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Extracellular vesicles derived from *Echinococcus granulosus* hydatid cyst fluid from patients: isolation, characterization and evaluation of immunomodulatory functions on T cells

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ABSTRACT

Cystic echinococcosis is a chronic and complex zoonotic disease. The mechanisms underlying the parasite's establishment, growth and persistence are not completely understood, and are thought be modulated by a crosstalk through extracellular vesicles. Here, EVs were isolated from the hydatid cyst fluid of patients with cystic echinococcosis and protoscolex culture supernatant. Proteomic analysis of these EVs revealed several parasite- and human-derived proteins. Very few studies have performed proteomic analysis of EVs isolated from HCF and PCS. Our proteomic analysis of the EVs derived from HCF and PCS facilitated identification of 1175 proteins, wherein 1026 and 38 proteins were exclusively identified in the EVs derived from HCF (HCF-EVs) and PCS (PCS-EVs), respectively, and 111 proteins were shared in both. The results of co-culture of PCS-EVs with murine peripheral blood mononuclear cells showed that PCS-EVs significantly regulated T lymphocyte functions in a dose-dependent manner. Collectively, our results provide valuable information on parasite survival strategies and new insights into the role of these EVs in the establishment and persistence of hydatid cysts.

1. Introduction

Cyst echinococcosis (CE) is a zoonosis caused by the larval form (metacestode) of parasites from the genus *Echinococcus granulosus*, and is typically transmitted in a pastoral life cycle involving sheep and dogs (Cucher et al., 2016). Globally, CE leads to severe social and economic burdens owing to the costs associated with human treatment and losses in livestock production (Budke et al., 2006). Ingestion of parasite eggs by humans allows the hatching of oncospheres and the subsequent development of metacestodes (or hydatid cysts) in the viscera (mostly the liver and lungs) (Santos et al., 2016).

Anatomical evaluation shows that the hydatid cyst is a uniloc-ular structure filled with hydatid cyst fluid (HCF). The cyst wall is delimited by an acellular and parasite-secreted laminar layer (LL) of variable thickness, and is internally lined with a thin germinal layer (GL) (Diaz et al., 2011a,b). The LL is a microfibrillar matrix comprising high molecular weight carbohydrates and represents the first line of defense for mechanical and immunological survival of the metacestode both mechanically and immunologically (Diaz et al., 2011a,b).

The GL is composed of a tegument and several cell types includ-ing proliferative undifferentiated cells responsible for the forma-tion of brood capsules containing protoscoleces (Galindo et al., 2008), which grow into new and fully developed metacestodes in the intermediate host and mature into adult worms in the definitive host (e.g. dog or wolf) (Monteiro et al., 2010). Therefore, protoscoleces could be considered the most important active component of metacestodes. HCF contains different molecules pro-duced by GL and protoscoleces (Siles-Lucas et al., 2017), and host proteins, which permeate into the cyst and cross the adventitial and LL barriers through unknown mechanisms (Silva-Alvarez et al., 2016; Virginio et al., 2012). The proteins secreted by protoscoleces are thought to play fundamental roles in the host-parasite relationship (Virginio et al., 2012).

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Comparing the differences between PCS and HCF components is necessary to understand the parasite biology, which may elucidate the mechanisms underlying parasite establishment, development and persistence during the progression of CE. Accordingly, some potential factors are crucial for parasite survival and could serve as new markers of diagnosis, treatment, and prognosis for patients with CE. A novel mechanism underlying the parasite-to-host communication via extracellular vesicles (EVs) was demonstrated (Buck et al., 2014). EVs from some parasites (e.g. *Leishmania, Trypanosoma cruzi, Fasciola hepatica* and *Echinostoma caproni*) are known to transport parasite molecules (e.g. miRNAs, proteins, and soluble factors) to different host cells, wherein these factors play a variety of functions, inducing specific cytokine secretion and immunosuppression (Coakley et al., 2015; Evans-Osses et al., 2015; Schorey et al., 2015). In recent years, plenty of publications have described the importance of helminth-derived EVs in host-parasite interactions and showed that helminth-derived EVs have complex and diverse immunomodulatory properties in host-parasite interactions including immunostimulation and immunosuppression (Eichenberger et al., 2018; Tritten and Geary, 2018).

Recently, studies have been conducted on the isolation and characterization of the exosomes derived from fertile sheep hydatid cysts, and protoscoleces and GLs were shown to secrete exosomes carrying virulence factors associated with cyst survival, including highly immunogenic and tolerogenic antigens and peptidases, into the HCF (Siles-Lucas et al., 2017). EVs carry a wide variety of molecules, but proteins seem to play a direct role. The proteomic analyses of the HCF-EVs from fertile sheep hydatid cysts (Siles-Lucas et al., 2017) and PCS-EVs from the lung and liver of infected cattle (Nicolao et al., 2019) have been conducted. However, no study has compared the proteomics of the two EVs derived from HCF and PCS isolated from a human patient's body.

Many reports have shown that the Th2-type responses are associated with susceptibility to *E. granulosus* infection in both humans (Rigano et al., 1999, 2001, 2004) and mice (Rogan, 1998; Dematteis et al., 1999, 2003), but the immunity against cestodes requires Th1-type responses to induce protection (Terrazas, 2008; Jenkins and Allen, 2010; Rostami-Rad et al., 2018). Thus, T lymphocytes play important roles in the prevention and development of CE (Pan et al., 2013). Until now, more and more studies have proved that EVs play important roles in immune regulation in helminth infection. A study has proved that *E. granulosus* exosome-like vesicles could be internalized by murine dendritic cells, inducing their maturation with an increase in CD86 and with down-regulation of the expression of MHC-II molecules (Nicolao et al., 2019). Besides, EVs from helminths were proven to modulate immune responses in dextran sulfate sodium-induced colitis, exerting a protective effect (Roig et al., 2018). Some researchers have certified that EVs secreted by *Heligmosomoides polygyrus* are internalized by macrophages, causing down-regulation of type 1 and type 2 immune response-associated molecules and inhibiting expression of the IL-33 receptor subunit ST2 (Coakley et al., 2017). In this study, proteomic analysis of EVs from *E. granulosus* was performed, combined with the effects of PCS-EVS on T lymphocyte proliferation and function as demonstrated by in vitro experiments on PCS-EVs and murine peripheral blood mononuclear cells (PBMCs), which contribute to revealing the host -parasite relationship and related mechanisms.

2. Materials and methods

2.1. Parasite materials

Echinococcus granulosus hydatid cysts were obtained from three CE patients with informed consent at the General Hospital of Ningxia Medical University, Yinchuan, China. The hydatid cysts obtained from each patient with CE were used individually as one group for proteomic sequencing to meet three biological replicates. The intact daughter cysts were removed into sterile round cell culture dishes (Corning-Costaer, USA) after gently opening the ectocyst (host fibrous tissue) of the entire hydatid cysts. The liquid in daughter cysts was aseptically transferred into 50 ml centrifuge tubes (Kirgen, USA) with a syringe and kept on ice until the hydatid sank to the bottom of the tube. The HCF was collected for the separation of EVs, and then the precipitate containing protoscoleces was collected. The daughter cysts without HCF were cut into small pieces and washed several times with sterile PBS (Biological Industries, Israel). The washing solution was filtered through an 80-mesh cell sieve, followed by aseptic collection of the filtrate in 50 ml centrifuge tubes. The precipitate containing protoscoleces was also collected after naturally precipitating on ice for 30 min. The obtained protoscoleces were combined and washed several times with sterile PBS. The viability of the protoscoleces was confirmed from their motility characteristics, and protoscoleces were counted under an ordinary light microscope.

2.2. Cultivation of protoscoleces

Protoscoleces were plated on 24-well flat-bottom culture plates (Corning-Costar, USA) at a density of 3,000 protoscoleces/well and cultured in RPMI-1640 medium supplemented with 30% exosome-depleted fetal bovine serum (FBS; ViVaCell-Biotechnology, Germany) and 1% penicillin-streptomycin solution (Gibco, USA) at 37 °C and 5% CO_2 for 1 month. The medium was changed every 3 days and the collected cultivate supernatant would be immediately used to extract EVs.

2.3. EV isolation and purification

The EVs were isolated and purified from fresh HCF and PCS using an exoEasy Maxi Kit (Qiagen, Germany), a membrane-based affinity binding step to isolate EVs, according to the manufacturer's instructions. Briefly, the supernatant was filtered to exclude particles larger than 0.8 μm with syringe filters (Sartorius Minisart NML, Germany). The pre-filtered samples were mixed with binding buffer XBP and allowed to bind to an exoEasy membrane affinity spin column. The bound EVs were washed with washing buffer XWP and eluted with elution buffer XE (an aqueous buffer containing primarily inorganic salts) for further analysis.

2.4. Detection of protein concentration

The concentration of EV total proteins was quantified with the bicinchoninic acid assay (Thermo Fisher, USA) according to the manufacturer's instructions.

2.5. Transmission electron microscopy (TEM)

Freshly isolated EVs were layered on copper grids with 0.125% formvar in chloroform and stained with 1% uranyl acetate in double-distilled water. EVs on the grids were immediately visualized under a transmission electron microscope (HITACHI-H7650, Japan).

2.6. Protein staining

In total, 10 μg/lane of EVs were loaded onto 12% acrylamide gels, subjected to SDS-PAGE, and subsequently stained with Coomassie Brilliant blue G250 (Sigma-Aldrich, USA) at room temperature for 2 h. The gel was then destained until the background was colorless and the strips were clear.

2.7. Western blot (WB) analysis

For WBs, 15 μg/lane of EVs were separated with 12% SDS-PAGE and transferred onto 0.45 μm polyvinylidene difluoride membranes (Merck Millipore, USA). The membranes were blocked with 5% w/v skimmed milk in TBST (0.05% v/v Tween-20 in Tris-buffered saline) for 2 h at room temperature, followed by an overnight incubation at 4 °C with mouse anti-human CD63 monoclonal antibody (dilution 1:200; Santa Cruz Biotechnology, USA). The membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (dilution 1:20,000; Abcam, USA) for 1 h at room temperature for chemiluminescence imaging with Piece Fast Western Blot Kit, ECL Substrate (Thermo Fisher, USA). The resulting blots were analyzed with Image Lab software (Bio-Rad, USA). The antibodies were diluted with Western BLoT Booster (Takara, Japan).

2.8. Nanoparticle tracking analysis (NTA)

We measured the particle sizes of EVs with NTA using ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and its corresponding software ZetaView 8.04.02.

EVs were diluted in sterile PBS (Biological Industries, Israel) before NTA analysis. NTA measurement was recorded and analyzed at 11 positions. The ZetaView system was calibrated using 110 nm polystyrene particles. The temperature was maintained at approximately 30 °C.

2.9. PBMC preparation

Five normal female BALB/c mice, 6–8 weeks of age, were purchased from Peking University Health Science Center (Department of Laboratory Animal Science), China, and maintained under specific pathogen-free conditions. Then the murine blood was collected in disposable EDTA anticoagulation vacuum blood collection tubes (Kang Jian, China). The anticoagulated whole blood samples were diluted 1:1 in RPMI-1640 and subjected to density gradient centrifugation using Lymphoprep (Solarbio, China) according to the manufacturer's instructions. The experiment was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. All efforts were made to minimize animal suffering. All animal procedures were approved by Ningxia Medical University Medical Ethical Committee, China.

2.10. Co-culture assays

PBMCs were labeled with 2 μM of 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, USA) at 37 °C for 10 min in the dark. A total of 3×10^5 PBMCs were cultured on 96-well flat-bottom culture plates (Corning-Costaer, USA) containing 300 μl of complete medium (10% exosome-depleted FBS and 1% penicillin–streptomycin solution dissolved in RPMI-1640 medium) in the presence of anti-CD3 (0.1 μg/ml; R&D Systems, USA) and anti-CD28 (0.25 μg/ml; R&D Systems, USA) antibodies at 37 °C and 5% CO_2. Different doses of PCS-EVs (0, 6, 12 and 24 μg) were added to the culture system, and the group without anti-CD3 and anti-CD28 antibodies was treated as a control and each group was performed in three replicates. After 3 days, all cells were harvested and stained with allophycocyanin (APC)-conjugated anti-CD3 (Biolegend, USA) and peridinin-chlorophyll-protein complex (PerCP)-conjugated anti-CD4 for the detection of the proliferation of T lymphocytes, $CD4^+$ T cells and $CD8^+$ T cells with BD Accuri C6 (BD Biosciences, USA). The cell culture supernatant was collected and frozen at −80 °C for cytokine measurement with a Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, USA) according to the manufacturer's protocol.

2.11. LC-MS/MS analysis

Firstly, the solutions containing EVs were freeze-concentrated to meet the requirements of proteomic sequencing for EV protein concentration. The proteins were digested with trypsin. The protein solution was reduced with 5 mM dithiothreitol for 30 min at 56 °C and alkylated with 11 mM iodoacetamide for 15 min at room temperature in the dark. The sample was then diluted with 100 mM NH4HCO3 at a urea concentration of less than 2 M. Finally, trypsin was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratio for a second 4 h digestion step.

The tryptic peptides were dissolved in 0.1% solvent A (formic acid) and directly loaded onto a home-made reverse-phase analytical column (15 cm length, 75 μm inner diameter (i.d.)). The gradient comprised an increase from 6% to 23% of solvent B (0.1% formic acid in 98% acetonitrile) over 26 min, 23% to 35% in 8 min and climbing to 80% in 3 min, followed by a hold at 80% for the last 3 min. The flow rate was maintained at 400 nl/min on an EASY-nLC 1000 UPLC system.

The peptides were subjected to NSI source followed by MS/MS in Q Exactive™ Plus (Thermo Fisher, USA) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The m/z scan range was 350–1800 for a full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. The peptides were selected for MS/MS using the NCE setting as 28 and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans with 15.0 s dynamic exclusion was used. Automatic gain control (AGC) was set at 5E4.

2.12. Database search

The resulting MS/MS data were processed with the MaxQuant search engine (v.1.5.2.8). Tandem mass spectra were searched against the 'Human' (20,317 sequences) database in Uniprot and *E. granulosus* (11,319 sequences) database in NCBI, and concatenated with the reverse decoy database to calculate the false discovery rate (FDR) caused by random matching, and some common contamination databases to eliminating the effects of contaminating proteins in the identification results. Trypsin/P was specified as the cleavage enzyme and up to two missing cleavages were permitted. The mass tolerance for precursor ions was set as 20 ppm in the first search and 5 ppm in the main search, while the mass tolerance for fragment ions was set as 0.02 Da. Carbamidomethyl on Cys was specified as a fixed modification, and oxidation on Met was specified as a variable modification. The label-free quantification method was LFQ, and the FDR was adjusted to < 1%. The minimum score for peptides was set at > 40.

The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (PRoteomics IDEntifications) partner repository with the dataset identifier **PXD014354**.

2.13. Bioinformatics analysis

Protein annotation, functional enrichment of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway were performed.

The UniProt-GOA database (www.http://www.ebi.ac.uk/GOA/), InterProScan (a sequence analysis application) based on protein sequence alignment method combined with the KEGG online service tool KAAS were used to annotate GO annotation pro-

teome and KEGG database description, respectively. If some identified proteins were not annotated with the UniProt-GOA database, InterProScansoft was used to annotate the GO function of the protein according to the sequence alignment method. Proteins were classified by GO annotation of the molecular function (MF) categories. UniProt-GOA, InterPro and KEGG databases were used for the enrichment analysis of GO and KEGG, respectively.

2.14. Statistical analysis

GraphPad Prism 5.0 software was used for statistical analysis. Images were processed with Adobe Photoshop CS3 to make the pictures clearer.

The data are expressed as mean ± S.D. For co-culture assays, one-way ANOVA was used to analyze the difference among groups. A value of $P < 0.05$ was considered statistically significant. For functional enrichment, a two-tailed Fisher's exact test was used to evaluate the enrichment of the differentially expressed proteins against all identified proteins. The corrected P value < 0.05 indicated significance. Standardization of the sample was achieved by controlling the amount of protein loaded. The relative quantification of shared proteins was obtained according to the protein LFQ intensity between different samples. A protein with a portion satisfying ratio (HCF-EV/PCS-EV) greater than 1.5 or less than 1/1.5, and a P value < 0.05 in the t-test was regarded as a differentially expressed protein.

3. Results

3.1. Identification of HCF- and PCS-EVs

To confirm the successful extraction of EVs, we characterized them using TEM. The representative micrographs revealed vesicles with round or oval membranes, and a diameter of <200 nm under TEM (Fig. 1A). To further characterize EVs, we used NTA and found that the main peak size of vesicles centered around 30–200 nm (Fig. 1B). The expression of tetraspanins CD63, an EV-specific marker ubiquitously present on EVs from most cell types (Kim et al., 2016; Kowal et al., 2016; Wiklander et al., 2018), was verified with WBs (Fig. 1C), and only HCF-EVs showed the band.

Collectively, these observations suggest the successful isolation of EVs from HCF and PCS.

3.2. The proteome of HCF- and PCS-EVs: identified proteins

Protein staining was performed to observe the differences in the distribution of HCF- and PCS-EV proteins. The results showed that two EV types showed protein bands in a wide molecular weight range from 15 to 250 kDa. While the HCF-EVs contained two major bands between 35 and 55 kDa, PCS-EVs exhibited two main bands between 55 and 130 kDa (Fig. 2A).

We performed LC-MS/MS proteomic analysis for HCF-EVs and PCS-EVs to identify and analyze these proteins. A total of 1175 (38 + 111 + 825 + 201) proteins were identified, and 1137

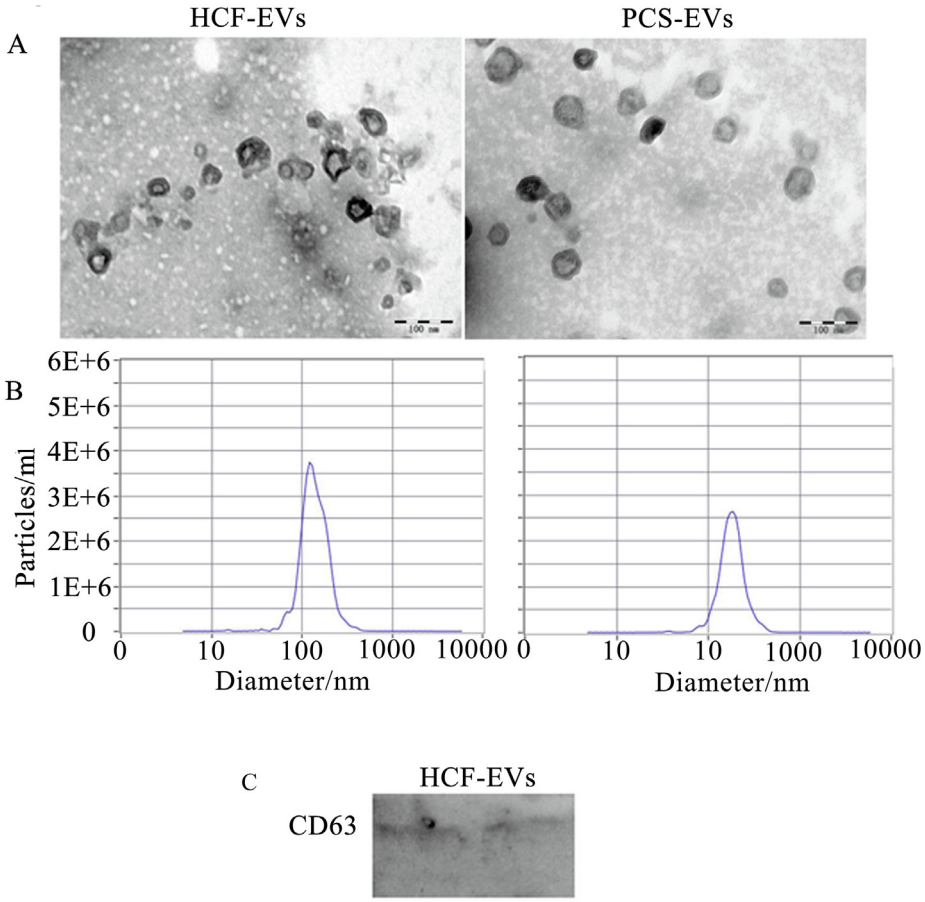

Fig. 1. Identification of extracellular vesicles isolated from hydatid cyst fluid (HCF-EVs) and protoscolex culture supernatant (PCS-EVs). (A) Transmission electron microscopy images of HCF- and PCS-EVs. Scale bars = 100 nm. Direct magnification: 30,000 ×. (B) Size distribution of EVs was analyzed by nanoparticle tracking analysis. (C) The diameter detection of EVs using NTA. (D) Western blot analysis of CD63 in HCF-EVs.

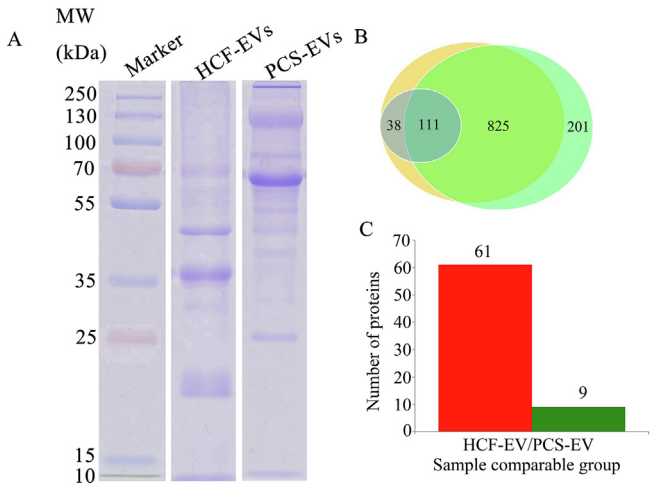

Fig. 2. Proteomic analysis of extracellular vesicles isolated from hydatid cyst fluid (HCF-EVs) and protoscolex culture supernatant (PCS-EVs). (A) SDS-PAGE profiles of HCF-EV and PCS-EV proteins were stained using coomassie brilliant blue. Marker, HCF-EVs and PCS-EVs are indicated. (B) Venn diagram of protein components contained in HCF-EVs and PCS-EVs. The blue area represents the proteins of PCS-EVs, the yellow area represents the *Echinococcus granulosus* proteins, and the green area represents the proteins of HCF-EVs. (C) Histogram analysis of 70 proteins with statistical significance among the proteins shared in HCF- and PCS-EVs. The red color represents up-regulated proteins in HCF-EVs compared with PCS-EVs, while the green color represents down-regulated proteins. A protein with a portion satisfying ratio (HCF-EV/PCS-EV) greater than 1.5 or less than 1/1.5, and a *P* value < 0.05 is regarded as a differential protein.

(111 + 825 + 201) and 149 (38 + 111) proteins were detected in HCF-EVs and PCS-EVs, respectively (Fig. 2B). A total of 1026 (825 + 201) proteins were exclusively identified in HCF-EVs, including 825 proteins of parasitic origin and 201 proteins of human origin (Fig. 2B), consistent with previous studies, in which many host proteins were found in EVs isolated from fertile sheep hydatid cysts (Siles-Lucas et al., 2017). The proteins with intensity values in HCF-EVs are listed in Supplementary Table S1. Contrarily, a total of 38 proteins were exclusively identified in PCS-EVs (Fig. 2B). Supplementary Table S2 shows the proteins with intensity values in PCS-EVs. Only 111 proteins, including 61 up-regulated proteins and nine down-regulated proteins in HCF-EVs as evident from HCF-EV/PCS-EV values, were shared between HCF-EVs and PCS-EVs (Fig. 2B, C and Supplementary Table S3).

In summary, the protein compositions of HCF-EVs and PCS-EVs were very different. The proteins from HCF-EVs were not only derived from the parasite, but also derived from the human. The number of parasite proteins identified in HCF-EVs far exceeds that of PCS-EVs.

3.3. Comparison proteins identified in HCF- and PCS-EVs

Most of the proteins (936 proteins) identified in HCF-EVs were from parasites, but some were of human origin (201 proteins) (Supplementary Table S1). And the human- and parasite-derived proteins annotated with MF both indicated that the two most populated GO terms were binding and catalytic activity (Fig. 3A, B). The high KEGG enrichment in HCF-EVs was mainly related to parasites and contained egl03050 proteasome and egl00020 citrate cycle (TCA cycle), which are included in the three most KEGG-enriched terms for HCF-EV parasite proteins (Fig. 3C). This observation predicts that the parasite proteins in HCF-EVs determine the enrichment of the KEGG pathway. Similarly, the proteins shared in HCF-EVs and PCS-EVs, including the up- and down-regulated proteins, were mostly distributed into binding and catalytic activity categories (Fig. 4A, B). For enrichment in up-regulated proteins,

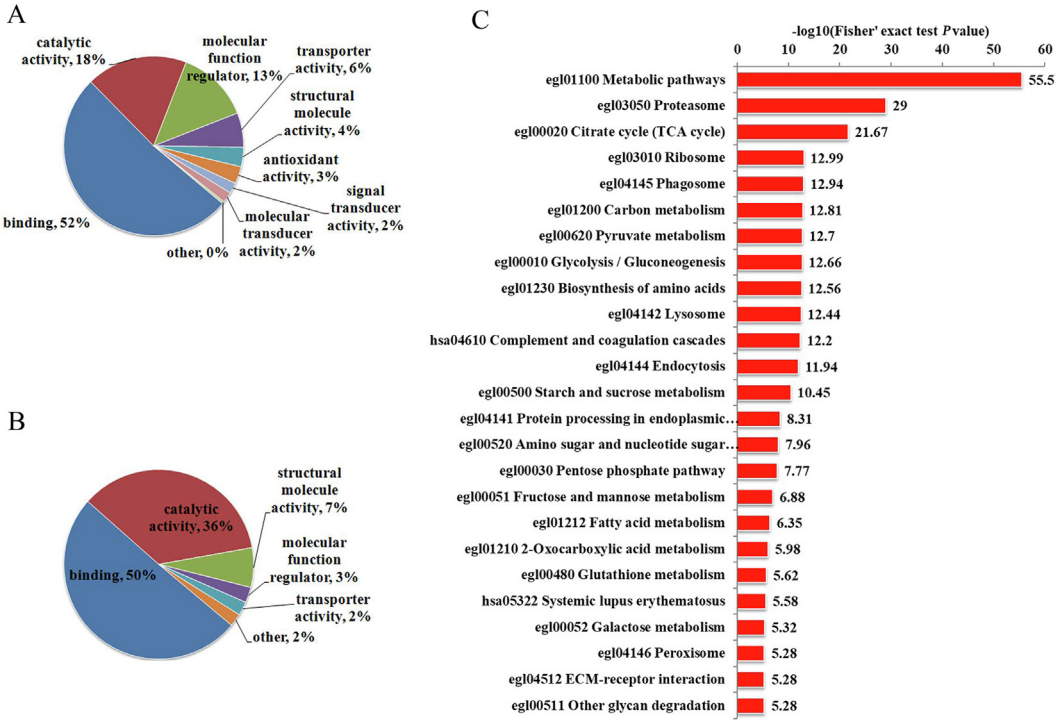

Fig. 3. Proteomic analysis of proteins identified in extracellular vesicles isolated from hydatid cyst fluid (HCF-EVs). The pie chart of molecular function (MF) annotation classification of (A) the human-(B) and parasite-derived proteins that were present in HCF-EVs. (C) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of the proteins identified in HCF-EVs. The *P* value obtained by the enrichment test (Fisher's exact test) is subjected to a negative logarithm (−log10) conversion. This indicated that the larger the converted value is, the more significant the enrichment of this functional type is. The horizontal axis value was a significant *P* value ($P < 0.05$) for negative logarithmic transformation and was then plotted as a bar graph.

the egl00020 citrate cycle (TCA cycle) was the most abundant terms (Fig. 4C).

It is worth noting that proteins closely related to EVs were also identified successfully, including proteins present in specific EVs such as Annexin A6, proteins present in multiple EVs such as annexins and tubulin alpha-1C, and proteins present in large EVs such as actinin. Besides, heat shock 70 kDa proteins, prominin-1 (CD133, a pentaspan protein) and tetraspanins CD63 were present in HCF-EVs (Supplementary Tables S1, S2).

Equally important is that some of the most relevant diagnostic antigens, including antigen B and hydatid disease diagnostic antigen P-29, were contained in HCF- and PCS-EVs. Moreover, antigen Eg13 was found in HCF-EVs.

3.4. PCS-EVs have immunomodulatory functions

The immune system plays an important role in eliciting a defense response against parasitic infection. To understand the role of EVs in the T lymphocyte immune response, we conducted a co-culture experiment in vitro. The results of co-culture of murine PBMCs and PCS-EVs showed that PCS-EVs could significantly inhibit the proliferation of murine lymphocyte, $CD4^+$ T cells, and $CD8^+$ T cells in a dose-dependent manner (Fig. 5).

We also explored the effect of PCS-EVs on T lymphocyte function. According to the results of cytokines detected by CBA in the culture supernatant, we concluded that PCS-EVs could significantly inhibit IL-10 secretion and significantly promote IL-2 secretion in a dose-dependent manner. However, the effect of PCS-EVs on the secretion of other cytokines was not observed at low doses (6 μg). The high dose (24 μg) resulted in the inhibition of the secretion of interferon -γ, IL-6, IL-17A, and tumor necrosis factor, and promoted the production of IL-4 (Fig. 6).

4. Discussion

In the present study, we focused on the protein differences between HCF- and PCS-EVs to determine the effects of EVs on host-parasite interactions. Our proteomic analysis found that in addition to parasite-derived proteins, human-derived proteins were also in HCF-EVs, which may mean that human EVs pass through the cyst wall, because this was reflected by the proteomics results and the WB data somewhat support such a hypothesis. Previous studies have demonstrated that host proteins were present in protoscoleces and GL (Monteiro et al., 2010) and in HCF (Monteiro et al., 2010; Aziz et al., 2011). The EVs from *E. caproni* and *F. hepatica* adults contain many host-derived proteins, suggesting that the EVs may play an important role in the host-parasite communication (Marcilla et al., 2012). In addition, the HCF-EVs expressed far more parasitic proteins (936 proteins) than the PCS-EVs (149 proteins), possibly because EVs are not only secreted by the protoscoleces, but also by GL (Siles-Lucas et al., 2017).

In addition to the exclusive proteins in HCF-EVs and PCS-EVs, we also analyzed the differentially expressed proteins shared in both. There were 61 up-regulated proteins and nine down-regulated proteins in HCF-EVs compared with PCS-EVs. The host-parasite interaction is a complex process, and the proteins in HCF are regulated by both host and parasite. It is inevitable that the shared proteins are changed in HCF- EVs compared with PCS-EVs.

We verified the isolated EVs with NTA, TEM and WB analysis. The results of the first two analyses confirmed that the characteristics of the isolated vesicles were consistent with those of EVs; however WB results showed that only the HCF-EVs revealed the band of EV marker CD63 (Fig. 1D), probably due to the use of antibodies against human proteins that may not efficiently bind to the proteins secreted by the protoscoleces. Nevertheless, even without

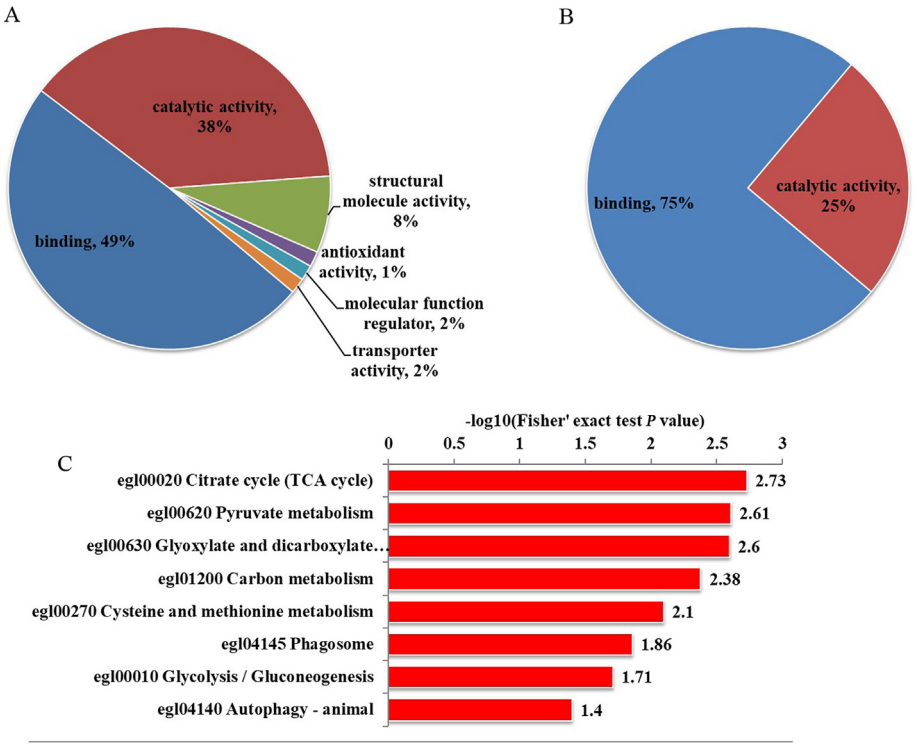

Fig. 4. Proteomic analysis of proteins shared in extracellular vesicles isolated from hydatid cyst fluid (HCF-EVs) and protoscolex culture supernatant (PCS-EVs). The pie chart of molecular function annotation classification of (A) the up-regulated and (B) the down-regulated proteins that were present in HCF-EVs as evident from the HCF-EV/PCS-EV values. (C) Kyoto Encyclopedia of Genes and Genomes pathway enrichment of the up-regulated proteins that were present in HCF-EVs. A protein with a portion satisfying ratio (HCF-EV/PCS-EV) greater than 1.5 or less than 1/1.5, and a P value < 0.05 is regarded as a differential protein. The P value obtained by the enrichment test (Fisher's exact test) is subjected to a negative logarithm (−log10) conversion. This indicated that the larger the converted value is, the more significant the enrichment of this functional type is. The horizontal axis value was a significant P value (P < 0.05) for negative logarithmic transformation and was then plotted as a bar graph.

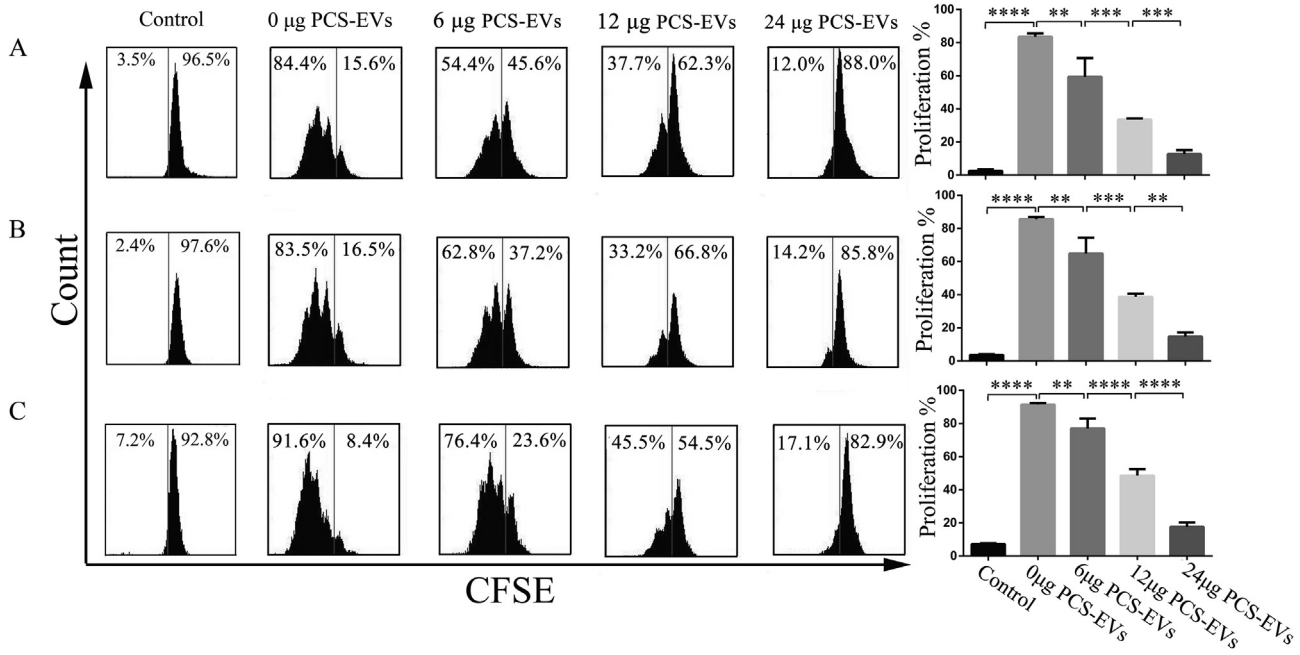

Fig. 5. The effect of extracellular vesicles isolated from protoscolex culture supernatant (PCS-EVs) on the proliferation of murine PBMCs. 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester-labeled PBMCs, stimulated with purified anti-mouse CD3 and CD28 antibodies, were co-cultured with different doses of PCS-EVs for three days. And then PBMCs were gated by staining with APC-CD3 antibody and PerCP-CD4 antibody, and the fluorescence of CFSE was detected by flow cytometry. (A) Proliferation of total lymphocyte; (B) proliferation of $CD4^+$ T cells; (C) proliferation of $CD8^+$ T cells. Comparisons between groups were performed with one-way analysis of variance; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Data are mean ± S.D. of at least three independent experiments.

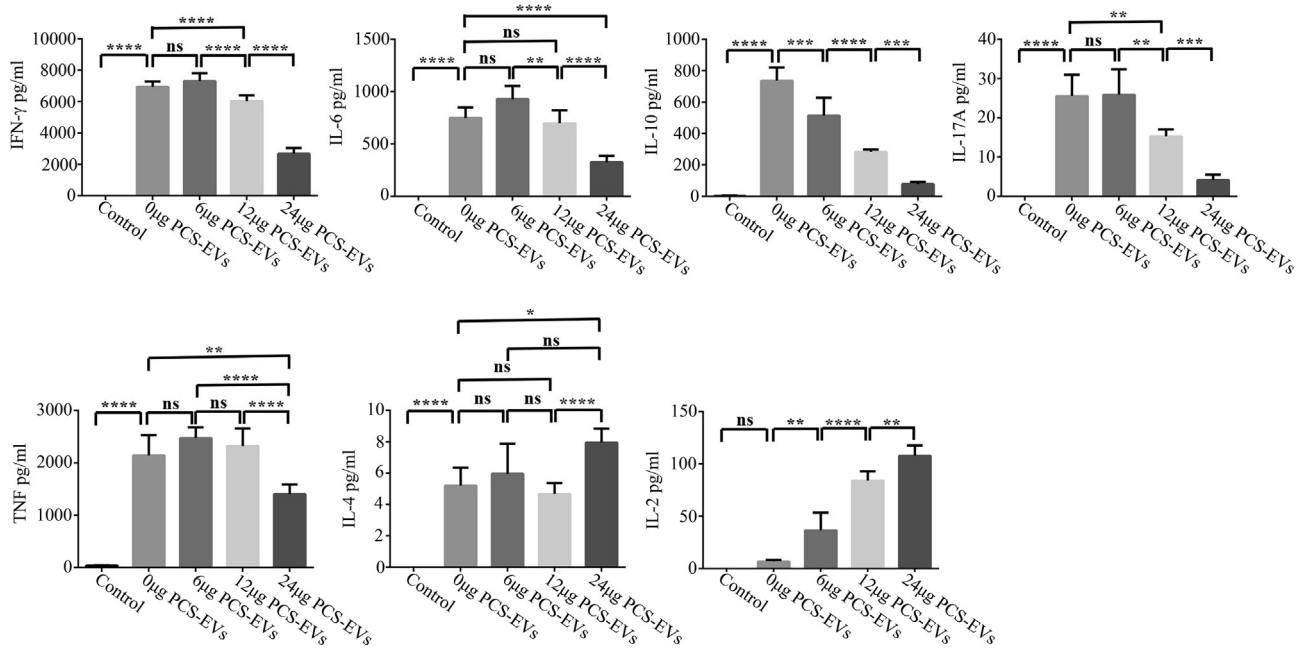

Fig. 6. The effect of extracellular vesicles isolated from protoscolex culture supernatant (PCS-EVs) on the secretion of cytokines in murine peripheral blood mononuclear cells. Murine PBMCs, stimulated with purified anti-mouse CD3 and CD28 antibodies, was co-cultured with different doses of PCS-EVs for 3 days. Then the level of cytokines in the culture supernatant was detected by Cytometric Bead Array Mouse Th1/Th2/Th17 Cytokine Kit. Comparisons between groups were performed with one-way analysis of variance; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Data are mean ± S.D. of at least three independent experiments. ns, not significant.

the results of WB available for PCS-EVs, we believe that the EVs were successfully isolated, since positive results were obtained for the other two analyses.

In the present study, we performed proteomic analysis of EVs and evaluated their immune functions. Next, we co-cultured PCS-EVs with murine PBMCs in vitro to explore the effect of PCS-EVs on the proliferation and functions of lymphocytes including $CD4^+$ T and $CD8^+$ T cells. However, the quantity of available HCF from the patients was insufficient to allow separation of enough HCF-EVs; hence, we were unable to co-culture HCF-EVs with PBMCs in vitro. Based on the results of co-culture assays, we found that PCS-EVs could significantly inhibit the proliferation of T lymphocytes (including $CD4^+$ T and $CD8^+$ T cells) in a dose-dependent manner (Fig. 5), thereby effectively promoting the establishment

and development of echinococcosis. Treg cells, a subtype of Th cells, are known potent immunosuppressive cells involved in the progression of echinococcosis. For instance, Treg cells can induce immune tolerance in hosts (Wang et al., 2018) and suppress allergic reactions caused during CE (Zhang et al., 2016). Whether the immunosuppressive function of PCS-EVs is related to Treg cells is questionable and warrants further study.

It is worth noting that our results also showed that the PSC-EV dataset contains serum proteins such as complement factors, immunoglobulins and fibrinogen. Since FBS is essential for cultivating protoscoleces, the serum protein in the PSC-EV dataset may be derived from FBS that has not been completely removed. Unfortunately, in order to ensure the vitality of protoscoleces, we cannot use the serum-free medium to culture protoscoleces. But in the separation of specimens and the extraction of EVs, there are multiple washing steps, and a previous study has shown that multiple washing steps can effectively reduce EV contamination by serum proteins (Hassani and Olivier, 2013). If FBS is really present in the PCS-EVs solution, it should be immuno-promoted for PBMCs in vitro, contrary to our experimental results, indicating that these proteins did not interfere in the immunomodulatory effects of PCS-EVs on PBMCs. Therefore, we can conclude that PCS-EVs do exert immunosuppressive effects on T lymphocytes proliferation.

In addition to proliferation analysis, we also detected the cytokine levels of the culture supernatant and found that PCS-EVs significantly inhibited the secretion of interferon-γ and promoted the production of IL-4. This observation may be associated with Th1 function to inhibit the growth of metacestodes and Th2 function to promote the progression of CE. Interestingly, PCS-EVs could also inhibit the secretion of other cytokines, but it significantly elevated the level of IL-2, a cytokine that promotes and maintains long-term cultures of T cells. Thus, we hypothesize that PCS-EVs promote IL-2 secretion and inhibit or block the expression of other substances required for IL-2-mediated T lymphocyte proliferation, such as IL-2R. Further studies are needed to validate these hypotheses.

EVs contain various molecules with immunomodulatory properties, including proteins, carbohydrates, lipids, miRNAs and other small RNAs (Coakley et al., 2015). Therefore, in the co-culture of PCS-EVs and PBMCs, the effect of PCS-EVs on PBMCs may not only depend on proteins but also on other molecules. In order to solve this problem, it is necessary to separate and purify different molecules in PCS-EVs, indicating that a large number of PCS-EVs are needed. Unfortunately, the PCS-EVs we obtained were not enough to complete this research, which is our interest in the future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2019.08.003.

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