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Original Article

Anti-inflammatory effect of pristimerin on TNFα-induced inflammatory responses in murine macrophages

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Abstract: Purpose: To investigate the anti-inflammatory effect of pristimerin using RAW 264.7 macrophage cells. Methods: The effect of pristimerin on the RAW 264.7 cells proliferation was determined by CCK8 test. The expression of cyclin D1 and cyclin E1 in RAW 264.7 cells treated with TNF-α and pristimerin was measured by qRT-PCR. The regulation of cytokine IL-1β, IL-6, IL-8, and PGE2 in RAW 264.7 cells treated by pristimerin was measured by ELISA. Results: TNF-α can induce the proliferation of RAW 264.7 cells in a dose-dependent manner, co-treatment with pristimerin results in a significant suppression in the proliferation of RAW 264.7 cells. Besides, pristimerin suppressed the expression of cyclin D1 and cyclin E1 induced by TNF-α. TNF-α had a dose-dependent induction on the production of IL-1β, IL-6, IL-8, and PGE2. Pristimerin inhibited the production of IL-1β, IL-6, IL-8, and PGE2 in RAW 264.7 cells. TNF-α increased the production of NO and iNOS in RAW 264.7 cells, which can be blocked by pristimerin. Conclusion: Pristimerin is an anti-inflammatory agent on macrophage, it realize this function through the following ways, first pristimerin reduce the production of pro-inflammatory cytokines; then pristimerin inhibit the expression of inflammatory mediators NO and iNOS; finally pristimerin inhibits the proliferation of RAW 264.7 cells through cell cycle arrest.

Keywords: Pristimerin, RAW 264.7 cells, TNF-α, inflammation

Introduction

Inflammation is defined as an initial host immune response to foreign challenge or tissue injury to protect normal tissue structure and function. Inflammation has been involved in numerous diseases, so great effort has been put into studying the mechanism of inflammation and development of anti-inflammatory agents. There are lots of inflammation-related mediators, including NO, PGE2, COX-2 and cytokines, which are over-expressed in the condition of inflammatory disorders and autoimmune diseases [1]. Macrophage is one of the essential immune cells to regulate inflammatory response [2]. The activated macrophages can induce antigen processing and present antigen specific T cells. So the regulation of macrophages plays an important role in the control of inflammation.

Various compounds isolated from plants have been reported to have anti-inflammatory efficacy to treat inflammation-related disorders. Pristimerin is a naturally quinone-methide triterpenoid compound, which has been used as an anti inflammatory, antioxidant, anti-malarial and insecticidal agent [5]. Recent studies reported pristimerin had apoptosis-inducing and anti-proliferation activity in diverse type of tumor cells [6, 7]. Pristimerin can also inhibit cell cycle progression [8]. It has been reported that pristimerin inhibit inducible nitric oxide synthase (iNOS), which catalyze the production of nitric oxide (NO) at mRNA and protein level [9]. INOS is an important enzyme to regulate inflammatory and NO production, which implicate that pristimerin have anti-inflammatory effect [10]. However, the anti-inflammatory effects of pristimerin on macrophage are not fully investigated.

TNF-α, which is mainly secreted by T lymphocytes and activated macrophages, is correlated with many physiological effects, including inflammation, proliferation, and apoptosis [3].

So TNF-α was used as pro-inflammatory agent in many studies [4]. In this study, we evaluated anti-inflammatory effect of pristimerin in RAW 264.7 macrophage cells stimulated by TNF-α. Furthermore, we investigated the role of pristimerin on RAW 264.7 macrophage cells proliferation and cell cycling. Results showed that pristimerin suppressed IL-1β, IL-6, IL-8, and PGE2 production in TNFα-treated macrophages, together with decreased NO production. Pristimerin inhibits the proliferation of RAW 264.7 cells, with decreased cyclin D1 and cyclin E1 expression.

Material and methods

Pristimerin were purchased from Sigma-Aldrich, Inc. The primary antibodies used in this experiment, unless otherwise stated, were obtained from Cell Signaling Biotechnology (USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin used in this study were obtained from Life Technology (USA).

Cell culture and cell viability assay

RAW 264.7 murine cell line were obtained from Sigma-91062702 and maintained in Dulbeccos Modified Eagles Medium (DMEM) and 10% fetal bovine serum (FBS) supplemented with antibiotics. Cell proliferation assay from Promega (Madison, WI) is used to count the number of viable cells. RAW 264.7 cells were plated at a density of 5×10^3/well in a 96-well plate, and pristimerin was added to each plate at the indicated doses of 0 to 0.8 μM. The number of viable cells was counted according to the manufacturer's instructions. To investigate effect of cytokines, cells were treated with TNF-α (25-50 ng/ml) for 24 h with or without pristimerin.

CCK-8 assay

Cell proliferation in the treated cells was determined by using Cell Counting Kit-8 (CCK-8) kit (Kumamoto, Japan) following the instructions of the manufacturer. Briefly, cells were plated at a density of 5×10^3/well with 100 μL of medium in 96-well plates. After treatment, 10 μL of CCK-8 was added to each well and the plates were incubated at 37°C under 5% CO_2 for 90 min. The absorbance of the cell suspension was measured with a microplate reader at a wavelength of 450 nm. Medium containing 10% CCK-8 served as a control. The percentage of inhibition of proliferation was calculated by (1-mean OD for drug group/mean OD for control group) ×100%.

ELISA for IL-1β, IL-6, IL-8, and PGE2 production

The amount of IL-1β, IL-6, IL-8, and PGE2 production was measured using an enzyme-linked immunosorbent assay (ELISA) kit from R&D (Minnea-polis, MI, USA) according to the manufacturer's instructions. RAW 264.7 cells were plated at a density of 1×10^4/well in a 96-well cell culture plate with 200 μL of culture medium and incubated for 12 h. Then treated by TNF-α (25-50 ng/ml) with or without pristimerin for 24 h. After washing, bound antibody was visualized by a Streptavidin-HRP incubation and reaction in the dark, and absorbance was measured at 450 nm. A blank value was subtracted for each plate, positive and negative control sera were included. To normalize and calibrate different plates, three serum samples were used as repeat controls on each plate (intra-assay coefficient of variation, <6%; inter-assay coefficient of variation, <15%).

NO concentration assay

Nitric oxide (NO) contents were assayed by method of nitrate reductase. Cells were plated at a density of 5×10^3/well in 96-well plates. After treatment, the supernatant was taken for NO determination. NO was assayed spectrophotometrically by measuring total nitrate plus nitrite (NO_3- plus NO_2-) and the stable end products of NO metabolism. The procedure was performed by the manufacturer's instructions. Results were expressed as μmol/g protein.

Real-time RT-PCR analysis

Total RNA was extracted from RAW 264.7 cells using RNAeasy mini columns (Qiagen) Followed by treatment. RNA was treated with RNase free DNAse I before elution from the column. 2 μg of purified DNA-free RNA was used to synthesize cDNA using SuperScipt III cDNA synthesis kit (Invitrogen). Reactions were set up in triplicate in 96 well plates using 1 μl cDNA with Fast SYBR Green PCR Master Mix (Applied Biosystems) to which gene-specific primers were added. Every set of samples was duplicated together with a template-free control. MRNA expression was quantified using the Step-One-Plus real-time PCR system (Applied Biosystems) according to the manufacturer's instructions.

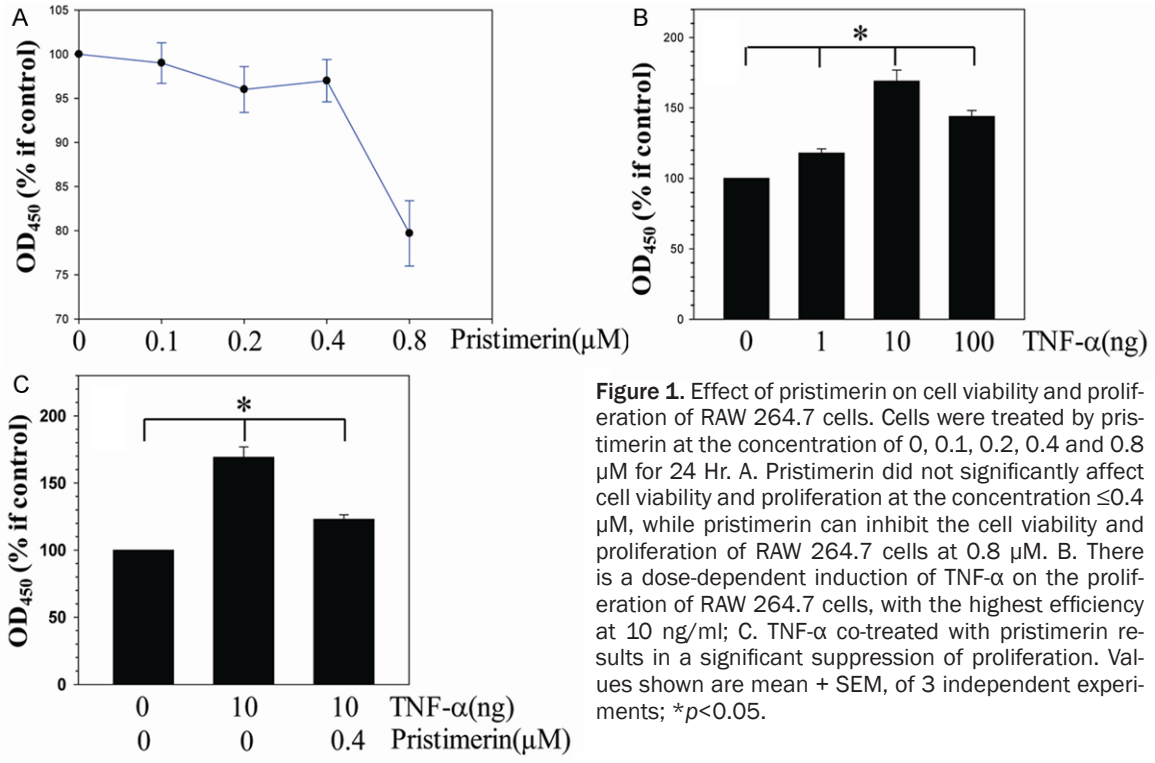

Figure 1. Effect of pristimerin on cell viability and proliferation of RAW 264.7 cells. Cells were treated by pristimerin at the concentration of 0, 0.1, 0.2, 0.4 and 0.8 μM for 24 Hr. A. Pristimerin did not significantly affect cell viability and proliferation at the concentration ≤0.4 μM, while pristimerin can inhibit the cell viability and proliferation of RAW 264.7 cells at 0.8 μM. B. There is a dose-dependent induction of TNF-α on the proliferation of RAW 264.7 cells, with the highest efficiency at 10 ng/ml; C. TNF-α co-treated with pristimerin results in a significant suppression of proliferation. Values shown are mean + SEM, of 3 independent experiments; *p<0.05.

HPRT was used to normalize the mRNA expression. Melting curves were analyzed to verify the specificity of the RT-PCR and the absence of primer dimmer formation. All the primers were synthesized by Integrated DNA Technologies, Inc. Gene-specific primer pairs used for amplification were as follows: inducible nitric oxide synthase, F 5'-GCC CTG CTT TGT GCG AAG-3', R 5'-GCC CTT TGT GCT GGG AGT C-3'; Cyclin D1 F 5'-ATT TCC AAC CCA CCC TCC AT-3', R 5'-GGC TTC AAT CTG TTC CTG GC-3'; Cyclin E1 F 5'-AGC CTC GGA AAA TCA GAC CA-3', R 5'-CTT CGC ACA CCT CCA TTA GC-3'.

Statistical analysis

All measurements were performed in triplicate. Results are presented as the mean ± SEM. Differences between groups were assessed by analysis of variance. P<0.05 were considered statistically significant.

Results

Effect of pristimerin on cell viability

The cell cytotoxicity of pristimerin was assayed, RAW 264.7 cells was treated by pristimerin at the concentration of 0, 0.1, 0.2, 0.4 and 0.8 μM for 24 Hr. Results showed pristimerin with concentration ≤0.4 μM did not significantly suppress cell viability and proliferation. While pristimerin can inhibit RAW 264.7 cells viability and proliferation at 0.8 μM. Therefore, pristimerin was used at concentrations of 0.4 μM in the following experiment (**Figure 1A**).

Pristimerin inhibits proliferation of RAW 264.7 cells

The effect of pristimerin on RAW 264.7 cells proliferation was measured by CCK8 assay. First, RAW 264.7 cells were treated with various concentrations of TNF-α to confirm the induction of TNF-α on RAW 264.7 cells proliferation. Results showed a dose-dependent induction of TNF-α on the proliferation of RAW 264.7 cells. Then, RAW 264.7 cells were incubated with pristimerin of 0.4 μM in the presence TNF-α at 10 μM. Results showed co-treatment with pristimerin resulted in a significant suppression in proliferation of RAW 264.7 cells (**Figure 1B**, **1C**).

Pristimerin inhibits TNFα-induced production of proinflammatory cytokines in RAW 264.7 cells

To investigate the regulation of pristimerin on pro-inflammatory cytokines production. RAW

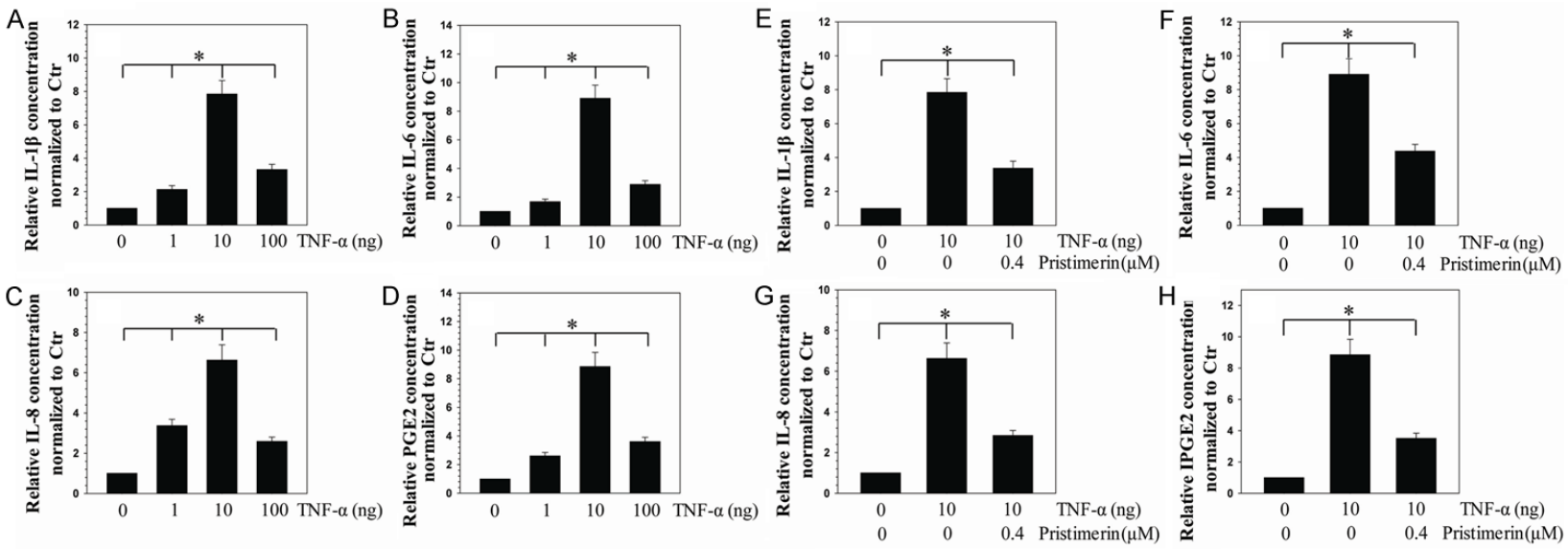

Figure 2. Pristimerin inhibits TNFα-induced production of proinflammatory cytokines in RAW 264.7 cells. RAW 264.7 cells were treated with various concentrations of TNF-α, which showed a dose-dependent induction effect on pro-inflammatory cytokines production, IL-1β, IL-6, IL-8, and PGE2. A-D. RAW 264.7 cells treated with pristimerin inhibited IL-1β, IL-6, IL-8, and PGE2 secretion into the supernatants stimulated by TNF-α for 24 h. E-H. Values shown are mean ± SEM, of 3 independent experiments; *P<0.05.

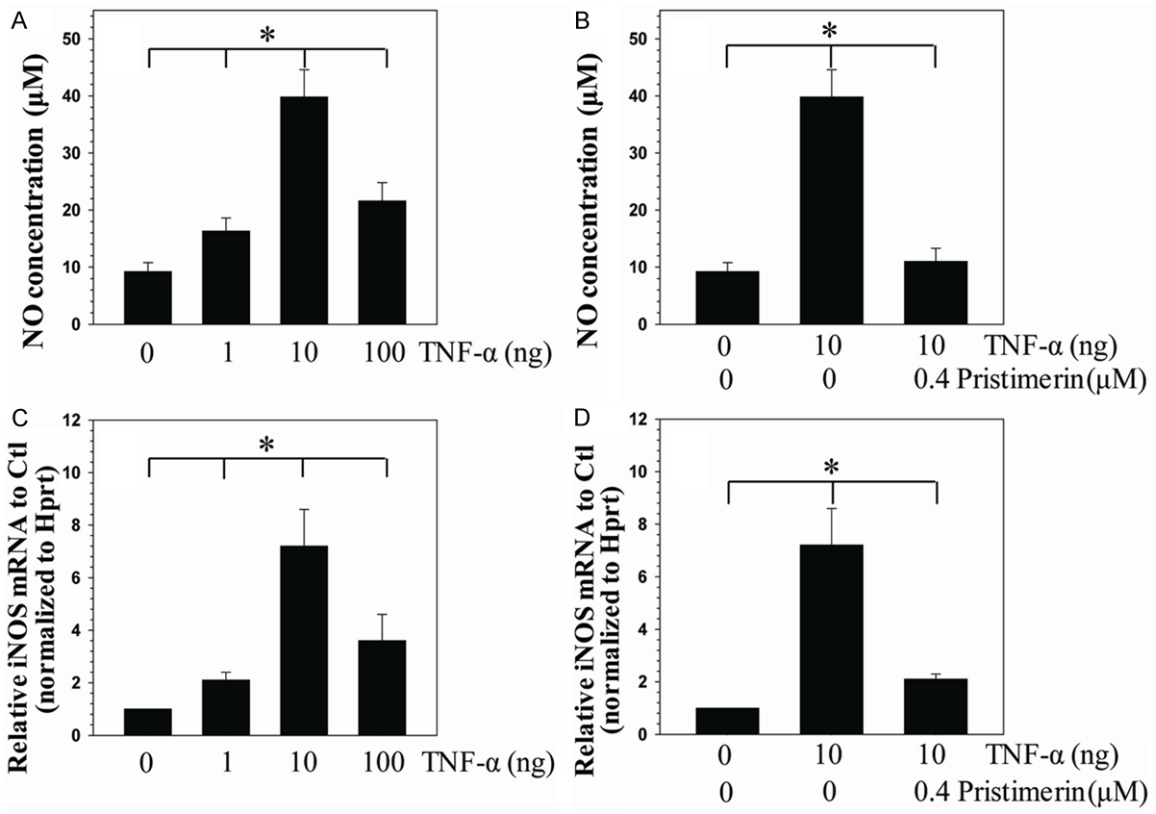

Figure 3. Pristimerin suppresses TNF-α induced NO and iNOS Production in RAW 264.7 cells. TNF-α led to up-regulation of NO production in RAW 264.7 cells, with the highest level at 10 ng/ml of TNF-α (A); Co-treatment with pristimerin inhibit the production of NO (B); TNF-α can stimulate the up-regulation of iNOS mRNA expression, with the highest level at 10 ng/ml (C); Co-treatment with pristimerin decrease the production of iNOS (D). Values shown are mean ± SEM, of 3 independent experiments; *P<0.05.

264.7 cells were treated with various concentrations of TNF-α. The level of IL-1β, IL-6, IL-8, and PGE2 in the supernatants was evaluated using ELISA. We found TNF-α had a dose-dependent induction on the production of pro-inflammatory cytokines, IL-1β, IL-6, IL-8, and PGE2 (**Figure 2A-D**). Then, RAW 264.7 cells were incubated with pristimerin of 0.4 μM in the presence TNF-α at 10 μM for 24 h. Results showed treatment with pristimerin inhibited the production IL-1β, IL-6, IL-8, and PGE2 in cultured RAW 264.7 cells induced by TNF-α (**Figure 2E-H**). This indicated pristimerin can inhibit pro-inflammatory cytokines in RAW 264.7 cells.

Inhibitory effect of pristimerin on no production

To investigate the mechanism of pristimerin on inflammation, we checked the expression level of NO in TNF-α stimulated RAW 264.7 cells. The amount of produced NO was indicated by accumulated nitrite (a stable metabolite of NO). First, RAW 264.7 cells were treated with various concentrations of TNF-α, which led to up-regulation of NO production in RAW 264.7 cells. When Co-treated with pristimerin of 0.4 μM, the production of NO was significantly inhibited (**Figure 3A**, **3B**).

Pristimerin reduce iNOS expression in TNF-α stimulated RAW 264.7 cells

We next investigated whether the inhibition of NO by pristimerin was caused by the regulation of iNOS in RAW 264.7 cells. Results showed that TNF-α can increase the expression of iNOS mRNA; while pristimerin can inhibit the induction of iNOS by TNF-α. This indicates that the suppression of NO expression by pristimerin was through the inhibition of iNOS (**Figure 3C**, **3D**).

Pristimerin suppresses TNF-α induced cyclin D1 and cyclin E1 expression in RAW 264.7 cells

Cyclin D1 and cyclin E1 plays a key role in the G0/G1-S phase of the cell cycle. We have proved that pristimerin can inhibit the prolifera-

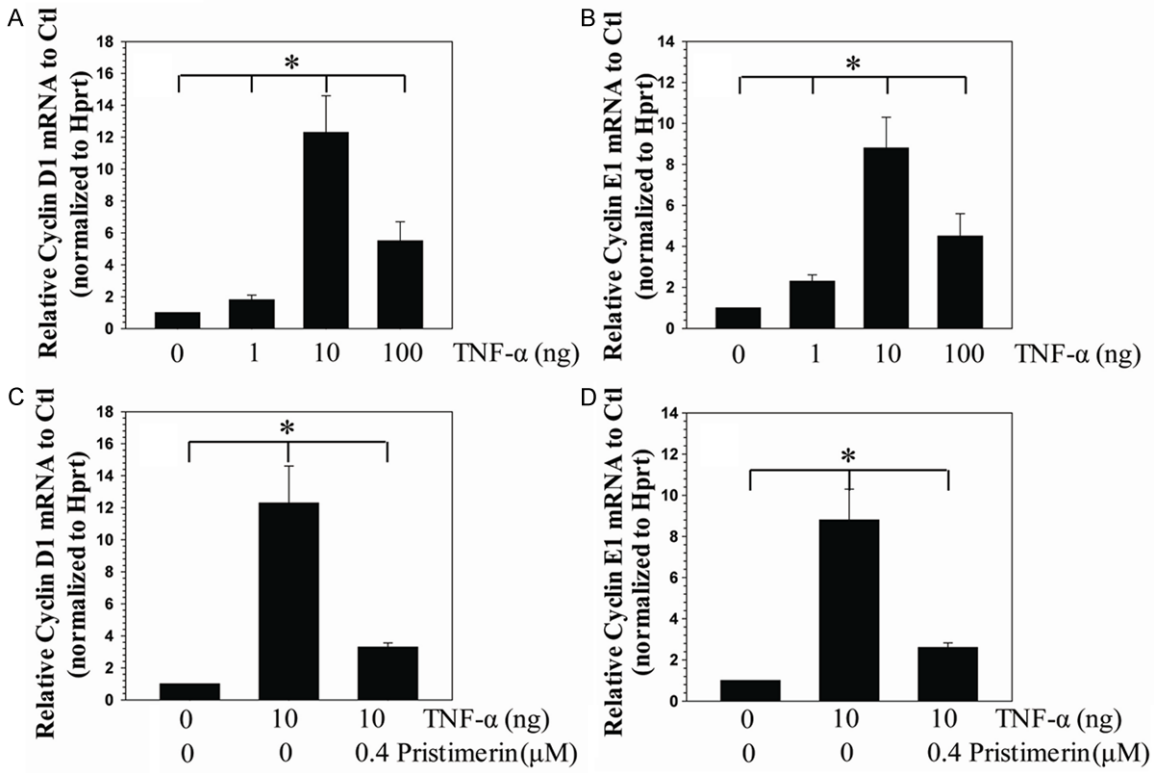

Figure 4. Pristimerin suppresses TNF-α induced cyclin D1 and cyclin E1 expression in RAW 264.7 cells. TNF-α treatment increased the expression of cyclin D1 and cyclin E1, with the highest level observed in 10 ng/ml (A, B); Co-treatment with pristimerin inhibit the production of cyclin D1 and cyclin E1 (C, D). Values shown are mean ± SEM, of 3 independent experiments; **P*<0.05.

tion of RAW 264.7 cells above, so we also analyzed the mechanism of pristimerin on regulation of cell cycle in RAW 264.7 cells. As shown in **Figure 4A**, **4B**, TNF-α treatment increased the expression of cyclin D1 and cyclin E1 mRNA. When cells were pretreated with pristimerin, the expression of cyclin D1 and cyclin E1 were blocked to almost base level (**Figure 4C**, **4D**).

Discussion

Inflammation is the body's first response to an injury or disease, while chronic inflammation promote the development and progression of various diseases, it starts to kill the organism and might just be the root cause of all degenerative disease [11]. A lot of diseases have a suspected root cause of chronic inflammation, such as allergy is the inflammatory cytokines induced autoimmune reactions [12], arthritis is the inflammatory cytokines induced joint cartilage destroy [13], lupus is caused by inflammatory cytokines induced autoimmune attack [14], pancreatitis is caused by inflammatory cytokines induce pancreatic cell injury [15], many cancers is caused by chronic inflammation [16], Alzheimer's is caused by the destroy of brain cells by chronic inflammation [17], damages of heart valves by chronic inflammation lead to aortic valve stenosis [18], inflammation leads to bone loss and osteoporosis [19], and so on. This indicates that the inflammation is harmful at least in these diseases, and should take into control.

Macrophages synthesize and release a variety of cytokines and other proteins that play a key role in the development of acute and chronic inflammation. During inflammation, antigen-activated $CD4^+$ T cells stimulate macrophages to produce cytokines such as IL-1β, IL-6, TNF-α, and PGE2. These pro-inflammatory cytokines are master regulator of inflammatory diseases. TNF-α, mainly produced by monocytes and macrophages, acts as a potent inducer of inflammatory responses through the up-regulation of many genes, including cytokines, chemokines, and adhesion molecules [20]. PGE2 induced by LPS plays a key role in the migration of macrophage cells to the inflammatory sites [21]. In this study, we found that TNF-α stimulation can enhance the production of cytokines

IL-1β, IL-6, IL-8, and PGE2 in RAW 264.7 cells, which is similar with previous report. Pristimerin is a natural qunionemethide triterpenoid compound, with the effect of anti-inflammatory [5], anti-proliferation [6] and apoptosis-inducing [7]. In the present study, we found pristimerin suppressed IL-1β, IL-6, IL-8, and PGE2 production in RAW 264.7 cells induced by TNF-α. This confirms the anti-inflammatory activity of pristimerin. The exact mechanism of its anti-inflammatory activity needs further research. In addition to the regulation of pristimerin in cytokine production, we have investigated the role of pristimerin on NO production and RAW 264.7 cells proliferation.

NO is a simple but unstable free radical and is an important cellular signaling molecule involved in many physiological and pathological processes [22]. During inflammation, NO is produced by inducible nitric oxide synthase (iNOS), over-expression of NO can lead to various inflammatory disease. INOS is an important enzyme to regulate inflammation and NO production. TNF-α could induce iNOS over-expression in activated macrophages to release more NO. It has been reported that pristimerin inhibit iNOS, which catalyze the production of NO at mRNA and protein level9. Another study showed that pristimerin suppressed NO, prostaglandin E2, and cyclooxygenase-2 expression induced by lipopolysacharide (LPS) in murine RAW 264.7 cells [23]. In this study, we found that pristimerin can inhibit the expression of both NO and iNOS, which implicate that the anti-inflammatory effect of pristimerin in RAW 264.7 cells may partially due to the inhibition of iNOS.

Proliferation of macrophages is an essential part of chronic inflammatory disease development and progression [24]. In this study, we found that TNF-α enhance the proliferation of RAW 264.7 cells, which can promote the progression of inflammation. However, co-treatment with pristimerin resulted in a significant suppression in proliferation of RAW 264.7 cells. Because dis-regulation of the cell cycle is associated with aberrant cell proliferation, then we detected the effect of pristimerin on RAW 264.7 cell proliferation following the aspect of cell cycle. Cyclins D and E play an important role in the progression of cell growth through G1 phase of cell cycle, over-expression of cyclins and cdks can provide cell with selective growth advantage [25]. It is well established that cyclin D1 and cyclin E1 played a key role in the G0/G1-S phase of the cell cycle [26]. Disregulation of *cyclin D1*, either by silence or by over-expression, will induce abnormal proliferation in various cell lines. Transgenic mice with over-expression of cyclin D1 in the esophageal epithelium develop epithelial dysplasia [27]. Cyclin E1 is indispensable for activation, proliferation and survival of hepatic satellite cells [28]. In this study, we found that TNF-α treatment increased the expression of cyclin D1 and cyclin E1, while pristimerin significantly inhibited the expression of cyclin D1 and cyclin E1 mRNA in RAW 264.7 cells induced by TNF-α. The mechanism of pristimerin in anti-proliferative activity isn't fully understood. It has been reported pristimerin caused cell cycle arrest in G1 phase in pancreatic cancer cells [8]. Therefore, the possible mechanism of pristimerin to inhibit the proliferation of RAW 264.7 cells may go through cell cycle arrest. So pristimerin is a potent chemotherapeutic agent to suppress inflammation by promoting cell cycle arrest.

A lot of Chinese traditional herbs have showed the role of anti-inflammatory and immune-suppressing effect, most of these Chinese herbal monomer can suppress inflammation by decreasing the secretion of inflammatory cytokines, which can relieve the symptom of disease. However, the anti-inflammatory mechanism of these herbal monomer is not clear, further research needed to explore exact anti-inflammatory mechanism of traditional Chinese medicine, which can provide scientific proof for clinical application.

In the current study, we demonstrated that pristimerin is an anti-inflammation agent on macrophage, it realize this function through the following ways, first pristimerin decrease the production of pro-inflammatory cytokines; then pristimerin inhibit the expression of inflammatory mediators NO and iNOS; finally pristimerin inhibits the proliferation of RAW 264.7 cells through cell cycle arrest. These results indicate that pristimerin may be useful for the treatment of chronic inflammatory diseases.

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Disclosure of conflict of interest

None.

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