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Resolvin D1 Induces mTOR-independent and ATG5-dependent Autophagy in BV-2 Microglial Cells*

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[Abstract] Objective: The activation state of microglia is known to occupy a central position in the pathophysiological process of cerebral inflammation. Autophagy is a catabolic process responsible for maintaining cellular homeostasis. In recent years, autophagy has been demonstrated to play an important role in neuroinflammation. Resolvin D1 (RvD1) is a promising therapeutic mediator that has been shown to exert substantial anti-inflammatory and proresolving activities. However, whether RvD1-mediated resolution of inflammation in microglia is related to autophagy regulation needs further investigation. The present study aimed to explore the effect of RvD1 on microglial autophagy and its corresponding pathways. **Methods:** Mouse microglial cells (BV-2) were cultured, treated with RvD1, and examined by Western blotting, confocal immunofluorescence microscopy, transmission electron microscopy, and flow cytometry. **Results:** RvD1 promoted autophagy in both BV-2 cells and mouse primary microglia by favoring the maturation of autophagosomes and their fusion with lysosomes. Importantly, RvD1 had no significant effect on the activation of mammalian target of rapamycin (mTOR) signaling. Furthermore, RvD1-induced mTOR-independent autophagy was confirmed by observing reduced cytoplasmic calcium levels and suppressed calcium/calmodulin-dependent protein kinase Ⅱ (CaMKⅡ) activation. Moreover, by downregulating ATG5, the increased phagocytic activity induced by RvD1 was demonstrated to be tightly controlled by ATG5-dependent autophagy. **Conclusion:** The present work identified a previously unreported mechanism responsible for the role of RvD1 in microglial autophagy, highlighting its therapeutic potential against neuroinflammation.

Key words: resolvin D1; microglia; mTOR-independent autophagy; ATG5-dependent autophagy; phagocytosis

In the past few decades, accumulating evidence indicates the pivotal role of neuroinflammation in an array of neurological disorders and degenerative processes, including multiple sclerosis, cerebral ischemia, Parkinson's disease, Alzheimer's disease, sepsis-associated encephalopathy, depression, and brain hemorrhage[1–7]. As the native immune cells in the brain, microglia serve as the first line of defense. It has been previously established that under normal physiological conditions, microglia exert a critical influence on the maintenance of cerebral homeostasis, which is mediated *via* immune surveillance and host defense. During brain pathologies, microglia become activated upon stimulation by various cellular factors and secrete high levels of inflammatory factors, leading to the activation of nearby/surrounding microglia and the propagation of neuroinflammation[8, 9].

The resolution of the inflammatory response is further driven by specialized proresolving lipid mediators (SPMs), which are usually comprised of resolvins, lipoxins, protectins, and maresins[10]. Several

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preclinical studies have emphasized the importance of SPMs in neuroinflammation[11–18]. D-series resolvins are derived from docosahexaenoic acid and are known to exert anti-inflammatory and proresolving actions during inflammatory processes[19]. Among these, resolvin D1 (RvD1) is most widely studied. Importantly, previous studies have reported its role as a mediator in limiting the resolution of inflammation, both *in vitro* and *in vivo*[20]. Several studies have reported specific effects of RvD1 on microglia, which involve regulation of microglial polarization[11], downregulation of β-amyloid 42-induced inflammation[21], and inhibition of microglial activation[22, 23].

Autophagy is a catabolic process that is known to be evolutionarily conserved. In fact, it is necessary for the maintenance of cellular homeostasis. In general, autophagy sequesters the cytoplasm, including protein aggregates and damaged organelles, into double-membrane vesicles known as autophagosomes, which are delivered to lysosomes for further degradation[24, 25]. BECN1 and ATG5 represent essential partners that signal the onset of autophagy[26]. During the elongation stage of the autophagosome, conversion of the LC3-Ⅱ/Ⅰ ratio and a decrease in P62 act as classical markers for autophagic activity[27, 28]. Multiple signaling pathways, including the classical mammalian target of rapamycin (mTOR) pathway and variations in the cytosolic calcium concentration, are known to be involved in the modulation of autophagy[29–31]. In particular, mTOR is an autophagy-suppressive regulator, and its inhibition results in the induction of autophagy, which is presented by the reduced activity of downstream substrates, including ULK1 (Ser 757), p70S6 kinase (S6K), and 4E-BP1[32, 33].

Microglia are major phagocytes in the brain that engulf and degrade various brain-derived debris as well as microbes. Microglial phagocytic activity is known to be tightly related to autophagy[34]. As a representative protein of both phagocytosis and autophagy, LC3 is recruited to the phagosomes depending on the activity of a series of autophagy-related proteins including ATG5[35]. However, the detailed roles of autophagy on phagocytosis in microglia remain to be elucidated.

Recent studies have indicated a crucial role of microglial autophagy in the modulation of neuroinflammation[36]. Previously accumulated evidence also suggests that autophagy negatively regulates inflammation in macrophages[37]. In fact, one study has reported that SPMs promote autophagy in macrophages[38]. Considering that microglia exhibit macrophage-like properties in the brain, it might be assumed that RvD1 is involved in the induction of microglial autophagy. Nevertheless, no relevant study has been previously reported on this subject. Therefore, the present study aimed to assess the role of RvD1 in microglial autophagy and attempted to unravel the underlying mechanism involved. The findings of this study will provide a foundation for the development of effective strategies for the efficient management of neuroinflammation.

1 MATERIALS AND METHODS

1.1 Cell Culture

The mouse microglial cell line BV-2 was purchased from the China Center for Type Culture Collection (GDC0311) and propagated in DMEM/F12 (Gibco, USA) with 10% heat-inactivated fetal bovine serum (Gibco, USA), 100 μg/mL streptomycin, and 100 U/mL penicillin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Mouse primary microglia cells were purchased from SAIOS (China) and cultured according to the manufacturer's instructions.

1.2 Drugs and Treatment

BV-2 cells were incubated with RvD1 (Cayman Chemical, USA) for different time lengths and at various concentrations as indicated or vehicle (0.038% ethanol). Prior to the administration of RvD1, 20 μmol/L chloroquine (CQ, Sigma-Aldrich, China) and 10 μmol/L LY294002 (LY, MedChem Express, USA) were added to BV-2 cells for 22 and 2 h, respectively. BV-2 cells were treated with 50 μmol/L arachidonic acid (AA, Sigma-Aldrich, China) for 2 h prior to RvD1 treatment. For the phagocytic activity assay, BV-2 cells were incubated with 50 μg/mL zymosan A (Thermo Fisher, USA) for 2 h following RvD1 treatment.

1.3 Protein Extraction

Following the experimental treatments, BV-2 cells were rinsed in PBS and lysed in radioimmune precipitation assay buffer (Beyotime Institute of Biotechnology, China) with protease and phosphatase inhibitor cocktails (Roche, Germany). The lysates were centrifuged for 15 min at 13 000 *g*. The supernatants were collected for protein concentration assessment with a BCA Protein Assay kit (Beyotime Biotechnology, China) before being denatured with 5× loading buffer and stored at –20°C.

1.4 Western Blotting

Equal amounts of total proteins were electrophoresed on 8%–12% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes. The membranes were saturated with QuickBlock™ Blocking Buffer (Beyotime Biotechnology, China) for 15 min and incubated at 4°C overnight with the following primary antibodies: P62 (R&D Systems, USA), LC3-Ⅱ/Ⅰ, ATG5, P-mTOR, mTOR, P-ULK1, ULK1, P-S6K, S6K, P-4E-BP1, 4E-BP-1, P-CaMKⅡ, CaMKⅡ (1:1000, all from Cell Signaling Technology, USA), β-actin (1:2000, Antgene Biotechnology, China). Following washing three times with TBST for 10 min each time, the membranes were incubated with HRP goat-anti-rabbit IgG or

HRP goat-anti-mouse IgG (1:3000, all from Antgene Biotechnology, China) at room temperature for 1 h. The membranes were washed another three times as previously mentioned, and the protein signals were detected by an ECL kit (Thermo Fisher Scientific, USA). The quantitative analysis of protein bands was carried out by using Image J software (National Institutes of Health, USA).

1.5 Confocal Immunofluorescence Microscopy

BV-2 cells were seeded on sterile glass coverslips precoated with polylysine (Sigma-Aldrich, China) and treated as indicated in the legend. The cells were subsequently washed with PBS three times, fixed with 4% paraformaldehyde, and permeabilized with 0.3% Triton X-100 in PBS at room temperature for 5 min. Following rinsing three times and blocking with 3% BSA in PBS for 1 h, the cells were incubated with the following primary antibodies: LC3-Ⅱ/Ⅰ (1:200, Cell Signaling Technology, USA), P62, LAMP1 (both from R&D Systems, USA). After washing, the cells were reacted with FITC- or Texas Red-conjugated secondary antibodies (1:200, all from Antgene Biotechnology, China) for 1 h at 37°C. After washing another three times, the cells were covered with an antifade mounting medium containing DAPI dye (Beyotime, China). The cells were observed by a confocal microscope (Nikon, Japan). Image-Pro Plus software was used to perform quantification of LC3 puncta.

1.6 Transmission Electron Microscopy

After washing twice in PBS, BV-2 cells were fixed in 2.5% glutaraldehyde at 4°C and then 1% osmium tetroxide at room temperature for 2 h. After dehydration in gradient ethanol and embedding in a 1:1 mixture of 812 embedding agents and acetone, the cells were cut into ultrathin sections, followed by staining with 2% aqueous uranium acetate and lead citrate for 15 min each. The sections were examined under a transmission electron microscope (Hitachi Scientific Instruments, Japan).

1.7 Flow Cytometry

BV-2 cells were stimulated according to the experimental design, harvested, and incubated with the Ca^{2+} fluorescent probe Fluo-4-AM (Yeasen Biotech, China) at 37°C for 40 min away from direct light. Then the cells were washed three times, followed by incubation protected from light for 30 min at 37°C. Lastly, after centrifugation and discarding the supernatants, the cells were resuspended and analyzed by flow cytometry (BD Biosciences, USA).

1.8 Establishment of a Stable ATG5-knockdown Cell Line

A stable ATG5-knockdown cell line was established by infecting BV-2 cells with lentiviral particles containing ATG5 shRNA (Santa Cruz Biotechnology, USA). BV-2 cells were seeded in a 6-well plate at a density of 1.0×10^5 cells/well. When the cells reached 50% confluence, 20 μL of lentiviral solution was added into the medium along with 5 μg/mL polybrene (Solarbio, China). The medium was changed 24 h later with fresh complete medium. By 72 h after the start of the infection, the cells were maintained in selective medium containing 5 μg/mL puromycin (Solarbio, China) henceforward. The cells were washed with PBS three times before changing the medium. Surviving cell colonies were digested and plated into new dishes for propagation and subsequent experiments.

1.9 Phagocytosis Assay

The cells were plated on sterile glass coverslips precoated with polylysine. After the treatments, the cells were incubated with zymosan A for 2 h. The subsequent procedures of confocal fluorescence microscopy were performed as previously described.

1.10 Statistical Analysis

Statistical analysis was performed by using GraphPad Prism 5.0 software (GraphPad Software Inc., USA). Tests for differences between two groups were performed by using the unpaired *t* test. One-way ANOVA with the Newman-Keuls test was applied for multiple group comparisons. Data are presented as mean ± SEM. A difference was deemed as statistically significant at $P<0.05$.

2 RESULTS

2.1 Autophagy Is Enhanced in RvD1-treated BV-2 Microglia

A previous study conducted in our laboratory reported that RvD1 significantly promoted IL-4-induced M2 polarization in BV-2 cells at concentrations varying from 10 nmol/L to 100 nmol/L[11]. Besides, treatment with RvD1 for 1 or 2 h was observed to induce autophagy effectively in macrophages in another study[38]. To investigate the potential effects of RvD1 on autophagy and to identify the optimal dose for subsequent experiments, BV-2 cells were first treated with RvD1 at doses of 10, 50, and 100 nmol/L, respectively, for 2 h. Next, Western blot analysis was used to determine the expression of P62 and LC3-Ⅱ. As shown in fig. 1A–1C, RvD1 treatment (50 and 100 nmol/L) resulted in a decrease in P62 and an increase in LC3-Ⅱ, compared to the control, vehicle, and 10 nmol/L RvD1 group, and it reached a maximum value at the dose of 50 nmol/L. Hence, 50 nmol/L was chosen as the optimal dose of RvD1 for the subsequent experiments. Subsequently, the optimal time point for autophagy activation was explored by detecting the levels of P62 and LC3-Ⅱ. During the early stages of RvD1 administration (15–30 min postadministration), no significant changes were reported among groups (fig. 1D–1F). During the later stages, however, RvD1 treatment resulted in the promotion of autophagy in

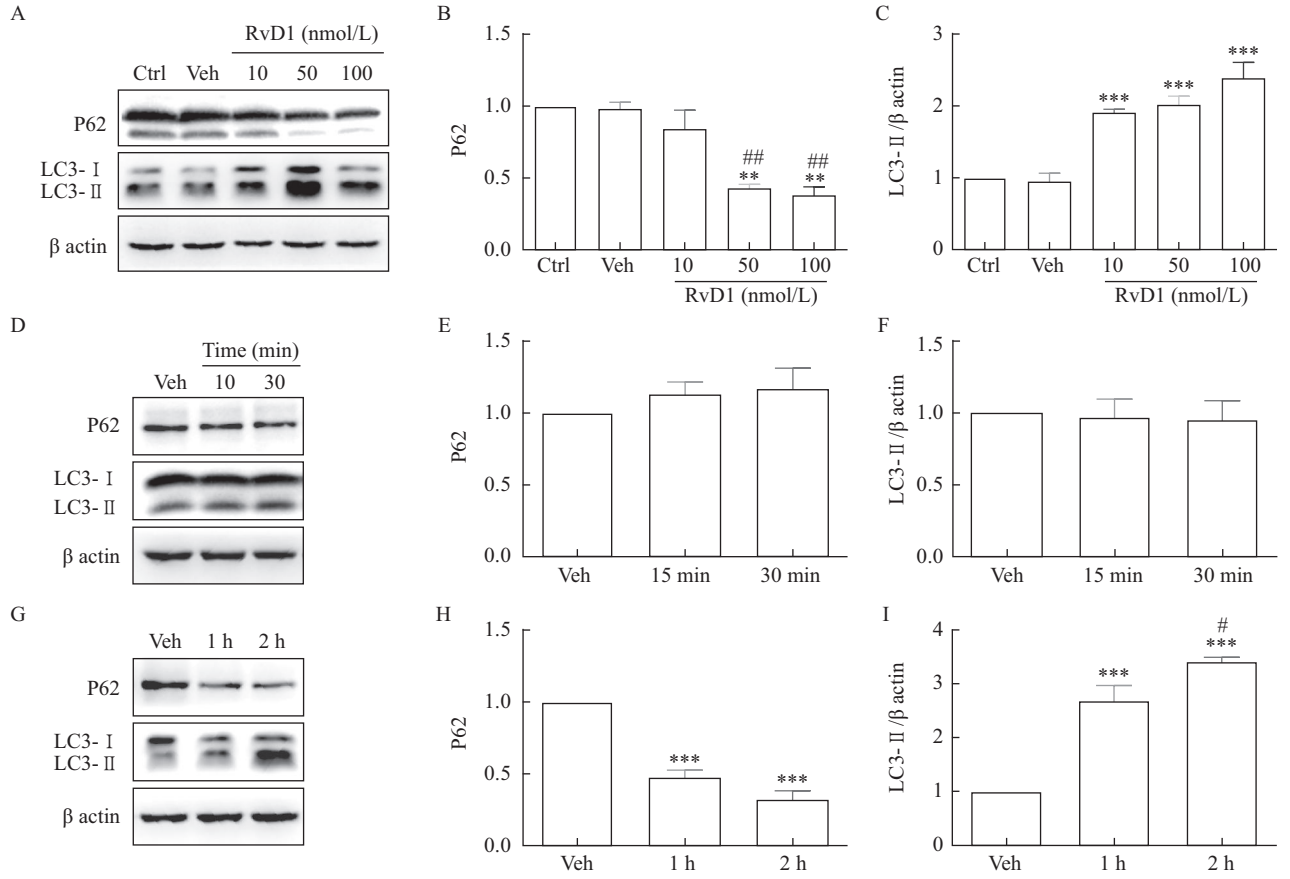

Fig. 1 RvD1 promotes autophagy in BV-2 cells

A–C: BV-2 cells were administered with vehicle (Veh) or indicated concentrations of RvD1 (10, 50, 100 nmol/L) for 2 h. The expression of P62 and LC3-Ⅱ was assessed by Western blotting. The histograms show the corresponding quantitative analysis (n=3). $^{**}P$<0.01, $^{***}P$<0.001 *vs*. control (Ctrl); $^{\#\#}P$<0.01 *vs*. 10 nmol/L RvD1. D–F: BV-2 cells were treated with RvD1 (50 nmol/L) for 15 or 30 min. The expression of P62 and LC3-Ⅱ was assessed by Western blotting. The band intensities were analyzed and are shown in the histograms (n=4). G–I: BV-2 cells were treated with RvD1 (50 nmol/L) for 1 or 2 h. The levels of P62 and LC3-Ⅱ were detected by Western blotting. The histograms show the quantitative analysis of P62 and LC3-Ⅱ (n=4). $^{***}P$<0.001 *vs*. Veh, $^{\#}P$<0.05 *vs*. 1 h. All the immunoblots were normalized by β-actin. Data are presented as mean ± SEM.

a time-dependent manner, and it reached the highest level at 2 h postadministration, which was presented in terms of degradation of P62 and increased LC3-Ⅱ expression (fig. 1G–1I). Thus, these results showed that RvD1 exerted the maximum autophagy effect on BV-2 cells at a dose of 50 nmol/L and a treatment time of 2 h.

2.2 RvD1 Mainly Promotes Late Stages of Autophagy

Autophagic flux usually involves the formation of autophagosomes, the fusion of autophagosomes with lysosomes, and subsequent degradation. Given that RvD1 induces microglial autophagy, the present study next aimed to identify the stage at which RvD1 induced autophagy flux. LY294002 (LY) is an autophagy inhibitor that is known to block the initial steps of autophagosome formation. In comparison, chloroquine (CQ) is widely applied to inhibit the fusion of autophagosomes and lysosomes. In the present study, BV-2 cells were incubated with vehicle or RvD1, with or without pretreatment with LY or CQ. The presence of LY in the treatment medium inhibited the degradation of P62 and the conversion of LC3-Ⅰ to LC3-Ⅱ, compared to treatment with RvD1 alone. In comparison, CQ exerted an inverse effect (fig. 2A). Compared with treatment with RvD1 or CQ alone, pretreatment with CQ resulted in a significant accumulation of autophagosomes or autolysosomes, which was visualized by using transmission electron microscopy (fig. 2B). P62 usually binds to LC3 in autophagosomes. Subsequently, it gets cleared in autolysosomes. LC3 usually colocalizes with LAMP1, a lysosomal marker. This is indicative of the formation of autolysosomes. In the present study, immunofluorescence double staining was used to analyze the colocalization of LC3-P62 (fig. 2C) and LC3-LAMP1 (fig. 2D). Both of these experiments reported a significant increase in double-positive stained puncta in BV-2 cells exposed to RvD1, suggesting that RvD1 can promote the formation of autophagosomes and autolysosomes. To further strengthen our findings, we performed these experiments on primary microglia and achieved similar results (fig. 3). Altogether, these findings indicate that RvD1 modulates the maturation of autophagosomes and their fusion with lysosomes.

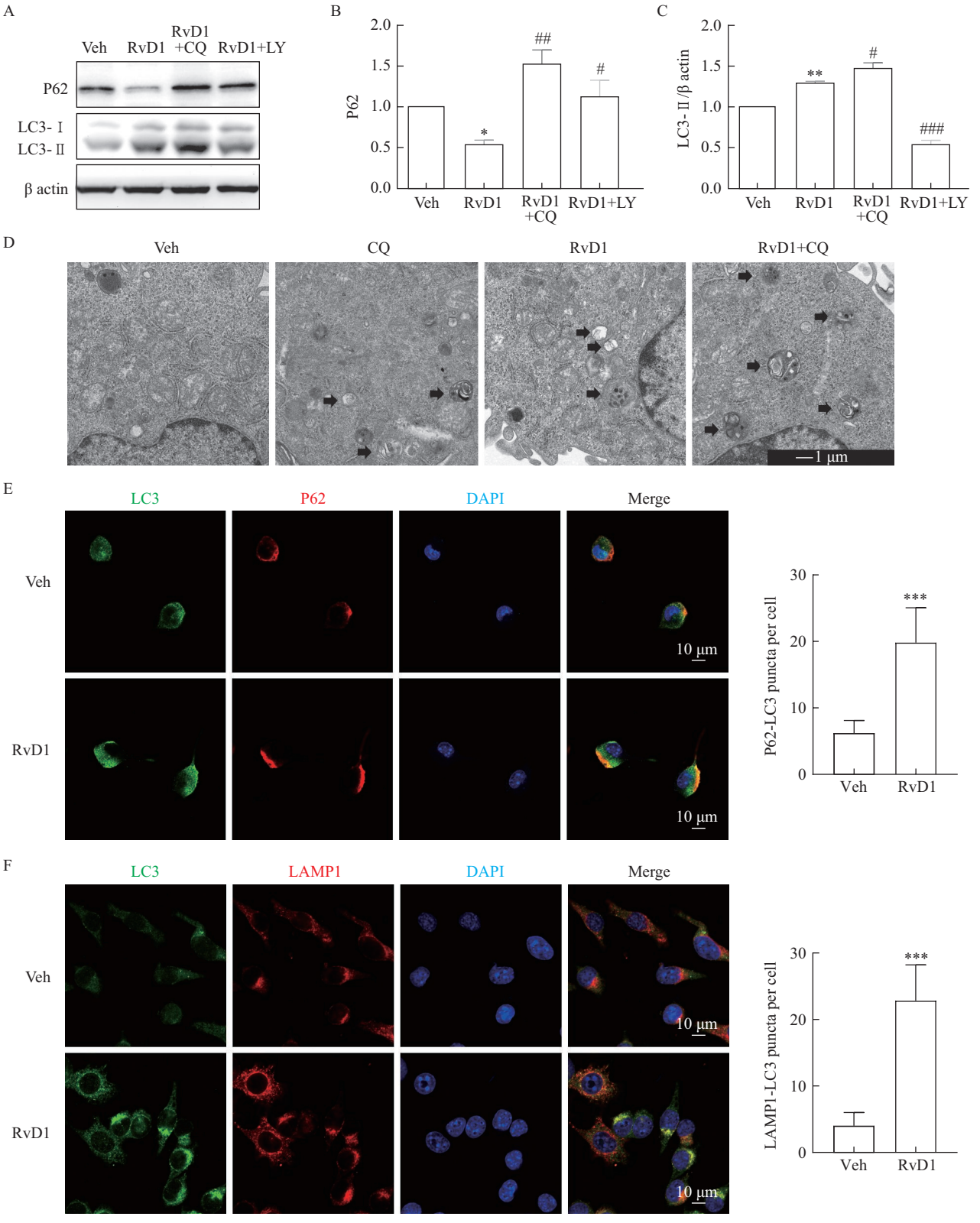

Fig. 2 RvD1 treatment promotes late stages of autophagy by inducing the formation of autolysosomes in BV-2 cells

A–C: BV-2 cells were treated with vehicle (Veh) or RvD1 (50 nmol/L, 2 h) with or without a pretreatment of chloroquine (CQ, 20 μmol/L, 22 h) or LY294002 (LY, 10 μmol/L, 2 h). The expression of P62 and LC3-Ⅱ was measured by Western blotting. Bands were normalized by β-actin. Bar charts indicate the quantitative analysis of P62 and LC3-Ⅱ (n=3). D: Representative transmission electron microscopy images of BV-2 cells treated with Veh or RvD1 (50 nmol/L, 2 h) with or without a pretreatment of CQ (20 μmol/L, 22 h). Black arrows indicate an autophagosome or autolysosome. E, F: Confocal immunofluorescence microscopy images show LC3 (green) and P62 (red) or LC3 (green) and LAMP1 (red) double staining of BV-2 cells treated with Veh or RvD1 (50 nmol/L, 2 h). Nuclei (blue) were labeled with DAPI. LC3-P62 and LC3-LAMP1 puncta quantification was conducted and shown in the bar graph (n=3 per group). Data are presented as the mean ± SEM. $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 *vs*. Veh; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 *vs*. RvD1

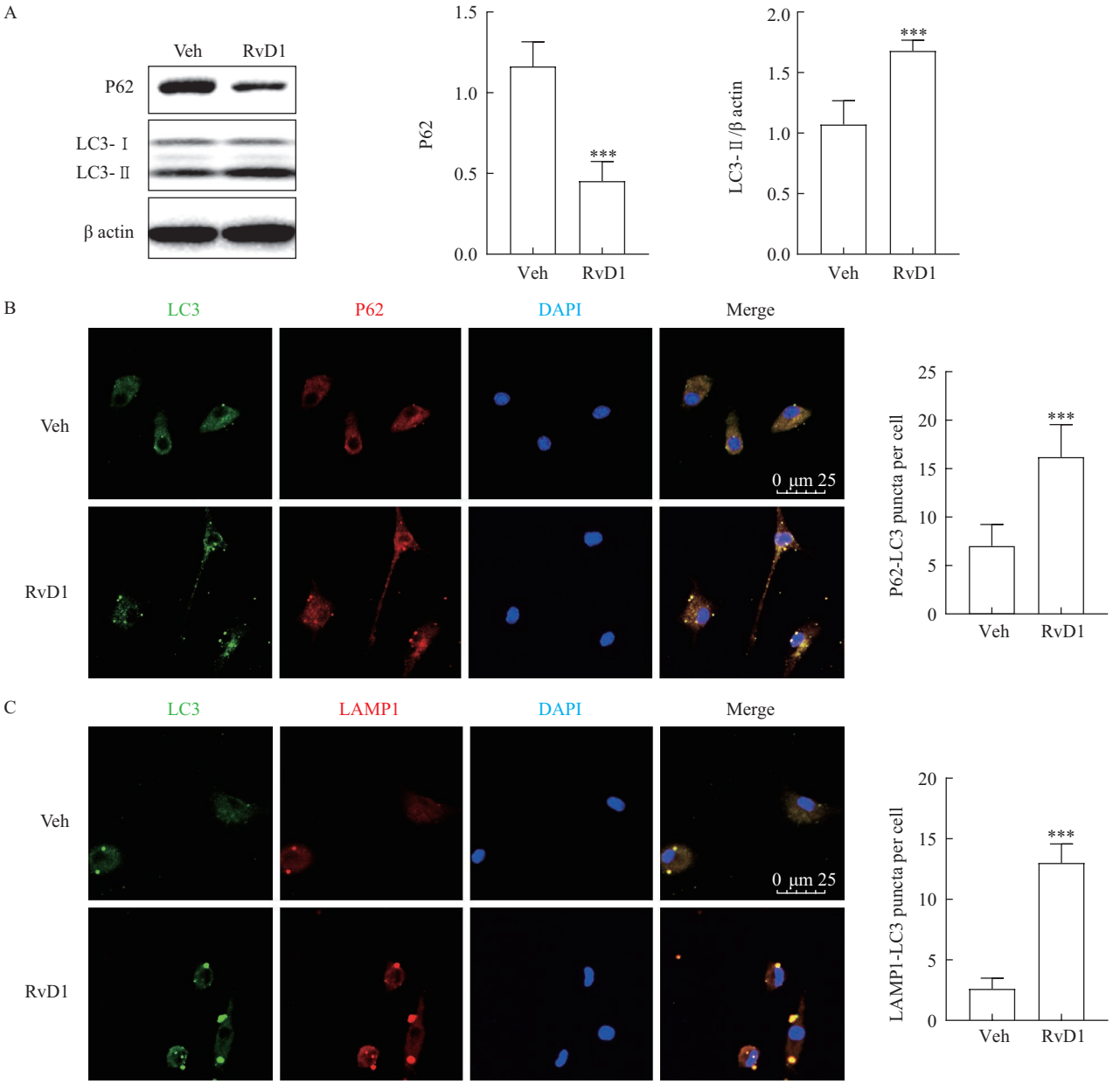

Fig. 3 RvD1 treatment promotes late stages of autophagy by inducing the formation of autolysosomes in primary microglia
A: Primary microglia were treated with vehicle (Veh) or RvD1 (50 nmol/L, 2 h). The expression of P62 and LC3-Ⅱ was measured by Western blotting. Bands were normalized by β-actin. Bar charts indicate the quantitative analysis of P62 and LC3-Ⅱ (n=6). B, C: Confocal immunofluorescence microscopy images show LC3 (green) and P62 (red) or LC3 (green) and LAMP1 (red) double staining of primary microglia treated with Veh or RvD1 (50 nmol/L, 2 h). Nuclei (blue) were labeled with DAPI. LC3-P62 and LC3-LAMP1 puncta quantification was conducted and is shown in the bar graphs (n=5 per group). Data are presented as mean ± SEM. $^{***}P$<0.001 *vs*. Veh. Scale bar = 25 μm

2.3 RvD1 Plays No Significant Role in the Activation of mTOR or Its Downstream Substrates

The mTOR signaling pathway is known to play a central role in a series of cellular processes, including early-stage autophagy. To further probe its involvement in RvD1-induced autophagy, BV-2 cells were incubated with vehicle or RvD1 for 1 and 2 h, respectively, following which the cells were lysed for Western blot analysis. Interestingly, no significant changes were detected in the expression of mTOR or its corresponding substrates, including ULK1, S6K, and 4E-BP1 (fig. 4A), regardless of the levels of total proteins, phosphorylated proteins, or phosphorylated-to-total protein ratio (fig. 4B–4D). All of these results suggest that RvD1 exerts no significant effect on the mTOR signaling pathway.

2.4 RvD1 Activates Autophagy by Reducing Cytoplasmic Calcium Levels and Suppressing the Activation of Calcium/Calmodulin-dependent Protein Kinase Ⅱ (CaMKⅡ)

A previous study has confirmed that increased cytoplasmic calcium levels negatively regulate autophagy, which represents one of the mTOR-independent pathways[31]. Moreover, it has been

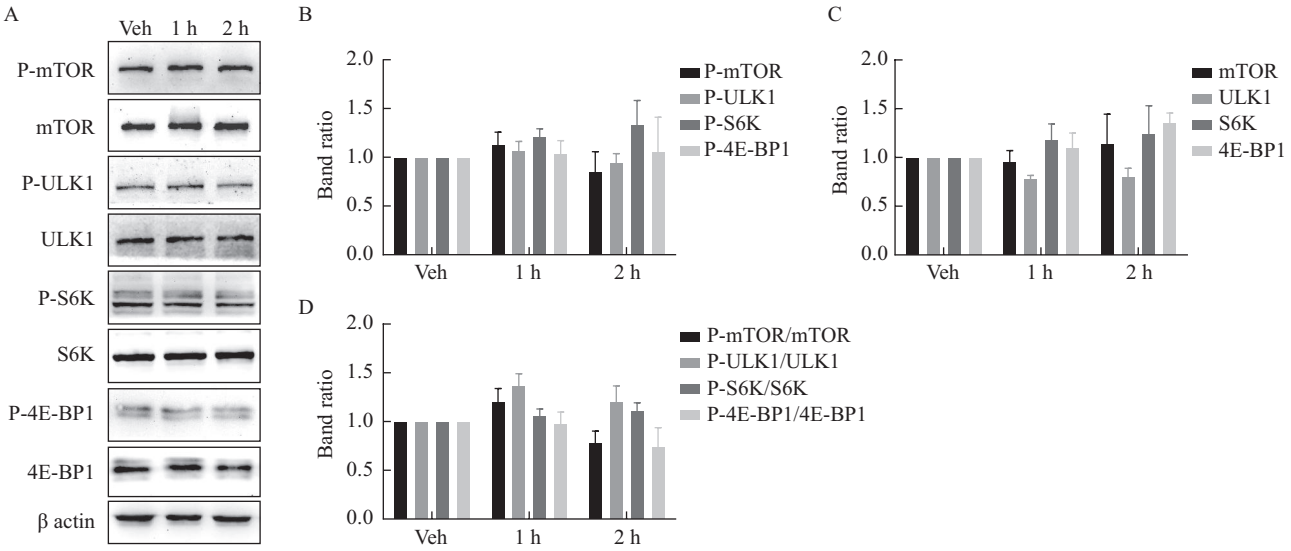

Fig. 4 RvD1 plays no significant roles in activation of mTOR or its downstream substrates

A: BV-2 cells were treated with vehicle (Veh) or RvD1 (50 nmol/L) for 1 or 2 h. The levels of mTOR, ULK1, S6K and 4E-BP1 and the corresponding phosphorylated forms were detected by Western blotting. B–D: Quantification of phosphorylated (B), total (C), and phosphorylated-to-total (D) protein expression is shown as bar charts (n=4). Data are presented as mean ± SEM.

previously reported that RvD1 exerts its proresolving actions/effects *via* blockage of calcium influx and suppression of CaMK Ⅱ activation in macrophages[39]. Next, the present study assessed whether the above conclusions would apply to BV-2 cells and further clarified the underlying mechanism involved in RvD1-induced autophagy. In particular, AA was used to increase the cytoplasmic calcium concentration. Furthermore, Western blot analysis was used to detect the protein expression, and the Ca^{2+} fluorescent probe Fluo-4-AM was applied to measure the intracellular calcium levels by flow cytometry. Treatment with AA significantly increased the phosphorylation of CaMK Ⅱ and the intracellular calcium levels, which were remarkably decreased following cotreatment with RvD1 (fig. 5A, 5B, 5E and 5F). However, no apparent differences were observed among various groups in terms of total CaMK Ⅱ or the phosphorylated-to-total ratio (fig. 5C and 5D). Cotreatment with RvD1 dramatically restored the autophagy suppressed by AA administration, which manifested as an increase of P62 degradation and LC3-Ⅱ expression (fig. 5G–5I). The aforementioned results corroborated the hypothesis that RvD1 promotes autophagy by decreasing cytoplasmic calcium and suppressing CaMK Ⅱ activation in BV-2 cells, further confirming that RvD1 induces mTOR-independent autophagy.

2.5 ATG5 Knockdown Abolishes RvD1-induced Promotion of Phagocytic Activity and Autophagy

Considering that phagocytic activity is closely related to autophagy, we next explored the effect of autophagy on the phagocytosis in BV-2 cells through knocking down the ATG5 level. Following RvD1 administration, wild-type (WT) BV-2 cells showed increased degradation of P62 and elevated expression of ATG5 and LC3-Ⅱ, indicating the activation of autophagy. This effect of RvD1 was abolished by knocking down ATG5 (fig. 6A–6D). During the phagocytosis assay, the WT group presented a dramatic increase in the capacity to phagocytose zymosan A particles following RvD1 treatment. However, this phagocytosis-inducing effect of RvD1 disappeared in the ATG5-knockdown (ATG5-KD) groups (fig. 6E and 6F). These results demonstrate that RvD1 induces ATG5-dependent autophagy in microglia, which exerts a major effect on the microglial phagocytic activity.

3 DISCUSSION

The present study unraveled a new role of RvD1 in the activation of microglial autophagy and provided the first insight into the underlying mechanism. In particular, the administration of RvD1 increased autophagic flux in both BV-2 cells and primary microglia as well as increased P62 degradation and LC3-Ⅱ expression. Interestingly, RvD1 showed no significant effect on the activation of mTOR signaling following drug addition, which indicated the involvement of an mTOR-independent pathway. Moreover, the AA-mediated increase in calcium influx and phosphorylated CaMK Ⅱ was remarkably alleviated by RvD1 treatment. Meanwhile, knockdown of ATG5 abolished the promotion of phagocytic activity induced by RvD1. These results suggest that RvD1 induces mTOR-independent and ATG5-dependent autophagy in BV-2 cells.

The significance of neuroinflammation has been previously illustrated by several studies[1–6]. Microglia are key immune cells present in the brain that are known to be involved in the regulation of

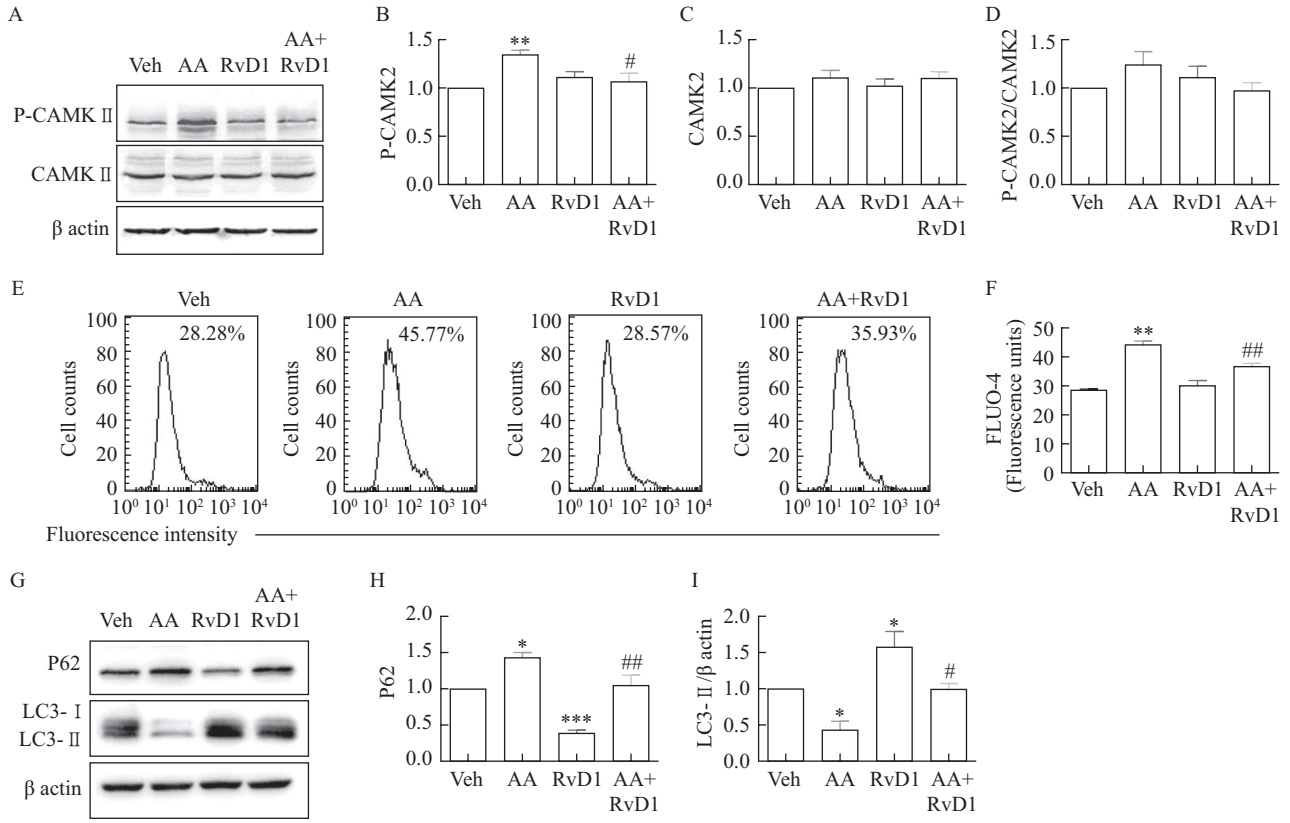

Fig. 5 RvD1 activates autophagy by reducing the cytoplasmic calcium levels and suppressing the activation of CaMK Ⅱ

BV-2 cells were administered with vehicle (Veh) , arachidonic acid (AA, 50 μmol/L, 4 h), RvD1 (50 nmol/L, 2 h), or a combination of AA (2 h ahead of RvD1) and RvD1. A: Representative immunoblots of CaMK Ⅱ and the corresponding phosphorylated form were detected by Western blotting. B–D: Quantitative analysis of phosphorylated (B), total (C), and phosphorylated-to-total (D) protein is presented as bar graphs (n=3). Bands were normalized by β-actin. E, F: flow cytometric analysis of specific fluorescence of the FLUO-4 calcium assay. Histograms show the quantitative analysis of the intracellular calcium levels (n=4). G–I: The expression of P62 and LC3- Ⅱ was measured by Western blotting. Bands were normalized by β-actin. Bar charts indicate the quantitative analysis of P62 and LC3- Ⅱ (n=3). Data are presented as mean ± SEM. $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 *vs*. Veh; $^{\#}P$<0.05, $^{\#\#}P$<0.01 *vs*. AA

inflammatory responses elicited by an array of stimuli, involving injuries and pathogens, and the release of soluble inflammatory mediators[40]. In addition, it has been previously reported that excessive activation of microglia by inflammatory factors results in a change in the balance between the proinflammatory and proresolving phenotype, which further leads to a vicious cycle of neuroinflammation[41]. Furthermore, the activation state of microglia is known to occupy a core status in the pathophysiological process of cerebral inflammation, to a certain degree.

RvD1 has been previously shown to exhibit anti-inflammatory effects in microglia[11, 22, 23, 42–44]. As one of the best-studied SPMs, RvD1 has been reported to induce the activation of autophagy in macrophages[38]. In addition, recent studies have unraveled the critical role of autophagy in microglia function[9, 45]. Hence, it was postulated that RvD1 might play an important role in the activation of microglial autophagy. As expected, RvD1 promoted autophagy in both BV-2 cells and primary microglia, which was confirmed using Western blotting, confocal immunofluorescence microscopy, and transmission electron microscopy. The present study is the first to report that RvD1 exerts an activation effect on microglial autophagy.

The mTOR pathway is one of the important and classical pathways that are related to autophagy. In particular, mTOR is a suppressive regulator of autophagy, and it serves as a mechanistic target for rapamycin and a downstream signaling molecule for the phosphoinositide 3-kinase pathway, thereby regulating various fundamental cell processes, including metabolism and homeostasis[30]. In a previous study, RvD1 was shown to be involved in transient activation of mTOR in macrophages at the initial stages of drug addition. Importantly, these effects disappeared at a longer exposure time[38]. In comparison, no significant changes were observed in the expression or activation of mTOR in BV-2 cells stimulated with RvD1 in our study. This finding was further confirmed in terms of mTOR substrates, ULK1, S6K, and 4E-BP1. The above results suggest that RvD1 exerts its effect *via* mTOR-independent autophagy.

It is well known that apart from mTOR signaling,

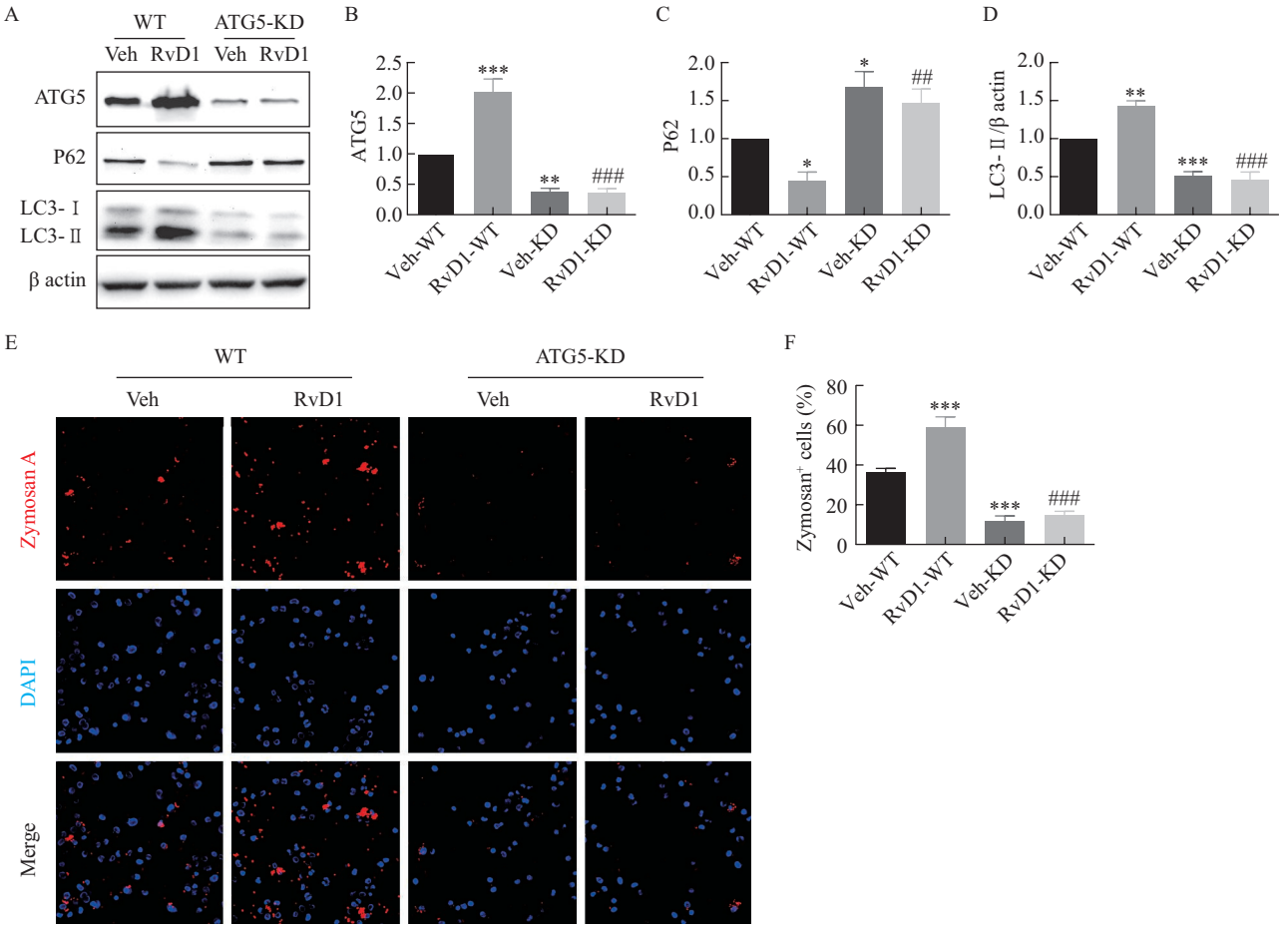

Fig. 6 RvD1 promotes phagocytic activity through ATG-5-dependent autophagy
Wild-type (WT) and ATG5-knockdown (ATG5-KD) BV-2 cells were treated with vehicle (Veh) or RvD1 (50 nmol/L, 2 h), respectively. A: The levels of ATG5, P62, and LC3- Ⅱ were detected by Western blotting. B–D: Quantification of ATG5 (B), P62 (C), and LC3- Ⅱ (D) protein levels is presented as bar graphs (n=3). All of the immunoblots were normalized by β-actin. E, F: Representative confocal fluorescence microscopy images show BV-2 cells incubated with zymosan A particles. The percentage of zymosan$^+$ cells was quantified and presented as histograms (n=3). Data are presented as mean ± SEM. $^*P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 *vs*. Veh-WT; $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 *vs*. RvD1-KD

autophagy can also be regulated by multiple pathways, in an mTOR-independent manner, such as variation in the cytoplasmic calcium level. A previous study has indicated that elevated cytosolic calcium levels strongly inhibited autophagy in rat hepatocytes[29]. It also has been reported that variations in the cytosolic calcium concentration can generate/exert complex effects on the formation of autophagosomes and autolysosomes. Interestingly, certain agents or drug carriers that promote calcium influx can lead to blockage of autophagic flux. In particular, treatment with an L-type Ca^{2+} channel blocker has been reported to markedly lower cytosolic calcium levels as well as increase the number of autophagosomes and the degradation of autophagy substrate[31]. Moreover, it has been demonstrated that RvD1 promoted inflammation resolution, which was mediated *via* the suppression of activation of CaMK Ⅱ[39]. In view of the aforementioned information, it might be expected that RvD1 regulates microglial autophagy by affecting cytosolic calcium levels. Consistent with the current hypothesis, treatment of BV-2 cells with RvD1 resulted in the downregulation of cytoplasmic calcium levels and inhibited CaMK Ⅱ activity. Thus, the identification of a novel role of RvD1 in the regulation of cytosolic calcium provided new insights into the potential mechanism underlying mTOR-independent autophagy in microglia.

Autophagy and phagocytosis are the two basic lysosomal clearance processes sharing mechanistic and functional similarities. ATG5 is an essential autophagy protein. The sequential and coordinated recruitment of LC3 and autophagy-related proteins, including ATG5, is an indispensable step in autophagy and certain types of phagocytosis[45]. Here, we showed that ATG5 knockdown inhibited autophagy and impaired the phagocytotic activity induced by RvD1 in BV-2 cells, manifesting a regulatory interaction between autophagy and phagocytosis. These results indicate that RvD1 promotes phagocytic activity and autophagy of microglia in an ATG5-dependent manner.

Our work has some limitations. First, this research was mainly carried out on BV-2 mouse microglia

cells instead of mouse primary microglia. Although the BV-2 cell line is a commonly used substitute in many experimental settings and we did achieve similar results from primary microglia on some key points of this study, whether the present conclusions would apply to *in-vivo* models remains to be determined. Second, the current study did not address the effect of RvD1-induced autophagy on neuroinflammation. Follow-up research is required to clarify the role of microglial autophagy in the context of inflammatory neurological diseases. Moreover, it is noteworthy that the natural microenvironment of microglia contributes to changes in the microglia signature. The connection and communication between microglia and the other surrounding cell types should not be underestimated[46].

For the past many years, the major thrust of autophagy research in the brain has been primarily focused on neurons. In recent years, emerging studies have identified autophagy modulation in microglia as a substantial regulator of neuroinflammation. The present study established that RvD1 exerted an activation effect on microglial autophagy. Importantly, identification of the functional consequences of microglial autophagy on neuroinflammation would provide new insights, which would form the basis for the next experimental study. It is believed that in-depth elucidation of RvD1-induced microglial autophagy and its impact on overall cerebral pathologies would assist in novel discoveries, which would eventually extend our understanding regarding the driving mechanism involved in inflammatory diseases of the central nervous system.

In conclusion, the current study revealed that RvD1 induced autophagy in microglia, which was mediated *via* the promotion of the formation of autophagosomes and autolysosomes. RvD1 exerted no significant activation effect on mTOR or its corresponding substrates. Importantly, an AA-induced increase in cytoplasmic calcium levels could be attenuated by cotreatment with RvD1. Thus, RvD1 reversed the autophagy inhibitory effect of calcium influx (fig. 7). Interestingly, knocking down ATG5 abolished the phagocytic activity and autophagy induced by RvD1. Altogether, the present study revealed that RvD1 induced activation of autophagy in microglia in an mTOR-independent and ATG5-dependent manner.

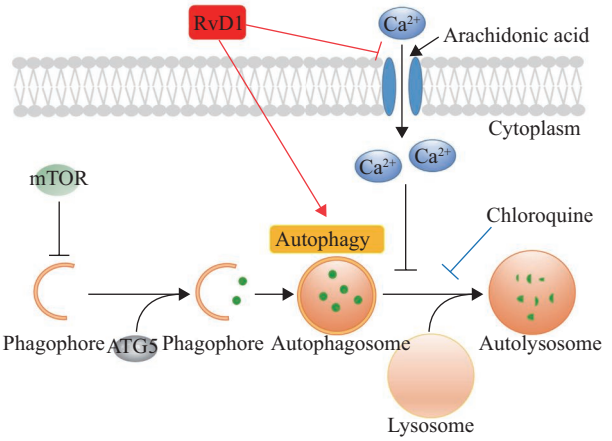

Fig. 7 Schematic diagram of the RvD1 induced mTOR-independent and ATG5-dependent autophagy in BV-2 cells

RvD1 induces ATG5-dependent autophagy in BV-2 cells by promoting maturation of autophagosomes and their fusion with lysosomes. Chloroquine inhibits autophagy through blockade of autophagosome-lysosome fusion. RvD1 exerts no significant effect on mTOR or its corresponding substrates. Increased cytoplasmic calcium induced by arachidonic acid could be attenuated by cotreatment with RvD1, reversing the autophagy inhibition effect of calcium influx.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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