RESVERATROL INHIBITS CYTOKINE PRODUCTION IN LPS-STIMULATED RAW264.7 CELLS POTENTIALLY THROUGH TLR4/MYD88/NF-κB PATHWAY

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SUMMARY

Resveratrol is a naturally occurring compound with anti-inflammatory properties. However, the protective molecular mechanisms of resveratrol against LPS-induced inflammation have not been thoroughly known. In the present study, we examined the anti-inflammatory effect of resveratrol in inflammatory model using murine macrophage-like cell RAW264.7 stimulated with LPS. Resveratrol suppressed the production of inflammatory cytokines in LPS-stimulated RAW264.7 cells with the IC_{50} value as 17.5 ± 0.7 μM for IL-6, 14.2 ± 1.9 μM for IL-10, and 18.9 ± 0.6 μM for TNF-α. Gene expression of *TLR4*, *MyD88* and *NF-κB* were significantly suppressed by resveratrol treatment in LPS-stimulated RAW264.7 cells. In conclusion, the anti-inflammatory property of resveratrol is potentially related to its inhibitory effect on TLR4/MyD88/NF-κB signaling pathway in macrophages.

Keywords: Macrophages, inflammatory cytokines, lipopolysaccharide, resveratrol

INTRODUCTION

Inflammation is the spontaneous defense response of human body tissue to any kind of injury and also a response to stimuli including bacteria and viruses that can promote the release of inflammatory cytokines from macrophages or dendritic cells. The primary indicators of inflammation are redness, heat swelling, and pain. During inflammation, white blood cells release substances into the blood or tissues to protect the affected tissue/organ injured or infected. This required increased blood flow to the areas of injury or infection, resulting in redness and warmth. Swelling occurs by the leakage of fluid into the affected tissue, which is caused by some of these compounds from white blood cells. This protective swelling process may trigger nerves to cause

pain. While acute inflammation is an initial response of the body to harmful stimuli, chronic inflammatory response endangers the body tissue involved. The uncontrolled inflammatory response is involved in various immune diseases (Ahmed, 2011).

Lipopolysaccharides (LPS) in the outer wall of Gram-negative bacteria can cause an inflammatory response by activating the production of inflammatory cytokines in different cell types including macrophages (Takashiba *et al.*, 1999; Liang *et al.*, 2013). LPS has been used to examine inflammation because of the affluence of the inflammatory effect, which is generated through Toll-like receptors 4 (TLR4) signaling pathways. TLR4 is a cellular receptor for bacterial LPS, which is by far the most extensively studied member of the TLR family. After being activated by LPS, TLR4 signaling has been divided into MyD88-dependent and MyD88-independent pathways. Its downstream signaling molecules, including nuclear factor kappa B (NF-κB), p38 mitogen activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) and activator of transcription (STAT), leading to the production of inflammatory cytokines (Lu *et al.*, 2008).

Resveratrol (Res) is a polyphenolic compound found naturally in many common food sources. This substance has been proposed to have a variety of therapeutic properties, including antioxidant, cardioprotective, antiviral, anti-aging, and anti-inflammatory effects (Holthoff *et al.*, 2010).Many studies have indicated that Res can inhibit molecules related to the TLR4 signaling pathway, decrease pro-inflammatory cytokines and prevent inflammatory responses (Lundahl *et al.*, 2022; Ma *et al.*, 2017; Tong *et al.*, 2019; Youn *et al.*, 2005). NF-κB activation is strongly associated with inflammatory

responses and other chronic diseases (Hayden & Ghosh, 2004). Despite numerous reports indicating that Res can inhibit NF-κB activation and target gene expression induced by various proinflammatory stimuli (Xu *et al.*, 2018), the direct molecular targets and the mechanisms for such inhibition are unknown.

Thus, in this study, we aimed to identify the molecular targets of Res in downstream signaling pathways activated by LPS-TLR4.

MATERIALS AND METHODS

Materials

Res was purchased from Sigma (CA, USA). RAW264.7 cell line was provided by Dr. T. Kishimoto, Osaka University, Japan. These cells were cultured in RPMI 1640 medium (10% FBS) with 100 μg/mL penicillin, 100 μg/mL streptomycin. Cells were plated into a 90 mm×20 mm petri dish. The culture medium is changed after 1-2 days. Proceed with cell passaging when the cell density reaches 80% confluence. The RAW264.7 cells were grown in a 24-well plate, containing $5×10^5$ cells/mL The cells were seeded in 24-well culture plates (Corning, USA), after adhesion, the cells were pre-stimulated with 2 μg/mL LPS (Sigma) for 1 h and then, treated with Res (1, 5, 10, 20 μM). The cells were harvested for qRT-PCR after 6 h and supernatants were harvested for ELISA after 24 h as described previously (Masuda *et al.*, 2011).

Cell viability assay

For cell viability assays, the cells ($5×10^5$ cells/well) were seeded in 96-well plates (Corning, USA), treated with 1, 5, 10, 20 μM Res for 24 h, control well contain only cells

and blank contain medium. Cell viability was assessed by MTT assay. The plate was then placed in an OD reader to record the absorbance of samples at 540 nm. The results were expressed as fold changes relative to the control. Three replicates were performed for each treatment. Cell viability was calculated as described previously (da Luz *et al.*, 2022).

% Cell viability =

$$\left(\frac{OD_{treated\ sample} - OD_{blank}}{OD_{control} - OD_{blank}}\right) X\ 100\ (\%)$$

Enzyme-linked immunosorbent assay

The cell culture media were collected and the levels of IL-6, IL-10, and TNF-α were measured using ELISA kits (Mabtech, Sweden) following the manufacturer's protocol. The absorbance was read at 450 nm using an OD reader, the cytokine levels were calculated from standard curves. Three replicates were performed for each treatment. The IC_{50} value was determined using ImageJ 1.50i computer software (NIH, Maryland).

Real-Time Quantitative RT-PCR

Total RNA was isolated from RAW264.7 cells using easy-spin™ Total RNA Extraction Kit (iNtRON, Korea) according to the manufacturer's instructions. A total of 1 µg of RNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, USA) according to the manufacturer's instructions. Relative gene expression was measured using PowerUp SYBR Green Master Mix (ThermoFisher Scientific, USA). The primers used for Real-Time qRT-PCR were synthesized by Phusa Genomics Co., Ltd. (Vietnam). The primer sequences are referenced from the previous article (Hsieh *et al.*, 2017) listed in table 1. Real-Time qRT-PCR reactions and analyses were performed using the QuantStudio™ 6 Pro Real-Time PCR System with Design & Analysis Software v2.6.0. The relative expression levels of the genes were calculated based on the $2^{-\Delta Ct}$ method (Livak & Schmittgen, 2001). β-actin gene was used as an endogenous control to normalize gene expression levels as described previously (Masuda *et al.*, 2011).

Statistical analysis

The data were expressed as the mean ± standard deviation (SD). The ImageJ software was used to analyze the IC_{50} value. Each experiment was performed at least three times, statistical analysis was performed using two-tailed Student's *t* test. Otherwise, representative data were shown, and $p < 0.05$ was considered significant.

Table 1. Primer sequences used in RT-qPCR.

Genes	Forward primers	Reverse primers
TLR4	5'- ATGGCATGGCTTACACCACC - 3'	5'- GAGGCCAATTTTGTCTCCACA - 3'
MyD88	5'- TCATGTTCTCCATACCCTTGGT- 3'	5'- AAACTGCGAGTGGGGTCAG - 3'
NF-κB	5'- ATGGCAGACGATGATCCCTAC - 3'	5'- TGTTGACAGTGGTATTTCTGGTG - 3'
β-actin	5'- TCATGAAGTGTGACGTGGACATC - 3'	5'- CAGGAGGAGCAATGATCTTGATCT - 3'

RESULTS AND DISCUSSION

Effect of Res on RAW264.7 cell viability

The MTT assay was performed to determine the effect of Res on the cell viability of the RAW264.7 cells. Figure 1 showed that RAW264.7 cell viability was not affected by Res at the concentrations 1, 5, 10, and 20 μM as 100, 102 ± 9, 105 ± 9, 93 ± 6, 90 ± 10 (%), respectively. Res exhibited no effect on RAW264.7 cells and was used for next experiments. Our results are in agreement with the previous findings which showed that Res cells with the concentration from 1 to 20 μM was not harmful to RAW264.7 cells (Ma *et al.*, 2017) while the other results showed that 20 μM of Res can caused toxicity on RAW264.7 cells (Son *et al.*, 2014).
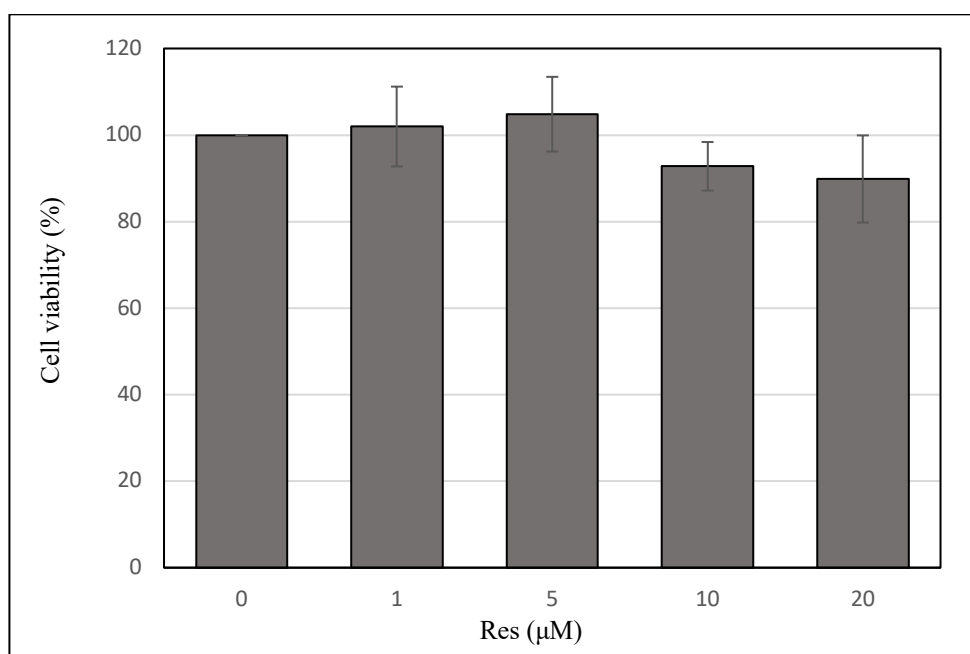

Figure 1. Effect of Res treatment on the viability of RAW264.7 cells. Cells were treated with different concentrations of Res. The cell viability was determined by MTT assay. The results are the means ± SD of 3 replicated experiments.

Res inhibited the release of cytokines in LPS-stimulated RAW264.7 cells

The cells were pre-stimulated with LPS (2 μg/mL) for 1 h and then with Res (1, 5, 10, 20 μM). Protein levels of three cytokines IL-6, IL-10, and TNF-α was detected by ELISA after 24 h. The results in figure 2 indicated that Res suppressed the production of IL-6 and TNF-α with the IC$_{50}$ value are 17.5 ± 0.7 μM and 18.9 ± 0.6 μM in LPS-stimulated RAW264.7 cells, respectively. Our results are in agreement with the previous findings showed that Res can reduce IL-6 and TNF-α production in agreement with the adjustment of IL-6 and TNF-α production affected by Res can be observed in the results of earlier studies (Ma *et al.*, 2017; Tong *et al.*, 2019).
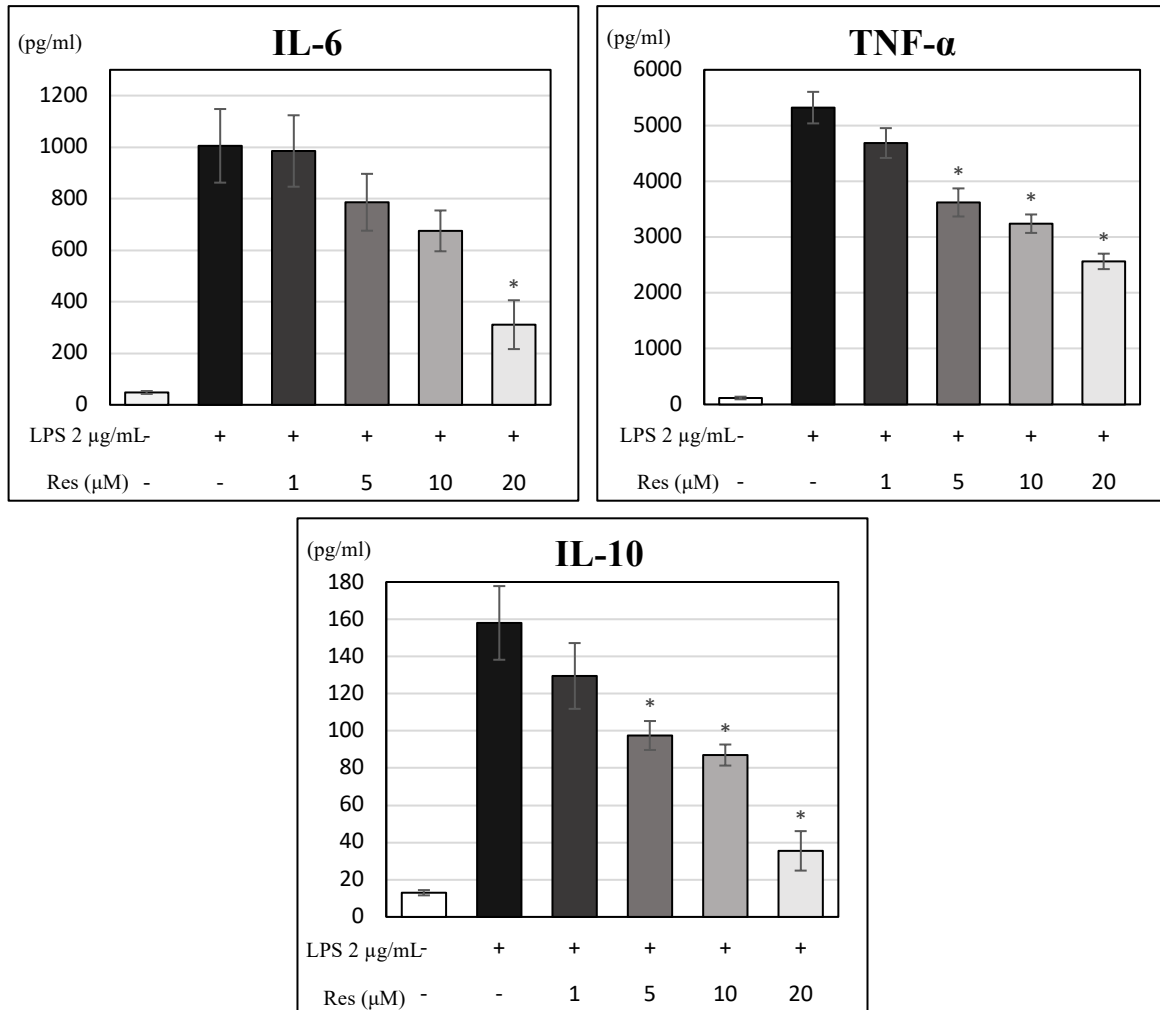
Figure 2. Res decreased IL-6, TNF-α, and IL-10 production in LPS-stimulated RAW264.7 cells. The cells were treated with various concentrations of Res in the presence of 2 µg/ml LPS for 24 h before the ELISA assays. The results are the means ± SD of 3 replicated experiments. *$p < 0.05$ vs only LPS stimulated sample.

The results from figure 2 also showed that Res reduced the production of IL-10 with the IC$_{50}$ value is 14.2 ± 1.9 μM. However, this result is different from the previous study. Compared to the previous research, the cells were cultured in DMEM medium, with the cell density of 5×10^5 cells/mL after being pre-treated with 25 μM Res and 12 h stimulated with LPS, the concentration of IL-10 was increased (Tong *et al*., 2019). This difference may be due to the inflammation model, density of cells, the medium of the cell culture, the origin/concentration of LPS, or the order of adding stimuli and compounds. For example, during this study, we stimulated the RAW264.7 cells with LPS for 30 min and then added Res with different concentrations. In contrast, according to other protocol of the previous study, the cells were pre-treated with Res for 2 h prior to the addition of LPS (Tong *et al*., 2019).
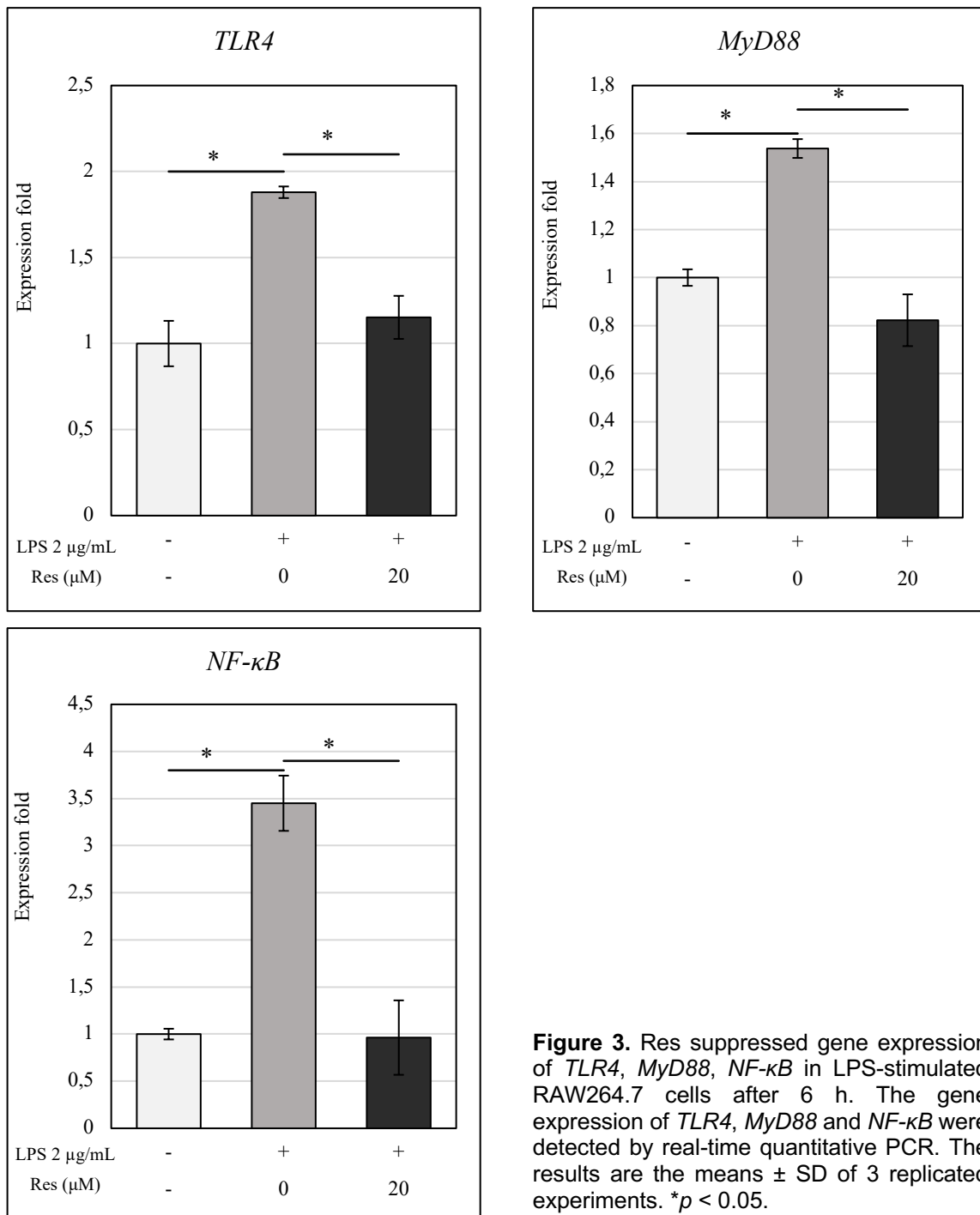
Figure 3. Res suppressed gene expression of *TLR4*, *MyD88*, *NF-κB* in LPS-stimulated RAW264.7 cells after 6 h. The gene expression of *TLR4*, *MyD88* and *NF-κB* were detected by real-time quantitative PCR. The results are the means ± SD of 3 replicated experiments. *$p < 0.05$.
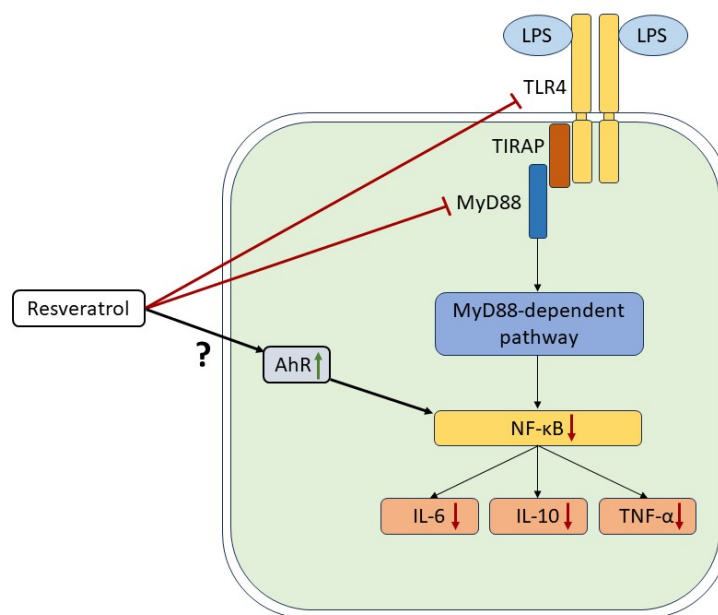
616

Figure 4. Resveratrol reduced inflammatory cytokine production in LPS-stimulated RAW264.7 cells potentially via inhibited gene expression of *TLR4*, *MyD88* and *NF-κB* in NF-κB signaling pathway.

Res inhibited NF-κB signaling pathway in LPS-induced RAW264.7 cells

To determine the anti-inflammatory effect of Res on the NF-κB signaling pathway in LPS-induced RAW264.7 cells, gene expression of *TLR4*, *MyD88*, and *NF-κB* has been examined. All three genes were detected by qPCR in 3 h after LPS stimulation and peaked at 6 h before decreasing at 24 h (data not shown). Then, the effect of 20 μM Res on gene expression of *TLR4*, *MyD88*, and *NF-κB* was examined after 6 h LPS stimulation by qPCR. The results in figure 3 showed that gene expression of *TLR4*, *MyD88*, and *NF-κB* increased in LPS-stimulated RAW264.7 cells (control sample *vs* only LPS-induced sample, *TLR4*: 1.00 ± 0.13 *vs* 1.88 ± 0.03; *MyD88*: 1.00 ± 0.03 *vs* 1.54 ± 0.04; *NF-κB*: 1.00 ± 0.06 *vs* 3.45 ± 0.29, $*p < 0.05$). Treatment with Res significantly decreased the gene expression of *TLR4*, *MyD88*, and *NF-κB* in LPS-stimulated RAW264.7 cells (only LPS-induced *vs* LPS + Res 20 μM, *TLR4*: 1.88 ± 0.03 *vs* 1.15 ± 0.13; *MyD88*: 1.54 ± 0.04 *vs* 0.82 ± 0.11; *NF-κB*: 3.45 ± 0.29 *vs* 0.96 ± 0.39, $*p < 0.05$). The results indicated that Res sufficiently suppressed the *TLR4*/*MyD88*/*NF-κB* expression in LPS-stimulated RAW264.7 cells, then potentially inhibited cytokine production. Besides, the production of cytokines in macrophages in the stimulation of LPS can be regulated by MyD88-independent pathway via TLR4/TRIF/Type 1 interferon pathway or Ahr signaling pathway (Lu *et al*., 2008; Masuda *et al*., 2011). Recently, we reported that several natural compounds activated Ahr signaling pathway and consequently resulted in anti-inflammatory effects in LPS-stimulated RAW264.7 cells (Tran *et al.,* 2022). Whether Res also activated Ahr signaling in LPS-stimulated RAW264.7

cells is under investigation (Figure 4).

CONCLUSION

Our results indicated that Res decreased the cytokine production of IL-6, IL-10, and TNF-α in RAW264.7 cells stimulated with LPS. Res inhibited the gene expression of *TLR4*, *MyD88*, and *NF-κB* in NF-κB signaling pathway in RAW264.7 cells stimulated by LPS. Our results contribute to the knowledge of potentially molecular mechanisms of Res against bacterial infection.

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