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Desoxyrhaponticin attenuates M1 Macrophage Polarization via targeting PI3K/AKT/mTOR signaling pathway in RAW264.7 macrophage cells

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Article

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Abstract

Background:

Macrophages play a critical role in the inflammatory response and excessive activation of M1-type macrophages is detrimental to the repair following acute myocardial infarction (AMI). Desoxyrhaponticin is an extract of Rheum tanguticum Maxim, a Chinese traditional nutrition food. Previous studies revealed that stilbene compounds of rhubarb possess anti-inflammatory activity, but no study has addressed whether Desoxyrhaponticin can regulate the polarization of macrophages to exert anti-inflammatory effects. Therefore, this study was designed to investigate the anti-inflammatory activity of Desoxyrhaponticin and the underlying mechanism.

Methods:

RAW264.7 cells were polarized to M1 macrophage by lipopolysaccharide (LPS). A cell counting kit-8 (CCK-8) assay was used to assess the cytotoxicity of Desoxyrhaponticin. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) and Western blotting were used to determine the mRNA and protein expression level of M1 macrophage marker. Western blotting was used to evaluate the activation of the PI3K/AKT/mTOR signaling pathway. PI3K inhibitor LY294002 were used to inhibit PI3K/AKT/mTOR signaling pathway.

Results:

The obtained results revealed that Desoxyrhaponticin inhibits the M1 polarization of RAW264.7 macrophages via the PI3K/AKT/mTOR signaling pathway. Conversely, PI3K inhibition by LY294002 exacerbated RAW264.7 macrophages polarization to the M1 type.

Conclusion:

Our study demonstrated the anti-inflammatory effects of Desoxyrhaponticin via PI3K/AKT/mTOR signaling pathway downregulation in RAW264.7 macrophages.

1.Introduction

Acute myocardial infarction (AMI) is a common cardiovascular disease with high incidence and mortality[1]. Recent research has revealed that inflammatory response plays a major role in the pathogenesis of myocardial infarction, including tissue repair and scar formation. Monocytes in circulating blood rapidly infiltrate into the infarcted region after MI and then differentiate into macrophages[2]. Native macrophage (M0-type) can be polarized into two major subtypes, classically

activated macrophages (M1-type) and classically activated macrophages (M2-type) with different surface markers and functions. The M1-type macrophages is the main subtypes in the

early phase of MI, characterized by increased phagocytic activity and enhanced levels of iNOS and TNF-α, but prolonged inflammatory response results in adverse cardiac remodeling and dysfunction.

The M2-type macrophages gradually predominate in the late phase of MI to inhibit excessive inflammatory as well as promote tissue repair and angiogenesis[3]. Therefore, regulation of macrophage polarization is a promising therapy for MI treatment.

Desoxyrhaponticin (DC) is an extract of Rheum tanguticum Maxim (also known as rhubarb), a Chinese traditional nutrition food and herb. Previous studies reported that Desoxyrhaponticin exert various bioactivities, including anti-allergic effect[4], antioxidant effect [5] and the apoptotic effect on Fatty acid synthase (FAS) over-expressed human breast cancer MCF-7 cells[6]. Many stilbene compounds of rhubarb possess anti-inflammatory activity, such as Resveratrol[7] and Rhaponticin[8]. However, whether Desoxyrhaponticin can regulate the polarization of macrophages to exert anti-inflammatory effects remains unknown.

The regulation of macrophage polarization is complex and involves multiple pathways. Phosphoinositide 3-kinase (PI3K) is an important transmission signal and activated PI3K recruits protein kinase B (AKT) and mammalian target of rapamycin (mTOR) [9]. Previous studies showed that PI3K/AKT/mTOR signaling pathway was up-regulated in LPS-induced M1 macrophages and down-regulation of PI3K/AKT/mTOR signaling pathway inhibited M1 macrophage polarization[10]. In this study, we hypothesized that Desoxyrhaponticin reduced LPS-induced RAW264.7 cells polarization to the M1 type via PI3K/AKT/mTOR signaling pathway downregulation.

2.Results

2.1 Effect of Desoxyrhaponticin on cell viability

The chemical structure of Desoxyrhaponticin were shown in Fig. 1A. To assess Desoxyrhaponticin toxicity, we treated RAW264.7 cells with different concentrations of Desoxyrhaponticin for 24 h. The CCK-8 result demonstrated that RAW264.7 cell viability displayed a trend of dose dependence, and Desoxyrhaponticin exhibit no obvious cytotoxicity under a concentration of 50μM comparing with the control group (Fig. 1B). A Desoxyrhaponticin concentration of 30 μM was selected for further investigation.

2.2 Desoxyrhaponticin inhibits RAW264.7 macrophages polarization to the M1 type

iNOS and TNF-α are widely known as M1 macrophage marker genes. In the LPS treated group, the mRNA expression level of iNOS and TNF-α as well as the protein expression level of iNOS were significantly

increased in RAW264.7 cells, indicating the successful establishment of the M1 macrophages polarization model (Fig. 2A-D). The real-time PCR assay demonstrated that macrophages polarization to the M1 type were attenuated through the administration of Desoxyrhaponticin (Fig. 2A-B). Similar results were observed on iNOS protein expression assessed via western blot (Fig. 2C-D). These results suggested that Desoxyrhaponticin exert anti-inflammatory activity in LPS treated RAW264.7 cells.

2.3 Desoxyrhaponticin reduces LPS-induced PI_3K/AKT/mTOR signaling pathway activation

Given that PI3K/AKT/mTOR signaling pathway is one of the key pathways regulating macrophage polarization[9], the activation of PI3K/AKT/mTOR signaling pathway was determined. As expected, the extended protein levels of the PI3K/AKT/mTOR signaling pathway were confirmed in M1 macrophages via western blot analysis (Fig. 3). Administration of Desoxyrhaponticin significantly reduced the phosphorylation of PI3K and its downstream signal molecules AKT and mTOR. Collectively, PI3K/AKT/mTOR signaling pathway is involved in LPS-induced M1 macrophages polarization and decreased by Desoxyrhaponticin pretreatment.

2.4 Desoxyrhaponticin inhibits RAW264.7 macrophage M1 polarization by inhibiting PI3K/AKT/mTOR signaling pathway activation

To further assess the role of the PI3K/AKT/mTOR signaling pathway in the anti-inflammatory effect of Desoxyrhaponticin on RAW264.7 cells stimulated with LPS, we treated RAW264.7 cells with the PI3K inhibitor LY294002. As shown in Fig. 4, Desoxyrhaponticin significantly reduced M1 macrophage related gene expression, while PI3K inhibitor LY294002 reversed the anti-inflammatory effect of Desoxyrhaponticin.

Furthermore, application of LY294002 markedly reversed the effects of Desoxyrhaponticin on anti-M1-type polarization and aggravate the expression of phospho-PI3K, phospho-AKT and phospho-mTOR (Fig. 5), suggesting that Desoxyrhaponticin exert anti-inflammation activity through PI3K/AKT/mTOR signaling pathway.

3.Discussion

This study was designed to verify the hypothesis that Desoxyrhaponticin inhibits macrophages M1 polarization to ameliorate LPS-induced inflammation and to clarify the molecular mechanisms of this effect. In this study, we showed that administration of Desoxyrhaponticin inhibits the RAW264.7 macrophages M1 polarization, suggesting that Desoxyrhaponticin possess anti-inflammatory ability. Our results demonstrated that PI_3K/AKT/mTOR signaling pathway was upregulated in LPS induced M1 macrophages in vitro and can be inhibited by Desoxyrhaponticin pre-treated.

Accumulating evidence has suggested that macrophage-mediated inflammation plays a crucial role in multiple inflammation related diseases, such as inflammatory bowel diseases[11], osteoimmunity and osteo-related diseases[12], and myocardial infarction[13]. Myocardial infarction is caused by sudden occlusion of the coronary artery. The repair of infarcted heart can be divided into three distinct but overlapping phases: the inflammatory phase, the proliferative phase and the maturation phase. The involvement of macrophages is crucial in all stages of myocardial infarction recovery. In the early stage, monocytes in circulating blood rapidly infiltrate into the infarcted region triggered by proinflammatory cytokines and chemokines, and then differentiate into macrophages. These monocyte-derived macrophages exert phagocytosis and efferocytosis effect to clear dying cells as well as matrix debris. The process of phagocytosis enables macrophages to adopt an anti-inflammatory phenotype and the repair of infarcted heart transited from the inflammatory phase to the proliferative phase. Polarized macrophages can be divided into proinflammatory M1 macrophages (classically activated macrophages) and inflammatory M2 cells macrophages (classically activated macrophages). Mouton et al. [14] assessed macrophage polarization transcriptomic signatures of macrophages isolated from the infarct region and reported that macrophages exhibited increased proliferation and phagocytosis at Day 3 while displayed a pro-reparative signature enriched for genes involved in ECM remodeling and scar formation at Day 7.

Altering M1/M2 macrophage polarization is necessary to replace dying cells and matrix debris with granulation tissue and protect the infarcted heart from adverse remodeling[15, 16]. A prolonged inflammatory response may promote left ventricle dilation and excessive scar formation leading to adverse remodeling[17]. Thus, timely regulation of macrophage polarization is a promising therapy for MI treatment.

It has been reported that several compounds isolated from rhubarb possess anti-inflammatory activity. Hu et al.[18] found that Rhein, emodin and aloe-emodin inhibit LPS-stimulated RAW264.7 macrophages by downregulating NF-κB phosphorylation and iNOS production. Rhubarb was also shown to prevent atherosclerosis via regulatory effects on foam cell formation and macrophage polarization as an inhibitor of M1 macrophage[19]. However, whether Desoxyrhaponticin affects macrophage polarization remains unknown. Herein, we demonstrated that Desoxyrhaponticin inhibit LPS-stimulated RAW264.7 macrophages M1 polarization, suggesting that Desoxyrhaponticin is a promising drug for inflammation related diseases.

Studies have reported that PI3K/AKT/mTOR signaling pathway was activated in multiple inflammation related diseases[20]. Li et.al[21] reported that Artemisinin relieves Osteoarthritis via inhibiting the PI3K/AKT/mTOR signaling pathway. Arranz et.al[22] reported that AKT1 ablation giving rise to an M1 while AKT2 ablation resulting in an M2 phenotype. Liu et al[23]reported that activation of AKT1/SOCS1/NF-κB inhibit M1 polarization. Wu et al [24] reported that the overexpression of AKT2 inducing M1 and decreasing M2, while knockdown AKT2 exerting an opposite effect. All of these suggesting that AKT molecule converges multiple signals to regulate macrophage pro- and anti-inflammatory effects. The role of PI3K/AKT/mTOR signaling pathway in myocardial infarction including

regulation of macrophage phenotypic transformation[25], autophagy[26] and angiogenesis[27]. Our results investigated that Desoxyrhaponticin inhibited macrophages M1 polarization by downregulating PI3K/AKT/mTOR signaling pathway. This result may be because it have adjusted the ratio of AKT1 in AKT, thereby inhibiting the polarization of M1 macrophages.

However, this study has some limitations. We lack evidence of in vivo experiments and we will carry out experiments to further explore and verify these findings in vivo in the future.

4.Material and Methods

4.1 Cell cultures and treatment

The murine RAW264.7 macrophage cells were obtained from the American Type Culture Collection. We cultured the cells in RPMI 1640 (Gibco) supplemented with 10% (v/v) fetal bovine serum (Gibco). The cells were incubated in humidified atmosphere at 37°C with 5% CO_2. All cells were in a logarithmic growth phase when they were intervened. RAW264.7 cells were induced to polarize with RPMI 1640 solution and 1μg/mL LPS for 24 h as previously described[26]. PI_3K inhibitor LY294002 (30 μM) was administered 30 min prior to treatment with LPS as previously described[28].

4.2 Cell Counting Kit-8 (CCK-8) Assay

The cytotoxicity of Desoxyrhaponticin was determined by CCK-8 assay (Beyotime, C0038). RAW264.7 macrophage cells were seeded in 96-well plates at 1×10^4 cells/well. After treatments with different concentrations (0-100 μM) of Desoxyrhaponticin for 24 h, we added 10 μL of CCK-8 reagent to each well and incubated the cells at 37°C for 1.5 h in the dark. The absorbance at 450 nm of each well was detected using a microplate reader (BioTek, United States).

4.3 RNA isolation and quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

Total cellular RNA was extracted using the RNA Purification Kit (EZBioscience, B0004DP). RNA concentration was determined using a NanoDrop UV-Vis spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized with 1 μg of total RNA using the PrimeScript RT Reagent Kit (Takara, RR037A). The qRT-PCR was conducted using LightCycler from Roche Molecular Biochemicals and TB Green Premix Ex Taq (TaKaRa, RR420A). Moreover, GAPDH acted as a housekeeping gen. The gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Table 1 lists the primers used in the experiment.

Table 1
Sequence information of all qRT-PCR primers.

Gene		Sequence of primer (5’ to 3’)
iNOS	Forward	GTTCTCAGCCCAACAATACAAGA
iNOS	Reverse	GTGGACGGGTCGATGTCAC
TNF-α	Forward	CAGGCGGTGCCTATGTCTC
TNF-α	Reverse	CGATCACCCCGAAGTTCAGTAG
GAPDH	Forward	AGGTCGGTGTGAACGGATTTG
GAPDH	Reverse	GGGGTCGTTGATGGCAACA

4.4 Protein preparation and western blot

RAW264.7 macrophage cells were lysed for 15 min in RIPA buffer (Beyotime, P0013B) containing protease and phosphatase inhibitors (Beyotime, P1048) on ice. The Lysates were centrifuged at 12,000 g for 15 min and the supernatant were collected. The protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Fisher, 23,227). In total, 10 μg of protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferredto polyvinylidene fluoride membrane. The membranes were blocked and incubated with primary antibodies against iNOS, PI_3K, Phospho-PI_3K, AKT, Phospho-AKT, mTOR, Phospho-mTOR and GAPDH overnight at 4℃. After washing with Tris-buffered saline with Tween20, the membrane were incubated with an HRP-conjugated secondary antibody at room temperature for 1 h. The bands were visualized with a chemiluminescence system (Bio-Rad), and band intensity was quantified using the ImageJ software (version 1.54e).

4.5 Statistical analysis

Data are expressed as mean ± standard error of the mean. All statistical data were analyzed using GraphPad Prism 9.4.1. Differences among groups were tested by one-way ANOVA followed by post hoc comparisons with Tukey’s post hoc tests (test level α = 0.05).

5.Conclusion

In summary, we verified that Desoxyrhaponticin possess anti-inflammatory activity and inhibited LPS-induced M1 macrophages polarization in RAW264.7 cells. In this process, Desoxyrhaponticin inhibited the activation of PI_3K /AKT/mTOR signaling pathway and the anti-inflammatory activity was reversed by PI_3K inhibitor LY294002.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not Applicable

Availability of Data and Materials

The datasets in the current study are included in the published article or available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

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Authors' contributions

Li and Guo: Conceptualization, Methodology, Software, Investigation, Formal Analysis, Writing - Original Draft;Lu and Qi:Data Curation, Software, Validation.Lian and Xue :Conceptualization, Funding Acquisition, Resources, Supervision, Writing - Review & Editing.

All authors reviewed the manuscript.

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Figures

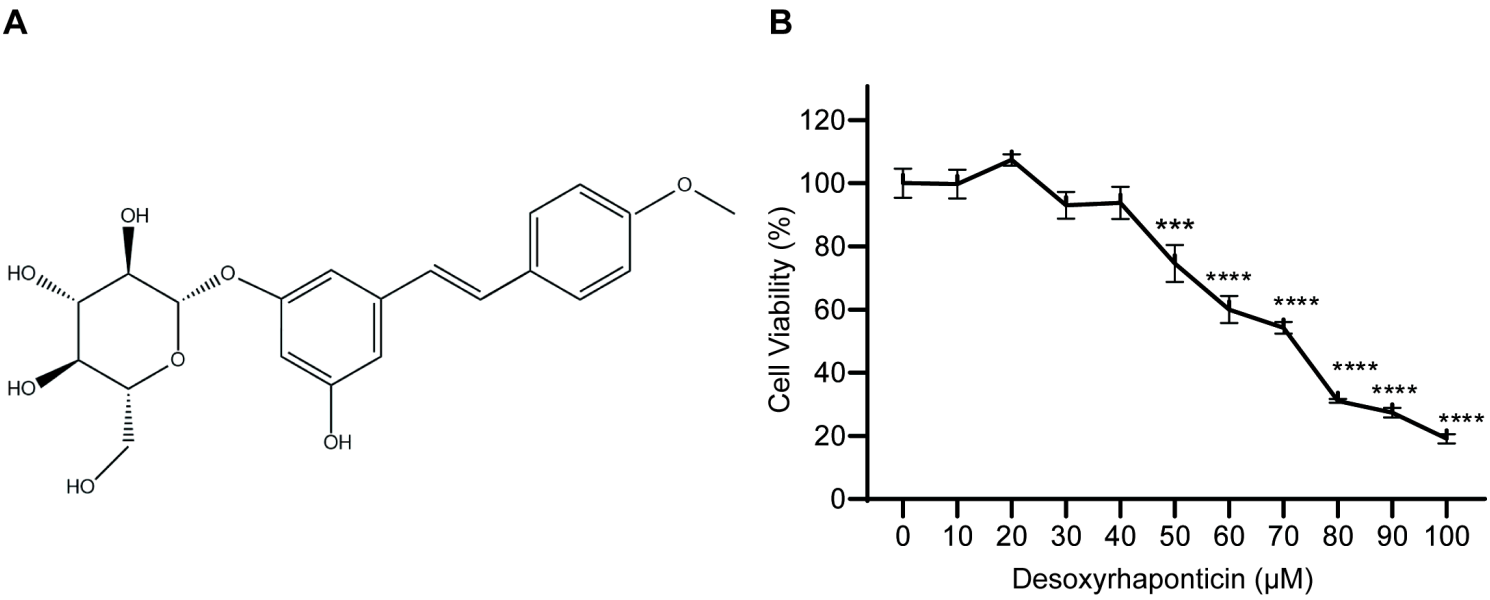


Figure 1

Effects of Desoxyrhaponticin on cell viability in murine macrophage RAW264.7 cells. (A) Chemical structure of Desoxyrhaponticin. (B) Viability of RAW264.7 cells treated with different concentrations of Desoxyrhaponticin. Values are presented as the mean ± standard error of the mean (n = 3 per group). ***p < 0.001, ****p < 0.0001 vs. negative control.

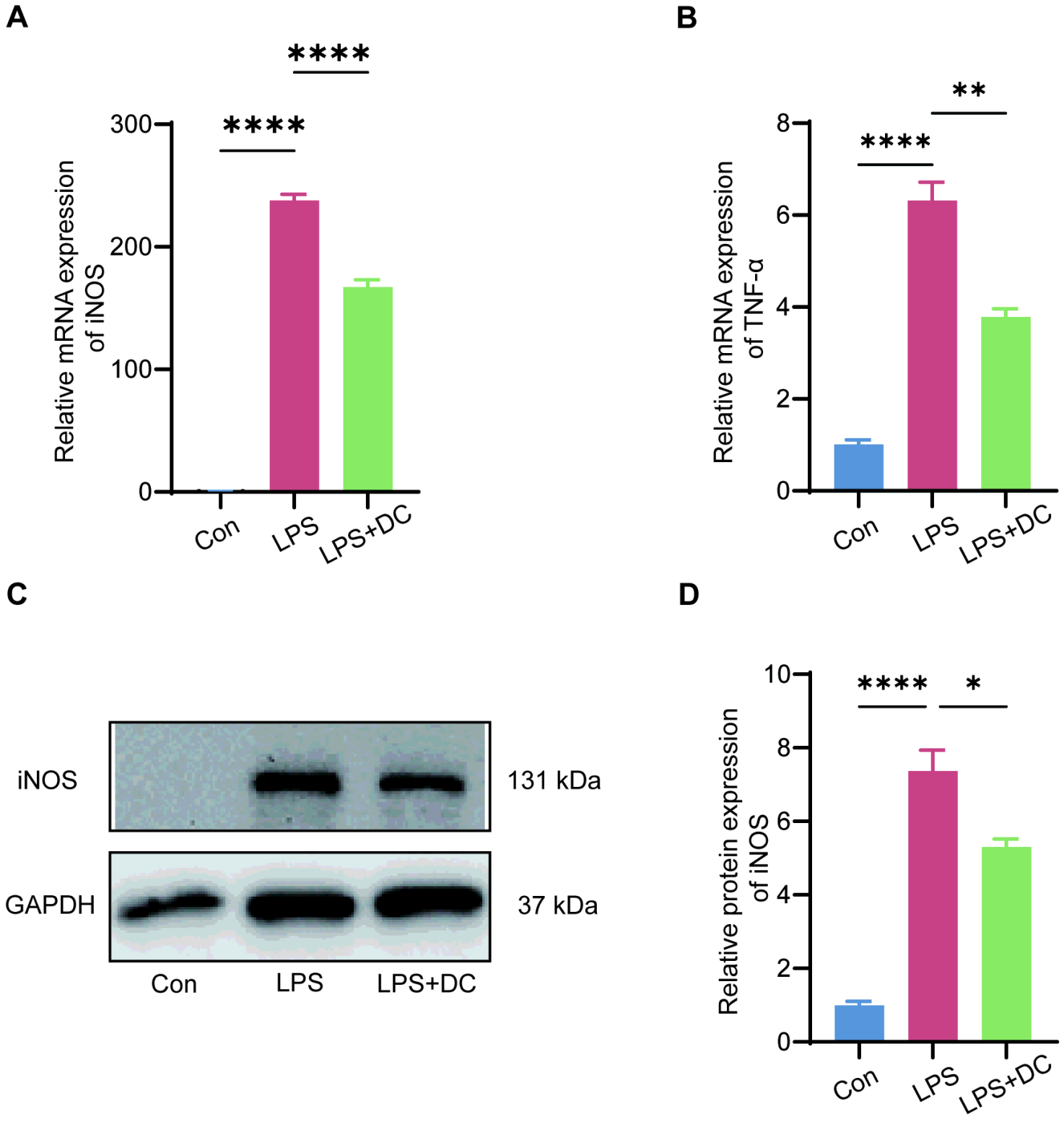


Figure 2

Desoxyrhaponticin suppresses M1 macrophage activation in RAW264.7 cells. (A) iNOS mRNA expression level in RAW264.7 cells. (B) TNF-α mRNA expression level in RAW264.7 cells. (C) Representative blots of iNOS. (D) iNOS quantification. Values are presented as the mean ± standard error of the mean (n = 3 per group). *p < 0.05, **p < 0.01, ****p < 0.0001.

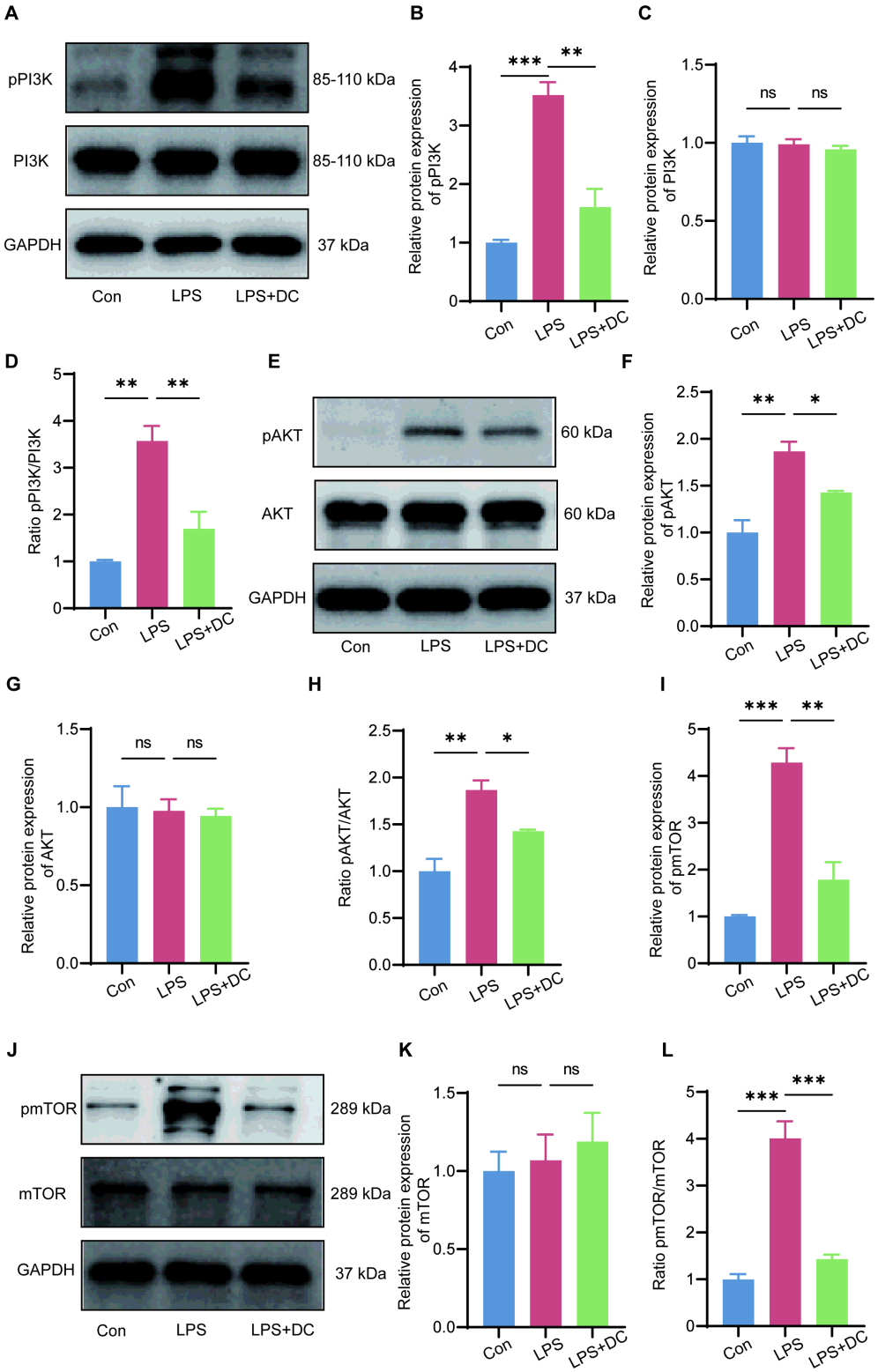


Figure 3

Effect of Desoxyrhaponticin on the PI3K/AKT/mTOR signaling pathway in M1 macrophages. (A) Representative blots of Phospho-PI3K and PI3K. (B) Phospho-PI3K quantification. (C) PI3K quantification. (D) Ratio of Phospho-PI3K/ PI3K expression. (E) Representative blots of Phospho-AKT and AKT. (F) Phospho-AKT quantification. (G) AKT quantification. (H) Ratio of Phospho- AKT / AKT expression. (I) Phospho-mTOR quantification. (J) Representative blots of Phospho-mTOR and mTOR. (K)

mTOR quantification. (L) Ratio of Phospho-mTOR / mTOR expression. Values are presented as the mean ± standard error of the mean (n = 3 per group). ns, not significant. *p < 0.05, **p < 0.01, ***p < 0.001.

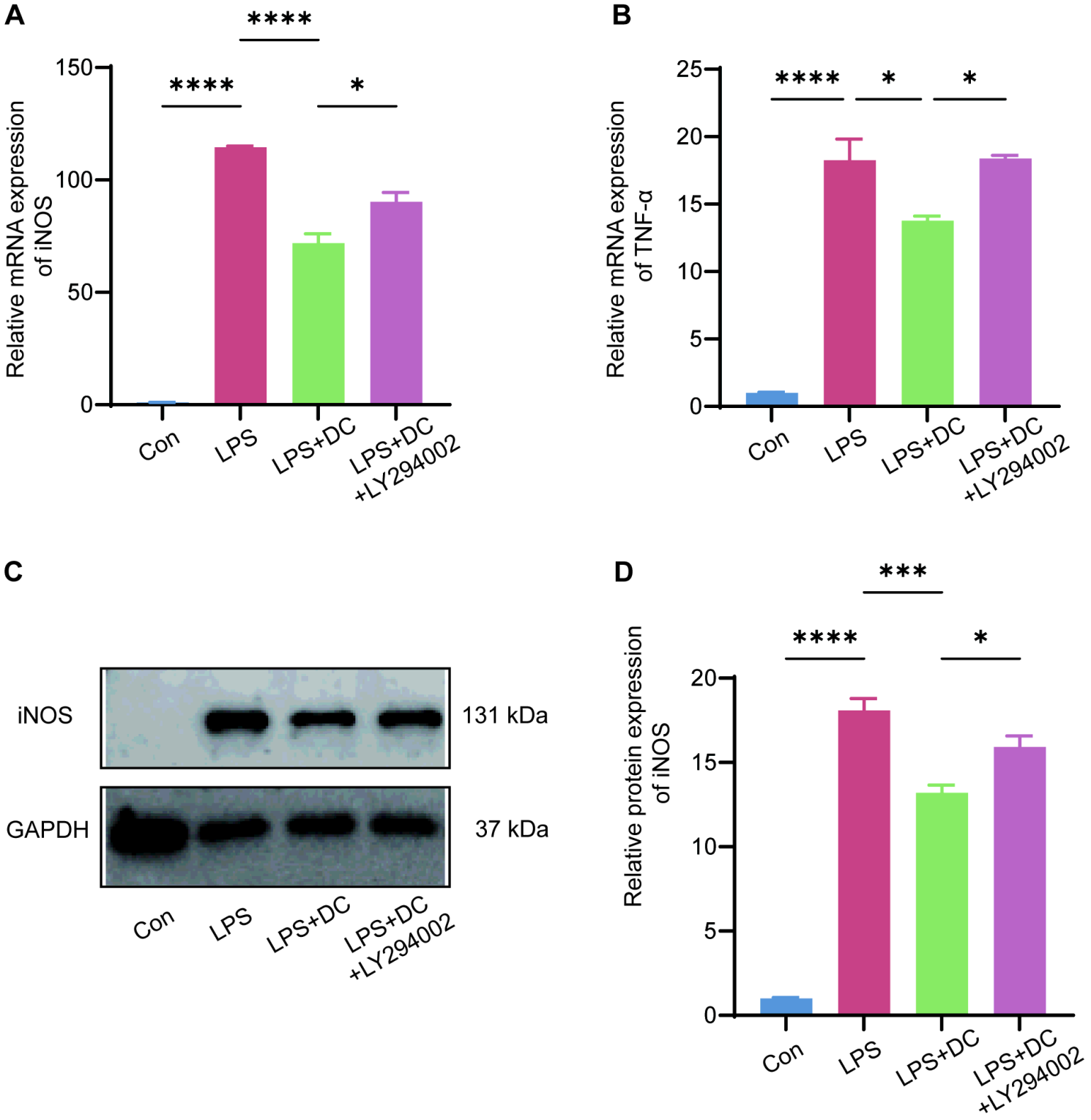


Figure 4

PI3K inhibition clearly decreased anti-inflammatory activity of Desoxyrhaponticin. (A) iNOS mRNA expression level in RAW264.7 cells. (B) TNF-α mRNA expression level in RAW264.7 cells. (C)

Representative blots of iNOS. (D) iNOS quantification. Values are presented as the mean ± standard error of the mean (n = 3 per group). *p < 0.05, ***p < 0.001, ****p < 0.0001.

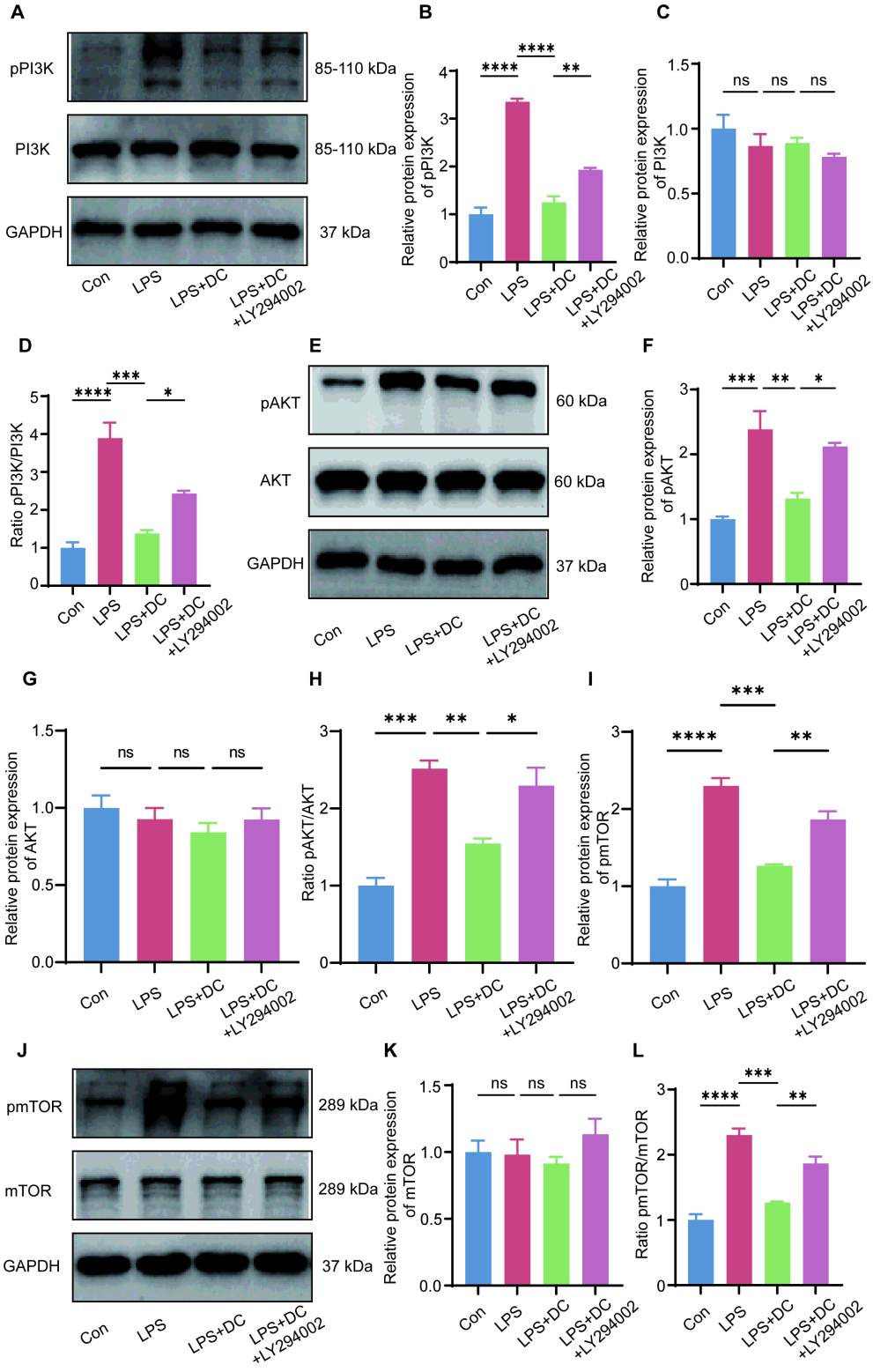


Figure 5

Administration of Desoxyrhaponticin alleviates macrophage M1 polarization through PI3K/AKT/mTOR signaling pathway in RAW264.7 macrophage cells. (A) Representative blots of Phospho-PI3K and PI3K. (B) Phospho-PI3K quantification. (C) PI3K quantification. (D) Ratio of Phospho-PI3K / PI3K expression.

(E) Representative blots of Phospho-AKT and AKT. (F) Phospho-AKT quantification. (G) AKT quantification. (H) Ratio of Phospho- AKT / AKT expression. (I) Phospho-mTOR quantification. (J) Representative blots of Phospho-mTOR and mTOR. (K) mTOR quantification. (L) Ratio of Phospho-mTOR / mTOR expression. Values are presented as the mean ± standard error of the mean (n = 3 per group). ns, not significant. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

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