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Activated toll-like receptor 4 is involved in oridonin-induced phagocytosis via promotion of migration and autophagy-lysosome pathway in RAW264.7 macrophages

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ABSTRACT

In our previous study, we demonstrated that oridonin enhances phagocytosis of apoptotic bodies by macrophage-like cells by inducing autophagy. However, the direct sensor of autophagy and the key event controlling phagocytosis remains unknown. Herein, we showed that Toll-like receptor 4 (TLR4), known to mediate immune responses, was activated by oridonin. Activated TLR4 contributes to phagocytosis of apoptotic cells by RAW264.7 macrophages. Indeed, inhibition or small interfering RNA (siRNA) silencing of TLR4 significantly attenuated oridonin-induced phagocytosis. Inhibition of TLR4 also decreased the level of autophagy and its associated proteins, Beclin-1 and light chain 3 (LC3), suggesting that activated TLR4 is involved in activation of autophagy. LPS-induced activation of TLR4 promoted phagocytosis and autophagy progression. Activation of TLR4 accompany increase in activities of lysosome acid phosphatase and cathepsin B as well as in up-regulation of lysosomal-associated membrane protein (LAMP 1 and 2) levels. Furthermore, TLR4 in association with translocation to cytoplasm leads to macrophage motility or migration through increased plasticity of skeleton and/or membrane structure. These results suggest that oridonin-induced phagocytosis of apoptotic bodies by macrophages is TLR4 signal pathway-mediated, via activation of the autophagy-lysosome pathway as well as increase of cell migration.

1. Introduction

Induction of cellular apoptosis is a common and effective strategy for antitumour therapy. Following apoptosis, cells release signals that lead to engulfment and phagocytosis by neighbouring macrophages. Effective recognition and removal of apoptotic cells are essential for both immune defences and tissue homeostasis. This dynamic process is also important for protecting against tumour recurrence and autoimmune diseases [1]. During initiation and execution of phagocytosis, activated membrane engulfment receptors may play an important role in uptake and digestion of dying or expendable cells. Toll-like receptors (TLRs) are important pattern recognition receptors (PRRs) that sense specific pathogen-associated molecular patterns (PAMPs) and lead to the triggering of intracellular pro-inflammatory or innate immunity responses [2]. TLR family member TLR4 is a lipopolysaccharide protein receptor that recognises pathogen-specific surface antigens such as lipopolysaccharide (LPS), heat shock proteins (HSPs), microbe-associated opsonins, and other components [3]. Accumulating evidence indicates that TLR4 signalling plays a crucial role in host defences against Gram-negative bacteria by recognising the outer membrane component and activating of variety of defence mechanisms within endocytosis [4,5]. Recent findings indicate that agonists of TLR4 increase phagocytosis

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and intracellular killing of *Escherichia coli*, and mediate host defences against *Salmonella typhimurium* [6,7]. Higher levels of surface TLR4 of macrophages increase phagocytosis of amyloid beta (Aβ) deposit or necrotic cells, playing a protective role in neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) [8,9]. However, TLR4 is still poorly understood in terms of the phagocytosis functions of apoptotic cells and the intracellular machinery involved. TLR4 is a sensor for autophagy associated with innate immunity. TLR4 mediates numerous critical biological processes such as host defences, clearance of inclusion bodies, and catabolism via coordination of autophagy-lysosome pathways [10,11].

Autophagy is an essential 'self-eating' process that removes damaged organelles and long-lived proteins from the cytoplasm. Autophagy plays an important role in cell survival and homoeostasis via degradation of unnecessary or dysfunctional cellular components, as well as invading microorganisms. Recently, autophagy was found to be involved in both innate and adaptive immunity against intracellular bacteria, viruses and inflammatory factors [12]. Activation of macroautophagy leads to degradation of intracellular components by engulfment into double-membrane vesicles or autophagosomes, followed by phagocytosis through highly-conserved, homeoplastic processes. Both autophagosomes and phagosomes are fused with lysosomal membranes to phagolysosomes and eventually cleared within.

Our previous studies revealed that oridonin extracted from the Chinese traditional medicine *Herba Rabdosiae* can promote phagocytosis of apoptotic bodies by macrophage-like U937 cells by inducing autophagy. Proteins associated with autophagosomes such as Beclin-1 and LC3 are involved in oridonin-induced phagocytosis of apoptotic cells [13]. Oridonin is a pharmacologically and physiologically active diterpenoid compound used widely in the clinic. However, it is not certain whether oridonin can target TLR4, or whether TLR4 is an initial factor upstream of autophagy that participates in the regulation of oridonin-induced phagocytosis. In the present study, we investigated the influence of oridonin on TLR4, and explored the mechanisms relating the TLR4 signalling pathway to oridonin-induced phagocytosis of apoptotic cells. In particular, the influence of TLR4 on autophagy and lysosome pathways was illuminated. For recognition and engulfment of the apoptotic cells by macrophages requires cell migration or approach to the target as an essential process. Therefore, we also investigate the effect of activation TLR4 on macrophage migration.

2. Materials and methods

2.1. Reagents

Oridonin (purity 99.7%) was provided by the Kunming Institute of Botany, The Chinese Academy of Sciences (Kunming, China). 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI), propidium iodode (PI), monodansylcadaverine (MDC), 3-methyladenine (3-MA), TLR4 inhibitor (IN-C34, abbreviated as IN in the text), and lipopolysaccharide (LPS) were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Polyclonal antibodies against TLR4, toll-interleukin-1 receptor domain-containing adapter molecule (TRIF), myeloid differentiation primary response protein 88 (MyD88), tumour necrosis factor receptor-associated factor 6 (TRAF6) were purchased from cell signalling. Beclin-1, LC3, LAMP1, LAMP2, β-actin and horseradish peroxidase-conjugated secondary antibodies obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

2.2. Cell culture

RAW264.7 cells were obtained from American Type Culture Collection (Manasas, VA, U.S.A.). The cells were cultured in DMEM (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% FBS, 0.03% L-glutamine (Gibco), penicillin (100 U/ml) and streptomycin (100 mg/ml) and maintained at 37 °C with 5% CO_2 in a humidified atmosphere.

2.3. Molecular dynamics computer simulation of oridonin binding site

The crystal structures of TLR4 prepared by DS 3.0. AutoDock Tools 1.5.4 (ADT) was used. The number 3fxi protein was chosen as research items. Discovery Studio 2017 Client was used for molecular interaction analysis. To improve the accuracy of molecular docking calculation, Autodock Vina score was utilized to predict the binding free energies of oridonin with TLR4.

2.4. Endotoxin removal

Endotoxins were removed from oridonin using Endotoxin Removal Agarose Resin Polymyxin B (PMB)-columns (Yeasen, Shang Hai, China) and the presence of endotoxins was assessed prior to and after removing endotoxins using ELISA kit (DRG, NJ, U.S.A) according to the manufacturer's instructions.

2.5. siRNA and transfection

The control (NS) and TLR4 siRNA (siRNA) were purchased from Ji Ma Biochemical (5′-GTCTCAGATATCTAGATCT-3′, Shang Hai, China). Transient transfection of RAW264.7 cells with siRNAs were performed using transfection agent TranSmarter (Abmart). According to the manufacturer's protocol, 48 h after transfection, cells were harvested and treated with oridonin for 24 h prior to analysis.

2.6. Immunofluorescence and confocal microscopy

Treatment with 2.5 μM oridonin for 24 h, Raw264.7 cells were fixed with 4% paraformaldehyde at room temperature for 10 min. After washing three times with ice-cold PBS, the cells were blocked in 2% BSA in PBS-T (0.1% Tween 20) at room temperature for 30 min. Sections were incubated with FITC labeled TLR4 antibody prepared in fresh blocking buffer at room temperature for 1 h. Then, the cells washed three times with PBS, and the coverslips were stained with DAPI for 10 min. Images were visualized using confocal microscopy (Nikon ECLIPSE Ti).

2.7. Fluorescent microscopy and flow cytometric analysis of phagocytosis

UV irradiated (52.1 J/m^2, 4 min) apoptotic Raw264.7 cells were labeled with PI solution (1 ml, 50 mg/L) in the dark for 30 min. After washing twice with PBS, the labeled cells were co-incubated with live RAW264.7 (pretreated with oridonin or LPS in the presence or absence of inhibitor or siRNA for 24 h) for another 2.5 h. Then, the cells were washed and stained with DAPI for 10 min. After washing with PBS, the cells on the plates were washed with PBS before detection under fluorescent microscopy (Olympus, Tokyo, Japan) with the emission wavelength at 525 nm. Then the cells were scraped and suspended in PBS, and 10,000 cells were analyzed by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

2.8. Fluorescent microscopy of autophagy with (MDC) staining

RAW264.7 cells (2×10^5/well) were inoculated in 6-well culture plates and were treated with oridonin or LPS with or without IN or siRNA for 24 h. Then the cells were incubated with 0.05 mM MDC, autophagy vacuole specific dye, at 37 °C for 40 min. After washing, the fluorescent changes were observed by Olympus IX70 reverse fluorescence microscopy (Olympus, Tokyo, Japan) with the emission wavelength at 525 nm.

2.9. Flow cytometric analysis of autophagy

RAW264.7 cells (2×10^5/well) were inoculated in 25 ml culture flasks and were treated with oridonin or LPS with or without IN or

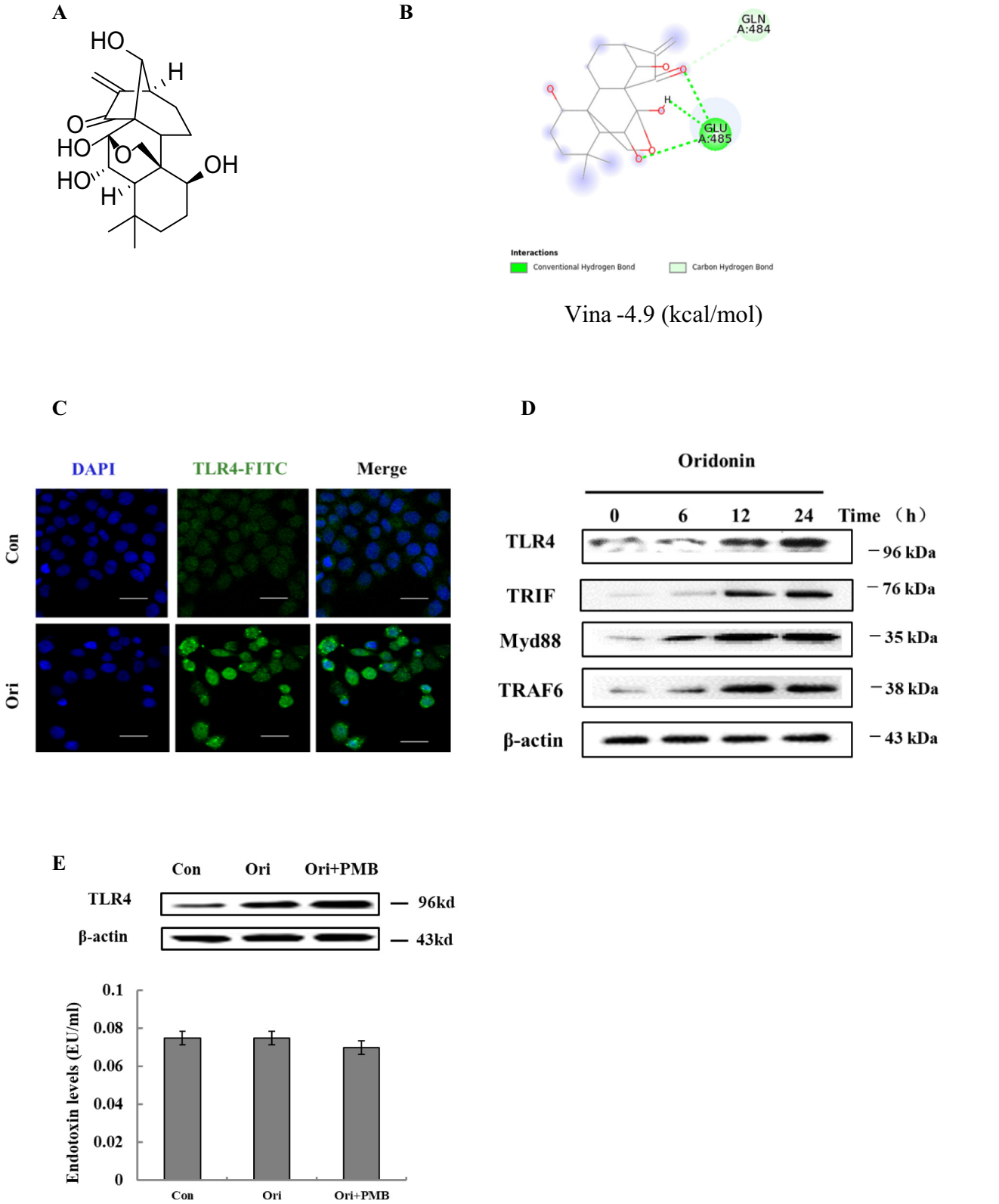

Fig. 1. Oridonin potentially binds with TLR4 and activates TLR4 signalling in RAW264.7 cells. (A) Chemical structure of oridonin. (B) Molecular dynamics simulation reveals that oridonin potentially binds to TLR4 via conventional Hydrogen bond (GLU A: 485) or Carbon Hydrogen bond (GLU A: 484). (C-D) Oridonin activates TLR4 pathway in RAW264.7 cells. (C) Representative images of immunofluorescent staining of TLR4 in oridonin-treated RAW264.7 cells. (×400 magnification, bar = 20 mm). (D) After incubation with 2.5 μM oridonin for the indicated time points, TLR4, TRIF, MyD88 and TRAF6 proteins expression was detected by Western blot. β-Actin serves as a loading control. (E) The presence of endotoxins and TLR4 expression were assessed after endotoxins purification of oridonin (2.5 μM) in this study.

siRNA for 24 h. The collected cells were suspended in 0.05 mM MDC at 37 °C for 40 min. Then cells were analyzed with flow cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) with the emission wavelength at 525 nm. The fluorescent intensity of intracellular MDC reflects the number of autophagic cells (autophagic ratio).

2.10. Western blot analysis

The RAW264.7 cells were harvested, washed twice with ice-cold PBS, and then lysed in lysis buffer (50 mM Hepes pH 7.4, 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin) at 4 °C for 60 min. The lysate was centrifuged at 15,000 × *g* for 10 min and the supernatants were used for Western blot analysis. Protein concentration was determined by the Folin assay. Equal amounts of total protein were separated by 8 or 15% SDSPAGE and electro-transferred to a PVDF membrane (Bio-Rad). After blocking with 5% w/v non-fat dry milk in TBST (0.1% Tween 20) at room temperature for 1 h, membrane was probed with the primary antibodies against TLR4, TRIF, MyD88, TRAF6, Beclin-1, LC3, LAMP 1, LAMP 2 and β-actin overnight at 4 °C, followed by horseradish peroxidase (HRP)-conjugated secondary antibody and visualization was performed with chemiluminescence (ECL) detection kit followed by autoradiography.

2.11. Acid phosphatase 2 (ACP2) activity measurement

RAW264.7 cells (2×10^5/well) were inoculated in 6-well culture plates and were treated with oridonin or LPS with or without IN or siRNA for 24 h. Then the cells were collected and homogenized in PBS. Protein concentration was determined by the Folin assay. Equal amounts of the total protein were applied. ACP2 activity was measured using acid phosphatase 2, lysosomal ELISA kit following the instruction (Nan Jing Jian Cheng Biochemical Company). ACP2 concentration was calculated according to the standard curve and normalized to control group.

2.12. Cathepsin B activity measurement

RAW264.7 cells (2×10^5/well) were inoculated in 6-well culture plates and were treated with oridonin or LPS with or without IN or siRNA for 24 h. Then the cells were collected and homogenized in CB cell lysis buffer. Protein concentration was determined by the Folin assay. A hundred micrograms of the total protein in 50 μl lysis buffer were applied. Cathepsin B (CTSB) activity was measured using Cathepsin B activity fluorometric assay kit (Biovision, LA, U.S.A.). The relative fluorescence units (RFU) were compared with control group.

2.13. Cell migration

Migration of RAW264.7 was determined using a 24-well transwell insert (Corning Costar), without fibronectin. Briefly, UV irradiated apoptotic Raw 264.7 cells were seeded on the bottom of the well's solution. RAW 264.7 cells treated with the indicated reagents were seeded in the upper side of the transwell insert. Cell migration was carried out at 37 °C for 48 h. Then the inserts were fixed with 20% methanol and stained with crystal violet for 5 min. After washing out the extra dye, the dried inserts were imaged under a microscope.

2.14. Statistical analysis

All data represent at least three independent experiments and were expressed as means ± S.D. Statistical comparisons were made using Student's *t*-test and One-way AVOVA followed by Tukey's post-hoc test. *P* Values of < 0.05 were considered to represent a statistically significant difference.

3. Results

3.1. Oridonin binds to TLR4 and activates TLR4 signalling in Raw264.7 macrophages

Oridonin (Fig. 1A) is an active diterpenoid isolated from *Rabdosia rubescens*. Computer-aided docking can be useful for exploring receptor-ligand interactions and is commonly used to predict ligand binding location and affinity. Molecular dynamics simulations revealed at least two possible TLR4 binding sites for oridonin, a conventional hydrogen bond (GLU A: 485), and a carbon hydrogen bond (GLU A: 484). Additionally, AutoDock Vina calculated a binding affinity of −4.9 kcal/mol (Fig. 1B), indicating that oridonin might activate the TLR4 signalling pathway. To examine this, we treated RAW264.7 cells with 2.5 μM oridonin for 24 h, and subsequent immunofluorescence staining of TLR4 indicated that oridonin promoted TLR4 expression on the surface of the cell membrane (Fig. 1C). Meanwhile, western blot analysis showed that oridonin up-regulated TLR4 and its downstream proteins TRIF, MyD88 and TRAF6 in a time-dependent manner (Fig. 1D), suggesting that oridonin induced both MyD88-dependent and independent TLR4 signalling pathways. Endotoxin level of the oridonin (2.5 μM) in this study was lower than 0.1 EU/ml, which is similar to background levels and to complete RPMI medium, so the oridonin used in experiments was considered to be endotoxin free. Moreover, there is no endotoxin to effect oridonin induced up-regulation of TLR4 expression (Fig. 1E).

3.2. Activated TLR4 is involved in endocytosis of apoptotic bodies

We next examined whether TLR4 activation was sufficient for oridonin-induced endocytosis of apoptotic bodies. Macrophage treatment with a TLR4 inhibitor or silencing with a small interfering RNA (siRNA) caused a significant decrease in endocytosis of apoptotic cells following oridonin induction (Fig. 2A, B). We observed less colocalisation of apoptotic bodies and macrophage intracytoplasm, and a lower phagocytosis fluorescence intensity, in TLR4 inhibition groups. The phagocytosis ratio was decreased from 33.73% with oridonin alone to ~25% with oridonin and the TLR4 inhibitor, and 21% with oridonin and the TLR4-specific siRNA, according to flow cytometry analysis. For further indication of the involvement of TLR4 in efferocytosis, we investigated the effect of lipopolysaccharide (LPS), a natural ligand of TLR4. Using LPS as an agonist of TLR4 instead of oridonin resulted in a similar enhancement of engulfment. These results suggest that activation of TLR4 is involved in oridonin-induced enhancement of endocytosis of apoptotic bodies (Fig. 2A-D).

3.3. TLR4 mediates oridonin-induced phagocytosis by stimulating autophagy

Growing evidence suggests that autophagy plays an important role in immune and inflammatory responses. Our previous studies showed that oridonin induces autophagy in U937 macrophage-like cells [13]. To test whether oridonin induces autophagy in RAW 264.7 cells and to reveal the potential association with phagocytosis, cells were treated with oridonin for 24 h, and we observed autophagic vacuoles by florescence microscopy following MDC staining (Fig. 3A). Additionally, flow cytometry analysis showed that the autophagic ratio was increased up to 46.7% after oridonin treatment (Fig. 3B-C). Furthermore, Beclin-1 expression was increased, as was LC3 II conversion from LC3 I (Fig. 3D), and LPS had the same effect as oridonin. Inhibition of TLR4 by IN or siRNA significantly decreased the autophagic ratio induced by oridonin (Fig. 3A-C), and crucially, inhibition of autophagy by 3MA inhibited both oridonin- and LPS-induced phagocytosis (Fig. 3E), indicating that autophagy likely enhances endocytosis via TLR4 activation.

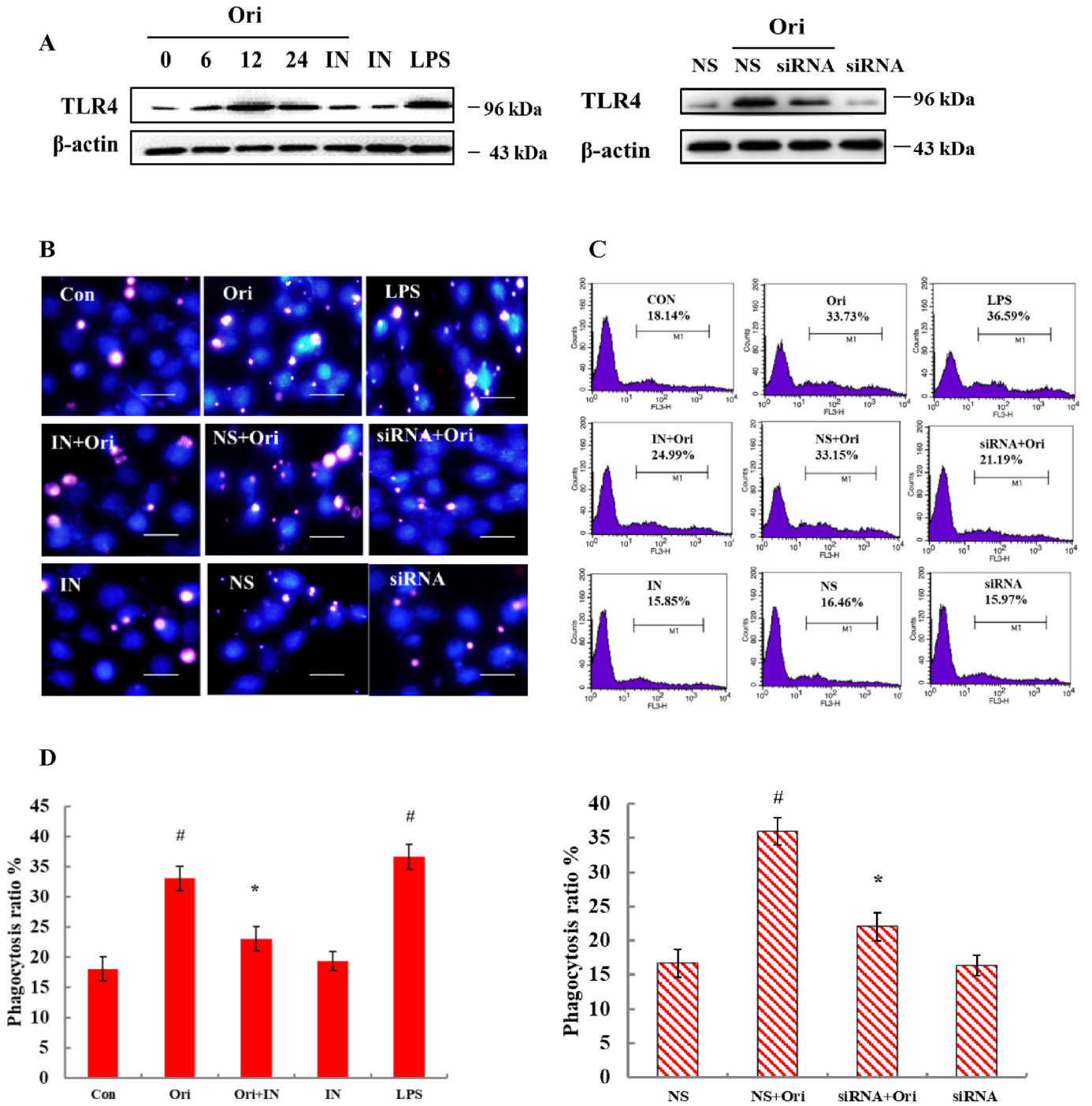

Fig. 2. Oridonin induced phagocytosis of apoptotic cells via TLR4 activation in RAW264.7 cells. Cells were incubated with 2.5 μM oridonin or LPS (250 ng/ml) for the indicated time points in the presence or absence of IN (10 μM) (left). After transiently transfected with a control siRNA (NS) or siRNA targeting TLR4, the cells were incubated with oridonin for 24 h (right). (A) TLR4 expression was detected by Western blot analysis. (B) Phagocytosis of apoptotic cells was determined by immunofluorescent staining. Red particles are PI labeled apoptotic bodies. Blue is DAPI marking RAW264.7 macrophage cells (×400 magnification, bar = 20 μm). Ratios of PI positive cells with phagocytosis were measured by flow cytometry (C). Phagocytosis ratios were quantified from C (D). # $P < 0.05$ VS. control; * $P < 0.05$ VS. oridonin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. TLR4 mediates oridonin-induced endocytosis by increasing the number of lysosomes and promoting lysosome activity

Fusion with lysosomes and breakdown of cellular macromolecules into simple compounds is the final step of endocytosis. To examine the involvement of lysosomes in this process, we measured the activity of lysosomal acid phosphatase 2 (ACP2) and cathepsin B (CTSB), both important enzymes in lysosomes. As shown in Fig. 4A and B, oridonin significantly increased the activation of both ACP2 and CTSB. Following inhibition of TLR4 by IN or siRNA, activity of both ACP2 and CTSB was dramatically decreased. Conversely, stimulation of TLR4 activation by LPS markedly enhanced the activity of these enzymes.

Lysosomal-associated membrane protein 1 and 2 (LAMP1 and LAMP2) reside primarily across lysosomal membranes, and these proteins can indirectly reflect the total number of lysosomes. Western blot analysis indicated that oridonin increased their expression, but this was

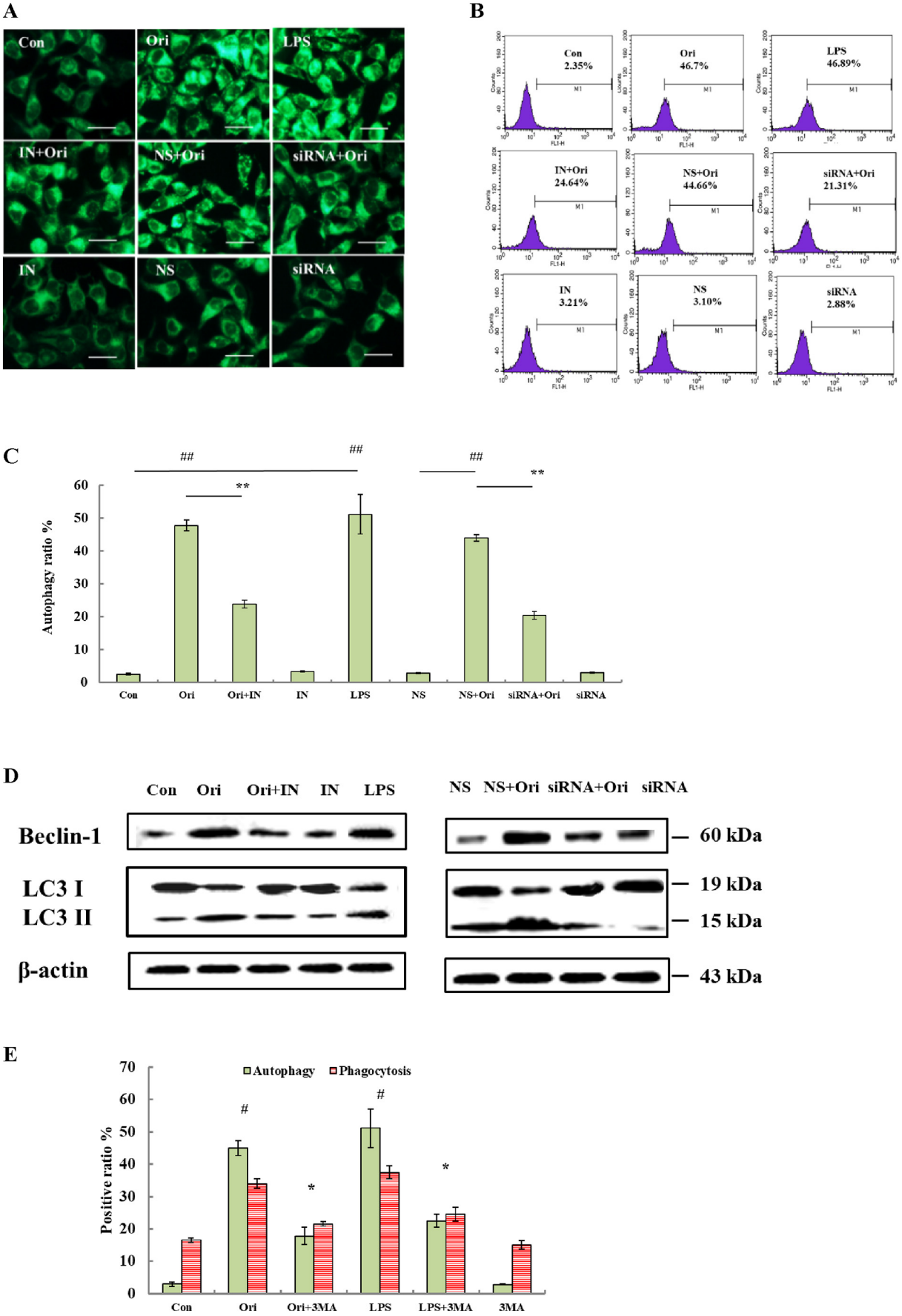

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Fig. 3. Autophagy was accompanied by phagocytosis in oridonin-treated RAW 264.7. Cells were incubated with 2.5 μM oridonin or 250 ng/ml of LPS for 24 h in the presence or absence of 10 μM IN or siRNA. (A) The autophagic vacuoles were observed under a fluorescence microscope after MDC staining (×400 magnification, bar = 20 mm). (B-C) Flow cytometric analyses of autophagic cell ratios after MDC staining. (D) Beclin-1 and LC3 expression was detected by Western blot analysis. (E) Autophagic ratios and phagocytosis ratios were quantified from B and from Fig. 1C, respectively. # $P < 0.05$, ## $P < 0.01$ VS. control; * $P < 0.05$, ** $P < 0.01$ VS. oridonin/LPS.

decreased by the TLR4 inhibitor or siRNA, and increased by LPS treatment (Fig. 4C). Together, these results provide further evidence that TLR4 mediates oridonin-induced phagocytosis by increasing the number of lysosomes and promoting lysosome activity.

3.5. TLR4 activation stimulates the migration of macrophages

TLR4 is a type I transmembrane protein located on the cell membrane. We previously showed that oridonin induced TLR4 expression and stimulated phagocytosis. Herein, we found that TLR4 expression was elevated in cytoplasm after co-incubation with apoptotic bodies, implying that activated TLR4 entered the cytoplasm along with phagocytotic vesicles (Fig. 5A). Activated TLR4 in the cytoplasm may well be involved in the activity, location, and motility or migration of macrophages. One feature of macrophages is the migration toward cellular debris, foreign substances, and non-healthy cells prior to

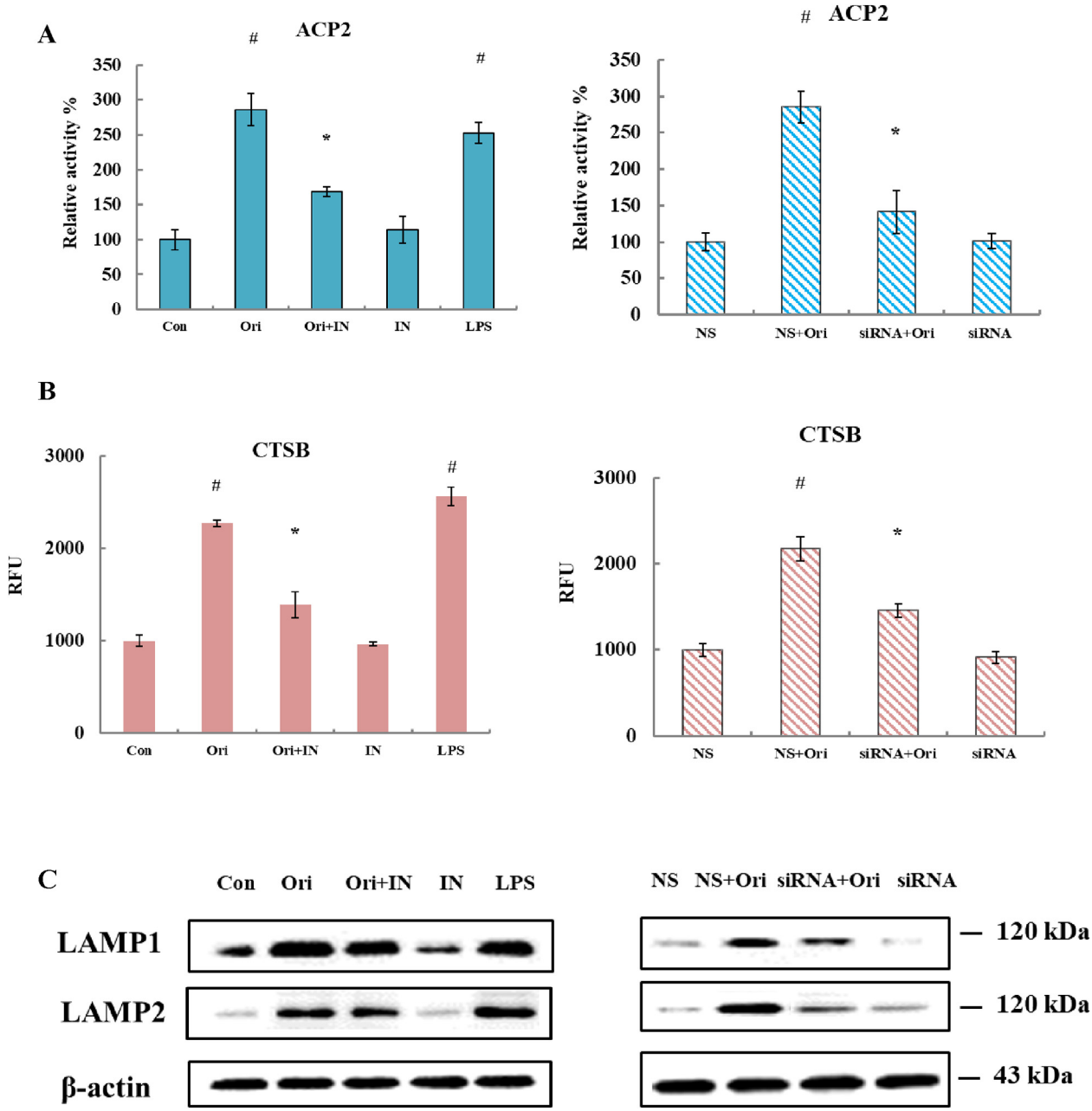

Fig. 4. TLR4 activation promotes activity and number of lysosomes in oridonin-induced phagocytosis. Cells were incubated with 2.5 μM oridonin or 250 ng/ml of LPS for 24 h in the presence or absence of 10 μM IN or siRNA. (A) Lysosomal acid phosphatase 2 (ACP2) activity was measured using ELISA kit. (B) Cathepsin B (CTSB) activity was measured by CTSB activity fluorometric assay kit. Relative fluorometric units (RFU) were recorded. (C) Western blot analysis of LAMP 1 and LAMP 2 expression. β-Actin is a loading control. # $P < 0.05$ VS. control; * $P < 0.05$ VS. oridonin.

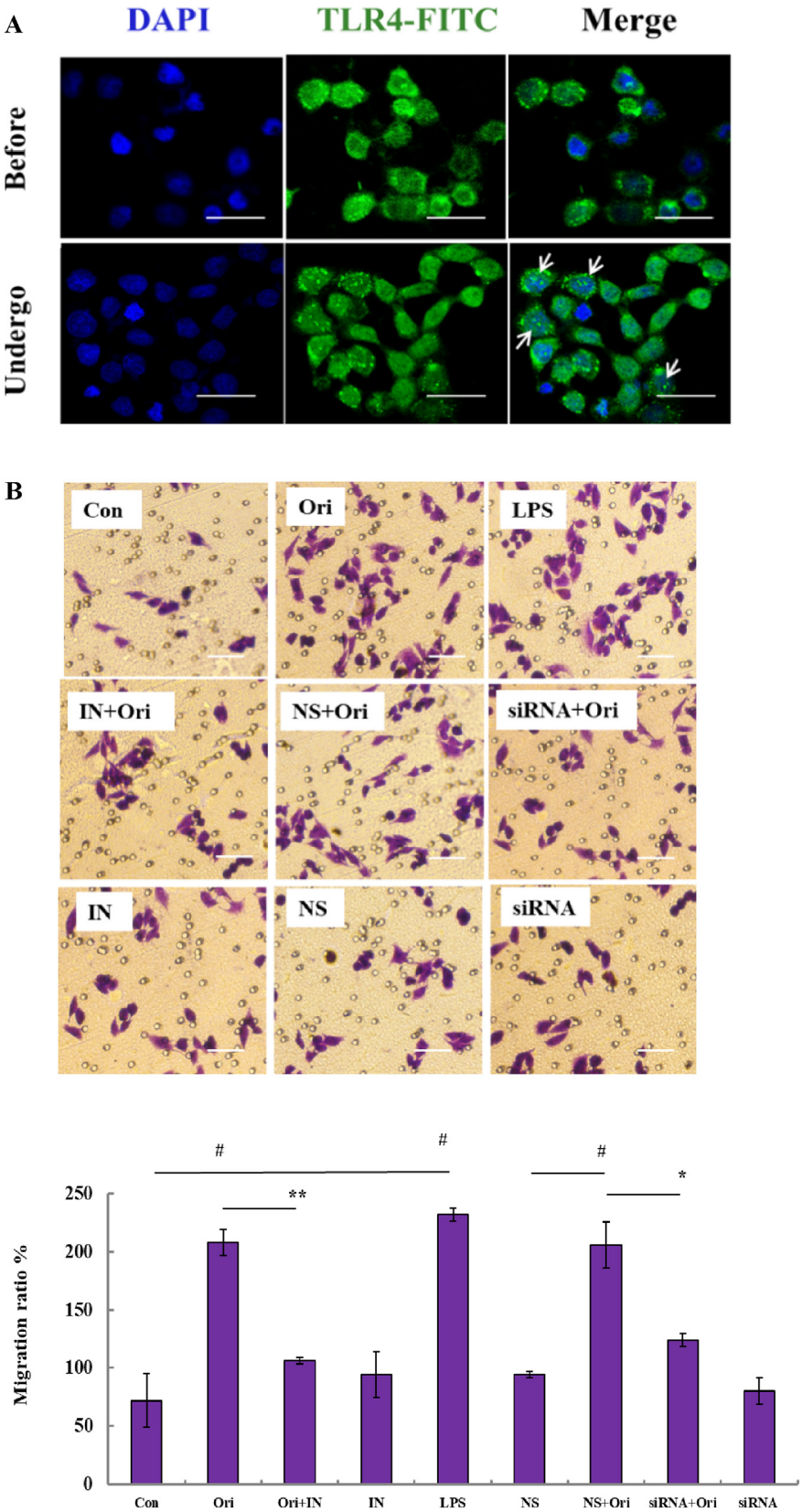

Fig. 5. Activated TLR4 translocates into cytoplasm with phagosomes, leading to the enhanced migration of macrophages. (A) Immunofluorescent staining of TLR4 when Raw264.7 cells undergo phagocytosis (×400 magnification, bar = 40 mm). (B) RAW 264.7 cells were treated with the indicated reagents, transwell assay was used to determine the migration of cells (×400 magnification, bar = 20 mm). UV-irradiated apoptotic Raw 264.7 cells were used as inducers placed on the bottom of the well. # $P < 0.05$, ## $P < 0.01$ VS. control; * $P < 0.05$, ** $P < 0.01$ VS. oridonin.

engulfment and digestion. To verify whether TLR4 participates in oridonin-induced migration of macrophages, RAW264.7 cells were treated with 2.5 μM oridonin with or without IN or siRNA, or LPS. The results of transwell assays showed that oridonin- or LPS-induced activation of TLR4 promoted RAW264.7 cell migration toward apoptotic cells, and the cell migration was inhibited by TLR4 inhibitor (IN), or TLR4-siRNA (Fig. 5B).

4. Discussion

Phagocytosis is an important part of the innate immune response that benefits tissue homeostasis by rapidly clearing dying cells, preventing the spillover of pro-inflammatory and neurotoxic molecules. Phagocytes achieve clearance of apoptotic or necrotic cells by contributing to antigen presentation [14]. Different receptors trigger different signalling pathways that stimulate phagosome formation. The mechanistic features of TLR4-mediated phagocytosis of bacteria, viruses and unfolded proteins have been extensively investigated in recent years, but the exact role of TLR4 and the molecular machinery coordinating engulfment and digestion of apoptotic cells remain elusive. In order to identify compounds that stimulate phagocytosis of apoptosis cells and could therefore improve resistance to cancer and autoimmune diseases, we investigated the effects of oridonin on TLR4-mediated phagocytosis of apoptotic cells. Oridonin is a diterpenoid compound that is widely used owing to its anti-tumour, anti-inflammation and anti-bacterial activities, and its ability to scavenge reactive oxygen species (ROS). Oridonin has a similar structure to paclitaxel that is considered a potential ligand of TLR4 [3]. Interestingly, paclitaxel enhances macrophage macropinocytosis via TLR4 activation [15]. In this study, oridonin was found to modulate phagocytosis of apoptotic cells by activating TLR4 pathways. Oridonin appears to interact directly with TLR4 via a conventional hydrogen bond and a carbon hydrogen bond, according to computational docking simulations. The effect of oridonin on activation of TLR4 and its enhancement of phagocytosis were detected in human macrophage-like mononuclear leucocyte U937 cells and mouse peritoneal macrophage, similar to our another experiment (manuscript in preparation). Meanwhile, TLR4 was reported to express in M1 and M2 macrophages, THP-1 macrophage, myeloid granulocyte et al., which were involved in phagocytosis and immune response [16–18]. We also found that oridonin might act on TLR7, 8 and 9 (manuscript in preparation). Thus, oridonin could be counted as an agonist or a ligand of TLR family members involved in immune responses, implying oridonin as a potential drug with anti-tumour activity.

After activation of TLR4, myeloid differentiation factor 88 (MyD88) is recruited to the intracellular portion of TLR4, and the TLR4-MyD88 signal leads to the activation of TRAF6 and downstream pathways such as autophagy [19]. It is assumed that these signalling processes are similar, if not identical, between phagocytosis and autophagy [20]. Autophagy-associated proteins Beclin-1 and LC3 belong to the two main pathways controlling phagocytosis. The Beclin-1 complex operates in association with class III phosphatidylinositol 3-kinase (PI3K) and some other enzymes involved in the production of phosphatidylinositol 3-phosphate (PI3P) that is required for phagosome maturation [20]. The LC3-associated pathway involves target recognition receptors and induces the translocation of the autophagy machinery to the cargo-containing single-membrane phagosome [21,22]. Our study shows that, oridonin activates TLR4, leading to enhanced autophagy in Raw264.7 macrophages. Inhibition of TLR4 down-regulated Beclin-1 and LC3 autophagy-associated proteins embedded in phagosomes to facilitate their fusion with lysosomes. Certain phagosomes recruit conjugating LC3 family proteins to lipids in the phagosome membrane, where the autophagic machinery interacts with lysosomes [23]. Evidence indicates that LC3-associated phagocytosis is induced by TLR1/2, TLR2/6, and TLR4, but not by TLR9 [24]. The activation of Beclin-1- and LC3-mediated autophagy mechanisms is necessary for activity of TLR-induced phagocytes. Mice that are deficient in LC3-associated autophagy accumulate apoptotic bodies in their tissues, and within the cytosol of phagocytic cells [25]. Autophagy plays a prominent role in facilitating these primary energetic pathways through the production of metabolic substrates and/or the quality control of organelles.

TLR4 recognises and combines with ligands, resulting in cytoskeletal rearrangements that mechanically pull particles into cells. This leads to the formation of phagosomes that subsequently acquire hydrolytic capacity through fusion with lysosomes, resulting in the destruction and degradation of the engulfed materials [26]. Herein, we show that oridonin possibly binds to TLR4. Oridonin-bound TLR4 or activated TLR4 is translocated into cytoplasm with couple cellular membrane which may fuse to formation of early phagosomes. Early phagosomes may recruit members of the autophagic machinery such as Beclin-1 or LC3 and aggregated double cargo-membrane is transformed into autophagosomes. Finally, late phagosome or phagolysosome is formed by the fusion of phagosomes to lysosomes [11,27]. Thus, TLP4 might act as a key tethering factor to ensure the fidelity and efficiency of lysosome maturation and activation. The lysosomal acid phosphatase (ACP2) performs a crucial role in membrane fusion, and is widely used as a lysosome marker. After delivery to lysosomes, ACP2 is converted from a membrane-bound precursor into a mature phosphatase by proteolytic cleavage [28]. CTSB, another well-studied enzyme in lysosomes, regulates cytokine exocytosis, protein cleavage inside lysosomes, and cell death [29]. Loss of function of TLR4 following treatment with an inhibitor or siRNA significantly decreased the activity of lysosomal enzymes including APC2 and CTSB. The observed increase in the number of lysosomes is also likely associated with TLR4 presentation on targeting particles.

We also observed that activation of TLR4 following treatment with oridonin or LPS led to an increase in the migration of macrophages. Apoptotic tumour cells are a rich source of antigens and can attract macrophages and discriminate them by releasing 'find me' and 'eat me' signals. Activation of TLR4 increases migration of macrophages to apoptotic bodies. The elevated fluidity of cell membrane and/or motility due to increased plasticity of cytoskeleton may avail to the frequencies to approach or touch of macrophages to apoptotic cells in transwell assays [30]. It was validated that cytoskeleton is regulated by TLR4-induced macrophage activation, cell migration, cell division, and localisation [31].

Activated TLR4 contributes to the progression and promotion of chemotactic migration in various tumours [31,32], which is detrimental for oncotherapy. TLR4-induced migration of macrophages is beneficial for tissue remodelling and repair, clearance of cell debris, and other processes. Oridonin- or LPS-induced TLR4 expression might decrease the levels of cytoskeleton proteins such as tubulin, leading to cytoskeletal rearrangement or cell motility [32,33].

Thus, given the double-edged sword nature of the effects on cell activation and migration, it may be preferable for TLR4 to exert its effects after inducing tumour cell apoptosis, and targeting macrophages. Meanwhile, autophagy and phagosome fusion with lysosomes is regulated by TLR4 degradation via proteasomal pathways to prevent excessive inflammation and tissue injury. Many studies evidenced that defects in clearance of apoptotic cells induced mouse and human cells susceptible to the autoimmune disease such as systemic lupus erythematosus, chronic inflammation and even deadly cancers. Oridonin-activated TLR4-autophagy pathway promotes phagocytosis of apoptotic cells, supporting a new strategy for therapy of immune disease and cancer [34,35]. Emerging evidence has revealed that ginseng-enhanced phagocytosis of apoptotic Jurkat cells by TLR4 in macrophages contributes to inhibition of melanoma cells growth [36]. Activated TLR4 also promotes phagocytosis of monosodium urate monohydrate (MSU) crystals' joint deposition to benefit for gout treatment [37]. TLR4-deficient mice display decreased circulating C-C chemokine ligand 2 (CCL2) levels and lower macrophage infiltration into adipose tissue, leading to metabolic restriction [33].

In summary, our findings indicate a positive and rapid enhancement of TLR4-mediated phagocytosis following oridonin treatment. Oridonin efficacy on macrophage activation is dependent on the autophagy/lysosome pathway in association with cell migration. This mechanism provides protection against the accumulation of apoptotic cells, and modulates the effector functions of the immune competent system.

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