < 0.05$ compared with control. Data are mean $\pm$ SEM from three experiments ($n > 15$). Scale bars, 10 μ m.

Dynein Is Necessary for MTOC Reorientation during Phagocytosis

The capture-sliding mechanism for MTOC reorientation suggests that dynein/dynactin complexes are bound to the plasma membrane and associate with MT (+) ends at the cell cortex (Gundersen, 2002; Kuhn and Poenie, 2002; Combs *et al.*, 2006). We first examined whether dynein inhibition affects phagocytosis by overexpressing p50 dynamitin-GFP in RAW 264.7 cells and examining the number of sRBCs internalized per macrophage. Phagocytosis was not suppressed in cells overexpressing p50 dynamitin-GFP (Supplementary Figure 2A). We examined the requirement for dynein in MTOC reorientation during phagocytosis in macrophages by both overexpressing p50 dynamitin-GFP and microinjecting RAW264.7 cells with a dynein-blocking antibody (mAb 70.1; Echeverri *et al.*, 1996; Burkhardt *et al.*, 1997; Leopold *et al.*, 2000). Figure 4A shows a representative image of a cell microinjected with anti-dynein mAb 70.1, followed by phagocytosis of IgG beads and immunostaining for pericentrin. Although phagocytosis is not inhibited, there is a marked reduction in cells displaying fully oriented MTOCs toward phagosomes in mAb 70.1-microinjected cells (Figure 4B). Similarly, overexpression of p50 dynamitin-GFP suppressed MTOC reorientation, visualized with anti- γ -tubulin antibodies, toward internalized sRBCs (Figure 4, A and B). Inhibition of MTOC reorientation was also observed in p50 dynamitin-GFP-transfected cells using frustrated phagocytosis assays (Figure 4, D and E). Although the majority of control, untransfected cells had fully oriented MTOCs after 15 min of plating on IgG-coated coverslips (Figure 4, C and E), only 16.2% of cells overexpressing

dynamitin-GFP showed fully oriented MTOCs toward the IgG coverslips at the same time point (Figure 4, D and E).

Signaling Molecules Involved in MTOC Reorientation during Phagocytosis

Recent initiatives have unveiled numerous signaling molecules involved in MTOC reorientation. One such molecule is the Rho family GTPase, cdc42, which is a diverse signal transducer and a central player in cell polarity including MTOC reorientation (Palazzo *et al.*, 2001). Expression of dominant negative cdc42 constructs in macrophages potentially inhibits Fc γ R-mediated phagocytosis (Caron and Hall, 1998). A representative image of the typical positioning of the MTOC with respect to a bound sRBC in cdc42 N17-GFP-transfected cells is shown in Figure 5A. Quantitative analysis of MTOC reorientation using immunostaining shows a significant decline in the percentage of cells with fully oriented MTOCs toward the site of bound sRBCs in cells transfected with cdc42 N17-GFP, compared with rac1 N17-GFP-transfected cells (Figure 5C).

PI3K activity is essential for MTOC reorientation in T-cells (Stowers *et al.*, 1995). Pretreatment of cells with LY294002 before phagocytosis inhibited internalization of particles (Figure 5A), in accordance with previous studies (Cox *et al.*, 1999). The percentage of LY294002 treated cells that exhibited fully oriented MTOCs toward the site of bound particles was greatly reduced compared with rac1 N17-GFP-transfected cells (Figure 5, A and C). Overexpression of a full-length mPAR-6-FLAG-tagged construct and pericentrin immunostaining after 30 min of phagocytosis of IgG beads is shown in Figure 5A. mPAR-6-FLAG overexpression did not

Figure 5. MTOC reorientation during phagocytosis requires cdc42, PI3K and mPAR-6. (A) Fluorescent images of RAW264.7 cells transfected with cdc42 N17-GFP (green, inset) or mPAR-6-FLAG (green) constructs, or treated with 100 μ M LY294002. After 30 min of exposure to IgG-sRBCs or IgG-beads (DIC, inset), cells were fixed and immunostained for pericentrin (red, transfected cells) or γ -tubulin (red, nontransfected cells) and sRBCs (blue). Arrow indicates internalized IgG-bead location within the cell. (B) XZ confocal reconstructions of untreated RAW264.7 cells, or cells transfected with cdc42 N17-GFP or mPAR-6-FLAG, or treated with 100 μ M LY294002 before plating on IgG coverslips for 15 min. (C) MTOC reorientation was quantified with respect to single, bound, or internalized IgG-sRBCs/beads in RAW264.7 cells. * $p < 0.05$ compared with control. Data are mean $\pm$ SEM from three replicate experiments ($n > 30$). (D) Quantification of MTOC reorientation during frustrated phagocytosis in control (untreated) cells, LY294002-treated, or cdc42 N17-GFP-, or mPAR-6-FLAG-transfected RAW264.7 cells. * $p < 0.05$ compared with control. Data are mean $\pm$ SEM from three replicate experiments ($n > 30$). Scale bars, 10 μ m.

inhibit phagocytosis or retrograde movement of particles (Supplementary Figure 2, B and C), but a reduced percentage of cells exhibited MTOCs completely facing the phagosome compared with control, untransfected cells (Figure 5, A and C).

Frustrated phagocytosis assays were also used to access the contribution of cdc42, PI3K, and mPAR-6 on MTOC reorientation toward IgG-coated coverslips. RAW264.7 cells were transfected with cdc42 N17-GFP or mPAR-6-FLAG or pretreated with LY294002 before plating on IgG-coated coverslips for 15 min. Representative XZ confocal reconstructions of treated cells and control cells are shown in Figure 5B. Only 7.2% of cdc42 N17-GFP-transfected cells had fully oriented MTOCs after plating on IgG-coated coverslips for 15 min (Figure 5, B and D), compared with 64.3% of control, untreated cells (Figure 5, B and D). cdc42 N17-GFP expression also inhibited cell spreading on IgG coverslips (Figure 5B). Inhibition of PI3K with LY294002 inhibited both cell spreading and MTOC reorientation toward IgG-coated coverslips compared with control, untransfected cells plated on IgG-coated coverslips for 15 min (Figure 5, B and D). mPAR-6-FLAG overexpression in RAW264.7 cells reduced cell

Figure 6. MTOC reorientation is not required for phagolysosome formation. Fluorescent images of RAW264.7 cells immunostained for LAMP-1 and γ -tubulin after 10 (A) and 30 min (B) of phagocytosis of IgG-sRBCs. Arrows indicate LAMP-1 accumulation around phagosomes. Arrowheads indicate sRBC fragments that are toward the MTOC, detected with anti-rabbit antibodies. Scale bars, 10 μ m.

spreading on IgG coverslips and reorientation of the MTOC to the site of coverslip contact (Figure 5B). Quantitative analysis of MTOC reorientation revealed that only 18.7% of cells overexpressing mPAR-6-FLAG had fully oriented MTOCs toward the IgG coverslips after 15 min of plating (Figure 5D).

MTOC Reorientation Is Not Required for Phagolysosome Formation

After establishing key regulators of MTOC reorientation, we proceeded to examine its possible role(s) during phagocytosis. Because the bulk of MTOC reorientation occurs post-particle internalization (see Figures 1 and 2), it is likely not involved in phagocytic cup elaboration. We addressed whether maturation of the phagosome requires reorientation of the MTOC. To assess whether MTOC reorientation is required for or facilitates phagolysosome formation, we monitored the acquisition of LAMP-1 on phagosomes at early time points of phagocytosis. LAMP-1 and γ -tubulin immunostaining was performed on RAW264.7 cells after 10 and 30 min of phagocytosis (Figure 6, A and B). After 10 min of phagocytosis, phagosomes had already accumulated visible amounts of LAMP-1, even in cells where γ -tubulin staining revealed MTOCs that were not reoriented toward the phagosome (Figure 6A). Quantification of LAMP-1 acquisition in phagosomes of RAW 264.7 cells after exposure to IgG-sRBCs for 10 min for the three zonal classification categories of MTOC reorientation revealed that >90% of the cells had acquired LAMP-1, regardless of the positioning of the MTOC ($n > 40$, data not shown). LAMP-1 positive phagosomes were tightly adjacent to the MTOC, after 30 min of phagocytosis of IgG-sRBCs (Figure 6B). Interestingly, staining for sRBCs frequently revealed minute sRBC fragments that were ahead of the phagosome, in close proximity to the MTOC (Figure 6, A and B).

The Golgi Complex Is Targeted toward Phagosomes

Our observation that small phagosomal fragments were directed toward the MTOC prompted us to look at the role of MTOC reorientation in facilitating phagosome interactions with the Golgi, a key organelle in antigen presentation. We

Figure 7. Golgi polarization occurs toward the phagosome. (A) RAW264.7 cells were exposed to IgG-beads (asterisk, and DIC image in insets) and fixed at given time intervals. Cells were immunostained for pericentrin (red) and giantin (green). Arrow indicates Golgi tubules extending toward the phagosome, and the arrowhead indicates Golgi tubules moving over the nucleus toward the phagosome. (B) Epifluorescent and DIC stills of a RAW264.7 cell transfected with luminal-GFP to label the Golgi, undergoing phagocytosis of an IgG-sRBC. Times are indicated from the time of particle contact. Asterisk marks initial site of bound sRBC. Scale bars, 10 μ m.

did phagocytosis immunostaining assays in RAW264.7 cells and imaged the MTOC and Golgi at fixed time intervals during phagocytosis (Figure 7A). After 5 min of phagocytosis, giantin staining showed central Golgi clustering around the MTOC, at a distance from the internalized IgG-bead (Figure 7A). After 15 min of phagocytosis, both the MTOC and Golgi are closer to the phagosome, and Golgi tubules precede the MTOC, occasionally extending over the nucleus toward the phagosome (Figure 7A). Both the Golgi and the MTOC were fully oriented toward phagosomes, after 30 min of phagocytosis of IgG beads (Figure 7A).

To more fully understand Golgi dynamics during phagocytosis, we used live fluorescent imaging of phagocytosis in RAW264.7 cells transfected with luminal-GFP (Figure 7B). Luminal-GFP labels the Golgi and Golgi-derived vesicles (Gromley *et al.*, 2005). Along with bulk Golgi reorientation toward the internalized sRBC, Golgi tubules were observed to extend toward and around the sides of phagosomes (Figure 7B). These findings give compelling evidence for a close spatial relationship between the Golgi and the phagosome.

DISCUSSION

The rapid and dramatic cellular rearrangements that occur at the plasma membrane during phagocytosis make it an ideal system to study cell polarization. Here we describe for the first time that MTOC reorientation occurs during Fc γ R-mediated phagocytosis in macrophages. We documented MTOC polarization during phagocytosis using three complementary approaches. Full polarization of the MTOC from the most distal region of the cell to sites of particle internalization took 48.1 min on average, using live fluorescent

imaging. MTOC reorientation was faster in cells that were fixed and stained for γ -tubulin and during frustrated phagocytosis (where 60% of the cells showed fully reoriented MTOCs at 27.3 and 12.4 min, respectively). Fc γ R engagement to IgG appears to motivate MTOC reorientation in macrophages. MTOC reorientation was not observed during Mac-1-mediated phagocytosis. In addition, MTOC reorientation is slower and less frequent in cells plated on uncoated glass coverslips, compared with cells plated on IgG-coated coverslips. Frustrated phagocytosis on IgG-coated coverslips provides ligand for countless Fc γ receptors and this robust stimulus may explain the accelerated reorientation of the MTOC using this assay, compared with the MTOC immunostaining and live fluorescent imaging assays. MTOC reorientation occurs within 2 h in wounded fibroblast monolayers and within 8 h of wounding in astrocyte cultures (Kupfer *et al.*, 1982; Etienne-Manneville and Hall, 2001; Palazzo *et al.*, 2001). MTOC reorientation is relatively quick during phagocytosis and more similar to MTOC reorientation in T-cells, which occurs within 30 min (Kupfer and Dennert, 1984; Stowers *et al.*, 1995; Lowin-Kropf *et al.*, 1998). The acute responses required by macrophages and T-cells during the immune response may explain their capacity for rapid MTOC polarization. The cytoskeletal mechanics of MTOC mobilization may also be easier in the smaller immune cells (with cell diameters of $\approx$ 30–40 μ m) versus larger cells such as fibroblasts that can span 80 μ m in diameter.

MTOC reorientation during Fc γ R-mediated phagocytosis requires intact MTs and the MT motor dynein. Disruption of the MT cytoskeleton in RAW264.7 cells with colchicine or nocodazole inhibited MTOC reorientation but not spreading in frustrated phagocytosis assays. Conversely, the inhibition of actin polymerization by cytochalasin D inhibited cell spreading on IgG coverslips, whereas MTOC reorientation proceeded normally. Together, these results suggest that cell spreading and MTOC reorientation during frustrated phagocytosis are independent events. MTOC reorientation was reduced in cytochalasin D-treated cells during our fixed phagocytosis assay using 3- μ m sRBCs, yet occurred normally when large, 8- μ m beads, were used for the assay. This, combined with our frustrated phagocytosis result, suggests that actin is not required for MTOC reorientation during phagocytosis of large particles, but is necessary for MTOC reorientation toward smaller particles, perhaps because of the reduced level of Fc γ R-signaling. Live imaging of RAW264.7 cells transfected with CLIP-170-head-GFP showed MTs penetrating the region of the phagocytic cup at the time of particle internalization. MTs persisted in this region for several minutes, suggesting that they may be captured at cortical regions. Dynein is required for MTOC reorientation during phagocytosis and plasma membrane-bound dynein may serve as a mechanism for tethering MTs to the cell cortex and pulling the MTOC toward the site of phagocytosis. Interestingly, 10 min after particle internalization, cortical MTs were observed posterior regions of the cell at the time of MTOC reorientation. This suggests that undetermined (+)-end directed “pushing” forces may also contribute to MTOC reorientation during phagocytosis. The mechanism for MT cortical capture in macrophages is not known. Fukata and colleagues showed that MT(+) ends associate with actin via CLIP-170-IQGAP1 interactions (Fukata *et al.*, 2002). IQGAP1 is a cdc42/rac1 effector, and the strong requirement of cdc42 for MTOC reorientation during phagocytosis hints that a similar mechanism may drive MT-cortical associations during phagocytosis.

Our signaling analysis also revealed that PI3K was a necessary regulator of MTOC reorientation in addition to its

Figure 8. A model of MTOC reorientation during FcγR-phagocytosis in macrophages. Shown in the illustration are the major receptor, cytoskeletal and intracellular signaling regulators of MTOC reorientation during phagocytosis. MTOC reorientation during phagocytosis is proposed to accelerate interactions of the phagosome with the Golgi complex.

established role in particle internalization (Cox *et al.*, 1999). Although both cdc42 and PI3K have a dual role in particle internalization and MTOC reorientation in macrophages, our research indicates that these events are mutually exclusive. Together with cdc42 and PI3K, we observed that mPAR-6 is an important contributor to MTOC reorientation during phagocytosis. Despite the fact that phagocytosis is a highly polarized event, this is the first documentation of a PAR protein involvement in macrophage function. It will be of interest to determine whether FcγR-stimulation directly activates mPAR-6 and how its activities are coordinated with cdc42 and PI3K to mobilize the centrosome during phagocytosis. A summary of required receptor and signaling events for MTOC reorientation during phagocytosis is depicted in Figure 8.

What is the role of MTOC reorientation during phagocytosis? The timing of reorientation of the MTOC suggests it is too slow to contribute to plasma membrane remodeling events for particle internalization. MTOC reorientation occurred during frustrated phagocytosis on IgG-coated coverslips, where target uptake is impossible. Furthermore, rac1 N17 expression in RAW264.7 cells impaired phagocytosis, yet MTOC reorientation toward the bound particle occurred normally, indicating that reorientation of the centrosome occurs independently of particle internalization. MTOC polarization may focus polymerization of MTs toward nascent phagosomes to mediate their retrograde translocation. However, MT penetration at the phagocytic cup preceded MTOC reorientation suggesting that MT targeting to the cup is upstream of MTOC polarization during phagocytosis.

The delayed reorientation of the MTOC during phagocytosis indicates that it may be involved in polarized intracellular events. There was no observable defect in LAMP acquisition on phagosomes in cells where MTOCs were located at a distance from the phagosome. Although lysosomes in RAW264.7 cells are often clustered around the MTOC, subpopulations of lysosomes remain dispersed throughout the cell and likely account for lysosomes that fuse with phagosomes in the absence of reoriented MTOCs. The retrograde movement of phagosomes along MTs mediates phagolysosome formation (Harrison *et al.*, 2003) and appears to be sufficient for this early maturation event, regardless of the location of the MTOC.

Comigration of the Golgi complex with the MTOC was evident during phagocytosis. Golgi tubules were frequently observed extending toward the phagosome in live fluorescent imaging analysis. This supports studies of phagocytosis in *Dictyostelium*, where an interaction of Golgi tubules with early phagosomes occurred within 5 min of internalization (Gerisch *et al.*, 2004). We did not see direct fusion of Golgi tubules with the phagosomes during our epifluorescent imaging. This agrees with previous quantitative epifluorescence analysis of organelle contributions to the phagosome, where the Golgi was not a detectable component of the phagosomal membrane (Henry *et al.*, 2004). Our phagolysosome studies also argue against a role for MTOC reorientation in mediating fusion of Golgi-derived MHC II endocytic organelles with the phagosomes. Instead, Golgi reorientation may facilitate movement of antigenic particles toward the Golgi for antigen cross-presentation. Several models have been proposed for MHC I loading of antigenic peptides in the ER. One proposal is that the ER directly engulfs the particle, thereby allowing direct loading of exogenous peptides with MHC I molecules (Gagnon *et al.*, 2002). More recently, it was suggested that internalized antigens move in a retrograde direction through the Golgi into the ER (Ackerman *et al.*, 2005). Accumulation of particulate antigens has been observed in the *trans*-Golgi in macrophages and dendritic cells and this movement requires MTs (Rao *et al.*, 1997; Peachman *et al.*, 2004). Our findings support these latter studies and provide microscopic evidence for a close phagosome/Golgi interaction. Additional work is needed to directly assess the role of MTOC/Golgi reorientation in cross-presentation and MTOC reorientation toward bacteria phagosomes that are capable of circumventing antigen-processing machinery in macrophages.

Directional reorientation of the MTOC/Golgi toward the phagosome will obviously accelerate interactions between the phagosome and the Golgi over retrograde transport of the phagosome alone. Although phagocytosis can occur on multiple regions of a macrophage surface, this mechanism likely evolved to tackle the intracellular processing of a single particle or a large cell, e.g., an apoptotic or infected cell. MTOC reorientation is thought to enable delivery of vesicles to active plasma membrane regions in T-cells and migrating fibroblasts; however, MTOC reorientation in

phagocytosis appears important for intracellular events. This is the first description of a role for MTOC reorientation in spatially driving two organelles together, namely the phagosome and the Golgi network.

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