A Combination of Pirfenidone and Inhibition of TGF- β Mitigates Cystic Echinococcosis–Associated Hepatic Injury

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Research

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Abstract

Background & Aims: TGF- β not only inhibits the function of NK cells, but also promotes liver fibrosis. It is unclear whether the inhibition of TGF- β signaling pathway could relieve the CE-related liver fibrosis.

Methods: By using the model of CE infected mouse liver, the effect of the infection of CE exerted on NK cells was identified. In vivo, SB525334 and Pirfenidone were applied to intervene the TGF- β signaling pathway.

Results: During the infection of *Echinococcus granulosus*, the inhibitory receptors on the surface of NK cells increased, while the activated receptors decreased. TGF- β 1 secretion was increased in liver tissues and mainly derived from macrophages. At the same time, the combination of drugs could also reduce the expression of TGF- β 1 signaling pathway-related proteins and collagen. For the secretion of TGF- β 1, only the pirfenidone group had an depressing effect. In addition, the combination of drugs can reduce liver cell damage and restore liver function.

Conclusions: During the infection of *Echinococcus granulosus*, the dysfunction of NK cells may be caused by increased secretion of TGF- β 1 from macrophages. *Echinococcus granulosus* infection was a process of occupying lesions. This persistent pressure was accompanied by senescent hepatocytes. Senescent hepatocytes induced the secretion of TGF- β 1 derived from macrophages, and promoted the activation of hepatic stellate cells and accelerated *Echinococcus granulosus*-associated liver fibrosis. By combining pirfenidone and SB525334, liver function, senescent hepatocytes, fibrosis associated with *Echinococcus granulosus* infection could be alleviated. Moreover, the functional status of NK cells also received partially amelioration. In summary, our work offered an experimental basis for clinical treatment.

Introduction

Cystic echinococcosis (CE) is a zoonotic disease caused by the larvae of *Echinococcus granulosus* when the parasite colonizes its intermediate host[1]. It is a life threatening disease that causes periparasitic granuloma and irreversible fibrosis in the liver. The disease is characterized

persistent space-occupying lesions, which induces obstruction of the bile duct as well as vessels and ultimately lead to liver failure[2]. In addition, it is associated with fibrous cysts which are formed from persistent chronic injury. Notably, fibrous cysts arise due to excessive accumulation of the extracellular matrix(ECM)[3], which is mainly produced by activated hepatic stellate cells (HSCs)[4]. As CE progresses, fibrous capsules may increase in both number and volume and finally form space-occupying lesions in the liver[5].

The liver harbors numerous immune cells and is particularly rich in natural killer (NK) cells,

Which account for approximately 30–50% of total lymphocytes in the organ[6]. NK cells are effector lymphocytes of the innate immune system that are able to prevent disease progression and formation of tissue lesions[7]. Additionally, NK cells alleviate liver fibrosis through NKG2D-dependent cytotoxicity and interferon- γ (IFN- γ)—related apoptosis of HSCs. This was observed in both in experimental animal models and patients[8]. Therefore, it is important to explore the influence CE on NK cells in the liver.

Members of transforming growth factor- β (TGF- β) family are cytokines that play multiple roles in cell proliferation, differentiation, apoptosis and migration[9]. Notably, TGF- β 1 is the most extensively investigated subtype in liver fibrogenesis and is considered an effective profibrotic cytokine[10]. TGF- β blocks the effector function of NK cells by inhibiting the activation of mTOR which is induced by either IL-2 or IL-15[11]. In addition, continuously high levels of serum TGF- β 1 were reported in CE compared to the control[12]. Therefore, TGF- β may be a potential target for the treatment of CE-associated liver fibrosis.

Senescence refers to a situation where the cell cycle undergoes a state of arrest due to continuous exposure to stressful conditions[13]. Senescent cells are characterized by an increased activity of senescence-associated β -galactosidase (SA- β -gal) and an up-regulated expression of proteins p16, p21 and p53 which mediate irreversible growth arrest[13]. Additionally, cellular senescence is characterized by the Senescence-associated Secretory Phenotype (SASP), which releases a variety of cytokines including TGF- β [14]. It was shown that TGF- β could induce senescence in multiple cell types including skin fibroblasts[15], bronchial epithelial cells[16] and hepatocellular carcinoma cells[17]. Moreover, *in vitro* studies showed that TGF- β could release ROS through non-smad pathways, promoted the secretion of SASP-related cytokines and induced senescence in hepatocytes. On the other hand, *in vivo* studies using a hepatocyte-specific senescence model showed that senescence could spread between hepatocytes and this relied on macrophage-derived TGF- β . In addition, Targeting TGF- β signaling could reduce the spread of senescence between hepatocytes[18]. CE leads to the development of space-occupying lesions, which may trigger hepatocytes to undergo senescence. Senescent hepatocytes in turn exacerbate liver fibrosis and promote the activation of HSCs[19]. Therefore, it is possible that TGF- β can be used as a target for the treatment of CE-related liver damage.

Furthermore, Pirfenidone is a pyridone compound that reduces the synthesis of TGF- β 1[20]. On the other hand, SB525334 is a potent and selective inhibitor of the TGF β 1 receptor (ALK5), which was shown to hinder the progress of TGF- β -induced hepatocyte senescence[18]. In this study, it was speculated that TGF- β played a key role in CE-related liver fibrosis. Therefore, a mouse model was used to investigate the effect of CE on liver NK cells. Additionally, Pirfenidone and SB525334 were used to block TGF- β , a potential therapeutic target.

Materials And Methods

Human Tissue

The CE patients signed written informed consent to participate in the study after which 4 specimens were collected during surgery. With regard to hepatic hemangioma, impaired liver tissues larger than 2 cm were considered as normal and this was confirmed by the pathologist. The liver tissues of 5 patients with hepatic hemangioma were collected in the study.

Proscolices Collection and culture

Sheep liver infected with *Echinococcus granulosus* were obtained from a slaughterhouse in Changji, Xinjiang province, China. First, hydatid fluid containing protoscolices was extracted from cysts using a 50 ml sterile

syringe before being transferred to a sterile console. Afterwards, the protoscolices were cleaned with sterile Phosphate Buffered Saline (PBS) (Gibico) until there was a color change from ivory-white to white. Finally, the cleaned protoscolices were cultivated in the RPMI-1640 medium (Gibco, containing L-glutamine and supplemented with 8% FBS (BI) and 100 U/ml penicillin/streptomycin). All Protoscolices were incubated at 37 °C under humidified conditions with 5% CO2.

Animal models and medication

All animal experiments were conducted in accordance with the procedural guidelines of animal welfare and ethical review treaties. C57BL/6 mice were purchased from the Xinjiang Medical

University Experimental Animal Center. The mice used ranged between 8 to 12 weeks old (tand weighed 18 to 20 g) at the beginning of the experiments. They were reared in standard conditions devoid of pathogenic bacteria with equal day and night times. Afterwards, the mice were anesthetized with 0.5% pentobarbital sodium salt (Sigma) and then fixed on a surgical table. Following this, the abdominal skin was disinfected before injecting the protoscolices into the glisson capsule using a 1 ml syringe. The wound was then sewed up. Pathological changes in the liver of mice infected with *Echinococcus granulosus* were observed. 4 mice each were used in the control and experimental groups. Moreover, liver tissues were extracted on day 30, 60, 90, 120 and 150 after infection. A total of 60 mice were used.

While detecting the expression of receptors on the surface of mouse liver NK cells, the control and experimental groups each used 8 mice. In addition, mouse liver lymphocytes were extracted at 7, 14, 21, 30, 60 and 90 days respectively, and a total of 144 mice were used. In the administration stage, 12 model mice were used in both the control and experimental groups and a total of 48 animals were used.

SB525334 (10 mg/kg; MedChemExpress) and Pirfenidone (300 mg/kg; MedChemExpress) were administered using the oral gavage once a day in 0.5% methylethercellulose (the solvent consisted of 10% polyethylene glycol 400, 5% dimethyl sulfoxide and 85% saline). The study chose an initial medication time of 30 days after infection, in the infection model and the number of protoscolices used was 5000. In addition, liver tissue and peripheral blood were extracted from the mice one month after medication. It is noteworthy that a large amount of loose ECM was deposited around the lesions within 30 days of infection although this diminished after day 30. This could have been due to the healing effect of the treatment. Moreover, the success rate in the 3000 Protoscolices modeling group was 66.7% while that in the 5000 Protoscolices group 90%.

Table 1 The success rate of modeling with different inoculation numbers of Protoscolices

numbers of Protoscolices inoculated	Total number of mice	Number of successful models	rate of success
2000 Proscolices	30	7	23.3%
3000 Proscolices	30	20	66.7%
5000 Proscolices	30	27	90%

Harvesting of animal tissue harvesting and serum analysis

The mice were anesthetized with 0.5% pentobarbital sodium salt and blood was extracted through cardiac puncture. Afterwards, they were euthanized using CO₂ before liver tissue was harvested. In addition, commercial kits were used for serum analysis (ALT (Elabscience), AST(Elabscience), SOD(Elabscience), DBL(Direct bilirubin, Nanjing Jiancheng), IL-15/IL-15R (Multi Science)) according to the manufacturers' instructions.

Isolation of hepatic leukomonocytes

First, extracted liver was cut into small pieces and then ground continuously on a 200-gauge stainless steel mesh. PBS was then used to rinse the mesh until the whole tissue was suspended in the solvent. Following this, the tissue homogenate was centrifuged, the supernatant discarded then the pellet re-suspended in 40% Percoll (GE Healthcare). Finally, the cell suspension was gently overlaid on 70% Percoll and centrifuged at 600 g for 30 min at room temperature. Hepatic leukomonocytes were extracted from the interphase and rinsed twice with PBS.

Flow Cytometry analysis

Hepatic leukomonocytes were washed with PBS containing 2% BSA. The FACS Aria III flow cytometer (BD Biosciences) was then used to detect the expression of each surface antigen. Unstained and isotype control groups were used as references for the staining category. NK cells were gated as NK1.1 + CD3- and the data was processed with FlowJo V10. 5.3. The antibodies used included the NK1.1 Monoclonal Antibody (clone:PK136, PE-Cyanine7, eBioscience), CD3 Monoclonal Antibody (clone:17A2, FITC, eBioscience), NKG2D Monoclonal Antibody (clone:CX5, PE, eBioscience), NKG2AB6 Monoclonal Antibody (clone:16a11, PE, eBioscience), TIM3 Monoclonal Antibody (clone:RMT3-23, PE, eBioscience) and CD49b (Integrin alpha 2) Monoclonal Antibody (clone:DX5, PE, eBioscience).

Histological Analysis

Human and mouse liver tissues were fixed in 4% polyoxymethylene for 24 hours and then embedded in paraffin. Afterwards, tissue sections were deparaffinized, stained with hematoxylin–eosin and examined under a light microscope. In addition, four-micrometer-thick paraffin sections were stained with hematoxylin-eosin (Solarbio) for examination of

general morphology or with Sirius Red (Solarbio)for assessment of collagen deposition.

The activity of SA- β -gal was detected based on the manufacturer's protocol (Solarbio). Briefly, liver tissue was fixed in 4% Polyoxymethylene (Biosharp) for 4 hours and then soaked in the β -Galactosidase staining solution for 24 hours in a constant shaker at 37°C.

Afterwards, stained tissue was embedded in paraffin, sliced and counterstained with eosin. All the stained sections were photographed with the microscope camera system (ZEISS, Axio Imager 2). **Note:** A 40x magnification was used for imaging all the sections in this article.

Immunohistochemistry and Immunofluorescence

Experiments were performed following the procedures recommended in the Abcam official website(<https://www.abcam.cn/protocols>). The antibodies included the Anti-alpha smooth muscle Actin Rabbit Monoclonal Antibody (#ab124964, clone: EPR5368, Abcam), Anti-p53 Mouse monoclonal antibody (#ab26, clone: PAb 240, Abcam) and Goat Anti-Mouse IgG H&L (HRP) (#ab205719, Abcam).

The difference in the immunofluorescence protocol was the conjugation of the secondary antibodies and the application of DAPI Fluoromount-GTM (Southern Biotechnology). The antibodies used included; Anti-p53 Mouse Monoclonal Antibody (#ab26, clone: PAb 240, Abcam), Anti-p21 Mouse Monoclonal Antibody (#AP021, clone F-5, Beyotime), Anti-Cytochrome P450 2D6 Rabbit Polyclonal Antibody (#DF4758, Affinity), Anti-TGF- β 1 Rabbit Polyclonal antibody (#ab92486, Abcam), Anti-NKp46 Rabbit Polyclonal Antibody (#orb13333, Biorbyt), Anti-F4/80 Rat Monoclonal Antibody (#14-4801-82, clone: BM8, eBioscience), Anti-Ki-67 Rat Monoclonal Antibody (#14-5698-82, clone: SolA15, eBioscience), Goat anti-Rat IgG (H + L) Secondary Antibody (#A18866, FITC, Invitrogen), Goat anti-Rabbit IgG (H + L) Secondary Antibody (#37670, Rhodamine, Invitrogen) and Goat anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody (#A16079, FITC, Invitrogen). Sections were scanned using the ZEISS Laser Scanning Microscope (LSM 800, ZEISS).

Immunoblotting and ELISA.

Whole mouse liver tissue was ground using the tissue homogenizer and re-suspended in lysis buffer (containing RIPA, PMSF and Protease Inhibitor Cocktail at a ratio of 100:1:1) for 30 minutes. All the procedures were performed at 4°C. After centrifuging the tissue homogenate, the supernatant consisted of the required protein mixture and concentration was determined using the NanoDrop 2000 (Thermo Fisher Scientific). Afterwards, equal amounts of total protein (~ 20 μ g) were electrophoresed in 8–15% SDS–polyacrylamide gels (Solarbio) and then transferred onto a Immobilon-P PVDF (Millipore, 0.22 μ m and 0.45 μ m). Protein bands were visualized on the ChemiDoc XRS + imager (Bio-Rad). The following antibodies were used and were diluted according to the manufactures' recommendations: Anti-p53 Rabbit Polyclonal Antibody (# AF0879, Affinity), Anti-p21 Mouse Monoclonal Antibody(#AP021, clone F-5, Beyotime), Anti-CDKN2A/p16INK4a Rabbit Monoclonal Antibody (#ab108349, clone: EPR1473, Abcam), Anti-alpha smooth muscle Actin Rabbit Monoclonal Antibody (#ab124964, clone: EPR5368, Abcam), Anti-TGF beta Receptor I Rabbit Polyclonal Antibody (#ab31013, Abcam), Anti-TGF beta Receptor II Rabbit Polyclonal Antibody (#ab186838, Abcam), Anti-Collagen I Rabbit Monoclonal Antibody (#ab6308, clone: COL-1, Abcam), Anti-Collagen III Rabbit Polyclonal Antibody (#ab7778, Abcam), Anti-MMP1 Rabbit Polyclonal Antibody (#ab137332, Abcam), Anti-TIMP1 Monoclonal Antibody (#MA1-773, clone: EPR5368, Thermo Fisher Scientific), Goat Anti-Mouse IgG H&L (HRP) (#ab205719, Abcam), Goat Anti-Mouse IgG H&L (HRP) (#ab205719, Abcam) and Anti- β -Actin Mouse mAb (#AM4302, clone: AC-15, Cell Signaling Technology).

The ELISA kit (Multi science) was used to detect the secretion of cytokine TGF- β 1 in liver tissue protein. The concentration of protein mixtures was adjusted to 50 μ g/ μ L. All procedures were performed according to the manufacturer's instructions. Finally, optical density was measured using the Thermo Scientific Varioskan LUX.

Statistical analysis

Two-way ANOVA was used to assess significance between multiple groups and samples (> 2). All statistical analyses were performed using the IBM SPSS Statistics 20 and GraphPad Prism 8.3.0 (538) utilized to draw the diagrams.. Statistical significance levels was set at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Results

CE causes infiltration of lymphocytes and dense fibrous tissue

Sirius Red and HE staining were used to investigate histomorphology. The HE staining results showed that numerous lymphocytes were infiltrated in the liver of CE patients compared to the normal samples. In addition, Sirius Red staining revealed that a substantial amount of collagen was deposited in CE liver tissues compared to the normal samples(Fig. 1A). Moreover, mouse models were used to systematically and comprehensively study the entire process involved in the course of liver infection. Therefore, 3000 and 5000 protoscolices, respectively were injected into the glisson capsule. Within an infection period of 90 days, there was limited progression of CE vesicles at the site of injection. However, when focus was expanded to the surrounding areas as infection progressed, the results showed that CE was a prolonged and progressive disease (Fig. 1B).

Furthermore, HE staining revealed that a substantial amount of lymphocytes infiltrated around lesions in the 3000 and 5000 protoscolices groups after 30 days of infection. However, the number of lymphocytes infiltrated around the lesions continuously decreased with the progression of CE. Additionally, scattered lymphocytes and closely arranged fibroblasts could be seen around the injured areas after 120 and 150 days of infection. The HE staining results also showed that the degree of inflammatory response assumed a downward trend, and tissue congestion presented was pronounced 120 days after of infection (Fig. 1C).

On the other hand, the Sirius Red stained liver sections revealed the formation of loose collagen fibrils after 30 days of infection. Afterwards, the fibrous tissue increased, became denser and eventually formed a monolayer (Fig. 1D).

CE alters the expression of NK cells surface receptor in the liver

In order to detect the impact of CE on NK cells in the liver, gate NK1.1 + CD3 was selected as the NK phenotype (Fig. 2A). Generally, murine intrahepatic NK cells can be divided into two subsets based on the relative expression of CD49a and DX5 respectively. They include circulating conditional NK cells and liver-resident NK cells[21]. The findings showed that the proportion of liver-resident NK cells increased in both the 3000 and 5000 protoscolices groups 7 days after infection but were afterwards steadily maintained at low levels and reached the lowest point on the 90th day. Notably, a novel cell subset persisted between day 14 and 90 and this may have been due to the migration of NK cells from the spleen to the damaged parts of the liver (Fig. 2B). The bar chart in Fig. 2F was therefore drawn to clearly highlight this change (Fig. 2F).

NKG2D is a marker of NK cell activation and it triggers apoptosis in HSCs by interacting with ligands on their surface to alleviate liver fibrosis[22]. In this study, expression of NKG2D reached its peak on the 7th day but then assumed a decreasing trend except on the 60th day in both the 3000 and 5000 protoscolices groups. On the 90th day, expression of NKG2D reached its lowest point in both groups, indicating the diminished ability of NK cells to limit liver fibrosis (Fig. 2C, G). Moreover, the up-regulation of NKG2D on the 60th day may have been because the migrating NK cells were at their maximum proportion (Fig. 2F).

Previous reports showed that the expression levels of NKG2A had an inhibitory effect on NK cells[23]. In this study, it was shown that the expression of NKG2A declined in CE 7 to 21 days after infection, compared to the controls in both the 3000 and 5000 protoscolices groups. However, expression of NKG2A's was continuously up-regulated between day 30 and 90 in both groups (Fig. 2D, H).

Moreover, TIM-3 is an inhibitory receptor to NK cells[24] and can hinder NK cell-mediated cytotoxicity [25]. In addition, it was reported that blocking the TIM-3 pathway could enhance the ability of NK cells to produce IFN- γ [26]. In the present research, it was shown that the inhibitory signal TIM-3 was gradually down-regulated between the 7th and 30th day but was up-regulated between the 60th and 90th day in both the 3000 and 5000 protoscolices groups (Fig. 2E, I).

Ce Boosts The Secretion Of Cytokines Il-15 And Tgf- β 1

Previous reports demonstrated that IL-15 could impede liver fibrosis [27], while TGF- β exerted the inverse effect [28]. In this study, there were increased levels of IL-15 secretion from day 7 with maximum levels on day 30 in both groups, compared to the control. However, a downward trend in the secretion levels of IL-15 in CE was observed afterwards in both groups, although the levels were persistently higher than that of the control group during the entire duration of infection (Fig. 3A).

Similar to findings from previous studies [12] the secretion levels of TGF- β 1 were continuously higher than that of the control group and reached a peak on the 60th day after infection (Fig. 3B). Given that elevated secretion of TGF- β in the liver mainly occurred between day 30 and 90 after infection, immunofluorescence was used to show that this excessive secretion was mainly derived from the macrophages in both patients and mice with CE (Fig. 3C-F).

CE induces the activation of HSCs and senescence in hepatocytes

Immunohistochemical staining revealed that α -SMA was over-expressed in the liver tissue sections of CE patients. α -SMA is a marker of activated HSCs and is mainly located around the region of focus (Fig. 4A). Activated hepatic stellate cells in turn produce excessive ECM that is deposited around vesicles hence maintaining/promoting CE-related fibrosis. In addition, senescent hepatocytes induce the activation of hepatic stellate cells[29]. In this study, liver tissues from CE patients were used to investigate the presence of senescent hepatocytes during infection. Generally, cell senescence arises due to the alteration of lysosome activity and involves the expression of senescence-associated- β -galactosidase (SA- β -Gal), which is a marker of cell senescence. Moreover, senescent hepatocytes were reported to express similar genes, including p53

(TRP53) and p21 (WAF1)[14]. The results of immunofluorescent staining revealed that markers of senescence including SA- β -Gal (Fig. 4B), p53 (Fig. 4C, D), p21 (Fig. 4E) were up-regulated during infection.

The use of inhibitors does not aggravate liver damage and can relieve the dysfunction of NK cells in CE mouse models

After using the inhibitor, ELISA results showed that only Pirfenidone and combined medication (Pirfenidone + SB525334) reduced the secretion of TGF- β in liver tissues in the CE mouse model. However, SB525334 had no such effect (Fig. 5A).

In addition, liver function was assessed by testing such markers as serum ALT, AST and direct bilirubin[30]. The results suggested that inhibitors had no detrimental effect on liver function (Fig. 5B-D).

Moreover, flow cytometry revealed changes in the expression of receptors on the surface of NK cells. In the Pirfenidone medication group, there was an increase in the expression of the activating receptor NKG2D and a down-regulation of the inhibitory receptor TIM3. There was however no effect on the expression of the inhibitory receptor NKG2A. In contrast, the SB525334 medication group showed an increase in the expression of the activating receptor NKG2D and a down-regulation of both the inhibitory receptors TIM3 and NKG2A. A combination of both drugs effectively merged the efficacy of the two inhibitors. In addition, all the three medications could up-regulate the expression of DX5. However, there was no statistical difference in the therapeutic effect of combined medication and the individual drugs (Fig. 5E). Moreover, combined medication could significantly increase the expression of proliferation index Ki67 in NK cells compared to the individual drugs (Fig. 5F).

The use of inhibitors reduces hepatocyte senescence in CE mouse models Similar results were obtained from the Western blotting technique. Pirfenidone inhibited the expression p16, p21 and p53 although SB525334 merely reduced the expression of p21 and p53. A combination of Pirfenidone and SB525334 showed better results with regard to curbing senescence in hepatocytes (Fig. 6A). In addition, combined medication could significantly decrease the expression of aging-related markers compared to single medication (Fig. 6B, C, D).

Use of inhibitors relieves CE-associated fibrosis in mouse models Collagen is a major component of the ECM and is usually degraded by Matrixmetalloproteinases (MMPs). However, during the process of fibrogenesis degradation is inhibited by Tissue Inhibitor of Metalloproteinases (TIMPs) [31]. In this study, Pirfenidone promoted the production of MMP1 and reduced the expression of TIMP1. On the other hand, SB525334 down-regulated the expression of TIMP1 but had no effect on the expression of MMP1. However, a combination of Pirfenidone and SB525334 substantially reduced the expression of TIMP1 and elevated the expression of MMP1. Based on these results, combination medicine altered the composition of enzymes and this may greatly have impacted the deposition of ECM.

Compared to the control group, combined medication was superior to each of the individual medications in reducing the production of collagen I and III. Pirfenidone had no effect on the expression of TGF- β receptors I and II. In addition, combined medication more effectively reduced the expression of TGF- β receptors I and II

compared to SB525334. With regard to markers of HSCs activation, a combination of Pirfenidone and SB525334 had a more potent effect than the individual drugs in curbing the expression of α -SMA (Fig. 7).

Discussion

CE is a globally distributed disease that has a negative impact on human health and wellbeing[1]. Therefore, it is important to investigate changes in pathology of CE in order to provide potential treatment strategies. By examining CE in liver tissues, this study revealed a decrease in infiltrated lymphocytes and an increase in both quantity and density of collagen fibers. Therefore, a decrease in inflammatory response and an increase in fibrous tissue were the main changes observed during infection.

In addition, histological examination revealed that the fibrous tissue around the capsule changed from loose to dense and the amount of fibrous tissue continuously increased in the course of infection. This fibrous tissue resulted from excessive deposition of ECM[32] resulting to the host-derived fibrous capsule[2]. This fibrous capsule might lead to immune escape by hindering the host's immune response against the parasite[33]. It was therefore concluded that the main treatment strategy would be to inhibit fibrosis as well reduce infiltration of lymphocytes around the cysts. The results are discussed based on two aspects, anti-fibrosis and pro-fibrosis.

NK cells possess antifibrotic properties since they facilitate the clearance of activated HSCs through NKG2D-dependent cytotoxicity and interferon- γ (IFN- γ)—related apoptosis[34]. NK cells preferentially reverse senescence in activated HSCs *in vitro* and *in vivo*, thereby inhibiting the progression of fibrosis[35]. In addition, NK cells generally perceive changes in their surroundings through a group of innate-receptors. Based on their effects on the function of NK cells, they can be divided into inhibitory and activating receptors. By combining the receptors, integrated signals give rise to cytotoxicity and cytokine production[36]. Moreover, activated NK cells tend to show elevated levels of activating receptors and reduced quantities of inhibitory receptors[37].

Therefore, a dysfunction of NK cells was characterized by an up-regulation of inhibitory receptors and a down-regulation of activating receptors in some chronic diseases[8]. In this study, it was shown that there was an increase in the inhibitory receptors-Tim-3 and NKG2A while the activating receptors-NKG2D-decreased. Therefore, it is possible that NK cells were in a dysfunctional state. In addition, blocking the TIM-3 pathway was able to enhance the ability of NK cells to produce IFN- γ .

IL-15 is a secretory cytokine that plays an essential role in the growth and development of NK cells[38]. Previous reports showed that the IL-15 / IL-15 receptor α could not only act on HSCs to reduce the production of collagen but also prime NK cells to remove activated HSCs[27]. However, TGF- β blocks the effector function of NK cells by inhibiting the activation of mTOR which is induced by IL-2 or IL-15[11]. Although increased secretion of IL-15 was reported in this study, continuously high levels of TGF- β could ultimately hinder the effector function of NK cells. Therefore, it was speculated that excess macrophage-derived TGF- β inhibited the anti-fibrotic effect of NK cells in the course of CE infection.

The role of TGF- β in liver fibrosis is based on the fact that it effectively stimulates the activation of HSC and the secretion of ECM[39]. Combined with activated TGF- β , type II receptors recruit and phosphorylate type I receptors. This transmitted signal then leads to phosphorylated SMAD2/3 proteins. Therefore, pSMAD2/3 binds to Smad4 and transfers to the nucleus eventually leading to the expression of related genes[40]. In liver fibrosis, type IV collagen and the laminin of constituent ECM shift toward type I and III collagen[3] and are the main changes in pathological fibrous tissues[31]. Through Sirius Red Staining, the study revealed that type I and III collagen were the main components surrounding the cysts. In addition, TGF- β can inhibit the secretion of IFN- γ and expression of NKG2D on NK cells hence reducing the anti-fibrotic function of NK cells[22]. Consequently, it was concluded that the TGF- β pathway may be crucial in liver fibrogenesis.

Senescent hepatocytes may be alternative pro-fibrotic mediators of CE-related liver fibrosis. Senescence is characterized by permanent DNA damage and activation of the Senescence-associated Secretory Phenotype (SASP). SASP involves the expression of interleukin-1 α (IL-1 α) and TGF- β [13], which can induce and maintain the senescent phenotype in cells[41]. Additionally, *in vitro* studies showed that TGF- β could release ROS through non-smad pathways, promote the secretion of SASP-related cytokines and enhance senescence in hepatocytes[42]. Moreover, in *in vivo* studies using a hepatocyte-specific senescence model showed that senescent hepatocytes substantially enhanced the secretion of macrophage-derived TGF- β that could result to further senescence hence forming a feedback loop. However, SB525334, an inhibitor of the TGF- β signaling pathway could reverse this feedback loop[43]. Senescent hepatocytes also boost the activation of HSCs[29]. In the present study, a substantial number of hepatocytes became senescent and the secretion of macrophage-derived TGF- β was maintained at a high level. It is therefore possible that there was also positive feedback in the course of CE. Based on this evidence, it is highly likely that TGF- β was an essential factor in the progression of CE-related liver fibrosis.

Furthermore, the study selected Pirfenidone and SB525334 as inhibitors of the TGF- β pathway. Pirfenidone is a pyridone chemical compound and is approved by the Food and Drug Administration (FDA) for the treatment of idiopathic pulmonary fibrosis. More importantly, Pirfenidone reduces collagen production related to the TGF- β signaling pathway[44]. On the other hand, SB525334 inhibits the TGF- β receptor I (ALK5) and was shown to restore hepatocellular senescence in acute liver injury [43]. A combination of Pirfenidone and SB525334 could not only reduce TGF- β secretion but also inhibited the TGF- β signaling pathway.

In vivo studies showed that Pirfenidone could alleviate the progression of liver fibrosis in humans[45]. Similarly, this study revealed that Pirfenidone reduced the secretion of TGF- β 1, restricted the activation of hepatic stellate cells and the production of collagen. To some extent, the decreased levels of TGF- β might have partially reversed senescence in hepatocytes and restored both liver and NK-cell function.

Matrix Metalloproteinases (MMPs) are the primary enzymes in the degradation of collagen and are mainly produced by macrophages. However, the Tissue Inhibitor of Metalloproteinases (TIMP) family, secreted by HSCs reversibly inhibit the activity of MMPs[46]. Therefore, the ratio of MMP-to-TIMP determines the progression of liver fibrosis[47]. In this study, it was shown that the levels of MMP1 increased while that of TIMP1 decreased and this was favorable for fibrogenesis. However, administration of Pirfenidone and SB525334, caused the ratio of MMP1-to-TIMP1 to assume a reverse trend. Moreover, a combination of both

drugs had the most potent effect in restricting the production of collagen. A combination of Pirfenidone and SB525334 could not only reduce the secretion of TGF- β but also inhibited the TGF- β signaling pathway. In addition, the markers related to hepatocyte senescence and activated HSCs were down-regulated. Notably, only Pirfenidone reduced the expression of p16 and the secretion of TGF- β 1 while no statistically significant difference was observed in SB525334, compared to the control group. Furthermore, the expression of activation and inhibitory receptors on NK cells showed some extent of a reverse trend. Compared to the control group, the therapeutic effect of combined medication was better than that of the individual drugs. This may be because combined medication was able to reduce the secretion of TGF in serum as well as block the TGF- β signaling pathway.

In study, a mouse model was used to conduct a complete and systematic analysis of the occurrence and development CE in Echinococcus granulosus infected liver. It was shown that Pirfenidone and inhibition of TGF- β reduced senescence in hepatocytes, restored the function of NK cells and relieved CE-associated fibrosis. This process was therapeutically feasible and provided a baseline for the treatment of CE-related fibrosis. However, more clinical studies are needed to validate its effectiveness and feasibility in patients with liver fibrosis.

However, given the short period of treatment, the side effects of the drugs could not be clearly elucidated. Moreover, considering the limited time, we only explored the efficacy of the drugs in vivo. Therefore, the underlying mechanisms need to be explored further in future experiments.

Abbreviations

CE- Cystic Echinococcosis; NK- Natural Killer cell; HSCs- Hepatic Stellate cells; ALT- Alanine Aminotransferase; AST- Aspartate Aminotransferase; PBS- Phosphate Buffered Saline; mAb- Monoclonal Antibody; FITC- Fluorescein Isothiocyanate; IFN- γ - Interferon- γ ; NKG2D- Natural Killer cell Group 2D; NKG2A- Natural Killer cell Group 2A; DAPI- 4,6-diamidino-2-phenylindole; PE- Phycoerythrin; TGF- β 1- Transforming Growth Factor- β 1; APC- Allophycocyanin; Tim-3- T-cell immunoglobulin and mucin-domain containing-3; α -SMA- α -Smooth Muscle Actin; IL-15- Interleukin-15; TIMP-1- Tissue Inhibitor of Metalloproteinase-1; MMP-1- Matrix metalloproteinase-1; DAB- Diaminobenzidine; ELISA- Enzyme-linked Immunosorbent Assay; PVDF- Polyvinylidene Fluoride; RIPA- Radio Immunoprecipitation Assay; DMSO- Dimethyl Sulfoxide; DBL- Direct Bilirubin; FBS- Fetal Bovine Serum; ECM- Extracellular Matrix.

Declarations

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Availability of data and materials

Contained data in this article supports the conclusions. All data generated or analyzed during the research can be gained from the corresponding authors upon request.

Ethics approval and consent to participate

Ethical approval to conduct both human and animal experiments was granted by the First Affiliated Hospital of Shihezi University, Xinjiang, China [Approval Number: A2017-068-01]. Moreover, consent was granted by the China Clinical Trial Registry, and the registration number was ChiCTR1900024122.

Consent for publication

Not applicable.

Competing interests

The authors declare that no conflicts of interest exist.

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Figures

Figure 1

Morphological characteristics of liver and fibrosis in CE patients and mouse models (A) Compared to normal human liver tissues, HE staining revealed that hepatocytes were disarranged, cell volume decreased and the nucleus shrank in the course of CE. In addition, Sirius red staining showed that massive fibrous tissue was deposited round the CE-related focus. (B) In CE mouse liver, there was an increase in semi-transparent vesicles. The number and volume of the vesicles increased in both the 3000 and 5000 protoscolices groups. (C) HE staining showed that lymphocyte infiltration initially increased but later decreased. Additionally, the

lesion area progressively expanded in both the 3000 and 5000 protoscolices groups. (D) Sirius Red staining revealed that collagen fibers increased constantly in both number and density during the course of CE in both the 3000 and 5000 protoscolices groups. Note: All the sections were imaged using a microscope at 40x magnification. Scale bars, 50 μ m.

Figure 2

Up-regulated activation receptors and down-regulated inhibitory receptors in NK cells from mice with CE (A) NK cells were detected from extracted liver lymphocytes through flow cytometry and NK1.1+CD3- chosen as

the gate. (B) A histogram showing the distribution of intensity in fluorescence revealed that the proportion of intrinsic NK cells in the liver decreased continuously in both the 3000 and 5000 protoscolices groups, compared to the controls. (C) A histogram depicting the distribution of intensity in fluorescence showed that the expression of activated receptor NKG2D in NK cells increased on day 7 after infection but remained at low levels afterwards in both the 3000 and 5000 protoscolices groups, compared to the control. (D and E) Histograms displaying the distribution of intensity in fluorescence indicated that expression of the inhibitory receptor TIM3 and NKG2A initially decreased and then increased in both the 3000 and 5000 protoscolices groups, compared to the control. (F, G, H and I) In order to intuitively highlight the change in trend, bar graphs corresponding to Figures B, C, D, and E respectively, were drawn. Three independent measurements were made to compare statistical significance between different groups (*P < 0.05, **P < 0.01 and ***P < 0.001).

Figure 3

CE causes increased secretion of IL-15 and TGF-β and TGF-β is mainly secreted by macrophages in the liver (A) Secretion of IL-15 in serum reached a peak on the 30th day after infection and was maintained at high levels 90 days after infection in both the 3000 and 5000 protoscolices groups, compared to the control. (B) Secretion of TGF-β in liver tissues reached a maximum 60 days after infection and was up-regulated within 90 days of infection in both the 3000 and 5000 protoscolices groups, compared to the control. (C-F) Immunofluorescence showed that TGF-β1 mainly originated from macrophages in both mouse and human

liver after developing CE. Note: All of the sections were imaged using a microscope at 40x magnification. Scale bars, 50 μ m.

Figure 4

Substantial hepatocyte senescence and activation of hepatic stellate cells in CE patients (A) The high expression of α -SMA showed that activation of HSCs was enhanced in CE patients. (B) Increased activity of SA- β -galactosidase implied that several hepatocytes became senescent in the course of CE. (C) Immunohistochemistry revealed that expression of the hepatocyte senescence marker, p53 was up-regulated in CE patients. (D-E) Immunofluorescence confirmed that there was an increase in the expression of the senescence markers p21 and p53 in CE patients. Note: All of the figures were obtained from a microscopic imaging system at 40x magnification except for Figure C where a magnification of 100x was used. Scale bars, 50 μ m.

Figure 5

Combination of Pirfenidone and SB525334 altered the surface receptors of NK cells in mice with CE (A) Only the Pirfenidone and combination medication groups showed decreased secretion of TGF- β in CE liver tissues. (B and C) Compared to the other groups, serum ALT and AST levels were lowest in the combined medication category. Similar results were observed with the levels of serum DBL. (D) Only SB525334 could reduce the secretion of DBL in the plasma of CE mice. (E) The three treatments could increase the expression of NK cell surface receptors DX5 and NKG2D and reduce the expression of NK cell surface receptor TIM3.

Both SB525334 and combined medication could reduce expression of the NKG2A receptor on the surface of NK cells although Pirfenidone was not able to. (E) There was an evident increase in the proliferative capacity of NK cells in the combined drug group compared to both the individual drugs and control groups.

Figure 6

Use of Pirfenidone and SB525334 reduced senescence in the hepatocytes of mice with CE (A) Both Pirfenidone and combined medication could reduce the expression of p16, p21 and p53 (markers of hepatocyte aging). However, SB525334 could only reduce the expression of p21 and p53. (B and C) Immunofluorescence showed that combined medication could reduce the expression of p21 and p53 more effectively compared to both individual drugs and controls. (D) SA-β-Gal staining showed that combined treatment could more effectively alleviate senescence in hepatocytes compared to the individual drugs. All the figures were obtained from a microscopic imaging system at 40x magnification. Measurements were

repeated 3 times to compare statistical significance between different groups. Scale bars, 50 μ m. (Statistical significance: *P < 0.05, **P < 0.01 and ***P < 0.001).

Figure 7

Use of Pirfenidone and SB525334 proved to be better in relieving CE-related liver fibrosis. Using inhibitors of the TGF- β signaling pathway, immunoblot experiments highlighted the expression of fibrosis-related proteins in the liver tissues of CE mice treated with different groups of drugs (Statistical significance: *P < 0.05, **P < 0.01 and ***P < 0.001).