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ORIGINAL PAPER

Clonorchis sinensis lysophospholipase inhibits TGF-β1-induced expression of pro-fibrogenic genes through attenuating the activations of Smad3, JNK2, and ERK1/2 in hepatic stellate cell line LX-2

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Abstract Liver fibrosis is a wound healing response associated with chronic liver injury. Hepatic stellate cells (HSCs) activation is a key event in the development of liver fibrosis. Since helminths have the ability to live for decades in the host by establishing an adaptive relationship in the interplay with its hosts, we hypothesize that whether *Clonochis sinensis* LysophospholipaseA (*Cs*LysoPLA), a component of excretory/secretory proteins, can attenuate the fibrogenic response by inhibiting activation of LX-2 cells, thereby balancing the pro-fibrotic and anti-fibrotic response during the *Clonochis sinensis* (*C. sinensis*) infection. In the present study, LX-2 cells were stimulated with *Cs*LysoPLA in the presence of TGF-β1, and the expressions of collagen type I (COL1A1), α-smooth muscle actin (α-SMA), and matrix metalloproteinase 2 (MMP2) were decreased. In addition, *Cs*LysoPLA significantly inhibited the proliferation and migration of LX-2 cells stimulated by TGF-β1. Pretreatment of LX-2 cells with *Cs*LysoPLA attenuated the phosphorylation of Smad3 as well as JNK2 and ERK1/2 in response to the stimulation of TGF-β1. For the first time, our results showed an anti-fibrogenic effect of *Cs*LysoPLA by attenuating the response of LX-2 cells to TGF-β1 through inhibiting the activations of Smad3, ERK1/2, and JNK2.

Keywords Liver fibrosis · *Clonochis sinensis* · *Cs*LysoPLA · LX-2 cells

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Introduction

Chronic liver injury caused by viruses and parasite infection, alcohol abuse, metabolic and autoimmune diseases, and the relative chronic activation of the wound healing reaction results in accumulation of extracellular matrix (ECM), destruction of the normal structure, and the alteration of the normal function, finally leading to liver fibrosis and failure (Pinzani and Macias-Barragan 2010; Anthony et al. 2010). Activated HSCs play a pivotal role in the fibrosis process (Yin et al. 2013). In response to chronic liver injury, HSCs are activated by inflammatory stimulus, such as oxidant stress, apoptotic bodies, and cytokines including TGF-β1, platelet derived growth factor (PDGF) released by hepatocytes, Kupffer cells, and sinusoidal endothelium (Lee and Friedman 2011). Upon activation, HSCs transform to myofibroblast-like cells, proliferate, and migrate to the site of injury, characterized by enhanced α-smooth muscle actin (α-SMA), MMP2 and collagen type I (COL1A1) expressions, ECM production, and pro- and anti-inflammatory cytokines production, such as TGF-β1 (Pellicoro et al. 2014). TGF-β1 is considered as a quite potent pro-fibrogenic cytokine in HSCs. TGF-β1 plays a key role in the process of transdifferentiation of HSCs into myofibroblast-like cells (Gressner et al. 2002). When TGF-β1 activity is enhanced, more collagen is secreted by HSCs to be deposited to the site of injury, aggravating fibrosis

progression in liver (Gressner and Weiskirchen 2006). It is known that TGF-β1 mediates intracellular signaling by sequentially phosphorylating ser/thr kinase type I and type II receptor, which then directly phosphorylates Smad2 and Smad3 for final nucleus translocation. In addition, TGF-β1 enhances ECM synthesis and inhibits its degradation, which is considered as a Smad3-dependent process (Flanders 2004).

Clonorchiasis is a food-borne parasitic disease caused by *C. sinensis* (Zhang et al. 2013; He et al. 2014). *C. sinensis* adults mainly parasitize in intra-hepatic bile duct. The long-term stimulation of mechanicalness and *C. sinensis* excretory–secretory products (*Cs*ESPs) triggers chronic liver injury, resulting in inflammation, fibrosis, and even cholangiocarcinoma (Choi et al. 2004). In particular, ESPs greatly contribute to these pathological impairments (Wang et al. 2011).

LysophospholipaseA (LysoPLA) is one of the most important enzymes that deacylate lysophospholipids (Wang et al. 2000). The low molecular mass LysoPLAs, around 25 kDa, have been found in and/or purified from a number of tissue and cells (Wang and Dennis 1999). Previously, we cloned, expressed, and characterized LysoPLA from *C. sinensis* with a molecular mass of around 25 kDa (Ma et al. 2007). In vitro, it has been demonstrated that *Cs*LysoPLA can directly activate LX-2 cells by upregulating α-SMA expression (Zhang et al. 2013), which means that *Cs*LysoPLA may play a pro-fibrogenic role. However, in the current study, we found that *Cs*LysoPLA attenuated the TGF-β1-induced pro-fibrogenic effect. For further research, we investigated the role and molecular mechanisms of *Cs*LysoPLA in the regulation of fibrogenic gene expression in LX-2 cells.

Materials and methods

Cell culture and stimulation

The human HSC line LX-2 was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) containing 10 % fetal bovine serum (FBS) (NQBB, Australia), 100 U/ml penicillin, and 100 ug/ml streptomycin (Gbico, USA) at 37 °C in an atmosphere of 5 % CO_2. For all experiments, LX-2 cells were seeded at 5×10^4 cells/well in a 96-well plate, 1×10^5 cells / well in a 12-well plate, or 2×10^5 cells / well in a six-well plate. When LX-2 cells reached 70 % confluence, they were serum-starved for 24 h prior to be stimulated by different concentrations of *Cs*LysoPLA for 2 h and subsequently activated by TGF-β1 (5 ng/ml) (Peprotech, USA) for 12 or 24 h.

Quantitative real-time q-RT-PCR

Total RNA of LX-2 cells was extracted using Trizol reagent (Life Technologies, USA) according to the manufacturer's protocol. cDNAs were synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) and amplified on a Bio-Rad CFX96 Real-Time system (Bio-Rad, USA) with SYBR Green I (TaKaRa, Japan) for quantitative analysis. α-SMA, COL1A1, and MMP2 were measured and normalized by β-actin expression. Relative multiples of change in mRNA expression were analyzed by calculating $2^{-\Delta\Delta Ct}$. Primer sequences are listed in Table 1.

Western blot analysis

Cells were stimulated as described in the above. After stimulation, cells were washed with cold PBS and lysed with RIPA buffer (Beyotime, China) containing protease and phosphatase inhibitor (KeyGEN, China). Equal total proteins (30 μg) were loaded on polyacrylamide gel and transferred to a PVDF membrane (Millipore, USA). The membrane was incubated with primary antibody against α-SMA (Proteintech, China), Phospho-Smad3 (Santa Cruz, USA), Phospho-JNK (Santa Cruz, USA), Phospho-ERK1/2 (Cell Signalling Technology, USA), or GADPH (Proteintech, China) overnight at 4 °C, followed by secondary HRP-conjugated antibody (Proteintech, China) at room temperature for 60 min. Blots were visualized with ECL kit (Advansta, USA). GADPH was used as a loading control.

Immunofluorescence staining

LX-2 cells were cultured on slides. Cells were serum-starved for 24 h and then treated with *Cs*LysoPLA (10 μg/ml) for 2 h followed by with or without TGF-β1 (5 ng/ml) stimulation for 24 h. Then, cells were washed in PBS and fixed by 4 % paraformaldehyde for 20 min at room temperature. Similarly, cells were washed with PBS and then permeabilized in PBS containing 0.3 % Triton X-100 for 10 min. Sliders were blocked in PBS containing 1 % Bull Serum Albumin (BSA) for 1 h and incubated with primary antibody against α-SMA (Proteintech, China) overnight at 4 °C. Secondary Cy3-conjugated goat anti-rabbit (Proteintech, China) antibody was incubated for 1 h in the darkness. Then, the slides were stained with DAPI and mounted with antifade reagent (Beyotime, China). Images were obtained using Olympus BX63 and cellSens Dimension (Version1.8) sofTware (Olympus, Japan).

Cell proliferation assay

Cell proliferation was evaluated by Cell Counting Kit-8 (CCK-8) assay (DOJINDO, Japan). Briefly, LX-2 cells were seeded in 96-well plates and incubated with 0–10 μg/ml *Cs*LysoPLA for 2 h prior to the TGF-β1 (5 ng/ml) stimulation for 12 or 24 h. Then, 10 μl of CCK-8 reagent was added into wells for another 2 h at 37 °C in the darkness. Cell

Table 1 Primer sequences for quantitative real-time q-RT-PCR

Genes	Primer sequence (5′→3′)		Accession no.
α-SMA	Forward primer	CCAGGGCTGTTTTCCCATCC	NM_001613.2
	Reverse primer	GCTCTGTGCTTCGTCACCCA	
COL1A1	Forward primer	TGTTCAGCTTTGTGGACCTC	NM_000088.3
	Reverse primer	TCTGTACGCAGGTGATTGGT	
MMP2	Forward primer	CAGCTGGCCTAGTGATGATG	NM_001127891.2
	Reverse primer	GGTGTTCAGGTATTGCATGTG	
β-Actin	Forward primer	TGGACTTCGAGCAAGAGATG	NM_001101.3
	Reverse primer	GAAGGAAGGCTGGAAGAGTG	

proliferation was analyzed by measuring the absorbance change at 450 nm using a Benchmark Plus plate reader (BioRad, USA).

Cell migration assay

The migration of LX-2 cells was assayed using a 24-well Transwell plate with 8.0 μm pore polycarbornate membrane insert (Corning, USA). LX-2 cells were cultured in normal medium, and then they were serum-starved for 24 h, harvested, and resuspended in serum-free medium. The up wells were loaded with 100 μl serum-free medium containing 5×10^4 LX-2 cells. The down wells were filled with 600 μl serum-free medium containing *Cs*LysoPLA (10 μg/ml) in the presence of TGF-β1 or TGF-β1 alone. The chamber was incubated for 24 h at 37 °C. Non-migration cells located in the inside of each insert were gently swabbed using cotton swabs. Migration cells adhering to the underside of inserts were fixed with 100 % methanol for 30 min and then stained with 0.1 % crystal violet stain (Leagene, China) for 15 min. The numbers of migration cells were counted by using a light microscope (Leica, Germany) to enumerate the number of stained cells in five random fields.

Statistical analysis

All results were expressed as mean±SEM. Statistical analysis of the data was performed by the independent Student's *t* test and one-way ANOVA followed by Bonferoni's multiple comparison tests using SPSS software for Windows (version 16.0; SPSS, Inc., IL, USA). A p value 0.05 was considered statistically significant.

Results

*Cs*LysoPLA attenuated the TGF-β1-induced expression of fibrogenic genes in LX-2 cells

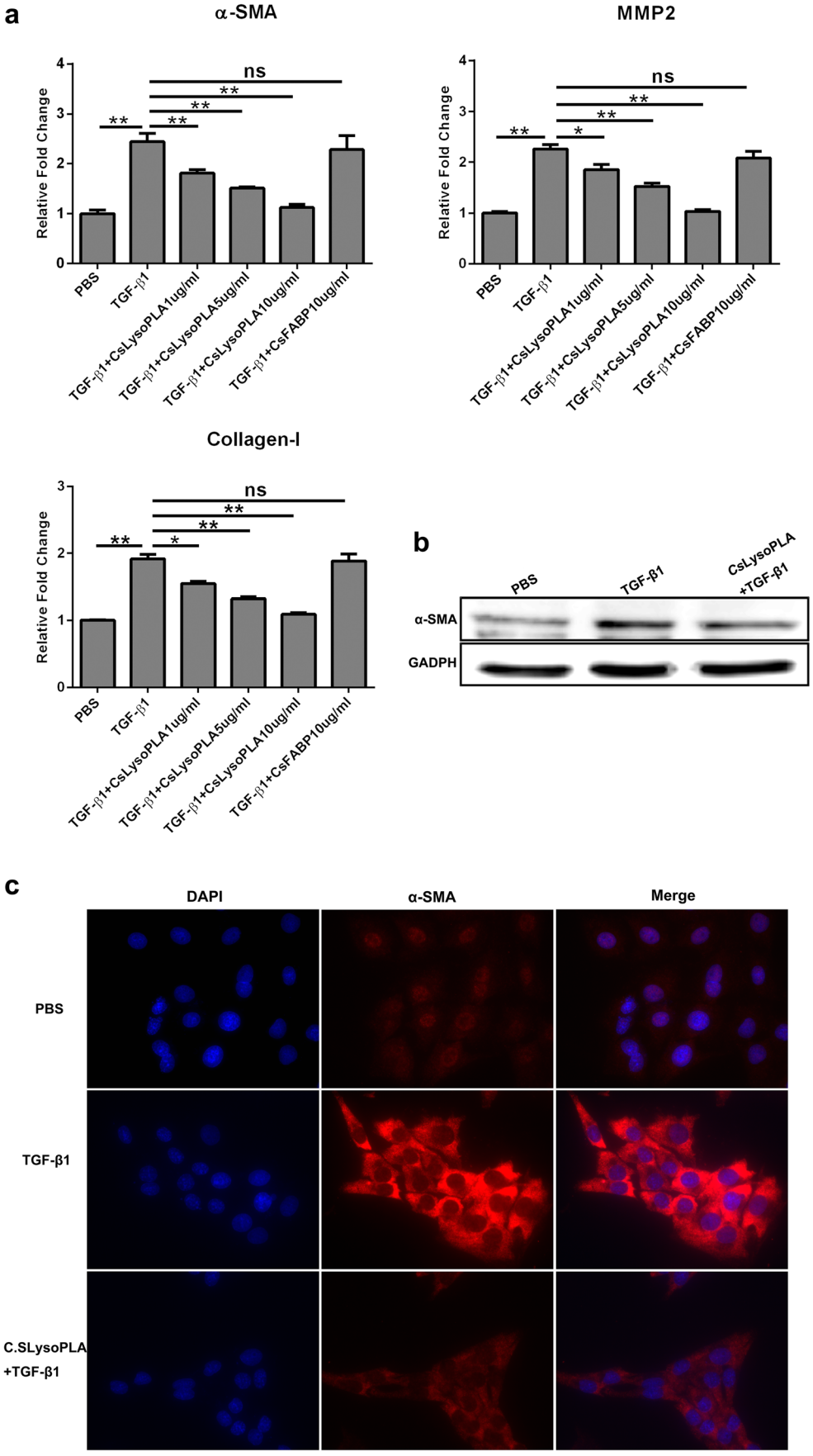
To investigate the effect of *Cs*LysoPLA on TGF-β1-induced LX-2 cells activation, *Cs*LysoPLA was used to treat LX-2 cells with or without the stimulation of TGF-β1. As previously described (Fabre et al. 2014), TGF-β1 induced a significant increase in the expressions of α-SMA, MMP2, and COL1A1 genes. In this study, α-SMA mRNA expression was strongly induced by TGF-β1 in LX-2 cells as compared with PBS treatment. *Cs*LysoPLA significantly inhibited the expression of α-SMA in a dose-dependent manner. MMP2 secreted by activated HSCs functions to degrade components of ECM (Siefert and Sarkar 2012), whose gene was upregulated in mRNA level in LX-2 cells after TGF-β1 treatment. This elevation was markedly attenuated by *Cs*LysoPLA in a dose-dependent manner, too. The level of COL1A1 mRNA expression in LX-2 cells treated with TGF-β1 with or without *Cs*LysoPLA was analogous to that of MMP2. No significant increase in the abovementioned three genes was found in LX-2 cells treated with TGF-β1 in the presence of *C. sinensis* fatty acid-binding protein (*Cs*FABP), a component of *Cs*ESPs, which was used as a negative control protein (Fig. 1a). We also detected the protein expression of α-SMA by Western blot. TGF-β1 increased the production of α-SMA protein in LX-2 cells, which was inhibited by *Cs*LysoPLA (Fig. 1b). Similarly, immunofluorescence showed the same result in α-SMA protein expression (Fig. 1c).

*Cs*LysoPLA weakened the proliferation of LX-2 cells induced by TGF-β1

The proliferation and migration of activated HSCs are important to liver fibrosis (Chiu et al. 2014). Here, we analyzed the effect of *Cs*LysoPLA on the TGF-β1-induced proliferation of LX-2 cells using a Cell Counting Kit-8 (CCK-8) assay. LX-2 cells significantly proliferated after stimulation with TGF-β1 compared to that of PBS-treated cells at both 12 and 24 h, while the elevation was remarkably decreased by the addition of *Cs*LysoPLA in a dose-dependent manner (Fig. 2).

*Cs*LysoPLA restrained the migration of LX-2 cells caused by TGF-β1

To analyze the migration, a Transwell plate was used. As shown in Fig. 3, compared with PBS-treated cells, TGF-β1


a
α-SMA
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PBS
TGF-β1
TGF-β1+CsLysoPLA1ug/ml
TGF-β1+CsLysoPLA5ug/ml
TGF-β1+CsLysoPLA10ug/ml
TGF-β1+CsFABP10ug/ml
b
PBS
TGF-β1
CsLysoPLA +TGF-β1
α-SMA
GADPH
c
DAPI
α-SMA
Merge
PBS
TGF-β1
C.SLysoPLA +TGF-β1

◀ **Fig. 1** Effect of *Cs*LysoPLA on TGF-β1-induced pro-fibrogenic response to LX-2 cells. **a** Relative expressions of the pro-fibrogenic genes COL1A1, ACTA2, and MMP2 were detected by quantitative real-time q-RT-PCR in LX-2 cells stimulated by TGF-β1 in the presence and absence of *Cs*LysoPLA. **b** Protein expression of α-SMA was examined by Western blot in LX-2 cells stimulated by TGF-β1 with or without *Cs*LysoPLA (GADPH was used as a loading control). **c** α-SMA expression was determined by immunofluorescence staining in LX-2 cells stimulated by TGF-β1 with or without *Cs*LysoPLA (×400). PBS and *Cs*FABP are considered as negative control. Figure is representative of three independent experiments. Data are shown as mean±SEM. * $p<0.05$, ** $p<0.01$

stimulation obviously increased the number of LX-2 cells adhering to the underside of inserts, but *Cs*LysoPLA treatment significantly decreased the migration amount of LX-2 cells induced by TGF-β1.

*Cs*LysoPLA inhibited the phosphorylations of Smad3, JNK, and ERK in response to TGF-β1 in LX-2 cells

As Smad3 protein has been proved as a pivotal mediator in TGF-β1 signaling pathway for fibrogenic responses (Liu et al. 2006), we evaluated the effect of *Cs*LysoPLA on the phosphorylation of Smad3. As shown in Fig. 4a, b, TGF-β1 stimulation presented increased levels of phosphorylated Smad3 at 30 min, but *Cs*LysoPLA treatment significantly reduced phosphorylation of Smad3 induced by TGF-β1. It was previously demonstrated that ERK and JNK modulated Smad3 activity by enhancing its phosphorylation (Liu et al. 2012; Matsuura et al. 2005). We next investigated whether Smad3 phosphorylation was associated with ERK and JNK. As expected,

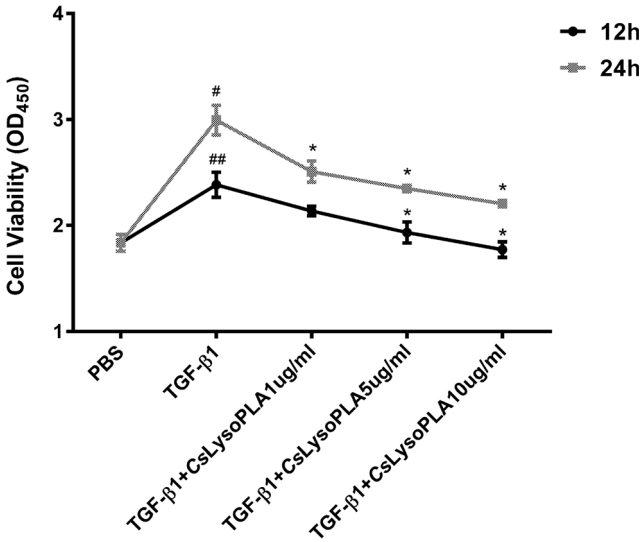

Fig. 2 Effect of *Cs*LysoPLA on the proliferation of LX-2 cells to TGF-β1 by CCK-8 assay. LX-2 cells were treated with different concentrations of *Cs*LysoPLA for 2 h followed by TGF-β1 stimulation for 12 or 24 h. PBS is considered as negative control. The levels were determined by OD_{450} value. Figure is representative of three independent experiments. Data are shown as mean±SEM. *, # $p<0.05$, ## $p<0.01$ (* comparing with TGF-β1, # comparing with PBS)

TGF-β1 alone induced ERK1/2 and JNK2 phosphorylations in LX-2 cells. Interestingly, the phosphorylations of ERK1/2 and JNK2 were inhibited in the presence of *Cs*LysoPLA (Fig. 4c).

Discussion

ESPs are mainly composed of a large quantity of proteases and lipases, especially phospholipases, and play an important role in the pathological impairment processes caused by bacteria or parasites (Hu 2008). ESPs of *C. sinensis* are associated with liver fibrosis caused by *C. sinensis* infection (Wang et al. 2011). It is well known that the activation of HSCs is a central event in liver fibrosis (Pellicoro et al. 2014). Indeed ESPs were found to activate HSCs and induce liver fibrosis in rats (Hu et al. 2009). As a component of ESPs, *Cs*LysoPLA has been well expressed and characterized, but the roles of *Cs*LysoPLA in liver fibrosis remain unclear. In this study, we found that *Cs*LysoPLA could attenuate LX-2 cells response to TGF-β1 in pro-fibrogenic genes and protein expressions, proliferation, and migration.

TGF-β1, a quite potent pro-fibrogenic cytokine, exerts the pro-fibrogenic effects mainly by activating HSCs. The activated HSCs can secrete collagen type I, fibronectin, and matrix metalloproteinases (MMPs) that can degrade extracellular matrix (Gauldie et al. 2007; Siefert and Sarkar 2012). In this study, we found that *Cs*LysoPLA was able to inhibit the TGF-β1-induced expressions of pro-fibrogenic genes, including α-SMA, COL1A1, and MMP2, suggesting that *Cs*LysoPLA had an influence on TGF-β1 activity. Furthermore, we demonstrated that the protein expression of α-SMA was attenuated by *Cs*LysoPLA in LX-2 cells, suggesting that *Cs*LysoPLA had the ability of inhibiting HSC activation. Although the previous data showed that *Cs*LysoPLA had a direct effect on activating LX-2 cells through upregulation of pro-fibrogenic genes (Zhang et al. 2013), our results have no contradiction with it. Helminths are complex eukaryotic organisms with the ability to modulate the immune system for strengthening their longevity in the infected host (Maizels and Yazdanbakhsh 2003). Helminths infection is characterized by a Th2-dominated immune response, which have protective effects in inflammatory diseases, such as allergic airway inflammation, inflammatory bowel disease, and multiple sclerosis, by producing immune regulatory products and promoting regulatory circuits to keep their niche (Maizels et al. 2004; Elliott and Weinstock 2012). In *C. sinensis*-infected mouse model, the early infective stages reflect a dominant Th1-cell response accompanied by increasing Th1 cytokines production. When the infection progresses, Th1-cell responses switch into Th2-cell responses and Th2 cytokines occupy the dominant position (Choi et al. 2003). This may reflect that helminths accommodate infection into an injury limitation

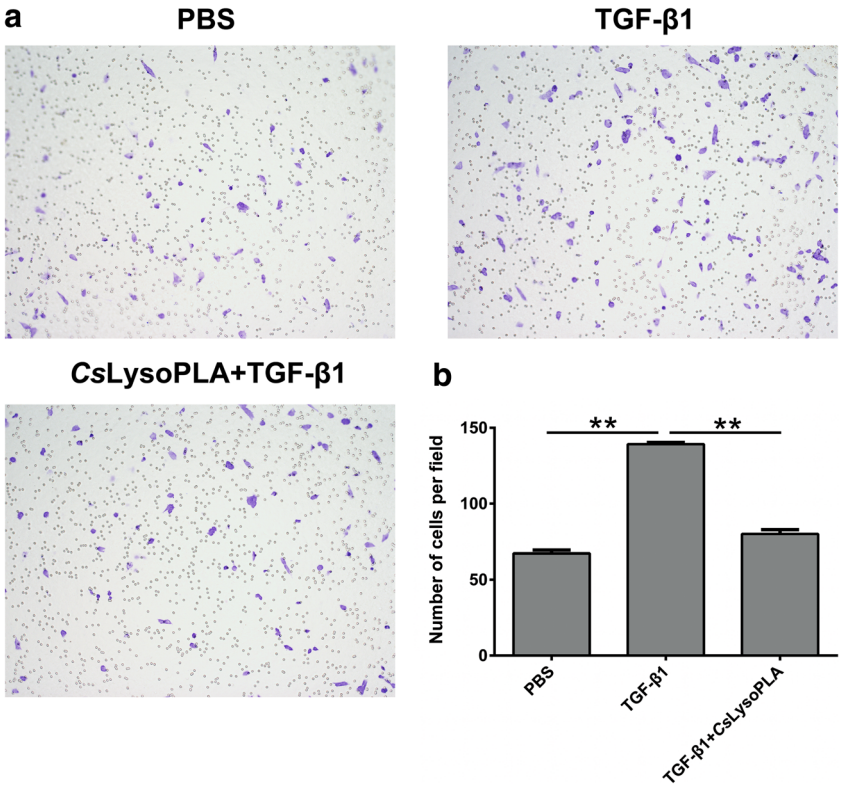

Fig. 3 Effect of *Cs*LysoPLA on the migration of LX-2 cells to TGF-β1. **a** The migration was analyzed using a Transwell plate. LX-2 cells were treated with *Cs*LysoPLA for 2 h followed by TGF-β1 stimulation for 12 or 24 h. Light microscopy was used to collect images of migrated cells. **b** The number of migration cells was counted as a mean of five randomly selected fields. PBS is considered as negative control. Figure is representative of three independent experiments. Data are shown as mean±SEM. ** $p<0.01$

state for the purpose of minimizing pathological impairment (Allen and Maizels 2011). Liver fibrosis is a consequence of inflammatory processes with immunomodulatory as well as *C. sinensis* infection (Pellicoro et al. 2014; Wick et al. 2013). The adult *C. sinensis* has a life span of 20 to 25 years in intrahepatic bile ducts, resulting in persistent infection (Choi et al. 2004). This suggests that the *C. sinensis* not only induces the initiation of inflammation in liver but also confines the development, reflecting the harmonious interplay between host and *C. sinensis*. This adaptive interplay may be the result of modulation of various molecules from ESPs. In addition, TGF-β1 has shown no significant increase in the early infection, but it presents a gradually increasing trend with increased degree of liver fibrosis (Yan et al. 2015). Our current work

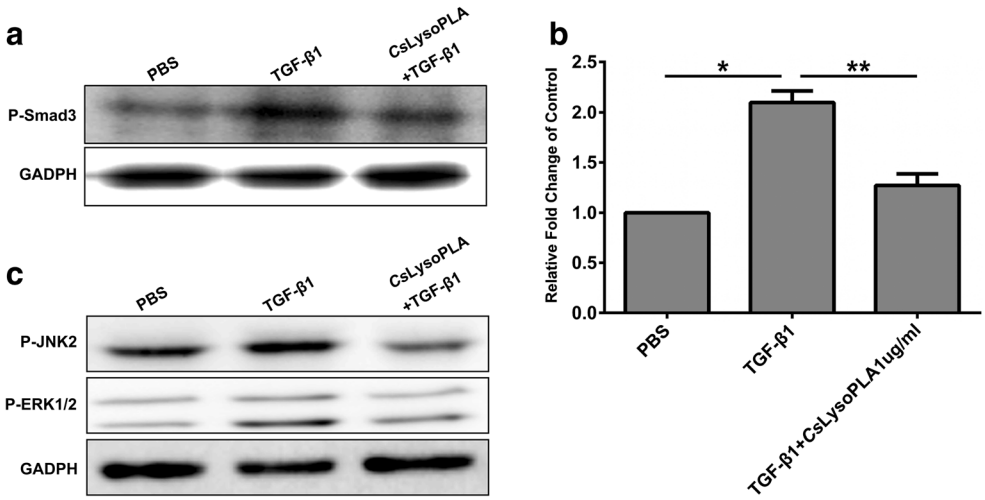

Fig. 4 Effects of *Cs*LysoPLA on the activation of Smad3, ERK1/2, and JNK2 in TGF-β1-activated LX-2 cells. LX-2 cells were stimulated with TGF-β1 in the presence or absence of *Cs*LysoPLA (10 μg/ml) for 30 min. **a** Protein expression of phosphorylation of Smad3 was detected by Western blot. GADPH was used as a loading control. PBS is used as negative control. **b** The relative phosphorylation of Smad3 quantification of Western blot was performed to view the difference between different stimulation groups. **c** Protein expressions of phosphorylation of JNK2 and ERK1/2 were detected by Western blot. Figure is representative of three independent experiments. Data are shown as mean±SEM. * $p<0.05$ ** $p<0.01$

showed the opposite result to the previous report that *Cs*LysoPLA promoted pro-fibrogenic genes expression directly in LX-2 cells. We hypothesize that *Cs*LysoPLA plays different roles largely depending on the stage of *C. sinensis* infection. *Cs*LysoPLA induces directly HSC activation in the initial infection stage when TGF-β1 have not yet been elicited by HSC or other immune cells. In the natural progression of infection, increased production of TGF-β1 makes its pro-fibrotic roles increasingly prominent. To limit the effects of TGF-β1, *Cs*LysoPLA plays the inhibitory role in HSCs activation, and TGF-β1 itself converts into anti-inflammatory effects (Lan 2011). This well illustrates the adaptive relationship between host and *C. sinensis*.

We also investigated whether *Cs*LysoPLA has an inhibiting effect on the proliferation and migration of LX-2 cells. We observed that TGF-β1 promoted the proliferation and migration of LX-2 cells, which was inhibited by the treatment of *Cs*LysoPLA, suggesting that *Cs*LysoPLA can limit the expansion of injury areas to retard the development of fibrosis. Proliferation and migration of HSCs can increase the number of cells to the sites of injury, which are known as important events in the progression of liver fibrosis (Hernandez-Gea and Friedman 2011). The activated HSCs secrete excessive ECM; in return, ECM provide signals for proliferation and migration of activated HSCs by specific membrane adhesion receptors, such as integrin family, ADAM molecules, discoidin domain recptors as well as chemokines, like PDGF-BB, TGF-β1, in a MMP-independent manner (Hernandez-Gea and Friedman 2011; Yang et al. 2003).

TGF-β/Smad signaling pathway is one of the key fibrogenic and inflammatory pathways in the liver (Gressner and Weiskirchen 2006; Flanders 2004). Among the Smad proteins, Smad3 seems to be a prominent one. Smad3 knock-out mice exhibited reduced fibrosis after the fibrogenic stimulus CCL_4, and HSCs from the mice showed higher proliferation and a reduction of collagen type I expression (Schnabl et al. 2001). We observed decreased Smad3 phosphorylation in TGF-β1-stimulated LX-2 cells with the treatment of *Cs*LysoPLA. In addition to Smad3, the reduction of phosphorylations of JNK2 and ERK1/2 was also observed. Our results are consistent with the previous reports that the Smad pathway is not the unique way for TGF-β to regulate cellular functions, so are the ERK and JNK pathways (Liu et al. 2012; Javelaud and Mauviel 2005; Guo and Wang 2009). Thus, *Cs*LysoPLA decreased the response of LX-2 cells to TGF-β1 by inhibiting both of the Smad3 and MAPK pathways.

In conclusion, for the first time, our work provided a strong evidence for that *Cs*LysoPLA can act as an indirect anti-fibrogenic regulator of LX-2 cells. Our results also indicate an important function of *Cs*LysoPLA in coordinating *C. sinensis*–host interactions. Further studies should investigate in vivo models to validate our in vitro data. Nonetheless, *Cs*LysoPLA may be the potential target for liver fibrosis therapy.

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Conflict of interest The authors declare that they have no competing interests.

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