The nucleoside diphosphate kinase NDK-1/NME1 promotes phagocytosis in concert with DYN-1/Dynam

Zsolt Farkas,* Metka Petric†,‡,§ Xianghua Liu,† Floriane Herit†,‡,§ Éva Rajnavölgyi,† Zsuzsa Szondy,*,§ Zsófia Budai,† Tamás I. Orbán,*,** Sára Sándor,*,** Anil Mehta‡,### Zsuzsa Bajtay†,†† Tibor Kovács,§§ Sung Yun Jung,*†,§,†† Muhammed Afaq Shakir,§ Jun Qin,*‡‡ Zheng Zhou,†,§ Florence Niedergang,†‡,‡,§,† Mathieu Boissan,†††,§‡ and Krisztina Takács-Vellai*†,‡,§

*Department of Biological Anthropology, Eötvös Loránd University, Budapest, Hungary; ‡Department of Pathology, Duke University Medical Center, Durham, North Carolina, USA; †Department of Genetics, Eötvös Loránd University, Budapest, Hungary; ‡‡Department of Immunology and MTA-ELTE Immunology Research Group, and ‡§Department of Pathology, Faculty of Medicine, Eötvös Loránd University, Budapest, Hungary; ††Department of Medical Sciences, Ninewells Hospital Medical School, Dundee, United Kingdom; ‡‡‡Sorbonne Université, Université Pierre et Marie Curie (UPMC) Paris 06, INSERM, UMR 9393, Saint-Antoine Research Center, Paris, France; and †††Assistance Publique–Hôpitaux de Paris (AP-HP), Hospital Tenon, Service de Biochimie et Hormonologie, Paris, France

ABSTRACT: Phagocytosis of various targets, such as apoptotic cells or opsonized pathogens, by macrophages is coordinated by a complex signaling network initiated by distinct phagocytic receptors. Despite the different initial signaling pathways, each pathway ends up regulating the actin cytoskeletal network, phagosome formation and closure, and phagosome maturation leading to degradation of the engulfed particle. Herein, we describe a new phagocytic function for the nucleoside diphosphate kinase NDK-1 (NDK-1), the nematode counterpart of the first identified metastasis inhibitor NM23-H1 (nonmetastatic clone number 23) or nonmetastatic isoform 1 (NME1). We reveal by coimmunoprecipitation, Duolink proximity ligation assay, and mass spectrometry that NDK-1/NME1 works in a complex with DYN-1/Dynamin. Together, our data demonstrate that NDK-1/NME1 is an evolutionarily conserved element of successful phagocytosis.

Macrophages are professional phagocytes that play a critical role in innate and acquired immunity because of their special ability to internalize and degrade both pathogens and apoptotic cells. Phagocytosis is initiated by the interaction of specific receptors on the surface of the phagocyte with ligands on the targeted particle. Although all phagocytosis requires actin polymerization, phagocytosis mediated through different receptors uses common and distinct mechanisms to regulate actin dynamics (1). Thus, DYN-1/Dynamin (Caenorhabditis elegans/human homolog proteins), an atypical GTPase, has been shown to participate in the uptake of apoptotic cells (2), zymozan, IgG-opsonized, and C3b-opsonized particles, although each of them is taken up by different

ABBREVIATIONS: BMDM, bone marrow–derived macrophage; DPLA, Duolink PLA; FCS, fetal calf serum; GFP, green fluorescent protein; hMMDM, human macrocyte–derived macrophages; IP, immunoprecipitation; NDK-1, NDK-like 1; NDPK, nucleoside diphosphate kinase; NM23, nonmetastatic clone number 23; NME1, nonmetastatic isoform 1; PLA, proximity ligation assay; RBC, red blood cell; siRNA, small interfering RNA

† These authors contributed equally to this work.
‡ Correspondence: Department of Biological Anthropology, Eötvös Loránd University, Pázmány Péter stny. 1/C, H-1117 Budapest, Hungary. E-mail: takacs@falco.elte.hu
§ This is an Open Access article distributed under the terms of the Creative Commons Attribution 4.0 International (CC BY 4.0) (http://creativecommons.org/licenses/by/4.0/) which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

doi: 10.1096/fj.201900220R
phagocytic receptors (3). In the engulfment phase, in which particles are rapidly encircled and internalized by the phagocyte, DYN-1 contributes to the membrane extensions necessary for pseudopod formation (2, 3) and to the scission of the phagosome (4). During corpse degradation, DYN-1 function is once again essential for phagosome maturation (2).

DYN-1/Dynamin was originally identified in the fly as a determining factor in endocytosis-mediated neurotransmitter uptake (5). In the fly, awd (abnormal wing discs) was found in multiple mutagenesis screens as a surprisingly exclusive genetic interacting partner for dynamin (6). AWD is a member of the nonmetastatic clone number 23 (NM23) protein family and is responsible for about 98% of the fly nucleoside diphosphate kinase (NDPK) activity. NDPKs are primarily known to ping-pong from phosphate to nucleoside diphosphates through a highly conserved phosphohistidine intermediate to generate their cognate nucleoside triphosphates, thus providing energy for different cellular processes (7). The best characterized member of NM23 (known as NME in the international nomenclature) protein family is NM23-H1 [nonmetastatic isoform 1 (NME1)] (8). To explain the exclusivity of the NDPK–Dynamin interaction, Boissan et al. (9) showed that the cytosolic NM23-H1 Caenorhabditis or NME1 and the mitochondrial NM23-H4/NME4 isoforms function in one complex with the cytosolic Dynamin at the plasma membrane clathrin-coated pits and with the mitochondrial DYN-1–like GTPase at the mitochondrial inner membrane, respectively. NDPKs fuel GTP locally to Dynamin superfamily GTPases in order to permit them to work with the highest thermodynamic efficiency during membrane remodeling (10–12).

Here, we show that a worm NDPK, NDK-1, which recently emerged as a new player in apoptotic cell elimination playing a role in the apoptotic cell internalization (13, 14), is also an essential factor promoting phagosome maturation through its direct interaction with DYN-1/Dynamin in the model organism C. elegans.

We also found by testing various phagocytosis models that the positive effect exerted on phagocytosis by NDK-1 is evolutionarily conserved because mouse and human NDPK homologs NM23-M1 and NM23-H1, respectively, are also factors promoting phagocytosis.

MATERIALS AND METHODS

C. elegans strains

C. elegans strains were maintained at 20°C on OP50 bacteria–seeded nematode growth media plates (15). Wild-type worms were C. elegans variant Bristol (N2). Strains used were TTV2 eluEx[1::NDK-1::gfp; unc-119(+); unc-119(ed3)III] and TTV3 eluSi[NDK-1::GFP + cb-unc-119(+)]III.

Plasmid construction and generation of the transgenic array

The Pcad::ndk-1::mCherry plasmid was constructed by PCR amplifying the ndk-1 genomic DNA between the start and the stop codon (13) and inserting it into the SalI and Xmal sites of a vector that carries the cad-1 promoter (16) and the mCherry reporter cassette (unpublished results). Transgenic lines were generated by microinjection of Pcd::dyn-1::gfp and Pcad::ndk-1::mCherry together as previously described by Jin et al. (17). Plasmids were injected alongside the coinjection marker pUNC76 [unc-76(+)III] into unc-76(e911) mutant adult hermaphrodites as previously described by Bloom et al. (18), with non-unc animals being identified as transgenic animals.

Duolink in situ proximity ligation assay

To detect the NDK-1/DYN-1 protein complex at single molecule resolution in C. elegans embryos, an in situ proximity ligation assay (PLA) was performed as previously described by Söderberg et al. (19) according to the manufacturer’s protocol (Olink Biosciences, Uppsala, Sweden).

Extracted C. elegans comma-stage embryos from strain TTV2 were freeze-cracked then fixed with acetone and incubated with primary antibodies: anti–green fluorescent protein (GFP) (Merck monoclonal antibody 3580, diluted to 1:500) and anti–DYN-1 (kindly provided by ZZ.Z., diluted to 1:200). Secondary antibodies tagged with short DNA oligonucleotides were added. Hybridization, ligation, amplification, and detection were realized according to the manufacturer’s protocol (Olink Biosciences). The PLA signal corresponds to the Cy3 fluorescence. Coverslips were analyzed on an inverted wide-field microscope. For human macrophages, cells grown on coverslips were fixed with cold methanol, incubated with primary antibodies, and processed as previously described.

Coimmunoprecipitation

Co-immunoprecipitation (co-IP) was performed as previously described by Rocheleau et al. (20). Mixed-stage worms from the strains TTV3 and N2 were homogenized with Ultra-Turrax homogenizer (Ika, Staufen, Germany) in lysis buffer: 25 mM HEPES NaOH (pH 7.4), 150 mM NaCl, 1 mM DTT, 0.5% Triton X-100, 1 mM EDTA NaOH, and protease inhibitor cocktail tablet (Roche, Basel, Switzerland). Two micromicrograms of monoclonal anti-GFP antibody (3E6; Thermo Fisher Scientific, Waltham, MA, USA) was used to capture NDK-1:GFP from worm extracts. The adequate complexes were detected by immunoblotting using anti–DYN-1 polyclonal antibodies (provided by ZZ.Z., diluted to 1:1000). For detection, alkaline phosphatase-conjugated anti-rabbit secondary antibody (MilliporeSigma, Burlington, MA, USA) with nitro-blue tetrazolium and 5-bromo-4-chloro-3′-indolylphosphate substrate (1:50; MilliporeSigma) was applied.

IP and mass spectrometry analysis

Mixed-stage C. elegans was suspended in 5 vol of NETN solution (50 mM Tris/pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40) and lysed using a bead beater homogenizer at 4°C for 1 min. Lysate was cleared by ultracentrifugation (100 kg, 20 min, 4°C). Five microliters of affinity purified pAb against DYN-1 (21) or the control antibodies (pAb against CED-2, affinity purified) (unpublished results) was added to the clear lysate and incubated at 4°C for 1 h.

The antibody and lysate mixture was cleared by the ultracentrifugation (100 kg, 20 min, 4°C). Twenty microliters of protein A agarose beads was added and incubated for 1 h at 4°C. The beads were collected by 500 g spin and washed 3 times with NETN. The washed beads were loaded to 4–20% precast Novex Tris-Glycine gel (Thermo Fisher Scientific), and the gel was stopped when the dye front reached half-length. Gels were minimally stained with...
Institute (RPMI) 1640 medium supplemented with 100 U/ml Pseudomonas aeruginosa lipopolysaccharide. Then, blood mononuclear cells were isolated by density-gradient sedimentation in Ficoll (GE Healthcare, Waukesha, WI, USA). Primary human peripheral blood was obtained from healthy volunteers with informed consent and provided anonymized samples. Blood of healthy donors was used (Etablissement Français du Sang, IlélédeFrance, Site Trinité, France) with the appropriate ethics approval as stated in the Etablissement Français du Sang, IlélédeFrance, Site Trinité, France. Bone marrow progenitors were obtained from the femurs of 2–4- mo-old mice and isolated by density-gradient sedimentation in Ficoll (GE Healthcare, Waukesha, WI, USA). Then, monocytes were selected by adherence to dishes for 2 h at 37°C in fetal calf serum (FCS)-free medium [Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 100 U/ml penicillin 100 µg/ml streptomycin and 2 mM L-glutamine (Thermo Fisher Scientific)]. They were differentiated into macrophages for 8 d in adhesion medium supplemented with 10% FCS and 10 ng/ml recombinant human macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN, USA) as in (22, 23).

Small interfering RNA transfection of primary macrophages

Small interfering RNA (siRNA) transfection of human monocyte-derived macrophages (hMDMs) was as previously described by Marion et al. (24). Briefly, macrophages at d 4–5 were washed twice and kept in complete medium at 37°C. The siRNA solution was prepared in OptiMEM medium (GlutaMax supplemented; Thermo Fisher Scientific) containing Lipofectamine RNAiMax reagent (Thermo Fisher Scientific) and siRNA at a final concentration of 240 nM. Cells were incubated at 37°C for the indicated time. siRNA were 5'-GCCUGUAGGAAACGUAGUU-3' for NME1 and control siRNA 5'-CGUACGGGGAUUCCUGA-3' for Luciferase (Eurogentec, Liège, Belgium).

Five-day–matured bone marrow–derived macrophages (BMDMs) were transfected with On-TargetPLUS Smartpool siRNA specific for mouse Nme1 and On-TargetPLUS Non-targeting Control Pool (Dharmacon, Lafayette, CO, USA) using the Dharmafect 1 Transfection Reagent (Dharmacon) according to Dharmafect’s Transfection Protocol. At 48 h after transfection, cells were harvested for detecting the protein level of Nme1 of transfected BMDMs by Western blot analysis.

Experimental animals

The experiments were carried out with 4-wk-old or 2–4-mo-old C57B6 mice. Mice were maintained in specific pathogen-free conditions in the Central Animal Facility, and all animal experiments were approved by the Animal Care and Use Committee of University of Debrecen.

Bone marrow–derived macrophage cell culture

Bone marrow progenitors were obtained from the femurs of 2–4-mo-old mice lavaged with sterile physiologic saline. Cells were allowed to differentiate for 5 d in DMEM supplemented with 10% fetal bovine serum, 20% conditioned medium derived from L929 cells, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in 5% CO2. Nonadherent cells were washed away after 3 d.

Cell culture of primary human monocyte–derived macrophages

Blood of healthy donors was used (Etablissement Français du Sang, IlélédeFrance, Site Trinité, France) with the appropriate prior ethics approval as stated in the Etablissement Français du Sang and INSEM agreements 15/EFS/012 and 18/EFS/030, ensuring that all donors gave a written informed consent and providing anonymized samples. Primary human peripheral blood mononuclear cells were isolated by density-gradient sedimentation in Ficoll (GE Healthcare, Waukesha, WI, USA). Then, monocytes were selected by adherence to dishes for 2 h at 37°C in fetal calf serum (FCS)-free medium [Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 100 U/ml penicillin 100 µg/ml streptomycin and 2 mM L-glutamine (Thermo Fisher Scientific)]. They were differentiated into macrophages for 8 d in adhesion medium supplemented with 10% FCS and 10 ng/ml recombinant human macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN, USA) as in (22, 23).

In vitro apoptotic cell phagocytosis

To determine the effect of Nme1 gene silencing on the phagocytosis of apoptotic thymocytes, at 48 h after transfection, an in vitro apoptotic cell phagocytosis assay was performed. To generate apoptotic thymocytes, thymi were collected from 4-wk-old C57B6 mice, and thymocytes were isolated and cultured for 24 h (10^7 cells/ml) in RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.5 µM CellTracker Deep Red Dye (Thermo Fisher Scientific) in the absence of serum. Stained apoptotic thymocytes were added to the BMDMs in a 5:1 (apoptotic cells:macrophage) ratio for 1 h. After coculture, apoptotic cells were washed away, and macrophages were detached by trypsinization. The percentage of macrophages engulfing apoptotic cells was analyzed on a Becton Dickinson FACSCalibur (Becton Dickinson, San Diego, CA, USA).

In vitro phagocytosis assays with hMDMs

Phagocytosis assays were performed with adherent cells plated on glass coverslips (25). For microscopy, RBC were washed in PBS1X and incubated with anti-RBC antibodies for 30 min at room temperature, then washed and resuspended in serum-free medium. Zymosan particles were washed in PBS1X and resuspended in serum-free medium. After internalization of the IgG-RBC for the indicated times, cells were fixed in 4% paraformaldehyde (MilliporeSigma) and 4% sucrose for 45 min at 4°C, and external RBC were labeled for 30 min with labeled
F(ab')2 anti-rabbit IgG Alexa Fluor 488 in PBS and FCS 2%. Cells were then permeabilized with 0.05% saponin before intracellular labeling with the indicated antibodies or fluorescent phalloidin in PBS and saponin 0.05% or FCS 2%.

To quantify phagocytosis, the number of internalized RBC per cell was counted in 50 cells randomly chosen on the coverslips (phagosomes are identified by combination of phase contrast and fluorescent images) corresponding to the phagocytic index. To quantify association, the number of external + internal RBC per cell was counted in 50 cells randomly chosen on the coverslips corresponding to the association index. The indexes obtained were divided by the index obtained for control cells and expressed as a percentage of control cells.

Quantification of F-actin or other proteins’ recruitment in the phagocytic cups was performed as previously described by Braun et al. (25). Briefly, quantification was performed on ImageJ 64-bit software (NIH) on a selected region in 1 section of a 16-bit stack. Primary fluorescence intensities through the phagocytic cup and in the cell cortex were measured and background corrected. Ratio values (enrichment indexes) were calculated by dividing the fluorescence intensities in the phagocytic cups by the fluorescence intensities in the cell cortex and plotted. Image acquisition was performed on an inverted wide-field microscope (DMI6000; Leica Microsystems, Buffalo Grove, IL, USA) with a ×100 (1.4 NA) objective and an Orika Flash4.0 (Hamamatsu Photonics, Shizuoka, Japan). Z-series of images were taken at 0.3-μm increments.

**Statistical analyses**

Statistical analyses were performed using unpaired, 2-sided Student’s t test in Microsoft Excel software except for the phagocytosis assay of hMDMs, in which a 1-sided t test was used. The level of statistical significance was set at \( P < 0.05 \).

**RESULTS**

**The NM23-H1/NME1 homolog NDK-1 physically interacts with DYN-1/Dynamin in the worm**

Previously, we investigated defects of apoptosis in ndk-1(ok314)–null mutant worms (13), finding that embryos and germ cells defective for NDK-1 accumulated apoptotic cell corpses significantly in excess of wild-type worms. Around the dying germ cells, NDK-1::GFP expression was observed in the gonadal sheath cells that are specialized for engulfment and clearance of germ line corpses. We recently reported that ndk-1 exhibits a genetic interaction with dyn-1/dynamycin, whose role is well documented in apoptotic cell elimination (2, 13).

In order to further characterize the significance of the Dynamin/NDPK interaction in the worm during engulfment and corpse removal, we investigated the potential physical interaction between NDK-1 and DYN-1 by multiple approaches, including IP, IP–mass spectrometry, and Duolink PLA (DPLA). We found that DYN-1/Dynamin and NDK-1/NDPK co-immunoprecipitated in worms transgenic for NDK-1::GFP using a monoclonal anti-GFP antibody and probed with a highly specific polyclonal anti–DYN-1 antibody (21) (Fig. 1A).

Furthermore, we performed an IP–mass spectrometry experiment in wild-type, mixed-stage worm extracts using the above-mentioned anti–DYN-1 antibody. A partial list of the proteins identified in the DYN-1 complex is presented in Table 1. The full mass spectrometry data are deposited in the ProteomeXchange Consortium. Dynamin was first identified as a microtubule-interacting protein (26). The presence of DYN-1, the original bait, and 3 tubulins (tubulins 1, 2, and 4), which are C. elegans microtubule components, validated that our experiments were properly conducted. In the same assay, we have also identified NDK-1 as a specific member of the DYN-1-complex but not of the control complex.

During C. elegans development, 131 somatic cells are eliminated by apoptosis (27), and the vast majority (109) die during embryogenesis (28). The dying cells are cleared by well-documented neighboring cells. This information enabled us to further analyze the colocalization of NDK-1 and DYN-1 in vivo through a DPLA performed on fixed C. elegans comma-stage embryos (Fig. 1B–D). In the comma stage, which is characteristic for mid embryogenesis, we previously reported a mean of 10.9 cell corpses per wild-type embryo (13). In the DPLA, which gives a positive signal only in case the antibodies recognizing the proteins of interest are in close proximity, we detected a mean of 8.2 positive signals per comma-stage embryo (Fig. 1D, E). This correlates well with the number of dying cells mentioned above (13) because apoptotic cells are eliminated by given neighboring cells. These data show that NDK-1 and DYN-1 function in the same complex in cells that have the potential to engulf apoptotic corpses in nematode embryos.

**Time-lapse microscopy shows that NDK-1 and DYN-1 are located on phagosomal surfaces at the early stage of phagosome maturation**

To further characterize the dynamics of NDK-1 and DYN-1 during apoptotic cell corpse removal in embryogenesis, we generated transgenic lines that coexpressed the DYN-1::GFP and NDK-1::mCherry reporters in engulfing cells under the control of the ced-1 promoter. A time-lapse fluorescent microscopic imaging technique was used in living C. elegans embryos to monitor the engulfment and the subsequent phagosome maturation processes (29). Previously, DYN-1 was reported to be enriched on the surface of extending pseudopods and maturing phagosomes (2). Using this technique, we observed the enrichment of NDK-1::mCherry on the surface of a phagosome during the phagosome maturation process (Fig. 2). We further observed the partial colocalization of DYN-1::GFP and NDK-1::mCherry, in the punctate form, on the surface of a phagosome during the maturation process (Fig. 2). These results are consistent with cooperation of DYN-1 and NDK-1 during apoptotic clearance in a living organism.

**NM23-M1/NME1 is implicated in apoptotic cell engulfment and phagocytosis by mouse BMDMs**

Genetic pathways of apoptosis and apoptotic cell elimination are highly conserved between worms and
mammals (14). Hence, we were interested to determine whether the involvement of NDK-1 in apoptotic cell engulfment is evolutionarily conserved. NM23-M1, the mouse homolog of NDK-1 was studied using BMDMs. Macrophages were treated by Nm23-M1–specific or nontargeting siRNAs and compared with reagent controls. Western blots showed that Nm23-M1–specific silencing resulted in a 55% decrease in NM23-M1 protein levels (Fig. 3A). Next, an in vitro apoptotic cell phagocytosis assay was performed, in which apoptotic thymocytes were incubated with macrophages, and the phagocytic capacity of mouse macrophages was examined after Nm23-M1 silencing. Decreased NM23-M1 level in specific or targeting siRNA-treated macrophages caused a 40% loss in their phagocytic activity (Fig. 3B).

**TABLE 1. Partial list of proteins coimmunoprecipitated with C. elegans DYN-1 and detected with mass spectrometry**

| Name                        | Control IP         | DYN-1 IP | Peptides identified (n) |
|-----------------------------|--------------------|----------|-------------------------|
| 71981891 Dynamin (C. elegans) | dyn-1              | 0        | 291                     |
| 17552540 Tubulin β-2 chain (C. elegans) | tbb-2              | 0        | 191                     |
| 17553980 Tubulin β-chain (C. elegans)   | tbb-1              | 0        | 177                     |
| 17549915 Tubulin β-4 chain (C. elegans) | tbb-4              | 0        | 90                      |
| 17506807 Nucleoside diphosphate kinase (C. elegans) | ndk-1              | 0        | 11                      |
| 193209657 Vitellogenin-2 (C. elegans) | vit-2              | 51       | 505                     |
| 17570193 Vitellogenin-1 (C. elegans) | vit-1              | 32       | 339                     |

Control IP are proteins pulled down using polyclonal antibodies against C. elegans CED-2.
NM23-H1/NME1 is crucial to promote efficient phagosome formation in human macrophages

Next, we analyzed the interaction of NM23-H1/NME1 and Dynamin in primary human macrophages derived in vitro from blood monocytes using DPLA (Fig. 4A, B). The interaction was specifically detected in cells labeled with both the anti-NME1 and anti-Dynamin antibodies. The latter was a highly specific polyclonal anti-Dynamin antibody working in immunofluorescence and DPLA (9). To gain further insight into the role of NM23-H1/NME1 NME1 in human phagocytes, we examined the recruitment of endogenous NM23-H1/NME1 and Dynamin during phagocytosis of nonopsonized zymosan chosen to allow staining with specific antibodies against both proteins (Fig. 4C). Confocal sections revealed corecruitment of both NME1 and Dynamin in phagocytic cups, defined by the presence of F-actin (Fig. 4C). We calculated the enrichment of F-actin around the particles as compared with nonphagocytosing parts of the cell cortex and found that actin was enriched more than 4-fold at sites of phagocytosis (Fig. 4D). Similarly, NME1 and Dynamin were recruited in phagocytic cups with indexes of more than 2, showing clear enrichment at sites of phagocytosis in human macrophages of both NME1 and Dynamin.

Next, we depleted NME1 by RNA interference (Fig. 4E–H). The depletion is never complete in macrophages (24), but there was a clear and significant reduction in the expression of NME1 compared with a control siRNA sequence (Fig. 4E, F). We then performed phagocytosis of IgG-opsonized particles on siRNA-treated cells and monitored the efficiency of initial particle binding (2 min, Fig. 4G) and internalization (60 min, Fig. 4H). The results obtained on cells from 6 donors revealed a significant impairment of phagocytosis in cells depleted from NME1 as compared with the control, whereas there was no significant modification of particle association to the cells. These results show that NME1 is important for an efficient receptor-mediated phagocytosis in human macrophages.

DISCUSSION

Phagocytosis is the mechanism of internalization of large particles used to clear microorganisms but also to remove cell debris, which is important during development (4). The process of plasma membrane extension around the
Dynamin is important for actin remodeling during phagocytosis of apoptotic cells by mouse BMDMs. Macrophages were transfected with nontargeting siRNA or Nme1 siRNA by Dharmafect transfection reagent. At 48 h after transfection, cells were collected to determine protein levels of Nme1 by Western blot analysis (A) and to determine their phagocytic capacity (B) as described in Materials and Methods. Data represent the mean ± SD of 5 independent experiments. *Significantly different from respective control \( P < 0.05 \) determined by Student’s \( t \) test (\( n = 4 \)).

NDK-1 might provide GTP locally to DYN-1/Dynamin. Thus, we report that NDK-1, as a potential GTP supplier for DYN-1, promotes both the engulfment and phagosome maturation phase of the apoptotic clearance process in the worm. DYN-1 is known to act downstream of the phagocytic receptor CED-1 in the clearance of apoptotic cells (2). Recently, the pathway led by CED-1 was reported to promote both the clearance of damaged axonal debris but also the regeneration of new axons by acting in muscle-type engulfing cells (30). The expression of dyn-1 and ndk-1 has been detected in body wall muscles (2, 31). We propose that NDK-1 and DYN-1 might also participate in these 2 new activities.

In C. elegans embryos, careful time-lapse recording and genetic analyses have demonstrated that the removal of apoptotic cells is a multistage process (32). Within phagosome maturation, the last step of dying cell clearance, there are also multistages, each of which is marked by a distinct set of protein and lipid molecules on phagosomal surfaces. In particular, upstream regulators, such as receptor CED-1, DYN-1, the small GTPase RAB-5, and the class-II PI3-kinase PIKI-1, are the first group of proteins to be recruited to the surface of nascent phagosomes, followed by the subsequent production of PtdIns(3)P and the small GTPases RAB-2 and RAB-7 and the sorting nexins, which are the PtdIns(3)P effectors, to phagosomal surface (32). These sequential events eventually lead to the recruitment of early endosomes and lysosomes, the intracellular organelles that are essential for the degradation of apoptotic cells, to fuse to phagosomes (32). The recruitment of RAB-5, RAB-7, and PIKI-1 requires the phagosomal enrichment and the function of DYN-1, indicating that DYN-1 is an early factor (33, 34). The dynamic enrichment pattern of DYN-1 on nascent phagosomes is consistent with the stage in which its function is needed (2). Based on the above evidence, we hypothesize that DYN-1, as an upstream regulator, acts to prime phagosomal surfaces for the downstream events and dissociates from the phagosomal membrane once its job is done. As a positive regulator of DYN-1, NDK-1 is likely to follow the dynamic enrichment pattern of DYN-1 on the phagosomal surface.

We were interested to see whether NME1 plays a similar role during phagocytosis in mammalian cells and focused first on the Dynamin/NME1 interaction during internalization of phagocytic particles in primary human macrophages. We confirmed the presence of the Dynamin/NME1 complex in hMDM cells by the highly specific DPLA method. Localization studies of endogenous NME1 and Dynamin during phagocytosis of insert particles revealed corecruitment of the 2 proteins to the phagocytic cups. Next, phagocytosis of IgG-opsonized particles was used to monitor the effect of NME1 knockdown in human macrophages. Depletion of NME1 resulted in a significant impairment of phagocytosis.

Besides hMDMs, we also examined the function of the mouse NME1 homolog, NM23-M1, in mouse BMDMs during elimination of apoptotic thymocytes. Silencing of NM23-M1 in BMDM cells resulted in decreased phagocytosis of apoptotic thymocytes.

Importantly, we demonstrate that the phagocytosis-promoting function of the mammalian homologs NM23-M1 and NM23-H1/NME1 is evolutionarily conserved.
because silencing of the appropriate NME1 homologs caused a decreased phagocytic capacity in worms as well as in mouse BMDMs and hMDMs tested in various phagocytosis models. Thus, we propose NME1/Dynamin cooperation as a global mechanism of successful phagocytosis.

ACKNOWLEDGMENTS

This work was supported by the Hungarian Scientific Research Fund (OTKA) Grants K115587 (to K.T.-V.), K112112 (to T.I.O.), and K124244 (to Z.S.); the GNP02.3.2-15-2016-00006 Project (cofinanced by the European Union and the European Regional Development Fund) (to Z.S.); and the UNKP-17-3 New National Excellence Program of the Ministry of Human Capacities grant (to Z.F.). This work was completed in the ELTE Institutional Excellence Program (1783-3/2018/FEKUTSRAT) supported by the Hungarian Ministry of Human Capacities. Work in the F.N. laboratory was supported by Centre National de la Recherche Scientifique (CNRS), INSERM, Université Paris Descartes, and the Agence Nationale de la Recherche (ANR 16-CE13-0007-01). The work of the Z.Z. laboratory was financed by U.S. National Institutes of Health, National Institute of General Medical Sciences Grant R01GM067848. Work in the M.B. laboratory was supported by INSERM, Sorbonne Université, and Groupe des Entreprises Françaises de Lutte Contre le Cancer (GEFLUC). Z.Z., F.N., M.B., and K.T.-V. are colast authors. The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Z. Farkas, M. Petric, X. Liu, F. Herit, Z. Szondy, Z. Budai, S. Sándor, T. Kovács, M. Afq Shakir, J. Qin, S. Y. Jung, Z. Zhou, F. Niedergang, M. Boissan, and K. Takács-Vellai

Figure 4. Role of NME1 in phagosome formation in human. Primary human macrophages were differentiated from blood monocytes. A, B) PLA was performed with anti-NME1 and anti-Dynamin antibodies. Images were acquired (A), and quantification of spots was performed and analyzed by ANOVA (B). Phagocytosis of zymosan was performed for 10 min before fixation, permeabilization, and labeling with phalloidin Alexa 635 and anti-NME1 followed by Cy3-coupled anti-mouse IgG antibodies and anti-Dynamin antibodies followed by Alexa 488–coupled anti-rabbit IgG antibodies. One confocal section is shown. C) Asterisks label the phagocytic cups. D) Protein enrichment in phagocytic cups was determined as described in Materials and Methods. E–H) Cells were treated with siRNA against NME1 (siNME1) or luciferase (siLuc) as a control for 72 h before cell lysis and Western blot analysis (E, F) or phagocytosis assay with IgG-opsonized red blood cells (G–H). Association (G) and phagocytosis (H) efficiencies were calculated as indicated in Materials and Methods and expressed as a percentage of control cells treated with siLuc for 6 different donors. Error bars represent SEM. For depletion, association, and phagocytosis, we used 1-sided Student’s t tests. Scale bar, 5 μm. Error bars represent SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
analyzed data; Z. Farkas, M. Petric, X. Liu, F. Herit, Z. Budai, S. Sándor, Z. Bajtay, T. Kovács, M. Afaq Shakir, J. Qin, and M. Boissan performed research; Z. Farkas, Z. Szondy, A. Mehta, S. Y. Jung, Z. Zhou, F. Niedergang, M. Boissan, and K. Takács-Vellai designed research; Z. Farkas, Z. Szondy, T. I. Orbán, Z. Zhou, F. Niedergang, M. Boissan, and K. Takács-Vellai contributed new reagents or analytic tools; and Z. Farkas, E. Rajnavölgyi, Z. Szondy, A. Mehta, Z. Bajtay, Z. Zhou, F. Niedergang, M. Boissan, and K. Takács-Vellai wrote the manuscript.

REFERENCES

1. Allen, I. A., and Adereum, A. (1996) Molecular definition of distinct cytoskeletal structures involved in complement and Fc receptor-mediated phagocytosis in macrophages. J. Exp. Med. 184, 627–637
2. Yu, X., Odera, S., Chuang, C.-H., Lu, N., and Zhou, Z. (2006) C. elegans dynamin mediates the signaling of phagocytic receptor CED-1 for the engulfment and degradation of apoptotic cells. Dev. Cell 10, 743–757
3. Gold, E. S., Underhill, D. M., Morrissette, N. S., Guo, J., McNiven, M. A., and Adereum, A. (1999) Dynamin 2 is required for phagocytosis in macrophages. J. Exp. Med. 190, 1849–1856
4. Marie-Anais, F., Mazzolini, J., Herit, F., and Niedergang, F. (2016) Dynamin-actin cross talk contributes to phagosome formation and closure. Traffic 17, 487–499
5. Van der Bliek, A. M., and Meyerowitz, E. M. (1991) Dynamin-like protein encoded by the Dro sophila shibire gene associated with vesicular traffic. Nature 351, 411–414
6. Krishnan, K. S., Rikhy, R., Rao, S., ShivaKirakan, K. S., Rikhy, R., Rao, S., Shivalkar, M., Mosk, M., Boissan, M., Montagnac, G., Shen, Q., Griparic, L., Guitton, J., Romao, J., Leuchowius, K., Jarvius, M., Ridderdstrale, K., Leuchowius, K.-J., Jarvius, K., Wester, K., Hylbring, P., Bahram, F., Larson, L.-G., and Landegren, U. (2006) Direct observation of individual endogenous protein complexes in situ by proximity ligation. Nat. Methods 3, 995–1000
7. Steeg, P. S., Palmieri, D., Ouatas, T., and Salerno, M. (2003) Histidine diphosphate kinases fuel dynamin superfamily proteins with GTP. EMBO J. 22, 3466–3476
8. Boissan, M., Montagnac, G., Shen, Q., Griparic, L., Guitton, J., Romao, M., Sauvonnè, N., Lagache, T., Lascu, I., Raposo, G., Desbordes, C., Schlatter, U., Lacombe, M.-L., Polo, S., van der Bliek, A. M., Roux, A., and Chavrier, P. (2014) Membrane trafficking. Nucleoside diphosphate kinases fuel dynamin superfamily proteins with GTP for membrane remodeling. Science 344, 1510–1515
9. Takai, K., McPherson, P. S., Schmidt, S. L., and De Camilli, P. (1995) Tubular membrane invaginations coated by dynamin rings are induced by GTP-gamma S in nerve terminals. Nature 374, 186–190
10. Sweitzer, S. M., and Hinshaw, J. E. (2007) Dynamin undergoes a GTP-dependent conformational change causing vesiculation. Cell 93, 1021–1029
11. Roux, A., Uyhazi, K., Frost, A., and De Camilli, P. (2006) GTP-dependent twisting of dynamin implicates constriction and tension in membrane fission. Nature 441, 529–531
12. Fancsalszky, L., Monostori, E., Farkas, Z., Poukarimé, E., Masoudi, N., Hargitai, B., Bosna, M. H., Dezeljin, M., Zsáki, A., Vellai, T., Mehta, A., and Takács-Vellai, K. (2014) NDK-1, the homolog of NM23-H1/H2 regulates cell migration and apoptotic engulfment in C. elegans. PLoS One 9, e92967
13. Conrad, B., Wu, Y.-C., and Xue, D. (2016) Programmed cell death during Caenorhabditis elegans development. Genetics 203, 1533–1562
14. Brenner, S. (1974) The genetics of Caenorhabditis elegans. Genetics 77, 71–94
15. Zhou, Z., Hartwig, E., and Horvitz, H. R. (2001) CED-1 is a transmembrane receptor that mediates cell corpse engulfment in C. elegans. Cell 104, 43–56
16. Jin, Y. (1999) Transformation. In C. elegans, a Practical Approach (Hope, I. A., ed.), pp. 69–96, Oxford University Press, Oxford, United Kingdom
17. Bloom, L., and Horvitz, H. R. (1997) The Caenorhabditis elegans gene unc-76 and its human homologs define a new gene family involved in axonal outgrowth and fasciculation. Proc. Natl. Acad. Sci. USA 94, 3414–3419
18. Söderberg, O., Gullberg, M., Jarviis, M., Ridderdstrale, K., Leuchowius, K.-J., Jarvius, K., Wester, K., Hylbring, P., Bahram, F., Larson, L.-G., and Landegren, U. (2006) Direct observation of individual endogenous protein complexes in situ by proximity ligation. Nat. Methods 3, 995–1000
19. Rocheleau, C. E., Yasuda, J., Shin, T. H., Lin, R., Sawa, H., Okano, H., Priess, J. R., Davis, R. J., and Mello, C. C. (1999) WRM-1 activates the LIT-1 protein kinase to transduce anterior/posterior polarity signals in C. elegans. Cell 97, 717–726
20. He, B., Yu, X., Margolis, M., Liu, X., Leng, X., Etzion, Y., Zheng, F., Lu, N., Qiao, F. A., Danino, D., and Zhou, Z. (2010) Live-cell imaging in Caenorhabditis elegans reveals the distinct roles of dynamin self-assembly and guanosine triphosphate hydrolysis in the removal of apoptotic cells. Mol. Biol. Cell 21, 610–629
21. Boissan, M., Montagnac, G., Shen, Q., Griparic, L., Guitton, J., Romao, J., Sauvonnè, N., Lagache, T., Lascu, I., Raposo, G., Desbordes, C., Schlatter, U., Lacombe, M.-L., Polo, S., van der Bliek, A. M., Roux, A., and Chavrier, P. (2014) Membrane trafficking. Nucleoside diphosphate kinases fuel dynamin superfamily proteins with GTP for membrane remodeling. Science 344, 1510–1515
22. Shpetner, H. S., and Valler, R. B. (1989) Identification of dynamin, a novel mechanochemical enzyme that mediates interactions between microtubules. Cell 59, 421–432
23. Subston, J. E., and Horvitz, H. R. (1977) Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Dev. Biol. 56, 110–156
24. Subston, J. E., Schierenberg, E., White, J. G., and Thomson, J. N. (1985) The embryonic cell lineage of the nematode Caenorhabditis elegans. Dev. Biol. 100, 64–119
25. Li, Z., Lu, N., He, X., and Zhou, Z. (2013) Monitoring the clearance of apoptotic and necrotic cells in the nematode Caenorhabditis elegans. Methods Mol. Biol. 1004, 183–202
26. Chiu, H., Zou, Y., Suzuki, N., Hsieh, Y.-W., Chuang, C.-F., Wu, Y.-C., and Chang, C. (2018) Engulfing cells promote neuronal regeneration and remove neuronal debris through distinct biochemical functions of CED-1. Nat. Commun. 9, 4842
27. Masoudi, N., Fancsalszky, L., Poukarimé, E., Vellai, T., Alexa, A., Reményi, A., Gartner, A., Mehta, A., and Takács-Vellai, K. (2013) The NM23-H1/H2 homolog NDK-1 is required for full activation of Ras signaling in C. elegans. Development 140, 3486–3495
28. Lu, N., and Zhou, Z. (2012) Membrane trafficking and phagosome maturation during the clearance of apoptotic cells. Int. Rev. Cell Mol. Biol. 293, 269–309
29. Yu, X., Lu, N., and Zhou, Z. (2008) Phagocytic receptor CED-1 initiates a signaling pathway for degrading engulfed apoptotic cells. PLoS Biol. 6, e161
30. Lu, N., Shen, Q., Mahoney, T. R., Neukom, L. J., Wang, Y., and Zhou, Z. (2012) Two PI3-kinases and one PI3-phosphatase together establish the cyclic waves of phagosomal PtdIns(3)P critical for the degradation of apoptotic cells. PLoS Biol. 10, e1001245

Received for publication January 23, 2019. Accepted for publication May 21, 2019.