

Lupeol alleviates coxsackievirus B3-induced viral myocarditis in mice via downregulating toll-like receptor 4

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Abstract

Objectives: To investigate the effect of lupeol in a mouse model of viral myocarditis induced by coxsackie virus B3 (CVB3).

Methods: Mice were separated into controls (DMEM, $n = 20$) and CVB3 infected groups (i.e., untreated CVB3 [$n = 40$]; CVB3 + lupeol 50 mg/kg [$n = 40$]; CVB3 + lupeol 100 mg/kg [$n = 40$]; CVB3 + small interfering RNA (siRNA)- toll-like receptor 4 (TLR4) [$n = 20$]; siRNA + EXP-H mice [$n = 20$]). Reverse transcription polymerase chain reaction (RT-PCR), western-blot assay, immunohistochemistry, enzyme-linked immunosorbent (ELISA) assay and histopathology were performed to investigate the cardioprotective role of lupeol.

Results: The elevated pro-inflammatory cytokines in CVB3-infected mice (i.e., interleukin- 1β [IL- 1β]; interleukin-6 [IL-6]; tumour necrosis factor- α [TNF- α]) were significantly reduced by lupeol 50 or 100 mg/kg. Interestingly, the mRNA level and protein level of toll-like receptor 4 (TLR4) were inhibited by lupeol.

Conclusions: Lupeol alleviates CVB3-induced viral myocarditis and myocardial damage in mice. The underlying mechanism may due to downregulation of TLR4.

Keywords

Lupeol, inflammation, myocarditis, toll-like receptor 4, coxsackievirus B3

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Introduction

Viral myocarditis is an inflammatory disease of the myocardium, which can cause heart failure in severe cases.¹ It is well known that coxsackie virus B3 (CVB3) is the predominant cause of viral myocarditis, and the pathogen has been shown to be the most common cause of heart failure in young adults.^{2,3} Viral myocarditis murine models established by Coxsackie virus B3 (CVB3) have been widely studied.^{4,5} The pathogenesis of viral myocarditis is complex and some researchers have proposed that excessive innate immune response-induced inflammation is the major mechanism.⁶ Toll-like receptor 4/nuclear factor κ B (TLR4/NF- κ B) signal pathway is closely related to the inflammatory response.^{7,8} and several investigators have suggested that there is a positive correlation between TLR4 activation and viral myocarditis. For example, TLR4 was reported to be overexpressed in CVB3 induced viral myocarditis,⁹ whereas TLR4 silencing was shown to ameliorate viral myocarditis in the CVB3 infected mouse model.¹⁰ Another study showed that viral myocarditis could be exacerbated by enhancing the activation of TLR4.¹¹ Therefore, inhibiting TLR4 activation may be a promising target for alleviating inflammation and myocardial damage in viral myocarditis.

Lupeol, is a pentacyclic triterpenoid found in a variety of edible vegetables, fruits and other plants.¹² It has been suggested to have beneficial pharmacological activities including antioxidant, anti-inflammatory and anti-cancer effects.^{12,13} Interestingly, lupeol was observed to inhibit TLR4/MyD88 (myeloid differentiation primary response gene 88) pathway in D-galactosamine and lipopolysaccharide-induced hepatic failure.¹⁴ In addition, lupeol has been shown to have cardioprotective effects.^{15,16} However, the effect of lupeol in viral myocarditis is unknown.

Therefore, the aim of this present study was to investigate the effect of lupeol on the standard mouse model of CVB3 induced viral myocarditis.

Materials and methods

Animals

Specific pathogen free six-week-old BALB/C mice (male, 14–16 g) were purchased from Hubei Experimental animal research centre (Wuhan, Hubei, China). Mice were housed under constant conditions and had free access to food and water during the experimental period. All animal procedures were performed according to the NIH guidelines for the Care and Use of Laboratory Animals. The study was approved by the Institutional Animal Care and Use Committee of Wuhan Asia Heart Hospital.

Establishment of the viral myocarditis mouse model

In total, 180 mice were used in the study and separated into six groups:

1. normal mice treated with the same volume of Dulbecco's Modified Eagle Medium (DMEM) (DNEM group, $n = 20$)
2. CVB3-induced myocarditis (CVB3 group, $n = 40$)
3. CVB3-induced myocarditis treated with 50 mg/kg lupeol (St. Louis, MO, USA) by intraperitoneal injection (EXP-L group, $n = 40$)
4. CVB3-induced myocarditis mice treated with 100 mg/kg lupeol by intraperitoneal injection. (EXP-H group, $n = 40$).¹⁴
5. CVB3-induced myocarditis mice treated with small interfering RNA (siRNA)-TLR4 (CVB3-siRNA group, $n = 20$)
6. CVB3-induced myocarditis mice treated with siRNA-TLR4 and 100 mg/kg lupeol

(St. Louis, MO, USA) by intraperitoneal injection (siRNA + EXP-H group, $n = 20$)

The CVB3-induced myocarditis mouse models were established by using intraperitoneal injections of 10^2 TCID₅₀ (50% tissue culture infectious dose) CVB3 on Day 0.¹⁰ After 24 h, all mice received intraperitoneal injections every 24 h for 21 days. The mice in the DMEM and CVB3 groups received normal saline (NS), and the mice in the EXP-L and EXP-H groups received lupeol. The mice were monitored and survival noted every two days until they were sacrificed at 21 days.

Detection of cardiac function index

Heart rate (HR) and left ventricular systolic pressure (LVSP) were measured via a Power Lab recording instrument (AD Instruments, Castle Hill, Australia). The left ventricle end-diastolic pressure (LVEDP) and left ventricular ejection fraction (LVEF) were detected by transthoracic echocardiographic using an Agilent Sonos 5500 ultrasound machine (Philips Medical Systems, Andover, Mass, USA).

Histopathology

Heart tissues from the mice were harvested and fixed in 4% paraformaldehyde for 20 h. Thereafter, the specimens were embedded in paraffin, cut into 4 μ m sections and stained with haematoxylin and eosin (H&E). The sections were examined by the same investigator under an optical microscope (OLYMPUS, Japan). Foci of mononuclear infiltration and myocardial necrosis were quantified from 5 high-power fields per section and each specimen was graded as follows: 0: no inflammation or injury; 1: <5% involvement; 2: 5% – 25% involvement; 3: 25% – 75% involvement; 4: >75% involvement.

Virus titre assay

For the virus titre assay, 50 mg of myocardial tissue was placed into 500 μ l of phosphate buffered saline (PBS) to form an homogenate. The supernatant was serially diluted 10-fold using DMEM and HeLa cells were infected with the supernatant on a 96-well culture plate. Cultured HeLa cells were incubated at 37°C with 5% CO₂ for 96 hours. Tissue culture infective dose at 50% (TCID₅₀) was calculated as previously described.¹⁷ The result was expressed as $-\log_{10}$ TCID₅₀.

Cytokines and biomarkers

Levels of muscle haemoglobin (Mb), MB isoenzyme of creatine kinase (CK-MB), cardiac troponin I (cTn I), serum tumour necrosis factor alpha (TNF- α) and serum interleukin (IL)- β , were measured using commercially available enzyme-linked immunosorbent (ELISA) kits (R&D Systems, Minneapolis, MN).

The immunohistochemistry for interleukin (IL)-6 was performed according to manufacturer's instructions for SP immunohistochemistry kits (I003-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Positive cells were detected as brown staining and specimens were analysed using a microscope (OLYMPUS, Japan).

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from tissues using the Trizol reagent (Invitrogen, Carlsbad, USA) and 2 μ g total RNA was reverse transcribed into cDNAs via the Prime Script TM RT Master Mix kit (Takara Bio, Kyoto, Japan). Fast Real-time PCR 7300 System (Applied Biosystems, Foster City, USA) was used to amplify the cDNA through the SYBR Green (Takara) detection method.

Table 1. Specific primers sequences for reverse transcription polymerase chain reaction (RT-PCR).

Gene	Forward primer sequence	Reverse primer sequence
β -actin	5'-TCACCGAGCGCGGCT-3'	5'-TAATGTCACGCACGATTTCCC-3'
COX2	5'-CCTCTGCGATGCTCTTCC-3'	5'-TCACACTTATACTGGTCAAATCC-3'
TLR4	5'-TGATTGTTGGTGTCCCA-3'	5'-TGTCCTCCCACTCCAGGTAA-3'

TLR4, toll like receptor 4; COX2, cyclooxygenase-2.

The mRNA expression of TLR4 and cyclooxygenase-2 (COX2) transcripts were normalized to the corresponding β -actin expression using the $2^{-\Delta\Delta C_t}$ method. The specific primers sequences are shown in Table 1.

Western-blot analysis

Protein was extracted using a total protein extraction kit (W034, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and quantified using a bicinchoninic acid (BCA) protein assay. Thereafter, 40 μ g of protein was resolved on gel for separation using SDS-PAGE loading buffer. The samples were subsequently transferred to a PVDF membrane (Millipore Millex, USA) that was blocked and washed. The membranes were incubated with primary antibodies (i.e., toll-like receptor (TLR) 4, MyD88, phosphorylated NF- κ B P65 [P-NF- κ B-P65] and NF- κ B P65 (all purchased from Abcam, Cambridge, UK) at 4°C overnight. The following day, antibodies were washed and incubated with HRP-conjugated secondary antibodies (Abcam) for 1.5 h at room temperature. Finally, the immunoreactive bands were visualized by enhanced chemiluminescence (ECL) and detected using a ChemiDoc XRS imaging system. B-actin was used as control.

To investigate the function of TLR4 in CVB3 caused viral myocardial injury, small interfering RNA (siRNA) of TLR4 was used to block TLR4 expression.

Statistical analysis

Data were analysed using Graphpad Prism, version 5.0 (GraphPad Software, La Jolla, CA) and a P -value <0.05 was considered to indicate statistical significance. Differences between groups were determined using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for comparison.

Results

By comparison with mice treated with DMEM, mice that received the CVB3 virus had reduced HR, LVSP and LVEF and an elevated LVEDP (Table 2). Interestingly, mice with the CVB3 virus who received lupeol treatment (i.e., EXP-L and EXP-H groups) were significantly less affected than the non-treated CVB3 group.

Heart tissues from the CVB3 group showed the most serious myocardial damage compared with the other three groups as evidenced by the irregularly arranged myocardial cells (Figure 1a). All CVB3-infected mice showed lesions of mononuclear cellular infiltration and necrosis. However, the two groups treated with lupeol showed less myocardial damage than the non-treated CVB3 group. All mice in the DMEM group survived until the end of the experiment (Figure 1b). By contrast, survival rates in the non-treated CVB3 group were significantly decreased ($P < 0.01$) but survival rates in the lupeol groups were markedly elevated from the CVB3 level ($P < 0.01$). By comparison with the non-treated CVB3 group, the two

Table 2. Haemodynamic and cardiac function parameters in the coxsackievirus B3 (CVB3) mouse myocarditis model.

Parameters	GROUPS			
	DMEM (n = 20)	CVB3 (n = 40)	EXP-L (n = 40)	EXP-H (n = 40)
Heart rate, bpm	473 ± 14	337 ± 12**	389 ± 16* [#]	421 ± 13* [#]
LVEDP, mm Hg	3.6 ± 0.8	12.3 ± 1.0**	6.0 ± 0.5* [#]	4.7 ± 0.4* [#]
LVSP, mm Hg	155 ± 14	102 ± 8**	125 ± 6* [#]	146 ± 13* [#]
LVEF, %	45 ± 3	30 ± 4**	35 ± 4* [#]	39 ± 4* [#]

Values are shown as mean ± SD.

DMEM, Dulbecco's Modified Eagle Medium; EXP-L, CVB3 + 50 mg/kg lupeol by intraperitoneal injection; EXP-H, CVB3 + 100 mg/kg lupeol by intraperitoneal injection; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure; LVEF, left ventricular ejection fraction.

* P < 0.05 compared with DMEM group, **P < 0.01 compared with DMEM group, [#]P < 0.05 compared with CVB3 group.

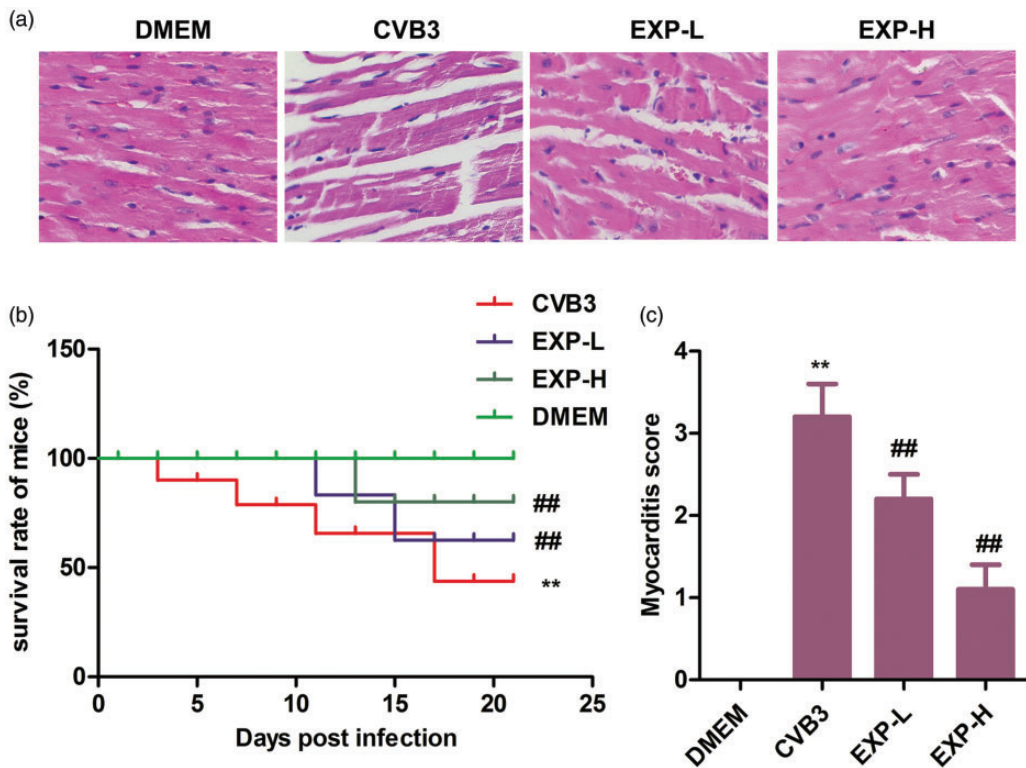


Figure 1. Effects of lupeol on myocardial histopathology and survival rates. (a) Cardiac injury as demonstrated by haematoxylin and eosin (H&E) staining (magnification 400×). (b) Survival rate of the four groups of mice. (c) Myocarditis scores (mean ± SD) of the four groups of mice. Foci of mononuclear infiltration and myocardial necrosis were quantified from 5 high-power fields per section and each specimen was graded as follows: 0: no inflammation or injury; 1: <5% involvement; 2: 5% – 25% involvement; 3: 25% – 75% involvement; 4: >75% involvement. DMEM (Dulbecco's Modified Eagle Medium) treated mice (n = 20); CVB3 (coxsackie virus B3) infected mice (n = 40); EXP-L mice (CVB3 + 50 mg/kg lupeol [n = 40]); EXP-H mice (CVB3 + 100 mg/kg lupeol [n = 40]).

**P < 0.01 compared with DMEM group, ###P < 0.01 compared with CVB3 group.

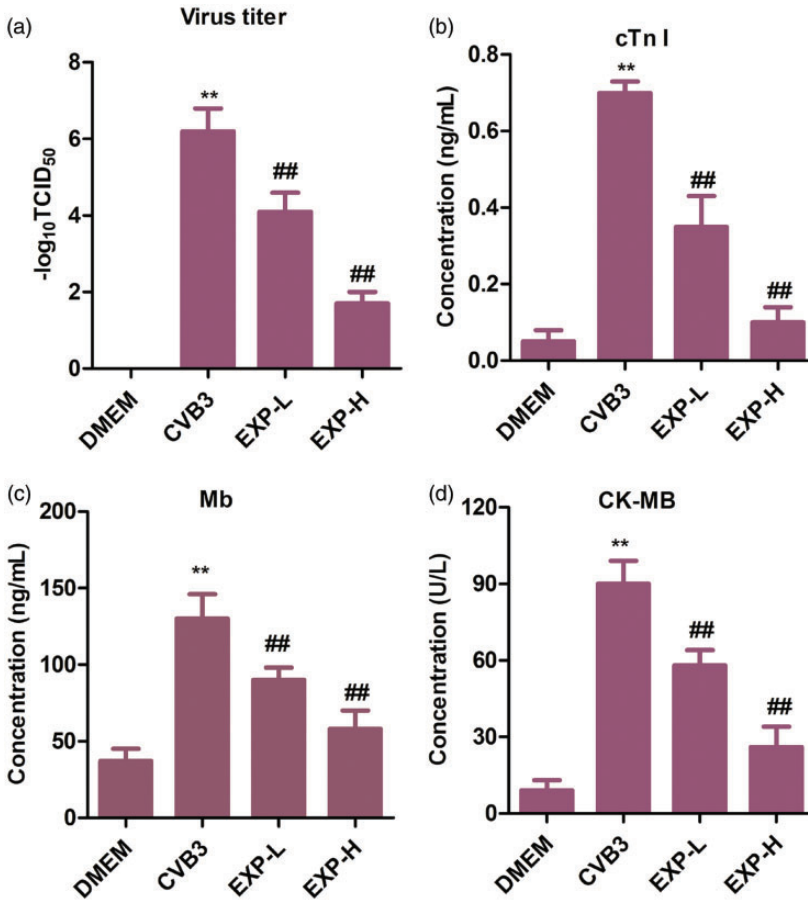


Figure 2. Effect of lupeol on virus titres and myocardial damage markers. (a) Virus titres; (b) cardiac troponin I (cTn I) levels; (c) muscle haemoglobin (Mb) levels; (d) MB isoenzyme of creatine kinase (CK-MB) levels. Values are shown as mean \pm SD. DMEM treated mice ($n = 20$); CVB3 (coxsackie virus B3) infected mice ($n = 40$); EXP-L mice (CVB3 + 50 mg/kg lupeol [$n = 40$]); EXP-H mice (CVB3 + 100 mg/kg lupeol [$n = 40$]). ** $P < 0.01$ compared with DMEM group, ## $P < 0.01$ compared with CVB3 group. TCID, tissue culture infective dose.

lupeol groups had significantly smaller myocarditis scores as assessed from histological analysis (Figure 1c).

Virus titres in each group were expressed as $-\log_{10}\text{TCID}_{50}$. The viral titre of CVB3 in the heart tissues was significantly reduced in lupeol groups compared with the non-treated CVB3 group (Figure 2a). In addition, the levels of myocardial damage markers (i.e., cTn I, Mb and CK-MB)

were significantly elevated in the non-treated CVB3 group ($P < 0.01$). By comparison with the non-treated CVB3 group, 50 mg/kg (EXP-L) and 100 mg/kg lupeol (EXP-H) significantly reduced the levels of the three markers ($P < 0.01$) (Figure 2b, 2c and 2d).

In contrast with the DMEM group, the levels of pro-inflammation cytokines (i.e., IL-1 β , TNF- α and COX2) were

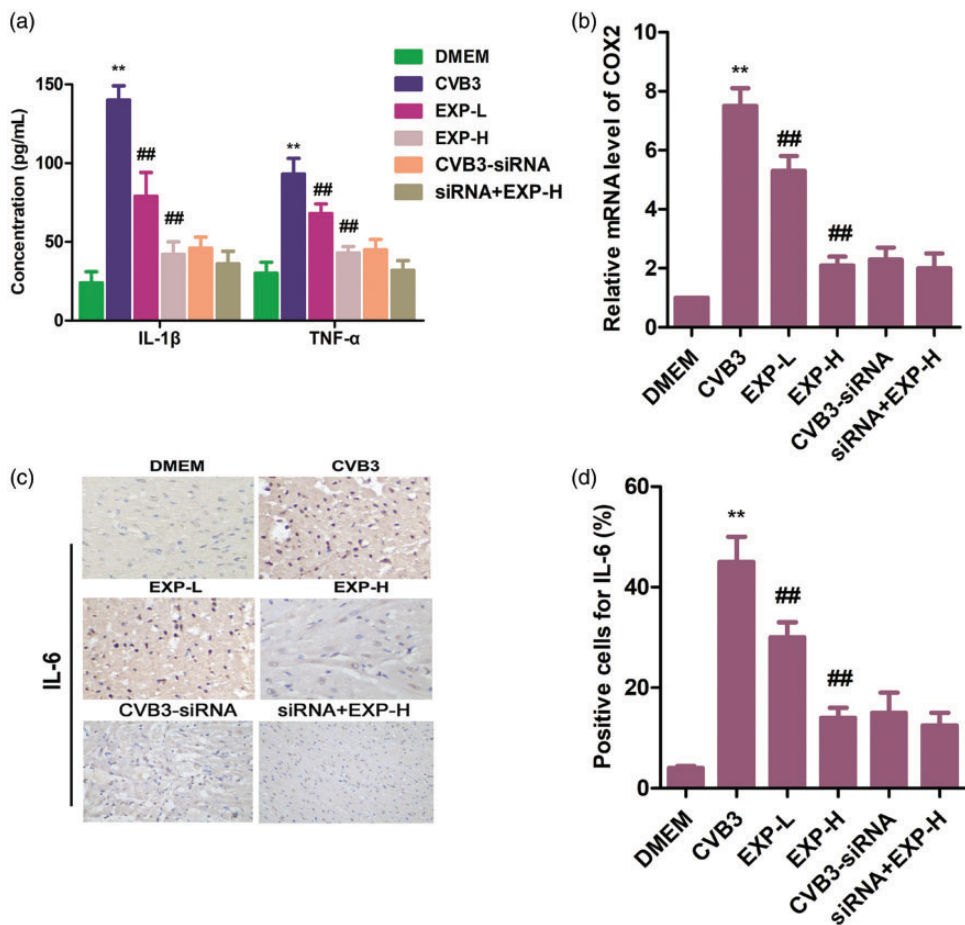


Figure 3. Effect of lupeol on pro-inflammation cytokines. (a) Levels of interleukin (IL)- β and tumour necrosis factor alpha (TNF- α) were detected by commercially available enzyme-linked immunosorbent (ELISA) kits. Values are shown as mean \pm SD. (b) Expression of cyclooxygenase-2 (COX2) was determined by reverse transcription polymerase chain reaction (RT-PCR). Values are shown as mean \pm SD. (c) Expression of interleukin (IL)-6 detected by immunohistochemical staining. (d) Percentage of IL-6 positive cells shown by immunohistochemical staining. Values are shown as mean \pm SD. DMEM treated mice ($n = 20$); CVB3 (coxsackie virus B3) infected mice ($n = 40$); EXP-L mice (CVB3 + 50 mg/kg lupeol [$n = 40$]); EXP-H mice (CVB3 + 100 mg/kg lupeol [$n = 40$]); CVB3-siRNA mice (CVB3 + small interfering RNA (siRNA)- toll-like receptor 4 (TLR4), [$n = 20$]); siRNA + EXP-H mice (CVB3 + siRNA-TLR4 + 100 mg/kg lupeol [$n = 20$]); ** $P < 0.01$ compared with DMEM group, ## $P < 0.01$ compared with CVB3 group.

significantly increased in the non-treated CVB3 group ($P < 0.01$) (Figure 3a and 3b). As expected, 50 mg/kg (EXP-L) and 100 mg/kg lupeol (EXP-H) significantly reduced the levels of these three cytokines compared with the non-treated CVB3

group ($P < 0.01$). Interestingly, the levels of IL-1 β , TNF- α and COX2 in the CVB3-siRNA, siRNA+EXP-H and EXP-H groups were similar.

Using immunohistochemical staining, IL-6 was shown to be upregulated in the

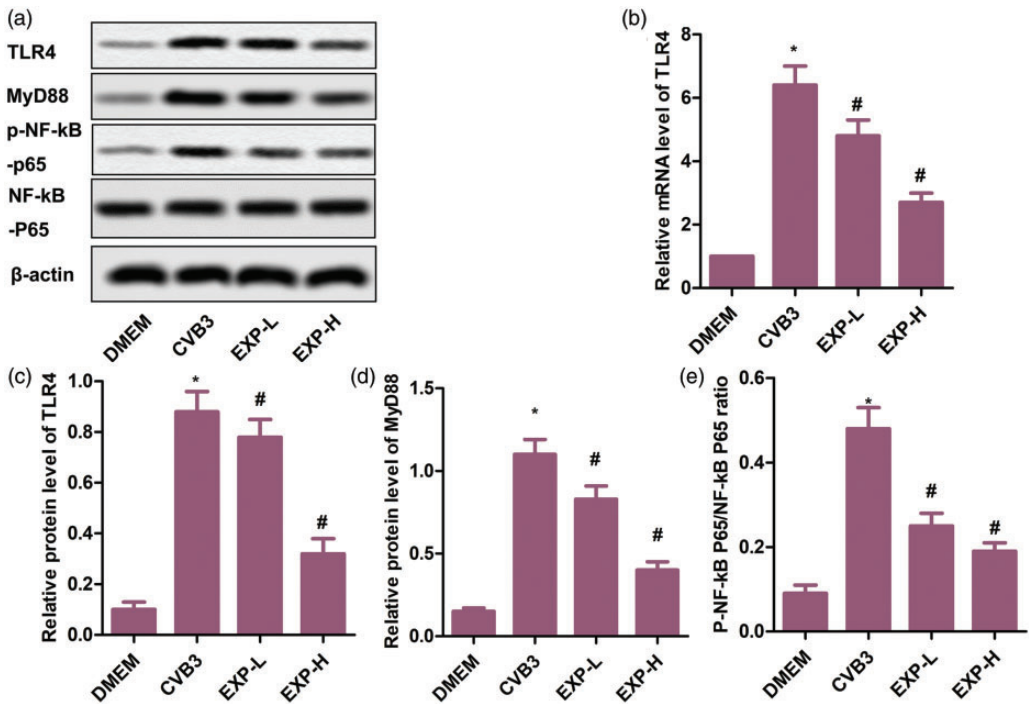


Figure 4. Effect of lupeol on the expression of toll-like receptor (TLR) 4, MyD88, phosphorylated NF-κB P65 [P-NF-κb-P65] and NF-κB P65. (a) The protein expressions of TLR4, MyD88, NF-κB P65 and p-NF-κB P65 determined by western blotting. (b) mRNA levels of TLR4 determined by the reverse transcription polymerase chain reaction (RT-PCR). Values are shown as mean ± SD. (c–e) Quantitative analysis of the expression of TLR4, MyD88, NF-κB P65 and p-NF-κB P65. Values are shown as mean ± SD. DMEM treated mice ($n = 20$); CVB3 (coxsackie virus B3) infected mice ($n = 40$); EXP-L mice (CVB3 + 50 mg/kg lupeol [$n = 40$]); EXP-H mice (CVB3 + 100 mg/kg lupeol [$n = 40$]). ** $P < 0.01$ compared with DMEM group, ### $P < 0.01$ compared with CVB3 group.

non-treated CVB3 group but significantly downregulated in the 50 mg/kg (EXP-L) and 100 mg/kg lupeol (EXP-H) groups ($P < 0.01$) (Figure 3c and 3d). The results following gene silencing of TLR4 by siRNA were similar to those in the EXP-H group.

The protein expressions of TLR4, MyD88, NF-κB P65 and p-NF-κB P65 were shown by western blotting (Figure 4a). The mRNA level of TLR4 was significantly elevated in the non-treated CVB3 group and by comparison with this group, mRNA levels were reduced

in the EXP-L and EXP-H groups ($P < 0.01$; Figure 4b). Similarly, protein levels of TLR4, MyD88 and phosphorylated NF-κB P65 (P-NF-κb-P65) were significantly upregulated in non-treated CVB3 group and by comparison with this group the levels were downregulated in EXP-L and EXP-H groups ($P < 0.01$; Figure 4c, 4d and 4e).

Discussion

Excessive inflammation and cardiac dysfunction are observed during CVB3-

induced myocarditis and so an effective therapeutic approach to combat viral myocarditis would be to restrain the inflammatory process.¹⁸ Reports have suggested that lupeol may have anticancer, antioxidant, anti-hyperglycaemic, anti-dyslipidaemia and anti-mutagenic properties.¹⁹ Although the compound has not been investigated previously in viral myocarditis, several animal studies have found that it has anti-inflammatory and cardioprotective effects.^{14,20–23}

In our experiments we used a standard viral myocarditis mouse model established by CVB3.^{4,5} Our results showed that the model had been established successfully as evidenced by decreased cardiac function, increased viral titres and levels of inflammatory cytokines and biomarkers. In addition, the survival rate for the CVB3 infected mice was reduced. We found that although lupeol at 50 or 100mg/kg alleviated the inflammatory response induced during the viral myocarditis, the 100 mg/kg dose had a higher efficacy.

TLR4 is an important member of TLR family which are a group of proteins that are important mediators of innate immunity and recognize many inflammatory inducers.^{9,24} There are two types of TLR4 signalling pathways (i.e., MyD88-dependent and MyD88-independent).²⁴ In the MyD88-dependent pathway, following TLR4 activation MyD88, an adaptor molecule, is recruited to activate the NF- κ B pathway which results in the production of inflammatory cytokines including IL-1 β , COX2 and TNF- α .²⁵ Therefore, affecting the TLR4/MyD88/NF- κ B signalling pathway will undoubtedly affect the inflammatory response.^{7,8} Consistent with the results from a previous study using the CVB3-induced myocarditis mouse model,¹⁰ we found that the expression of TLR4, MyD88 and p-NF- κ B P65 were increased in CVB3-induced mice. These results indicated that the MyD88-

dependent pathway had been activated during viral induced myocarditis. We found that lupeol at 50 mg/kg or 100 mg/kg inhibited the activation of the MyD88-dependent pathway as shown by the reduced protein expression of TLR4, MyD88 and p-NF- κ B P65. In addition, gene silencing of TLR4 reduced the levels of pro-inflammatory cytokines including IL-1 β , COX2 and TNF- α . Therefore, CVB3 may induce viral myocarditis by upregulating TLR4 expression. Interestingly, we found that gene silencing of TLR4 by using siRNA provided similar results to those achieved in the EXP-H group (CVB3+ lupeol 100mg/kg).

To our knowledge, our study is the first to demonstrate the anti-inflammatory and cardioprotective effects of lupeol in CVB3-induced viral myocarditis in mice. The underlying mechanism for lupeol may due to inhibition of the TLR4/MyD88/NF- κ B P65 signalling pathway. However, there may be other possible target molecules for lupeol and involvement of other downstream molecules of TLR4. Further studies are required to confirm our results but these preliminary data suggest that lupeol may be a potential new therapeutic target for the treatment for viral-induced myocarditis.

Declaration of conflicting interests

The authors declare that there are no conflicts of interest.

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