Kinesin 5B Is Necessary for Delivery of Membrane and Receptors during Fcγ R-Mediated Phagocytosis

Kristen E. Silver and Rene E. Harrison

*J Immunol* published online 13 December 2010
http://www.jimmunol.org/content/early/2010/12/11/jimmunol.1002161

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/12/13/jimmunol.1002161.1.DC1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Kinesin 5B Is Necessary for Delivery of Membrane and Receptors during FcγR-Mediated Phagocytosis

Kristen E. Silver and Rene E. Harrison

FcγR-mediated phagocytosis is a cellular event that is evolutionary conserved to digest IgG-opsonized pathogens. Pseudopod formation during phagocytosis is a limiting step in managing the uptake of particles, and in this paper, we show that the conventional kinesin is involved in both receptor and membrane delivery to the phagocytic cup. Expression of a mutant kinesin isoform (GFP dominant negative mutant of kinesin H chain [EGFP-Kif5B-DN]) in RAW264.7 cells significantly reduced binding of IgG–sheep RBCs when macrophages were faced with multiple encounters with opsonized particles. Scanning electron microscopy analysis of EGFP-Kif5B-DN–expressing cells challenged with two rounds of IgG–sheep RBCs showed sparse, extremely thin pseudopods. We saw disrupted Rab11 trafficking to the phagocytic cup in EGFP-Kif5B-DN–transfected cells. Our particle overload assays also implicated phagosome membrane recycling in pseudopod formation. We observed reduced phagosome fission and trafficking in mutant kinesin-expressing cells, as well as reduced cell surface expression of FcγRs and Mac-1 receptors. In conclusion, anterograde trafficking via kinesin is essential for both receptor recycling from the phagosome and delivery of Rab11-containing membrane stores to effect broad and functional pseudopods during FcγR-mediated phagocytosis. The Journal of Immunology, 2011, 186: 000–000.

P

hagocytosis is a cellular process that is evolutionary conserved and used by a variety of cell types including the hematopoietic cell lineage to digest pathogens, apoptotic cells, and necrotic material (1). Phagocytosis has been largely studied in macrophages and involves a complex set of sequential events initiating when a ligand binds to the surface receptor FcγR, in the case of FcγR-mediated phagocytosis. The FcγR is the best-studied receptor involved in phagocytosis, and it recognizes particles opsonized with IgG (2). Cellular events that are triggered after binding include clustering of receptors, kinase activation, phospholipid alterations, and trafficking of endomembrane components to the site of particle attachment (3). These localized cortical events cause membrane protrusions called pseudopods that encircle opsonized particles (4). Concurrently, actin cytoskeleton remodeling and polymerization results in the formation of an actin cup that supports and surrounds the IgG particle. The end result of actin cup and pseudopod formation is fusion of the pseudopod tips, releasing the particle into an intracellular membrane-bound organelle, the phagosome. The nascent phagosome is located near the cell periphery, on which the phagosome matures through a series of fusion events with the endocytic pathway (5). Maturation terminates in a hybrid organelle called the phagolysosome, which includes a low pH, a number of hydrolytic enzymes, and toxic oxidative compounds, which makes it an ideal environment for the digestion of the particle. Transport through the microtubule (MT) motor dynein moves the phagosome centripetally toward the perinuclear region where fusion with lysosomes occurs (6, 7).

Phagocytosis of large and/or multiple particles requires delivery of intracellular membrane sources to the site of growing pseudopods; this process has been coined “focal exocytosis” (8). Early endomembranes such as recycling endosomes bearing Rab11 and late endosomes containing vesicle-associated membrane protein 7 (VAMP7) are key players in focal exocytosis by providing membrane materials to the localized site of particle attachment and increasing the surface area for localized growth during pseudopod formation (9, 10). The main function of focal exocytosis is to minimize plasma membrane loss during the formation of the nascent phagosome. The cell surface increases during phagocytosis; therefore, there must be a compensation for any loss of plasma membrane through the recruitment of vesicles (8).

MT motors mediate long-range vesicle trafficking through the cell. MT accumulation within F-actin–rich pseudopods has triggered studies that aim to determine a role for MTs at the site of particle-membrane engagement during phagocytosis (11, 12). It is well-known that pseudopodia activity in neutrophils and T cells is highly dependent on MTs (13, 14). Also, drugs such as colchicine and nocodazole, which depolymerize MTs, result in suppressed phagocytosis because of a reduction in pseudopod formation and extension (11). To date, no results are available regarding the role of the anterograde MT motor, kinesin, during phagocytosis.

For this study, we used a mouse macrophage cell line, RAW264.7 expressing a GFP dominant negative mutant of kinesin H chain (EGFP-Kif5B-DN), to test the hypothesis that kinesin is necessary for efficient phagocytosis. We chose to study Kif5B because our recent MT proteome screen identified Kif5B as the most abundant kinesin bound to MTs in macrophages (15). In this study, we have analyzed in detail the role of kinesin in the early events of phago-
cytosis before particle internalization. We focused our studies on visualizing pseudopod formation, the delivery of endomembranes to the localized site of particle–membrane engagement, and the recycling of phagosome membrane components such as the FcγR to the cell surface through the use of both fixed and live cell imaging techniques. The results from this study show that conventional kinesin Kif5B is the major MT motor involved in trafficking materials required for the early events in phagocytosis. We found that, in the absence of functional kinesin, specific pathways involved in particle binding, “focal exocytosis”, pseudopod formation, and phagosome membrane fission during FcγR-mediated phagocytosis are inhibited. This study provides novel insight into mutant, EGFP-Kif5B-DN, or RFP-Kif5B dominant negative (DN) constructs are a gift from Dr. John Brumwell (Rockville, MD) and cultured in DMEM with 10% heat-inactivated FBS at 37°C in a T-25 tissue culture flask. RAW264.7 cells were grown to 37°C in DMEM with 10% FBS in a T-25 tissue culture flask to a confluency of 70%. AMAXA Cell Line Nucleofector Kit V solution (Lonza, Cologne, Germany) and siRNA were allowed to reach room temperature. One milliliter DMEM with 10% FBS was added to six-well tissue culture plates with or without glass coverslips and placed in a 37°C incubator. Cells were scraped from a T-25 tissue culture and centrifuged in a 15-ml falcon tube at 6000 rpm for 10 min. During this time, 5.6 μl Kif5B siRNA and scrambled siRNA were added to 100 μl AMAXA solution V. Media was aspirated, and the pellet was resuspended in the AMAXA/αRNA mix. From this mixture, 100 μl was added to electroporation cuvettes and electroporated. Five hundred microliters DMEM with 10% FBS was added to the cuvette, and this was added to each six-well tissue culture plate. After 48 h, phagocytosis was performed, and cells were fixed and immunostained.

**FcγR-mediated binding and phagocytosis assays**

For single-round FcγR-mediated phagocytosis, RAW264.7 cells grown on coverslips in six-well tissue culture plates were initially transfected with EGFP-Kif5B-DN. The following day, SRBCs were opsonized with rabbit anti-SRBC IgG at room temperature for 1 h proceeded by three washes with PBS (7). After a 5-min incubation with IgG-SRBCs at 37°C, RAW264.7 cells were vigorously washed with PBS to remove any unbound particles. Cells were fixed at this time point and the initial binding index was quantified. In the double-round assays of FcγR-mediated phagocytosis, RAW264.7 cells in six-well tissue culture plates were initially transfected with EGFP-Kif5B-DN, RFP-Kif5B-DN, EGFP-Rab11-DN, or YFP-Kif5B rigor mutant overnight. Macrophages were then allowed to bind an excess of IgG–SRBCs for 5 min (typically 3–4 IgG–SRBCs per macrophage) followed by washing to remove unbound particles and then incubated a further 15 min at 37°C to allow particle internalization. The cells were then treated with an additional round of IgG–SRBCs for 5 min at 37°C. Cells were washed three times with PBS and fixed in 4% paraformaldehyde (PFA) in PBS at room temperature for 15 min, followed by two washes with PBS (7). Cells were then permeabilized in 0.5% Triton X-100 (Sigma-Aldrich) for 15 min at room temperature, washed with PBS (7), and block Ab, and rat IgG2b anti-mouse Ab isotype control was from BD Pharmingen (San Jose, CA). Cy3- and Cy5-conjugated donkey anti-rabbit, anti-rat, and anti-human IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). PE-conjugated F(ab′)2 fragments, or fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Zymed Laboratories), and Alexa Fluor 488-conjugated goat anti-rat IgG (Invitrogen) were used as secondary Ab. For double-labeling experiments, cells were fixed and immunostained using primary Abs against phagocytosed SRBCs (IgG–SRBCs), and then incubated with a secondary Ab for the same fluorophore, or with an isotype-matched secondary Ab for a different fluorophore. Controls were performed, and cells were fixed and immunostained.

**Constructs**

The dominant negative (DN) EGFP-Kif5B-DN cDNA construct was a gift from Dr. Don Arnold (University of Southern California, Los Angeles, CA), EGFP-Rab11 (wild type [WT] and DN) cDNA was provided by Dr. John Brumwell (Rockville, MD). The following day, SRBCs were opsonized with rabbit anti-SRBC IgG at room temperature for 1 h proceeded by three washes with PBS (7). After a 5-min incubation with IgG-SRBCs at 37°C, RAW264.7 cells were vigorously washed with PBS to remove any unbound particles. Cells were fixed at this time point and the initial binding index was quantified. In the double-round assays of FcγR-mediated phagocytosis, RAW264.7 cells in six-well tissue culture plates were initially transfected with EGFP-Kif5B-DN, RFP-Kif5B-DN, EGFP-Rab11-DN, or YFP-Kif5B rigor mutant overnight. Macrophages were then allowed to bind an excess of IgG–SRBCs for 5 min (typically 3–4 IgG–SRBCs per macrophage) followed by washing to remove unbound particles and then incubated a further 15 min at 37°C to allow particle internalization. The cells were then treated with an additional round of IgG–SRBCs for 5 min at 37°C. Cells were washed three times with PBS and fixed in 4% paraformaldehyde (PFA) in PBS at room temperature for 15 min, followed by two washes with PBS (7). Cells were then permeabilized in 0.5% Triton X-100 (Sigma-Aldrich) for 15 min at room temperature, washed with PBS (7), and block Ab, and rat IgG2b anti-mouse Ab isotype control was from BD Pharmingen (San Jose, CA). Cy3- and Cy5-conjugated donkey anti-rabbit, anti-rat, and anti-human IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). PE-conjugated F(ab′)2 fragments, or fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Zymed Laboratories), and Alexa Fluor 488-conjugated goat anti-rat IgG (Invitrogen) were used as secondary Ab. For double-labeling experiments, cells were fixed and immunostained using primary Abs against phagocytosed SRBCs (IgG–SRBCs), and then incubated with a secondary Ab for the same fluorophore, or with an isotype-matched secondary Ab for a different fluorophore. Controls were performed, and cells were fixed and immunostained.

**Small interfering RNA of Kif5B**

Kif5B small interfering (siRNA) was purchased from Invitrogen. The following sequence was used: 5′-GAGCAGCGAGAGAUGAUGGCAU-3′. RAW264.7 cells were transfected with 100 nM siRNA using FuGENE HD according to the manufacturer’s instructions. For flow cytometry experiments, cells were plated on six-well tissue culture plates and transfected with 2 μg cDNA of EGFP-Kif5B-DN or EGFP-Rab11-DN overnight.
Scanning electron microscopy
RAW264.7 cells grown in tissue culture dishes were transfected with EGFP-Kif5B-DN or EGFP overnight. Cells were then scraped from tissue culture dishes and resuspended in 2 ml HBSS in 2% FBS in FACS polystyrene tubes. Cells were filtered and sorted using FACS into polypropylene tubes, which cells were washed and transduced control cells, EGFP-transfected control cells, and EGFP-Kif5B-DN cells. Each group of sorted cells was plated on 13-mm diameter Thermopen plastic coverslips (Nalge Nunc International, Naperville, IL). For binding experiments, cells were exposed to IgG–SRBCs for 5 min followed by PBS washing and fixation with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Cells were also exposed to a double round of IgG–SRBCs, as described earlier, followed by fixation. Or, cells underwent “frustrated phagocytosis,” where 13-mm plastic coverslips were coated with human IgG, and cells were incubated for 60 min and then fixed in 2.5% glutaraldehyde. Subsequently, cells were postfixed in 1% OsO4 in sodium cacodylate buffer for 1 h. Cells were then dehydrated in ethanol, critical point-dried with liquid CO2, and sputter coated with 15 nm thick gold. Images were visualized using a JEOL JSM 820 scanning electron microscope (JEOL). Pseudocoloring of electron micrographs was accomplished using Adobe Photoshop 7.0 (Adobe Systems).

Live “frustrated phagocytosis” assay
Control and transfected (EGFP-Kif5B-DN) RAW264.7 cells were grown to 80% confluency, mechanically scraped, and centrifuged at 1000 rpm for 5 min. The cells were then resuspended in 1.5 ml HPMI and spun for 3 h at room temperature. Cells (400 μl) were plated on human IgG-opsonized 2.5-cm coverslips in an Attofluor chamber and bathed in HPMI and held in a thermal chamber at 37˚C on an inverted epifluorescent Zeiss microscope (Axiovert 200M). Images were captured using an AxioCam MRm camera (Carl Zeiss Microimaging, Germany). Times between frames were acquired at 15-s intervals for a period of 30 min to visualize frustrated pseudopod membrane dynamics using DIC and fluorescent imaging.

Imaging of EGFP-VAMP7 WT and EGFP-Rab11 WT transfected cells with spinning disk confocal microscopy
Control cells, EGFP-VAMP7 or EGFP-Rab11-transfected cells, and cotransfected with EGFP-VAMP7 or EGFP-Rab11 and RFP-Kif5B-DN were placed in an Attofluor chamber, immersed in HPMI, and kept at 37˚C in a Zeiss stage. Spinning disk confocal microscopy was performed on a Quorum confocal microscope (DMIRE2; Zeiss) with Velocity software (Improvision). Images were captured using an electron multiplying charge-coupled device camera (Hamamatsu Photonics). IgG–SRBCs (20 μl) were added to the cells; once these IgG–SRBCs were internalized, a second round of IgG–SRBCs were added. DIC and fluorescent images were acquired at 1- to 5-s intervals for a duration up to 10 min.

IgG recycling assay
RAW264.7 cells were incubated with rhodamine IgG-coated SRBCs for a period of 20 min at 37˚C to allow for internalization. Coverslips were then placed into a Attofluor chamber, immersed in HPMI, and kept in a thermal chamber at 37˚C on a Zeiss inverted microscope (Axiovert 200M). Epifluorescent images were acquired at 15-s intervals for a duration of 20 min, to follow dissociation and trafficking of rhodamine IgG-containing vesicles.

FACS Calibration flow cytometry
RAW264.7 macrophage cells were scraped from tissue culture flasks and tissue culture dishes, and immunostained with the FcγRIII (Fc block) Ab or the rat anti-mouse CD16/CD32 Ab. Cells (1 × 10^6) were collected into falcon tubes and washed twice with ice-cold HBSS containing 10 mM HEPES, pH 7.0, 0.02% sodium azide, and 1% FCS (HHF) before blocking with 5% FCS for 15 min and stained with 1 μg/ml primary Ab anti-rat mouse (CD16/CD32) or rat IgG2b isotype control for 30 min on ice. Cells were washed twice with HHF and incubated with 1 μg PE or Cy5-conjugated F(ab′)2 fragments of anti-rat IgG secondary Ab on ice for 30 min. Then cells were washed and resuspended in 500 μl HHF buffer and analyzed for surface staining of FcγR or agg-IgG and isotype control with FACS Calibur (Becton Dickinson) and CellQuest software (BD Biosciences, San Jose, CA). Live cells were gated in each sample totaling 10,000 events, including control samples. Cells were sorted into untransfected control cells, EGFP-transfected control cells, and EGFP-Kif5B-DN–transfected cells. Experimental results represent the relative fluorescent intensity of EGFP-Kif5B-DN cells or EGFP-Rab11-DN–transfected cells compared with untransfected PE* or Cy5* cells. Untransfected PE* stained cells were used as a positive control, whereas PE* stained isotype IgG2b untransfected cells were used as a negative control. The FACSCalibur was replicated three times, and the fluorescent expression intensity was expressed as the mean ± SEM. For measuring cell area using FACScalibur, the forward scatter profile of control and EGFP-Kif5B-DN RAW264.7 cells was compared with 3- and 8-μm beads. Each micrometer represents 25 forward scatter; therefore, a 3-μm bead would represent a forward scatter of 75. Cell diameters were extrapolated from the results and were expressed as the mean ± SEM from replicate experiments. Statistical significance was determined using the Student’s t test.

Viability assay
RAW264.7 cells were transiently transfected with EGFP, YFP-Kif5B rigor mutant, EGFP-Kif5B-DN overnight, or transfected with siRNA Kif5B for 48 h to ensure transfected cell viability was not compromised. DMEM with serum was aspirated out and replaced with 1 ml PBS. PI was added at 1:1000, and DAPI was added as a cell marker at 1:10,000 for 5 min. Cells positive for PI were counted on an epifluorescent Zeiss microscope (Axiovert 200M).

Data quantification and statistical analysis
Experiments were done in triplets (n > 30). The binding indices were calculated by counting the number of binding IgG–SRBCs per 100 macrophages. The phagocytic index represents the number of internalized IgG–SRBCs per 100 cells. For quantifying “frustrated phagocytosis” the maximal pseudopod area was measured using Java image processing software. Image 1.32 (National Institutes of Health). Phalloidin staining was used as the following marker for measuring the cell area. Quantification of abnormal pseudopods by scanning electron microscopy was performed by analyzing cells for the presence of thin, abnormal protrusions. In total, 100 cells were analyzed from three independent experiments. All quantification was performed using the Student’s t test, with the p values < 0.05 considered significant. Error bars represented in graphs represent the mean ± SEM. All experimental results were repeated in at least three independent experiments.

Results
Kinesin DN expression in RAW264.7 cells alters pseudopod formation when macrophages are faced with consecutive particle challenge and reduces IgG–SRBC binding
It is well established that targeted delivery of membrane and F-actin protein modulators occurs at the site of phagocytosis. However, the intracellular transport mechanism behind this focal recruitment has not been resolved. We investigated whether the MT motor protein kinesin is the underlying driving force of protein and vesicular targeting during FcγR-mediated phagocytosis. For these analyses, we transiently transfected RAW264.7 macrophages with EGFP-Kif5B-DN, a DN construct that lacks the kinesin ATPase motor domain located in the kinesin I chain region (16). Overexpression of this construct overrides and impedes conventional kinesin activity in mammalian cells (16). To more fully understand the IgG–particle–binding defect seen with epifluorescence in EGFP-Kif5B-DN–transfected cells, we turned to scanning electron microscopy, which enables the analysis of pseudopod surface topology with high resolution. Macrophages normally encounter multiple particles in succession and are equipped to undergo sequential rounds of phagocytosis (17). We examined the kinesin requirement in pseudopod formation when RAW264.7 cells were challenged with a second round of IgG–SRBCs. FACS was used to sort the EGFP-Kif5B-DN cells from untransfected cells before analysis with scanning electron microscopy. RAW264.7 cells were transiently transfected with EGFP-Kif5B-DN or EGFP alone overnight, then sorted into either untransfected control cells, EGFP-transfected control cells, or EGFP-Kif5B-DN–transfected cells using FACS. The retrieved sorted cells were separately seeded onto coverslips in six-well tissue culture plates. For this assay, we exposed transfected, sorted cells with IgG–SRBCs for 5 min at 37˚C, washed unbound particles, and allowed RAW264.7 cells to continue the in-
ternalization process at 37°C for 20 min. Then, cells were incubated with an additional round of IgG–SRBCs for 5 min at 37°C, fixed, and either immunostained or processed for scanning electron microscopy. In EGFP-Kif5B-DN–expressing cells, the amount of IgG–SRBC binding in the second round of challenge was significantly reduced compared with control cells, as seen in immunofluorescent images (Fig. 1A). The binding index was quantified by totaling the number of bound IgG–SRBCs per 100 RAW264.7 cells. The binding index was significantly depressed in 100 EGFP-Kif5B-DN–transfected macrophages by almost 50% compared with control cells (Fig. 1A). We next examined the pseudopods of the IgG–SRBCs on these cells. Ultrastructural surface analysis showed less binding of IgG–SRBCs in EGFP-Kif5B-DN–expressing cells compared with EGFP-transfected and untransfected control cells (Fig. 1B). Interestingly, unlike the typical pseudopods observed by scanning electron microscopy in the control cell populations, the mutant kinesin-expressing cells instead displayed thin, finger-like pseudopods that tightly associated with the particle (Fig. 1B). To ensure that the overexpression of EGFP-Kif5B-DN does not induce the bundling of MTs, we stained acetylated α-tubulin in both control and EGFP-Kif5B-DN–transfected cells. We saw no obvious bundling or anomalies with the organization of MTs in the transfected cells (results not shown). We also repeated this experiment in cells transiently transfected with the YFP-Kif5B-DN rigor mutant (18) to ensure that Kif5B MT-binding activity is responsible for this observed decrease in IgG–SRBC binding. In YFP-Kif5B-DN–transfected cells, IgG–SRBC binding was reduced significantly by ∼40% (Fig. 1C). Because ectopic overexpression of constructs can scavenge important cofactors or regulatory molecules, the double round binding assay was performed in RAW264.7 cells transfected with siRNA against Kif5B. After 48 h of siRNA exposure, RAW264.7 cells had a significant reduction in capacity to bind a second round of IgG–SRBCs, mirroring the mutant kinesin overexpression assays (Fig. 1D). None of these strategies of mutant kinesin expression or protein knockdown significantly affected cell viability (data not shown). Thus, Kif5B expression and activity are essential for macrophages challenged with multiple particles. Kinesin was particularly essential in macrophages that were actively processing multiple particles, because analysis of particle binding of the initial round of IgG–SRBC exposure showed no significant difference between control and Kif5B-DN–transfected cells (Supplemental Fig. 1A). We also examined whether kinesin plays a role in two internalization processes that do not require elaborate pseudopods. RAW264.7 cells were allowed to engulf a single round of IgG–SRBCs and then challenged with 0.8-μm beads. As shown in Supplemental Fig. 1B, there was no significant difference in the number of bound 0.8-μm beads in control versus Kif5B-DN–transfected cells. We next incubated RAW264.7 control and EGFP-Kif5B-DN–transfected cells with one round of IgG–SRBCs and then one round of agg–IgG complexes. We analyzed the amount of agg–IgG complexes on the surface of the cell using flow cytometry. The relative fluorescent intensity of agg–IgG on the surface of control RAW264.7 cells was similar to that of Kif5B-DN–transfected cells (Supplemental Fig. 1C).

DN kinesin interferes with pseudopod extension and dynamics in cells undergoing “frustrated phagocytosis”

Our particle overload assays indicated that anterograde MT motor delivery is particularly vital when macrophages are challenged with excessive amounts of IgG–SRBCs. We further tested this by exposing macrophages to an extreme target: an IgG-opsonized coverslip. This “frustrated phagocytosis” experimental strategy allows researchers to study pseudopod dynamics in isolation from maturation events and localizes molecular activities to the basal plasma membrane, which spreads rapidly across the coverslip (12, 19–24). DN kinesin-transfected cells and control cells were “dropped” onto opsonized coverslips and incubated for 30 min at 37°C to allow for maximal pseudopod spreading along the coverslip. Cells were fixed and F-actin was stained to demarcate the cell periphery. Total cell area was measured from immunofluorescent images using National Institutes of Health ImageJ software. Quantitative analysis demonstrated that control cells were able to spread over an area ∼2.16 times larger than cells transfected with EGFP-Kif5B-DN (Fig. 2A, 2B). We checked that the decrease in cell spreading was not just a result of overall reduced cell size, possibly induced by disrupting kinesin-based transport. Measurements of cell areas of EGFP-Kif5B-DN–transfected cells, plated on glass coverslips overnight, were taken and compared with untransfected control cells. As shown in Fig. 2C, there was not a substantial difference in the size of resting macrophages when kinesin DN constructs were expressed in RAW264.7 cells.

![FIGURE 1. Pseudopods are impaired EGFP-Kif5B-DN–transfected RAW264.7 cells. A. RAW264.7 cells were transfected with EGFP-Kif5B-DN overnight and treated with IgG–SRBCs for 20 min at 37°C, then treated with a second round of IgG–SRBCs for 5 min, before fixation and analysis. Results represent the mean ± SEM from three separate experiments. B. RAW264.7 cells were untransfected, or transfected with either EGFP-Kif5B-DN or EGFP, and sorted using FACS. Cells were plated and treated with two rounds of IgG–SRBCs, then fixed and processed for scanning electron microscopy analysis. Fewer IgG–SRBCs were observed bound to EGFP-Kif5B-DN–transfected cells, and pseudopods were highly defective in these cells (arrow), compared with control cells. C. Quantification of the number of bound IgG–SRBCs from the second round of IgG–SRBC addition per 100 RAW264.7 macrophages when RAW264.7 cells were transfected with YFP-Kif5B-DN. Results represent the mean ± SEM from three separate experiments. D. RAW264.7 cells treated with scrambled or Kif5B siRNA for 48 h were exposed to a double round of IgG–SRBCs and fixed and analyzed. The graph represents the number of bound IgG–SRBCs from the second round of IgG–SRBC addition per 100 RAW264.7 macrophages. Results represent the mean ± SEM from three separate experiments. *p < 0.05 compared with control cells. Scale bars, 10 μm.](http://www.jimmunol.org/)
Confirmatory quantification of cell area was done using flow cytometry analyses. A solution of suspended control and EGFP-Kif5B-DN–transfected cells was spiked with beads of known size before flow cytometry. On forward scatter analysis, it was revealed that the mean diameter of untransfected control cells was 24 μm, similar to the value of 25 μm obtained for EGFP-Kif5B-DN–transfected cells.

We then used scanning electron microscopy analysis to more closely inspect which aspects of pseudopod development during frustrated phagocytosis were kinesin dependent. Immunofluorescence imaging is insufficient to detect precise pseudopodia changes, and for this reason, scanning electron microscopy analysis was used to visualize minute alterations on the macrophage surface. Control, EGFP alone, and EGFP-Kif5B-DN–transfected RAW264.7 cells

![Image](https://via.placeholder.com/150)

**FIGURE 2.** A “frustrated phagocytic” assay reveals prominent actin and membrane defects in cells transfected with EGFP-Kif5B-DN. A, Staining of F-actin (red) in RAW264.7 cells transfected with EGFP-Kif5B-DN. Cells were dropped onto human IgG-coated glass coverslips and incubated at 37°C for 30 min. Asterisks denote EGFP-Kif5B-DN–transfected cell. B, Quantification of frustrated phagocytosis area in control and EGFP-Kif5B-DN–transfected cells. The frustrated pseudopods in control cell were 2.17-fold larger than in EGFP-Kif5B-DN–transfected cells. C, Below the graph, the numerical value of the total cell area of resting control and EGFP-Kif5B-DN–transfected RAW264.7 cells was also determined using FACS Calibur. No significant difference in the overall cell size was observed between control and EGFP-Kif5B-DN cells. Control cells, on average, are 24 μm, whereas EGFP-Kif5B-DN cells are 25 μm. D, EGFP-Kif5B-DN–transfected, untransfected control, and EGFP-transfected control cells were sorted using FACS. Sorted cells underwent “frustrated phagocytosis” for 60 min at 37°C and then were fixed and processed for scanning electron microscopy. Control cells display pronounced membrane ruffling and dorsal membrane protrusions (arrows) versus the EGFP-Kif5B-DN–transfected cells, which were comparably rounder with few broad, pseudopod-like extensions. E, The percentage of cells containing abnormal, thin membrane protrusions was quantified and was significantly greater in EGFP-Kif5B-DN–transfected cells, compared with untransfected and EGFP control cells. Results represent the mean ± SEM from three independent experiments. *p < 0.05 compared with control cells. Scale bars, 10 μm.
were sorted using FACS and engaged in “frustrated phagocytosis,” as described in Materials and Methods, for 60 min at 37°C. As shown in Fig. 2D, cell spreading was observable in untransfected control and EGFP-transfected control cells, which had a flat “fried egg” morphology. In contrast, EGFP-Kif5B-DN–transfected cells were much rounder with few spread pseudopods along the IgG coverslip. Instead, occasional thin filopodia-type structures were observed in kinesin mutant cells (Fig. 2D). In addition, both untransfected and EGFP-alone–transfected cells showed peripheral membrane ruffles and thin membrane protrusions that extended perpendicularly toward the plasma membrane (Fig. 2D). Membrane ruffles and other structures indicative of dynamic membrane events were not present in EGFP-Kif5B-DN–transfected cells (Fig. 2D). These thin membrane protrusions were observed in 17% of the untransfected and EGFP control cells compared with 75% of the EGFP-Kif5B-DN–transfected cells (Fig. 2E).

We next used live DIC imaging to more fully understand the requirement for kinesin in membrane pseudopod dynamics during frustrated phagocytosis. Cells were transfected with EGFP-Kif5B-DN overnight, scraped, and seeded onto a human IgG coverslip in a live cell imaging chamber held at 37°C, immersed in HPML. A complete set of DIC images of RAW264.7 cells undergoing “frustrated phagocytosis” were acquired at 10-s intervals for 30 min. As noted in the fixed “frustrated phagocytosis” assay (Fig. 2), EGFP-Kif5B-DN cells displayed decreased cell spreading compared with untransfected control cells (Fig. 3). In contrast with kinesin DN–transfected cells, control cells displayed filopodia-like extensions (Fig. 3, see arrows), together with pronounced membrane ruffling at the cell periphery (Fig. 3; see also Supplemental Video 1). These discrete membrane events were lacking in EGFP-Kif5B-DN–transfected cells (Fig. 3; see also Supplemental Video 1). Reduced plasma membrane dynamics in kinesin DN–transfected cells implies a role for kinesin in pseudopod development.

**Rab11 and VAMP7 show differential MT motor requirements during “focal exocytosis”**

Our results strongly implicated a role for kinesin in pseudopod formation during FcyR-mediated phagocytosis. Disruption of MTs blocks granule secretion in neutrophils (25, 26), which is thought to be kinesin mediated (27). Targeted delivery of intracellular membranes has been shown by several researchers to promote pseudopod formation. Intracellular endomembrane compartments that contribute to “focal exocytosis” during phagocytosis include recycling endosomes bearing the proteins Rab11 and a v-SNARE, VAMP3, late endosomes bearing the v-SNARE, VAMP7, lysosomes bearing lysosomal-associated membrane protein-1, and possibly endoplasmic reticulum (5, 8, 10). Because of the direction that these reserves are being transported (in an anterograde motion), kinesin is a putative transport mechanism. Thus, we analyzed VAMP7 and Rab11 recruitment during phagocytosis in RAW264.7 control and kinesin DN–transfected cells. The cells were cotransfected with either EGFP-VAMP7 or EGFP-Rab11 with RFP-Kif5B-DN overnight. The cells were then incubated with IgG–SRBCs at 37°C for 20 min. An additional round of IgG–SRBCs were added to the coverslip, which commenced imaging. Live spinning disk confocal imaging demonstrated rapid accumulation of both EGFP-Rab11 and EGFP-VAMP7 within 5 min of phagocytosis, during the second round of IgG–SRBCs (Fig. 4A; data for VAMP7 are not shown). In cells cotransfected with RFP-Kif5B-DN, a strong accumulation of EGFP-VAMP7 was also observed around incoming IgG–SRBCs, indicating that the recruitment of VAMP7 vesicles is kinesin independent (data not shown). In contrast, EGFP-Rab11 recruitment to the phagocytic cup was attenuated in cells cotransfected with RFP-Kif5B-DN in the second round of IgG–SRBCs (Fig. 4B). In these cells, no apparent localized accumulation of EGFP-Rab11 at the pseudopod was observed compared with control cells (Fig. 4B). The second round IgG–SRBCs was unsuccessful in binding to the cotransfected cell. Also, the Rab11 distribution on the periphery of the cell was different between the RFP-Kif5B-DN–transfected and control cell. Rab11 vesicles were found along the plasma membrane in the control cells, which was diminished in the transfected cell. This suggests that there are different pathways of delivery for both recycling and late endosomes during focal exocytosis, and that the trafficking of recycling endosomes to the pseudopod is via the kinesin motor. Also, a conglomeration of Rab11 vesicles was seen around the internalized IgG–SRBC in the cotransfected cell, which may implicate a fission defect. RAW264.7 cells were transiently transfected with EGFP-Rab11-DN overnight and then exposed to a double round of IgG–SRBCs, as described in Materials and Methods, to determine whether Rab11 is essential for the binding of IgG–SRBCs when macrophages are faced with multiple rounds of phagocytosis. Cells transfected with EGFP-Rab11-DN showed a significant reduction in the number of bound, second round IgG–SRBCs compared with control cells (Fig. 4C).

**Kinesin is required for recycling of membrane from phagosomes**

Our live epifluorescent imaging demonstrated a role for kinesin in delivering recycling endosomes to the forming pseudopod. Our studies also showed that mutant expression of kinesin had the most severe effect on pseudopod development when macrophages were faced with a second challenge of IgG–SRBCs. Together, this suggests that membranes internalized as part of the phagosome are also required to develop subsequent membrane pseudopods. To analyze the kinetics of recycling from the phagosome during phagocytosis in the presence of an overexpressed DN mutant, we followed trafficking of IgG-containing vesicles from the phagosome compartment in control and EGFP-Kif5B-DN–transfected cells. Previous studies have shown that treatment of RAW264.7 cells with nocodazole, an MT depolymerizing agent, reduced the recycling of IgG from the phagosome, implicating a role for MTs in long-range transport of recycling vesicles (28). To assess phagosome membrane recycling, SRBCs were prelabeled with rhodamine IgG. These fluorescent IgG–SRBCs were then incubated with
both untransfected and EGFP-Kif5B-DN RAW264.7 cells for 20 min at 37˚C to promote internalization, followed by extensive washing and hydrolytic lysis to remove any bound IgG–SRBCs. Coverslips were then placed into a live cell-imaging chamber and were visualized by epifluorescent microscopy. Time-lapse microscopy of control cells followed the dissociation of rhodamine-labeled IgG from the phagosome and the progressive movement of these vesicles toward the plasma membrane in a time-dependent manner (Fig. 5A; see also Supplemental Video 2). In contrast, in EGFP-Kif5B-DN–transfected cells, recycling of IgG from the phagosome was markedly inhibited. Rhodamine IgG vesicles were much fewer in these cells, and they showed minimal directional transport (Fig. 5B; see also Supplemental Video 3). Taken together, our results indicate that kinesin is involved in the formation and anterograde recycling of membrane compartments containing IgG from phagosomes to the plasma membrane.

**Mutant kinesin reduces FcγR display on the plasma membrane of macrophages**

As shown in Figs. 4 and 5, the trafficking of recycling endosomes and phagosomal membranes was impaired in EGFP-Kif5B-DN–transfected cells. The reduced delivery of these membrane compartments alone can explain the defective pseudopods observed in mutant kinesin-expressing cells. However, these results do not clarify why there is attenuated IgG–SRBC particle binding, which is upstream of pseudopod formation, in EGFP-Kif5B-DN–transfected cells. We speculated that the recycling membranes could additionally be bringing opsonin receptors back to the surface for subsequent encounters with particles. To assess this, we measured the levels of FcγR at the cell surface in resting cells and in cells that had already internalized particles. Normally, FcγRs bound to IgG–SRBCs are internalized into the cell, depleting the receptors at the plasma membrane. However, recycling of the receptors occurs during SRBC degradation (29). To detect surface FcγR, we stained cells with the Fc blocking Ab and a PE-labeled secondary Ab. Flow cytometry analysis of FcγR in resting cells demonstrated no significant difference between control and kinesin DN-transfected cells (Fig. 6A). We next compared the level of FcγRs on the plasma membrane in control and EGFP-Kif5B-DN–transfected cells after they had already internalized particles. Control and EGFP-Kif5B-DN cells were exposed to IgG–SRBCs for 20 min at 37˚C to allow for internalization, followed by a hydrolytic lysis of external IgG–SRBCs and several washes. Cells were then incubated at 37˚C for 1 h to allow for the recycling of FcγR to the plasma membrane. Flow cytometry quantification of FcγR
revealed a 41% reduction in surface FcγR in EGFP-Kif5B-DN cells, compared with untransfected control cells (Fig. 6B). To analyze whether receptor recycling was also Rab11 dependent, we exposed EGFP-Rab11-DN–transfected cells to IgG–SRBCs, then gave a similar chase time to allow for receptor recycling. There was no significant difference in FcγR on the surface of EGFP-Rab11-DN–transfected cells compared with control, untransfected cells (Fig. 6C). Previously, it has been shown that the β2 integrin Mac-1 (CD11b/CD18) has a functional interaction with FcγR and is found in the phagocytic cup (23). Moreover, both EGFP-Kif5B-DN and EGFP-Rab11-DN expression in macrophages reduced C3bi-particle binding during Mac-1–mediated phagocytosis (30). Thus, we performed flow cytometry analysis for Mac-1 on RAW264.7 cells expressing EGFP-Kif5B-DN. Cells were treated with IgG–SRBCs for 20 min and incubated at 37˚C to allow time for the recycling of Mac-1. We found that cells transfected with EGFP-Kif5B-DN had a reduced surface expression of Mac-1 by ∼70% (Fig. 6D).

These results indicate that kinesin plays dual roles in recycling both phagosome-derived FcγRs and Mac-1 receptors and Rab11+ intracellular membranes to the cell surface for optimal particle binding and pseudopod formation, respectively (Fig. 6E).

**Discussion**

Pseudopod development during FcγR-mediated phagocytosis is essential to manage the uptake of particles ranging in size from relatively small bacteria to an equivalent or larger sized apoptotic mammalian cell (31). Furthermore, macrophages must be equipped to manage consecutive challenges of phagocytic target and readily possess intracellular transport mechanisms to deliver internalized plasma membrane back to the cell surface. Recent eloquent cell biology studies have detailed diverse sources of intracellular membrane resources that are trafficked to phagocytic

---

**FIGURE 6.** Kinesin DN but not Rab11 DN-transfected RAW264.7 cells after IgG–SRBC ingestion have decreased surface expression of FcγR compared with control cells. Cell surface expression of FcγR in RAW264.7 macrophages was analyzed by flow cytometry using a polyclonal Ab against FcγR. A, Basal levels of FcγR on the surface of control or EGFP-Kif5B-DN–transfected RAW264.7 cells. No significant difference in basal surface expression was noticed. B, Cells after internalizing IgG–SRBCs were stained for FcγR on the plasma membrane. EGFP-Kif5B-DN–transfected cells have a significant decrease of FcγR on the plasma membrane after one round of IgG–SRBCs. C, EGFP-Rab11-DN–transfected cells and control untransfected cells were stained for FcγR after internalization of IgG–SRBCs. No significant difference in surface FcγR was observed in EGFP-Rab11-DN–transfected RAW264.7 macrophages compared with control cells. D, EGFP-Kif5B-DN–transfected and control untransfected cells were stained for Mac-1 after internalization of IgG–SRBCs. Surface Mac-1 levels are reduced in mutant kinesin-expressing cells. A–D, Quantitative evaluation of the relative fluorescent intensities of control versus EGFP-Kif5B-DN– or EGFP-Rab11-DN–transfected RAW264.7 macrophages. Results represent mean ± SEM from three separate experiments. E, Model of the role of kinesins in FcγR-mediated phagocytosis. IgG–SRBCs interact with FcγR on the surface of macrophages, and downstream signaling events initiate the formation of the pseudopod. Pseudopod elaboration is accomplished by kinesin-mediated delivery of endosomes bearing Rab11. Also, internalized phagosome membranes containing IgG, FcγR, and Mac-1 are recycled back to the plasma membrane through kinesin to maintain a steady-state surface receptor display. *p < 0.05 compared with control cells.
cups. What has remained a mystery is how these membranes are specifically trafficked to focal membranes of engagement. In this article, we identify Kif5B as the molecular motor responsible for delivering key membranes and receptors necessary for particle binding and pseudopod elaboration during FcyR-mediated phagocytosis.

Kinesin has been shown to be important for pseudopod formation during cell polarization, although the cargo source and action(s) at the site of pseudopods has not been resolved (32). Our studies show that trafficking of recycling endosomes to the phagocytic cup requires the anterograde motor. We cannot be certain that phagocytosis initiates a global kinesin-dependent movement of recycling endosomes to the plasma membrane, but the reduced Rab11-EGFP on nascent phagosomes in kinesin-mutant cells suggest that localized trafficking of recycling endosomes may occur to active plasma membranes engaged in phagocytosis. Selective trafficking of recycling endosomes may occur on stabilized MT subsets, which have been shown to enhance kinesin-based transport (33). We have observed MTs penetrating into sites of phagocytosis, and these MTs may serve as tracks for kinesin-mediated recycling endosome delivery (11, 12, 34). VAMP7-GFP late endosome delivery to phagocytic cups showed no kinesin dependency, so it is curious how these compartments are rapidly delivered to the membrane.

We also observed abnormal vesicle trafficking from the phagosome in Kif5B-DN–transfected cells. The phagosome membrane is constantly being remodeled during maturation, because it sequentially fuses with components of the endocytic pathway (35). Fission events have also been reported that are believed to recycle receptors from the phagosome and to retain a constant membrane around the particle (35). Elegant studies with photoactivatable GFP-labeled FcyRIIA have shown that a proportion of the receptors pinch off of the phagosome (36). We see reduced trafficking of fluorescent IgG-containing vesicles from the phagosome in Kif5B-DN–transfected macrophages. The vesicles show reduced movement that is similar to what was reported from phagosomes in cells treated with nocodazole to depolymerize MTs. Interestingly, there are reduced vesicles overall, implicating kinesin activity in vesicle budding from the phagosome. The mechanism of vesicle budding from phagosomes has remained elusive, because clathrin and coat proteins do not appear to play a major role in fission of FcyRs from phagosomes (37). Dynein has been linked to membrane deformations, and kinesin may play a similar role in pulling membranes along MTs (38), in this case, to accelerate fission events. This cellular evidence suggests that kinesin may play a role vesicle formation or budding.

The neonatal FcR has been shown to be recycled to the plasma membrane via a Rab11-dependent step (39). Our flow cytometry results indicate that Rab11 is not involved in the kinesin-dependent recycling of FcyRs during phagocytosis. We cannot distinguish whether the kinesin-dependent membrane trafficking from phagosomes and recycling endosomes are coordinated or independent events. On first encounter with a large particle, kinesin appears to drive Rab11-vesicle delivery necessary for pseudopod formation. Rab11 is also required for adherence of subsequent particles, and the absence of sufficient membrane delivery for pseudopod elaboration may weaken the particle’s association with the cell surface. Kinesin also appears to become additionally activated to retrieve internalized phagosome receptors to maintain a steady-state of receptors for subsequent particle engagement.

The kinesin motor requirement during FcyR-mediated phagocytosis is particularly pronounced when macrophages are faced with multiple particles or an impossibly large target such as an IgG-coated coverslip. In macrophages facing a high membrane demand, live imaging showed a reduction in pseudopod membrane dynamics that is likely directly attributable to aberrant F-actin assembly in kinesin mutant cells. It is possible that kinesin is responsible for delivering F-actin regulating proteins to the phagocytic cup. The Rho family of small GTPases involved in actin polymerization and cell motility, including rhoA, rac1, and cdc42, has been associated with kinesin, a vesicle membrane protein that binds to kinesin (40, 41). Equally plausible is that reduced delivery of FeRs to the surface results in sparser receptor ligation and downstream signaling that causes attenuated actin cup formation. Our scanning electron microscopy analysis of Kif5B-DN–transfected cells faced with multiple rounds of IgG-SRBCs provided strong evidence that fewer receptors are engaged on particles resulting in finger-like extensions partially reaching around the particle. This is a unique phenotype that may help unveil the timing of pseudopod formation. A zippering mechanism has been postulated for decades, but details on the mechanics of its assembly have not been resolved. Imaging FeRs on target particles has not been feasible because specific Ab tags block binding sites, and heterologous expression of receptors often results in overexpression of proteins that compete with endogenous receptors for binding. In this study, we see that Fe ligation occurs on linear strips that extend upward along the particle. This suggests that cortical cytoskeletal elements may prearrange rows of FeRs within the resting plasma membrane or strengthen binding of engaged receptors in a linear fashion against the particle. The finger-like projections also suggest that kinesin-mediated membrane stores are delivered to fill in the gaps between adherence sites to form a continuous sheet of membrane around the particles.

Previous studies of primary macrophages have shown an important role for conventional kinesin in the intracellular remodeling of tubular lysosomes (42, 43). A recent study of human primary macrophages also revealed that Kif5B is essential for the delivery of matrix metalloproteinases to the cell surface MT1-MMP. It has been previously shown that tubular lysosomes undergo extension along the tracks of MTs and appear to be mediated by kinesin in murine macrophages. Later, it was shown this same phenomenon of tubular lysosomes moving along the tracks of MTs requires the motor protein kinesin (18). In this paper, we extend these kinesin studies to show an additional role in delivering key opsonin receptors, including Mac-1, to the cell surface. As we assayed for FcyR display using an Ab that recognizes both FcyRI and FcyRII, we cannot determine conclusively the cargo that is driven via kinesin to the plasma membrane. However, because FcyRII is an inhibitory phagocytosis receptor in mice (44), it is most plausible that delivery of the activating FcyRII (45) requires Kif5B. Another activating FcyR, FcyR1, undergoes receptor recycling to the plasma membrane when bound to IgG (46), although we did not test the recycling of this specific receptor in our assays.

In summary, we show that an MT motor is the driving mechanism behind intracellular membrane store delivery to the phagocytic cup. These findings also revealed a functional dependency of actin cup assembly on pseudopod elaboration, providing further evidence that these are not independent events. The involvement of conventional kinesin in FcyR-mediated phagocytosis is necessary for delivery of intracellular membrane stores for both particle adherence and pseudopod formation and dynamics.

**Acknowledgments**

We thank Don Arnold (University of Southern California) for the EGFP-Kif5B-DN construct, John Brumell (Hospital for Sick Children) for the EGFP-Rab11 constructs, Stefan Linder for the YFP-Kif5B-DN construct, and Sergio Grinstein (Hospital for Sick Children) for the VAMP7-GFP construct.
Disclosures
The authors have no financial conflicts of interest.

References