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SHORT COMMUNICATION

Dynamin-2 controls actin remodeling for efficient complement receptor 3-mediated phagocytosis

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Abstract

Background information: Phagocytosis is the mechanism of the internalization of large particles, microorganisms and cellular debris. The complement pathway represents one of the first mechanisms of defense against infection and the complement receptor 3 (CR3), which is highly expressed on macrophages, is a major receptor for many pathogens and debris. Key to dissecting the mechanisms by which CR3-mediated phagocytosis occurs, is understanding how the complex actin binding protein machinery and associated regulators interact with actin during phagocytosis, from triggering of receptor, through to phagosome formation and closure.

Results: Here, we reveal that Dynamin-2 is recruited concomitantly with polymerized actin at the phagocytic cup and during phagosome formation and closure. Inhibition of Dynamin activity leads to stalled phagocytic cups and a decrease in the amount of F-actin at the site of phagocytosis.

Conclusions: Dynamin-2 regulates the assembly of the F-actin phagocytic cup for successful CR3-mediated phagocytosis.

Significance: These results highlight an important role for Dynamin-2 in actin remodeling downstream of integrins.

KEYWORDS

actin, complement receptor, dynamin, phagocytosis

INTRODUCTION

Phagocytosis is the mechanism of the internalization of large particles, microorganisms, and cellular debris (Depierre et al., 2021; Uribe-Querol & Rosales, 2020). It has a nutritional role in Amoebae and, in the professional phagocytes of the immune system, phagocytosis plays a key role in the development of immune responses via cytokine secretion and presentation of antigen derived

from the internalized material. Phagocytosis is also important for normal turnover and remodeling of tissues via the disposal of dead cells. Phagocytosis is initiated by the triggering of surface receptors that bind directly to determinants of microorganisms or receptors for opsonins like immunoglobulins or complement that coat the particulate antigen. Signal transduction downstream of the phagocytic receptors involves local reorganization of the plasma membrane, and the underlying actin cytoskeleton, resulting in the internalization of the phagocytic target.

The complement receptor (CR) 3, which is highly expressed on macrophages, is a direct receptor for many pathogens and binds activated complement molecules deposited on microorganisms or cells (Torres-Gomez

Abbreviations: C³-IgM-RBCs, red blood cells opsonized with IgM followed by complement; CR3, Complement receptor 3; dSTORM, Direct stochastic optical reconstruction microscopy; FcR, receptors for the Fc portion of immunoglobulins; hMDMs, human monocyte-derived macrophages; RBCs, red blood cells; TIRFM, total internal reflection fluorescence microscopy.

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et al., 2020a). It is a phagocytic integrin also known as $\alpha M\beta 2$ that needs to be activated to bind to extracellular ligands. The actin dynamics play a key role first in the activation of the integrin receptors via inside-out signals and force-dependent conformation changes of the $\beta 2$ chain, that is, anchored to the cortical actin cytoskeleton (Torre-Gomez et al., 2020a). Second, actin polymerization is induced in downstream signaling pathways that are controlled by the small GTPase RhoA, the formin mDia1, and the Arp2/3 nucleator (Caron & Hall, 1998; Colucci-Guyon et al., 2005; May et al., 2000). In addition, a subtle interplay between the microtubules and the actin cytoskeleton is important for CR3-mediated phagocytosis (Allen & Aderem, 1996; Binker et al., 2007; Lewkowicz et al., 2008). The early steps of CR3 engagement and signaling have been dissected to show how the Syk kinase and focal adhesion proteins act as a molecular clutch to promote phagocytosis (Jaumouille et al., 2019). CR3-mediated phagocytosis was initially reported to rely on particle “sinking,” without extensive formation of membranous lamellipodia (Allen & Aderem, 1996; Kaplan et al., 1977). Some membrane extension and ruffling activity have however been associated with CR3-mediated phagocytosis (Jaumouille et al., 2019; Patel & Harrison, 2008). Overall, much less is known about the mechanisms of phagosome formation and closure downstream of CR3 than in the case of other phagocytic receptors such as FcR.

Dynamin-2 is an important mediator of budding and scission of small vesicles (Morlot & Roux, 2013). Dynamin participates in phagosome maturation in *Dicystostelium discoideum* via F-actin binding and endosome tubulation (Gopaldass et al., 2012). In addition, Dynamin-2 was found to be involved in two different steps of Receptors for Fc portion of Immunoglobulins (FcR)-mediated phagocytosis, both during pseudopod extension and actin polymerization, and during phagosome closure (Marie-Anais et al., 2016b). The role of Dynamin-2 in phagocytosis of zymosan and IgG-coated particles had already been reported previously without pointing to a precise mechanism (Gold et al., 1999). Given the strong requirement for Rho, ROCK, and Myosin II in actin assembly and particle engulfment in CR3-mediated phagocytosis (Olazabal et al., 2002), it could be hypothesized that CR3 phagosome formation and closure could rely on other molecular players than Dynamin-2. Here, we investigated whether Dynamin-2 plays a role in integrin-phagosome formation. Dynamin recruitment at the phagocytic cup during CR3-mediated phagocytosis was investigated in primary human monocyte-derived macrophages (hMDMs) using super resolution microscopy. Concomitant recruitment of F-actin and Dynamin-2 was monitored from the onset of CR3-phagosome formation until closure by live cell imaging with total internal reflection fluorescence microscopy (TIRFM). Actin quantification in phagocy-

tosis assays revealed that Dynamin-2 is vital for the formation of the phagocytic cup, and therefore, efficient CR3-mediated phagocytosis.

RESULTS

Dynamin-2 is recruited concomitantly with actin during CR3 phagosome formation and closure in macrophages

Direct stochastic optical reconstruction microscopy (dSTORM) was used to visualize primary human macrophages (hMDMs) in a “3D” arrangement as described previously (Marie-Anais et al., 2016a; Mularski et al., 2018). For this, hMDMs sediment onto coverslips coated with red blood cells (RBCs) opsonized with IgM followed by complement (C'-IgM-RBCs), and non-covalently bound to a poly-L-lysine activated surface (shown schematically in Figure 1a). Cells were allowed to phagocytose the opsonized RBCs for 10–15 min prior to fixation and staining to detect both F-actin and Dynamin-2 by dSTORM (Figure 1b). F-actin is accumulated in focal adhesion structures as well as at the site of nascent phagosomes (Figure 1b, left panel). Dynamin-2 is enriched in these F-actin rich phagocytic cups, although not exactly at the same place as F-actin accumulations (Figure 1b, middle panel). A tessellation-based colocalization analysis (Levet et al., 2015) was performed yielding a colocalization value for each single molecule localization of each species separately, minimizing false positive colocalization. The colocalization parameter, the Spearman coefficient, can have values from -1 (anticorrelation) through 0 (no correlation and therefore a low probability of colocalization) to 1 (perfect correlation and therefore a high probability of colocalization). The analysis allowed for a comparison of the actin-Dynamin and Dynamin-actin colocalization in the cell body (blue dashed line, Figure 1b) and the phagocytic cups (white dashed line, Figure 1b). The Spearman coefficients of actin with Dynamin and Dynamin with actin from analysis of regions of interest in the cell body are close to zero (means of 0.024 and 0.15 , respectively), indicating a low probability of colocalization (Figure 1c). The Spearman coefficients for actin-Dynamin and Dynamin-actin colocalization from regions of interest around phagocytic cups yield positive means (means of 0.30 and 0.28 , respectively), indicating a higher probability of colocalization in the phagocytic cups than in the cell body (Figure 1c). This result seems to indicate that Dynamin-2 is part of the CR3-phagosome formation process.

To better describe the role Dynamin-2 plays in CR3-dependent phagocytosis, we used TIRFM to observe the dynamics of recruitment of F-actin and Dynamin-2 in RAW264.7 macrophages transfected to transiently

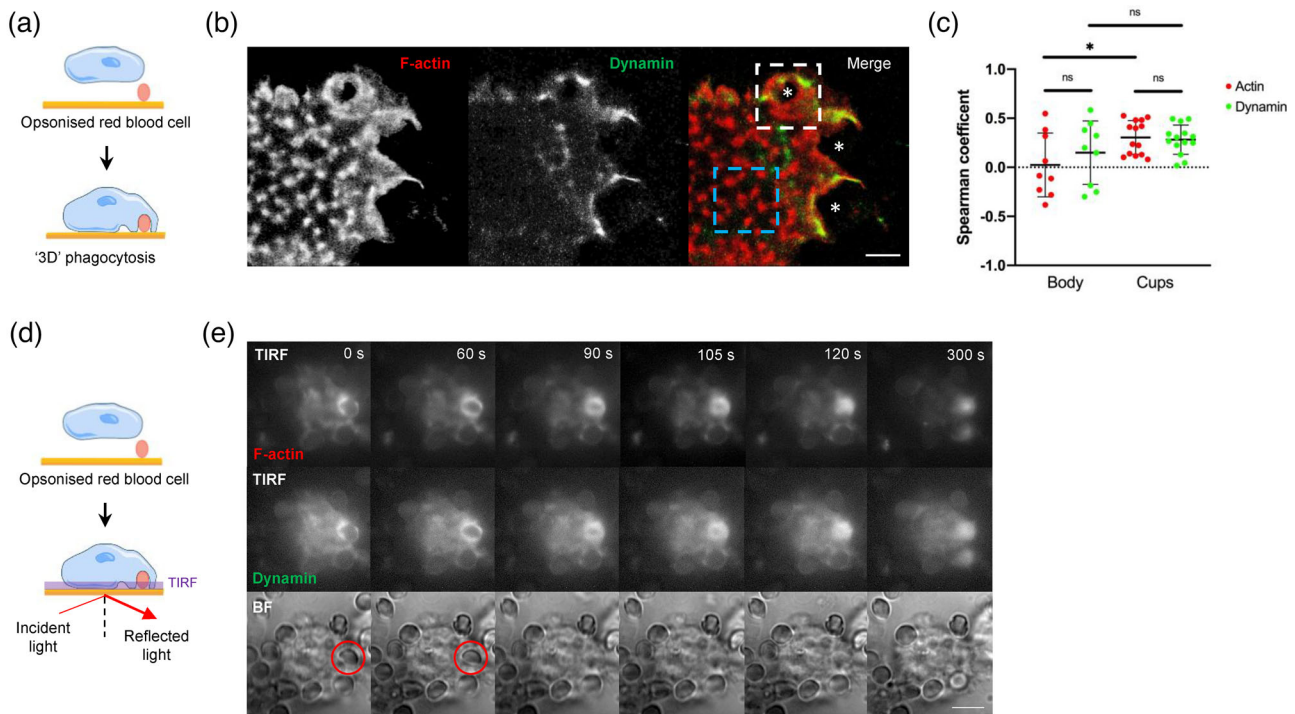


FIGURE 1 Dynamin-2 is recruited with actin during CR3 phagosome formation and closure. STORM visualization of hMDMs undergoing “3D” phagocytosis shows accumulation of F-actin and Dynamin-2. Schematic representation of a cell undergoing “3D” phagocytosis (a). Primary human macrophages are allowed to sediment on coverslips coated with loosely bound C'-IgM-RBCs and then fixed, permeabilized, and labeled. Actin was labeled with phalloidin-Alexa 647 and Dynamin-2 with Alexa-555. Cells were visualized with STORM (b), asterisks indicate the position of the RBCs, bar = 2.5 μm . Image analysis determined Spearman coefficients for actin (red circles) and dynamin (green circles) in regions of interest in cell body (b, blue dashed line, nine cell body regions) and phagocytic cups (b, white dashed line, 14 phagocytic cups) (c). Phagosome closure assay was performed using RAW 264.7 macrophages transiently expressing both LifeAct-mCherry and Dynamin-2-GFP. Shown schematically in d, cells are allowed to phagocytose opsonized particles that are non-covalently bound to the coverslip, allowing the site of closure to be observed using TIRFM. TIRFM images of a representative cell (of 10 replicate experiments) expressing LifeAct-mCherry (e, top row) and Dynamin-2-GFP (e, middle row) and brightfield images (e, bottom row) were captured every 0.5 s for 10 min at 37°C. The RBC phagocytosed is clearly visible in brightfield images at 0 and 60 s (b, bottom row, red circles), but pulled from the focal plane inside the cell by 90 s. Bar = 5 μm .

express LifeAct-mCherry and Dynamin-2-GFP. Cells were exposed to coverslips coated with C'-IgM-RBCs non-covalently bound to poly-L-lysine activated surface. After transfected cells were allowed to sediment to the surface, phagocytic cup extension and closure around the bound RBCs were observed in the TIRF region, as described in detail in (Mari-Anais et al., 2016a, 2016b; Mularski et al., 2018) and shown schematically in Figure 1d. A time-course of a representative macrophage (of 10 replicate experiments) undergoing CR3 mediated phagocytosis is shown in Figure 1e (movie available in Video S1). The RBC phagocytosed is clearly visible in brightfield images at 0 and 60 s (E, bottom row, red circles), but pulled from the focal plane inside the cell by 90 s. F-actin and Dynamin-2 are recruited concomitantly throughout the phagocytic event, from membrane extension around the RBC, through to fusion and scission, before disassembly. Dynamin-2 was recruited with actin at the tips of membrane extensions surrounding the RBC and accumulated at the site of phagosome closure and scission.

These results confirm that Dynamin-2 is present at different stages of CR3 phagosome formation and suggest a role for Dynamin-2 in phagosome completion from the plasma membrane.

Phagocytosis, but not association, is impaired by Dynamin-2 inhibition

To determine the role of Dynamin-2 in CR3-mediated phagocytosis, we inhibited its function prior to performing phagocytosis assays, as in (Mari-Anais et al., 2016a, 2016b). hMDMs were treated with DMSO (control) or with two different inhibitors of dynamin activity: dynasore and pyrimidin-7. After treatment, cells were centrifuged in medium containing C'-IgM-RBCs for 2 min to synchronize phagocytosis. Macrophages were either fixed at this point to assess the efficiency of RBC association (0 min), or, were allowed to phagocytose C'-IgM-RBCs for 60 min at 37°C to evaluate phagocytic efficiency. For both time points, following fixation, macrophages were

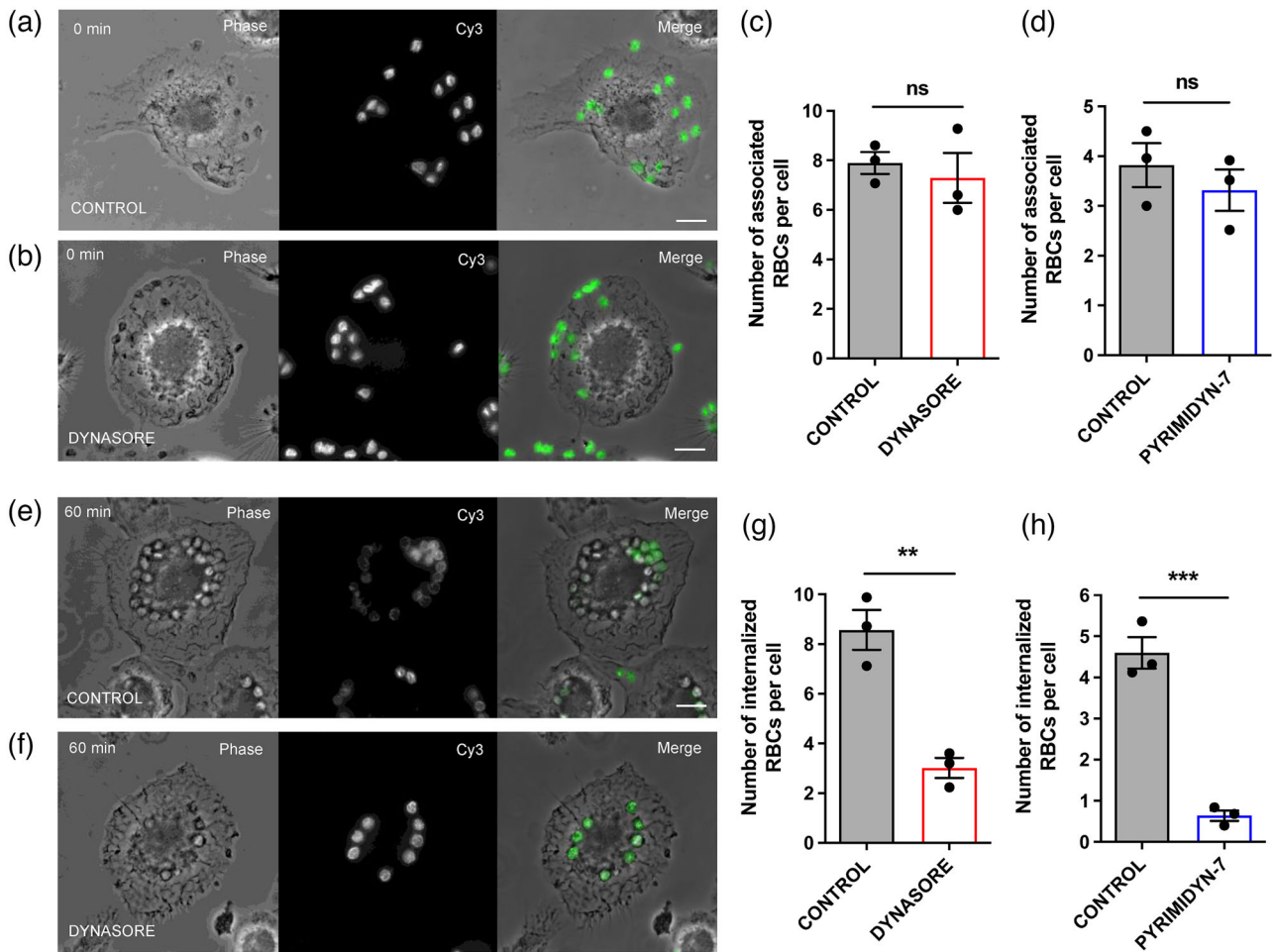


FIGURE 2 Pharmacological inhibition of Dynamin-2 impairs phagocytosis but not association. hMDMs were treated with DMSO (control, a and e), dynasore monohydrate (80 μ M, b and f) or Pyrimidin-7 (4 μ M). hMDMs were then allowed to phagocytose C'-IgM-RBCs for 0 (a and b) and 60 min (e and f), fixed and stained to detect bound but not internalized RBCs with Cy3-coupled anti-rabbit IgG antibodies. Internalized RBCs were detected using phase contrast. Average number of associated RBCs per cell (c and d) and internalized RBCs (g and h) were calculated in 25 cells per donor at 0 and 60 min, respectively. The means of three independent experiments are plotted ($* p < 0.01$). Bar = 10 μ m.

stained for RBCs (Figure 2a–e). The number of external (detected by fluorescence) and internal (detected by fluorescence and/or phase contrast) RBCs was quantified. The average number of associated (bound + internalized) RBCs per cell and internalized RBCs per cell were calculated in 25 cells per donor at 0 and 60 min, respectively. The average number of associated RBCs per cell from three independent experiments on different donors (Figure 2c,d), revealed no significant difference between cells treated with DMSO, dynasore, or pyrimidin-7, in their ability to engage with C'-IgM-RBCs. The average number of internalized RBCs per cell (Figure 2g,h), however, demonstrate that phagocytic efficiency is significantly reduced in cells treated with dynasore or pyrimidin-7 relative to control cells ($** p < 0.01$). This result indicates that inhibiting Dynamin-2 leads to the impairment of uptake without interfering with integrin-receptor binding to complement.

Inhibition of Dynamin-2 decreases F-actin recruitment to the phagocytic cup

Phagocytosis is an active process requiring F-actin polymerization to provide the force with which membranes are deformed to envelop the phagocytic target (Mularski & Niedergang, 2017; Torre-Gomez et al., 2020a). As CR3-mediated phagocytosis is impaired when Dynamin-2 is inhibited, F-actin quantification was performed at the site of phagocytosis in cells treated with DMSO (control) or dynasore. Phagocytosis was synchronized by centrifugation of hMDMs with C'-IgM-RBCs for 2 min. Macrophages were then allowed to phagocytose C'-IgM-RBCs for 15 min, then fixed and stained for RBCs and F-actin. Quantification was performed on four different macrophage preparations from four different donors. In control cells, many F-actin rich phagocytic cups under C'-IgM-RBCs were easily located (Figure 3a). When

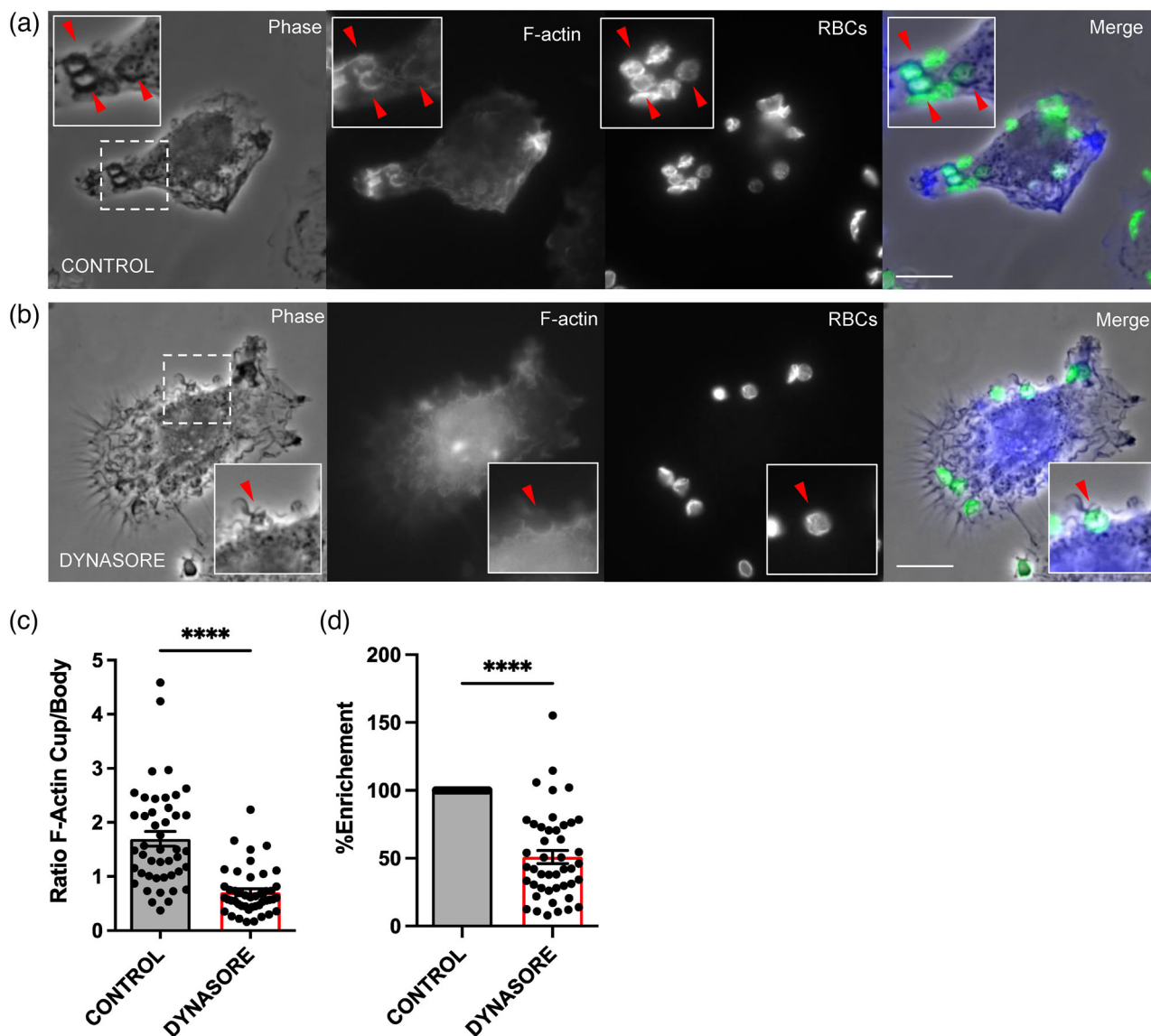


FIGURE 3 Pharmacological inhibition of Dynamin-2 decreases the F-actin recruitment to the phagocytic cup. hMDMs were treated with DMSO (control) or dynasore monohydrate (80 μ M). hMDMs were allowed to phagocytose C³-IgM-RBCs for 15 min, then fixed and stained to detect bound but not internalized RBCs with Cy3-coupled anti-rabbit IgG antibodies. Cells were then re-fixed, permeabilized and F-actin was stained with phalloidin-Alexa Fluor 488. Images for DMSO (a) and dynasore-treated (b) cells were acquired using an inverted wide-field microscope (Leica DMI6000) with a 100 \times (1.4 NA) objective. Bar = 10 μ m. Actin enrichment was quantified in phagocytic cups (a, b, red arrows): maximum fluorescence intensities of selected regions of interest were background corrected by subtracting the maximum intensity value from a membrane region in the same cell with no phagocytic event. Ratio values obtained by dividing the fluorescence intensities in the phagocytic cups by fluorescence intensities in the cell body were calculated (c). This ratio was expressed as a percentage of F-actin enrichment relative to control cells (d). The results of four independent experiments are plotted ($n = 15$ actin cups/condition, **** $p < 0.01$). Bar = 10 μ m, inset box = 12 μ m.

Dynamin-2 was inhibited, although RBCs were associated with the membrane, there were fewer phagocytic cups (Figure 3b). Regions of interest were selected where C³-IgM-RBCs were attached and a phagocytic cup was visible. F-actin recruitment was quantified as a ratio between the fluorescence in the phagocytic cup and the fluorescence in a cell body region without particle, as described in the Materials and Methods section. The index of F-actin enrichment at sites of phagocytosis was 1.8 ± 0.7 (Figure 3c) in the control cells treated

with DMSO. Macrophages pre-treated with dynasore monohydrate exhibited a significantly lower F-actin ratio cup to body index: 0.75 ± 0.4 (Figure 3c), representing 50% decrease in the F-actin recruitment at the site of CR3-phagocytosis in dynasore-treated macrophages as compared with control cells (Figure 3d). Therefore, F-actin enrichment in CR3 phagocytic cups is decreased when Dynamin-2 is inhibited by dynasore monohydrate, implicating Dynamin-2 in the actin assembly of the CR3 phagocytic cup.

DISCUSSION

In this study, we reveal that Dynamin-2 is crucial for CR3 integrin-mediated F-actin recruitment and phagosome completion. The CR3-mediated phagocytosis was often described as a “sinking” process relying on the formation of reduced phagocytic cups (Allen & Aderem, 1996; Kaplan et al., 1977). As pointed out recently (Jau-mouille et al., 2019; Patel & Harrison, 2008), when analyzed using live cell imaging methods, it becomes visible that this type of phagocytosis does also generate some membrane extensions. dSTORM and live TIRFM experiments used in this study also showed membrane protrusions containing F-actin that close over the phagocytic target (Figure 1). As CR3 is an integrin, formed by the $\alpha M\beta 2$ pair, the actin structures formed around the target particle are similar to focal adhesions, in that they contain talin, vinculin, and associated proteins (reviewed in [Torre-Gomez et al., 2020a]). Integrin activity is highly regulated by conformational changes. During the “inside-out” phase of integrin activation, there is an actomyosin-dependent stretching of talin that allows the release of RIAM and subsequent binding to vinculin (Torre-Gomez et al., 2020b; Vigouroux et al., 2020), which reinforces the association to the $\beta 2$ tail and its activation. Interestingly, it seems that Dynamin-2 was not implicated in regulating these actin-dependent steps of integrin activation in human macrophages that are required for efficient ligand binding prior to phagocytosis.

Instead, Dynamin-2 was critical for the “outside-in” signal downstream of CR3 leading to actin polymerization. This complex pathway involves the tyrosine kinase Syk, the small GTPases of the Rho family RhoG, and the actin nucleators VASP, Arp2/3 and Formin, as well as an actomyosin activity to orchestrate this integrin-mediated phagosome formation (Colucci-Guyon et al., 2005; Jaumouille et al., 2019; Olazabal et al., 2002; Torre-Gomez et al., 2020a). Here, we provide novel evidence that Dynamin-2 controls actin polymerization downstream of the phagocytic CR3. We used both a dynamin inhibitor, which might have some off-target effects in the absence of Dynamin (Preta et al., 2015), as well as transient expression of the Dynamin-2 K44A mutant (Orth et al., 2002), that is, unable to bind and hydrolyze GTP (not shown). Dynamin-2 has been shown to interact directly with actin *in vitro*, and its interaction with short F-actin filaments was found to promote its GTPase activity and its oligomerization, which was confirmed in cellulose (Gu et al., 2014). It was recently further deciphered how Dynamin bundles actin filaments *in vitro* and how this mechanism could play a role in the context of cell-cell fusion (Zhang et al., 2020). The assembly-stimulated GTPase activity of Dynamin triggers rapid GTP hydrolysis and helix disassembly. Of note, the nucleoside diphosphate kinase (NDPK), an

important regulator to fuel GTP to Dynamin, is recruited together with Dynamin-2 at the site of phagocytosis, thus ensuring efficient phagocytic uptake in various experimental models (Farkas et al., 2019). This mechanism of actin bundling could also be relevant in the context of CR3-phagocytosis. We have indeed shown previously that the integrity of the F-actin network is necessary for sustained recruitment and function of Dynamin-2 downstream of the phagocytic receptors for the FcR and, reversely, that Dynamin-2 activity was necessary for an efficient assembly of the F-actin cup upon FcR ligation (Mari-Anais et al., 2016a, 2016b). This is also in line with observations that actin polymerization precedes Dynamin recruitment at clathrin-coated pits in the last phases of clathrin-mediated endocytosis before the scission and release of vesicles (Grassart et al., 2014; Merrifield et al., 2002). Given the specificities of the CR3 integrin signaling and morphological features of the phagocytic cups, that Dynamin-2 plays a crucial role during CR3-phagosome shaping is particularly noteworthy.

Interestingly, the macropinocytic capture that shares many morphological features with CR3-phagocytosis has been described to be independent from Dynamin activity (Le Roux et al., 2012; Liberali et al., 2008). In the context of CR3-phagocytosis, F-actin could help control the membrane tension and therefore complement the constriction activity of Dynamin-2 for scission, as proposed (Morlot & Roux, 2013). Thus, Dynamin might contribute to the mechano-sensing and responding strategies used by the phagocytic cells to adapt their response to the physical properties of their targets, which is highly relevant to clear pathogens, debris, and tumor cells (Mularski & Niedergang, 2017).

MATERIALS AND METHODS

Antibodies, plasmids, and reagents

The following antibodies were used: rabbit anti-sheep RBCs (Accurate, AIAM6840); F(ab')₂-donkey anti-rabbit IgG-Cy3 (Jackson ImmunoResearch, 711-166-152); rabbit anti-Dynamin-2 (kindly provided, via Mathieu Boissan (Hospital Saint-Antoine, Paris), produced by the De Camilli Lab (Ferguson et al., 2009)); F(ab')₂-goat anti-rabbit IgG-Alexa Fluor 555 (Life Technologies, A21430); C5 deficient serum human (Sigma, C1163); Phalloidin-Alexa Fluor 488; and 647 (Molecular Probes, A12379 and A22287). The plasmid encoding LifeAct-mCherry was kindly provided by G. Montagnac (Institut Curie); the plasmids encoding DynWT-GFP was provided by Mark Mc Niven (Mayo Clinic). Dynasore monohydrate (Merck, D7693) was used at 80 μ M and Pyrimidin-7 (abcam, ab144501) at 4 μ M in phagocytosis assays.

Cell culture

RAW264.7 macrophages were maintained in complete medium consisting of RPMI 1640- GlutaMAX supplemented with 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μ M β -mercaptoethanol, and 10% fetal calf serum (all from Gibco).

Human peripheral blood mononuclear cells were isolated from whole blood of healthy donors (Etablissement Français du Sang Ile-de-France, Site Trinité, INSERM agreement #18/EFS/030 ensures all donors have provided written informed consent to provide anonymous samples) by density gradient sedimentation using Ficoll-Plaque (GE Healthcare). Cells were allowed to adhere in 6 or 24 well plates at 37°C for 2 h in adhesion medium (RPMI 1640-GlutaMAX supplemented with 2 mM L-glutamine and 100 μ g/mL streptomycin/penicillin) before cultivating them in complete medium (RPMI 1640- GlutaMAX supplemented with 10% FCS, 2 mM L-glutamine, and 100 μ g/mL streptomycin/penicillin). Every 2 days, complete medium was exchanged. The adherent monocytes were left to differentiate into macrophages (referred to as human monocyte-derived macrophages (hMDMs)) in the text as described previously (Jubrail et al., 2020) and used for experiments from day 5 to day 10 of differentiation.

Phagocytosis assay

Phagocytosis assays were performed with human monocyte-derived macrophages (hMDMs) on 12 mm glass coverslips before incubation with complement bound, IgM-opsonized sheep, red blood cells (C³-IgM-RBCs) as described elsewhere (Braun et al., 2004). C³-IgM-RBCs are resuspended in serum-free medium containing Dynamin-2 inhibitors: dynasore monohydrate (80 μ M), Pyrimidin-7 (4 μ M), or DMSO as a control. Optimal dynasore monohydrate concentration was determined in previous studies (Mari—Anais et al., 2016a, 2016b). hMDMs were exposed to inhibitors for 10 min prior to incubation with C³-IgM-RBCs. After 2 min of centrifugation (500 \times g, RT) to ensure sedimentation of C³-IgM-RBCs, macrophages were allowed to internalize C³-IgM-RBCs at 37°C for specified times then fixed in 4% PFA (paraformaldehyde 4%, PBS 1 \times) for 45 min at 4°C. Cells were then labeled for 45 min at room temperature with F(ab')₂ anti-rabbit IgG coupled to Cy3 in PBS1X/2% FCS to label bound, but not internalized RBCs. Cells were then washed three times with PBS1X/2% FCS and fixed again in 4% PFA for 15 min at room temperature, before being permeabilized in PBS1X/2% FCS/0.05% saponin for 15 min at room temperature. Actin was stained with phalloidin-Alexa Fluor 488 in PBS1X/2% FCS/0.05% saponin for 30 min at room temperature. After three washes in PBS1X/2% FCS/0.05% saponin, and two washes in PBS1X, nuclei

were stained with 0.3 μ g/mL DAPI (4',6-diamidino-2-phenylindole, Sigma) in PBS1X for 5 min and mounted using Fluoromount G (Interchim).

External RBCs are defined as particles positive for the fluorescent labeling and not detectable by phase contrast due to their physical properties. Internal RBCs appear weakly stained with the labeled F(ab')₂ anti-rabbit IgG because the cells are fixed prior to labeling. In addition, internal RBCs are detectable by phase contrast as holes in the cells.

External and internalized RBCs were counted in 25 randomly chosen cells, and the mean number of phagocytosed RBCs per cell was calculated. The number of cell-associated (bound + internalized) RBCs, was also counted, to calculate the mean number of associated RBCs per cell. Images were acquired using an inverted wide-field microscope (Leica DMI6000) with a 100 \times (1.4 NA) objective and a (MicroMAX Princeton Instruments) camera. Actin enrichment was quantified in phagocytic cups: maximum fluorescence intensities of a selected region of interest were background corrected by subtracting the mean value from a membrane region in the same cell with no phagocytosis event. Ratio values obtained by dividing the fluorescence intensities in the phagocytic cups by fluorescence intensities in the cell body were calculated as in (Braun et al., 2007).

Electroporation of RAW264.7 cells

Cells were transfected by electroporation with the ElectroBuffer kit from Cell Projects Ltd (EB-110). One 100 mm plate of cells, grown to sub-confluence, and 20 μ g of plasmid (10 μ g per plasmid for co-transfection) were used for each electroporation. Cells were electroporated in 4 mm cuvettes (Biorad) at 250 V, 900 μ F in an electroporation apparatus (X pulser Bio-Rad Laboratories) then immediately resuspended in complete culture medium and plated. Cells were used for experiments 15–18 h post electroporation.

Phagosome closure assay

Functionalized glass bottom dishes for phagosome closure assays were prepared according to the method described in (Marie—Anais et al., 2016a, 2016b; Marion et al., 2012; Mularski et al., 2018). Briefly, C³-IgM-SRBCs were centrifuged onto 35 mm glass bottom dishes (Mat-Tek) pre-treated with 0.01% poly-L-lysine in PBS for 30 min at RT. After washing once with 10% BSA in PBS, dishes were incubated in 10% BSA to saturate exposed poly-L-lysine. Dishes were then washed and filled with pre-warmed serum-free microscopy medium (RPMI 1640- GlutaMAX without phenol red, supplemented with 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μ M β -mercaptoethanol), and main-

tained at 37°C. Transfected cells were then allowed to sediment onto C'-IgM-RBCs adhered to the dish surface.

Total internal reflection fluorescence microscopy (TIRFM)

TIRFM was performed using a Till PHOTONICS iMIC microscope equipped with an oil immersion objective (Apo N 100×, NA1.49 Olympus America Inc.), a heating chamber maintained at 37°C and two cameras: a cooled iXonEM camera and an iXon3 897 Single Photon Detection EMCCD Camera (Andor Technology). The critical angle was determined prior to imaging by scanning through incident angles from 0° to 5° to maximize evanescent wave induced fluorescence (Mari-Anais et al., 2016a, 2016b). Excitation was performed with 491 and 561 nm lasers. For the phagosome closure assay, images were acquired at 50 ms per frame in bright field illumination, TIRF mode, and in epifluorescence mode with polychrome illumination at 3 μm increment. TIRFM image stacks were processed using ImageJ Color Profiler software (NIH).

Direct stochastic optical reconstruction microscopy (dSTORM)

Prior to fixation, coverslips were washed three times in PHEM buffer preheated to 37°C (PHEM buffer: 60 mM PIPES, 25 mM HEPES, 5 mM EGTA, 2 mM Mg(CH₃COO)₂ (Sigma), pH 6.9). Cells were then fixed for 15 min in 4% PFA and 0.02% glutaraldehyde (Sigma) preheated to 37°C. Permeabilization was then performed for 15 min at room temperature in 1XPBS/4% PFA, 0.02% glutaraldehyde, and 0.5% Triton X-100 (Sigma). Cells were incubated with the polyclonal anti-Dynamin-2, then with F(ab')₂-goat anti-rabbit IgG-Alexa Fluor 555 (Life Technologies). Coverslips were mounted on microscope slides with 80-μL cavities filled with optimized dSTORM buffer (Abbelight) containing phalloidin-Alexa Fluor 647 (Molecular Probes) for F-actin staining. Microscopy was performed on a Leica SR GSD (DMI 6000B) equipped with an oil immersion objective (HCX PL APO 160x, NA1.43, Leica), 532 and 642 nm lasers and an EMCCD Andor iXon Ultra 897. Protein enrichment and localization were quantified using ThunderSTORM (Ovesny et al., 2014) and SR-Tesseler (Levet et al., 2015). Tessellation based colocalization analysis was performed using Coloc-Tesseler software (Levet et al., 2015).

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CONFLICT OF INTEREST STATEMENT

No competing interests declared.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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