Pharmacogn. Mag.
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ORIGINAL ARTICLE

Resveratrol Attenuates Inflammation by Regulating Macrophage Polarization via Inhibition of Toll-Like Receptor 4/MyD88 Signaling Pathway

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Submitted: 17-Jul-2020 Revised: 04-Sep-2020 Accepted: 26-Feb-2021 Published: 12-Jul-2021

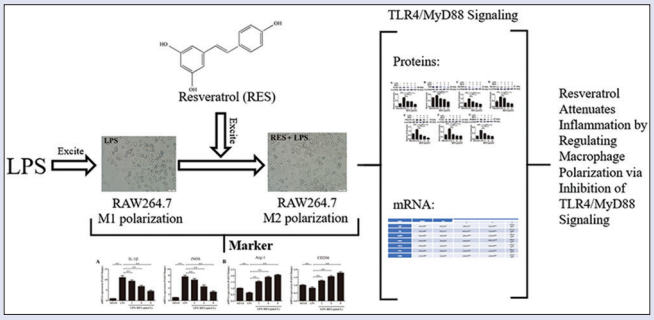
ABSTRACT

Background: Resveratrol (RES) can induce macrophage polarization to achieve the immune response. **Objectives:** In this study, we aimed to determine whether RES attenuates inflammation by regulating macrophage polarization through inhibition of toll-like receptor 4 (TLR4)/MyD88 signaling. **Materials and Methods:** We measured the effects of different concentrations of RES on cellular activity of RAW264.7 and measured it using the methyl thiazolyl blue tetrazolium bromide method. The immunomodulatory effects of RES on lipopolysaccharide (LPS)-induced RAW264.7 cells were detected by measuring the levels of nitric oxide (NO), interleukin (IL)-6, and tumor necrosis factor (TNF)-α. The quantitative real-time polymerase chain reaction (RT-qPCR) was used to detect the markers of M1 and M2 polarization of macrophages. The changes in the expression of both mRNA and proteins related to the TLR4/ myeloid differentiation factor 88 (MyD88) receptor pathway detected by Western blot (WB) and RT-qPCR analyses. **Results:** According to our results, 2, 4, and 8 μmol/L RES decreased the levels of NO, IL-6, and TNF-α in LPS-induced RAW264.7 cells, thereby reducing inflammation and increasing immunity. IL-1 and inducible NO synthase, which are the markers of M1-type macrophages, were increased by LPS, and arginase-1, CD206, which are the markers of M2-type macrophages, were decreased. However, in LPS-induced RAW264.7 cells incubated with RES, we observed the opposite results for both M1-and M2-type macrophage markers. Proteins and mRNA related to the TLR4 pathway were detected by WB and RT-qPCR analysis and TLR4, P65, MyD88, interleukin receptor-associated kinases 1, tumor necrosis factor receptor associated factor 6, activated kinase 1, and IKKβ were significantly increased by LPS. In contrast, when the cells were incubated with RES, the TLR4 pathway-related proteins and mRNA were significantly decreased and showed a volume-response relationship. **Conclusion:** RES can polarize M1-type macrophages to M2-type macrophages and regulate them through the TLR4/MyD88 receptor pathway. The polarization of macrophages can reduce the level of inflammation and regulate the immune system.

Key words: Lipopolysaccharide, macrophages, polarization, resveratrol, toll-like receptor 4/MyD88

SUMMARY

- Resveratrol attenuates inflammation by regulating macrophage polarization via toll like receptor 4/MyD88 receptor pathway.



Abbreviations used: RES: Resveratrol; MTT: Methyl thiazolyl blue tetrazolium bromide; WB: Western Blot; IL-1/6: Interleukin-1/6; TNF-α: Tumor necrosis factor-α; LPS: Lipopolysaccharide; iNOS: Inducible nitric oxide synthase; ARG-1: Arginase 1; TLR4: Toll like receptor 4; MyD88: Myeloid differential protein-88; IRAK1: Interleukin receptor associated kinases 1; TRAF6: Tumor necrosis factor receptor associated factor 6; TAK1: Activated kinase 1; VEGF: Vascular endothelial growth factor; PDGF: Platelet-derived growth factor; LTA: Lipoteichoic acid.

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DOI: 10.4103/pm.pm_312_20

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INTRODUCTION

Resveratrol (RES) was discovered in 1924 as a natural phytoalexin synthesized in various plants, such as grapes, wine, and soy. It plays an important role in response to biotic and abiotic stress in plants.[1] In humans, it prevents oxidation of low-density lipoprotein,[2] limits platelet aggregation,[3] and reduces the risk heart disease.[4] RES can promote the apoptosis of breast cancer cells through cyclooxygenase-2 (COX-2).[5] It also shows anti-inflammatory[6] and anti-allergic[7] effects. It mainly acts through polarization of the cells, which plays a therapeutic role in diseases. For example, RES improves cardiac function by promoting M2-like polarization of macrophages in mice with myocardial infarction.[8] Furthermore, it promotes the M2 polarization of microglia and reduces neuroinflammation after cerebral ischemia by inhibiting miR-155.[9] Under the conditions of neuroinflammatory injury, RES regulates microglia M1/M2 polarization through peroxisome proliferator-activated receptor γ coactivator-1α.[10]

Cite this article as: Fan Y, Huang SL, Li H, Cui YL, Li DY. Resveratrol attenuates inflammation by regulating macrophage polarization via inhibition of toll-like receptor 4/MyD88 signaling pathway. Phcog Mag 2021;17:321-6.

The polarization of macrophages confers vast phenotypic and functional plasticity. Macrophages can be divided into M0 (homeostatic), M1 (pro-inflammatory), and M2 (anti-inflammatory) forms by polarization.[11] Currently, in the construction of the macrophage polarization model, lipopolysaccharide (LPS) is often used to stimulate macrophages to build an M1-polarized cell model of macrophages.[12] Vascular endothelial growth factor (VEGF), arginase-1 (Arg-1), tumor growth factor (TGF)-β, interleukin (IL)-10, and other cytokines secreted by M2-type macrophages play an important role in inhibiting the progress of inflammation, as well as promoting tissue repair and angiogenesis.[11,13,14]

Toll-like receptors (TLRs) have been discovered as mammalian homologs of Toll. TLR2/4 has been shown to be crucial in identifying cell wall components in different bacteria. LPS, lipoteichoic acid, and taxol are recognized by TLR4. TLRs and the downstream signaling pathway play an essential role in innate immune recognition and in subsequent adaptive immune activation.[15] Myeloid differentiation factor 88 (MyD88) is a key adaptor protein of TLRs, which is the major pathway of TLR signal transduction.[16] LPS with TLR4 in mammals activates two downstream intracellular signaling pathways: MyD88-dependent and independent pathways.[17] Many drugs function through regulation of polarization of different cells through TLR pathway.[18-20] Mice immune cells attenuated inflammation by increasing the polarization of M1-to M2-type macrophages via TLR4 pathway,[21] causing macrophage polarization through the inhibition of TLR4 expression and its signaling pathways.[22]

Therefore, this study used LPS to stimulate macrophages to observe that RES attenuates inflammation by regulating macrophage polarization through inhibition of TLR4/MyD88 signaling.

MATERIALS AND METHODS

Reagents

RES (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution of 32 μmol/L which was further diluted as needed.

Cell culture and treatment

RAW264.7 macrophages were purchased in the Cell Bank at Shanghai Academy of Sciences. RAW264.7 cells were cultured in RPMI 1640 cell culture medium supplemented with (fetal bovine serum 10% [V/V]), 10 U/mL penicillin, and 100 U/mL streptomycin. The cell culture environment was 5% CO_2 at 37°C. The culture medium was changed every 3–4 days. The cells were treated with RES in the presence of LPS for further measurements.

Methyl thiazolyl blue tetrazolium bromide assay

RAW264.7 were seeded in 96-well plates at a density of 2×10^4 cells/well/100 μL culture media. RES was used at a concentration range of 1–32 μmol/L and LPS was used at a range of 0.1–10 μg/mL in the experiments. After incubating the cells with different concentrations of the study material for 24 h, we performed methyl thiazolyl blue tetrazolium bromide (MTT) assay. MTT solution (0.5 mg/mL) was added to each well and incubated for 4 h. Then, the supernatant was removed, and 150 μL of DMSO was added to each well. The OD value was determined at a wavelength of 570 nm and 630 nm using a microplate reader. The viability of the cells was calculated by the following formula:

Cell viability (%) = (A 570, 630[sample]/A [control]) ×100.

Measurement of cytokines

RAW264.7 cells were seeded at a density of 5×10^5 cells/mL in 6-well plates and were pretreated with RES (2–8 μmol/L) for 2 h prior to the addition of 1 μg/mL LPS for 12 h. Then, the supernatant was collected through centrifugation at × 1200g for 10 min at 4°C. The tumor necrosis factor-α (TNF-α), IL-6, and nitric oxide (NO) levels were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's specifications.

Western blot analysis

Pierce™ Bicinchoninic acid protein content determination kit (Thermo Fisher Scientific, USA) was used to measure the total protein content. TLR4, P65, MyD88, interleukin receptor associated kinases 1 (IRAK1), tumor necrosis factor receptor-associated factor 6 (TRAF6), activated kinase 1 (TAK1), and IKKβ (TaKaRa, Japan) were incubated overnight with their respective primary antibodies and subsequently with their respective secondary antibodies at 37°C for 1 h. The proteins were detected using a SignalFire™ ECL Reagent (Cell Signaling Technology, USA).

Quantitative real-time polymerase chain reaction

Trizol reagent was used to extract the total RNA in cells, and the RNA was reverse transcribed into cDNA (TaKaRa, Japan) according to the manufacturer's protocol. The gene expression levels were determined by the Applied Bio systems Vii ATM 7 real-time polymerase chain reaction system (Life Technologies, USA). Table 1 shows the primers used in the experiments. The reaction parameters were as follows: predenaturation of 95°C for 2 min; 40 cycles of 95°C for 10 s, 60°C for 3 s, 70°C for 45 s; then annealing at 0.5°C/s and a final extension at 65°C. GAPDH was the internal control to normalize the expression levels of genes.

Statistical analysis

All values are presented as the mean ± standard error (SE). A one-way analysis of variance was conducted using SPSS 18.0 software (IBM, USA) and differences between groups were defined as significant at $P < 0.05$.

Table 1: The sequences of the primers for quantitative real-time polymerase chain reaction

Name	The sequence of the primer (5`-3`)		bp
IL-1β	Forward	GTCTTCAGTGCTCAGGTTTCT	199
	Reverse	AAAGTTGGTGGGAGAATTGAAGC	
iNOS	Forward	TGGCAGTTTCTGTTCAAGGC	139
	Reverse	TGCTGAGGCTGTGACACTTG	
Arg-1	Forward	CAGAAGAATGGAAGAGTCAG	115
	Reverse	CAGATATGCAGGGAGTCACC	
CD206	Forward	GACTCCCGAACCCAAATGTCC	166
	Reverse	TCGCCATATTGTTTGCTGTTCC	
TLR4	Forward	CATGGATCAGAAACTCAGCAAAGTC	140
	Reverse	CATGCCATGCCTTGTCTTCA	
P65	Forward	TGCACAGACTGCCCTGATCC	153
	Reverse	TGCACAGACTGCCCTGATCC	
MyD88	Forward	TACAGGTGGCCAGAGTGGAA	134
	Reverse	GCAGTAGCAGATAAAGGCATCGAA	
IRAK1	Forward	CGGACTTCCACAGTTCGAGGTA	125
	Reverse	TGACCAGCAAGGGTCTCCAG	
TRAF6	Forward	TCATTATGATCTGGACTGCCCAAC	150
	Reverse	TTATGAACAGCCTGGGCCAAC	
TAK1	Forward	AGCAGAGTAGCTGCGGT	134
	Reverse	GAGGAGCTTGCTGCAGAT	
IKKβ	Forward	CAGAATCATCCATCGAGACCTGAA	122
	Reverse	TGCACAGACTGCCCTGATCC	
GAPDH	Forward	TGTGTCCGTCGTGGATCTGA	122
	Reverse	TTGCTGTTGAAGTCGCAGGAG	

Arg-1: Arginase-1; MyD88: Myeloid differentiation factor 88; TLR: Toll-like receptor

RESULTS

Effects of lipopolysaccharide and resveratrol on RAW264.7 cells

Based on the level of NO, LPS caused inflammatory reaction in RAW264.7 cells [Figure 1a]. After our initial experiments, 1 μg/mL LPS was used in subsequent experiments. The effect of RES on the activity of RAW264.7 cells was investigated using the MTT method. Compared with the blank control group, RES at 16 and 32 μmol/L concentrations caused cytotoxicity, whereas the other tested concentrations did not show any toxicity [Figure 1b]. Therefore, we selected 2, 4, and 8 μmol/L RES concentrations for subsequent experiments.

Resveratrol attenuated lipopolysaccharide-induced inflammation

After induction of inflammation with 1 μg/mL LPSRAW264.7, cells were incubated with 2, 4, and 8 μmol/L RES. The inflammatory cytokines NO, IL-6, and TNF-α were measured by ELISA. According to our results, RES significantly reduced the production of inflammatory cytokines in LPS-induced RAW264.7 cells, which was dose-dependent in nature [Figure 2].

Resveratrol-induced polarization of M1 to M2-type in RAW264.7 cells

Compared with the blank control group, the mRNA expression of *IL-1 β* and inducible nitric oxide synthase *(iNOS)* was significantly increased which are the markers of M1-type macrophages [Figure 3a]. The mRNA expression of *IL-1 β* and *iNOS* was significantly decreased after incubation of the cells with 2, 4, and 8 μmol/L RES. According to the results, LPS polarized RAW264.7 cells from M0-type to M1-type. The expression of the markers of M2-type namely, *CD206* and *Arg-1*, was significantly downregulated when treated with LPS. When the cells were treated with both RES and LPS, the mRNA expression of *Arg-1* and *CD206* was significantly increased [Figure 3b]. Furthermore, our results demonstrated that RES polarized the M1 macrophages to M2 macrophages.

Effect of resveratrol on the toll-like receptor 4/MyD88 pathway

In this study, we evaluated the effects of RES on the TLR4/MyD88 signaling pathway to improve the understanding of the potential mechanism by which RES regulated the polarization of macrophage cells. RAW264.7 cells were pretreated with LPS (1 μg/mL) and RES (2, 4, and 8 μmol/L) for 24 h. Subsequently, we analyzed the protein and mRNA expression of TLR4, P65, MyD88, IRAK1, TAK1, and IKKβ by Western blot (WB) and RT-qPCR analyses [Figure 4 and Table 2, respectively]. LPS activated the TLR4/MyD88 pathway. Compared with the blank control group, the protein and mRNA expression of the TLR4/MyD88 pathway was significantly increased in LPS-induced cells. Compared with LPS, the protein and mRNA expressions of TLR4/MyD88 pathway in LPS-induced cells treated with RES were significantly reduced. These results indicated that RES significantly inhibited the activation of TLR4/MyD88 signaling pathway in LPS-induced cells. In summary, the polarization of M1 macrophages was dependent on the activation of the TLR4/MyD88 signaling pathway, which is in agreement with our hypothesis that RES polarized M1 to M2 phenotype via inhibition of the TLR4/MyD88 signaling pathway.

DISCUSSION

Macrophages play an indispensable role in atherosclerosis through a variety of immune functions. Research has shown that macrophages can be divided into M1 and M2 types, which are activated by diverse stimuli.[23] LPS can simulate the macrophages from M0 phenotype to M1 phenotype, which has been applied in various studies.[24-27] LPS stimulated the activation of M1 macrophages, which has been associated with the production of pro-inflammatory cytokines, such as IL-1 β, TNF-α, and iNOS.[28] In contrast, polarization of M2 macrophages is involved in tissue repair and remodeling, which are associated with the expression of the mannose receptor, chitinase 3-like 3 (Ym 1), Arg-1, and IL-10.[29] RES can reduce the level of NO, IL-6, and TNF-α in RAW 264.7 cells which induced by LPS. LPS upregulated the expression of iNOS and IL-1 β mRNA in M1-type macrophages.[25,30] In this study, 1 μg/mL LPS was used to stimulate RAW264.7 cells for 24 h, which resulted in the

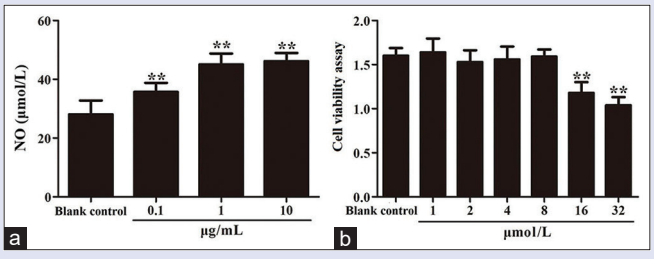

Figure 1: Effects of different concentrations of lipopolysaccharide and resveratrol on RAW264.7. (a) The acute inflammation degree of RAW264.7 induced by lipopolysaccharide was reflected by measuring nitric oxide production. (b) Effects of resveratrol on the activity of RAW264.7. Resveratrol showed cytotoxicity at 16 and 32 μmol/L. The concentration of nitric oxide was measured by using enzyme-linked immunosorbent assay kits, and the measurement of cytotoxicity in resveratrol-treated RAW264.7 cells was performed by methyl thiazolyl blue tetrazolium bromide method. The data are expressed as the mean ± standard error per group versus blank control group (*$P < 0.05$ and **$P < 0.01$)

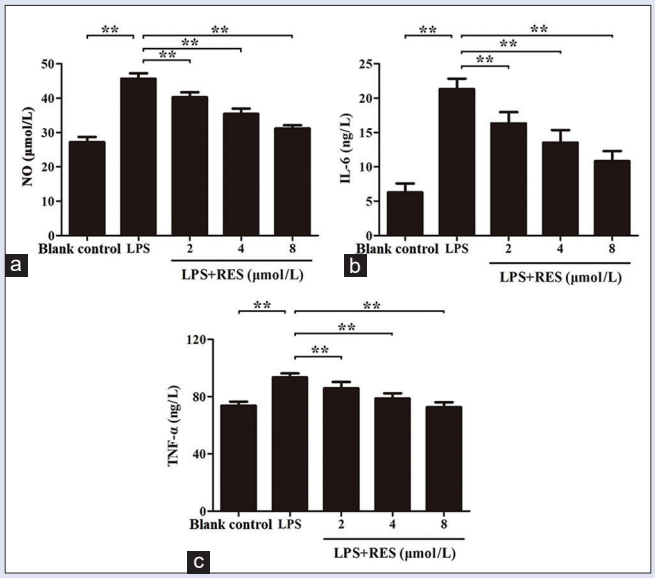

Figure 2: Resveratrol inhibits inflammatory cytokines produced by lipopolysaccharide-induced RAW264.7 cells. Treatment of lipopolysaccharide-induced RAW264.7 cells with 2, 4, and 8 μmol/L resveratrol inhibited the production of nitric oxide (a), interleukin-6 (b), and tumor necrosis factor-α (c). The levels of nitric oxide, interleukin-6, and tumor necrosis factor-α level were measured by enzyme-linked immunosorbent assay. The data are expressed as mean ± standard error per group versus blank control group (*$P < 0.05$ and **$P < 0.01$)

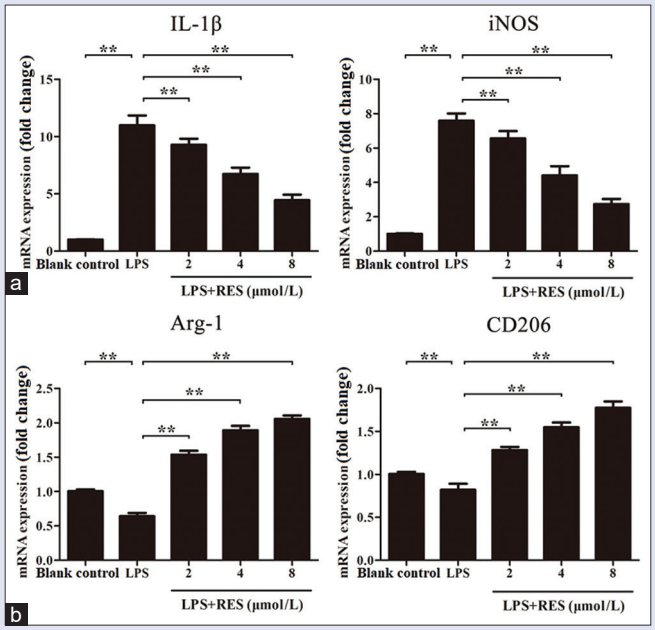

Figure 3: Effects of resveratrol on lipopolysaccharide -induced M1 macrophages. lipopolysaccharide-induced the macrophage polarized to M1-type macrophages and increased the mRNA expression of IL-1β and inducible nitric oxide synthase, which are the markers of M1 macrophages (a). Resveratrol-treated cells were treated with resveratrol (2, 4, or 8 μmol/L for 24 h) and the corresponding M2 phenotype makers Arg-1 and CD206 were analyzed. The levels of Arg-1 and CD206 were significantly increased by resveratrol (b). The mRNA level was measured by quantitative real-time polymerase chain reaction. The data are expressed as mean ± standard error per group versus blank control group (*$P < 0.05$ and **$P < 0.01$, respectively)

increased mRNA expression of *iNOS* and *IL-1 β*. The mRNA of *Arg-1* and *CD206* was downregulated, which are the markers of M2-type macrophages.[31] When RAW264.7 cells were treated with both LPS and RES, the mRNA expression of *iNOS*, *IL-1 β*, *Arg-1*, and *CD206* showed the opposite result, suggesting that LPS can influence the polarization of macrophages from M0 to M1 phenotype, and RES can influence the polarization of macrophages from M1 to M2 phenotype. RES attenuates inflammation by regulating macrophage polarization.

TLRs are the most important receptors for the immune system to sense pathogenic microorganisms or endogenous risks to the host. They play important roles in self or nonself-recognition, detection of pathogens, and innate and adaptive immunity.[32] TLRs activate a classical signaling pathway through a variety of cytokines and chemokines primarily via the MyD88-and non-MyD88-dependent pathways.[33] A previous investigation showed structural insights of RES with the inhibitor κB kinase, cyclooxygeanse-2 (COX-2), and tank-binding kinase I in the inflammatory TLR4 signaling pathway.[34] RES shows both anti-inflammatory and antioxidant activities which has been extensively studied both pharmacologically and biologically. Treatment with RES improves neuroimmune dysregulation through the inhibition of proinflammatory mediators and TLRs/NF-κB signaling pathway.[35] RES ameliorated the elevated levels of TLR4 and NF-κB and inhibited the translocation of NF-κB p65 after the stimulation of hypoxia/reoxygenation.[36] It markedly decreased the expression of TLR4/MyD88 signaling pathway, and decreased the concentration of IL-6 and COX2. It shows a protective effect against LPS-induced acute lung injury, at least in part by inhibiting the TLR4/MyD88-dependent signaling pathway.[37] This may also be true for inflammation, where RES has been shown to inhibit NF-κB light-chain enhancer of activated B cells inTLR4 pathway. There are multiple cellular targets that bind to RES; however, the mode and fundamental interactions involved remain elusive. In this study, LPS induction increased the protein and mRNA expression of TLR4,

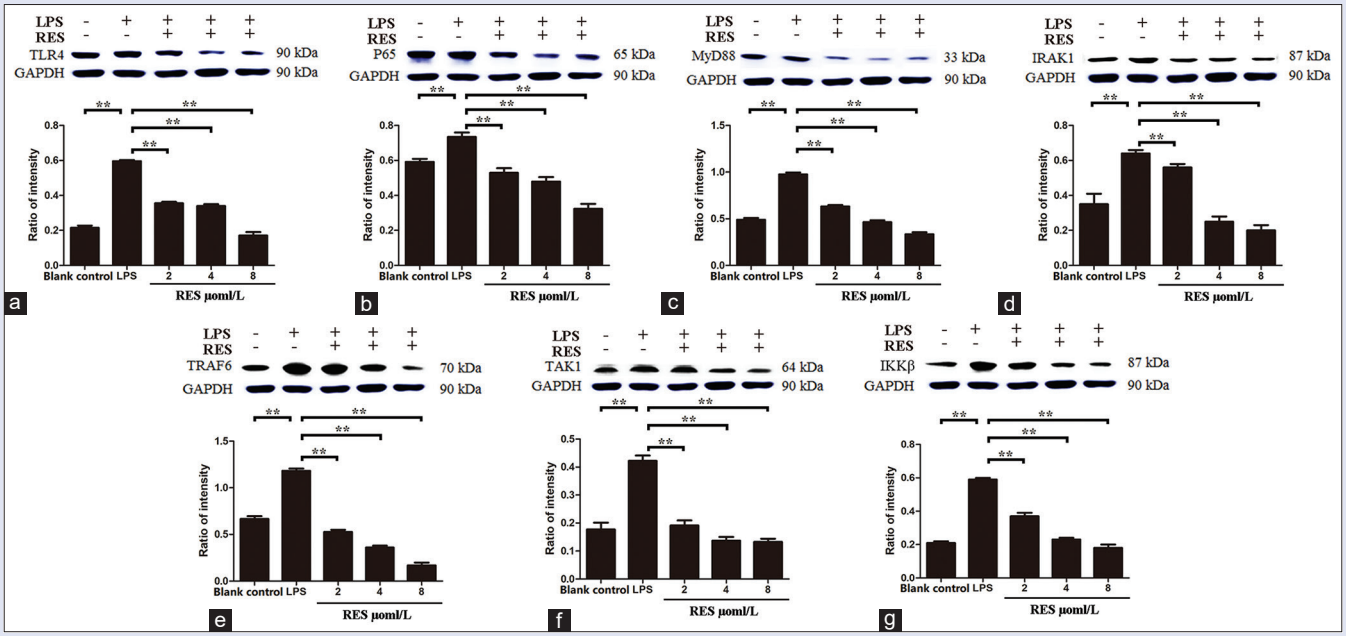

Figure 4: Effect of resveratrol on the protein expression of the toll like receptor 4/MyD88 signaling pathway. The toll like receptor 4/MyD88 signaling pathway regulated the macrophage polarization from M1 to M2 phenotype. LPS-induced macrophages were cultured in the presence of resveratrol for 24 h. Then, the cells were harvested for Western blot analysis. (a-g) Represented the protein expression of toll like receptor 4, P65, MyD88, interleukin receptor associated kinases 1, tumor necrosis factor receptor associated factor 6, activated kinase 1, and IKKβ, respectively. The data are expressed as mean ± standard error per group versus blank control group (*$P < 0.05$ and **$P < 0.01$)

Table 2: The mRNA expression in the toll-like receptor-4 pathway

Name	Blank control	LPS	RES (μmol/L)		
			2	4	8
TLR4	1.44±0.06	4.20±0.24**	3.08±0.10**	2.01±0.06**	1.32±0.05**
P65	1.85±0.08	6.02±0.11**	4.45±0.10**	2.76±0.08**	2.77±0.08**
MyD88	0.35±0.02	2.46±0.06**	2.00±0.04**	1.63±0.04**	0.98±0.04**
IRAK1	0.50±0.03	1.00±0.02**	0.93±0.05**	0.084±0.03**	0.58±0.04**
TRAF6	0.58±0.03	1.41±0.03**	1.31±0.01**	0.99±0.06**	0.74±0.05**
TAK1	0.67±0.05	2.00±0.07**	1.79±0.04**	1.22±0.05**	0.85±0.06**
IKKβ	1.13±0.04	3.22±0.07**	2.38±0.05**	1.98±0.05**	1.22±0.04**

The data were expressed as mean±SE per group versus blank control. **P<0.01. TLR: Toll-like receptor; Arg-1: Arginase-1; MyD88: Myeloid differentiation factor 88; LPS: Lipopolysaccharide; RES: Resveratrol; SE: Standard error

P65, MyD88, IRAK1, TAK1, and IKKβ, and the expression levels of the aforementioned proteins and mRNAs were significantly reduced after treatment with RES. These findings indicate that RES affects macrophage polarization through the TLR-4/MyD88 receptor pathway.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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