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*Original Article*

# Luteolin alleviates NLRP3 inflammasome activation and directs macrophage polarization in lipopolysaccharide-stimulated RAW264.7 cells

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**Abstract:** Pure plant extract luteolin has been demonstrated to possess numerous biological effects. However, the specific effect of luteolin on macrophage polarization and NOD-like receptor protein 3 (NLRP3) inflammasome activation has not been documented. In this study, Cultured RAW264.7 cells were treated with or without luteolin in the presence or absence of LPS. Subsequently, cell viability was tested by CCK-8 assay. Total reactive oxygen species (ROS) were measured by flow cytometry. NLRP3, apoptosis-associated speck-like protein containing CARD (ASC), caspase-1, inducible nitric oxide synthase (iNOS) and Arginase (Arg-1) protein expression was detected using western blotting. Enzyme-linked immunosorbent assay (ELISA) kits were used to detect the level of TNF-α, IL-18, and Interleukin-1β (IL-1β). Increased production of ROS and expression of NLRP3, ASC, caspase-1, IL-18 and IL-1β proteins were observed in RAW264.7 cells incubated with LPS and were effectively inhibited by 2 μM luteolin. Furthermore, 2 μM luteolin pretreatment enhanced the expression of M2 macrophage markers (Arg-1 and IL-10), and decreased the expression of markers associated with M1 macrophage polarization (TNF-α, IL-6 and iNOS). These results indicated that low-dose luteolin inhibits NLRP3 inflammasomes activation and promotes macrophage polarization toward an M2 phenotype, which provides new evidence for the anti-inflammation activity of luteolin.

**Keywords:** Macrophage polarization, NLRP3 inflammasome, atherosclerosis, luteolin

## Introduction

Atherosclerosis is an arterial inflammatory disease and the primary cause of cardiovascular disease. Macrophage, known as one of the most dominant and widely distributed inflammatory cells, is involved in the development of atherosclerosis [1]. Classically activated macrophages (M1) secrete various pro-inflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, and producing excessive reactive oxygen species (ROS), leading to the inflammatory cascade and accelerate the progression of atherosclerosis [2].

Accumulating evidence indicates that the NOD-like receptor protein 3 (NLRP3) inflammasome-mediated inflammation is a critical component element in the development of atherosclerosis, which was identified as a novel link between lipid metabolism and inflammation [3]. The NLRP3 inflammasome consists of NLRP3, apoptosis associated speck-like protein (ASC) and caspase-1. ASC regulates the interaction between NLRP3 and caspase-1. Previous study indicates that NLRP3 protein expression in macrophages was up-regulated by lipopolysaccharide (LPS) in M1 macrophages but not in M2 [4]. Besides, inhibition of the NLRP3 inflammasome and pro-inflammatory M1 macrophages has been shown to protect against atherosclerosis [5]. Thus, new drugs targeting NLRP3 inflammasome and/or macrophage polarization represent an attractive option for future therapy against atherosclerosis.

Luteolin (3,4,5,7-tetrahydroxy-flavone) from plant extracts has been demonstrated to possess numerous biological effects, including anti-inflammatory, anti-oxidative, and inhibits macrophage foam cells formation and apoptosis [6, 7]. However, whether luteolin directs macrophage polarization and limits NLRP3 inflammasome activation requires further

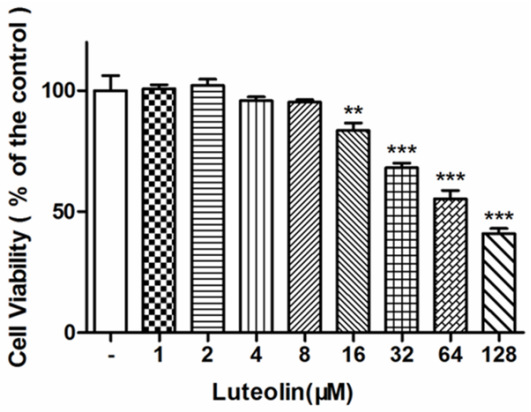


**Figure 1.** Effects of luteolin on the viability of RAW264.7 cells. RAW264.7 cells were treated with various concentrations of luteolin for 24 h, and the cell viability was then measured by CCK-8 assays. Data are expressed as mean ± SD (n=3), $^{**}P<0.01$, $^{***}P<0.001$ vs. control group alone.

investigation. In this study, RAW264.7 cells were treated with LPS to induce pro-inflammatory responses and the cells were then treated with luteolin to evaluate the anti-inflammatory activity of luteolin.

## Materials and methods

### *Reagents and antibodies*

Luteolin (purity >98%) was purchased from Sigma Chemical (St. Louis., MO, USA, Cat#: 491-70-3). It was dissolved in dehydrated alcohol as 50 mM stock solutions, and then serially diluted in PBS immediately prior to experiments. Lipopolysaccharide (LPS) was obtained from Sigma (St. Louis., MO, USA, Cat#: 297-473-0). Antibodies including Rabbit anti-Caspase-1 (#Ab108362), anti-iNOS (#Ab178945) and anti-Arg-1 (#Ab91279) was from Abcam (Cambridge, MA, USA). Rabbit anti-NLRP3 (#BA3677) was purchased from Boster Biological Technology (Wuhan, China); anti-ASC (#Sc-22514-R) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-GAPDH (#AP0063) and anti-β-Actin (#I102) were purchased from Bioworld Technology (Minnesota, USA); Mouse TNF-α (#EK0527), IL-6 (#EK0411), and IL-1β (#EK0394) ELISA kits were purchased from Boster Biological Technology (Wuhan, China); IL-18 (#CK-E11385M) was purchased from Calvin Biological Technology (Suzhou, China); IL-10 (#VAL605) ELISA kit was obtained from R&D Systems (Minneapolis, Minnesota, USA); The bicinchoninic acid (BCA) protein assay kit was from Beyotime Biotechnology (Shanghai, China). All other chemicals, if not stated, were acquired from Sigma Chemicals (St Louis, MO, USA).

### *Cell culture and treatment*

RAW264.7 murine macrophage cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified 5% $CO_2$ atmosphere. For experiments, the above mentioned cells (seeded at $3\times10^5$ cells/ml) were treated with 100 ng/mL LPS for 6 hours as described previously [8].

The RAW264.7 cells were randomly divided into the following four groups: (1) normal control group (cells treated with PBS were used as controls); (2) LPS group (100 ng/mL LPS group); (3) luteolin intervention + LPS group (luteolin + 100 ng/mL LPS group); (4) luteolin control group (luteolin group). In luteolin intervention + LPS group, cells were exposed to luteolin for an additional 24 hours in the presence of 100 ng/mL LPS. After the treatment, the cells were collected and analyzed as described below.

### *Cell viability assays*

Cell viability was detected using CCK-8 assay according to the manufacturer's instructions. Cells were grown and treated on 96-well plates and were incubated with CCK-8 reagent for 3 hours at 37°C. Absorbance was measured at 450 nm and expressed in arbitrary unit, being proportional to cell toxicity. For each of these experiments at least three parallel measurements were carried out.

### *ELISA of cytokines in cell culture supernatants*

RAW264.7 cells seeded in 24-well plates were pretreated with 100 ng/mL LPS for 6 hours followed by treated with 2 μM luteolin for another 24 hours. After stimulation, the culture media were collected and centrifuged at 1,000 g for 15 minutes. The levels of cytokines in the supernatants for tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-18 and IL-1β measured by using a commercially available Enzyme-linked immunosorbent assay (ELISA)

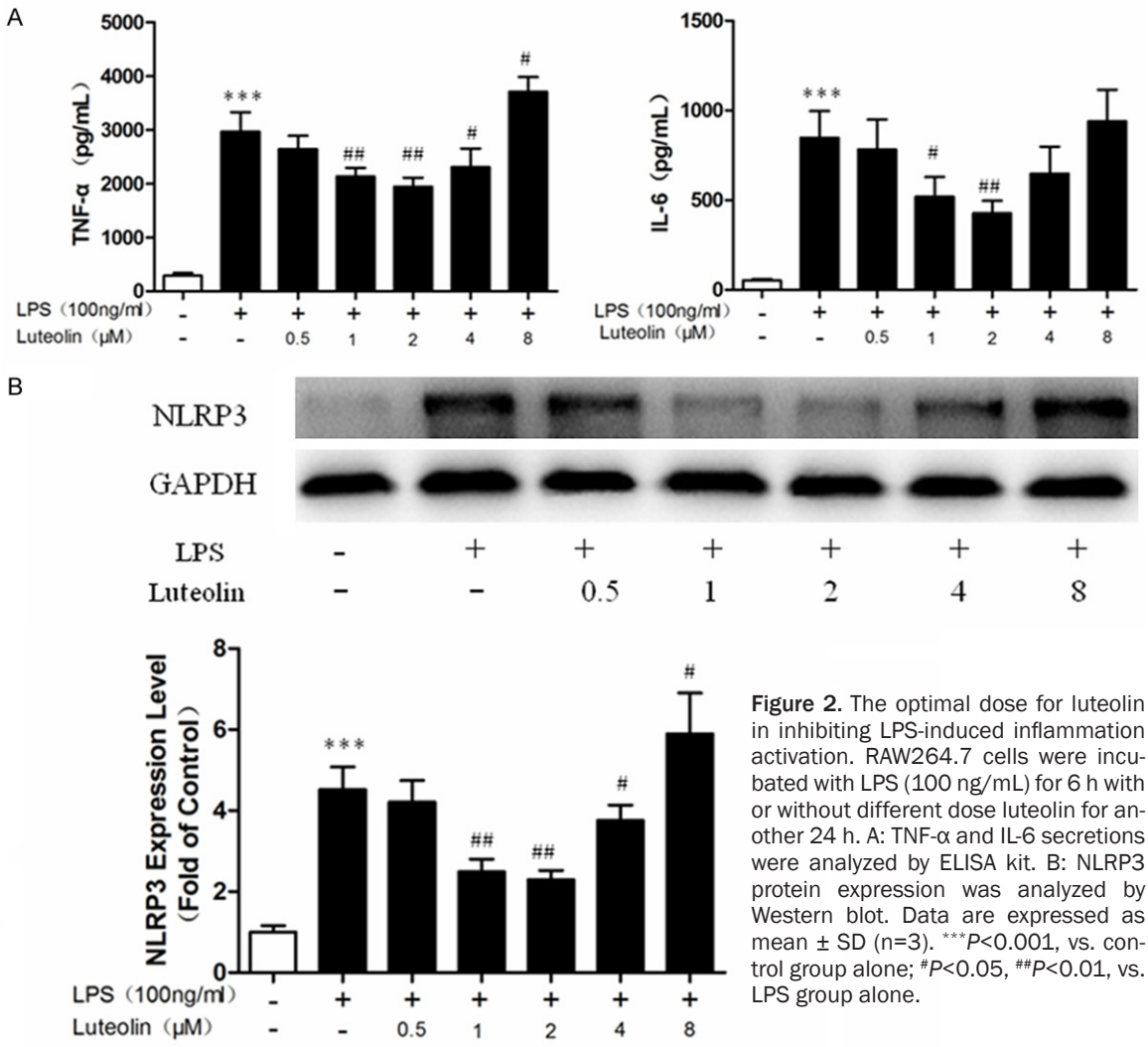


**Figure 2.** The optimal dose for luteolin in inhibiting LPS-induced inflammation activation. RAW264.7 cells were incubated with LPS (100 ng/mL) for 6 h with or without different dose luteolin for another 24 h. A: TNF-α and IL-6 secretions were analyzed by ELISA kit. B: NLRP3 protein expression was analyzed by Western blot. Data are expressed as mean ± SD (n=3). $^{***}P<0.001$, vs. control group alone; $^{\#}P<0.05$, $^{\#\#}P<0.01$, vs. LPS group alone.

kits according to the manufacturer's instructions. Three replicates were performed for each of the different treatments.

*Western blot analysis*

Protein samples were obtained from the lysates of cultured cells, and the protein concentration was determined. Protein samples were separated by SDS-PAGE, transferred to a PVDF membrane and blocked with TBS containing 0.05% Tween-20 (TBST) and 5% nonfat milk powder for 2 hours. The membranes were then incubated with appropriate primary antibodies overnight at 4°C. After 3 washes with TBST, membranes were incubated with secondary antibodies for 2 hours at room temperature. The protein samples were visualized using a chemiluminescence method (ECL Plus Western Blotting Detection System; Amersham Biosciences, Foster City, CA, USA).

*Detection of intracellular reactive oxygen species*

The RAW264.7 cells in the exponential phase were seeded at $2\times10^4$ cells per well in a 48-well plate until grown to confluence and then treated with luteolin (2 μM) with or without the presence of LPS (100 ng/mL) for 6 hours. After incubation, cells were washed twice with PBS, then incubated with reactive oxygen species (ROS)-specific fluorescent dye DCFH-DA (Best-Bio, Shanghai, China) at 37°C for 0.5 hours and kept in the dark. Cells were washed with PBS to rinse the unconjugated dye and fixed in 2% paraformaldehyde (v/v) at 4°C for 5 minutes. Intracellular ROS was observed under fluorescence microscope (Olympus, Tokyo, Japan) using appropriate filters, with excitation wave length at 480 nm and emission wave length at 525 nm. Cells then were washed with PBS and the intensity of emitted fluorescence was ana-

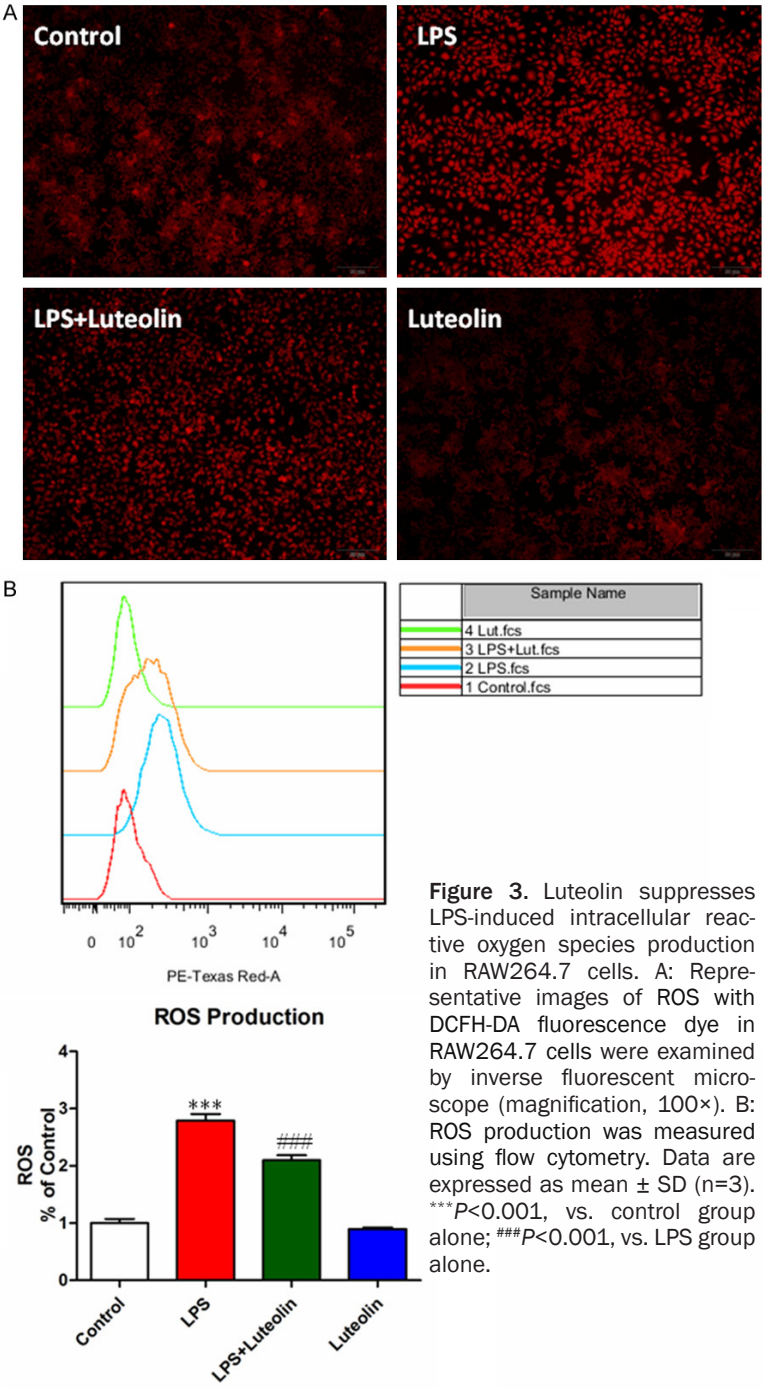


**Figure 3.** Luteolin suppresses LPS-induced intracellular reactive oxygen species production in RAW264.7 cells. A: Representative images of ROS with DCFH-DA fluorescence dye in RAW264.7 cells were examined by inverse fluorescent microscope (magnification, 100×). B: ROS production was measured using flow cytometry. Data are expressed as mean ± SD (n=3). $^{***}P<0.001$, vs. control group alone; $^{###}P<0.001$, vs. LPS group alone.

lyzed by flow cytometry using a FACScan flow cytometry system (Becton Dickinson, San Jose, CA, USA) at a wavelength of 525 nm.

*Statistical analysis*

For the statistical analyses, the values are expressed as the mean ± standard deviation (SD). The statistical significance was determined using a two-tailed Student's *t*-test for independent means, and a *p* value <0.05 was considered to indicate a statistically significant difference. Statistical calculations were performed using GraphPad PRISM 5.0 statistical software (San Diego, California, USA).

## Results

*The optimal dose for luteolin in LPS-induced RAW264.7 cells*

Cytotoxicity of luteolin on RAW264.7 cells was assessed by CCK-8 assay. RAW264.7 cells were incubated with different concentrations (0 μM, 1 μM, 2 μM, 4 μM, 8 μM, 16 μM, 32 μM, 64 μM, 128 μM) of luteolin for 24 hours. As shown in **Figure 1**, at the concentrations of 16 μM, 32 μM, 64 μM or 128 μM, luteolin significantly decreased cell viability, while the viability of RAW264.7 cells was not affected by luteolin at concentrations below 8 μM.

To further assess the optimal dose of luteolin in inhibiting LPS-induced inflammation activation, RAW264.7 cells were pretreated with 100 ng/mL LPS for an additional 6 hours before the application of luteolin for 24 hours. As shown in **Figure 2A**, **2B**, statistical analysis revealed 2 μM luteolin dramatically inhibited the expression of TNF-α, IL-6 and NLRP3 activation. Therefore, luteolin in a concentration of 2 μM was used in the subsequent studies.

*Luteolin reduces the production of intracellular reactive oxygen species in LPS-stimulated RAW264.7 cells*

It is known that ROS may have a central role in NLRP3 inflammasome activation [9], we then investigated the role of ROS in LPS-induced

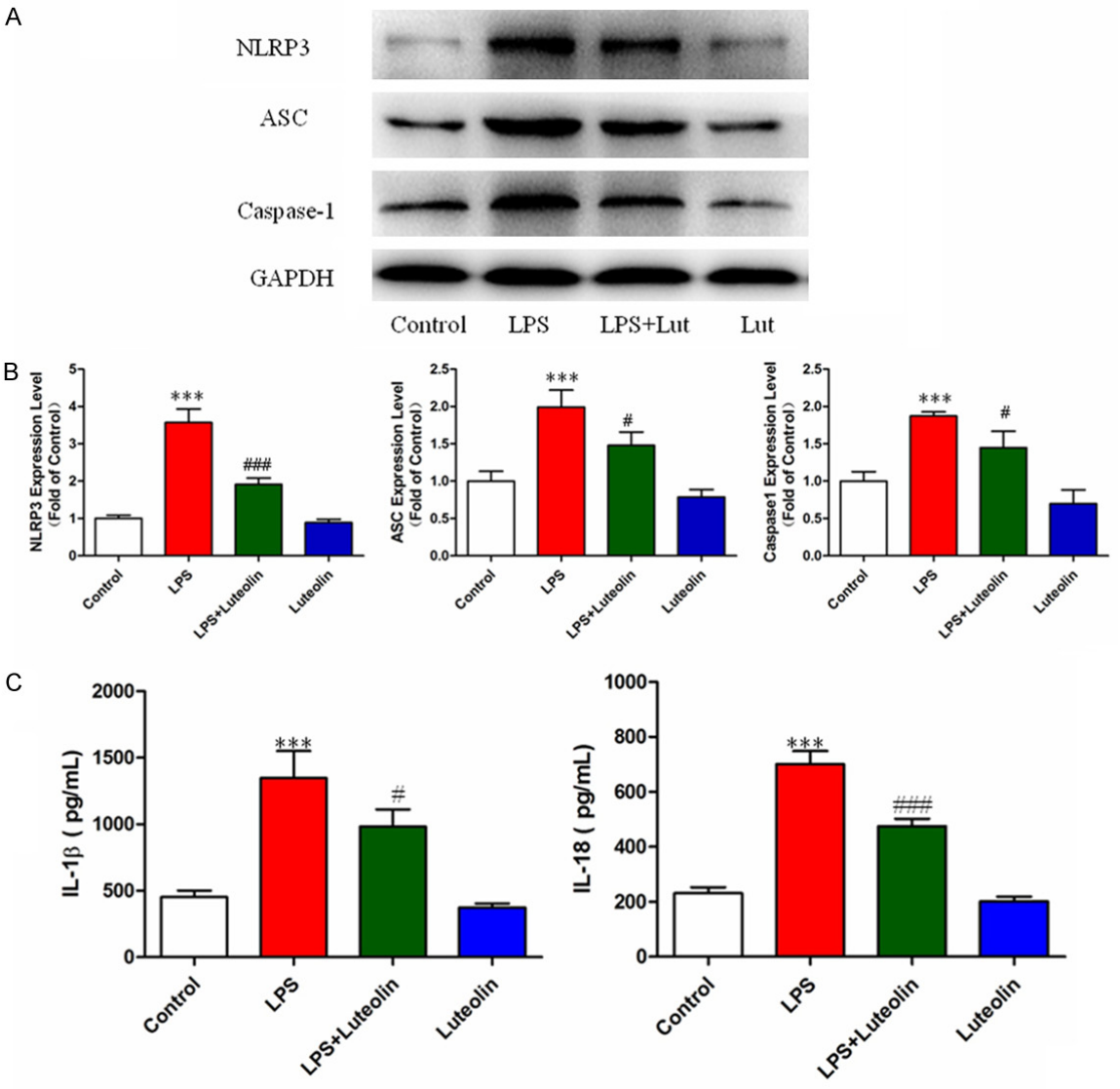


**Figure 4.** Luteolin suppresses LPS-induced NLRP3 inflammasome complex activation in RAW264.7 cells. A: Representative images of the western blot showing the protein expression of NLRP3, ASC and Caspase1. GAPDH was used as an equal loading control. B: Relative quantitation of NLRP3, ASC and Caspase1. C: IL-1β and IL-18 levels were measured using ELISA kit. Data are expressed as mean ± SD (n=3). $^{***}P<0.001$, vs. control group alone; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ vs. LPS group alone.

NLRP3 inflammasome activation and hypothesis that luteolin may prevent LPS-induced ROS production in RAW264.7 cells. As shown in the **Figure 3A**, little fluorescence was observed in the control group; ROS production increased rapidly in the LPS-treated group compared with the control group. However, increased ROS levels induced by LPS stimulation were effectively suppressed by luteolin treatment.

The effects of luteolin on LPS-induced ROS production assessed by flow cytometry with DCFH-DA were depicted in **Figure 3B**. We found that treatment with luteolin significantly inhibited ROS production in RAW264.7 cells compared with that of treatment with LPS alone. Based on these results, we could draw a conclusion that luteolin could suppress ROS production in LPS-induced RAW264.7 cells.

*Luteolin suppresses NLRP3 inflammasome complex activation in LPS-stimulated RAW264.7 cells*

To investigate the effects of luteolin on NLRP3 inflammasome complex activation, Western

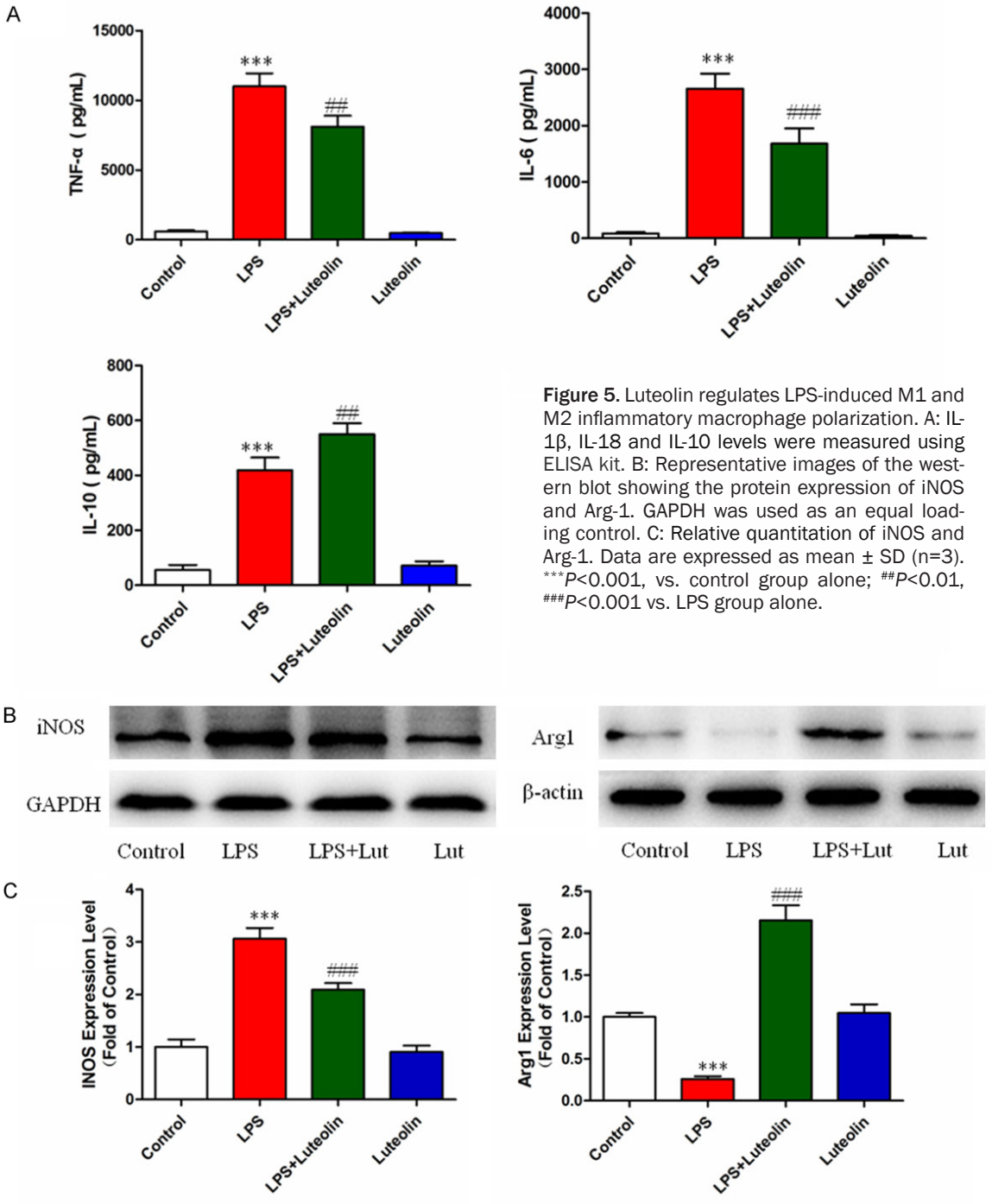


**Figure 5.** Luteolin regulates LPS-induced M1 and M2 inflammatory macrophage polarization. A: IL-1β, IL-18 and IL-10 levels were measured using ELISA kit. B: Representative images of the western blot showing the protein expression of iNOS and Arg-1. GAPDH was used as an equal loading control. C: Relative quantitation of iNOS and Arg-1. Data are expressed as mean ± SD (n=3). $^{***}P<0.001$, vs. control group alone; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. LPS group alone.

blot analysis of NLRP3, apoptosis associated speck-like protein (ASC), and caspase-1 was performed. The NLRP3, ASC, and caspase-1 levels were markedly increased in LPS-stimulated cells, whereas the luteolin-treated RAW264.7 cells stimulated with LPS, exhibited decreased expression of NLRP3 ASC, and caspase-1 (**Figure 4A**, **4B**).

*Luteolin reduces the release of pro-inflammatory cytokines in LPS-stimulated RAW264.7 cells*

To further estimate the inhibition of luteolin on NLRP3 inflammasome activation, we checked the expression of NLRP3 downstream cytokines IL-1β and IL-18. The ELISA test results also

showed that luteolin effectively inhibited IL-1β and IL-18 secretion triggered by LPS (**Figure 4C**).

*Luteolin regulates M1 and M2 inflammatory macrophage polarization in LPS-stimulated RAW264.7 cells*

As previous studies have shown that the NLRP3 inflammasome-related components links with the changes in the polarization state of macrophages under the inflammatory micro-environment [10, 11]. In the present study, pro-inflammatory cytokines TNF-α and IL-6 in M1 macrophages induced by LPS decreased significantly following treatment with luteolin, as shown by ELISA test. It is noteworthy that the secretion of anti-inflammatory cytokines IL-10 increased when the RAW264.7 cells were cultured in LPS medium compared with the control. However, luteolin still increased the level of IL-10 when the cells were treated with a combination of LPS and luteolin (**Figure 5A**). The results of western blot analysis for the protein expression of the macrophage polarization markers were similar to those obtained by ELISA. The level of M1 phenotype marker iNOS increased significantly by the LPS stimulation in RAW264.7 cells, LPS-induced iNOS generation was also inhibited by luteolin treatment. By contrast, luteolin significantly upregulated the M2 marker Arg-1 expression in LPS-induced RAW264.7 cells (**Figure 5B**, **5C**). The above results demonstrate that luteolin promoted M1 phenotype switching to M2 macrophage.

## Discussion

Luteolin, a natural flavonoid with a wide range of pharmacological activities, has been reported to inhibit the expression of pro-inflammatory cytokines and protects against oxidative damage in vitro and in vivo [12-14]. However, correlations among NLRP3 inflammasome, macrophage polarization and luteolin remain unknown. In the present study, The data clearly demonstrated that low-dose luteolin treatment suppresses the ROS generation, NLRP3-inflammasome activation, and pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-18) release by enhancing M2 macrophage polarization (mediated by increases in Arg-1 expression and IL-10 production) in RAW264.7 cells following LPS stimulation. Luteolin has both cytoprotective and cytotoxic effects. It has been reported that low concentrations of luteolin exerts its protective action against high glucose-induced vascular endothelial cells dysfunction [15]. Similarly, high concentrations of luteolin induces cell apoptosis by induction of oxidative stress [16]. In accordance with previous studies, we also provide evidence supporting that low-dose luteolin protects against LPS-related inflammation.

It is known that the NLRP3 inflammasome is a caspase-1-containing multi-protein complex that controls caspase-1 activity and the release of IL-1β and IL-18 in the innate immune system [17]. NLRP3 inflammasome activation may contribute to the development of atherosclerotic plaque progression [18]. Previous study using NLRP3 inflammasome knockout mice have shown that the NLRP3 inflammasome controls the pathogenesis of atherosclerosis [3]. A recent study also showed that NLRP3 expression elevated in the monocytes of patients with coronary artery disease [19]. Thus, the negative regulation of NLRP3 inflammasomes is a potential therapeutic target for atherosclerosis.

Macrophages are a heterogeneous population of cells that may undergo classical M1 or alternative M2 activation in response to various signals [20]. M1 macrophage polarization has been reported to promote NLRP3-inflammasome activation in atherosclerosis process, which contributes to an increased and sustained inflammatory response by producing high levels of pro-inflammatory cytokines, as well as ROS [4, 21]. Therefore, inhibiting M1 macrophage polarization is pivotal to limiting inflammation and thus to preventing atherosclerosis progression. As shown in our present study, luteolin treatment promotes alternative M2 macrophage polarization by inducing the expression of IL-10 and Arg-1, and decreasing the production of M1 inflammatory markers, such as TNF-α, IL-6 and iNOS in LPS-stimulated RAW264.7 cells. Therefore, we supposed that the inhibitory effect of luteolin on inflammation might be related to the alteration of macrophage phenotype.

Collectively, these results reveal that luteolin inhibits NLRP3 inflammasome activation and promoting M2 macrophage polarization in LPS-stimulated RAW264.7 cells. These findings provide new insights into the molecular mecha-

nism of luteolin and its therapeutic potential in the treatment of atherosclerosis.

## Acknowledgements

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## Disclosure of conflict of interest

None.

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