Eimeria falciformis secretes extracellular vesicles to modulate proinflammatory response during interaction with mouse intestinal epithelial cells

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Abstract

Background: Protozoan parasite secretions can be triggered by various modified media and diverse physicochemical stressors. Equally, host-parasite interactions are known to co-opt the exchange and secretion of soluble biochemical components. Analysis of Eimeria falciformis sporozoite secretions in response to interaction with mouse intestinal epithelial cells (MIECs) may reveal parasite secretory motifs, protein composition and inflammatory activities of E. falciformis extracellular vesicles (EVs).

Methods: Eimeria falciformis sporozoites were allowed to interact with inactivated MIECs. Parasite secretions were separated into EV and vesicle-free (VF) fractions by discontinuous centrifugation and ultracentrifugation. Secreted EVs were purified in an iodixanol density gradient medium and the protein composition of both EV and VF fractions were analyzed by liquid chromatography-tandem mass spectroscopy. The inflammatory activities of E. falciformis sporozoite EV on MIECs were then investigated.

Results: During the interaction of E. falciformis sporozoites with inactivated MIECs, the parasite secreted VF and vesicle-bound molecules. Eimeria falciformis vesicles are typical pathogenic protozoan EVs with a mean diameter of 264 ± 2 nm, and enclosed heat shock protein (Hsp) 70 as classical EV marker. Refractile body-associated aspartyl proteinase (or eimepsin), GAP45 and aminopeptidase were the main components of E. falciformis sporozoite EVs, while VF proteins include Hsp90, actin, Vps54 and kinases, among others. Proteomic data revealed that E. falciformis EV and VF proteins are aggregates of bioactive, antigenic and immunogenic molecules which act in concert for E. falciformis sporozoite motility, pathogenesis and survival. Moreover, in MIECs, E. falciformis EVs induced upregulation of gene expression and secretion of IL-1β, IL-6, IL-17, IL-18, MCP1 as well as pyroptosis-dependent caspase 11 and NLRP6 inflammasomes with the concomitant secretion of lactate dehydrogenase.

Conclusions: Eimeria falciformis sporozoite interaction with MIECs triggered the secretion of immunogenic and antigenic proteins. In addition, E. falciformis sporozoite EVs constitute parasite-associated molecular pattern that induced
inflammatory response and cell death. This study offers additional insight in the secretion and protein composition of *E. falciformis* secretomes as well as the proinflammatory functions of *E. falciformis* sporozoite EVs.

**Keywords:** *Eimeria falciformis*, Secretome, Extracellular vesicles, Inflammasomes, Pyroptosis, Host-parasite interactions

### Background

Host-parasite interaction is a dynamic and phenomenal feature of parasitism. It co-opts parasite development and pathogenesis by means of cellular signaling, inflammation, cell death and, essentially, the excretion or secretion of molecules. In general, parasite secretions (or secretomes) are formed along several secretory pathways and released during the host-parasite interplay [1]. Previous studies have described and characterized the secretion of extracellular vesicles (EVs) and vesicle-free (VF) molecules from parasitic protozoa and helminths [2, 3] as well as from parasite-infected host cells [4]. Similarly, there have been several studies on the secretion, characterization and proteomic profiling of *Eimeria* sporozoite proteins triggered by thermal, chemical and mechanical stressors [5]. In addition, VF molecules have been induced and characterized from protozoan parasites in various modified mediums and by physicochemical stressors [5, 6]. Pathogenic protozoan EVs activated by chemical stressors and serum-starved media have also been characterized and profiled [7]. A few studies have also indicated that host-parasite interactions could induce the secretion of vesicle-bound and VF molecules. In this regard, the formation of parasite secretomes could occur prior to [8] or during protozoan parasite attachment or invasion of the host cell [3, 8–10], but the description of distinct parasite secretions during interactions with the host cell, and characterization of such parasite-derived secretomes have not been demonstrated.

Extracellular vesicles are heterogeneous, sub-cellular, lipid-bound vesicles formed during pathophysiological processes and host-pathogen interactions [11]. Based on biogenesis and size, EVs are broadly classified as exosomes, microvesicles/microparticles and apoptotic bodies [7, 11–15]. EVs usually enclose soluble components of cellular origin, such as metabolic intermediates, glycoconjugates, lipids, proteins and nucleic acids [14]. Functionally, protozoan parasite EVs can deliver their cargoes to mediate parasite motility and development, differentiation, cytoadherence and pathogenesis [16–20]. During host–pathogen interaction, EVs are pivotal in such processes as intercellular communication, horizontal gene transfer, disease biomarker, antigen presentation, immune response and host cell death [14, 15, 21–23].

Typically, *Eimeria* spp. are obligate intracellular apicomplexan parasites [16]. More specifically, *Eimeria falciformis* naturally parasitizes caecal epithelial cells of wild and laboratory mice, causing catarrhal enteritis, hemorrhage and epithelial sloughing [17, 18]. The life-cycle of *E. falciformis* is similar to that of other coccidian parasites, with the sporozoites, after excystation from the sporocyst, actively migrate through the mouse gut before invading the host intestinal epithelial cells (IECs) [17, 18, 25]. In general, parasitization of the host cells by apicomplexan parasites involves gliding motility, cellular attachment, invasion and spontaneous formation of excretory/secretory products with which apicomplexans modulate host cell responses [10, 26–28]. However, *E. falciformis* sporozoite proteins secreted during interaction with the host IECs have not been described [5]. Also, the secretion of parasite-derived EVs has not been characterized in *Eimeria* spp. [7], nor have the inflammatory responses of host IECs, such as the secretion of cytokines and formation of inflammasome complexes, to *Eimeria*-derived EVs.

In this study, *E. falciformis* sporozoites were allowed to interact with inactivated mouse IECs (MIECs), and *E. falciformis* sporozoite VF and EV fractions were separated, purified and analyzed. In addition, MIECs were stimulated with *E. falciformis* sporozoite EVs (EfSEVs) to determine differential regulation and expression of inflammatory cytokines (interleukin [IL]-1β, IL-6, IL-17, IL-18), the chemokine monocyte chemoattractant protein 1 (MCP1) and pyroptosis-dependent caspase 11 and nucleotide-oligomerization domain (NOD)-like receptor pyrin 6 (NLRP6) inflammasomes, and to reveal underlying synergy between MIEC inflammatory response and lethal activity of EfSEVs.

### Methods

#### Parasite propagation

Parasite propagation was carried out as described earlier [17, 29, 30] with modification. In brief, 100 specific pathogen-free Balb/c mice aged between 6 and 8 weeks were raised in plastic cages placed in a pathogen-free room with adequate supply of food and sterile water. Each mouse was orally inoculated with 50 µl of $2 \times 10^5$ *E. falciformis* sporulated oocysts/ml phosphate-buffered saline (PBS). Oocysts shed in feces between 7- and 11-day post-infection were recovered from homogenized fecal solution, washed through a metal strainer and purified by salt flotation. Purified oocysts were washed and suspended as appropriate in 2.5% potassium dichromate followed by incubation at 28 °C and 120 rpm for approximately
10 days. Sporulated oocysts were stored at 5 °C and used within 30 days of storage.

Purification of *E. falciformis* sporozoites

*Eimeria falciformis* sporozoites were excystated and purified as earlier reported [31–33]. Briefly, *E. falciformis* sporulated oocysts were washed in PBS to remove potassium dichromate through discontinuous centrifugation. Purified oocysts were precipitated in 40 ml of antibiotic–antimycotic diluent (1 part diluent per 100 ml) (Solarbio, Beijing China). *Eimeria falciformis* sporulated oocysts were then suspended in 5% (v/v) sodium hypochlorite for 20 min on ice with intermittent stirring, and the oocysts were washed in PBS. The hypochlorite-treated sporulated oocysts were then vortexed with an equal volume of glass beads (diameter: 0.5 mm) for 3 min to release sporozoites and thereafter incubated with 0.75% (w/v) sodium taurodeoxycholate and 0.25% (w/v) trypsin in PBS for 1 h at 37 °C and 200 rpm for sporozoite excystation. The final purification step was vacuum filtration of the *E. falciformis* sporozoites [19, 32].

Cell and parasite culture

Mouse intestinal epithelial cells were cultivated in complete medium (Dulbecco’s modified Eagle’s medium [DMEM; Sigma-Aldrich, St. Louis, MO, USA] supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) (v/v) and 1% antibiotic diluent (Solarbio, Beijing, China)[26]. After confluence was reached, approximately 1×10^6 MIECs were plated with complete medium and incubated overnight. To inhibit exosome secretions [27], MIECs were inactivated with 4% paraformaldehyde (Solarbio, Beijing, China) for 20 min at room temperature and washed 5 times with sterile PBS. Inactivated MIECs were re-cultured in Exo-clear cell growth medium (System Biosciences, Palo Alto, CA, USA) followed by inoculation of 2×10^6 freshly excysted *E. falciformis* sporozoites [28]. The parasites were maintained in the culture plates for 18 h [30] at 37 °C in a 5% CO₂ humidified incubator. A non-treated control (NC) experiment was prepared in parallel in which inactivated MIECs were incubated as described above, but without *E. falciformis* sporozoites.

Separation of *E. falciformis* sporozoite secretomes

A 350-ml sample of culture supernatant was harvested without disrupting the MIEC monolayer and centrifuged at 4 °C sequentially at: 1000 g, 5 min; 2000 g, 10 min; 5000 g, 20 min; 10,000 g, 30 min. The supernatant was then transferred into polycarbonate ultracentrifugation tubes (Thermo Fisher Scientific, Waltham, MA, USA) and centrifuged at 120,000 g for 18 h in a Sorvall WX+ Ultra Series centrifuge (T-890 fixed angle rotor, k-factor 25.1; Thermo Fisher Scientific). The resulting supernatant from the ultracentrifugation was subjected to ultrafiltration through a 0.22-µm Corning sterile filter (Corning, Corning, NY, USA) at 4 °C under gravity, followed by 10-fold concentration using an Ultra 10 kDa cut-off membrane (Amicon Ultrafiltration System; MilliporeSigma, Burlington, MA, USA) and stored at − 80 °C as the *E. falciformis* sporozoite VF fraction [31]. The cell culture supernatant from the NCs was prepared in the same manner.

The resulting pellets from the ultracentrifugation process were pooled, reconstituted in 8 ml PBS (pH 7.2) and resolved on discontinuous iodixanol gradients solutions prepared by diluting OptiPrep™ density gradient (ODG) medium (Sigma-Aldrich) in 60% (v/v) aqueous iodixanol with sterilized 0.3 M sucrose/10 mM Tris, pH 7.2 [32, 33]. The gradient was formed by adding 1.6 ml each of 40, 20, 10 and 5% iodixanol solution into a 9-ml polycarbonate tube (Thermo Fisher Scientific) and 1 ml of *E*SEV suspension was overlaid and subjected to ultracentrifugation for 20 h at 120,000 g and 4 °C in a Sorvall WX+ Ultra Series centrifuge (T-890 fixed angle rotor, k-factor 25.1; Thermo Fisher Scientific). Seven gradient layers were collected, resuspended in PBS (pH 7.2) and washed twice for 4 h at 120,000 g and 4 °C. *E*SEV pellets were pooled, concentrated in 400 µl of PBS and stored at − 80 °C. Likewise, NC pellets were collected and washed appropriately. The density of *E*SEVs was measured from the corresponding ODG layer after appropriate dilution at an optical density of 244 nm [32, 33] with a UV–Visible spectrophotometer (Biomates 3S; Thermo Fisher Scientific) (Additional file 6 : Data set).

Transmission electron microscopy

For transmission electron microscopy (TEM), 10 µl of *E*SEVs was added to the a formvar carbon film-coated copper grid for 3 min. Excess liquid was removed with filter paper followed by staining with 3% phosphotungstic acid [34, 35]. An equal volume of NC particles was also stained. The grids were observed in model HT770 transmission electron microscope (Hitachi, Tokyo, Japan).

Nanoparticle tracking analysis

The size of *E*SEVs was determined using the Zetasizer Nano-Zs instrument (Malvern, Worcestershire, UK). Briefly, 10 µg of *E*SEVs and an equal volume of NC pellets were dissolved in an appropriate volume of PBS to make a total volume of 1 ml in each case. The size and distribution of the particles were measured in triplicate [13].
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and liquid chromatography-tandem mass spectrometry

The protein components of the EfSEV and VF fractions were quantified using the BCA kit (Solarbio, Beijing, China). An equivalent of 10 µg of EfSEV and VF fractions, as well as the NC and NCs were separately mixed with lysis buffer (Solarbio, Beijing, China) according to the manufacturer’s instruction, heated in a water bath at 100 °C for 10 min and resolved by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) [35, 36] at 100 V in a Mini-Cell electrophoresis system (BioRad Laboratories, Hercules, CA, USA). Following electrophoresis, the SDS-PAGE gel was stained with a Coomassie blue stain kit. Subsequently, the EfSEV and VF protein gel lanes were excised and pooled for mass spectrometry (MS).

Liquid chromatography-tandem MS (LC–MS/MS) was performed by the Lu-Ming Biotech Co., Ltd. (Shanghai, China). In brief, excised protein gel lanes were dehydrated and dried in a vacuum. Gel particles were reduced in 10 mM DTT/25 mM NH4HCO3 followed by treatment with alkylation buffer and recovered in 25 mM NH4HCO3. Reduced gel particles were digested in 20 µl of 0.02 µg/µl trypsin in 25 mM NH4HCO3. The supernatant was treated with 50 µl of 5% formic acid/67% acetonitrile followed by elution in 5% formic acid/67% ACN/H2O. After drying using low-pressure centrifugation, the sample was re-dissolved in 40 µl of 0.1% formic acid and centrifuged through a column. The digested peptide elute was adjusted to pH 7 by H3PO4 and the resulting peptides were lyophilized in 150 µl of 100% methanol. MS analyses were performed with a Q-Exactive mass spectrometer (Thermo Fisher Scientific) equipped with Nanospray Flex source (Thermo Fisher Scientific). MS/MS spectra were obtained with a resolution of 17, 500 and an AGC target of 2e5 at a maximum injection time of 50 ms. The Q-E dynamic exclusion was set at 15.0 s in positive mode.

Protein database search and bioinformatics

MaxQuant, a quantitative proteomics software package, was used to search peptide sequences against Eimeria species, Toxoplasma gondii and Plasmodium falciparum proteins in UniProt. Trypsin digestion specificity was used to search databases. For protein quantification, MS1 and MS2 tolerance were set at 10 ppm and 0.02 Da, respectively. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genome (KEGG) analyses were also performed.

Western blot

EfSEV and VF protein concentrations were measured using a BCA kit (Solarbio, Beijing, China). An equivalent volume (10 µg) of EfSEV, VF, NC and NCs was separately mixed with 4× Loading Buffer (Solarbio, Beijing, China) and vortexed. The mixture was heated in a water bath at 100 °C for 10 min. The protein components were resolved in a 12% SDS-PAGE gel, and the protein bands were transferred to a polyvinylidene difluoride membrane (Merck-Millipore, Germany). After 1 h of blocking with 0.01% Tween-20 in PBS (PBST) containing 5% skimmed milk, the membrane was incubated with anti-heat shock proteins 70 kDa (Hsp70; Proteintech Group Inc., Rosemont, IL, USA) overnight at 4 °C. Thereafter, the membrane was washed in PBST and incubated with horseradish protein-conjugated antibodies (Proteintech Group) for 1 h at room temperature. The membrane was treated with the WesternBright™ ECL enhanced chemiluminescent substrate kit (Advanta Inc., San Jose, CA, USA) according to the manufacturer’s instruction and visualized using the Luminescent Image Analyzer Amer sham Imager 600 series (GE Co., Boston, MA, USA).

Multiplex protein microarray

The MIECs were plated at a density of 1 × 10^6 in complete medium and incubated overnight at 37 °C and 5% C02. Plated MIECs were subsequently re-cultured in DMEM supplemented with 10% exosome-depleted fetal bovine serum [37] and stimulated with 10, 30, 50 and 100 µg of EfSEVs and PBS (0 µg of EfSEVs) was used as an experimental control treatment. Similarly, an equal density of MIECs was treated with 50 µg of EfSEVs for 0, 6, 12 and 18 h. Cell culture supernatants were collected for protein microarrays. IL-1β, IL-6, IL-17, IL-18 and MCP1 proteins were quantified by QAM-INF-1 (Quantibody® Mouse Inflammatory Array 1 Kit; RayBiotech, Peachtree Corners, GA, USA) [38]. Analyses were done in triplicate, and targeted cytokines were tested in quadruplicate. Each cytokine was quantified as mean fluorescence using the InnoScan 300 Microarray Scanner (Innopsys, Carbonne, France) at a wavelength of 532 nm and a resolution of 10 µm. Data were analyzed using QAM-INF-1 analysis software (RayBiotech).

RNA extraction and quantitative PCR

RNA was extracted from EfSEV-stimulated MIECs using TRIzol reagents (Invitrogen, Thermo Fisher Scientific). In parallel, the same density of MIEC was treated with NC particles. A 1-µg aliquot of total RNA was reverse transcribed with the Primerscript™ RT Reagent Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer’s instructions. PCR primers for IL-1β, IL-6, IL-17, IL-18, MCP1, caspase 11 and NLRP6 were synthesized by Tsingke Biological Technology (Xi’an, China) (Additional file 5). Real-time quantitative PCR (qPCR) was performed using TB Green pre-mix Ex Taq™ II (Takara)
Bio Inc.) and the CFX98 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Each reaction tube contained 1 µl each of forward and reverse primers, 2 µl of cDNA template, 10 µl of TB Green and 6 µl of nuclease-free water. Assays were performed in triplicate, and data were normalized by using β-actin as a reference gene.

**Lactate dehydrogenase assay**

Approximately $1 \times 10^6$ MIECs in DMEM supplemented with 10% exosome-depleted FBS were stimulated with 50 µg of EfSEVs for 0, 6, 12, 18 and 24 h. Likewise, 10, 30, 50, and 100 µg of EfSEVs were used to stimulate MIECs and PBS (0 µg of EfSEVs) as experimental control treatment. Also, an equivalent volume of NC particles was used as a negative control for EfSEV time- and dose-dependent production of lactate dehydrogenase (LDH) by MIECs [39, 40]. The culture plates were incubated at 37 °C and 5% CO₂. LDH was measured by Chekine™ LDH assay kit (Abbkine, Wuhan, China) as instructed in the product manual. Absorbance was measured at 450 nm on a SpectraMax M5e Multi-Mode Microplate Reader (Molecular Devices, LLC, San Jose, CA, USA).

**Statistical analyses**

Data were expressed as the mean with the standard deviation and analyzed by one-way analysis of variance. Turkey’s test was used for multiple comparisons. Linear regression analysis was performed using SPSS software version 21 (SPS IBM Corp., Armonk, NY, USA). Charts were generated in GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Differences among dose- and time-dependent activities of EfSEVs on MIECs relative to the experimental control were considered to be statistically significant at *$P<0.05$, **$P<0.001$ and ***$P<0.0001$.

![Fig. 1](image-url) **Eimeria falciformis** sporozoite and derived EVs. **a** E. falciformis sporozoite after excystation from sporocyst. **b** Bar graph from NTA analysis of 10 µg EfSEVs in 1 ml of PBS. EfSEV mean diameter was 246 ± 2 nm. **c** TEM image of EfSEV processed through negative staining with 3% phosphotungstic acid. **d** Western blot analysis of EfSEVs using anti-Hsp70. Abbreviations: EfSEV, *E. falciformis* sporozoite EVs; EVs, extracellular vesicles; Hsp70, heat shock protein 70; NC, non-treated control particles; NCs, non-treated control supernatant; TEM, transmission microscopy; VF, vesicle-free (see Additional files 1–5).
**Results**

*E. falciformis* sporozoite secretomes during interaction with MIECs

The *E. falciformis* sporozoite (Fig. 1a) is the motile, invasive stage of the parasite which navigates through the host gut before invading the host cell. The fractions of *E. falciformis* sporozoite secretome were separated from the culture medium by serial centrifugation and ultracentrifugation steps, and then the secreted EVs were characterized by nanoparticle tracking analysis (NTA) and TEM. Also, EfSEV and VF protein compositions were identified by electrophoresis (Additional file 5) and analyzed by MS (Additional file 6). Our findings revealed that EfSEVs had a buoyant density ranging from 1.12 to 1.179 g/cm$^3$ distributed between the second and the seventh ODG layers. The particles from NC had a buoyant density that ranged between 0.57 and 1.103 g/cm$^3$ (Additional file 5). NTA analysis revealed that EfSEVs were a heterogeneous population of EVs with a diameter of approximately 37.84–1,281 nm (Fig. 1b). TEM observations indicated that EfSEVs were spherical and circular in shape, similar to *Toxoplasma gondii* and *Neospora caninum* EVs [35, 41] (Fig. 1c). In addition, TEM revealed that NC particles (Additional file 2) did not contain secreted vesicles, indicating that the observed EVs were formed during *E. falciformis* sporozoite interaction with MIECs. Additionally, the vesicular property of EfSEVs and depletion of protein in the non-treated control experiment were also confirmed by targeting Hsp70 in EfSEVs, VF, NC, and NCs for western blotting (Fig. 1d).

**Protein composition of *E. falciformis* sporozoite EV and VF fractions**

Electrophoretic analyses of the EfSEV and VF fractions showed that *E. falciformis* sporozoites secreted proteins as components of its secretomes (Additional file 6) during interaction with inactivated MIECs. The protein composition of the EfSEV and VF fractions were analyzed by LC–MS/MS. MS/MS spectra were searched in the protein database for avian and bovine *Eimeria* species, *T. gondii* and *P. falciparum* because of the intractable genome of *E. falciformis* [9]. Forty-two proteins were identified from the *E. falciformis* sporozoite VF fraction (Additional file 6), including actin (putative), kinases, heat shock proteins (Hsp 70 and Hsp90), vacuolar protein sorting (Vps) 54, elongation factor 1 alpha (EF-1α), early gametocyte enriched phosphoprotein (EGXP), as well as some *P. falciparum* and *T. gondii* orthologous proteins (Fig. 2; Additional file 6). GO terms for *E. falciformis* sporozoite VF protein are shown in Fig. 3. Also, the protein composition of EfSEVS implies the involvement of these proteins in *E. falciformis* sporozoite binding/fusion to the host cell, endocytosis and metabolic processes (Fig. 3). KEGG analysis revealed that EfSEV proteins were enriched in pathways involving endocytosis, protein processing, RNA transport, spliceosome and ribosome, as found in *N. caninum* EV proteins [35], as well as the glutathione metabolism pathway, which is associated with gene expression, DNA and protein synthesis, signal transduction, cytokine production and immune responses (Fig. 3; Additional file 5).

**EfSEVs induced IL-1β, IL-6, IL-17, IL-18 and MCP1 production in MIECs**

Intestinal epithelial cells are known to secret cytokines and chemokines as well as cellular infiltration during inflammatory responses and parasite-host interactions [17, 42]. Also, *Eimeria* species can induce the secretion of diverse cytokines/chemokines in the host intestinal epithelium, including IL-6, IL-17, IL-18, IL-1β, interferon gamma (INFγ) [17, 44] and MCP1 [43]. However, the reactive antigenicity of *Eimeria*-derived EVs in host inflammatory responses during infection is not fully understood. Therefore, we examined the secretion of MIEC proinflammatory cytokines after EfSEV stimulation. The results showed that MIECs produced quantifiable IL-1β, IL-18, IL-17 and IL-6 in response to EfSEV dose and time stimulations. While the level of IL-6
significantly increased with increasing time of MIEC stimulation by ESEVs, the production of IL-17 reduced (Fig. 4). In addition, the production of IL-6 and IL-17 increased at high doses of ESEVs (Fig. 4). Also, the production of MCP1 significantly increased with increasing ESEV doses and time of stimulation (Fig. 4). The secretion of IL-18 by ESEVs-stimulated MIECs was initially low but increased with time and with increasing doses of ESEVs. Similarly, the production of IL-1β only increased significantly at a high dose of ESEVs (Fig. 4). Taken together, ESEV dose enhanced the production of proinflammatory cytokines in MIECs whereas the production of MCP1 was significantly increased with increasing time of stimulation and dosage of ESEVs (Fig. 4).

Gene expression profiles of the selected cytokines and chemokine also indicated steady and significant
upregulation of MCP1 and IL-6 mRNA expression with increasing time of stimulation and EfSEV doses, but the increased IL-17 mRNA expression was just significant at high concentration of EfSEVs (Fig. 5). IL-18 mRNA expression was also significantly upregulated with increasing dose of EfSEVs and time of treatment (Fig. 5). However, mRNA expression of INFγ remained unchanged with increasing time and EfSEV doses (data not shown). In essence, EfSEVs are parasite components that modulate proinflammatory cytokines and chemokine genes during Eimeria-host interactions (Fig. 5). The apparent inconsequential change in the expression of the cytokines with NC particles indicated that the observed relative expressions of IL-6, IL-17, IL-18, IL-1β and MCP1 were due to the MIEC responses to EfSEV bioactivities (Fig. 5&6).

**EFSEVs upregulated MIEC pyroptotic inflammasomes and induced the release of LDH**

The eventual outcomes of pathogen-host interactions are sometimes marked by inflammation and host cell death [45]. Given the functional association of IL-18 and IL-1β with inflammatory programmed cell death, we investigated the regulatory activities of caspase 11 and NLRP6 in EfSEV-stimulated MIECs by qPCR. The analysis showed that increasing EfSEV dose and increasing duration of stimulation significantly upregulated the mRNA expression of NLRP6 and caspase 11 in MIECs whereas NC particles showed no distinct effects on inflammasome activation (Fig. 6a, b).

Cytosolic protein ligands, such as pathogen-associated molecular patterns (PAMPs), have been shown to activate pyroptosis-dependent IEC death. Specifically, pyroptosis of IECs can occur via the activation of caspase 11 and NLRP6 with a concomitant secretion of IL-18 and IL-1β [46] with the release of cytosolic LDH [39]. Therefore, LDH production by EfSEV-stimulated MIECs was investigated. The increased production of LDH by MIECs was significant from 18 h of EfSEV treatment and with increasing doses of EfSEVs. The result indicated that EfSEVs induced significant production of LDH as a marker for proinflammatory MIEC death. This finding was supported by the observation that NC particles showed no significant involvement in the production of LDH by MIECs (Fig. 6c).

**Discussion**

Previous studies have demonstrated that protozoan parasite secretomes include vesicle-bound and VF molecules such as nucleic acids, carbohydrates or glycoconjugates, lipids and proteins [6, 7, 47]. In this study, *E. falciformis* sporozoite EV and VF proteins were resolved from the total secretome formed while the parasite interacted with inactivated MIECs, and the protein components were analyzed by gel electrophoresis and LC–MS/MS. The electrophoretic profiles revealed protein bands in EfSEV
and VF gel lanes, but no specific band was visible from NC and NCS, indicating that EfSEVs and VF preparations were free from MIEC secretions (Fig. 1d) (Additional file 5). This observation is in conformity with T. gondii tachyzoites which were unable to secrete vesicles after being treated with aldehyde [48]. Notwithstanding, EfSEVs are subpopulations of self-assembled spherical and circular-shaped EVs with an expression of Hsp70 as membrane marker (Fig. 1c, d).

The proteomic dataset showed that EfSEVs and the VF fraction comprised membrane-associated and bioactive proteins (Additional file 6). Functionally, the gliding motility of apicomplexan parasites requires actin polymerization [49], and E. falciformis sporozoite VF Hsp90 might be involved in host cell invasion, host-parasite interaction, signal transduction and parasite development similarly as found in Eimeria tenella, T. gondii, and P. falciparum Hsp90 [50]. Also, the Vps51-54 complex has been suggested to mediate host endocytosis of T. gondii and P. falciparum [51, 52]. Vps54 would possibly play a similar role during E. falciformis sporozoite invasion (Fig. 2). Additionally, Cryptosporidium parvum EF-1α has been associated with host cell invasion [53], and E. tenella protein kinases are immunogens [54] known to inhibit apoptosis and play major roles during stress [55, 56]. Congruently, E. falciformis VF proteins are likely to be important for sporozoite motility, survival, invasion and pathogenesis.

Moreover, EF-1α and Hsp70 have been identified as EV markers of Leishmania major and other parasitic protozoa [57]. The identification of Hsp70 and EF-1α in EfSEV and VF proteins (Fig. 2) suggests that specific protein could be secreted via two or more secretory pathways during host-parasite interactions. The secretion of Hsp70 by Eimeria species is associated with sporozoite formation, pathogenicity, protective immunity and response to stress [5, 58, 59]. Hsp70 has also been reported among EV proteins from C. parvum, N. caninum, T. gondii, Leishmania and Trypanosoma species [7]. EfSEV proteases are likely to play important roles in E. falciformis sporozoite invasion, evasion of immune cells [60], developmental regulation and virulence, as revealed in other Apicomplexa [61], as well as degrading the cell matrix of host tissue [62]. Also, proteases are transcriptional regulators of E. tenella, Plasmodium and T. gondii life-cycles [5, 63] and, more importantly, the invasion of Eimeria sporozoites is accompanied by proteolytic shedding of surface adhesins mediated by protease [64] (Fig. 2).
Reflected body (RB)-derived functional molecules include a number of proteins/enzymes, such as aspartyl proteinase (eimepsin), RB antigen (SO7), and several haloacid dehalogenases such as hydrolase, subtilase, tranhydrogenase and ubiquitin, as well as some carbohydrates [65, 66]. Eimepsin is an *Eimeria*-specific antigenic protein [67, 68] that is abundantly secreted in the sporozoite stage [5]. SO7 is also highly immunogenic with conserved antigenic epitopes among *Eimeria* species infecting chicken [69]. SO7 has been reported to effectively stimulate the immune response compared with single fragment vaccine [70], and it also plays important roles in host cell invasion, secretion of microneme proteins and *Eimeria* parasite intracellular survival [69]. In addition, the *E. falciformis* and *E. tenella* leucine aminopeptidase is an enzyme which preferentially catalyzes the hydrolysis of leucine residues at the N-terminus of peptides and proteins [71], which could be crucial for invasion, immune response, parasite excystment [72] and functions similar to the EV-bound aminopeptidase and serine protease of *Acanthamoeba castellanii* [6, 73]. Identification of *Ef*SEV GAP45 suggests that the *E. falciformis* sporozoite has a trail of EVs during gliding motility [74, 75] as reported in *C. parvum* infection [76]. Also, histones enclosed in EVs secreted by *Leishmania donovani*, *P. falciparum*, *Trypanosoma brucei* and *T. cruzi* could have functions in parasite viability and virulence [77–79], and *Ef*SEV histones could have similar roles. Although *Ef*SEV RNA content was low (data not shown), attending *Ef*SEV histones suggest that *E. falciformis* EV cargo contained nucleic acids. Also, inactivation might have resulted in the loss of MIEC receptors [10] for *E. falciformis* sporozoite attachment, which in turn could have affected the number and composition of proteins in *E. falciformis* sporozoite secretomes.

IL-1β, IL-6, IL-17, IL-18 and MCP1 secretions are known to play crucial roles in *T. tenella*, *C. parvum* and *T. gondii* infections [80, 81]. IEC IL-18 is constitutive and links innate and adaptive immunity during infection [82]. Similarly, MCP1 recruits monocytes and macrophages and co-regulates IL-1β in inflamed IECs [83]. Therefore, the expression of MCP1 (Figs. 4, 5) indicated that *Ef*SEVs could play critical roles in the transcytosis of monocytes to the site of *E. falciformis* infection [21]. Also, the production of IL-1β could accelerate IL-17 response to PAMPs and stimulate chemokines recruiting leukocytes, T lymphocytes and neutrophils to the intestinal epithelium [84]. Then, *E. falciformis* sporozoites possibly secrete EVs to prevent early infiltration of cytotoxic CD4+ T cells to the site of infection [84] as MIEC IL-1β and IL-17 expressions were low in time-dependent responses to *Ef*SEVs (Figs. 4, 5) and it is comparable to the expressions of the IL-17 subset and neutrophils in *T. cruzi*-infected mice [85]. However, at a later time and high concentrations, *Ef*SEVs induced significant expression of IL-17, as reported in mice infected with *Leishmania major* [86], suggesting that *Ef*SEVs are potent, dose-dependent activators of inflammation and that the observed latent inflammatory effect of *Ef*SEVs on MIEC might be associated with *E. falciformis* sporozoite behavior for intracellular survival.

It has been previously reported that *Trichomonas vaginalis*, *Leishmania infantum*, and *P. falciparum* EVs induced secretion of IL-6 from immune cells [7]. However, the secretion of IL-6 could be related to anti-inflammatory and anti-apoptotic responses in IECs [87, 88]. It is possible that *E. falciformis* sporozoites secrete EVs to suppress MIEC apoptotic activities because it has been suggested that *T. vaginalis* exosomes upregulate IL-6 to suppress other proinflammatory cytokines [89]. This is in contrast to extracellular acidosis that has been reported to transiently modulate IL-6 expression in rat epithelial cell [90]. Nevertheless, *Ef*SEVs enclosed potential danger/damage/stress-associated ligands that activated the release of endogenous IL-18 and IL-1β as proinflammatory cytokines associated with inflammasome complexes [91] (Figs. 5, 6). Also, *Ef*SEVs [35, 41] were able to bind green fluorescent dye and aggregate around the MIEC membrane with some degree of internalization at the later experimental time, as well as involvement in MIEC membrane disruption or sphericalization (Additional file 5). The cytoplasmic incorporation of *Ef*SEVs by MIECs might render them as cytosolic ligands that subsequently activate inflammasome complexes [92].

Inflammasomes are vital innate immune complexes in protozoa-host interactions [93] and are activated by intracellular pattern-recognition receptors (or sensors) that recognize pathogen- danger- and stress-associated ligands [95]. Inflammasomes include NLRPs, AIM2 and caspases [93, 96]. NLRP inflammasome families contribute to intestinal disease pathogenesis while caspases participate in proinflammatory processes and pathophysiology of enteric pathogens [20, 97, 98]. In the present study, upregulation of MIEC caspase 11 and NLRP6, as well as dose-dependent upregulation of IL-18 and IL-1β (Figs. 5, 6) are comparable with inflammatory cascades and NLRP6 activation during mice colonic inflammation and tumorigenesis [46, 96]. Likewise, the activation of NLRP6 during *Cryptosporidium tyzzeri* infection in mice caused a luminal release of IL-18 [99, 100]. It is therefore not unlikely that *C. tyzzeri*-derived EVs, though not characterized, might have played pivotal role in the activation of NLRP6 in *C. tyzzeri*-infected mice, as observed in this study (Fig. 6a, b). Mechanistically, non-canonical caspase 11 is activated when toxins (e.g. bacterial lipopolysaccharide [LPS]) find their way into the host cell cytoplasm [97,
In the absence of the bacteria, *Brucella melitensis* LPS induced caspase 11 in murine enterocytes [102] like *Ef* SEVs in this study. Moreover, the production of IL-1β, IL-18 and caspase 11 have been posited as markers for intestinal inflammation in response to pathogens [92, 103]. Additionally, caspase 11-dependent pyroptosis, in association with IL-18, is a particularly important host immune mechanism against intracellular pathogens [92, 102]. Also, the production of cytosolic LDH has been defined as a marker for inflammasome-dependent pyroptosis [98, 104, 105]. Upregulation of caspase 11 and NLRP6 with a significant release of LDH as well as dose- and time-dependent secretion of IL-1β and IL-18 [106] signified that *Ef* SEVs are potential activators of pyroptosis in MIECs (Figs. 5, 6). Comparatively, the MIEC inflammatory response and the release of the cell death signal induced by *Ef* SEVs were synchronous with the time of *E. falciformis* sporozoite intracellular egress [30]. However, the mechanism by which intracellular parasites secrete a regulated amount of EVs to temporarily delay host responses until the completion of intracellular development requires further investigation. It is therefore possible that inflammatory responses and host cell death during *Eimeria* infection are not entirely passive due to intraepithelial distress, but are prefigured by *Eimeria* parasite behavior for survival and successful completion of the life-cycle.

**Conclusion**

This study emphasized host-parasite interaction as an additional mechanism by which the protozoan parasite *E. falciformis* secretes EVs and VF molecules. The study unveils the protein compositions of *E. falciformis* sporozoite secretome as well as the MIEC inflammatory response to *E. falciformis* sporozoite EVs. Specifically, *E. falciformis* sporozoite EVs contained important antigenic and immunogenic proteins as well as other apicomplexan proteins that are implicated in motility, invasion, pathogenesis, survival and host cell degradation. Also, *E. falciformis* VF proteins encompassed proteins involved in pathogenesis, motility and vesicle formation. Furthermore, *E. falciformis* sporozoite EVs induced the release of proinflammatory molecules such as IL-6, IL-17, IL-1β and IL-18 as well as the upregulation of pyroptosis-dependent caspase 11 and NLRP6, at the least, in a dose-dependent manner. Essentially, *E. falciformis* EVs are components of danger-associated molecular patterns (DAMPs) with protein- and gene-regulatory activities that support pyroptosis-dependent response. Unlike parasite protein characterization through extraneous stimuli, the model of the study could be adapted to ascertain secretory motifs and constitutive antigens/immunogens in *Eimeria* species for the development of vaccines against poultry coccidiosis. Also, it will benefit our understanding to further describe the classical components of NLRP6- and caspase 11-mediated pyroptosis during *E. falciformis* infection.

**Abbreviations**

AIM2: Absent in melanoma 2; ECL: Enhanced chemiluminescence; *Ef*SEVs: *E. falciformis* sporozoite EVs; EVs: Extracellular vesicles; IL: Interleukin; LC–MS/MS: Liquid chromatography–tandem mass spectrometry; LPS: Lipopolysaccharide; MCP1: C–C motif chemokine 2/monocyte chemoattractant protein 1; MIECs: Mouse intestinal epithelial cells; NLRP6: Nucleotide-oligomerization domain (NOD)-like receptor pyrin 6; PAMP: Pathogen-associated molecular pattern; PBS: Phosphate-buffered saline; ODG: OptiPrep™ density gradient; SDS-PAGE: Sodium dodecyl sulphate–polyacrylamide gel electrophoresis; VF: Vesicle-free.

**Supplementary Information**

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**Author contributions**

JC designed the study. SY, ZQ and JO conceptualized the study. SY, ZQ, XX, XL, JW, BL and MX, provided briefings on protocols. YB processed the proteomic data. JO carried out the experiments. JC and SY appraised the experimental results. JO drafted the manuscript and JC revised the manuscript. All authors read and approved the final manuscript.

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**Declarations**

**Ethics approval and consent to participate**

Balb/c mice used in this work were handled in strict compliance with Animal Ethics Procedures and Guidelines of the People's Republic of China as approved by the Animal Administration and Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (LVRIAEAC-2020-035).
Consent for publication
The authors agree to the publication of the study.

Competing interests
All authors declare that there is no conflict of interest.

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