

Aloin suppresses lipopolysaccharide-induced inflammation by inhibiting JAK1-STAT1/3 activation and ROS production in RAW264.7 cells

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Abstract. The anti-inflammatory effects of aloin, a bioactive ingredient extracted from *Aloe vera*, have been described previously. The present study aimed to assess these effects and explore the underlying molecular mechanisms. RAW264.7 cells were incubated with different doses of aloin (100, 150 and 200 $\mu\text{g/ml}$) and lipopolysaccharide (LPS; 100 ng/ml) for the indicated times. Then, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 expression levels were detected by western blot analysis and reverse transcription polymerase chain reaction (RT-PCR). The concentrations of inflammatory cytokines in the cell culture supernatant were determined by ELISA. Total nitric oxide (NO) assay and reactive oxygen species (ROS) kits were used to detect NO and ROS levels, respectively. Mitogen-activated protein kinase, nuclear factor κB and Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway activation were verified by western blot analysis. Confocal and nucleocytoplasmic separation experiments were used to detect STAT nuclear translocation. It was identified that aloin decreased the level of LPS-induced iNOS expression, inhibiting the release of interleukin (IL)-1 β , IL-6, tumour necrosis factor- α and NO dose-dependently. Mechanistically, aloin suppressed LPS-induced JAK1-STAT1/3 activation and STAT1/3 nuclear translocation. Additionally, LPS-induced

ROS production was inhibited by aloin. Collectively, these data suggest that aloin attenuated LPS-induced inflammation by inhibiting ROS-mediated activation of the JAK1-STAT1/3 signalling pathway, thereby inhibiting the nuclear translocation of STAT1/3 in RAW264.7 cells. The present study provides an experimental basis for the clinical application of aloin in inflammatory-associated diseases.

Introduction

Inflammation is a protective immune response against bacterial infection or tissue injury. However, excessive inflammation often leads to diseases, including arthritis and sepsis (1,2). The principal components of the outer membrane of gram-negative bacteria are lipopolysaccharides (LPS), also termed endotoxins, which initiate inflammatory immune responses (3). LPS induce inflammatory cytokine release, including interleukin (IL)-6, IL-1 β and tumour necrosis factor (TNF)- α , via toll-like receptor 4 binding, activating the downstream inflammation-associated signalling pathways (2,4). The signalling pathways of mitogen-activated protein kinases (MAPKs), including extracellular signal-related kinase (ERK)-1/2, p38MAPK, c-Jun NH2-terminal kinase (JNK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κB), have been demonstrated to be involved in LPS-induced inflammatory responses (5,6). The Janus kinase-signal transducer and activator of transcription (JAK-STAT) signalling pathway is an additional important inflammatory signalling pathway activated by LPS (7,8). Following receptor binding, LPS induce JAK phosphorylation, effecting STAT phosphorylation. The phosphorylated STATs form homo- or heterodimers that translocate into the nucleus, regulating the transcription of a number of pro-inflammatory cytokines, chemokines and regulatory enzymes, including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (9,10). Increasing evidence has suggested that the inhibition of the JAK-STAT signalling pathway attenuates LPS-induced inflammation (10,11).

Attention has been paid to natural active products, due to their abundance and minimal side effects. The *Aloe vera* plant

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has been widely used in health and nutritional supplements in Chinese herbal medicine (12). Aloin, a bioactive ingredient extracted from *Aloe vera*, has been indicated to induce anti-inflammatory (13), antioxidant (12) and antitumour (14-16) effects, neuroprotection (17) and osteoclastogenesis (18,19). However, the anti-inflammatory mechanism of aloin remains unknown.

The present study evaluated the effects of aloin on the LPS-induced inflammatory response and then investigated the underlying molecular mechanism in RAW264.7 cells. It was determined that aloin inhibited LPS-induced TNF- α , IL-1 β , IL-6 and nitric oxide (NO) release, attenuating the iNOS expression induced by LPS. Mechanistically, aloin suppressed reactive oxygen species (ROS)-mediated JAK1-STAT1/3 signalling pathway activation, inhibiting the nuclear translocation of STAT1/3.

Materials and methods

Reagents and antibodies. LPS from *Escherichia coli* and aloin (purity $\geq 97\%$; Fig. 1A) were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) and Aladdin Industrial Corporation (Shanghai, China), respectively. The aloin was dissolved in dimethyl sulfoxide and diluted with sterile PBS. DAPI was obtained from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Anti-phosphorylated (phospho)-STAT3 (Tyr705, sc7993) and phospho-I κ B (B-9, sc8404) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). The antibodies against phospho-p38 MAPK (p-p38 MAPK; Thr180/Tyr182, 4511S), phospho-ERK (Thr202/Tyr204, 4376S), phospho-JNK (Thr183/Tyr185, 4668S), p38 MAPK (8690S), ERK (4695S), JNK (9258S), phospho-JAK1 (Tyr1034/1035, 3331S), phospho-JAK2 (Y1007/1008, 3771S), phospho-STAT1 (Tyr701, 9167S), JAK1 (3332S), JAK2 (3230S), STAT1 (14994S), STAT3 (12640S), COX-2 (4842S), iNOS (13120), GAPDH (5174S) and β -actin (4970S) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The anti-phospho-I κ B (IKK; S176/177, ab194528) antibody was purchased from Abcam (Cambridge, UK). Secondary antibodies coupled to IRDye 800 fluorophore used in the western blot analysis (926-3221 and 926-32210) were obtained from LI-COR Biosciences (Lincoln, NE, USA). The Alexa Fluor[®] 555 goat anti-rabbit IgG secondary antibody used in the confocal microscopy experiment was obtained from Invitrogen (Z25305; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell culture and passage. Murine macrophage RAW264.7 cells were purchased from Kunming Cell Bank of Type Culture Collection, Chinese Academy of Sciences (Kunming, China) and cultured in high glucose Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.), 100 μ g/m streptomycin and 100 U/ml penicillin at 37°C in 5% CO₂. The cells were passaged every 2 days.

Cell viability detection. Cell viability was detected using a Cell Counting Kit-8 (CCK-8; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) according to the manufacturer's protocol. Briefly, RAW264.7 cells were treated at 37°C with different

concentrations of aloin (20, 40, 80, 100, 200 and 400 μ g/ml) for 24 h and then incubated at 37°C with 10 μ l CCK-8 working solution for 2 h. The absorbance was detected using a Multiskan[™] GO spectrophotometer (Thermo Fisher Scientific, Inc.) at 450 nm. All experiments were repeated in triplicate, and the data are represented as mean \pm standard deviation (SD).

Pro-inflammatory cytokine detection. Following a 2 h pre-treatment at 37°C with different aloin concentrations (100, 150 and 200 μ g/ml), RAW264.7 cells were seeded at a density of 1×10^6 /well in 12-well cell culture plates and stimulated with LPS (100 ng/ml) for 16 h at 37°C. The levels of TNF- α , IL-1 β and IL-6 in the cell culture supernatants were detected using TNF- α (P06804), IL-1 β (P10749) or IL-6 (P08505) ELISA kits (RayBiotech, Inc., Norcross, GA, USA) according to the manufacturer's protocol. The experiments were repeated in triplicate for all aloin concentrations. The results are presented as mean \pm SD.

Nitric oxide detection. RAW264.7 cells were pre-treated at 37°C with aloin (100, 150 and 200 μ g/ml) for 2 h followed by a 16 h LPS treatment. NO production in the cell culture medium was determined using a Total Nitric Oxide Assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. The absorbance at 540 nm was measured using a Multiskan[™] GO spectrophotometer (Thermo Fisher Scientific, Inc.). Each experiment was repeated in triplicate for all aloin concentrations.

ROS determination. RAW264.7 cells were seeded at a density of 2×10^5 /well in 12-well cell culture plates. Following aloin (100, 150 and 200 μ g/ml) pre-treatment at 37°C, the cells were stimulated with LPS for 30 min, and intracellular total ROS was detected using a Reactive Oxygen Species Assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Briefly, following treatment, the cell culture medium was removed, and dichloro-dihydro-fluorescein diacetate (DCFH-DA) was added to a final concentration of 10 μ M. Then, the cells were incubated in a CO₂ incubator for 20 min at 37°C and washed 3 times with PBS to completely remove the DCFH-DA from the cells. ROS production was observed by inverted fluorescence microscopy (magnification, $\times 100$; Olympus Corporation, Tokyo, Japan) and quantified using ImageJ software version 1.46 (National Institutes of Health, Bethesda, MD, USA). The experiments were repeated in triplicate.

Nuclear and cytoplasmic protein separation and western blot analysis. The nuclear and cytoplasmic proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction kit (P0028; Beyotime Institute of Biotechnology) according to the manufacturer's protocol. The extraction of total intracellular protein was as follows: Cells were pretreated with aloin (100, 150, 200 μ g/ml) for 2 h, then stimulated with 100 ng/ml LPS for different times (30 min, 4 or 16 h) at 37°C. Pre-treated RAW264.7 cells were washed twice with ice-cold PBS and lysed in ice-cold cell lysis buffer (P0013; Beyotime Institute of Biotechnology) including 20 mM Tris (pH7.5), 150 mM NaCl, 1% Triton X-100, sodium pyrophosphate, β -glycerophosphate, EDTA, Na₃VO₄ and leupeptin. Following lysis on ice for 30 min,

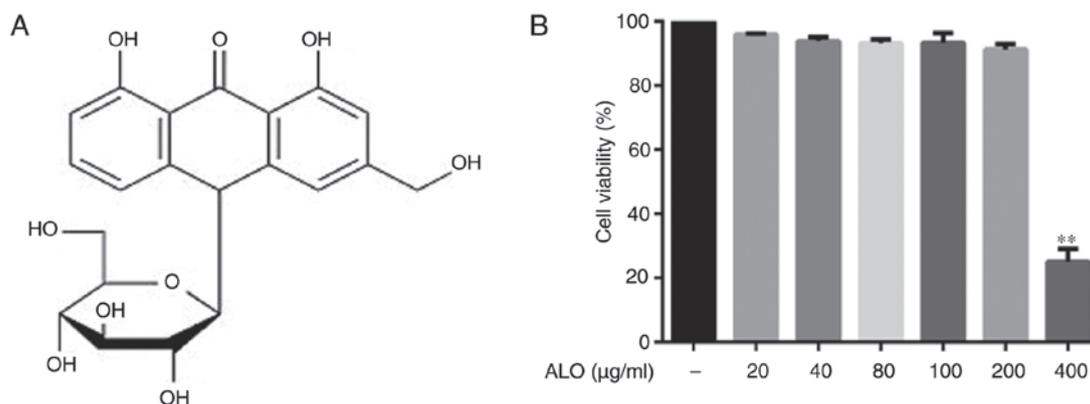


Figure 1. Effect of ALO on RAW264.7 cell viability. (A) Chemical structure of ALO. (B) Cells viability was detected by Cell Counting Kit-8 assay. Data are presented as mean \pm standard deviation of three independent experiments. ** $P < 0.01$ vs. the control group. ALO, aloin.

the lysates were centrifuged (14,300 \times g) for 15 min at 4°C, and the protein samples were quantified using a BCA/Bradford assay. Then, equal amounts (50 μ g) of protein were denatured in SDS and electrophoresed on a 12% SDS-PAGE prior to transferring to a nitrocellulose membrane (Pall Corporation, Port Washington, NY, USA). The membrane was blocked in 5% skimmed milk dissolved in TBST for 1 h at room temperature. Following washing with TBS and 0.1% Tween®-20 3 times, the membrane was incubated overnight at 4°C with primary antibodies diluted 1:500 with TBST, followed by incubation with IRDye 800-conjugated IgG secondary antibodies (1:5,000; LI-COR Biosciences) at room temperature for 1 h. The antigen-antibody complex was visualised using an Odyssey® infrared imaging system (LI-COR Biosciences). ImageJ version 1.46 software (National Institutes of Health) was used for the densitometry analysis.

Reverse transcription polymerase chain reaction (RT-PCR). Intracellular total RNA was extracted from RAW264.7 cells using TRIzol® reagent (Life Technologies; Thermo Fisher Scientific, Inc.), and a RevertAid™ First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) was used to synthesise cDNA. The PCR primers were: 5'-GGGTCTTGT TCACTCCACGG-3' (iNOS forward), and 5'-GCTCAGAAC AGCACAAGGGG-3' (iNOS reverse); 5'-GGAGAGTGTTC CTCGTCCC-3' (GAPDH forward), and 5'-ACTGTGCCGTTG AATTTGCC-3' (GAPDH reverse). The thermocycling conditions were as follows: 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min and 72°C for 1 min with a final extension step at 72°C for 10 min. The PCR products were detected by agarose gel (1.5%) electrophoresis and visualized with GoldView (Service Biological Technology Co., Ltd., Wuhan, China). The image was captured by the Gel Doc™ EZ imager (Bio-Rad Laboratories, Inc., Hercules, CA, USA). ImageJ version 1.46 software (National Institutes of Health) was used for densitometry analysis.

Confocal laser microscopy. RAW264.7 cells were seeded in a small confocal laser dish at 500 cells/well. RAW264.7 cells were pre-treated with 200 μ g/ml aloin for 2 h, and then stimulated with LPS for 4 h at 37°C. Following treatment, the cells were washed with PBS, fixed with 4% paraformaldehyde

for 30 min at room temperature, permeabilised with 0.2% Triton X-100, blocked with 3% bovine serum albumin in PBS for 1 h at room temperature and incubated with STAT1 and STAT3 primary antibodies diluted 1:100 with PBS overnight at 4°C. Following rinsing with PBS, the cells were incubated with a goat anti-rabbit IgG Alexa Fluor® 555 conjugated fluorescent secondary antibody diluted 1:200 with PBS for 1 h in the dark at room temperature. Finally, the cells were stained at room temperature with 0.1 μ g/ml DAPI for 3 min in the dark. Images were captured using a TCS SP8 confocal microscope (magnification, \times 200; Leica Microsystems GmbH, Wetzlar, Germany).

Statistical analysis. All data were presented as mean \pm standard deviation. Statistical analyses were performed using Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The results were analyzed by one-way analysis of variance followed by a post hoc Tukey's test for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Aloin inhibits LPS-induced iNOS expression in RAW264.7 cells. The present study investigated aloin cytotoxicity using a CCK-8 cell viability assay. Aloin did not affect cell viability, even at a high dose of 200 μ g/ml (Fig. 1B). As iNOS and COX-2 are considered to be associated with LPS stimulation (7), the effects of aloin on LPS-induced iNOS and COX-2 expression were investigated. RAW264.7 cells were pre-treated with different doses of aloin for 2 h, then treated with LPS for 16 h, and the iNOS and COX-2 levels were determined by western blot analysis. Aloin treatment suppressed iNOS expression dose-dependently but did not affect COX-2 expression (Fig. 2A). According to these results, aloin concentrations of 100, 150 and 200 μ g/ml were selected for subsequent experiments. RT-PCR and western blot analyses were used to detect iNOS expression levels in RAW264.7 cells pre-treated with different aloin doses (100, 150 and 200 μ g/ml) for 2 h and then LPS for 6 or 16 h. Aloin markedly attenuated iNOS expression at mRNA and protein levels in a dose-dependent manner (Fig. 2B and C).

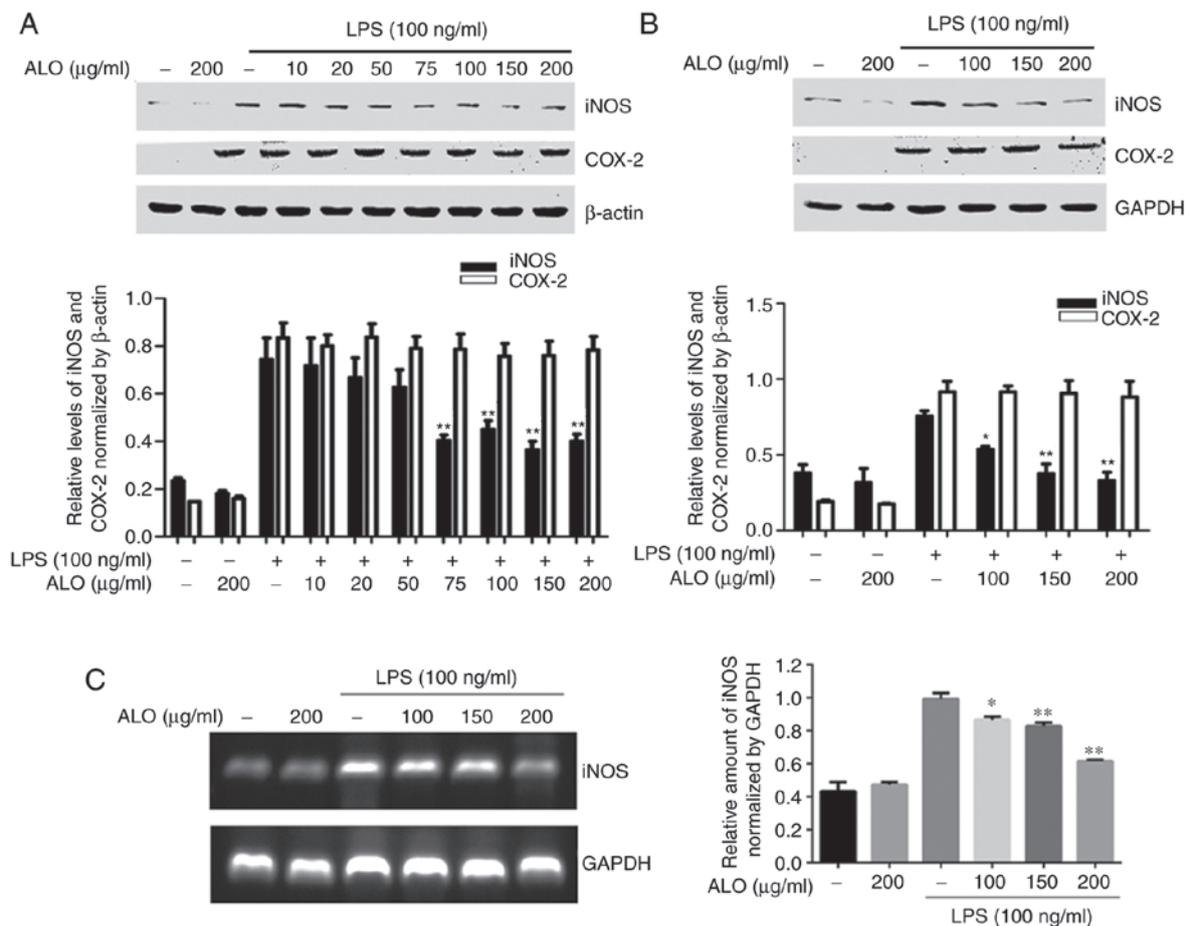


Figure 2. ALO inhibits LPS-induced expression of iNOS, but not COX-2. RAW264.7 cells were pre-treated with (A) different doses of ALO (10, 20, 50, 75, 100, 150 and 200 μ g/ml) for 2 h or (B) 100, 150 and 200 μ g/ml of ALO for 2 h, then stimulated with LPS (100 ng/ml) for 16 h. Following total protein extraction, iNOS and COX-2 levels were determined by western blot analysis. GAPDH or β -actin was used as a control. (C) RAW264.7 cells were pre-treated with ALO for 2 h, then treated with LPS for 6 h. Total cellular RNA was extracted using TRIzol[®] reagent, and reverse transcription-polymerase chain reaction was used to quantify the iNOS transcripts. GAPDH was used as a control. Data are presented as mean \pm standard deviation. * P <0.05 and ** P <0.01 vs. LPS-stimulated cells. ALO, aloin; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2.

Aloin inhibits the release of pro-inflammatory cytokines and mediators induced by LPS. To investigate the anti-inflammatory effect of aloin, the levels of pro-inflammatory cytokines and mediators, including TNF- α , IL-1 β , IL-6 and NO in LPS-stimulated RAW264.7 cells were first determined. The obvious increase of the levels of TNF- α , IL-1 β , IL-6 and NO resulting from the LPS stimulation were inhibited by aloin in a dose-dependent manner (Fig. 3A-D). Collectively, these results suggest that aloin inhibited pro-inflammatory cytokine and mediator release, exhibiting an anti-inflammatory effect.

Aloin suppresses the LPS-activated JAK1-STAT1/3 pathway, but not the MAPK signalling pathway. It has been suggested that the JAK-STAT, MAPK and NF- κ B signalling pathways are involved in LPS-triggered inflammatory responses (7,10,20). Therefore, to investigate the underlying molecular mechanism of aloin-based inhibition of LPS-induced inflammatory reactions, the effects of aloin on LPS-activated STAT and MAPK signalling pathways were determined. Firstly, the phosphorylation levels of MAPKs and STATs following LPS stimulation of RAW264.7 cells were assessed. LPS stimulation was performed for different time periods, and western blot

analysis was used to detect MAPK and STAT activation. At 10 min of LPS stimulation, p38 MAPK, ERK and JNK were activated, peaking at \sim 30 min visually (Fig. 4A). To additionally explore the effects of aloin on MAPK phosphorylation induced by LPS, the cells were pre-treated with aloin for 2 h and LPS for 30 min. Activation was detected by western blot analysis. LPS-induced phosphorylation of p38 MAPK, ERK and JNK were not affected by aloin treatment (Fig. 4B). A previous study demonstrated that NF- κ B became activated following the activation of its upstream kinase IKK and I κ B regions (21,22). To explore the effects of aloin on the LPS-induced NF- κ B signalling pathway, levels of I κ B kinase (IKK) and I κ B phosphorylation were determined by western blot analysis. The results of the present study demonstrated that aloin exhibited a minimal effect on the phosphorylation levels of upstream IKK and I κ B (Fig. 4C). Therefore, the transcriptional activity of downstream transcription factor NF- κ B was not examined. STAT1 and STAT3 phosphorylation increased at 0.5 h, peaked at 4 h and was sustained until 6 h following LPS stimulation as observed visually (Fig. 4D). The increased levels of STAT1 and STAT3 phosphorylation were markedly decreased by aloin in a dose-dependent manner (Fig. 4E). Collectively, these data

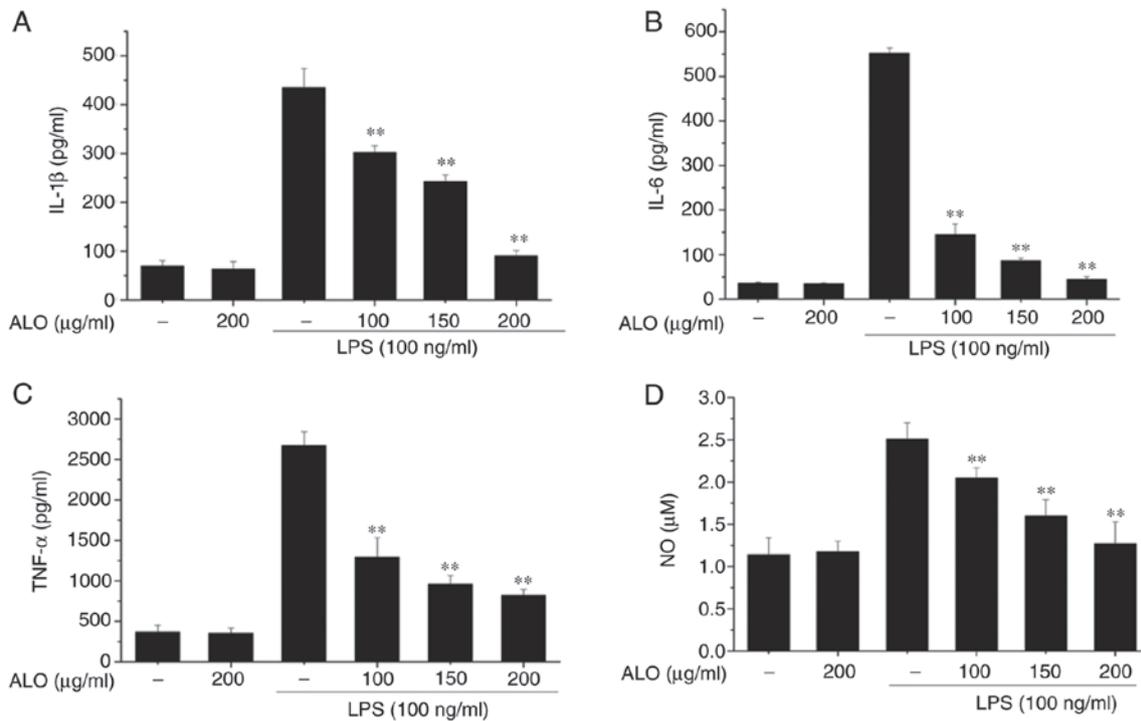


Figure 3. ALO decreased LPS-induced release of pro-inflammatory cytokines and mediators in RAW264.7 cells. RAW264.7 cells were pre-treated with different doses of ALO (100, 150 and 200 $\mu\text{g/ml}$) for 2 h and treated with LPS (100 ng/ml) for 16 h. The levels of (A) IL-1 β , (B) IL-6, (C) TNF- α and (D) NO in the cell culture supernatants were detected by ELISA or a total nitric oxide assay, respectively. Data are presented as mean \pm standard deviation of three independent experiments. ** $P < 0.01$ vs. the LPS-stimulated group. LPS, lipopolysaccharide; ALO, aloin; IL, interleukin; TNF- α , tumour necrosis factor- α ; NO, nitric oxide.

suggested that aloin attenuated the LPS-triggered inflammatory response by suppressing STAT1 and STAT3 activation, but not MAPKs, IKK or I κ B.

As STAT transcription factors have been determined to be activated by the JAKs (23), the effects of aloin on JAK signals were investigated. RAW264.7 cells were stimulated with LPS for different time intervals, and JAK1 and JAK2 phosphorylation was determined by western blot analysis. JAK1 and JAK2 phosphorylation increased at 5 min and peaked at \sim 15 min following LPS treatment (Fig. 5A). RAW264.7 cells were also incubated with aloin for 2 h and treated with LPS for 15 min to detect JAK1 and JAK2 activation by western blot analysis. Aloin pre-treatment suppressed LPS-induced JAK1 phosphorylation, whereas JAK2 phosphorylation was not affected (Fig. 5B). Collectively, these results indicated that aloin inhibited STAT1 and STAT3 phosphorylation, potentially via the inhibition of JAK1 in LPS-induced inflammatory responses.

Aloin suppresses LPS-induced STAT1 and STAT3 nucleocytoplasmic translocation. Activated STATs undergo dimerisation and translocate into the nucleus to initiate transcription. Therefore, the present study investigated whether aloin may inhibit the nuclear translocation of phosphorylated STAT1 and STAT3. The cytoplasmic and nuclear proteins of RAW264.7 cells pre-treated with aloin for 2 h and stimulated with LPS for 4 h were extracted to determine cytoplasmic and nuclear STAT1 and STAT3 levels. LPS stimulation induced the nuclear translocation of STAT1 and STAT3 and aloin pre-treatment significantly inhibited their nuclear translo-

cation (Fig. 6A). A confocal microscopy experiment was performed to detect the location of STAT1 and STAT3. In the control and aloin groups, STAT1 and STAT3 were primarily localised in the cytoplasm, indicated by red staining. In the LPS-treated cells STAT1 and STAT3 transferred into the nuclei, as indicated by blue staining patterns (Fig. 6B and C). However, the levels of nucleocytoplasmic translocation of STAT1 and STAT3 induced by LPS were markedly decreased by aloin. Collectively, the results indicated that aloin inhibited LPS-induced inflammatory responses by suppressing JAK1-STAT1/3 activation and the nuclear translocation of STAT1 and STAT3, at least partially.

Aloin inhibits LPS-induced ROS release. ROS are crucial to LPS-induced inflammation in RAW264.7 cells through the activation of STAT transcription factors (23), and Simon *et al* (24) suggested that ROS production contributing to JAK-STATs activation. Furthermore, a previous study has revealed that aloin exhibits an antioxidant effect (25). Therefore, the present study investigated whether the anti-inflammatory effect of aloin was due in part to its inhibition of ROS accumulation. RAW264.7 cells were pre-treated with aloin for 2 h and stimulated with LPS for 30 min. A ROS detection kit was used to assess ROS accumulation. Aloin significantly decreased LPS-induced ROS production in a dose-dependent manner (Fig. 7). The data of the present study demonstrated that aloin may function as an antioxidant. The anti-inflammatory mechanism of aloin may involve the inhibition of ROS-mediated JAK1-STAT1/3 signalling pathway activation.

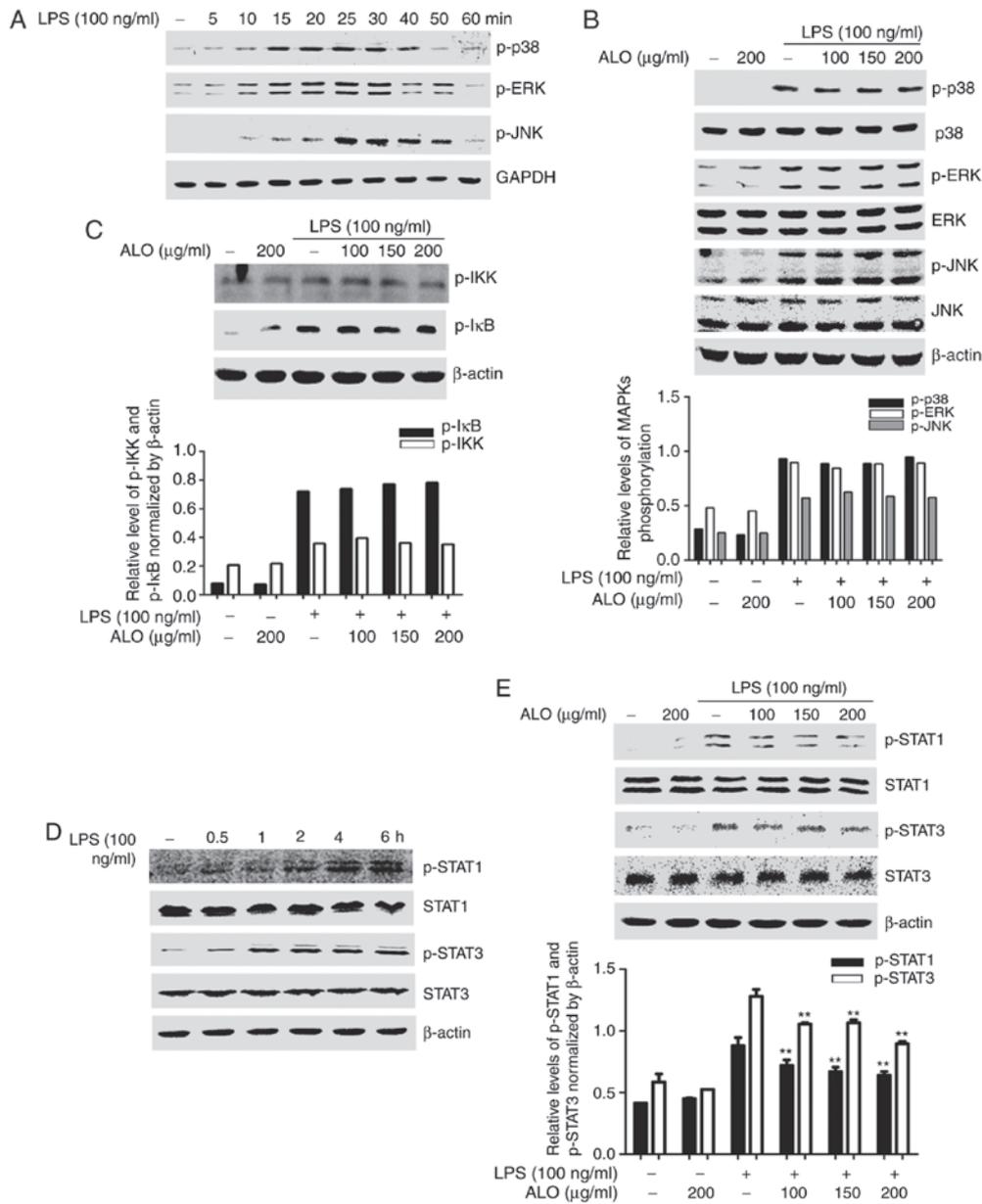


Figure 4. ALO inhibits LPS-activated STAT signalling pathways but does not affect MAPKs, IKK and I κ B activation. (A) RAW264.7 cells were stimulated with LPS for different time intervals. (B) RAW264.7 cells were pre-treated with the indicated ALO doses for 2 h and then stimulated with LPS for 30 min. Proteins were extracted, and p-p38, p-ERK, p-JNK, p-IKK and p-I κ B expression levels were detected by western blot analysis using target-specific antibodies. (C) Densitometric analysis of western blot analysis data from (B). The histogram indicates the relative expression of p-p38, p-ERK and p-JNK normalized to total p38, ERK and JNK respectively. (D) STAT1 and STAT3 phosphorylation in RAW264.7 cells stimulated with LPS for different time intervals. (E) STAT1 and STAT3 phosphorylation in RAW264.7 cells pre-treated with indicated doses of ALO for 2 h and then stimulated with LPS for 4 h. The levels of p-STAT1/3 and total STAT1/3 were measured by western blot analysis. Data are presented as mean \pm standard deviation. **P<0.01 vs. LPS-stimulated cells. LPS, lipopolysaccharide; STAT, signal transducer and activator of transcription; p, phosphorylated; MAPK, mitogen-activated protein kinase; p38, p38 MAPK; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; ALO, aloin; IKK, I κ B kinase.

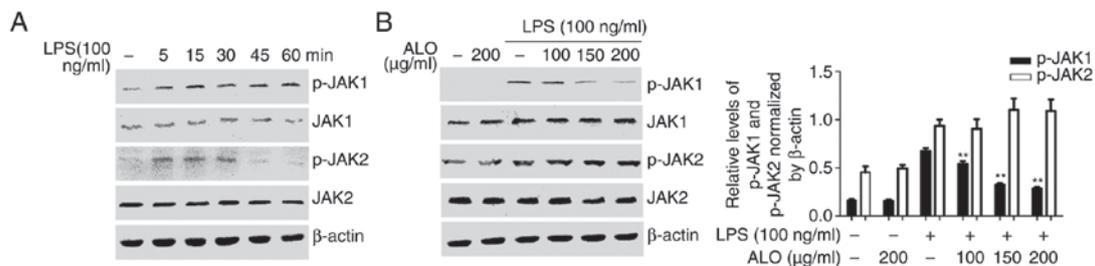


Figure 5. ALO decreases LPS-activated JAK1, but not JAK2. (A) RAW264.7 cells were stimulated with LPS for different time intervals. (B) RAW264.7 cells were pre-treated with indicated doses of ALO for 2 h and then stimulated with LPS for 15 min. Proteins were extracted, and p-JAK1, p-JAK2, total JAK1 and total JAK2 were detected by western blot analysis. Data are presented as mean \pm standard deviation. **P<0.01 vs. LPS-stimulated cells. LPS, lipopolysaccharide; JAK, Janus kinase; p, phosphorylated; ALO, aloin.

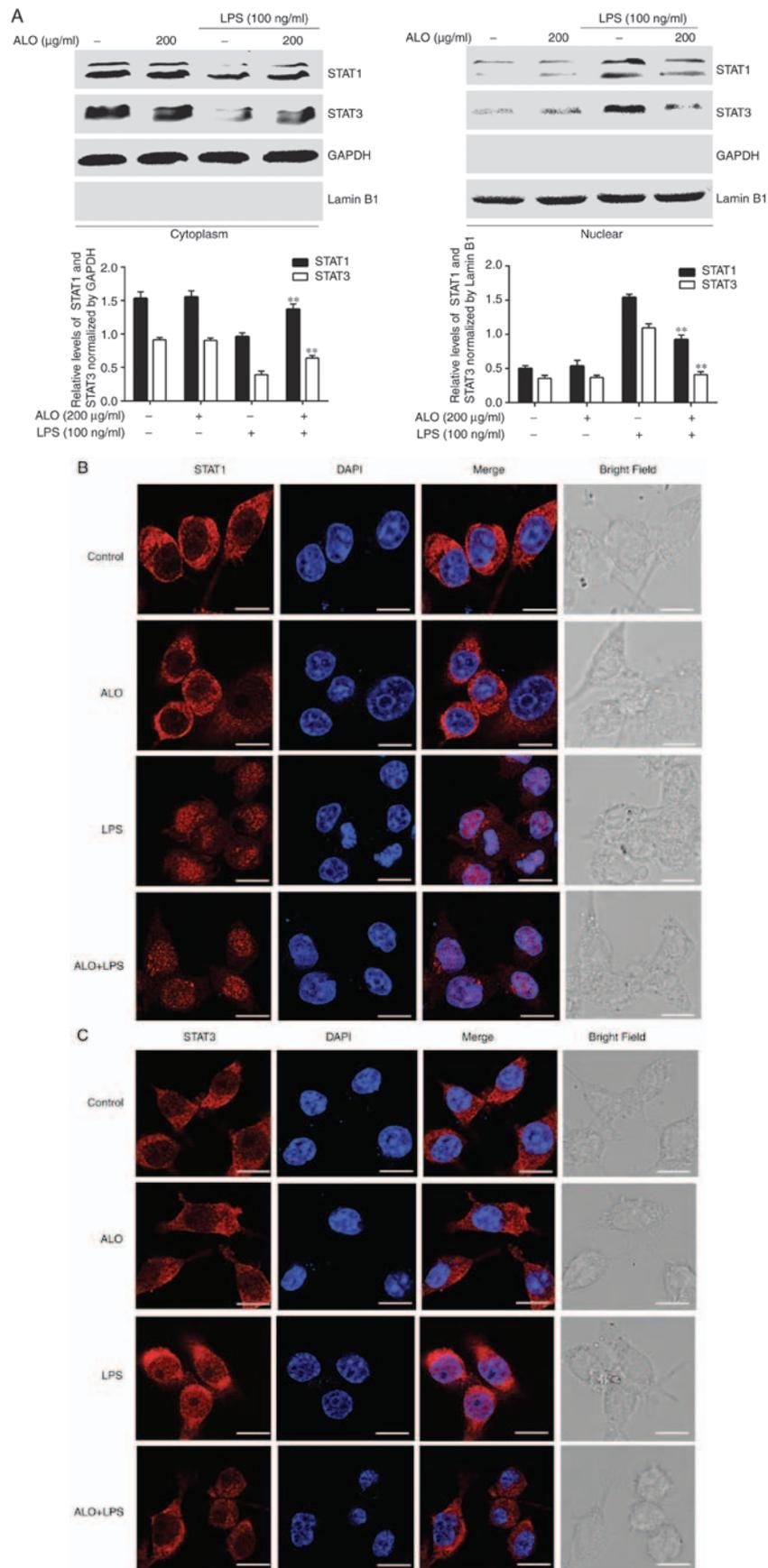


Figure 6. ALO suppresses LPS-induced nuclear translocation of STAT1 and STAT3. RAW264.7 cells were pre-treated with ALO for 2 h and then stimulated with LPS for 4 h. (A) Nuclear and cytoplasmic proteins were extracted, and STAT1/3 levels were detected by western blot analysis. Data are presented as mean \pm standard deviation. ** $P < 0.01$ vs. LPS-stimulated cells. The nuclear translocation of (B) STAT1 and (C) STAT3 was revealed using confocal laser microscopy. The cells were fixed, permeabilised and incubated with anti-STAT1 and STAT3 antibodies followed by incubation with Alexa Fluor[®] 555 goat anti-rabbit IgG antibody (red). The nuclei were stained with DAPI (blue). Scale bar = 25 µm. LPS, lipopolysaccharide; ALO, aloin; STAT, signal transducer and activator of transcription.

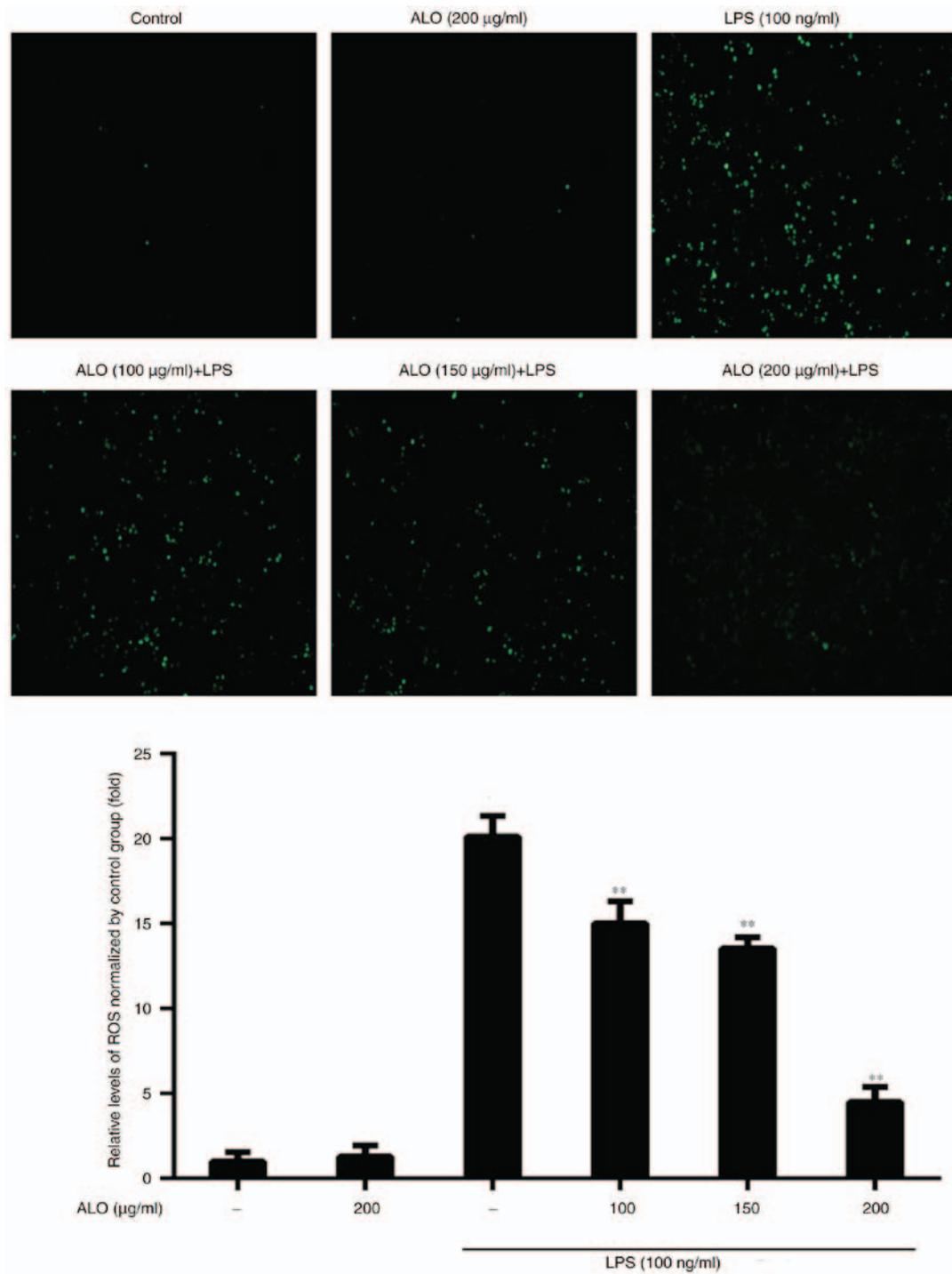


Figure 7. ALO attenuates ROS production induced by LPS. RAW264.7 cells were incubated with ALO for 2 h and then stimulated with LPS for 30 min. ROS accumulation was determined using a ROS detection kit (magnification, x100). The experiments were repeated in triplicate. ** $P < 0.01$ vs. the group stimulated with LPS. ALO, aloin; LPS, lipopolysaccharide; ROS, reactive oxygen species.

Discussion

Inflammation is a protective response. However, the excessive release of pro-inflammatory cytokines from activated macrophages and monocytes causes systemic inflammation (26). As LPS increase the release of pro-inflammatory cytokines, they have been used for several years in the study of this process (27).

Increasing evidence has revealed that a number of bioactive products may antagonise the inflammatory response

induced by LPS, having little or no side effects on the human body (28,29). The *Aloe vera* plant has been widely used in Chinese herbal medicine and extracts have been suggested to possess anti-inflammatory properties (30). Aloin, the bioactive compound obtained from the leaf exudates of *Aloe vera*, has been demonstrated to exhibit anti-inflammatory activity (25,31). However, the detailed molecular mechanisms remain unknown. Therefore, the present study investigated the effects of aloin on LPS-stimulated inflammation responses and additionally explored the potential molecular mechanisms.

To identify aloin cytotoxicity in RAW264.7 cells, the cells were treated for 24 h with different aloin doses prior to determining cell viability. Aloin did not affect cell viability in RAW264.7 cells at 200 $\mu\text{g}/\text{ml}$, suggesting that aloin cytotoxicity from 20-200 $\mu\text{g}/\text{ml}$ could be rejected in subsequent experiments.

iNOS and COX-2 are 2 key inflammatory factors, and TNF- α , IL-1 β , IL-6 and NO are important pro-inflammatory cytokines and mediators in inflammatory disease pathogenesis. The data from the present study revealed that aloin decreased LPS-enhanced iNOS expression at protein and transcript levels in a dose-dependent manner. The release of LPS-induced pro-inflammatory cytokines and mediators were markedly inhibited in a dose-dependent manner. A number of studies have demonstrated the interaction between COX-2 and iNOS, and that NO or its product may activate COX enzymes (32). However, the results of the present study indicated that aloin inhibited iNOS expression and NO release but had no inhibitory effect on COX-2 expression. A potential cause of this discrepancy maybe due to the purity of the aloin used.

To investigate the molecular mechanism, the effects of aloin on LPS-induced inflammatory signalling pathway activation were explored. The JAK-STAT, NF- κB and MAPK signalling pathways have been demonstrated to be involved in inflammation (33). Therefore, the present study aimed to determine whether aloin suppressed the activation of JAK-STAT, NF- κB and MAPK signalling pathways. It was identified that aloin inhibited JAK1, STAT1 and STAT3 phosphorylation in a dose-dependent manner. As STAT phosphorylation is required for their nuclear translocation and transcriptional activity (34), the effect of aloin on the nuclear distributions of STAT1 and STAT3 in RAW264.7 cells was determined. The nucleocytoplasmic separation experiment and confocal microscopy analysis of the present study revealed that aloin markedly suppressed STAT1 and STAT3 nuclear translocation. Unexpectedly, aloin treatment did not affect the phosphorylation of MAPKs, IKK and I κB induced by LPS. Collectively, the results of the present study suggested that aloin may inhibit the LPS-induced inflammatory response by suppressing the activation of the JAK1-STAT1/3 signalling pathway and the nuclear translocation of STAT1 and STAT3.

Luo *et al* (35) demonstrated that aloin attenuated LPS-induced NF- κB transcriptional activity by inhibiting its upstream kinase p38 MAPK and mitogen- and stress-activated protein kinase-1. However, the results of the present study demonstrated that aloin pretreatment had no effect on LPS-induced p38 activation. This result was different from that of Luo *et al* (35). In that study, the inhibitory effect of aloin on p38 MAPK activation was detected 2 h following LPS stimuli. However, the present study detected the inhibitory effect at 30 min. Therefore, it was hypothesized that the potential reason for the discrepancy is due to the different detection times. Additionally, the present study revealed a novel signal pathway for the anti-inflammatory mechanism of aloin.

It has been demonstrated that LPS stimulation promotes ROS production in macrophages (36), and that ROS serve as secondary messengers capable of regulating pro-inflammatory gene expression (37). Previous studies have indicated the

antioxidant properties of aloin (12,25). In the present study, it was identified that aloin decreased ROS accumulation in LPS-stimulated RAW264.7 cells. Furthermore, ROS are potent inducers of various signalling pathways, including MAPK and JAK-STAT pathways (38). Our previous studies demonstrated that N-acetyl-L-cysteine, a ROS inhibitor, suppressed the phosphorylation of JAK-STATs and the expression of iNOS (6,8). These data led us to hypothesize that the inhibitory effect of JAK1-STAT1/3 by aloin may be attributed to its antioxidant activity towards ROS in RAW264.7 cells.

In summary, the present study demonstrated that aloin may partly exert its anti-inflammatory activities through the inhibition of ROS-mediated JAK1-STAT1/3 signalling pathway activation in RAW264.7 macrophages. These results provide novel insight into the anti-inflammatory molecular mechanisms of aloin and provide experimental basis for the clinical application of aloin.

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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

ZQ and YZ designed the experiments, YM, TT, QZ and LS performed this study. ZW and HT analyzed the data. ZQ and YM wrote the paper. 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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