1 **Licocoumarone isolated from *Glycyrrhiza uralensis* selectively alters LPS-induced**
2 **inflammatory responses in RAW 264.7 macrophages**

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4 Lehao Wu^{a1}, Yunpeng Fan^{b1}, Chao Fan^a, Yang Yu^a, Lei Sun^a, Yu Jin^{b*}, Yan Zhang^{a*},
5 Richard D. Ye^{a,c*}

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7 ^aSchool of Pharmacy, Shanghai Jiao Tong University, Shanghai 200240, China

8 ^bEngineering Research Center of Pharmaceutical Process Chemistry, Ministry of
9 Education, School of Pharmacy, East China University of Science and Technology,
10 Shanghai 200237, China

11 ^cInstitute of Chinese Medical Sciences, University of Macau, Macau, SAR, China

12

13 *Correspondence: zhangyan_sjtu@sjtu.edu.cn (Y.Z); jiny@ecust.edu.cn (Y.J);
14 yedequan@sjtu.edu.cn (R.D.Y)

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16 ¹ These authors contribute equally to this work

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1 **Abstract:** The effects of licocoumarone (LC) isolated from *Glycyrrhizauralensis* were
2 studied in LPS-stimulated RAW 264.7 macrophages. Our study demonstrated that LC
3 dose-dependently attenuated LPS-induced NO production by down-regulating iNOS
4 expression. Additionally, the treatment with LC inhibited LPS-induced expression of
5 cytokines including IL-1 β , IL-6 and IL-10, but not TNF- α , at both mRNA and protein
6 levels. Similar suppressive effects of LC were observed on LPS-stimulated murine
7 peritoneal macrophages as well. Furthermore, LC significantly reduced LPS-stimulated
8 NF- κ B activation by inhibition of I κ B α degradation and p65 phosphorylation. The results
9 from NF- κ B-luc reporter gene assay further support the inhibitory effect of LC on NF- κ B
10 activation. Further studies showed that LC also interfered with the MAPKs and STAT3
11 signaling pathways, which are typical inflammatory signaling pathways triggered by LPS.
12 Taken together, these results show that LC attenuates LPS-induced cytokine gene
13 expression in RAW 264.7 macrophages through mechanisms that involve NF- κ B, MAPKs
14 and STAT3 signaling pathways, but the pattern of inhibition differs from that of a global
15 immunosuppressant. Our study indicates that LC is a functional constituent of
16 *Glycyrrhizauralensis* with potential implications in infectious and immune-related diseases.
17 **Key words:** licocoumarone; *Glycyrrhizauralensis*; NF- κ B; MAPKs; STAT3

1. Introduction

Inflammation is a natural defense response that plays a central role in protecting against bacteria, viral and fungal infections (Moretta et al., 2005). The ingress of leukocytes into sites of inflammation is an important aspect of the pathogenesis of inflammatory responses. For example, macrophages are recruited to inflammatory sites, and are activated by various signals that stimulate intracellular cascades of cytokines and chemokines (Mancino et al., 2015). In macrophages, lipopolysaccharide (LPS), a major form of endotoxin, induces the production of inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β), and inflammatory mediators, such as nitric oxide (NO), which is synthesized by inducible NO synthase (iNOS) and consequently cause clinical symptoms (Kierner et al., 2003).

Nuclear factor-Kappa B(NF- κ B), a nuclear transcription factor, is the crucial mediator in the inflammatory process and has been widely implicated in inflammatory diseases, largely based on the central role of NF- κ B in the expression of the genes encoding pro-inflammatory cytokines (Grilli et al., 1993; Pateras et al., 2014; Tak and Firestein, 2001). The mitogen-activated protein kinases(MAPKs), including extracellular signal regulated kinase (ERK), c-jun N terminal kinase (JNK) and p38 also mediate inflammatory and immune responses, which can be activated by LPS stimulation(Hatano et al., 1989; Li et al., 2015). Moreover, several studies have shown that MAPKs play critical roles for the activation of NF- κ B(Maulik et al., 1998; Pateras et al., 2014). The JAK-STAT (Janus kinase-signal transducers and activator of transcription) cascade is another essential inflammatory signaling pathway in response to LPS and releasing pro-inflammatory

1 cytokines in macrophage(Leonard and O'Shea, 1998; Murray, 2007; Park et al., 2003).
2 Thus,molecules that can regulate inflammatory response through either NF- κ B, MAPKs or
3 JAK-STATs pathways or in combination have the potential for the treatment of
4 inflammation-related disorders.

5 There is considerable research interest in the identification of new anti-inflammatory
6 agents from plants used in traditional Chinese medicines (TCMs). *Glycyrrhizauralensis* is
7 one of the oldest and the most frequently used herb in TCMs. *Glycyrrhizauralensis* is used
8 mainly for the treatment of coughs, bronchitis, and peptic ulcer. Moreover, clinical and
9 experimental studies suggest that it has anti-inflammatory, antiviral and
10 immunomodulatory effects (Kim et al., 2006; Peng et al., 2011; Wang et al., 2013).
11 Licorice-derived compounds such as glycyrrhizin, isoliquiritigenin and glycyrol are
12 considered to be the active constituents of *Glycyrrhizauralensis*(Adianti et al., 2014).
13 Glycyrrhizin, the major sweetening component, has been found to exhibit
14 hepatoprotective effects in chemical-induced liver injury models (Lee et al., 2007; Lin et al.,
15 1999). Isoliquiritigenin attenuates inflammatory responses mediated by suppressing
16 TLR4/MD-2 mediated NF- κ B and MAPK activation(Honda et al., 2012). Glycyrol has been
17 reported to alleviate COX-2 expression in LPS-stimulated RAW 264.7 macrophages and
18 reduced carrageenan-induced mouse-paw edema (Shin et al., 2008). However, the
19 information regarding the contribution of the other compounds to the anti-inflammatory
20 and immunomodulatory property of *Glycyrrhizauralensis*, which is important for the quality
21 control and clinical use is very limited. Licocoumarone (LC) (Structure see Fig. 1) is one of
22 the licorice-derived compounds isolated from *Glycyrrhizauralensis*(Hatano et al., 1989). In

the present study, as part of our on-going screening program to explore anti-inflammatory natural compounds, we evaluated the effects and its underlying mechanisms of LC in LPS-stimulated macrophages.

2. Material and Methods

2.1. Reagents

All ELISA kits were obtained from MultiSciences (Lianke) Biotechnology Corporate Limited (Zhejiang, China). The BCA protein assay kit and the NO detection kit were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). RPMI-1640 were purchased from HyClone (Logan, UT). Antibodies were obtained as following: Anti-ERK, JNK, P38, COX-2, iNOS, P65, I- κ B α , STAT3, and phosphor-ERK, -JNK, -P38, -P65, -STAT3 were purchased from Cell Signaling Technology (Danvers, MA); anti- β -actin was purchased from Sigma Chemical Company (St. Louis, MO). Mouse and rabbit control IgGs were purchased from Santa Cruz Biotechnology (Dallas, TX). IRDye[®]800CW secondary antibodies were purchased from LI-COR (Lincoln, NE). Other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

2.2. Cell culture

The RAW264.7 mouse macrophage cell line was obtained from China Cell Line Bank (Beijing, China). The cells were grown in RPMI-1640 supplemented with 10% heat-inactivated FBS, 2.05 mM L-Glutamine, penicillin (100 U/ml) and streptomycin (100 U/ml) at 37 °C under a humidified atmosphere of 5% CO₂. Murine peritoneal macrophages were elicited by i.p. injection of 4 ml of 4% thioglycolate into the peritoneal cavity of 6-10 weeks old C57BL/6 mice. After 3 days, cells in the peritoneal exudates were

1 obtained by lavage with ice-cold DMEM. The cells were washed twice, re-suspended in
2 HEPES-buffered DMEM supplemented with NaHCO₃, 10% FBS, penicillin (100 U/ml) and
3 streptomycin (100 U/ml). In all experiments, cells were allowed to acclimate for 24 h
4 before any treatment.

5 *2.3. Isolation of LC*

6 LC was isolated as our previous report(Fan et al., 2016). In brief, the dried and
7 crushed licorice roots (2.5 kg) were extracted with 95% ethanol (30 L) by refluxing. The
8 extract was evaporated to dryness under reduced pressure to yield crude extracts (300 g).
9 The crude extracts were suspended in water (10 L) and subjected to ultrasonic for 30 min,
10 then centrifuged. The solution was discarded and the residue was dissolved in ethyl
11 acetate. The filtered ethyl acetate fraction was concentrated and separated using SPE
12 column. After above pretreatment, 75 g ethyl acetate eluant was obtained. Subsequently,
13 pre-NPLC, pre- RPLC were used in turn to purify LC from ethyl acetate fraction. The purity
14 of LC was determined by HPLC and HRMS analysis, and confirmed to be more than 96%
15 (Fan et al., 2016).

16 *2.4. MTT assay for cell viability*

17 Cell viability was evaluated by the MTT reduction assay (Yang et al., 2016). Cells
18 were seeds in 96-well plates at 2×10^4 cell/well. After 24 h, the cells were treated with 150
19 μ l of different concentrations of LC (0-50 μ M) for 24 h. Subsequently, 15 μ l of 5 mg/ml MTT
20 in DD water was added to each well, and the cells were incubated for 4 h. The medium
21 was removed and resolved with 150 μ l/well DMSO. The optical density was measured at
22 490 nm on a Flexstation 3 (Molecular Devices, Silicon Valley, CA, USA).

2.5. Bioassay for NO production

Accumulation of nitrite, a metabolite of nitric oxide (NO) in the culture media was measured by Griess assay (Zhao et al., 2012). Briefly, cells were plated onto 96-well plates (5×10^4 cell/well) and pretreated with 0-50 μ M of LC for 1 h before stimulation with 100 ng/ml (for RAW 264.7) or 1000 ng/ml (for murine peritoneal macrophages) of LPS. After incubation for 24 h, an equal volume (50 μ L) of the supernatant was mixed with Griess Reagent in a 96-well plate. The mixture was allowed to react for 15 min, and the release of NO was measured at 540 nm on a Flexstation 3.

2.6. Real-time PCR

The complementary DNA (cDNA) was subsequently subjected to Real-Time PCR to quantify the transcripts of TNF- α , IL-6, IL-1 β and IL-10 using SYBR® Green Real-time PCR Master Mix (TOYOBO, Osaka, Japan). The following primers were used: TNF- α (5'-TTC TCA TTC CTG CTT GTG G-3'; 5'-ACT TGG TGG TTT GCT ACG-3'), IL-6 (5'-GAG GAT ACC ACT CCC AAC AGA CC-3'; 5'-AAG TGC ATC ATC GTT GTT CAT ACA-3'), IL-1 β (5'-AGA GCA TCC AGC TTC AAA T-3'; 5'-CAT CTC GGA GCC TGT AGT G-3'). IL-10 (5'-AGG GTT ACT TGG GTT GC-3'; 5'-TGA GGG TCT TCA GCT TC-3'). The primers for the mouse housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5'-CCT TCC GTG TTC CTA CCC-3'; 5'-CAA CCT GGT CCT CAG TGT AG-3'). PCR was performed according to the following conditions: 94 °C for 3 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. The quantified fold changes in messenger RNA (mRNA) in each sample were normalized to GAPDH expression and

1 calculated using the $2^{\text{exp}}(-\Delta\Delta\text{Ct})$ method.

2 2.7. Western blot analysis

3 RAW 264.7 cells were plated onto 6-well plates (8×10^5 cells/well) and pretreated with
4 different concentrations of LC for 1 h, and then stimulated with 100 ng/ml (for RAW 264.7)
5 or 1000 ng/ml (for murine peritoneal macrophages) of LPS for 24 h for iNOS and COX-2,
6 and 30 min for p65, MAPKs and I κ B α . Proteins were collected by SDS-PAGE Sample
7 Loading Buffer (Yeast, Shanghai, China) and were quantified using a BCA Protein
8 Assay Kit. Equal amounts of proteins for each sample were heated at 99 °C for 7 min.
9 Proteins in sample loading buffer were separated on 10% SDS-PAGE and transferred
10 onto nitrocellulose membranes (Whatman Protran, Dassel, Germany). Blots were blocked
11 with 5 % non-fat milk for 1 h at room temperature and incubated overnight at 4 °C with
12 primary antibodies, followed by the respective IRDye[®]800CW secondary antibodies
13 (LI-COR Biosciences, Lincoln, NE). The membranes were scanned using the 800 nm
14 channel on an Odyssey[®] CLX Infrared Imaging System. The immunoreactive bands were
15 quantified using the NIH Image J software.

16 2.8. Measurements of IL-1 β , IL-6, IL-10 and TNF- α production by ELISA

17 RAW 264.7 cells were plated onto 24-well plates (2×10^5 cells/well), and treated with
18 LC (0–50 μ M) for 1 h before stimulation with 100 ng/ml (for RAW 264.7) or 1000 ng/ml (for
19 murine peritoneal macrophages) of LPS. The supernatants of cultured medium were
20 collected and the production of IL-1 β , IL-6 and TNF- α production was measured after
21 incubation for 24h using an ELISA kit according to manufacturer's instructions.

22 2.9. Data analysis

All statistical calculations were performed using GraphPad Prism 5.0 (GraphPad, Avenida, CA, USA). Results are expressed as the mean±standard error. Data were analyzed by student's t-test. $P < 0.05$ was considered significant.

3. Results

3.1. Effects of LC on LPS-induced NO and cell viability in RAW 264.7 macrophages

LPS-stimulated synthesis of nitric oxide is an important part of the LPS signaling pathway in macrophages. In this study, NO production was measured with various concentrations of LC in LPS-stimulated RAW 264.7 macrophages. Parthenolide (PAR) was used as a positive control for the inhibition. The amount of nitrite, a stable metabolite of NO, was measured in the medium using the Griess reagent and used as an indicator of NO production. As shown in Fig. 2A, treatment of LPS significantly induced nitrite production, and this effect was potently inhibited by LC in a dose-dependent manner.

To evaluate the cytotoxic effects of LC on LPS-stimulated RAW 264.7 macrophages, cells were incubated with the indicated concentrations of LC (1.5, 3, 6, 12.5, 25, 50, 100 μM) for 24 h. The cell viability was analyzed using MTT assays. The result showed that LC at concentrations up to 50 μM did not affect cell viability (Fig.2B). Even the cells treated with 100 μM of LC showed little effect with a viability of $83.3 \pm 1.1\%$. Thus, the inhibitory effect of LC on NO production was not due to cytotoxic effects.

3.2. Effects of LC on LPS-induced iNOS mRNA and protein expression in RAW 264.7 cells

To investigate whether the inhibitory effect of LC on NO production was mediated through inhibition of corresponding iNOS modulation, the mRNA and protein expression of

1 iNOS was determined by RT-PCR and immunoblotting, respectively. As shown in Fig. 3A,
2 iNOS mRNA levels were markedly up-regulated in response to LPS, and LC clearly
3 attenuated the mRNA expression in a dose-dependent manner. Immunoblotting showed
4 that iNOS protein expression correlated with their mRNA expression level (Fig. 3B). These
5 data suggest that LC can down-regulate LPS-induced iNOS expression at the
6 transcription level.

7 *3.3. Effects of LC on LPS-induced expression of TNF- α , IL-1 β , IL-6 and IL-10 in RAW*
8 *264.7 cells*

9 Since inflammatory cytokines are mainly produced through transcriptional and
10 translational regulation, the effects of LC on LPS-induced cytokine production was
11 evaluated using RT-PCR and ELISA. As shown in Fig. 4 (A-C), LPS exposure alone
12 significantly increased the expression of the mRNAs for IL-1 β , IL-6 and IL-10. LC
13 dose-dependently reduced IL-1 β and IL-10 expression (Fig. 4A, C) and dramatically
14 decreased the level of IL-6 mRNA at 50 μ M (Fig. 4B). However, the level of TNF- α was only
15 slightly affected by LC (Fig. 4D). Similar results were observed when the effects of LC on
16 the protein levels of IL-1 β , IL-6, IL-10 and TNF- α were investigated (Fig. 4E-H). Compared
17 to the positive control (PAR), LC didn't produce a significant inhibitory effect on the
18 LPS-induced TNF- α secretion (Fig. 4H).

19 *3.4 Effects of LC on LPS-induced NO production and iNOS expression in mouse*
20 *peritoneal macrophages*

21 To determine whether these anti-inflammatory effects of LC occur in primary cells as
22 well, we further examined its effects on LPS-induced NO production and iNOS expression

1 in peritoneal macrophages isolated from mice. As shown in Fig. 5A, LC was found to
2 significantly inhibit LPS-induced NO production. Furthermore, in these cells LC markedly
3 down-regulated the expression of iNOS in a concentration-dependent manner (Fig. 5B).
4 These inhibitory effects of LC were not caused by a toxic effect as determined by MTT
5 reduction assay (Fig. 5C).

6 *3.5. Effects of LC on LPS-induced activation of NF- κ B signaling in RAW 264.7 cells*

7 To determine whether LC affects LPS-induced activation of NF- κ B signaling, the
8 degradation of I κ B and the phosphorylation of p65 subunit were examined by
9 immunoblotting. LPS induced marked p65 phosphorylation (Fig. 6A) and I κ B degradation
10 (Fig. 6B) compared with untreated control. Treatment with LC at 50 μ M significantly
11 inhibited the LPS-induced p65 phosphorylation and I κ B degradation (Fig. 6A-B).

12 In subsequent experiments, the inhibitory effects of LC on NF- κ B transcriptional
13 activity was confirmed in HeLa cells transfected with NF- κ B-luciferase reporter gene. As
14 shown in Fig. 6C, LC markedly inhibited TNF- α induced NF- κ B activation in a
15 concentration-dependent manner. Taken together, the anti-inflammatory effect of LC is
16 regulated, at least in part, via the NF- κ B signaling pathway.

17 *3.6. Effects of LC on LPS-induced activation of MAPKs and STAT3 signaling pathway in* 18 *RAW 264.7 cells*

19 To investigate if LC interferes with LPS-stimulated activation of MAPKs signaling, the
20 phosphorylation of key MAPK signaling proteins were examined by immunoblotting. As
21 shown in Fig. 7A-C, phosphorylation of ERK 1/2, JNK 1/2 and p38 MAPK was significantly
22 increased with LPS treatment, and all these were suppressed by LC in a dose-dependent

1 manner. These results suggest that LC exerts its anti-inflammatory effect also via the ERK
2 1/2, JNK 1/2 and p38 MAPK signaling pathway.

3 Subsequently, we investigated whether the anti-inflammatory effects of LC were
4 related to JAK-STAT3 signaling pathway. As shown in Fig.7D, LC at 50 μ M significantly
5 attenuated the LPS-induced phosphorylation of STAT3 without changing STAT3 protein
6 levels, suggesting that preventing STAT3 phosphorylation by LC attributable for its
7 anti-inflammatory effects.

9 4. Discussion

10 The pathology of inflammation is initiated by complex processes triggered by
11 microbial pathogens such as LPS, which is a prototypical endotoxin. LPS-stimulated
12 macrophages produce inflammatory mediators including NO, TNF- α , IL-1 β , IL-6 and IL-10
13 (Kiemer et al., 2003). Pharmacological inhibition of LPS-inducible inflammatory mediators
14 is regarded as key to alleviation of a variety of disorders caused by activation of
15 macrophages (Yi et al., 2013). Therefore, LPS-stimulated macrophages provide us with
16 an excellent model for investigating inflammatory response and subsequently for
17 screening anti-inflammatory drugs and their underlying mechanisms.

18 *Glycyrrhizauralensis* is a popular nutritional and therapeutic plant, which is used
19 worldwide as food and medicine (Drobnik, 2016). Previous investigations on the active
20 components of *Glycyrrhizauralensis* mainly focused on glycyrrhizin and its derivatives, as
21 they were thought to be responsible for the hepatic protective and anti-ulcer effects of
22 *Glycyrrhizauralensis*(Orlent et al., 2006).Recently, several reports have addressed the

1 licorice-derived phenolic compound, LC, with apoptosis-inducing effects and antibacterial
2 activity (Hatano et al., 2000; Watanabe et al., 2002). However, the anti-inflammatory
3 potential of LC and its underlying mechanisms have not been fully defined yet. Thus, our
4 study was designed to investigate the anti-inflammatory potential of LC on LPS-stimulated
5 RAW264.7 macrophages by determining the effects on the production and expression
6 of selected inflammatory mediators, and explore the mode of action involved. In the
7 present study, we for the first time report that LC exhibits significant suppression on
8 LPS-stimulated production of inflammatory cytokines and mediators, including IL-1 β , IL-6,
9 IL-10 and NO, in macrophages. Isoliquiritigenin and glycyrol, the other two active
10 components in *Glycyrrhiza uralensis* have been found to exert similar effects in
11 macrophages (Kim et al., 2008; Shin et al., 2008). The inhibition of IL-10, an
12 anti-inflammatory cytokine is not what we originally expected. However, given the
13 relatively low level of its induction compared to the level of IL-6 in LPS-induced
14 macrophages, the anti-inflammatory effect may be quite limited and its inhibition by LC
15 may not have a significantly impact on the anti-inflammatory effect of LC in a global
16 perspective.

17 LPS or inflammatory cytokines caused increased iNOS expression followed by a
18 significant production of NO (Wilson et al., 1996). Thus, decreased iNOS synthesis can
19 significantly inhibit LPS-stimulated inflammatory cytokine production in macrophages. Our
20 study demonstrated that LC decreased the transcription and protein expression of iNOS,
21 which supports the observation that LC inhibits NO production in macrophages induced
22 by LPS.

1 Subsequently, we investigated whether the inhibitory effects of LC on inflammatory
2 mediators were related to the suppression of NF- κ B, MAPK and JAK-STATs signaling
3 pathways since these pathways are the essential inflammatory signaling pathways
4 triggered by LPS. NF- κ B proteins reside in the cytoplasm in unstimulated cells, and its
5 activation is regulated by the degradation of I κ B α and the phosphorylation of p65
6 subunit(Baeuerle and Baichwal, 1997; Kumar et al., 1994). In the present study, LC was
7 demonstrated to inhibit the degradation of I κ B α and the phosphorylation of p65 subunit. In
8 an additional study, LC showed inhibitory effects on NF- κ B-dependent reporter gene
9 expression. All these results indicate that LC may decrease expression of these
10 pro-inflammatory mediators by inhibiting NF- κ B activation, similar to the action of
11 isoliquiritigenin and glycyrol(Honda et al., 2012; Shin et al., 2008). The phosphorylation of
12 MAPK including ERK, p38 and JNK is known to be part of the upstream signaling pathway
13 for the NF- κ B activation. We found that MAPK signaling pathway is largely involved in
14 LC-mediated anti-inflammatory effects in LPS-stimulated macrophages. The JAK-STATs
15 is an important signaling pathway activated by many extracellular signaling ligands
16 including LPS and IL-10(Donnelly et al., 1999; Murray, 2007; Park et al., 2003). In
17 particular, STAT3 plays a critical role in releasing pro-inflammatory cytokines in
18 macrophages. Macrophages secrete IL-10 in response to LPS stimuli, subsequently
19 binding to IL-10 receptors activates the IL-10/JAK1/STAT3 signaling cascade (Hutchins et
20 al., 2013; Tak and Firestein, 2001). Phosphorylated STAT3 then translocate into the
21 nucleus and regulate the transcription of inflammation-related genes (Wen et al., 1995).
22 Our study indicates that the suppression of STAT3 signaling pathway by LC may be due to

1 its suppressive action on IL-10 in RAW 264.7 macrophages. Collectively, our results
2 reveal that LC exerted its inhibitory effects on the LPS-induced up-regulation of
3 pro-inflammatory cytokines and mediators through blocking the signaling pathways of
4 NF- κ B, MAPK and STAT3.

5 Because LC produces an inhibitory effect on LPS-induced expression of
6 inflammatory cytokine as well as IL-10 in both RAW 264.7 and primary macrophages, it
7 gives a profile akin to a global immunosuppressant. Upon close examination, however, we
8 found selectivity of LC in its action. As reported in this study, LC does not suppress the
9 LPS-induced expression of TNF- α , a primary inflammatory cytokine involved in many
10 inflammatory diseases. The lack of inhibition is specific for LC, as parthenolide produced
11 potent inhibition on LPS-induced TNF- α expression under exactly the same experimental
12 conditions. Inhibition of LPS-induced expression of cytokines, including IL-10 but not
13 TNF- α , indicates that the LC effect is selective, although the range of its targets may be
14 broad. The inhibition profile of LC, therefore, is different from that of a global
15 immunosuppressant.

16 We also observe that LC inhibited TNF- α induced NF- κ B-luc activity in HeLa cells in a
17 very short concentration range, seemingly in an all-or-none response manner. This
18 feature may be explained by the fact that LC inhibited on multiple targets, many of which
19 contributing to NF- κ B activation. As the activity of luciferase that we monitored was
20 dependent on the activity of NF- κ B, inhibition at multiple upstream sites results in
21 inhibitory dose response kinetics that differ from the ones involving single targets or linear
22 pathways. Future studies are required to further explore this possibility.

1 In summary, the effects of LC was investigated in LPS-stimulated RAW264.7
2 macrophage cells. LC effectively reduced NO production, which was accompanied with
3 the down-regulation of iNOS and pro-inflammatory cytokines in both protein and mRNA
4 expression levels. The suppressive effects of LC on LPS-induced murine peritoneal
5 macrophages confirm that its effects observed in RAW 264.7 cells occur on primary cells
6 as well. Furthermore, this effect was observed in accordance with the reduced activation
7 of NF- κ B, MAPKs and STAT3. Together these findings suggest that LC is a functional
8 constituent of *Glycyrrhizauralensis*, which has the potential to be further developed as a
9 pharmaceutical agent for the prevention and treatment against infectious or
10 immune-related disorders. The precise underlying mechanism remains to be clarified and
11 the more potent compounds derived from its structure should be further characterized and
12 developed.

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Fig.1. Chemical structure of LC isolated from *Glycyrrhizauralensis*

Fig. 2. Effects of LC on LPS-induced NO production (A) and cell viability (B) in RAW 264.7 macrophages. (A) Cells were pre-treated with different concentrations of LC (0-50 μ M) for 1 h and then stimulated with LPS (100 ng/mL) for 24 h. The nitrite level was determined by Griess reagent. (B) Cells were treated with different concentrations of LC (0-100 μ M) for 24 h. Parthenolide (10 μ M) was used as a positive control. The cell viability was assessed by MTT reduction assays. The data were obtained from three independent experiments and expressed as the means $\pm$ S.E.M. **P < 0.01 vs the control group.

Fig. 3. Effects of LC on LPS-induced mRNA and protein expression levels of iNOS in RAW 264.7 cells. (A) iNOS was amplified by real-time PCR. Cells were treated with different concentrations of LC for 1 h and then stimulated with LPS (100 ng/mL) for 4 h. (B) iNOS protein levels were analyzed by Western blot. Cells were treated with different concentrations of LC for 1 h and then stimulated with LPS (100 ng/mL) for 24 h. The iNOS signal was normalized to the β -actin signal. The data were obtained from three independent experiments and expressed as the means $\pm$ S.E.M. **P < 0.01, ***P < 0.001 vs the LPS-treated group; ##P < 0.01 vs the control group.

Fig. 4. Effects of LC on LPS-induced mRNA expression and production of pro-inflammatory cytokines in RAW264.7 cells. (A-D) Inhibition at the mRNA level. Cells were treated with different concentrations of LC for 1 h and then stimulated with LPS (100

1 ng/mL) for 4 h. IL-1 β (A), IL-6 (B), TNF- α (C) and IL-10 (D) mRNA levels were determined
2 by real-time PCR analysis. (E-H) Inhibition at the protein level. Cells were pre-treated with
3 LC for 1 h and then stimulated with LPS (100 ng/mL) for 24 h. The protein levels of IL-1 β
4 (E), IL-6 (F), IL-10 (G) and TNF- α (H) were determined by ELISA according to
5 manufacturer's instructions. Parthenolide (PAR, 10 μ M) was used as a positive control.
6 The data were obtained from three independent experiments and expressed as the
7 means $\pm$ S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001 vs the LPS-treated group; ##P < 0.01,
8 ###P < 0.001 vs the control group.

10 **Fig. 5.** Effects of LC on LPS-induced NO production and iNOS expression in mouse
11 peritoneal macrophages. (A) The nitrite level was determined by Griess reagent. Cells
12 were pre-treated with different concentrations of LC (0-50 μ M) for 1 h and then stimulated
13 with LPS (1000 ng/mL) for 23 h. (B) iNOS protein levels were analyzed by Western blot. (C)
14 Cell viability was determined by MTT assay. Cells were treated with different
15 concentrations of LC for 1 h and then stimulated with LPS (1000 ng/mL) for 23 h. The
16 iNOS signal was normalized to the β -actin signal. The data were obtained from three
17 independent experiments and expressed as the means $\pm$ S.E.M. **P < 0.01, ***P < 0.001
18 vs the LPS-treated group; ##P < 0.01 vs the control group.

20 **Fig. 6.** Effects of LC on the activation of NF- κ B. (A) The protein expression levels of
21 phosphorylated p65 and (B) degraded I κ B α in LPS-stimulated RAW 264.7 were detected
22 by Western blot and normalized to the total p65 and the β -actin signal, respectively. Cells

1 were pre-treated with LC for 1 h and then stimulated with LPS (100 ng/mL) for 30 min. The
2 data were obtained from three independent experiments and expressed as the
3 means $\pm$ S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001 vs the LPS-treated group; #P < 0.05,
4 ##P < 0.01, ###P < 0.001 vs the control group. (C) Inhibitory effects of LC on NF- κ B activation
5 in Hela/NF- κ B-luc cells. Cells were treated with different concentrations of licocourmarone
6 1 h prior to the addition of TNF- α (5ng/ml) and were further incubated for 4 h. Parthenolide
7 (10 μ M) was used as a positive control. The produced luciferase activity was assayed as
8 described in Materials and Methods. Data points represent mean $\pm$ S.E.M. (n = 3) of
9 relative luminescence units (RLU), *P < 0.05 (ANOVA) compared with negative control.

10
11 **Fig.7.** Effects of LC on LPS-induced (A-C) MAPKs and (D) STAT3 signaling pathways in
12 RAW 264.7 cells. The cells were pretreated with LC for 1 h and subsequent stimulated
13 with LPS (100 ng/mL) for 30 min. The expression levels of phosphorylated ERK (P-ERK)
14 (A), JNK (P-JNK) (B), p38 (P-p38) (C), and STAT3 (p-STAT3) (D) were determined by
15 Western blot and normalized to the total ERK, JNK, p38 and STAT3 signal, respectively.
16 The data were obtained from three independent experiments and expressed as the
17 means $\pm$ S.E.M. *P < 0.05, ***P < 0.001 vs the LPS-treated group; ##P < 0.01, ###P < 0.001 vs
18 the control group.

Figure 1

Figure 2

Figure 4

Figure 6

A

B

C

D