# α-rhamnrtin-3-α-rhamnoside exerts anti-inflammatory effects on lipopolysaccharide-stimulated RAW264.7 cells by abrogating NF-κB and activating the Nrf2 signaling pathway

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**Abstract.** α-rhamnrtin-3-α-rhamnoside (ARR) is the principal compound extracted from *Loranthus tanakae* Franch. & Sav. However, its underlying pharmacological properties remain undetermined. Inflammation is a defense mechanism of the body; however, the excessive activation of the inflammatory response can result in physical injury. The present study aimed to investigate the effects of ARR on lipopolysaccharide (LPS)-induced RAW264.7 macrophages and to determine the underlying molecular mechanism. A Cell Counting Kit-8 assay was performed to assess cytotoxicity. Nitric oxide (NO) production was measured via a NO colorimetric kit. Levels of prostaglandin E2 ($PGE_2$) and proinflammatory cytokines, IL-1β and IL-6, were detected using ELISAs. Reverse transcription-quantitative (RT-q)PCR analysis was performed to detect the mRNA expression levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), IL-6 and IL-1β in LPS-induced RAW246.7 cells. Western blotting, immunofluorescence and immunohistochemistry analyses were performed to measure the expression levels of NF-κB and nuclear factor-erythroid 2-related factor 2 (Nrf2) signaling pathway-related proteins to elucidate the molecular mechanisms of the inflammatory response. The results of the cytotoxicity assay revealed that doses of ARR ≤200 μg/ml exhibited no significant effect on the viability of RAW264.7 cells. The results of the Griess assay demonstrated that ARR inhibited the production of NO. In addition, the results of the ELISAs and RT-qPCR analysis discovered that ARR reduced the production of the proinflammatory cytokines, IL-1β and IL-6, as well as the proinflammatory mediators, $PGE_2$, iNOS and COX-2, in LPS-induced RAW264.7 cells. Immunohistochemical analysis demonstrated that ARR inhibited LPS-induced activation of TNF-associated factor 6 (TRAF6) and NF-κB p65 signaling molecules, while reversing the downregulation of the NOD-like receptor family CARD domain containing 3 (NLRC3) signaling molecule, which was consistent with the results of the western blotting analysis. Immunofluorescence results indicated that ARR reduced the increase of NF-κB p65 nuclear expression induced by LPS. Furthermore, the results of the western blotting experiments also revealed that ARR upregulated heme oxygenase-1, NAD(P)H quinone dehydrogenase 1 and Nrf2 pathway molecules. In conclusion, the results of the present study suggested that ARR may exert anti-inflammatory effects by downregulating NF-κB and activating Nrf2-mediated inflammatory responses, suggesting that ARR may be an attractive anti-inflammatory candidate drug.

## Introduction

Inflammation is the body's defense response against various damage factors, including bacteria, viruses and tissue damage, and regulated inflammatory responses play a vital role in coping with pathogens and preventing tissue damage (1). However, abnormal inflammatory responses can facilitate the progression of a wide range of types of disease, including rheumatoid arthritis, chronic hepatitis, Alzheimer's disease, inflammatory bowel disease and cancer (2,3). Thus, effective control of inflammatory responses is pivotal for the prevention and treatment of several diseases, including cancer (3). Inflammatory diseases are complex and difficult to cure, thus inflammatory models are used to screen for anti-inflammatory drugs. For example, the lipopolysaccharide (LPS)-induced inflammatory response model is widely used in inflammation research (4-6).

NF-κB is an important, multi-directional, functional regulator of the anti-inflammatory response (7). The main method for preventing chronic inflammation-mediated disorders is to regulate the secretion of proinflammatory cytokines (8). The production or secretion of proinflammatory cytokines results

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in the activation of NF-κB, which in turn stimulates several transcription factors that control the gene expression of proinflammatory cytokines, including ILs, inducible nitric oxide synthase (iNOS) and cyclooxygenease-2 (COX-2) (9). The activation of NF-κB also plays an indispensable role in the development of several types of disease, including rheumatoid arthritis, inflammatory bowel disease and autoimmunity, as well as diseases comprising a significant inflammatory component, such as cancer and atherosclerosis (10).

TNF-associated factor 6 (TRAF6) is a key regulator of NF-κB and plays an important regulatory role in inflammation (11). NOD-like receptor family CARD domain containing 3 (NLRC3) has been found to exert inhibitory effects on proinflammatory signaling transduction, the ubiquitination of TRAF6 and nuclear translocation of NF-κB p65 (12). In addition, NLRC3 was discovered to inhibit a major inflammatory pathway controlled by NF-κB, which directly interacts with TRAF6 and forms a new protein complex called the 'TRAFasome' (13).

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a pivotal and significant transcription factor, which controls several antioxidant enzymes including heme oxygenase-1 (HO-1) and NAD(P)H quinone dehydrogenase 1 (NQO1) (14). HO-1 plays a key role in the antioxidant processes and suppressing the immune response (15). Nrf2, coupled with its target genes to act as an inflammation regulatory system, has been reported to downregulate the expression levels of several proinflammatory cytokines, which could antagonize NF-κB activation (16,17).

*Loranthus tanakae* Franch. & Sav, which grows on the trees of *Quercus L*. and *Betula*, has been found to have various biological properties, including anti-microbial, antitumor and antioxidant effects (18). It has been reported that its methanol extracts possess various antitumor activities (19). Natural products represent novel compounds that have been shown to prevent different types of disease, such as cancer, infectious diseases and cardiovascular diseases (20). Epigallocatechin, curcumin and other natural phenolic compounds have been established to possess anti-inflammatory and antioxidant activities (21-23). α-rhamnrtin-3-α-rhamnoside (ARR; Fig. 1A), a phenolic flavonoid compound, is the main active ingredient of *Loranthus tanakae* Franch. & Sav (24). However, to the best of our knowledge, the pharmacological activities and anti-inflammatory molecular mechanisms of ARR remain unknown. Thus, the present study aimed to investigate the anti-inflammatory effect of ARR in RAW264.7 cells to determine whether it occurred via the NF-κB and Nrf2 signaling pathway. In addition, the study sought to elucidate its underlying molecular mechanism of action to provide a preliminary basis for the development of ARR into an anti-inflammatory drug.

## Materials and methods

*Reagents and chemicals*. ARR (purity >95%) was isolated from *Loranthus tanakae* Franch. & Sav. in our laboratory, as previously described (21). The structure of ARR was elucidated by nuclear magnetic resonance. LPS and indomethacin (Indo) were purchased from Sigma-Aldrich; Merck KGaA. DMEM, FBS and penicillin-streptomycin were purchased from Gibco; Thermo Fisher Scientific, Inc. Primary rabbit monoclonal antibodies, including NF-κB p65 (cat. no. ab16502), phosphorylated-(p)-NF-κB-p65 (cat. no. ab76302), Nrf2 (cat. no. ab92946), NQO1 (cat. no. ab80588), HO-1 (cat. no. ab13243), TRAF6 (cat. no. ab137452), β-actin (cat. no. ab8227) and Histone H3 (cat. no. ab1791) were purchased from Abcam and the NLRC3 antibody (cat. no. DF13411) was obtained from Affinity Biosciences. The nitric oxide (NO) colorimetric kit (cat. no. E-BC-K035-M) and the cytokine mouse ELISA kits specific for prostaglandin E2 (PGE2; cat. no. E-EL-0034c), IL-6 (cat. no. E-EL-M0044c) and IL-1β (cat. no. E-EL-M0037c) were purchased from Elabscience.

*Cell lines and culture*. Leukemia cells from mouse mononuclear macrophages (RAW264.7; cat. no. CL-0190) were obtained from Procell Life Science & Technology Co. Ltd., and cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin, at 37˚C with 5% $CO_2$.

*Cell viability assay*. A Cell Counting Kit-8 (CCK-8) assay (which uses WST-8 for the colorimetric reaction) was performed to assess cell viability. Briefly, RAW264.7 macrophages ($4x10^4$ cells/ml) were seeded into 96-well plates (100 µl/well) and treated with different concentrations of ARR (5, 10, 20, 40, 80, 100 or 200 µg/ml) for 24 h at 37˚C. Following the incubation, 10 µl CCK-8 reagent (Sigma-Aldrich; Merck KGaA) was added to each well and incubated for an additional 2 h at 37˚C. In the presence of the electronic coupling reagent, 1-Methoxy PMS, WST-8 was transformed to orange-yellow water-soluble formazan. The optical density was determined using a microplate reader at a wavelength of 450 nm.

*NO assay*. For the NO assay, $1x10^6$/ml RAW264.7 macrophages were seeded into 6-well plates. Following incubation for 24 h with different concentrations of ARR (0, 25, 50 and 100 µg/ml) or the positive control drug, Indo (8 µg/ml; commonly used to treat inflammation) for 2 h, LPS (100 ng/ml) was added, and cells were incubated for an additional 24 h. According to the manufacturer's instructions, reagents were added into each well and cells were incubated at 37˚C for 30 min in the dark. The absorbance was measured using a microplate reader at a wavelength of 550 nm. The dosage of ARR used was determined according to the preliminary experiments (data not shown), and the dosage of LPS was selected based on our previous study (25).

*ELISA*. The levels of the cytokines, IL-6, IL-1β and $PGE_2$, in the macrophage supernatants (obtained by centrifugation at 1,000 x g at room temperature for 5 min) from each group were determined using IL-6, IL-1β and $PGE_2$ ELISA kits, according to the manufacturer's instructions. The absorbance was measured using a microplate reader at a wavelength of 450 nm.

*Reverse transcription-quantitative (RT-q)PCR*. Total RNA was extracted from RAW264.7 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and the quality and concentration of the isolated RNA were determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). First-strand cDNA was synthesized using an RT-qPCR synthesis kit (cat. no. AT311; Beijing TransGen Biotech Co., Ltd.), according to the manufacturer's protocol. Relative

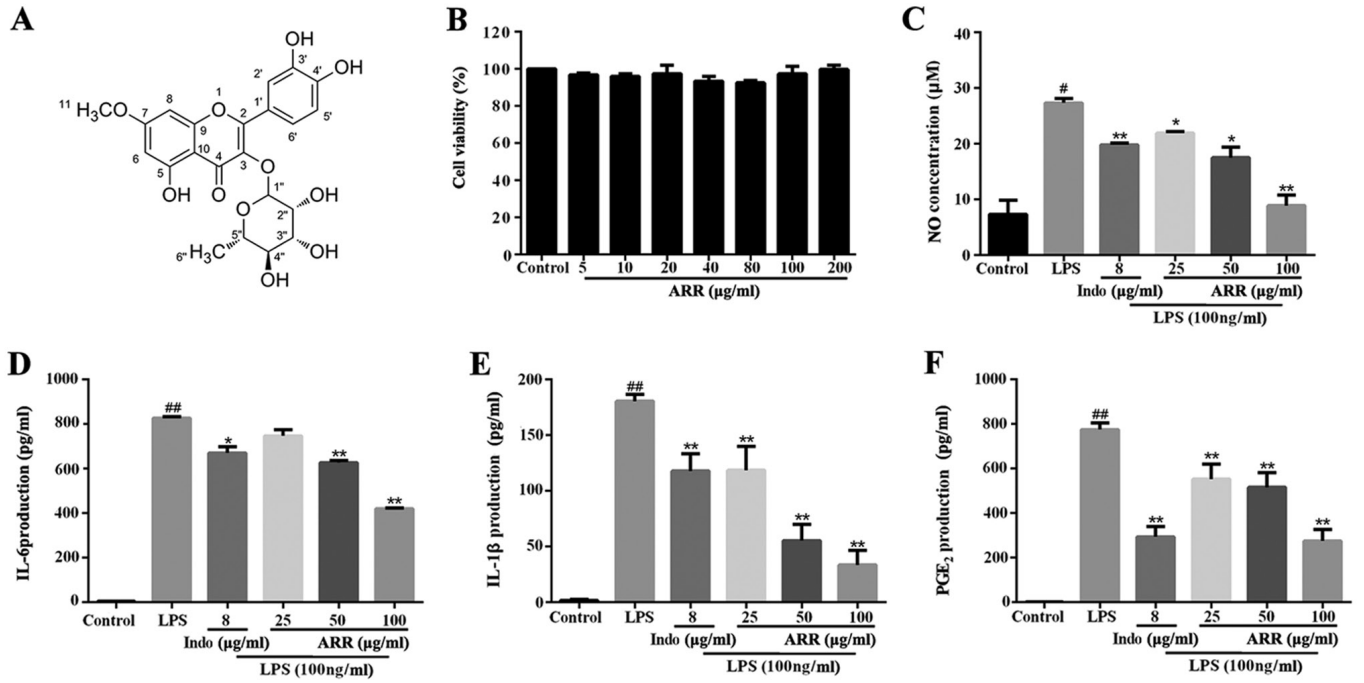


Figure 1. ARR inhibits the inflammatory response. (A) ARR is a flavonoid compound extracted from the *Loranthus tanakae* Franch. & Sav. (B) RAW264.7 cells were treated with different concentrations of ARR (0, 5, 10, 20, 40, 80, 100 or 200 $\mu$g/ml) for 24 h. A Cell Counting Kit-8 assay was performed to assess cell viability. RAW264.7 cells were pretreated with different concentrations of ARR (0, 25, 50 or 100 $\mu$g/ml) or Indo (positive control, 8 $\mu$g/ml) at 37˚C for 2 h, and incubated with or without LPS (100 ng/ml) at 37˚C for 24 h. (C) Levels of NO in the culture media were determined using a NO colorimetric assay kit. The effect of ARR on (D) IL-6, (E) IL-1β and (F) $PGE_2$ cytokine production was detected using ELISA kits. Data are presented as the mean ± SD. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. untreated control group; $^{*}$P<0.05, $^{**}$P<0.01 vs. LPS group. ARR, α-rhamnrtin-3-α-rhamnoside; Indo, indomethacin; LPS, lipopolysaccharide; NO, nitric oxide; $PGE_2$, prostaglandin E2.

expression levels of IL-6, IL-1β, COX-2, iNOS and GAPDH were determined using a PerfectStart™ Green qPCR SuperMix (Beijing TransGen Biotech Co., Ltd.). The following thermocycling conditions were used for the qPCR: Initial denaturation at 94˚C for 30 sec; followed by 45 cycles at 94˚C for 5 sec and 60˚C for 30 sec. The mRNA expression levels of IL-6, IL-1β, COX-2 and iNOS were calculated using the $2^{-\Delta\Delta Cq}$ method (26) and normalized to GAPDH. The primer sequences used for the qPCR are listed in Table I.

*Western blotting*. Total protein was extracted from RAW264.7 cells using a Whole Cell Lysis assay (Nanjing KeyGen Biotech Co., Ltd.). Total nuclear and cytoplasmic proteins were extracted using the Nuclear and Cytoplasmic Protein Extraction kit (Nanjing KeyGen Biotech Co., Ltd.). Protein concentration was measured using a BCA protein assay kit (Nanjing KeyGen Biotech Co., Ltd.) and 20 $\mu$g protein/lane was separated via 10% SDS-PAGE. The separated proteins were subsequently transferred onto PVDF membranes and blocked with 5% BSA (Thermo Fisher Scientific, Inc.) at room temperature for 1 h. The membranes were then incubated overnight at 4˚C with primary antibodies against NF-κB p65 (1:1,000), p-NF-κB p65 (1:1,000), Nrf2 (1:1,000), HO-1 (1:1,000), NQO1 (1:1,000), NLRC3 (1:1,000), TRAF6 (1:1,000), β-actin (1:2,000) or Histone H3 (1:2,000). Following the primary antibody incubation, the membranes were incubated with an HRP-conjugated anti-rabbit secondary antibody (Abcam; cat. no. ab6721; 1:5,000) at room temperature for 1 h. The membranes were washed multiple times with TBS-Tween-20 buffer and the protein bands were visualized using enhanced chemiluminescence reagent (cat. no. G2020; Wuhan Servicebio Technology Co., Ltd.) and a chemiluminescence imaging system (Bio-Rad Laboratories, Inc.). Densiometric analysis was performed using Image Lab software (version 6.0; Bio-Rad Laboratories, Inc.).

*Immunofluorescence staining*. RAW264.7 cells ($1x10^5$/ml) were seeded onto glass coverslips, plated into the bottom of 6-well plates and fixed with 4% paraformaldehyde at room temperature for 15 min. Cells were subsequently permeabilized with 0.1% Triton X-100 and blocked with 10% goat serum (Elabscience) at room temperature for 30 min. Cells were then incubated with a rabbit anti-NF-κB p65 antibody (1:1,000) at 37˚C for 1 h and Cy3-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Elabscience; cat. no. E-IR-R321; 1:5,000) at 37˚C for 1 h. Nuclei were stained with DAPI (Pierce; Thermo Fisher Scientific, Inc.) at 37˚C for 5 min, and a drop of anti-fluorescence quenching mounting solution was added prior to visualization using a fluorescence microscope (magnification, x400). Analysis was performed using ImageJ software (version 1.80; National Institutes of Health).

*Immunohistochemistry staining*. Immunohistochemistry analysis was performed as previously described (22). The primary antibodies used were as follows: Anti-NLRC3 (1:500), anti-TRAF6 (1:500) and anti-NF-κB p65 (1:500), and an

Table I. Primer sequences used for reverse transcription-quantitative PCR.

| Gene | Primer sequence (5'→3') |
|---|---|
| IL-6 | F: GAGTCCTTCAGAGAGATACAG |
| | R: CTGTGACTCCAGCTTATCTG |
| IL-1β | F: AAATACCTGTGGCCTTGGGC |
| | R: CTTGGGATCCACACTCTCCAG |
| COX-2 | F: GAAGATTCCCTCCGGTGTTT |
| | R: CCCTTCTCACTGGCTTATGTAG |
| iNOS | F: GCTTGGGTCTTGTTCACTCC |
| | R: GGCCTTGTGGTGAAGAGTGT |
| GAPDH | F: CCTTCCGTGTTCCTACCCCC |
| | R: AGCCCAAGATGCCCTTCAGT |

COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; F, forward; R, reverse.

HRP-conjugated anti-rabbit antibody was used as the secondary antibody (Abcam; cat. no. ab6721; 1:500). Following the antibody incubations, all samples were stained with DAB at room temperature for 2 min. Samples were observed using a fluorescence microscope (magnification, x400) and quantified using ImageJ software.

*Statistical analysis*. All experiments were repeated at least three times. Statistical analyses were performed using SPSS software (version 26.0; IBM Corp.) and GraphPad Prism software (version 6.0; GraphPad Software, Inc.). Data are presented as the mean ± SD. Significant differences between groups were determined using a one-way ANOVA followed by a Tukey's or Dunnett's T3 post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Effect of ARR on viability in LPS-stimulated RAW264.7 cells*. To investigate the effect of ARR on cell viability, RAW264.7 cells were incubated with 0-200 $\mu$g/ml ARR for 24 h. As presented in Fig. 1B, 5-200 $\mu$g/ml ARR exerted no significant effect on the viability of RAW264.7 cells. Thus, doses of 25-100 $\mu$g/ml ARR were used for subsequent experimentation. The doses of ARR were used according to our prior trial (data not shown).

*Effect of ARR on inflammatory mediators in LPS-stimulated RAW264.7 cells*. As the LPS-induced inflammatory response model is widely used in inflammation research of anti-inflammatory drugs (4-6), the present study established an LPS-induced inflammatory response model in RAW264.7 cells to evaluate the anti-inflammatory effect of ARR. Following incubation with LPS, NO expression was significantly increased compared with the control group; however, the addition of ARR at all doses significantly suppressed the LPS-induced secretion of NO (Fig. 1C). Among them, the high dose of ARR (100 $\mu$g/ml) exhibited the strongest inhibitory effect. These results suggested that ARR markedly suppresses NO production in RAW264.7 cells.

IL-6, IL-1β and $PGE_2$ are critical inflammatory cytokines involved in mediating inflammatory responses (27); thus, in the present study, the expression levels of IL-6, IL-1β and $PGE_2$ were detected in RAW264.7 cell supernatants using ELISA kits. As presented in Fig. 1D-F, LPS significantly upregulated the levels of IL-6, IL-1β and $PGE_2$ compared with the control group, while ARR treatment significantly decreased the expression levels of the three inflammatory cytokines compared with the LPS group. Taken together, these results suggested that ARR may exert anti-inflammatory effects by inhibiting the release of IL-6, IL-1β and $PGE_2$. In addition, the anti-inflammatory effect of ARR seems to occur in a dose-dependent manner, and works best at a concentration of 100 $\mu$g/ml.

*Effect of ARR on the gene transcription of proinflammatory factors in LPS-stimulated RAW264.7 cells*. To determine whether the regulation of inflammatory factors by ARR occurred at the mRNA level, the expression levels of various inflammatory factors were detected via RT-qPCR analysis. As presented in Fig. 2A-D, LPS significantly upregulated the mRNA expression levels of iNOS, IL-6, IL-1β and COX-2 compared with the control group. Compared with the LPS group, the mRNA levels of inflammatory factors, iNOS, IL-6, IL-1β and COX-2 (except 25 $\mu$g/ml ARR treatment), were significantly downregulated following the addition of ARR, whereby the effects of ARR were most notable at 100 $\mu$g/ml. These findings are consistent with the ELISA results, suggesting that ARR may exert an anti-inflammatory effect by inhibiting the expression of several inflammatory factors at both the mRNA and protein levels.

*Effect of ARR on the Nrf2 signaling pathway in LPS-stimulated RAW264.7 cells*. The effect of ARR on the Nrf2 signaling pathway in LPS-stimulated RAW264.7 cells was also investigated. The western blotting results demonstrated that, compared with the control group, LPS significantly upregulated the expression levels of NQO1, while the expression levels of Nrf2 and HO-1 were not significantly altered. Treatment with ARR notably induced the expression levels of Nrf2 protein and its target molecule, HO-1, compared with the LPS group (Fig. 3D-F). Taken together, these results suggest that ARR can also exert anti-inflammatory effects via the Nrf2 signaling pathway.

*Effect of ARR on NF-κB p65 translocation in LPS-stimulated RAW264.7 cells*. NF-κB is a well-known transcription factor that is involved in the inflammatory response (28). Western blotting analysis was performed to detect the phosphorylation of p65 and translocation of NF-κB p65 to the nucleus. LPS significantly increased the phosphorylation of NF-κB p65 compared with the control group, while the addition of ARR significantly suppressed the phosphorylation of NF-κB p65 in RAW264.7 cells (Fig. 3A). Stimulation with LPS also induced the translocation of NF-κB p65 to the nucleus (Fig. 3B-C). However, addition of ARR significantly suppressed LPS-induced NF-κB p65 nuclear translocation of RAW264.7 macrophages. Similar results for NF-κB p65 nuclear translocation were observed via immunofluorescence microscopy. As expected, LPS markedly increased

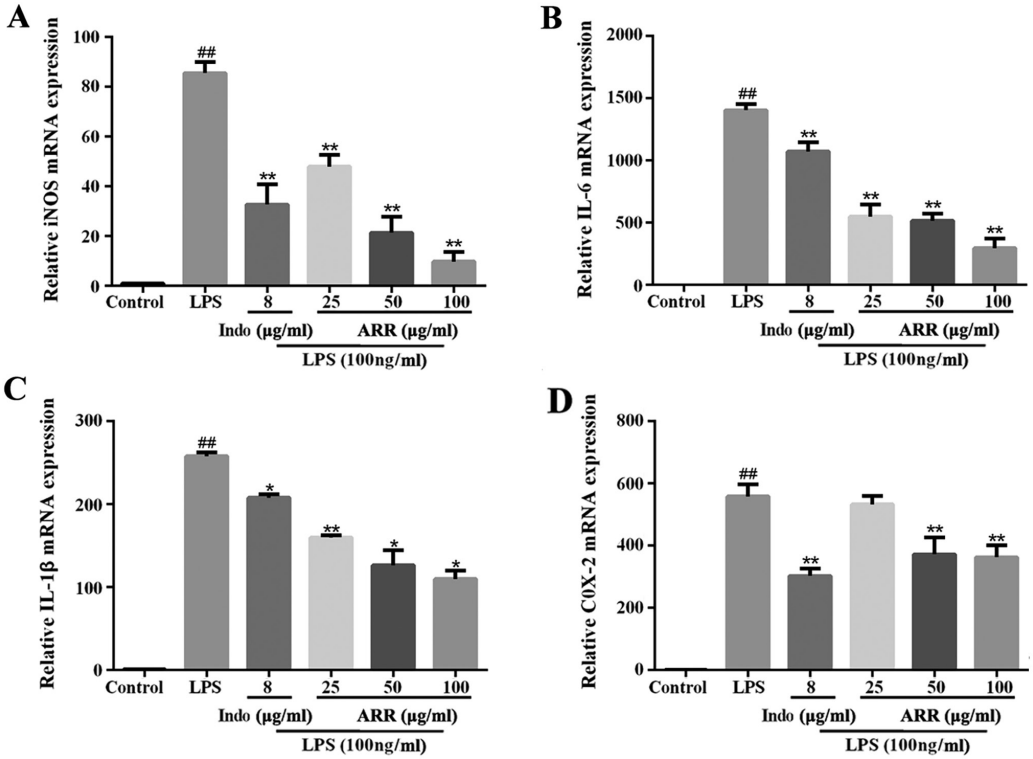


Figure 2. ARR decreases the levels of proinflammatory factors. RAW264.7 cells were pretreated with different concentrations of ARR (0, 25, 50 or 100 $\mu$g/ml) or Indo (positive control, 8 $\mu$g/ml) for 2 h, and incubated with or without LPS (100 ng/ml) for 24 h. Reverse transcription-quantitative PCR analysis was performed to detect the mRNA expression levels of (A) iNOS, (B) IL-6, (C) IL-1β and (D) COX-2. Data are presented as the mean ± SD. (n=3). $^{\#\#}$P<0.01 vs. untreated control group; $^{*}$P<0.05, $^{**}$P<0.01 vs. LPS group. ARR, α-rhamnrtin-3-α-rhamnoside; Indo, indomethacin; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2.

NF-κB p65 nuclear translocation compared with the control group (Fig. 4). Notably, ARR markedly suppressed the nuclear translocation of NF-κB p65 compared with the LPS group. Taken together, these results suggested that ARR may exert anti-inflammatory effects by inhibiting NF-κB p65 translocation.

*Effect of ARR on NLRC3, TRAF6 and NF-κB p65 protein expression in LPS-stimulated RAW264.7 cells*. To further investigate the effect of ARR on the NF-κB signaling pathway, immunohistochemistry staining and western blotting were performed to determine the expression levels of NLRC3, TRAF6 and NF-κB p65 in RAW264.7 cells. Treatment with LPS did not significantly affect NLRC3 expression, while the expression levels of TRAF6 and NF-κB p65 were upregulated compared with the control group (Fig. 5A-D). However, NLRC3 expression was markedly upregulated following the addition of ARR, while the expression levels of TRAF6 and NF-κB p65 were downregulated compared with the LPS group. Taken together, these results suggested that ARR may significantly upregulate NLRC3 and downregulate TRAF6 and NF-κB p65 expression levels in the inflammatory response.

## Discussion

Inflammation is a natural host defense reaction process, which is divided into acute and chronic inflammation according to the duration (29). The main symptoms of acute inflammation include redness, swelling and pain (30,31). Chronic inflammation is caused by the persistence of inflammatory factors and damage to the tissues, which is manifested by the degeneration, exudation and proliferation of local tissues (32,33). ARR is a flavonoid compound extracted from the *Loranthus tanakae* Franch. & Sav (19). Flavonoids have been reported to exert anti-inflammatory (34), anticancer (35) and cardioprotective effects (36). However, the effect of ARR on inflammation and its underlying molecular mechanism remain unclear.

When macrophages are activated, they produce various inflammatory cytokines that cause inflammation (9). LPS is a macrophage stimulus, which can cause macrophages to secrete proinflammatory factors, including NO, $PGE_2$, IL-6 and IL-1β (37). The present study established an LPS-induced inflammatory response *in vitro* model to evaluate the anti-inflammatory effect of ARR on RAW264.7 cells. The use of an LPS-induced macrophage line is a well-established anti-inflammatory *in vitro* model, which is widely deemed as a standard and reliable model to determine the potential of novel anti-inflammatory drug candidates, and therefore predominantly adopted by researchers of this field (38-41). Exposure to high levels of NO can cause an innate immune response and result in tissue disruption or cell injury (42). The cytokines, IL-6 and IL-1β, cause tissue damage and play an essential role in mediating various types of inflammatory disease (43). The results of the present study demonstrated that ARR notably suppressed the secretion of the proinflammatory factors, IL-6 and IL-1β.

Inflammatory responses are accompanied by the systematic activation of several signaling pathways (44). NF-κB is crucial to inflammatory responses as it releases

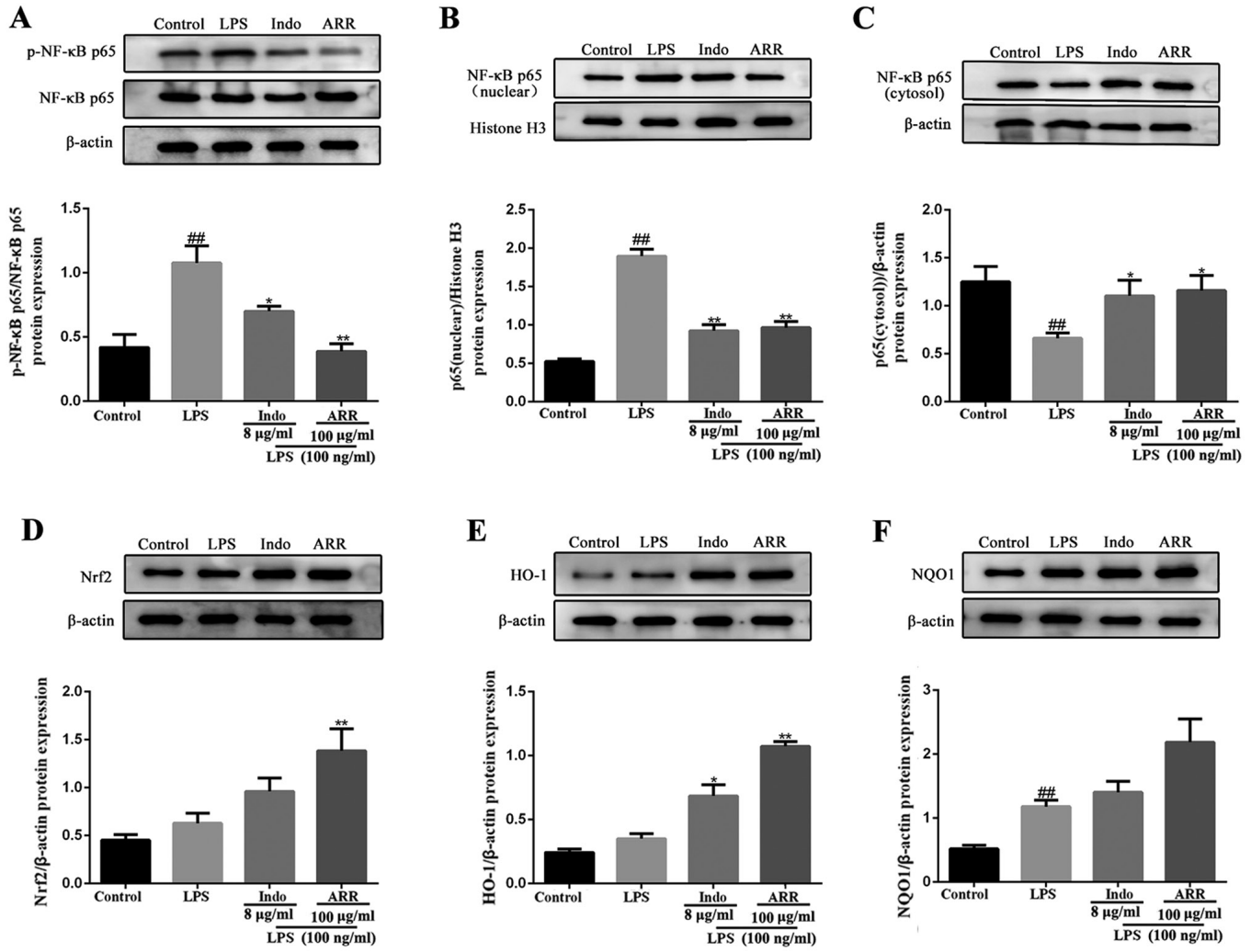


Figure 3. ARR inhibits the NF-κB signaling pathway and activates the Nrf2 signaling pathway. RAW264.7 cells were treated with ARR (100 $\mu$g/ml) or Indo (positive control, 8 $\mu$g/ml) at 37˚C for 24 h, with or without LPS (100 ng/ml). The protein expression levels of (A) total NF-κB p65 and p-NF-κB p65, (B) NF-κB p65 in the nucleus, (C) NF-κB p65 in the cytosol, (D) Nrf2, (E) HO-1 and (F) NQO1 were detected using western blotting and semi-quantified using Image Lab software. LPS represents proteins from the 100 ng/ml LPS-treated group. Data are presented as the mean ± SD. $^{\#\#}$P<0.01 vs. control group; $^{*}$P<0.05, $^{**}$P<0.01 vs. LPS group. ARR, α-rhamnrtin-3-α-rhamnoside; Nrf2, nuclear factor-erythroid 2-related factor 2; Indo, indomethacin; LPS, lipopolysaccharide; HO-1, heme oxygenase-1; NQO1, NAD(P)H quinone dehydrogenase 1; p-, phosphorylated.

proinflammatory cytokines and p65 translocation plays a key role in the activation of NF-κB (45), which was also discovered to be the main signaling pathway for LPS to induce inflammation in macrophages (46,47). Suppression of NF-κB activation has been found to represent a promising anti-inflammatory strategy (48). NF-κB downregulates the expression levels of iNOS, COX-2 and other inflammatory-related genes by activating transcription (49). NO is a free gaseous signaling molecule synthesized by iNOS, and excess production of NO mediated by iNOS induces an inflammatory response (50). COX-2 is known to generate proinflammatory prostaglandins, such as $PGE_2$, which induce inflammation (51). A variety of natural compounds, including flavonoids, quercetin, genistein and kaempferol have been considered as natural COX-2 inhibitors (52,53). Kim *et al* (54) demonstrated that formononetin-7-O-phosphate inhibited COX-2 expression by inhibiting NF-κB nuclear translocation.

The present study also investigated whether ARR exerts anti-inflammatory effects via the NF-κB signaling pathway. As expected, the results demonstrated that ARR not only downregulated iNOS and COX-2 mRNA expression levels, but also suppressed NO and $PGE_2$ content, in a dose-dependent manner. In addition, ARR markedly blocked NF-κB p65 translocation. To the best of our knowledge, the present study was the first to demonstrate that ARR can inhibit the inflammatory response via the NF-κB signaling pathway in LPS-induced RAW264.7 cells.

NLRC3 serves as a checkpoint to prevent dysregulated inflammation. Following stimulation of RAW264.7 cells with LPS, NLRC3 was observed to serve as a de-ubiquitinating enzyme to remove the ubiquitination of TRAF6 and inhibited the nuclear translocation of the NF-κB p65 subunit to reduce the release of IL-1β (55). The results of the present study demonstrated that ARR upregulated NLRC3 expression to inhibit the activation of the NF-κB pathway, which is consistent with previous findings (55,56).

The Nrf2 signaling pathway is another important regulator of inflammation. The activation of Nrf2 and its target

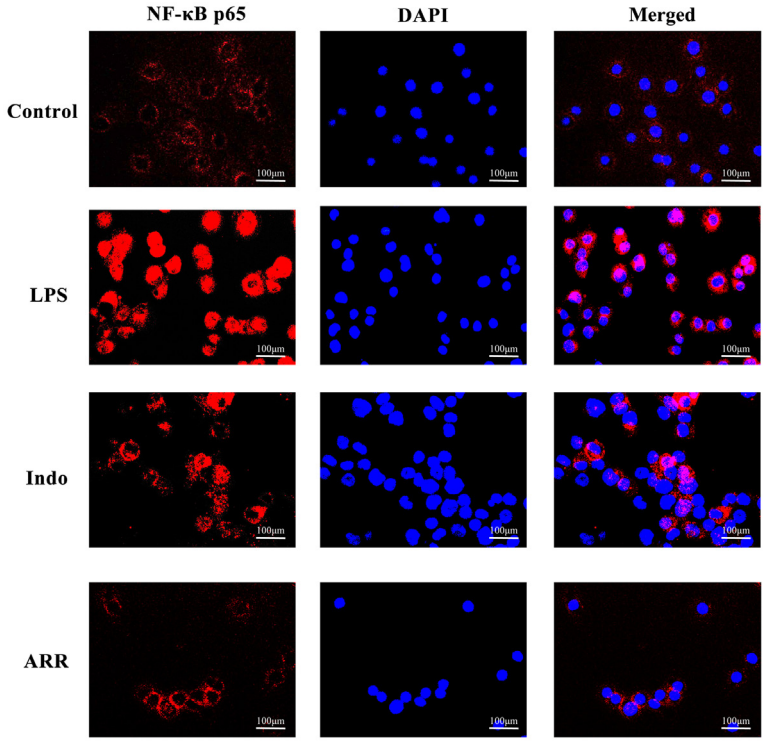


Figure 4. ARR blocks NF-κB p65 nuclear translocation. RAW264.7 cells were treated with ARR (100 $\mu$g/ml) or Indo (positive control, 8 $\mu$g/ml) at 37˚C for 24 h, with or without LPS (100 ng/ml). The nuclear translocation of NF-κB p65 was detected using immunofluorescence microscopy (magnification, x400; scale bar, 100-$\mu$m). Indo, ARR, α-rhamnrtin-3-α-rhamnoside; Indo, indomethacin.

molecules, such as HO-1 and NQO1, is considered an intracellular protective mechanism against oxidative stress and inflammatory responses (57). It has been reported that the activation of Nrf2 could disrupt the crosstalk between NF-κB and its target molecules, thereby controlling the inflammatory response (58). In addition, HO-1 and NQO1 were discovered to inhibit the transcription of inflammatory adhesive molecules mediated by NF-κB (41). Moreover, previous studies have revealed that the regulation of NF-κB may be associated with the Nrf2 signaling pathway, and it was reported that Nrf2 knockdown promoted the transcriptional activity of NF-κB (59-61). The results of the present study demonstrated that ARR upregulated Nrf2 expression and inhibited the nuclear translocation of NF-κB. Therefore, it was suggested that ARR-induced Nrf2 activation may prevent the increase of inflammatory cytokines mediated by NF-κB. However, the underlying molecular mechanism by which ARR affects the crosstalk between Nrf2 and NF-κB requires further investigation.

The aim of the present study was to explore the effects of ARR on LPS-induced RAW264.7 macrophages and to investigate the potential underlying mechanism. The western blotting, immunofluorescence and immunohistochemistry experimental results indicated that ARR inhibited the LPS-induced activation of TRAF6 and NF-κB p65 signaling molecules. Furthermore, ARR could upregulate NLRC3, HO-1, NQO1 and Nrf2 expression. The experiments performed and parameters evaluated in the present work suggested that ARR may exert anti-inflammatory effects, at least in part, by downregulating NF-κB and activating Nrf2-mediated inflammatory responses. In future studies, more in-depth investigations on the anti-inflammatory effect of ARR ,and the specific relationship between NLRC3, NLRC3 and NF-κB should be performed.

In conclusion, the results of the present study demonstrated that ARR exerted anti-inflammatory effects in LPS-stimulated RAW264.7 cells, at least partially through the modulation of NF-κB- and Nrf2-mediated inflammatory responses. These results suggested that ARR may be an attractive candidate for the treatment of inflammation-related diseases. However, as this study was only performed using one macrophage cell line, future studies should be conducted on a wider variety of cells to verify the current study findings. Currently, numerous studies have evaluated the biological activities and mechanisms of tested compounds by comparing the treatment group (tested compound plus challenge) with the model group (only challenge), seldom employing a group treated with the sole test compound without challenge (62-66). Following this experimental setup, this type of grouping was employed for the LPS-stimulated RAW264.7 cell model in the present work. Hence, in future studies, more in-depth investigations on the anti-inflammatory effects of ARR, including the involvement of an ARR group without LPS challenge and *in vivo* animal models, should be conducted to gain further insight into the mechanism of action. These future studies should broaden the current understanding of the anti-inflammatory mechanism and highlight the potential of ARR as anti-inflammatory candidate drug.

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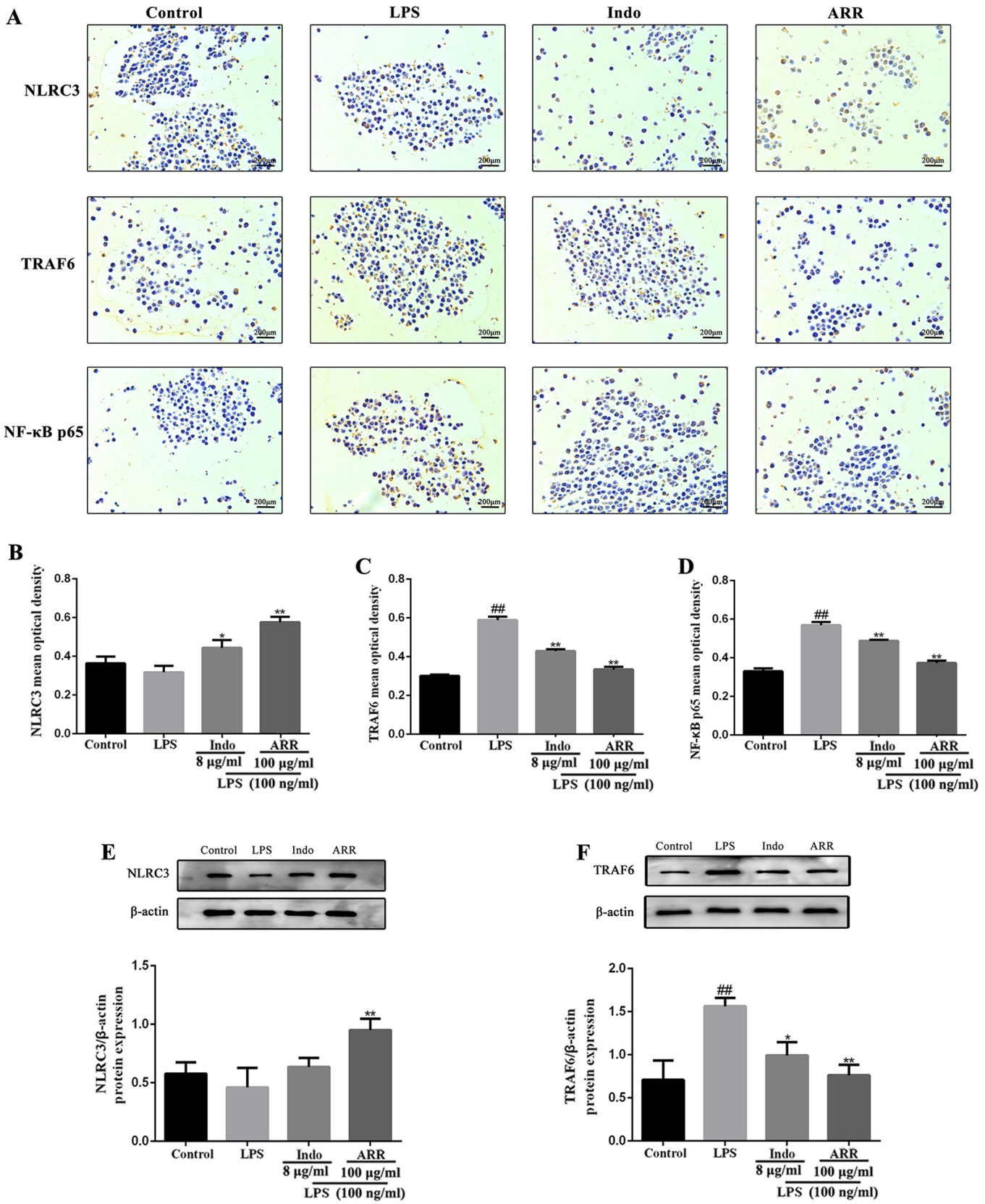


Figure 5. ARR downregulates the protein expression of TRAF6 and NF-κB p65 by increasing the content of NLRC3 protein molecules. (A) Expression levels of NLRC3, TRAF6 and NF-κB p65 in LPS-stimulated RAW264.7 cells were detected using immunohistochemistry staining (magnification, x400; scale bar, 200-$\mu$m). The mean optical density values of (B) NLRC3, (C) TRAF6 and (D) NF-κB p65 were quantified using ImageJ software. The protein expression levels of (E) NLRC3 and (F) TRAF6 were detected using western blotting and semi-quantified using Image Lab software. LPS represents protein from the 100 ng/ml LPS-treated group; Indo represents protein from the 8 $\mu$g/ml Indo and 100 ng/ml LPS-treated group; ARR represents protein from the 100 $\mu$g/ml ARR and 100 ng/ml LPS-treated group. Data are presented as the mean ± SD (n=3). $^{\#\#}$P<0.01 vs. control group; $^{*}$P<0.05, $^{**}$P<0.01 vs. LPS group. ARR, α-rham nrtin-3-α-rhamnoside; TRAF6, tumor necrosis factor-associated factor 6; NLRC3, NOD-like receptor family CARD domain containing 3; LPS, lipopolysaccharide; Indo, indomethacin.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

JTZ and GY conceived and designed the experiments. KDR, JC and JH performed the experiments and analyzed the data. JTZ and GY confirmed the authenticity of all the raw data. JTZ and KDR drafted the initial manuscript and prepared the figures. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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