

Echinococcus granulosus cyst fluid suppresses inflammatory responses by inhibiting TRAF6 signalling in macrophages

Research Article

*These authors contributed equally to this study.

Cite this article: Lin K *et al.* (2021). *Echinococcus granulosus* cyst fluid suppresses inflammatory responses by inhibiting TRAF6 signalling in macrophages. *Parasitology* **148**, 887–894. <https://doi.org/10.1017/S0031182021000548>




Received: 13 January 2021
Revised: 19 March 2021
Accepted: 21 March 2021
First published online: 29 March 2021

Keywords:

Cystic echinococcosis; EgCF; inflammation; macrophages; TRAF6

Authors for correspondence:

Xueling Chen,
E-mail: chenxueling@shzu.edu.cn;
Jun Hou, E-mail: houjun229@163.com

Ke Lin^{1,*}, Di Zhou^{2,*}, Min Li³, Jin Meng¹, Feiming He¹, Xiaofeng Yang¹,
Dan Dong¹ , Xian Wang¹, Xiangwei Wu⁴, Xueling Chen¹  and Jun Hou¹ 

¹Department of Immunology, Key Laboratory of Xinjiang Endemic and Ethnic Diseases, Shihezi University School of Medicine, Shihezi, Xinjiang, China; ²Medical Laboratory, the First Affiliated Hospital, Shihezi University School of Medicine, Shihezi, Xinjiang, China; ³Nursing School of Shihezi University, Shihezi, Xinjiang, China and ⁴Department of Hepatobiliary Surgery, the First Affiliated Hospital, Shihezi University School of Medicine, Shihezi, Xinjiang, China

Abstract

Echinococcus granulosus sensu lato has complex defence mechanisms that protect it from the anti-parasitic immune response for long periods. *Echinococcus granulosus* cyst fluid (EgCF) is involved in the immune escape. Nevertheless, whether and how EgCF modulates the inflammatory response in macrophages remains poorly understood. Here, real-time polymerase chain reaction and enzyme-linked immunosorbent assay revealed that EgCF could markedly attenuate the lipopolysaccharide (LPS)-induced production of pro-inflammatory factors including tumour necrosis factor- α , interleukin (IL)-12 and IL-6 but increase the expression of IL-10 at mRNA and protein levels in mouse peritoneal macrophages and RAW 264.7 cells. Mechanically, western blotting and immunofluorescence assay showed that EgCF abolished the activation of nuclear factor (NF)- κ B p65, p38 mitogen-activated protein kinase (MAPK) and ERK1/2 signalling pathways by LPS stimulation in mouse macrophages. EgCF's anti-inflammatory role was at least partly contributed by promoting proteasomal degradation of the critical adaptor TRAF6. Moreover, the EgCF-promoted anti-inflammatory response and TRAF6 proteasomal degradation were conserved in human THP-1 macrophages. These findings collectively reveal a novel mechanism by which EgCF suppresses inflammatory responses by inhibiting TRAF6 and the downstream activation of NF- κ B and MAPK signalling in both human and mouse macrophages, providing new insights into the molecular mechanisms underlying the *E. granulosus*-induced immune evasion.

Introduction

Echinococcosis refers principally to two severe chronic helminthic diseases, cystic echinococcosis and alveolar echinococcosis, caused by the cystic growth or the intrahepatic tumour-like growth of *Echinococcus granulosus* (*E. granulosus*) sensu lato and *Echinococcus multilocularis*, respectively (McManus *et al.*, 2012; Singh *et al.*, 2014; Gottstein *et al.*, 2017; Wen *et al.*, 2019). Human cystic echinococcosis (hydatid disease) most commonly occurs in internal organs, especially the liver and lung (da Silva, 2010). Hydatid disease continues to be a substantial cause of morbidity and mortality in many parts of the world and is challenging to be eliminated (BelhassenGarcía *et al.*, 2014). At present, the early diagnosis of echinococcosis is not precise, and the prevention effect is low. Surgical operation is the primary treatment for hydatidosis, but the injury is severe. Moreover, the cysts are easy to rupture during the surgery, and the protoscoleces in the cysts may enter the tissue and cause re-infection (Toumi *et al.*, 2017). Therefore, further exploring the interaction between *E. granulosus* sensu lato and the host in the early stage of infection is of great significance for developing novel immunotherapies.

Parasitic infections induce host immune responses that eliminate the invading parasites. However, parasites have evolved to develop effective strategies to evade host immune attacks and survive in a hostile environment for long periods (Shao *et al.*, 2019). *Echinococcus granulosus* sensu lato could escape host immunosurveillance by interfering with monocyte differentiation and DC maturation (Wang *et al.*, 2015a, b). In addition to the fibrous cyst's physical barrier, components in the *E. granulosus* cyst fluid (EgCF) are involved in the escape from the host's immune response (Li *et al.*, 2013; Zhang *et al.*, 2016). Hydatid fluid is a complex mixture of components derived from both the parasite and the host (Ahn *et al.*, 2015). Proteomic analysis of hydatid cyst fluid from different anatomical locations revealed variations in protein expression and the extensive exchange of proteins between the metacystode and surrounding host environment cross the cyst wall through unknown mechanisms (Monteiro *et al.*, 2010; Zeghir-Bouteldja *et al.*, 2017; Zhou *et al.*, 2019). Cyst fluid is a massive reservoir of immune-modulators, such as serodiagnostic antigens, low molecular mass metabolites and extracellular vesicles (Diaz, 2017; Zhou *et al.*, 2019). Besides protoscoleces, various immunomodulatory effects have been attributed to EgCF (Mokhtari Amirmajidi *et al.*, 2011; Wang *et al.*, 2015a; Zhou *et al.*, 2019; Yang *et al.*, 2021). We have previously demonstrated

glycomolecules in EgCF could interfere with the Toll-like receptor 4 (TLR4)-mediated activation of DCs and the downstream inflammatory factors (Hou *et al.*, 2020). Additionally, EgCF could promote T lymphocytes' differentiation into Treg cells and induce transforming growth factor- β secretion, subsequently impairing NK cell effector function (Yin *et al.*, 2014). Nevertheless, how EgCF modulates the inflammatory response in macrophages remains poorly understood.

Macrophages play a critical role in the initiation of inflammation by releasing inflammatory mediators and pro-inflammatory cytokines. Macrophages recognize microorganisms *via* a limited number of pattern recognition receptors, including TLRs, which play crucial roles in innate immune responses (Iwasaki and Medzhitov, 2015; Browne, 2020). Recognition of pathogen components such as lipopolysaccharides (LPS) by TLR4 on macrophages results in the activation of mitogen-activated protein kinases (MAPKs) and the transcription factor nuclear factor- κ B (NF- κ B) signalling pathways, leading to the production of pro-inflammatory cytokines (Schmid *et al.*, 2012; Vidya *et al.*, 2018). Tumour necrosis factor receptor-associated factors (TRAFs) form a family of adapter molecules, coupling the tumour necrosis factor receptor (TNFR) superfamily to intracellular signalling events (Yang and Sun, 2015; Park, 2018). Among them, TRAF6 plays a pivotal role in the TLR4-mediated signalling pathway. TRAF6 forms a homo-dimer and catalyses K63-linked ubiquitination; it can also be modified by K48-linked ubiquitination and proteasomal degraded to block the inflammatory process (Walsh *et al.*, 2015). For instance, *Leishmania donovani* infection impairs K63-linked ubiquitination of TRAF6 by induction of host deubiquitinating enzyme A20 and suppresses the production of interleukin (IL)-12 and TNF- α (Srivastav *et al.*, 2012). Death-associated protein kinase-related apoptosis-inducing kinase 1 inhibits the pro-inflammatory signalling pathway by targeting TRAF6 for degradation, thereby suppressing inflammatory signalling-mediated tumour growth and metastasis in advanced cervical cancer cells (Park *et al.*, 2020). However, the role of TRAF6 in macrophages during *E. granulosus* *sensu lato* infection remains poorly understood.

In this study, we speculated that EgCF might regulate inflammation response by targeting TRAF6 protein. Using three macrophage models, we provided evidence that EgCF could promote the proteasomal degradation of TRAF6 and suppress the downstream activation of NF- κ B and MAPK signalling pathways and subsequent cytokine production.

Materials and methods

Cells and cell culture

Mouse peritoneal macrophages were isolated from C57BL/6 female mice (6–8 weeks, 16–25 g). Briefly, mice were sacrificed by cervical dislocation. Subsequently, mice were injected intraperitoneally with Dulbecco's modified Eagle's medium (DMEM) using a 5 mL syringe. The abdomen was gently massaged without removing the needle, then most of the fluid from the peritoneum was slowly withdrawn with the syringe (You-Nian *et al.*, 2016). Collected peritoneal exudate cells were washed with phosphate-buffered saline (PBS) three times and centrifuged at 1500 rpm for 5 min. Cells were resuspended and plated in six-well plates at a density of 1×10^6 cells per well in DMEM containing L-glutamine supplemented with 10% fetal bovine serum (FBS) according to previous reports (Chen *et al.*, 2020; Gao *et al.*, 2020; He *et al.*, 2020). After overnight culture, the non-adherent cells were removed, and the attached cells (enriched in peritoneal macrophages) were treated with LPS ($1 \mu\text{g mL}^{-1}$) or LPS and

EgCF (2.15 mg mL^{-1}) for the indicated periods as a previous report (Hou *et al.*, 2020).

The mouse macrophage cell line RAW 264.7 and human monocytic cell line THP-1 were purchased from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. RAW 264.7 cells were cultured in DMEM (Gibco) supplemented with 10% FBS, 1% penicillin–streptomycin (Gibco) in a humidified incubator with 5% CO₂ at 37°C following the provider's instructions. THP-1 cells were maintained in RPMI 1640 medium (Gibco) containing 10% FBS, 1% penicillin–streptomycin and 0.05 mM β -mercaptoethanol (Gibco). THP-1 cells were differentiated into macrophages by 150 nM PMA treatment for 24 h.

Preparation of *Echinococcus granulosus* cyst fluid

Sheep liver hydatid cysts containing protoscoleces were acquired from a slaughterhouse in Shihezi, Xinjiang, China. After sterilizing the *E. granulosus sensu lato*-infected sheep liver with 75% alcohol, cyst fluid in the non-calcified translucent vesicles was extracted using a 50 mL sterile syringe. The upper layer mixture after standing was collected and centrifuged at 3000 rpm for 10 min. The resulting supernatant from different sheep livers was mixed, filtered through a 0.22- μm filter, quantified for protein concentration and stored in a -80°C refrigerator for later use.

Quantitative real-time polymerase chain reaction (PCR)

RNA was extracted using a total RNA kit (Omega) and converted into cDNA using the RevertAid RT Reverse Transcription Kit (Thermo Scientific). The primer sequences are listed in Table 1. Quantitative real-time PCR was performed on a CFX96 Touch detection system (Bio-Rad) using the TB Green Premix Ex Taq II (Tli RNaseH Plus; TaKaRa). Relative expression levels of target genes were calculated by normalizing to β -actin using the $2^{-\Delta\Delta\text{Ct}}$ method and expressed as fold-changes to that of the control group.

Enzyme-linked immunosorbent assays (ELISAs)

Cell culture medium from mouse peritoneal macrophages or RAW 264.7 cells treated with LPS with or without EgCF was collected and centrifuged at 3000 rpm for 10 min. The supernatants were collected and used for detecting TNF- α , IL-6 and IL-10 concentrations by mouse ELISA kits according to the manufacturer's instructions (Multisciences Biotech).

Western blot analysis

Cells were harvested at indicated times in the RIPA lysis buffer. Samples were electrophoretically separated on a 10% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% bovine serum albumin for 2 h at room temperature, followed by incubation at 4°C overnight with primary antibodies specific to phospho-NF- κ B p65 (p-p65, #3033), NF- κ B p65 (#3987), phospho-p38 MAPK (p-p38, #9211), p38 MAPK (#9212), phospho-extracellular signal-regulated kinase (ERK)1/2 (p-ERK1/2, #4370), ERK1/2 (#4695) from Cell Signalling Technology, TRAF6 (#ab137452) from Abcam and β -actin (#TA-09) from ZSGB-BIO. Following washing three times for 10 min with 0.1% Tween-20 in TBST, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing, the immune complexes were detected by an enhanced chemiluminescence assay (Thermo Fisher). β -Actin was served as a loading control.

Table 1. Primer sequences for real-time PCR

Gene	Forward	Reverse
Mouse IL-6	GAGGATACCCTCCCAACAGACC	AAGTGCATCATCGTTGTTTCATACA
Mouse TNF- α	TGATCCGCGACGTGGAA	ACCGCCTGGAGTTCTGGAA
Mouse IL-12	GGGGTGTAAACAGAAAG	CATGAGGAATTGTAATAGCG
Mouse IL-10	ACCTGGTAGAAGTGATGCC	CACCTTGGTCTTGGAGCT
Mouse β -actin	GGCTATGCTCTCCCTCACG	GAGCAACATAGCACAGCTTCTCTTT
Human IL-6	GATGGATGCTTCCAATCTGGAT	AGTTCTCCATAGAGAACAACATA
Human TNF- α	TTCCCCAGGGACCTCTCTAATC	GAGGGTTTGCTACAACATGGGCTAC
Human β -actin	GAGAAAATCTGGCACCACACC	GGATAGCACAGCCTGGATAGCAA

Immunofluorescence assay

Cells grown on glass coverslips were fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100. Then cells were washed three times with PBS and blocked with 1% bovine serum albumin (BSA) for 1 h at 25°C. Samples were incubated with the primary antibody against p65 in PBST and 1% BSA overnight at 4°C. Subsequently, cells were washed three times in PBS, incubated with Rhodamine-conjugated Goat anti-Rabbit IgG (H + L) Secondary Antibody (ZSGB-BIO #ZF-0316) for 1 h at room temperature, and counterstained with diamidino-2-phenylindole. The images were observed under a fluorescence microscope (Olympus).

Statistical analysis

All experiments were independently repeated with similar results. Data are represented as mean \pm S.E.M. of at least three replicates. Statistical analyses were performed using GraphPad Prism version 8.0 software. Comparison between groups was made using Student's *t* test or one-way analysis of variance. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; ns, not significant.

Results

EgCF inhibits the LPS-induced inflammatory response in mouse peritoneal macrophages

Production of inflammatory mediators is a crucial response of macrophages to LPS stimulation. To figure out whether EgCF exerts an inhibitory effect on LPS-induced macrophage activation, primary mouse peritoneal macrophages were treated with EgCF and LPS. Real-time PCR analysis showed that LPS highly induced the mRNA levels of TNF- α , IL-12 and IL-6. In contrast, EgCF exposure significantly attenuated the expression of the inflammatory cytokines evoked by LPS at 1 h post-treatment and lasted to 6 h post-treatment but enhanced IL-10 mRNA levels compared to the LPS alone group (Fig. 1A–D). Furthermore, we measured these cytokines in the cell culture supernatant by ELISA. The LPS-induced secretion of TNF- α and IL-6 protein in the cell culture supernatant was also dramatically inhibited, whereas IL-10 protein secretion was elevated in the presence of EgCF (Fig. 1E–G). Taken together, these results indicated that EgCF caused a shift from pro-inflammatory to anti-inflammatory cytokine production in mouse peritoneal macrophages.

EgCF inhibits the LPS-induced inflammatory response in RAW 264.7 cells

To further validate the immunomodulatory effect of EgCF in macrophages, the RAW 264.7 cell line was also treated with

EgCF and LPS. Results showed that EgCF could dramatically inhibit the LPS-induced mRNA expression of IL-6 and TNF- α (Fig. 2A and B). In contrast, the mRNA expression of IL-10 significantly increased at 6 h upon exposure to EgCF in RAW 264.7 cells (Fig. 2C). Exposure with EgCF also suppressed the LPS-induced IL-6 protein production while enhancing the secretion of IL-10 in RAW 264.7 cells (Fig. 2D and E). These results confirmed that EgCF suppressed the production of pro-inflammatory factors and exerted an anti-inflammatory potential in mouse macrophages.

EgCF suppresses the LPS-induced activation of NF- κ B and MAPK signalling and promotes the proteasomal degradation of TRAF6 in mouse macrophages

It is well established that NF- κ B and MAPKs have essential roles in the LPS-induced inflammation process. However, the signal transduction mechanisms involved in EgCF-exposed macrophages remain poorly understood. These cascades' activation was checked by detecting phosphorylation levels of NF- κ B p65, p38 MAPK and ERK1/2 using immunoblot. Intriguingly, results showed that the LPS-induced NF- κ B p65 phosphorylation was inhibited in RAW 264.7 cells post-EgCF treatment. Besides, EgCF also suppressed the LPS-induced phosphorylation of p38 MAPK and ERK1/2 in RAW 264.7 cells (Fig. 3A). Similar inhibition of the LPS-induced activation of p65, p38 and ERK1/2 signalling was also observed in mouse peritoneal macrophages (Fig. 3B). Furthermore, immunofluorescence assay indicated that the LPS-induced nuclear translocation of NF- κ B p65 was abolished in EgCF-treated mouse peritoneal macrophages (Fig. 3C). These findings collectively suggested that EgCF suppressed the LPS-induced activation of NF- κ B and MAPK pathways in mouse macrophages.

As previously reported, TRAF6 could mediate signal transduction from the TLR family, and it is a key adaptor for the activation of the NF- κ B signalling pathway. Therefore, we reasoned that EgCF might modulate the NF- κ B signalling through modulation of TRAF6. To verify the hypothesis, we further measured the TRAF6 expression level upon EgCF treatment by western blotting. The results showed that the expression of TRAF6 protein markedly decreased in both EgCF-treated RAW 264.7 cells and mouse peritoneal macrophages when compared to the LPS alone group (Fig. 4A and B), in concordance with the reduced NF- κ B p65 phosphorylation. To explore whether EgCF might affect the degradation of TRAF6 protein, we treated RAW 264.7 cells with the protein synthesis inhibitor cycloheximide (CHX), an inhibitor of protein synthesis. Indeed, results showed that EgCF could accelerate TRAF6 protein degradation (Fig. 4C), indicating the post-translational modification of TRAF6 by EgCF. To examine whether the shortened TRAF6

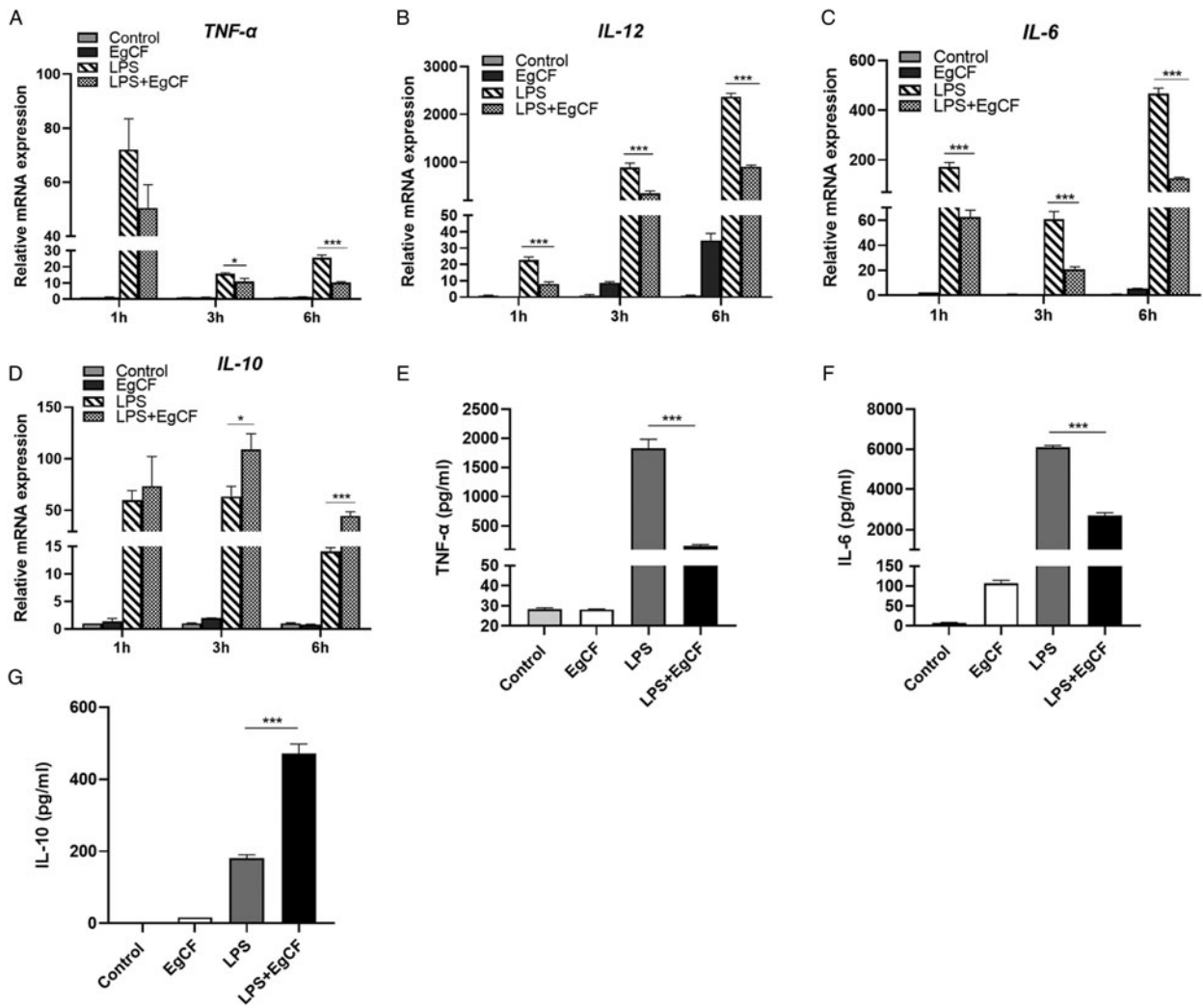


Fig. 1. EgCF inhibits LPS-induced inflammatory response in mouse peritoneal macrophages. Mouse peritoneal macrophages were treated with EgCF (2.15 mg mL⁻¹) and LPS (1 μg mL⁻¹) for the indicated time points. The mRNA expression levels of TNF-α (A), IL-12 (B), IL-6 (C) and IL-10 (D) were analysed by real-time PCR. Mouse peritoneal macrophages were incubated with LPS in the presence or absence of EgCF for 24 h. Secretion of TNF-α (E), IL-6 (F) and IL-10 (G) in the cell culture supernatants was measured by ELISA.

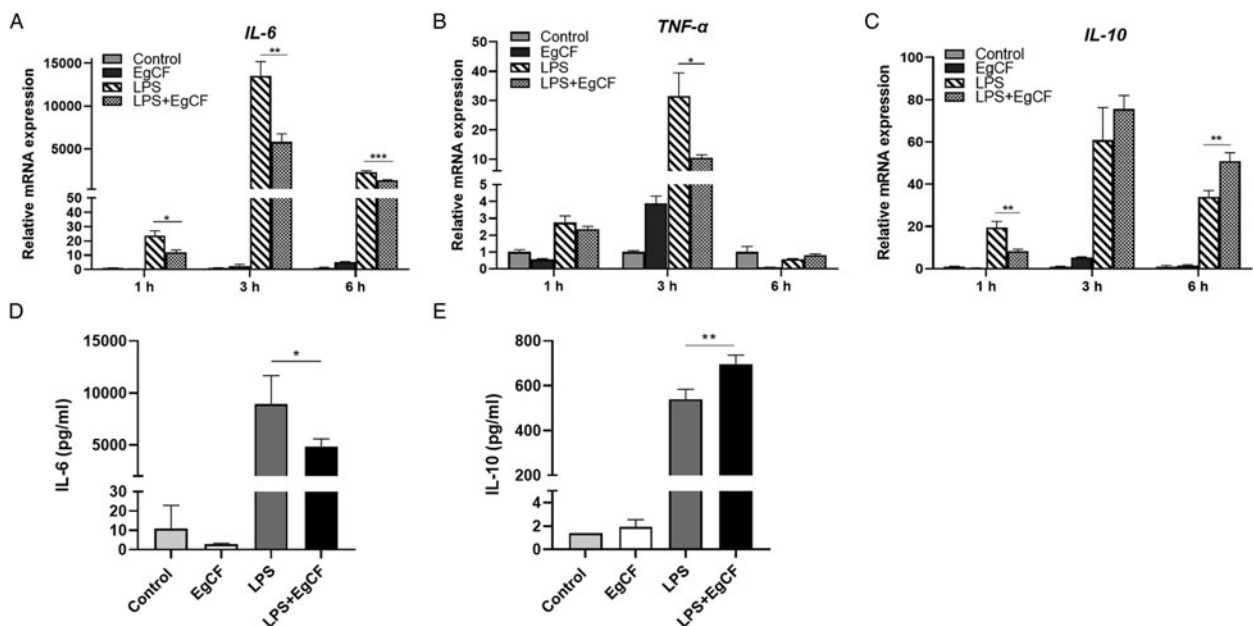


Fig. 2. EgCF suppresses LPS-induced inflammatory response in RAW 264.7 cells. RAW 264.7 cells were treated with EgCF (2.15 mg mL⁻¹) and LPS (1 μg mL⁻¹) for the periods indicated. The mRNA expression levels of IL-6 (A), TNF-α (B) and IL-10 (C) were analysed by real-time PCR. RAW 264.7 were incubated with LPS in the presence or absence of EgCF for 24 h. Secretion of IL-6 (D) and IL-10 (E) in cell culture supernatants was measured by ELISA.

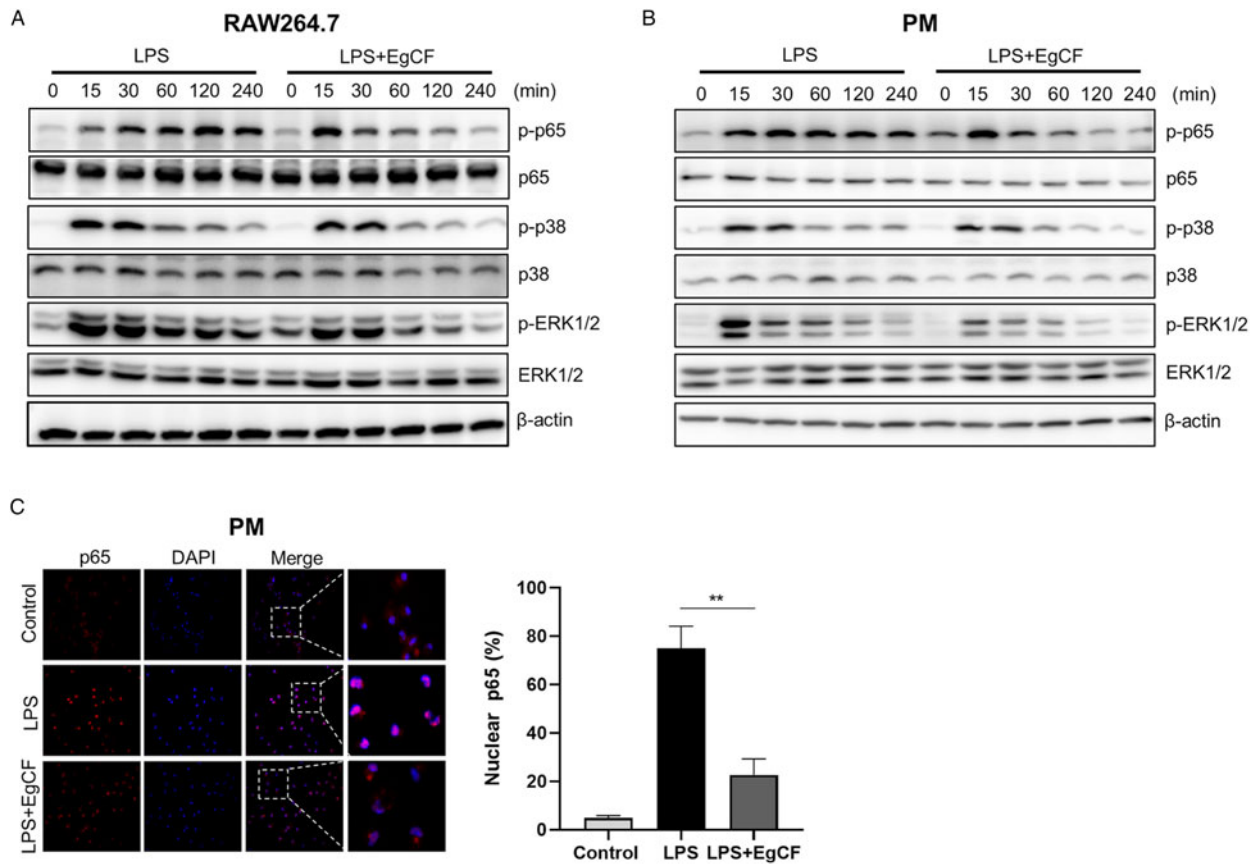


Fig. 3. EgCF inhibits LPS-induced NF- κ B and MAPK signalling activation in mouse macrophages. RAW 264.7 cells (A) and mouse peritoneal macrophages (B) were treated with EgCF and LPS for the indicated time points. Activation status of NF- κ B p65, p38 MAPK and ERK1/2 signalling was detected via western blotting. PM, peritoneal macrophages. (C) Mouse peritoneal macrophages were incubated with LPS in the presence or absence of EgCF for 4 h. Cells were subjected to immunofluorescence assay for NF- κ B p65 nuclear translocation, and the rates of peritoneal macrophages with nuclear p65 staining were quantified.

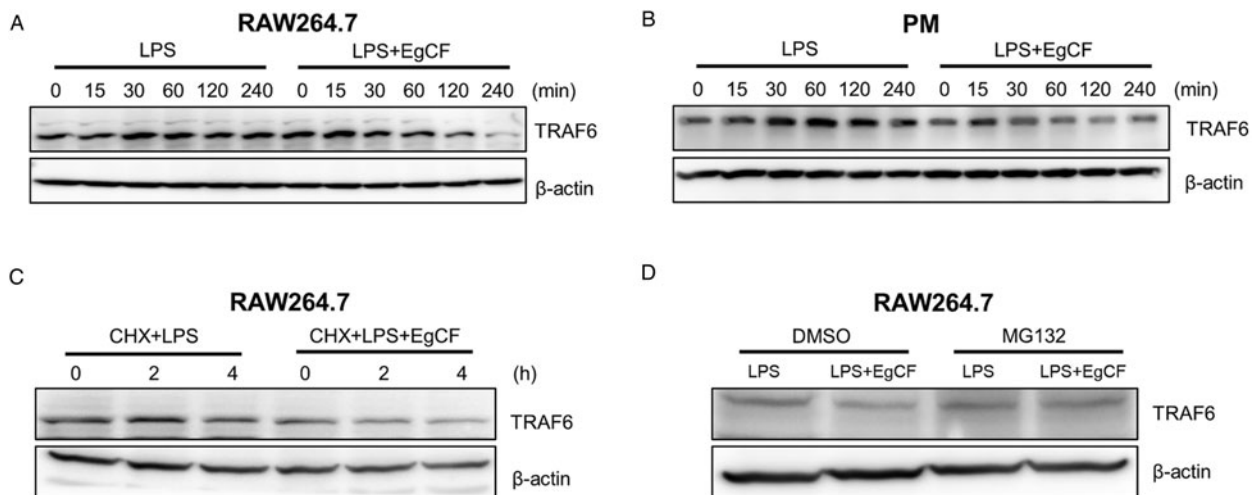


Fig. 4. EgCF promotes the proteasomal degradation of TRAF6 in mouse macrophages. RAW 264.7 cells (A) and mouse peritoneal macrophages (B) were treated with EgCF and LPS for the periods indicated. TRAF6 protein levels were evaluated by western blotting. PM, peritoneal macrophages. (C) The protein expression of TRAF6 was checked by western blotting in RAW 264.7 cells treated with EgCF and LPS in the presence of CHX ($50 \mu\text{g mL}^{-1}$) for 2 or 4 h. (D) RAW 264.7 cells were treated with EgCF and LPS in the presence of MG132 ($20 \mu\text{M}$) to block the proteasomal degradation for 4 h. Western blotting was used to analyse TRAF6 protein levels.

half-life was mediated through the ubiquitin–proteasome pathway, we treated RAW 264.7 cells with the proteasome inhibitor MG132. MG132 treatment rescued TRAF6 protein levels in EgCF-exposed mouse macrophages (Fig. 4D), suggesting that EgCF promoted TRAF6 degradation in a proteasome-dependent manner.

EgCF inhibits the LPS-induced inflammatory response by promoting the proteasomal degradation of TRAF6 in human macrophages

To determine whether the EgCF's anti-inflammatory effect observed in mouse macrophages also exists in human, we

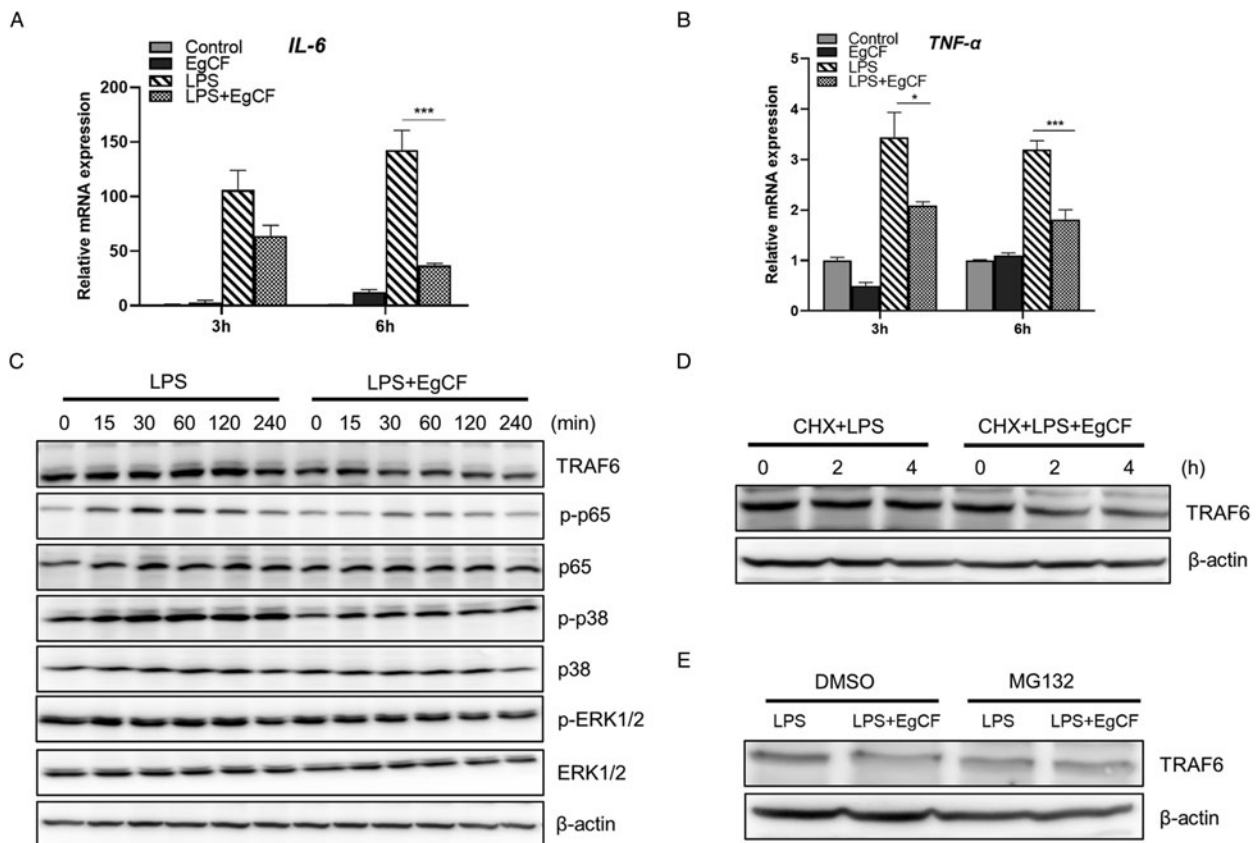


Fig. 5. EgCF inhibits LPS-induced inflammatory response by promoting proteasomal degradation of TRAF6 in human macrophages. Relative mRNA expression levels of IL-6 (A) and TNF- α (B) in THP-1 cells treated with EgCF and LPS for the indicated time points were measured by real-time PCR. (C) The protein levels of TRAF6 and the activation status of NF- κ B p65, p38 MAPK and ERK1/2 signalling in THP-1 cells stimulated with LPS and EgCF for the periods indicated were checked by western blotting. (D) THP-1 cells were treated with EgCF and LPS in the presence of CHX ($50 \mu\text{g mL}^{-1}$) for 2 or 4 h. Western blotting was used to analyse TRAF6 protein expression. (E) The protein levels of TRAF6 were evaluated by western blotting in THP-1 cells treated with EgCF and LPS in the presence of MG132 ($20 \mu\text{M}$) to block the proteasomal degradation for 4 h.

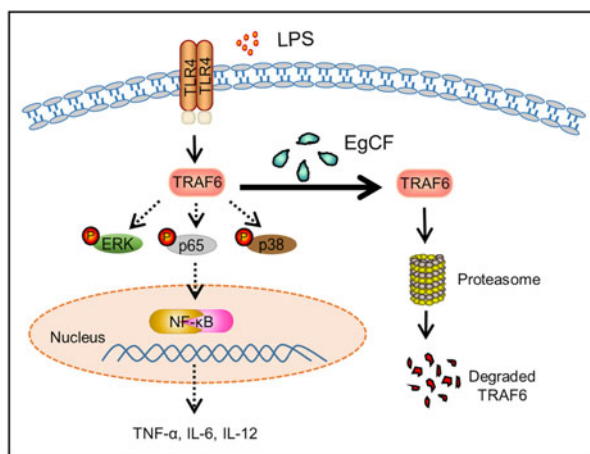


Fig. 6. Working model for EgCF suppressing LPS-induced inflammatory response in macrophages. EgCF promotes the proteasomal degradation of TRAF6 and subsequently impairs the LPS-induced activation of NF- κ B and MAPKs signalling pathways and thus the inflammatory response.

measured the inflammatory response in human THP-1 cells. As shown in Fig. 5A and B, a marked inhibitory effect of EgCF treatment on the mRNA expression of LPS-induced pro-inflammatory cytokines IL-6 and TNF- α was observed in THP-1 macrophages. We also examined the phosphorylation of critical components in the NF- κ B and MAPK signalling pathways in THP-1 cells. Compared to the LPS group, the phosphorylation of p65 in

NF- κ B signalling pathway and p38, ERK1/2 in MAPK signalling pathway was decreased in a time-dependent manner in the EgCF-treated group (Fig. 5C), consistent with the phenomenon observed in mouse RAW 264.7 cells and primary peritoneal macrophages. Western blotting showed that EgCF treatment decreased the protein stability of TRAF6 in the presence of CHX (Fig. 5D), whereas MG132 could rescue the expression of TRAF6 in THP-1 cells (Fig. 5E), suggesting that the EgCF-promoted anti-inflammatory response and TRAF6 proteasomal degradation were conserved in human macrophages.

Discussion

Cystic echinococcosis is a chronic, complex and neglected zoonotic infection. As a globally distributed parasitic disease, the infection of *E. granulosus sensu lato* poses a considerable threat to human life (OudniM'rad *et al.*, 2015). *Echinococcus granulosus sensu lato* has elaborate defence mechanisms, protecting it from the anti-parasite immune responses (Siracusano *et al.*, 2012). In this study, it was demonstrated that EgCF inhibited the activation of NF- κ B and MAPK signalling pathways and the pro-inflammatory responses by promoting the proteasomal degradation of TRAF6 in both human and mouse macrophages (Fig. 6).

Macrophages are essential cells for inflammatory processes involved in regulating acute inflammatory response and chronic pathological changes (Hasturk *et al.*, 2012). Bacterial LPS was a typical ligand for membrane protein TLR4, which can induce the host's innate immune response (Peri *et al.*, 2010). In this

study, we utilized three different macrophages to investigate the roles of EgCF in the inflammation process. Pro-inflammatory cytokines TNF- α , IL-12 and IL-6, which are regarded as biomarkers of inflammatory processes (Rossol *et al.*, 2011), increased dramatically over the following several hours following LPS stimulation. We provided sufficient and systemic evidence that EgCF reduced the LPS-induced production of pro-inflammatory mediators and cytokines (TNF- α , IL-6 and IL-12) at mRNA and protein levels not only in the mouse RAW 264.7 cell line and primary mouse peritoneal macrophages but also in human THP-1 cells. In line with our findings, *E. multilocularis* vesicles are also able to suppress the LPS-induced release of IL-12 and TNF- α by PBMC (Hübner *et al.*, 2006).

NF- κ B and MAPK pathways are two critical pathways that participated in the LPS/TLR4 signalling cascades. Here, we detected multiple key components in these signalling pathways and found that phosphorylation of NF- κ B p65 was significantly downregulated in all three macrophages after EgCF treatment. Consistently, the inactivation of NF- κ B signalling has been reported to be an immune evasion approach shared by other parasites. For instance, *Giardia lamblia* decreases NF- κ B p65 protein levels and impairs LPS-evoked pro-inflammatory response in mouse RAW 264.7 cells and human monocyte-derived macrophages (Faria *et al.*, 2020). Additionally, EgCF suppressed the LPS-induced phosphorylation of p38 MAPK and ERK1/2 in macrophages. In contrast, p38 MAPK and ERK1/2 are activated in rat hepatocytes upon exposure to *E. multilocularis* vesicle fluid (Lin *et al.*, 2009), suggesting context-dependent effects of these two metacestodes on the host environment.

A recent study shows that resveratrol could suppress NF- κ B activation by attenuating TRAF6 expression and ubiquitination (Jakus *et al.*, 2013; Wang *et al.*, 2017). As an essential molecule in the TLR-induced NF- κ B and MAPK signalling, TRAF6 has been demonstrated to be tightly regulated. TRAF6 can be ubiquitinated with both K48-linked degradative and K63-linked non-degradative chains at multiple locations. K63-linked autoubiquitination of TRAF6 is essential for the formation and activation of the TAK1 complex and NF- κ B signalling (Yanbao *et al.*, 2011). TRAF6 can also be modified by K48-linked ubiquitination, which promotes TRAF6 degradation through proteasome and blocks the inflammatory signal transduction in the TLR4 pathway (Zhou *et al.*, 2010; Zhao *et al.*, 2012). We assumed that EgCF negatively regulated inflammation by targeting TRAF6. Our study verified that EgCF could promote the degradation of TRAF6 in a proteasome-dependent manner, confirming that EgCF could inhibit the activation of NF- κ B and MAPK signalling pathways by promoting proteasomal degradation of TRAF6.

In conclusion, we demonstrate that EgCF could promote the proteasomal degradation of TRAF6 and then inhibit the activation of NF- κ B and MAPK signalling pathways and negatively regulate the inflammatory response. To better understand the *E. granulosus*-induced immune evasion, how *E. granulosus* cyst fluid makes contact with the immune system, and which component functions to suppress macrophages warrant further investigation.

Data

All data generated or analysed during this study are included in this published article.

Author contributions. JH, XC and KL conceived and designed the study. KL, DZ, ML, JM, FH, XY, DD, XW and XW conducted data gathering. KL performed statistical analyses. KL, XC and JH wrote the article.

Financial support. This study was supported by the National Natural Science Foundation of China (Nos. 82060579 and 82060297).

Conflict of interest. The authors declare there are no conflicts of interest.

Ethical standards. Animal study was performed with approval from the Animal Experimental Ethical Inspection of First Affiliated Hospital, Shihezi University School of Medicine.

References

- Ahn CS, Han X, Bae YA, Ma X, Kim JT, Cai H, Yang HJ, Kang I, Wang H and Kong Y (2015) Alteration of immunoproteome profile of *Echinococcus granulosus* hydatid fluid with progression of cystic echinococcosis. *Parasites & Vectors* **8**, 10.
- BelhassenGarcía M, Romeroalegria A, Velascotirado V, AlonsoSardón M, Lopezbernius A, Alvelasuares L, Del Villar LP, Carpioperez A, Galindoperez I and Corderosanchez M (2014) Study of hydatidosis-attributed mortality in endemic area. *PLoS One* **9**, e91342.
- Browne EP (2020) The role of toll-like receptors in retroviral infection. *Microorganisms* **8**, 1787.
- Chen S, Fuller KK, Dunlap JC and Loros JJ (2020) A pro- and anti-inflammatory axis modulates the macrophage circadian clock. *Frontiers in Immunology* **11**, 867.
- da Silva AM (2010) Human echinococcosis: a neglected disease. *Gastroenterology Research and Practice* **2010**, 583297.
- Diaz A (2017) Immunology of cystic echinococcosis (hydatid disease). *British Medical Bulletin* **124**, 121–133.
- Faria CP, Neves BM, Lourenço Á, Cruz MT, Martins JD, Silva A, Pereira S and Sousa MDC (2020) *Giardia lamblia* decreases NF- κ B p65(RelA) protein levels and modulates LPS-induced pro-inflammatory response in macrophages. *Scientific Reports* **10**, 6234.
- Gao Q, Wei A, Chen F, Chen X, Ding W, Ding Z, Wu Z, Du R and Cao W (2020) Enhancing PPARgamma by HDAC inhibition reduces foam cell formation and atherosclerosis in ApoE deficient mice. *Pharmacological Research* **160**, 105059.
- Gottstein B, Soboslay P, Ortona E, Wang J, Siracusano A and Vuitton D (2017) Immunology of alveolar and cystic echinococcosis (AE and CE). *Advances in Parasitology* **96**, 1–54.
- Hasturk H, Kantarci A and Van Dyke TE (2012) Oral inflammatory diseases and systemic inflammation: role of the macrophage. *Frontiers in Immunology* **3**, 118.
- He C, Jiang H, Song W, Riezman H, Tontonoz P, Weston TA, Guagliardo P, Kim PH, Jung R, Heizer P, Fong LG and Young SG (2020) Cultured macrophages transfer surplus cholesterol into adjacent cells in the absence of serum or high-density lipoproteins. *Proceedings of the National Academy of Sciences of the United States of America* **117**, 10476–10483.
- Hou J, Li L, Dong D, Wang L, Wang X, Yang K, Xu X, Chen C, Wu X and Chen X (2020) Glycomolecules in *Echinococcus granulosus* cyst fluid inhibit TLR4-mediated inflammatory responses via c-Raf. *Cellular & Molecular Immunology* **17**, 423–425.
- Hübner MP, Manfras BJ, Margos MC, Eiffler D, Hoffmann WH, Schulz-Key H, Kern P and Soboslay PT (2006) *Echinococcus multilocularis* metacestodes modulate cellular cytokine and chemokine release by peripheral blood mononuclear cells in alveolar echinococcosis patients. *Clinical & Experimental Immunology* **145**, 243–251.
- Iwasaki A and Medzhitov R (2015) Control of adaptive immunity by the innate immune system. *Nature Immunology* **16**, 343–353.
- Jakus PB, Kalman N, Antus C, Radnai B, Tucsek Z, Gallyas F Jr, Sumegi B and Veres B (2013) TRAF6 is functional in inhibition of TLR4-mediated NF- κ B activation by resveratrol. *Journal of Nutritional Biochemistry* **24**, 819–823.
- Li J, Ju Y, Wang X, Zhang Z, Li J, Zhu M and Zhao W (2013) Analysis of the chemical components of hydatid fluid from *Echinococcus granulosus*. *Revista da Sociedade Brasileira de Medicina Tropical* **46**, 605–610.
- Lin RY, Wang JH, Lu XM, Zhou XT, Mantion G, Wen H, Vuitton DA and Richert L (2009) Components of the mitogen-activated protein kinase cascade are activated in hepatic cells by *Echinococcus multilocularis* metacestode. *World Journal of Gastroenterology* **15**, 2116–2124.
- McManus DP, Gray DJ, Zhang W and Yang Y (2012) Diagnosis, treatment, and management of echinococcosis. *BMJ: British Medical Journal* **344**, e3866.
- Mokhtari Amirmajidi M, Sankian M, Eftekharzadeh Mashhadi I, Varasteh A, Vahedi F, Sadrizadeh A and Spotin A (2011) Apoptosis of human lymphocytes after exposure to hydatid fluid. *Iranian Journal of Parasitology* **6**, 9–16.

- Monteiro KM, de Carvalho MO, Zaha A and Ferreira HB (2010) Proteomic analysis of the *Echinococcus granulosus* metacestode during infection of its intermediate host. *Proteomics* **10**, 1985–1999.
- Oudni M^{rad} M, M^{rad} S, Ksia A, Lamiri R, Mekki M, Nouri A, Mezhoud H and Babba H (2015) First molecular evidence of the simultaneous human infection with two species of *Echinococcus granulosus* sensu lato: *Echinococcus granulosus* sensu stricto and *Echinococcus canadensis*. *Parasitology Research* **115**, 1065–1069.
- Park HH (2018) Structure of TRAF family: current understanding of receptor recognition. *Frontiers in Immunology* **9**, 1999.
- Park Y, Pang K, Park J, Hong E, Lee J, Ooshima A, Kim H, Cho J, Han Y, Lee C, Song Y, Park K, Yang K and Kim S (2020) Destabilization of TRAF6 by DRAK1 suppresses tumor growth and metastasis in cervical cancer cells. *Cancer Research* **80**, 2537–2549.
- Peri F, Piazza M, Calabrese V, Damore G and Cighetti R (2010) Exploring the LPS/TLR4 signal pathway with small molecules. *Biochemical Society Transactions* **38**, 1390.
- Rossol M, Heine H, Meusch U, Quandt D, Klein C, Sweet M and Hauschildt S (2011) LPS-induced cytokine production in human monocytes and macrophages. *Critical Reviews in Immunology* **31**, 379–446.
- Schmid D, Gruber M, Piskaty C, Woehs F, Renner A, Nagy Z, Kaltenboeck A, Wasserscheid T, Bazylko A and Kiss AK (2012) Inhibition of NF- κ B-dependent cytokine and inducible nitric oxide synthesis by the macrocyclic ellagitannin oenothien B in TLR-stimulated RAW 264.7 macrophages. *Journal of Natural Products* **75**, 870.
- Shao S, Sun X, Chen Y, Zhan B and Zhu X (2019) Complement evasion: an effective strategy that parasites utilize to survive in the host. *Frontiers in Microbiology* **10**, 532.
- Singh BB, Dhand NK, Ghatak S and Gill JPS (2014) Economic losses due to cystic echinococcosis in India: need for urgent action to control the disease. *Preventive Veterinary Medicine* **113**, 1–12.
- Siracusano A, Delunardo F, Teggi A and Ortona E (2012) Host-parasite relationship in cystic echinococcosis: an evolving story. *Clinical & Developmental Immunology* **2012**, 639362.
- Srivastav S, Kar S, Chande A, Mukhopadhyaya R and Das P (2012) *Leishmania donovani* exploits host deubiquitinating enzyme A20, a negative regulator of TLR signalling, to subvert host immune response. *Journal of immunology (Baltimore, MD: 1950)*, **189**, 924–934.
- Toumi O, Noomen F, Salem R, Rabeh H, Jabra SB, Korbi I, Bannani S, Nasr M, Zouari K, Mondher G and Hamdi A (2017) Intraperitoneal rupture of hydatid cysts. *European Journal of Trauma and Emergency Surgery* **43**, 387–391.
- Vidya MK, Kumar VG, Sejian V, Bagath M, Krishnan G and Bhatta R (2018) Toll-like receptors: significance, ligands, signalling pathways, and functions in mammals. *International Reviews of Immunology* **37**, 20–36.
- Walsh MC, Lee J and Choi Y (2015) Tumor necrosis factor receptor-associated factor 6 (TRAF6) regulation of development, function, and homeostasis of the immune system. *Immunological Reviews* **266**, 72–92.
- Wang Y, Wang Q, Lv S and Zhang S (2015a) Different protein of *Echinococcus granulosus* stimulates dendritic induced immune response. *Parasitology* **142**, 879–889.
- Wang Y, Zhou H, Shen Y, Wang Y, Wu W, Liu H, Yuan Z, Xu Y, Hu Y and Cao J (2015b) Impairment of dendritic cell function and induction of CD4 (+)CD25(+)Foxp3(+) T cells by excretory-secretory products: a potential mechanism of immune evasion adopted by *Echinococcus granulosus*. *BMC Immunology* **16**, 44.
- Wang G, Hu Z, Fu Q, Song X, Cui Q, Jia R, Zou Y, He C, Li L and Yin Z (2017) Resveratrol mitigates lipopolysaccharide-mediated acute inflammation in rats by inhibiting the TLR4/NF- κ Bp65/MAPKs signalling cascade. *Scientific Reports* **7**, 45006.
- Wen H, Vuitton L, Tuxun T, Li J, Vuitton DA, Zhang W and McManus DP (2019) Echinococcosis: advances in the 21st century. *Clinical Microbiology Reviews* **32**, e00075-18.
- Yanbao X, Fu Q, Wenji P, Chang S, Wahl LM, Medvedev AE and Jbc M (2011) Endotoxin tolerance impairs IL-1 receptor-associated kinase (IRAK) 4 and TGF- β -activated kinase 1 activation, K63-linked polyubiquitination and assembly of IRAK1, TNF receptor-associated factor 6, and κ B kinase γ and increases A20 expression. *Journal of Biological Chemistry* **286**, 7905–7916.
- Yang XD and Sun SC (2015) Targeting signalling factors for degradation, an emerging mechanism for TRAF functions. *Immunological Reviews* **266**, 56–71.
- Yang J, Wu J, Fu Y, Yan L, Li Y, Guo X, Zhang Y, Wang X, Shen Y, Cho WC and Zheng Y (2021) Identification of different extracellular vesicles in the hydatid fluid of *Echinococcus granulosus* and immunomodulatory effects of 110 K EVs on sheep PBMCs. *Frontiers in Immunology* **12**, 602717.
- Yin S, Chen X, Zhang J, Xu F, Hou J, Wu X and Chen X (2014) Initial studies on the role of hydatid fluid in the immune evasion strategies of *Echinococcus granulosus*. *Pakistan Journal of Zoology* **46**, 1711–1718.
- You-Nian XU, Liu GL, Meng C, Anesthesiology DO and Hospital U (2016) Highly effective extraction of mouse peritoneal macrophages by quick vibrating method. *Chinese General Practice* **19**, 303–306.
- Zeghir-Bouteldja R, Polome A, Bousbata S and Touil-Boukoffa C (2017) Comparative proteome profiling of hydatid fluid from Algerian patients reveals cyst location-related variation in *Echinococcus granulosus*. *Acta Tropica* **171**, 199–206.
- Zhang C, Wang L, Ali T, Li L, Bi X, Wang J, Lu G, Shao Y, Vuitton DA, Wen H and Lin R (2016) Hydatid cyst fluid promotes peri-cystic fibrosis in cystic echinococcosis by suppressing miR-19 expression. *Parasites & Vectors* **9**, 278.
- Zhao W, Wang L, Zhang M, Yuan C and Gao C (2012) E3 ubiquitin ligase tripartite motif 38 negatively regulates TLR-mediated immune responses by proteasomal degradation of TNF receptor-associated factor 6 in macrophages. *The Journal of Immunology* **188**, 2567–2574.
- Zhou L, Ma Q, Shi H and Huo K (2010) NUMBL interacts with TRAF6 and promotes the degradation of TRAF6. *Biochemical & Biophysical Research Communications* **392**, 409–414.
- Zhou X, Wang W, Cui F, Shi C, Ma Y, Yu Y, Zhao W and Zhao J (2019) Extracellular vesicles derived from *Echinococcus granulosus* hydatid cyst fluid from patients: isolation, characterization and evaluation of immunomodulatory functions on T cells. *International Journal for Parasitology* **49**, 1029–1037.