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EgCF mediates macrophage polarisation by influencing the glycolytic pathway

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Abstract

Human cystic echinococcosis (CE) is a zoonotic disorder triggered by the larval stage of *Echinococcus granulosus* (*E. granulosus*) and predominantly occurred in the liver and lungs. The M2 macrophage level is considerably elevated among the liver of patients with hepatic CE and performs an integral function in liver fibrosis. However, the mechanism of CE inducing polarisation of macrophage to an M2 phenotype is unknown. In this study, macrophage was treated with *E. granulosus* cyst fluid (EgCF) to explore the mechanism of macrophage polarisation. Consequently, the expression of the M2 macrophage and production of anti-inflammatory cytokines increased after 48 h treatment by EgCF. In addition, EgCF promoted polarisation of macrophage to an M2 phenotype by inhibiting the expression of transcriptional factor hypoxia-inducible factor 1-alpha (HIF-1α), which increased the expression of glycolysis-associated genes, including hexokinase 2 (HK2) and pyruvate kinase 2 (PKM2). The HIF-1α agonist ML228 also inhibited the induction of macrophage to an M2 phenotype by EgCF *in vitro*. Our findings indicate that *E. granulosus* inhibits glycolysis by suppressing the expression of HIF-1α.

Introduction

Echinococcosis is a zoonotic infection induced by the larval stage of cestode species in the genus *Echinococcus.* More commonly known as CE, it is caused by *Echinococcus granulosus.* CE is widely distributed, with a global burden of 188,000 new cases per year, and the Middle East, Russia and China are highly endemic areas (Deplazes et al. 2017; Torgerson et al. 2015). *E. granulosus* need two hosts to complete its life cycle, with humans as the intermediate host. The larval stage of *E. granulosus* predominantly dwells within the intermediate host's liver (75% of the cases) and lungs, where they grow into unilocular cysts that contain larval worms, resulting in chronic local inflammation (Wen et al. 2019). The cyst is surrounded by fibrous tissue, is filled with cyst fluid and protoscoleces and grows slowly in the human body. Cystic hydatids cause disability when they grow in the human liver (Deplazes et al. 2017), and hepatic hydatid cyst perforation, which is one of the most frequently occurring and severe complications, causes anaphylactic shock, which has a poor prognosis, leading to severe economic loss and posing a threat to human health worldwide. Although CE can be controlled by well-known measures (Velasco-Tirado et al. 2018) and depends on the overall improvement of the disease with the development of human society, immunological studies remain of great importance. It is certain that the immune escape mechanisms that accompany *E. granulosus* infections must involve complex immunoregulatory responses, and identifying the molecules that trigger or amplify these regulatory responses holds promise for the treatment of autoimmune and allergic diseases.

Macrophages play an active role in fighting pathogenic infections and exhibit significant plasticity. On the basis of their activity, surface molecules and cytokine synthesis and metabolism differences, macrophages may be classified into classic M1 as well as alternative M2 macrophages (Kimura et al. 2016; Wang et al. 2018). M1 macrophages express M1 macrophage markers including inducible nitric oxide synthase, tumour necrosis factor (TNF)-α and a cluster of differentiation 86 (CD86), which perform a crucial function in inflammation, pathogen elimination and anticancer immunity(Ahmed et al. 2019; Chen et al. 2019; Sakamoto et al. 2019). M2 macrophages appear to promote immune suppression and tissue repair by upregulating expressions of transforming growth factor (TGF)-β, interleukin (IL)-10 and CD206, which is well-recognised as an M2 biomarker (Shapouri-Moghaddam et al. 2018). *Echinococcus granulosus* develops cysts by interacting with human tissues, forming a lesion microenvironment where various immune cells accumulate, including macrophages that produce macrophage migration inhibitory factor, TGF-β1 and extracellular matrix protein 1. The proportion of M2 macrophages also increased significantly in patients with active cysts (Yasen et al. 2021). M2 macrophages lead

to hepatic fibrosis by producing profibrotic cytokines in chronic infection (Duan et al. 2019; Li et al. 2017). Moreover, M2 macrophages play an immunosuppressive role through arginase expression, IL-10 production and Treg recruitment (Kumar et al. 2018; Spence et al. 2013). *Echinococcus granulosus* antigen B inhibits the generation of TNF-α and IL-1β, and it induces differentiation of macrophage towards the M2 phenotype, which suppresses TLR4-mediated pro-inflammatory cytokine production and Th1-driven immune responses during helminthic infections (Silva-Álvarez et al. 2016; Vukman et al. 2013). The M2 macrophage could induce the production of extracellular matrix proteins, which appear during cyst formation, leading to the immune evasion of *E. granulosus* (Silva-Álvarez et al. 2016). A recent study showed that M1 macrophages may ameliorate liver fibrosis induced by persistent *E. granulosus* infection (Li et al. 2019). Therefore, inhibiting macrophage polarisation towards an M2 anti-inflammatory phenotype and inducing it to pro-inflammatory M1 phenotype may be new therapeutic strategies for hepatic CE.

HIF-1 is a heterodimeric transcriptional complex consisting mainly of an oxygen-modulated HIF-1α subunit and a HIF-1β subunit. The stability of HIF-1α protein is negatively regulated by O_2 concentration. Under high oxygen conditions, HIF-1α is hydroxylated, leading to its enhanced proteasomal degradation whereas, under hypoxia, the degradation of HIF-1α is suppressed and translocated in the nucleus to bind promoters of its target genes (Palazon et al. 2014). HIF1 modulates the transcription of hundreds of genes that play a role in various cellular activities, such as energy metabolism, cell survival, tumour invasion, angiogenesis and inflammation. Research has illustrated the fundamental function exerted by HIF-1α in modulating the glycolytic pathway by inducing the expression of its target genes lactate dehydrogenase (LDHA), pyruvate dehydrogenase kinase 1 (PDK1) and HK2 for glycolytic metabolism (Dabral et al. 2019; Fan et al. 2014; Xu et al. 2020). HIF-1α plays a role in the induction process of macrophage polarisation towards M1 phenotypes by increasing IL-1β secretion and enhancing the glycolysis of macrophages (Mills et al. 2016; Rodriguez et al. 2019). Research reports have shown that *E. granulosus* induced an anti-inflammatory phenotype and impaired presenting ability in macrophages (Sagasti et al. 2021; Silva-Álvarez et al. 2016). However, the mechanism of how *E. granulosus* induces the polarisation of macrophages to an M2 phenotype is unclear. Herein, we demonstrate that *E. granulosus* induce macrophage polarisation to an M2 phenotype by restraining HIF-1α expression, which would provide new treatments or interventions for hepatic hydatid disease.

Materials and methods

Production of E. granulosus and E. granulosus cyst fluid

Echinococcus granulosus was collected from the liver of diseased sheep in a slaughterhouse in Changji Prefecture, Xinjiang Uygur Autonomous Region. First, sheep livers infected with *E. granulosus* were disinfected and cleaned with alcohol. Second, the clear fluid from the capsule containing *E. granulosus* was extracted with a sterile 50-mL syringe and placed in a sterile container. The clear, uncontained EgCF was subjected to filtering with the aid of a 0.22-μm filter in a sterile station and dispensed for use. *Echinococcus granulosus* extract was washed with autoclaved phosphate-buffered solution (PBS; Item no.C20012500BT; Gibco, MA, USA) until the supernatant becomes clear. The extract was also stained with eosin (Item no. ZLI-9613, Beijing Zhongshan Jinqiao Biotechnology Co., China), and on microscopic examination, ≥90% of the worms were alive; if eosin does not stain, the worms are alive. Finally, the cleaned *E. granulosus* extract was subjected to incubation in Dulbecco's Modified Eagle Medium (DMEM) high-sugar medium (Item no. C11995500BT; Gibco) that contains 10% extra fetal bovine serum (FBS, Item no. 04-001-A; Gibco) and 2% penicillin-streptomycin (Item no. SV30010, Gibco).

Extraction and in vitro culture of peritoneal macrophage (PM) from C57BL/6 mice

Starch broth (0.5 g starch, 0.22 g broth powder) was prepared and administered via an injection method into the mice's peritoneal cavity once a day. Subsequently, the peritoneal cavity was fasted without water on day 3 and was lavaged with DMEM high-sugar medium on day 4. The obtained cells were transferred into a DMEM high-sugar medium that contained 10% special-grade FBS for walled culture and introduced into six-well plates at a density of $1{\times}10^6$ per well. The HIF-1α agonist ML228 (Item no. HY-12754, MCE Biotechnology, USA) was added at a concentration of 3 μM.

Animal studies

Animal model

Healthy 6–8-week-old C57BL/6 mice, with a bodyweight range of 18–22 g, were procured from the Animal Experimental Center of Xinjiang Medical University. They are reared in standard conditions in pathogenic-free conditions. The mice were classified into healthy control groups and CE infected groups. Moreover, each group was randomly divided into eight mice. Subsequently, the mice were allowed to fix on a manipulative table after being anaesthetised using 0.5% pentobarbital sodium salt (Sigma-Aldrich, MO, USA). Disinfection of the abdominal area was performed using 75% alcohol, and the abdomen was cut open with ophthalmic scissors. Then, 5,000 protoscoleces/each were introduced into the Glisson capsule with the aid of a 1-mL insulin needle (Wang et al. 2021), healthy group injected with PBS. Finally, the abdominal skin was closed with stitches, and animal modeling was performed for 45 days.

Isolation of mouse liver immunocytes

The isolated liver was sliced into smaller segments and continuously crushed on a 300-gauge mesh, which was then rinsed with 1X PBS till complete suspension of the whole sample was achieved. Subsequently, the obtained tissue cell suspension was centrifugated at 1500 g, followed by the removal of the supernatant. The cell layer was resuspended with 40% Percoll and then slowly added on top of the 70% Percoll isolate and centrifugated at 1500 g for 25 minutes. The intermediate immunocytes were extracted and rinsed using PBS.

Quantitative Reverse-transcription polymerase chain reaction (qRT-PCR)

We extracted total RNA from fully lysed macrophages using the Total RNA Extraction Kit (Item no. R6834-01, Omega Bio-tek, USA). Each sample's purity and concentration were determined with the aid of the NanoDrop 2000 (Thermo Fisher Scientific, MA, USA). About 3 μg of the total RNA were reverse-transcribed into cDNA using a reverse-transcription kit (Item no. K1622, Thermo Fisher Scientific) on a reverse-transcription instrument (11007136, TAKARA Bio, Inc., Japan), with the reverse-transcription

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conditions of 70°C in 5 min and 4°C in 2 min and amplification conditions of 42°C in 60 min, 70°C in 5 min and 4°C subsequently. Fluorescence quantification was performed using a qRT-PCR instrument (785BR11656, Bio-Rad, USA). The following were the reaction criteria: of the present research 95°C, 30 s predenaturation; 95°C, 5 s denaturation; 60°C, 30 s annealing/extension in 39 cycles. We used β-actin as the internal control, and the findings were examined utilizing Bio-Rad CFX96 Manager. Table 1 displays the primer sequences used.

Immunofluorescence

Here, 4% paraformaldehyde (Item no. BL539A, White Shark Biotechnology) was used to fix macrophages on glass slides; thereafter, they were blocked using 1–3% bovine serum albumin. Anti-rabbit HIF-1α (Item no. 36169, CST, TX, USA) (1:500) was added to each well and placed in a wet box throughout the night at 4°C. Samples were then stained using fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit (Item no. ZF-0311, Beijing Zhongshan Jinqiao Biotechnology Co.) (1:80), followed by 4',6-diamidino-2-phenylindole staining (Item no. C0065, Beijing Solaibao Technology Co., Ltd., Beijing, China). Samples were assessed via a fluorescence-inverted microscope (IX71, Olympus), and all samples were assessed with the same setup.

Flow cytometry

Macrophages were washed with PBS. Then, using a flow cytometer (45-2-2005-3352-1, Agilent Technologies, Inc., CA, USA), we detected the expression of each antigen. Macrophages were gated as F4/80+CD11b+. The antibodies included PE anti-mouse CD86 (Item no. 105007, Biolegend, Inc.) (1:200), APC anti-F4/80 (Item no. ab105080, Abcam, UK) (1:20), PE anti-anti-mouse CD11b (Item no. 101207, Biolegend, Inc.) (1:100), PE anti-mouse CD206 (Item no. 85-12-2061-80, eBioscience, Inc., CA, USA) (1:200), PE anti-mouse TNF-α (Item no. 12-7321-81, eBioscience, Inc.) (1:20), APC anti-mouse IL-10 (Item No. 505009, BioLegend, Inc., CA, USA) (1:20), anti-rabbit HIF-1α (1:200) and FITC-labelled goat anti-rabbit (1:100).

Enzyme-linked immunosorbent assay (ELISA)

Mouse IL-10 ELISA Kit (Item no. 70-EK2102/2, UNIQUE Group Co., Ltd., Gambia) was utilised for the purpose of determining the IL-10 levels in culture supernatants in accordance with the manufacturer's guidelines. Absorbance was determined at 450 nm and 560 nm with the aid of the Enzyme-Labelled Instrument (3001-2140, Thermo Fisher Scientific, Ltd.), a standard curve was generated and each sample's concentration was derived.

Statistical methods

GraphPad Prism 7.0 software (GraphPad Software Inc., LCC, USA) was utilised to perform analyses of statistical data. One-way analysis of variance test was utilised to identify significant differences across groups. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ mean that the difference is significant, whereas ns denoted no significant difference.

Results

Ratio of M2 macrophages increased in mouse liver with hepatic CE

To evaluate the effect of *E. granulosus* on hepatic macrophages, we injected 5000 protoscoleces into the Glisson capsule for 45 days. The extracted mouse liver immunocytes were then stained with the flow antibody APC anti-mouse F4/80, followed by the flow cytometer-gated liver macrophages and F4/80+ for macrophages (Figure 1A). The expressions of macrophages CD86 and CD206 in healthy mouse liver and proximal and distal liver of CE mice were detected by flow cytometry. We found that the expression level of M1 marker CD86 was lowered ($P < 0.05$) (Figure 1B), whereas the that of M2 marker CD206 was elevated ($P < 0.05$) (Figure 1C) in the proximal liver of CE mice, suggesting the polarisation of macrophages towards the M2 phenotype in the *E. granulosus* mouse liver model.

Table 1. Primers for qRT-PCR

Primer name	Forward (5'→3')	Reverse (5'→3')
β-actin	CGTGAAAAGACCCAGATCA	CACAGCCTGGATGGCTACGT
HIF-1α	ACCTTCATCGGAAACTCCAAAG	CTGTTAGGCTGGGAAAAGTTAGG
HK2	TGATCGCCTGCTTATTCACGG	AACCGCCTAGAAATCTCCAGA
PKM2	GCCGCCTGGACATTGACTC	CCATGAGAGAAATTCAGCCGAG
CD206	TTCGGTGGACTGTGGACGAGCA	ATAAGCCACCTGCCACTCCGGT
CD86	GACCGTTGTGTGTGTTCTGG	GATGAGCAGCATCACAAGGA
TNF-α	CCTGTAGCCCACGTCGTAG	GGGAGTGAGCAAGGTACAACCC
IL-10	GCTGGACAACATACTGCTAACC	ATTTCCGATAAGGCTTGGCAA
IL-4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
ARG1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
TGF-β	GAGCCCGAAGCGGACTACTA	TGGTTTTCTCATAGATGGCGTTG

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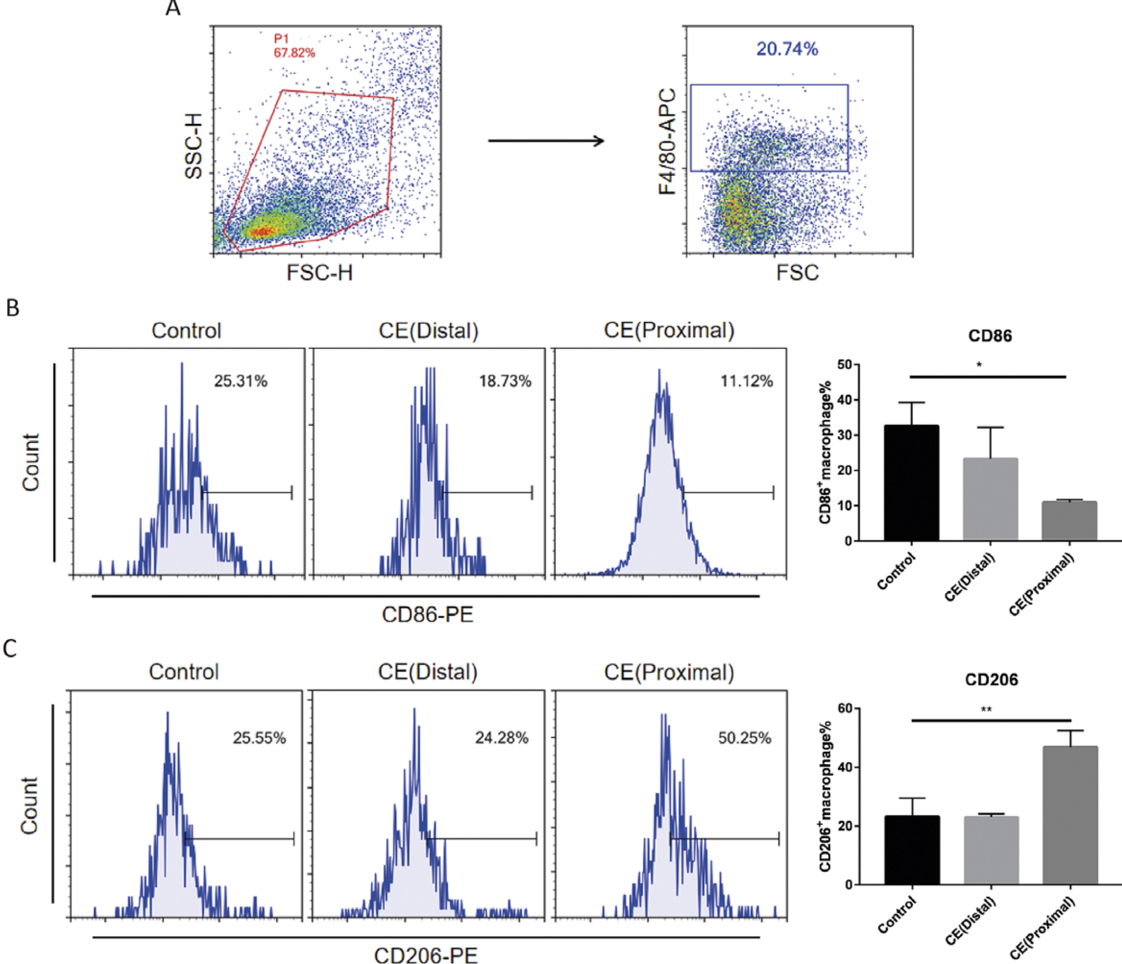

Figure 1. Increase in the ratio of M2 macrophages.
(A) Flow cytometry set gates to determine macrophage populations. (B) and (C) The expressions of CD86 and CD206 in proximal and distal liver tissues of CE mice were measured by flow cytometry and compared with healthy mouse liver tissues, which showed a lowered expression level of CD86 in the proximal liver tissues of CE mice and increased CD206 expression in proximal liver tissues of CE mice. All data are reported as mean ± SD of three separate experiments. **P* < 0. 05, ***P* < 0. 01. CE, cystic echinococcosis.

EgCF promotes the polarisation of macrophages to M2 phenotypes in vitro

To thoroughly examine the impacts of *E. granulosus* on the polarisation mechanism of mouse macrophages, we used extracted PM and EgCF for *in vitro* studies, with a final EgCF dosage of 0.5 mg/mL. EgCF was cocultured with PM for 0 h, 24 h and 48 h, and gate F4/80+CD11b+ was selected as the PM phenotype (Figure 2A). Consequently, the M1 marker CD86 elevated at 24 h, returning to basal level at 48 h ($P < 0.05$, Figures 2B and 2C); however, the M2 marker CD206 gradually increased ($P < 0.05$, Figures 2D and 2E). This demonstrated that EgCF can promote the polarisation of macrophages towards an M2 phenotype. Furthermore, we assessed the protein expression of inflammatory markers produced by macrophages. The findings showed that the protein percentage of the pro-inflammatory factor TNF-α secreted by M1 increased at 24 h, decreased at 48 h and remained above basal levels ($P < 0.05$, Figure 2F), but the protein percentage of the anti-inflammatory factor IL-10 reduced at 24 h, enhanced at 48 h, level with basal levels ($P < 0.05$, Figure 2G). ELISA showed a gradual increase in IL-10 expression ($P < 0.05$, Figure S1A). This indicated that EgCF can regulate the polarisation of macrophage phenotypes and the secretion of cytokines. This result was in line with the trend of phenotypic alterations in M1 and M2. We also analysed the mRNA relative expression of inflammatory factors and found that the mRNA relative expression of the pro-inflammatory markers TNF-α and IL-1β increased and then decreased but remained above basal levels. ($P < 0.05$, Figures 2H and 2I), but the mRNA relative expression of the anti-inflammatory markers IL-4 and IL-10 increased and then decreased to the same level as basal levels, while arginase 1 (ARG1) and TGF-β increased and then decreased but remained above basal levels ($P < 0.05$, Figures 2J–2M). This result was generally consistent with the inflammatory factor protein expression. Collectively, these results indicated that EgCF facilitated the polarisation of macrophages towards M2.

EgCF affects the expression of HIF-1α in macrophage

The HIF-1α can promote macrophage polarisation towards M1, which contributes to the formation of atherosclerosis (Wang et al. 2021). Therefore, we speculate that *E. granulosus* may suppress macrophage polarisation towards M1 macrophages by suppressing the expression of HIF-1α. In the present research, we examine if HIF-1α was involved in macrophage polarisation under *E. granulosus* infection. EgCF was co-cultured with PM for 0 h, 24 h and 48 h. Then,

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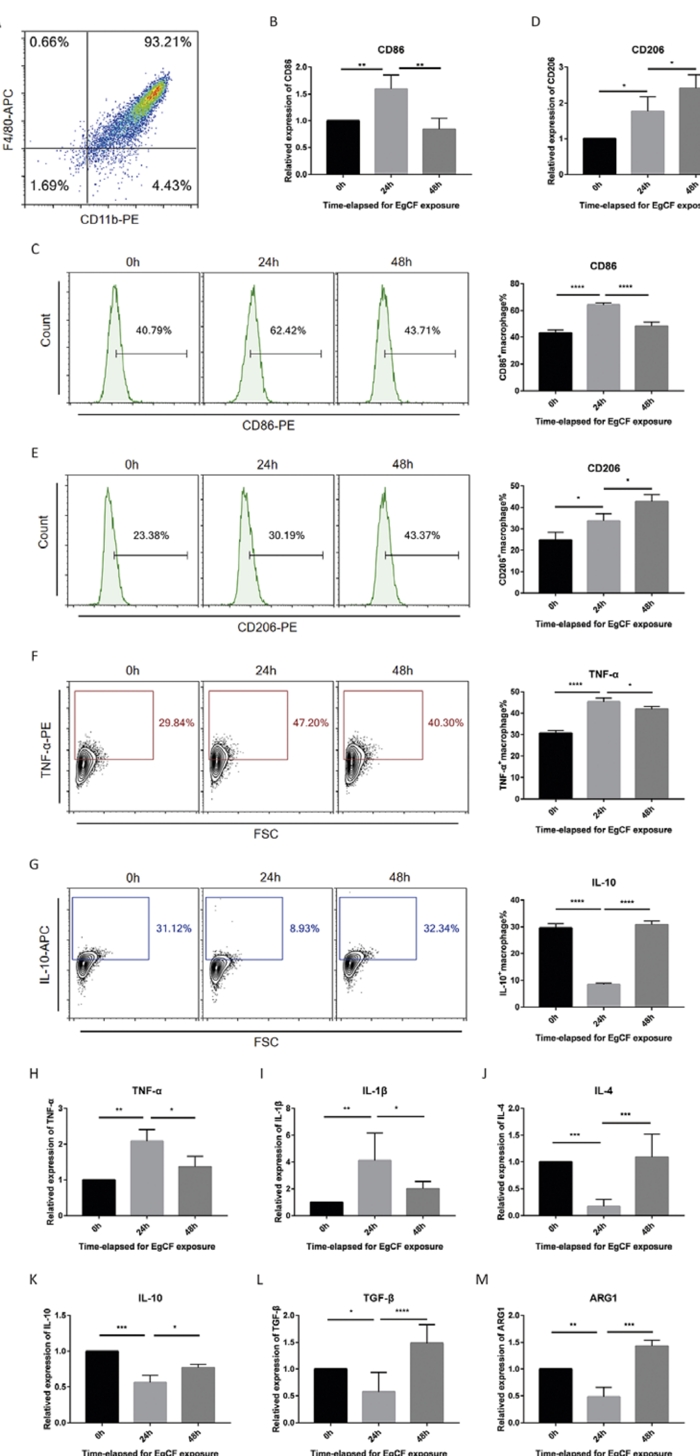

Figure 2. EgCF promotes the polarisation of macrophages to M2 phenotypes *in vitro.*
(A) Purity was detected from the extracted mouse peritoneum macrophages through flow cytometry, and F4/80+CD11b+ was chosen as the gate. (B)–(E) The expressions of CD86 and CD206 in macrophages were detected by qRT-PCR and flow cytometry, indicating the M1-CD86 expression elevated at 24 h, returning to basal level at 48 h, while the M2-CD206 expression enhanced after infection with EgCF. (F) and (G) The results of flow cytometry demonstrate the expression of the pro-inflammatory marker TNF-α secreted by M1 increased at 24 h, decreased at 48 h and remained above basal levels, and the expression of the anti-inflammatory factor IL-10 secreted by M2 reduced at 24 h, enhanced at 48 h, level with basal levels after infection with EgCF. (H)–(M) qRT-PCR further verified that the expressions of M1-associated markers TNF-α and IL-1β increased and then decreased but remained above basal levels, but the expression of M2-associated markers IL-4 and IL-10 increased and then decreased to the same level as basal levels, while arginase 1 (ARG1) and TGF-β increased and then decreased but remained above basal levels after infection with EgCF. All data are expressed as the mean ± SD of three independent experiments. *P < 0. 05, **P < 0. 01, ***P < 0. 001 and ****P < 0. 0001. EgCF, *E. granulosus* cyst fluid; TNF-α, tumour necrosis factor-alpha; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; TGF-β, transforming growth factor-beta; IL, interleukin; ARG1, arginase 1.

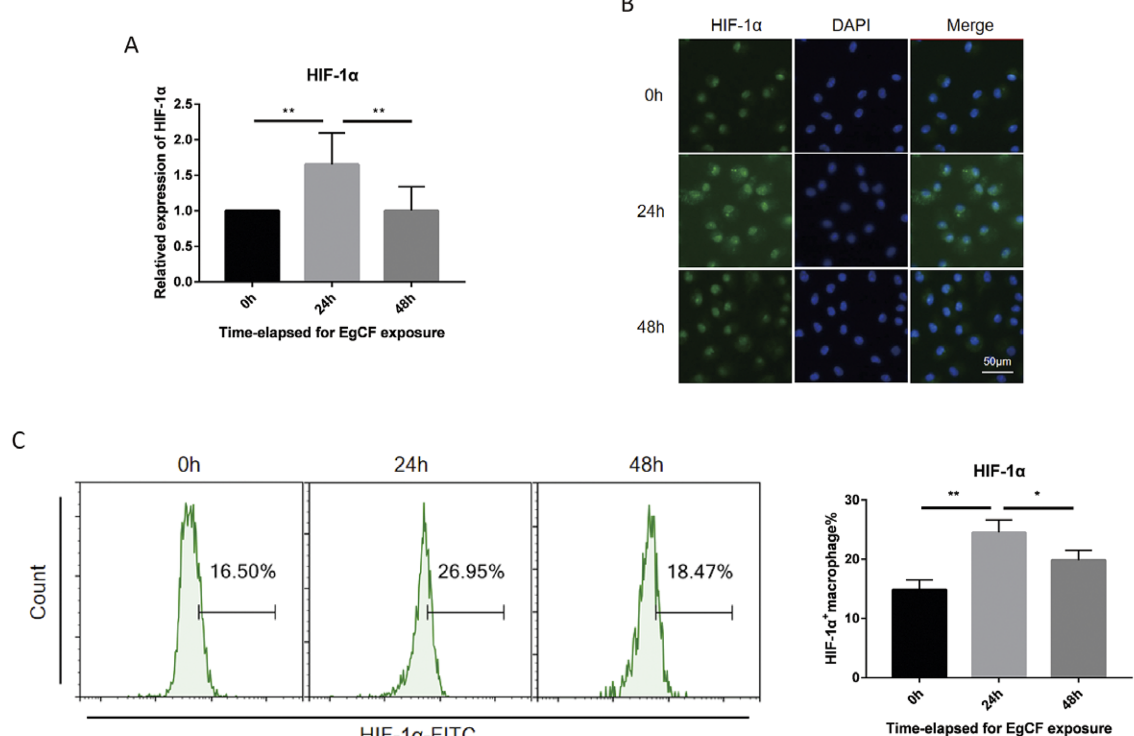

Figure 3. EgCF promotes macrophage polarisation to M2 phenotypes by suppressing the expression of HIF-1α. (A)–(C) qRT-PCR, Immunofluorescence and flow cytometry were employed to observe the expression of HIF-1α in macrophage, indicating suppressed expression of HIF-1α at 48 h compared to 24 h.

we evaluated the HIF-1α mRNA expression in the macrophages and further discovered that the expression level of HIF-1α elevated at 24 h, returning to basal level at 48 h ($P < 0.01$, Figure 3A). Additionally, we measured the HIF-1α protein expression in macrophages. The findings were in line with the mRNA expression level ($P < 0.01$, Figures 3B and 3C). This indicated that EgCF suppressed the expression of HIF-1α at 48 h compared to 24 h.

HIF-1α agonist inhibits the expression of M2 markers and the secretion of anti-inflammatory factors

Moreover, we co-cultured the HIF-1α agonist, EgCF and PM for 48 h; the HIF-1α agonist was introduced at a dosage of 3 μM. EgCF was introduced at a final concentration of 0.5 mg/mL. Then, we measured the HIF-1α protein expression in the macrophages. Consequently, the HIF-1α expression level was remarkably elevated in the 3 μM HIF-1α agonist groups as opposed to that in the control group ($P < 0.001$, Figures 4A and S1B). Then, we analysed whether the M1 marker CD86 and the M2 marker CD206 changed after the introduction of the HIF-1α agonist. As a result, the CD86 was significantly higher, whereas that of CD206 was significantly lower in the 3 μM HIF-1α agonist group as opposed to that of the control group ($P < 0.05$, Figures 4B and 4C). This suggests that the 3 μM HIF-1α agonist can facilitate the polarisation of macrophages towards M1.

In addition, we examined the protein and mRNA expression of inflammatory factors secreted by macrophages. As opposed to the control, the protein percentage of TNF-α secreted by M1 in the 3 μM HIF-1α agonist group was upregulated; however, IL-10 secreted by M2 was downregulated in the 3 μM HIF-1α agonist group ($P < 0.05$, Figures 4D and 4E). These findings illustrated that the mRNA relative expression of pro-inflammatory factors TNF-α, IL-1β secreted by M1 was enhanced ($P < 0.05$, Figures 4F and 4G) and the mRNA relative expression of anti-inflammatory factors IL-4, IL-10, ARG1 and TGF-β secreted by M2 was inhibited ($P < 0.05$, Figures 4H–4K) compared with the control, DMSO groups. These results suggested that the 3 μM HIF-1α agonist promotes the production of pro-inflammatory markers by macrophages. In summary, these findings revealed that HIF-1α promotes macrophage phenotype polarisation towards M1, whereas EgCF polarises macrophages towards M2 by restraining HIF-1α expression.

EgCF suppresses the expression PKM2 and HK2 by inhibiting HIF-1α

Our previous results suggest that HIF-1α can facilitate macrophage phenotypic polarisation towards M1. M1 macrophages are essentially energised by glycolysis (Nonnenmacher and Hiller 2018). We wanted to continue our investigation on whether HIF-1α could regulate macrophage polarisation by glycolysis. We co-cultured EgCF and PM for 0 h, 24 h and 48 h and evaluated the expressions of HK2 and PKM2, which are key enzymes of the glycolytic process, in macrophages infected with *E. granulosus*. The results showed that their expression enhanced at 24 h, reduced at 48 h, level with basal levels ($P < 0.05$, Figures 5A and 5B).

Then, we co-cultured the HIF-1α agonist, EgCF and PM for 48 h (the HIF-1α agonist was introduced at a dosage of 3 μM; EgCF was introduced at a final concentration of 0.5 mg/mL) and measured the expression of key enzymes of glycolysis, namely, HK2 and PKM2, in macrophages. As opposed to the control and DMSO groups, the

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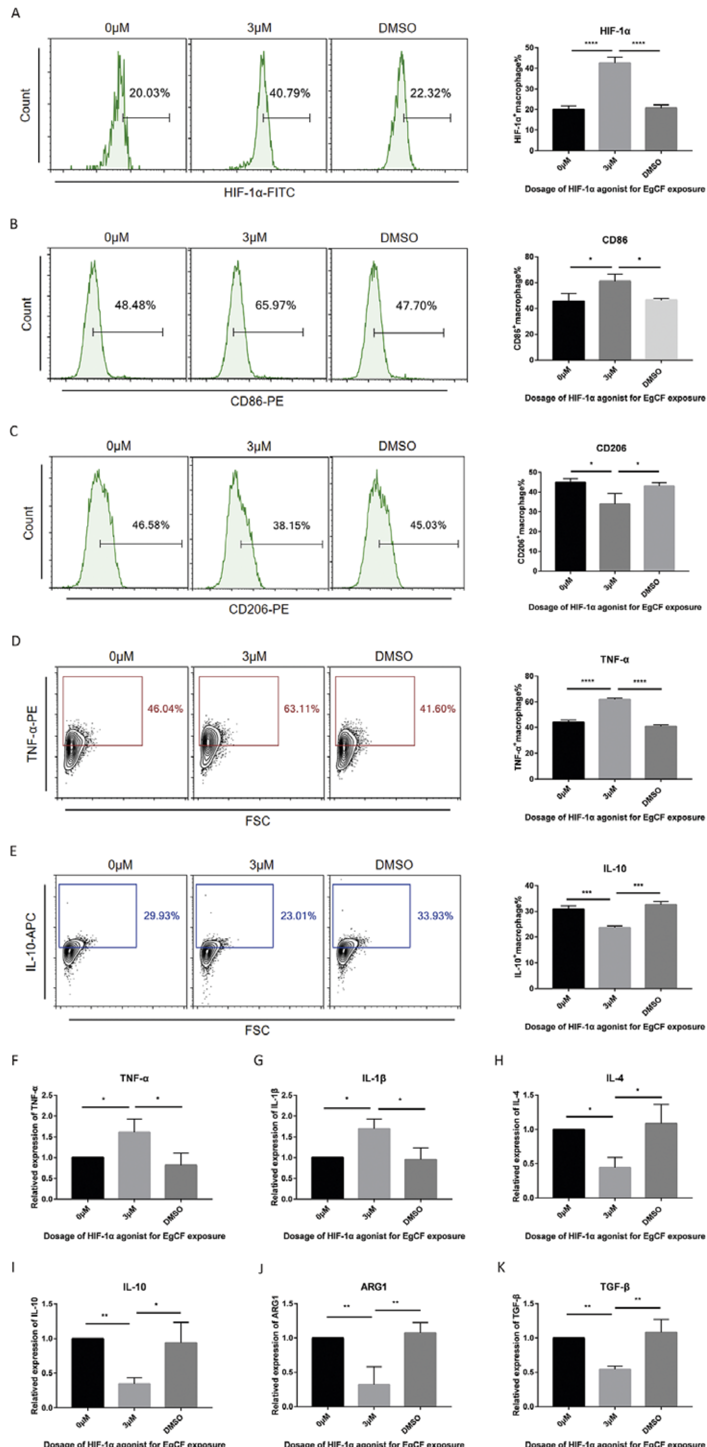

Figure 4. EgCF promotes macrophage polarisation to M2 phenotypes by suppressing the expression of HIF-1α.
(A) Flow cytometry demonstrated that 3 μM HIF-1α agonists can elevate the expression of HIF-1α when macrophages were co-cultured with EgCF and HIF-1α agonist for 48 h. (B)–(E) Flow cytometry indicated that the 3 μM HIF-1α agonist can improve the protein expression of CD86 and TNF-α and inhibit the protein expression of CD206 and IL-10. (F)–(K) qRT-PCR showed that the 3 μM HIF-1α agonist can improve the mRNA expression of M1-associated factors TNF-α and IL-1β and inhibit the mRNA expression of M2-associated markers IL-4, IL-10, ARG1 and TGF-β. All data are expressed as the mean ± SD of three separate experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$. EgCF, *E. granulosus* cyst fluid; TNF-α, tumour necrosis factor-alpha; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; TGF-β, transforming growth factor-beta; IL, interleukin; ARG1, arginase 1;.

expression levels of HK2 and PKM2 were significantly higher in the 3 μM HIF-1α agonist group ($P < 0.05$, Figures 5C and 5D). The above findings demonstrate that HIF-1α may enhance the expression of key enzymes of glycolysis; thus, HIF-1α can mediate the polarisation of macrophages towards M1 by promoting the glycolytic pathway.

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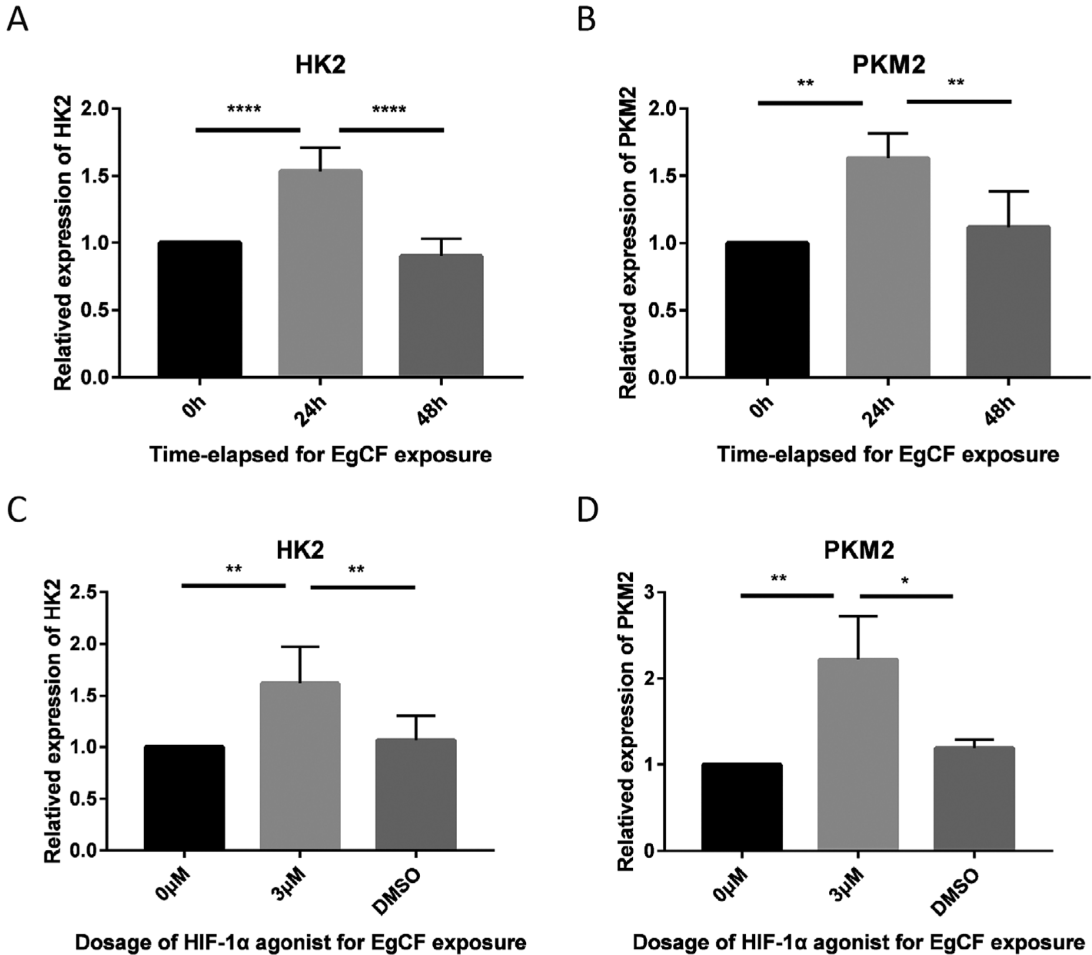

Figure 5. EgCF suppresses the expressions PKM2 and HK2 by inhibiting HIF-α.
(A) and (B) The expressions of HK2 and PKM2 in macrophages were detected by qRT-PCR, indicating suppressed expression of HK2 and PKM2 at 48 h compared to 24 h. (C)–(D) qRT-PCR demonstrated that the 3 μM HIF-1α agonist can improve the expression of HK2 and PKM2 when macrophages were co-cultured with EgCF and HIF-1α agonist for 48 h. All data are reported as the mean ± SD of 3 separate experiments. *P<0. 05 and **P<0. 01. EgCF, *E. granulosus* cyst fluid; qRT-PCR, quantitative reverse-transcription polymerase chain reaction.

Discussion and conclusions

Cystic echinococcosis (CE, also called hydatid disease) is a neglected zoonotic tropical disease estimated to be responsible for the yearly loss of 1 million disability-adjusted life years (Agudelo Higuita et al. 2016). The prevalence of the disease is estimated to be 1–7% in infected areas and up to 9% in parts of Xinjiang, China (Budke et al. 2013).

90% of human CE is caused by *E. granulosus* (Kern et al. 2017), and persistent *E. granulosus* infections can span many years due to 'avoidance strategies'. Indeed, *E. granulosus* has evolved complex strategies to evade host immune responses and establish a delicate balance between host protection and long-term parasite growth (Gottstein et al. 2017; Jafari et al. 2019; Jiang et al. 2022; Vuitton 2003). Macrophages are widely distributed innate immune cells that are widely dispersed found in the human body play a role in multiple pathophysiological processes, including organ development, host defense, chronic and acute inflammatory processes and tissue remodelling as well as homeostasis. M2 macrophages secrete large amounts of anti-inflammatory cytokines to suppress inflammation. Previous studies (Silva-Álvarez et al. 2016; Yasen et al. 2021) have found CD68+ macrophages expanding in diseased liver samples from patients with active CE, with M2 macrophages as the dominant macrophage phenotype, which was confirmed by single-cell data (Jiang et al. 2022) that showed macrophage expansion in the livers of late-infected mice with *E. granulosus* infections, which may be more analogous to M2 macrophages with immunosuppressive functions.

EgCF, the environment in which *E. granulosus* larvae live, has a complex composition. Researchers isolated antigen B, a 120 kDa polymeric lipoprotein composed of various 8 kDa subunits, from EgCF, which inhibits elastase activity and neutrophil chemotaxis and triggers a non-protective Th2 cellular response through inhibition of elastase activity and neutrophil chemotaxis. In addition, antigen B can modulate the inflammatory response by binding to monocytes and macrophage (Siracusano et al. 2008).

Our previous studies have shown that *E. granulosus* promotes macrophage polarisation towards the M2 type (Chen et al. 2017). Based on this, we conducted further studies and found that *E. granulosus* infection increased the proportion of M2-type macrophages in the mouse liver. Subsequently, EgCF was co-cultured with peritoneal macrophages, and it was found that EgCF not only induced peritoneal macrophages to be M2-polarised, but also induced macrophages to secrete more anti-inflammatory factors.

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Nuclear transcriptional factors have recently been found to perform a fundamental function in macrophage polarisation. HIF-1α is a nuclear transcription factor that regulates a wide range of target genes. It has been shown in the literature (Ambade et al. 2016; Cheng et al. 2017; Werno et al. 2010) that HIF-1α can mediate macrophage polarisation, and macrophages lacking HIF-1α have a diminished ability to ingest and destroy pathogens (Santos and Andrade 2017), so we speculated that EgCF may be regulating HIF-1α to affect macrophages. To confirm the above speculation, we examined the expression of HIF-1α in PM cells after co-culture with EgCF and found that EgCF stimulation did inhibit the expression of HIF-1α, whereas the expression of M2 polarisation and anti-inflammatory factors was reduced and the expression of M1-associated factors was elevated after the addition of a HIF-1α agonist, which suggests that HIF-1α is indeed involved in macrophage polarisation induced by EgCF.

Glycolysis performs an integral function in macrophages, particularly for M1 macrophages involved in pro-inflammatory responses, and glycolysis rapidly provides energy to promote inflammation. Therefore, we explored the relationship between glycolysis and M1/M2 polarisation in *E. granulosus* infections. We also observed whether HIF1-α regulates macrophage polarisation and further exerts an immune escape effect by controlling the glycolytic pathway, results revealed that EgCF inhibited HK2 and PKM2, key enzymes of the glycolytic pathway, whereas the addition of HIF-1α agonist restored the expression of some HK2 and PKM2.

Unfortunately, there are still many shortcomings in our experiments. First, whether HIF-1α still mediates EgCF-induced macrophage polarisation in mice *in vivo*; second, whether the glycolytic pathway is involved in whether HIF-1α mediates EgCF-induced macrophage polarisation still needs to be further investigated; and third, whether the HIF-1α agonist can be combined with albendazole in the treatment of CE patients. Various shortcomings still need to be further explored in future studies. The findings of the present research demonstrate that EgCF inhibits the HIF-1α expression, which inhibits the glycolytic pathway and eventually polarises macrophages towards M2. Macrophages change their phenotype and function accordingly. M2 exerts an immunosuppressive effect, leading to the immune escape of *E. granulosus* and thus enabling long-term survival *in vivo*. Therefore, we want to reduce the formation of M2. We used an HIF1-α agonist, which promoted the expression of HIF1-α, thus promoting the glycolytic pathway and reducing the formation of M2. Essentially, the present research supports the hypothesis that HIF1-α may serve as a therapeutic target for *E. granulosus* infection. This provides the theoretical and experimental bases for the development of new drugs against *E. granulosus* infections. Moreover, we need to conduct further experiments, such as increasing the concentration of HIF1-α agonist and combining HIF-1α with albendazole in the treatment of CE.

Supplementary material. The supplementary material for this article can be found at http://doi.org/10.1017/S0022149X23000548.

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Competing interest. The authors declare that there are no conflicts of interest associated with this study.

Statement of ethics. All experimental animals were handled in a rational manner, and all protocols were approved by the Medical Ethics Committee of the First Affiliated Hospital of Shihezi University School of Medicine (Approval Number A2020-123-01).

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