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11-2015

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### Recommended Citation

Chaidayet, S., Sotillo, J., Smout, M., Cantacessi, C., Jones, M, et al. (2015). Carcinogenic Liver Fluke Secretes Extracellular Vesicles That Promote Cholangiocytes to Adopt a Tumorigenic Phenotype. *Journal of Infectious Diseases*, 212(10), 1636-1645 doi:10.1093/infdis/jiv291

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## **Carcinogenic liver fluke secretes extracellular vesicles that promote cholangiocytes to adopt a tumorigenic phenotype**

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## ABSTRACT

### Background

Throughout Asia there is an unprecedented link between cholangiocarcinoma and infection with the liver fluke *Opisthorchis viverrini*. Multiple processes including chronic inflammation and secretion of parasite proteins into the biliary epithelium drive infection towards cancer. Until now, the mechanism and effects of parasite protein entry into cholangiocytes was unknown.

### Methods

Various microscopy techniques were used to identify *O. viverrini* extracellular vesicles (EVs) and their internalization by human cholangiocytes. Using mass spectrometry we characterised the EV proteome and associated changes in cholangiocytes after EV uptake, and detected EV proteins in bile of infected hamsters and humans. Cholangiocyte proliferation and IL-6 secretion was measured to assess the impact of EV internalization.

### Results

EVs were identified in fluke culture medium and bile of infected hosts. EVs internalized by cholangiocytes drove cell proliferation and IL-6 secretion and induced changes in protein expression associated with endocytosis, wound repair and cancer. Antibodies to an *O. viverrini* tetraspanin blocked EV uptake and IL-6 secretion by cholangiocytes.

### Conclusions

This is the first time that EVs from a multicellular pathogen have been identified in host tissues. Our findings imply a role for *O. viverrini* EVs in pathogenesis and highlight an approach to vaccine development for this infectious cancer.

## Introduction

The liver fluke, *Opisthorchis viverrini*, is classified as a group 1 carcinogen by the International Agency for Research on Cancer, and is a major public health problem in many parts of Southeast Asia. In Northeast Thailand alone, more than 8 million people harbour the parasite [1] due to traditional dietary preferences for eating uncooked fish that harbour the infective stage of the fluke [2]. Upon ingestion of infected fish, the metacercariae excyst in the duodenum and migrate to the bile ducts of the definitive host where they feed on the biliary epithelia. Infection is associated with a spectrum of hepatobiliary abnormalities including bile duct cancer or cholangiocarcinoma (CCA) [2, 3]. The incidence of CCA in Northeast Thailand is substantially higher than elsewhere in the world, and associates strongly with the prevalence of *O. viverrini* infection [4].

The mechanisms involved in liver fluke-driven tumorigenesis are multi-factorial, with apparent roles for (i) mechanical damage caused by parasites grazing on the biliary epithelium; (ii) chronic immunopathologic processes that are dominated by pro-inflammatory cytokines such as IL-6 [1]; (iii) the active release of parasite-derived excretory/secretory (ES) products into the bile ducts that drive unchecked cell proliferation [2, 5-7]. Intriguingly, some of these ES products have been identified inside cholangiocytes of experimentally infected hamsters [6, 8, 9], akin to the intracellular presence of the CagA protein from another carcinogenic pathogen, *Helicobacter pylori* [10]. Until now, the mechanisms by which liver fluke ES proteins are internalized by cholangiocytes, and the ramifications of this process for the host cell, have remained unknown.

Recent reports have highlighted the presence of secreted extracellular vesicles (EVs) from parasites of both unicellular [11, 12] and multicellular (helminths) [13-15] origin, and provide a plausible explanation for the abundance in helminth ES products of apparently “intracellular” proteins [16]. EVs are small membrane-enclosed structures that are released by many different cell types [17, 18]. EVs from unicellular parasites have been shown to

influence host physiological processes, including immunomodulation, and adherence and communication between host and parasite [12, 19, 20]. A recent report described the uptake of parasitic platyhelminth EVs by host cell lines *in vitro* using low resolution fluorescence microscopy [13], but the molecular impact of EV uptake on the recipient cell, and detection of helminth EVs *in vivo* has until now, not been reported.

Here we show that *O. viverrini* secretes EVs that induce a pro-inflammatory/tumorigenic phenotype in human cholangiocytes. We also show that cellular uptake of *O. viverrini* EVs can be blocked by antibodies to an EV recombinant tetraspanin, highlighting the potential utility of EV proteins as vaccines to prevent fluke infection and associated cholangiocarcinogenesis.

## Materials and Methods

### *Ethics statement*

Hamsters were maintained at Khon Kaen University (KKU), Thailand. The study was approved by the KKU Animal Ethics Committee (AEKKU 55/2554). Human bile was collected under an approved protocol (HE 521209) from the KKU Institutional Review Board.

### *Parasite material, isolation of ES products and EV purification*

Parasites were obtained from experimentally infected hamsters and ES products were isolated as described [8]. EVs were purified from ES products using differential ultracentrifugation following a modified protocol [21]. Briefly, parasite culture media was centrifuged at 2,000 g for 30 min at 4°C to remove larger debris; supernatant was further centrifuged at 15,000 g for 30 min at 4°C. ES supernatants were filtered using a 0.2 µm membrane (Schleicher and Schuell Bioscience) then ultracentrifuged at 110,000 g for 2 h at 4°C. The pellet was washed in PBS containing protease inhibitor cocktail (Roche) and

ultracentrifuged for 1 h. Crude pellet was resuspended in 200  $\mu$ l PBS/ protease inhibitor cocktail and stored at 4°C.

#### *Preparation of EVs for Transmission Electron Microscopy*

Five  $\mu$ l of purified EVs was applied to a carbon-formvar coated grid and air dried. Grids were negatively stained in 2% uranyl acetate for 15 secs. Stained grids were viewed in a JEM1011 transmission electron microscope (JEOL) equipped with a Morada side-mounted digital camera (Olympus).

#### *Fluorescence labelling of EVs membranes*

EVs were labelled with Alexa Fluor 488 5-SDP Ester (AF488) (Life Technologies) following the manufacturer's protocol. EV pellets were resuspended in 400  $\mu$ l of PBS and mixed with 50  $\mu$ g of AF488 for 1 h at 4°C. The samples were collected by ultracentrifugation at 110,000 g for 2 h to remove excess dye, washed twice and resuspended in 400  $\mu$ l of PBS.

#### *Cell co-cultures and fluorescence microscopy*

To investigate internalization of EVs by mammalian cells, normal immortalized human cholangiocytes (H69) were grown to 80% confluence as described [22]. Cells were starved of FCS overnight, incubated with AF488-EVs for 6 h, and analysed by fluorescence microscopy. Cell nuclei were stained with Hoechst dye and visualized using an AxioImager M2 ApoTome fluorescence microscope (Zeiss). Two technical and 2 biological replicates were performed and at least 5 fields were analysed in each replicate. Fluorescence was quantified using ImageJ.

### *Ultra-resolution microscopy*

To convincingly demonstrate internalization of *O. viverrini* EVs by cholangiocytes, we used 3D-Structured Illumination microscopy (3D-SIM). H69 cholangiocytes were grown to 50% confluence and co-cultured with AF488-EVs or an equivalent amount (based on fluorescence) of ES products (10 µg/ml) for up to 6 h. Treated cells were fixed in 4% paraformaldehyde, permeabilised in 0.1% Triton X-100 in PBS and stained with Alexa Fluor 568 Phalloidin (Life Technologies). Specimens were mounted in 5% N-propyl-Gallate (Sigma) and 3D-SIM was performed on isolated single cells using a DeltaVision OMX 3D-SIM system (Applied Precision Inc, GE Healthcare). Solid state lasers (405, 488, 593 nm) provided wide-field illumination and multi-channel images were captured simultaneously using three 512× 512 pixel size PCO edge scientific CMOS cameras. All data capture used a 60× 1.4 NA oil objective and standard excitation and emission filter sets (in nm, 405 EX / 419-465 EM, 488 EX / 500-550 EM and 592.5 EX / 608-648 EM). 3D-SIM images were sectioned using a 125 nm Z-step size. Raw 3-phase images were reconstructed as previously described [23]. Reconstructed images were rendered in 3D using IMARIS version 7.X (Bitplane Scientific).

### *Proteomic analysis of EVs*

Sample preparation for proteomic analysis was performed as described [13]. Peptides were resuspended in 5% formic acid and analysed by LC-MS/MS. Ten microliters was injected onto a C18 trap column (Thermo Scientific Acclaim) and flushed into an analytical column (Agilent) and eluted via a mobile phase gradient: 5–80% solvent B over 120 min. The eluted material was directly applied to the nanospray source of a QSTAR Elite instrument (Applied Biosystems), and data analysis was conducted with Analyst 2.0 (Applied Biosystems).



### *iTRAQ analysis*

H69 cells were co-cultured with the quantity of EVs that corresponded to 1.2 µg/ml of ES protein in PBS for 30 min, 3 h and 16 h. Cells were washed using PBS containing protease inhibitor cocktail and lysed in 5 M urea, 2 M thiourea, 0.1% SDS, 1% triton X-100 and 40 mM Tris (pH 7.4). Each sample was ground with a TissueLyser II (QIAGEN) and centrifuged at 12,000 g for 20 min. The protein supernatant was precipitated with cold methanol, centrifuged at 8,000 g and air dried pellets re-dissolved in 0.5 M triethylammonium bicarbonate (TEAB)/0.05% SDS, then centrifuged at 12,000 g. Samples were resuspended in 0.5 M TEAB prior to reduction, alkylation, digestion and iTRAQ labelling (AB Sciex) then analysed on a 5600 TripleTOF mass spectrometer as described [24].

### *Bioinformatic analysis of protein expression data*

For proteomic characterization of *O. viverrini* EV proteins, the *O. viverrini* genome database [25] was interrogated using MASCOT (Matrix-Science) allowing two missed cleavages and mass tolerance of 1.2 Da in MS mode and 0.8 Da on MS/MS product ions. Probability ( $P < 0.05$ ) of FDR was calculated for every search using the MASCOT decoy database facility. For iTRAQ, two biological and two technical replicates were conducted. Database searches were performed against NCBI nr using MASCOT allowing two missed cleavages and a mass tolerance of 0.1 Da on MS and MS/MS modes, and results were validated using Scaffold v.4.2.1 (Proteome Software). Differentially expressed (dysregulated) proteins were determined using a Kruskal-Wallis test and results expressed in log<sub>2</sub> ratios. Only proteins whose expression level underwent a significant ( $P < 0.05$ ) log<sub>2</sub> fold-change of >0.6 or <-0.6 for upregulated and downregulated proteins respectively were further considered. KEGG and Reactome functional enrichment analyses for dysregulated proteins were performed using KEGGMapper and DAVID [26]. Protein-protein interaction analysis was performed using String [27].

### *Detection of EV proteins in bile of O. viverrini-infected humans and hamsters*

Bile was collected from *O. viverrini*-infected humans by aspiration of resected gallbladders, and experimentally infected and uninfected hamsters (controls) by puncture of the gallbladder from euthanized animals, and EVs isolated as described above. Selected Reaction monitoring (SRM) was performed on a 5600 TripleTOF to identify *O. viverrini* EV proteins in bile. All analyses were performed using SRM-initiated IDA experiments in which a SRM survey scan triggered the acquisition of MS/MS spectra. Skyline 3.1 was used to generate SRM transitions and for data analysis.

### *Cell proliferation*

H69 cholangiocytes were cultured as described above until 80% confluent. Cells were starved of FCS overnight and cultured with the amount of EVs that corresponded to 1.2 µg/ml of ES protein over 72 h at 37°C. Control cells were incubated with 1.2 µg/ml of ES products (not ultra-centrifuged) or 1.2 µg/ml BSA for the negative control group. Cell proliferation was assessed by measuring cell index (CI) values in real time using an xCELLigence SP system and E plates (Acea). Three biological replicates were analysed and a 2-way ANOVA was performed.

### *Blocking internalization of EVs with a recombinant Ov-TSP-1*

AF488-EVs were incubated with mouse anti-Ov-TSP-1 serum [28] or normal mouse serum (NMS) at different dilutions (1:2.5, 1:5, 1:20) at RT for 1 h. EV:antibody complexes were added to H69 cells and co-cultured for 6 h. Images were obtained using an ECLIPSE Ni-u confocal microscope (Nikon) at 60× magnification. Thirty fields from 2 biological replicates were analysed and a 1-way ANOVA was performed. Cell fluorescence was quantified using ImageJ.

### *IL-6 detection*

H69 cholangiocytes were cultured as above to monitor cell proliferation over 24 h. Culture media were collected and centrifuged at 2000 g for 10 min to remove cell debris. IL-6 levels were quantified using an IL6-ELISA kit (R&D System) following the manufacturer's instructions. Three biological replicates were analysed and statistical analysis was performed using a 2-way ANOVA.

## **Results**

### *Excretory/secretory products from *O. viverrini* contain EV-like vesicles*

After filtration and ultracentrifugation of ES products from adult *O. viverrini* we identified microvesicular structures using negative staining in transmission electron microscopy (Figure 1). These spherical vesicles had an external diameter of 40-180 nm, resembling EVs recently reported from other trematodes [13, 14].

### *Proteomic analysis of *O. viverrini* EVs reveals conserved and novel proteins*

*O. viverrini* EVs were digested with trypsin and submitted to LC-MS/MS. We identified 108 different proteins (Supplementary Table 1), 42 of which were homologous to mammalian EV proteins in Exocarta [29], including proteins that are diagnostic of mammalian exosomes such as tetraspanins, HSP-70, cytoskeletal, regulatory and trafficking proteins. Gene Ontology (GO) analysis of the proteins (Supplementary Figure 1) returned 212 biological process terms. Pfam analysis identified proteins with an EF hand domain as the most frequently represented (Supplementary Figure 1).

*O. viverrini* exosome proteins are identified in bile of infected hosts

SRM analysis confirmed the presence of specific EV proteins in the bile of *O. viverrini* infected humans and hamsters. Seven of the top 15 proteins identified in the EV proteomic analysis (based on number of peptides identified) were successfully quantified (Supplementary Figure 2).

*O. viverrini* EVs are internalized by human cholangiocytes

We monitored the uptake of AF488-EVs by human cholangiocytes *in vitro*. Within 60 min punctate fluorescent structures were detected, the number of which increased markedly over 6 h (Figure 2A). Cell fluorescence of all fields from the two biological replicates revealed fluorescence coverage of  $62.44 \pm 9.52\%$  and  $90.57 \pm 4.58\%$  at 0.5 h and 6 h respectively. 3D-SIM analysis confirmed the intracellular presence of AF488-EVs within the cytoskeletal network of well-separated individual cells as evidenced by their position between the basal and apical membranes outlined with phalloidin (Figure 2B, C, Supplementary Movie). We attempted to co-localize EVs to defined organelles using specific fluorescent markers but did not observe co-localization (not shown), suggestive of a cytoplasmic location.

*O. viverrini* EVs promote cell proliferation and stimulate wound healing and tumorigenic pathways in cholangiocytes

H69 cholangiocytes were incubated for 72 h with (i) crude ES products, (ii) EVs purified from crude ES products, or (iii) an irrelevant protein control, Bovine Serum Albumin (BSA). We chose BSA as a control rather than EVs from another source because H69 cholangiocytes produce (and uptake) their own exosomes in culture [30]. ES and EVs both induced significant ( $P < 0.05$ ) cell proliferation compared to BSA-treated control cells (Figure 3). A total of 705 proteins containing at least 2 peptides were identified and

quantified with a 99.9% protein threshold and 1.2% false discovery rate (FDR) from 6,863 unique peptides (peptide threshold = 95% and 0.82% FDR) from H69 cholangiocytes that were co-cultured with EVs (Supplementary Table 2); a total of 396 proteins from cholangiocytes co-cultured with EVs were differentially expressed ( $P < 0.05$  as determined using a Kruskal-Wallis test) and presented a fold-difference  $\geq 1.5$  ( $\log_2 \geq 0.6$ ) at  $\geq 1$  time point (Supplementary Table 2). The greatest changes in protein expression in EV-co-cultured cells compared to control cells occurred after 3 h, where 238 (60.1%) proteins were differentially expressed (fold-difference  $\geq 1.5$  ( $\log_2 \geq 0.6$ )) in cells incubated with EVs (Figure 4A).

A total of 274 proteins from EV-treated cells matched homologous proteins in the KEGG database associated with 186 distinct biological pathways; 171 of these proteins were assigned to 14 Reactome pathways (Supplementary Table 3). The 25 KEGG and Reactome pathways associated with the largest numbers of proteins differentially expressed after incubation with EVs are shown in Figure 4B and C respectively. The expression of proteins linked to four pathways implicated in cancer and wound healing was significantly modified by EVs (Figure 4B). Supplementary Table 4 highlights all cholangiocyte proteins and KEGG pathways related to cancer and wound healing and their expression following co-culture with EVs. A total of 7 proteins involved in the Phagosome pathway were differentially expressed following exposure of human cholangiocytes to EVs (Supplementary Figure 3, Supplementary Table 5).

When differentially expressed proteins were assigned to Reactome pathways, those associated with Apoptosis, Regulation of activated PAK-2p34 by proteasome, and Cdh1-mediated degradation of Skp2 pathways were differentially regulated in cholangiocytes co-cultured with EVs (Figure 4C). Expression of 17 proteins associated with Apoptosis was regulated by EVs, and protein-protein interactions revealed that the majority of dysregulated

cholangiocyte proteins formed a cluster belonging to the proteasome complex (Supplementary Figure 4).

*Uptake of EVs and pro-inflammatory cytokine production by cholangiocytes can be blocked with antibodies to an O. viverrini EV tetraspanin*

Due to the roles of tetraspanins in the formation of EVs by mammalian cells and their subsequent uptake by recipient cells [31], and the recent identification of the *Ov*-TSP-1 tetraspanin on the surface of *O. viverrini* [28], we explored the role of *Ov*-TSP-1 in the uptake of EVs by cholangiocytes. Antiserum to recombinant *Ov*-TSP-1 blocked the uptake of EVs by cholangiocytes compared to normal mouse serum ( $P < 0.001 - P < 0.0001$ ) (Figure 5). ES and EVs both stimulated the secretion of significantly more IL-6 from cholangiocytes than did control cells incubated with media alone ( $P < 0.01 - P < 0.001$ ; Figure 7), and IL-6 secretion was significantly reduced when EV internalisation was blocked by antiserum to *Ov*-TSP-1 ( $P < 0.05$ ; Figure 6).

## Discussion

While the mechanisms that drive chronic liver fluke infection towards cancer are multi-factorial, a pivotal role for fluke ES products in promoting a tumorigenic phenotype has been demonstrated [7]. The intracellular localization of *O. viverrini* ES proteins in cholangiocytes of infected hosts has prompted speculation on the mechanisms underlying this process of parasite protein internalization and its potential pathologic and carcinogenic consequences [6, 7]. Herein we show that *O. viverrini* secretes EVs that are internalized by human cholangiocytes *in vitro* and trigger a cascade of inflammatory and pro-tumorigenic changes within the cell, thereby providing a plausible mechanism by which ES proteins are taken up by biliary cells of infected hosts and contribute to the development of CCA in infected individuals.

Investigations on the interactions between EVs derived from single celled parasites and the cells that they encounter in an infected host have shed light on a novel means of host-parasite cellular communication [11, 12]. Protozoan parasite EVs are also capable of modulating pro-inflammatory immune responses and inducing production of regulatory cytokines in recipient cells [19]. EV production by helminth parasites however has received considerably less attention. A seminal study recently showed that the parasitic nematode *Heligmosomoides polygyrus* secretes exosomes that contain microRNAs which suppress type 2 innate responses [15]. However, little is known about the production and particularly the biological effects of EVs from parasitic flatworms (platyhelminths). EVs were reported in the ES products from the flatworms *Fasciola hepatica* and *Echinostoma caproni* [13], and more recently *Dicrocoelium dendriticum* [14], and while the authors showed binding of fluke EVs to cell lines, neither the functional implications nor *in vivo* evidence of EV uptake by tissues of infected hosts were addressed. We show here for the first time that helminth EVs are unequivocally internalized by host cells, and major EV proteins can be detected in the bile of naturally infected humans and experimentally infected hamsters. From proteomic analyses, we identified 108 proteins from *O. viverrini* EVs, and only a handful (13) of these had been previously identified in a proteomic analysis of the ES products of the fluke [32].

Cholangiocytes line the bile ducts and provide the first line of defence against pathogens in the biliary system. We show here that *O. viverrini* EVs drive production of IL-6 from recipient human cholangiocytes, implicating these EVs in the hepatic disease process. IL-6 has been associated with chronic periductal fibrosis and CCA in opisthorchiasis patients [33], and IL-6 has been implicated in the maintenance of a chronic inflammatory milieu that could lead to tumorigenesis [34]. *O. viverrini* EVs were also capable of driving proliferation of cholangiocytes, a phenomenon that has been convincingly documented in the hamster infection model [35]. This relentless cell proliferation, coupled with other carcinogenic insults

such as chronic immunopathology and elevated intake of dietary nitrosamines [2] culminates in the establishment of an environment that is conducive to malignant changes.

Uptake of EVs by cholangiocytes resulted in dysregulated expression of proteins with documented roles in wound healing and cancer. Indeed, wound repair has long been implicated in tumorigenesis, and there are striking histological and molecular similarities between tumor stroma and wounded tissues [36]. Nineteen KEGG pathways related to cancer and wound healing were affected by EVs in human cholangiocytes. Among the proteins significantly regulated we found different tropomyosin isoforms, PAK-2 and the tight junction protein ZO-2. PAK-2 is an important kinase that mediates tumor cell invasion [37]. We also noted that PAK-2 could play an important role in tumour progression by interacting with different components of the proteasome and with vimentin, a protein involved in maintaining cellular integrity and providing resistance against stress in several epithelial cancers [37]. Moreover, tight junction proteins play important roles in cell-cell junction assembly and organization, and are up-regulated during cell proliferation and wound healing [38].

Other important cholangiocyte proteins underwent dysregulated expression after co-culture with EVs, notably proteins involved in the proteasome complex. Most of the proteins involved in this complex were upregulated in cholangiocytes exposed to EVs and are known to interact with other proteins involved in cancer and wound healing processes. The proteasome complex regulates directly or indirectly many important cellular processes, and has been suggested as a therapeutic target for cancer [39, 40].

EV uptake by dendritic cells can be blocked with antibodies to EV tetraspanins [41]. Tetraspanins are thought to be involved in the regulation of protein assembly and microRNA recruitment in mammalian exosomes [31] and influence cell selectivity [42]. Our findings highlight an important role for *Ov*-TSP-1 in *O. viverrini* EV uptake by cholangiocytes. Although tetraspanins are found on the surface membranes of all exosomes, their discovery



here is particularly pertinent due to their abundance on the surface membranes of parasitic helminths [43] and their importance in the development of the *O. viverrini* tegument [28]. Tetraspanins are efficacious helminth vaccine antigens [44-46], and our findings here suggest that the mechanism of vaccine efficacy is linked to interruption of parasite EV uptake by recipient host cells *in vivo* and subsequent disruption of key physiological (and pathological) processes.

Our study describes for the first time the release of EVs in the secreted products of the carcinogenic liver fluke, *O. viverrini*, and highlights the role of fluke EVs in promoting an inflammatory yet simultaneously modulatory (wound healing) environment that ultimately facilitates the development of biliary cancer. Our findings do however offer hope for the eventual control of this debilitating neglected tropical disease by highlighting a key physiological process that can be potentially interrupted via subunit vaccines targeting key EV surface molecules.

## **FUNDING**

This work was supported by a Project Grant (APP1085309) from the National Health and Medical Research Council of Australia (NHMRC). AL is supported by a NHMRC principal research fellowship. SC was supported by the Thailand Research Fund (TRF)-the Royal Golden Jubilee PhD scholarship (RGJ) through Dr. Banchob Sripa. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## **ACKNOWLEDGMENTS**

We thank Drs Antonio Marcilla and Rafael Toledo for helpful discussions and Mr Alun Jones for assistance with proteomics.

## **CONFLICT OF INTEREST**

The authors have no conflicts of interest to declare.

## **FOOTNOTES**

### **Conflict of interest**

All authors: No reported conflicts.

### **Funding statement**

This work was supported by a Project Grant (APP1085309) from the National Health and Medical Research Council of Australia (NHMRC). AL is supported by a NHMRC principal research fellowship. SC was supported by the Thailand Research Fund (TRF)-the Royal Golden Jubilee PhD scholarship (RGJ) through Dr. Banchob Sripa. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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## FIGURE LEGENDS

**Figure 1. Extracellular vesicles (EVs) secreted by *Opisthorchis viverrini*.** Transmission electron micrograph showing the presence of microvesicles after ultracentrifugation of *O. viverrini* excretory/secretory products. EV-like microvesicles of 40-100 nm can be observed.

**Figure 2. Internalization of *Opisthorchis viverrini* secreted extracellular vesicles (EVs) by human cholangiocytes.** Fluorescence images of Alexa Fluor 488-labeled EVs (green) internalized by H69 cholangiocytes (A). Cholangiocytes internalized the EVs within 1 h of co-culture and maximum internalization was observed by 6 h. Control cells were incubated with PBS. Hoescht dye (blue) was employed to label cell nuclei (A). 3D-Structured Illumination microscopy (3D-SIM) fluorescence image of the edge of a well-separated individual cholangiocyte after 6 h incubation with Alexa Fluor 488-stained EVs (green) (B). Lateral (xy) overview of cell showing EVs present within the cytoskeletal actin network (red) of the cell (B). Rendered axial (xz) view of inset in panel A revealed EVs between the apical and basal surfaces of the cell that were stained by phalloidin (red) (C).

**Figure 3. *Opisthorchis viverrini* extracellular vesicles (EVs) drive proliferation of human cholangiocytes.** *O. viverrini* EVs (open triangles) and excretory/secretory (ES) products (closed circles) promoted the proliferation of human cholangiocytes. Asterisks represent the time-point from which cell growth remained significantly different between test and control groups after each treatment. \*  $P < 0.05$ .

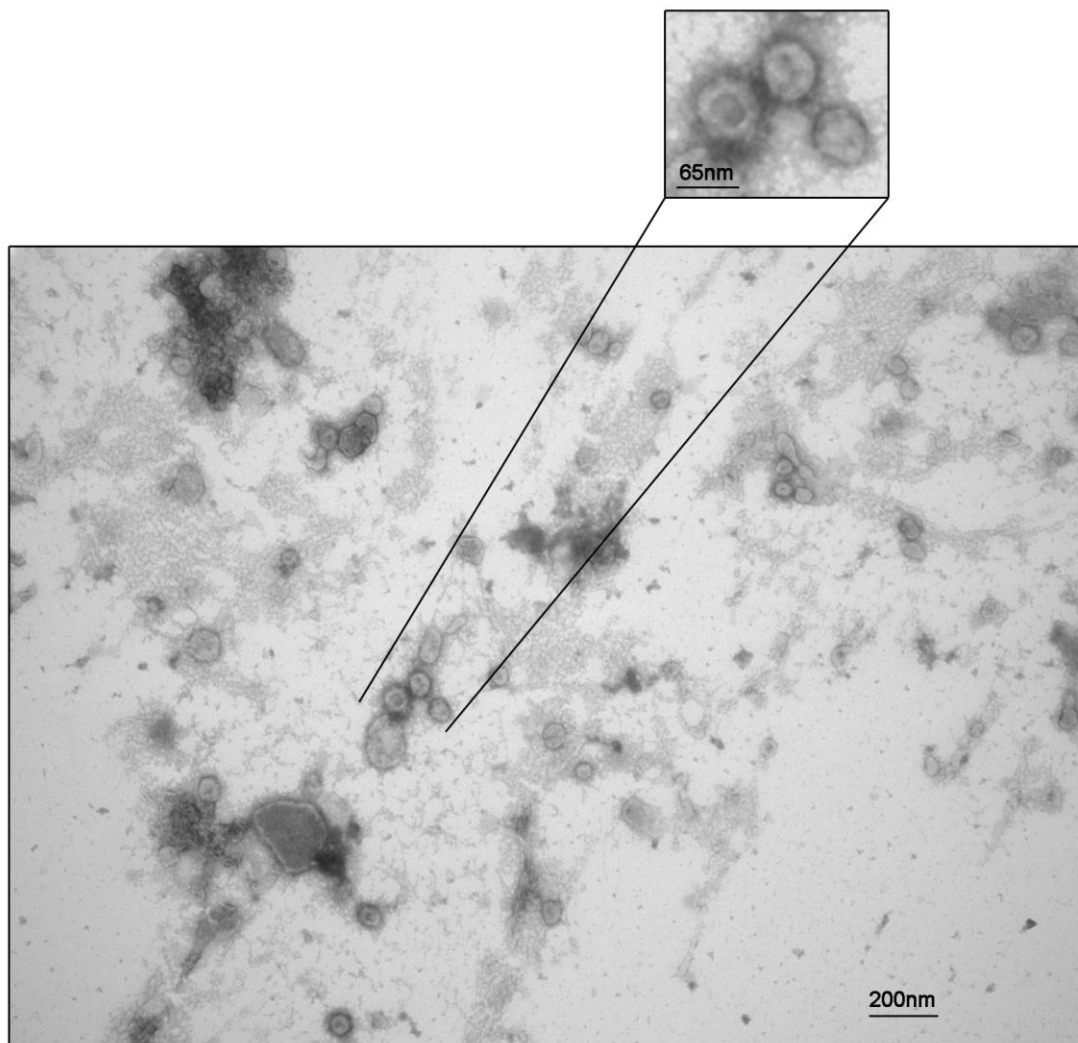
**Figure 4. Comparison of proteins that were significantly regulated, and biological pathways regulated in cholangiocytes co-cultured with *Opisthorchis viverrini* extracellular vesicles (EVs).** (A) Heatmap of the proteins from human cholangiocytes with a



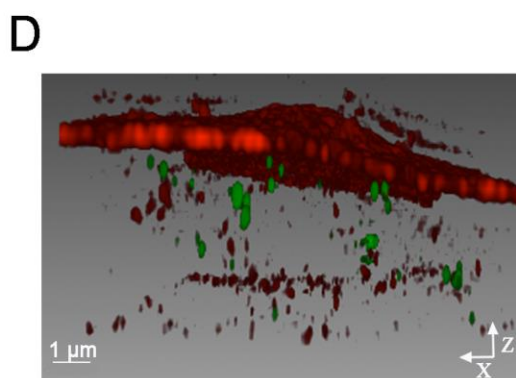
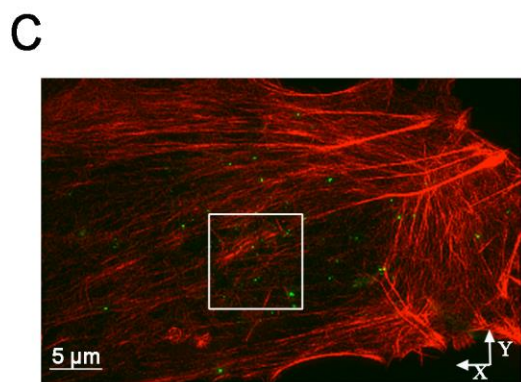
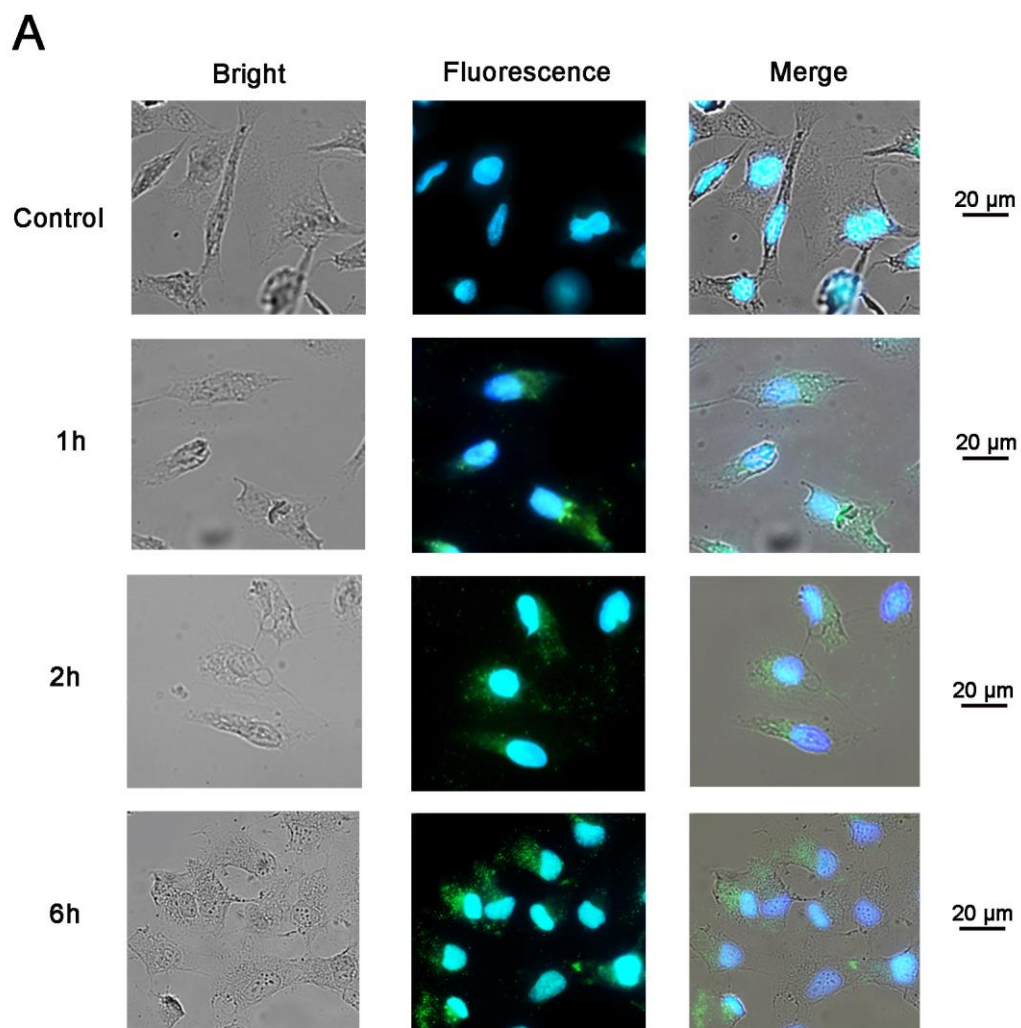
significant fold regulation after Kruskal-Wallis test and that underwent a 2-fold-change ( $\log_2=1.5$ ) in at least one of the time-points assessed after incubation with EVs from *O. viverrini*. (B) Top 25 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways regulated after incubation with EVs. (C) Reactome pathways regulated after incubation with EVs. Pathways that are involved in wound healing and cancer are indicated by red font.

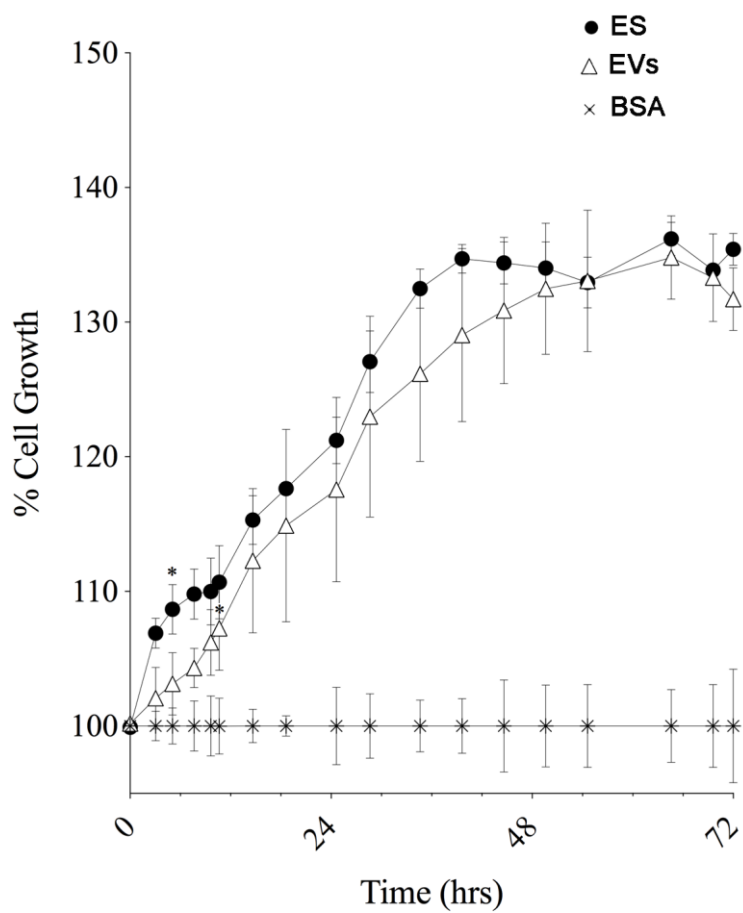
**Figure 5. Blockade of uptake of *Opisthorchis viverrini* extracellular vesicles (EVs) by antibodies to recombinant *Ov*-TSP-1.** Mouse antibodies raised to recombinant *Ov*-TSP-1 blocked the uptake of Alexa Fluor 488-labeled *O. viverrini* EVs by H69 cholangiocytes. Fluorescence micrographs of internalized EVs co-cultured with H69 cholangiocytes in the presence of anti-*Ov*-TSP-1 serum (A) or normal mouse serum (NMS) (B) at different dilutions. A  $\square\square\square$ -*Ov*-TSP-1 significantly reduced the binding and internalization of EVs at all serum dilutions when measured as fluorescence intensity (C) when compared to NMS (D). \*\*\* $P<0.001$ ; \*\*\*\* $P<0.0001$ .

**Figure 6. IL-6 production by human cholangiocytes after internalisation of *Opisthorchis viverrini* extracellular vesicles (EVs).** Human cholangiocytes produce significantly greater levels of IL-6 after co-culture with *O. viverrini* EVs. IL-6 production was partially blocked when EVs were incubated with antibodies against recombinant *Ov*-TSP-1 prior to cell culture. \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$ .

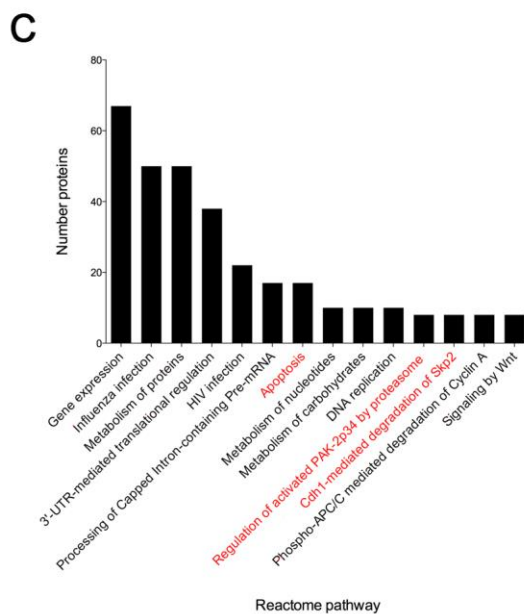
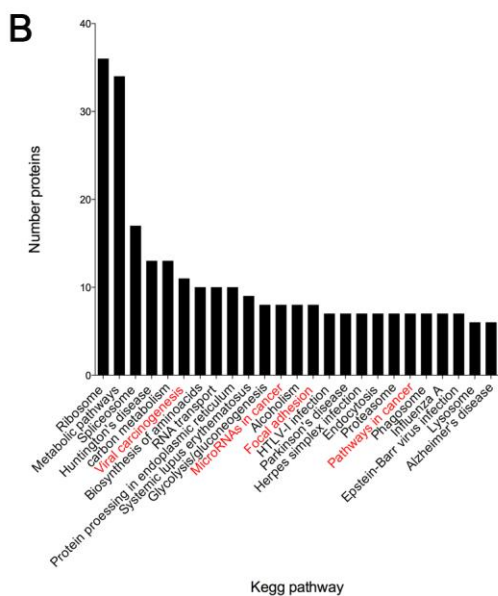
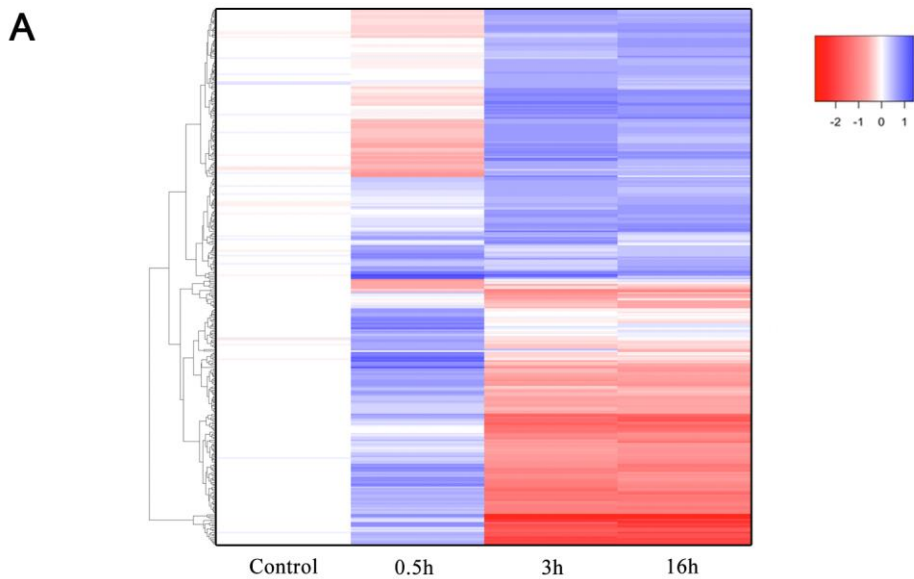


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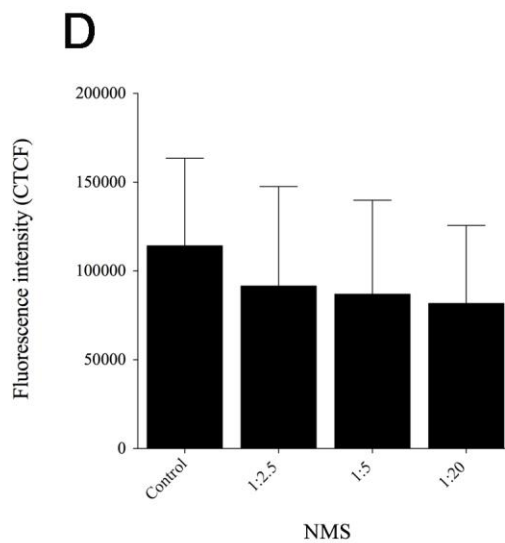
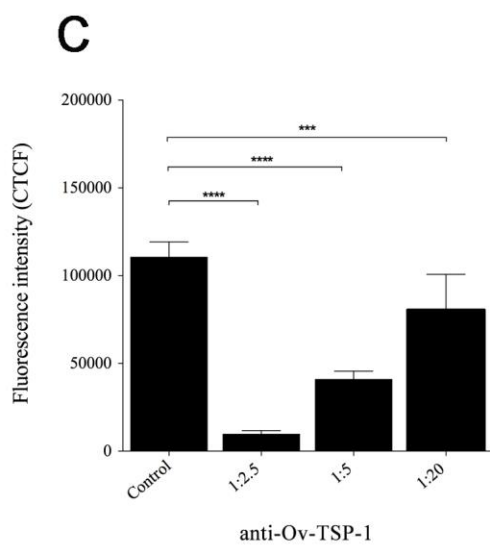
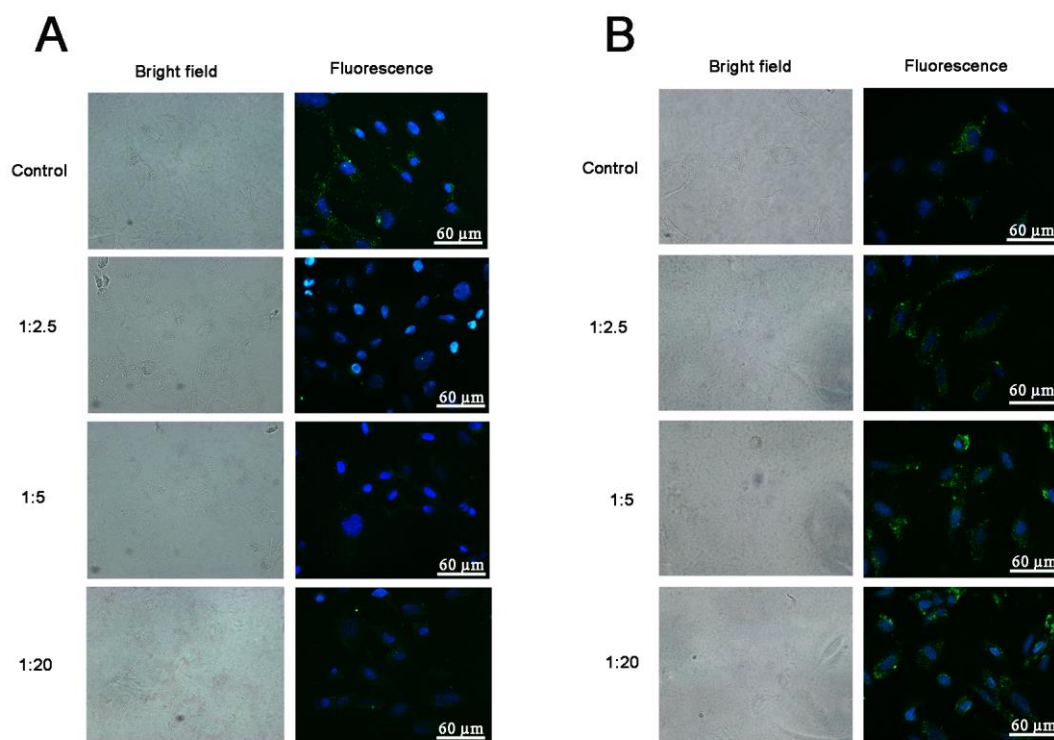




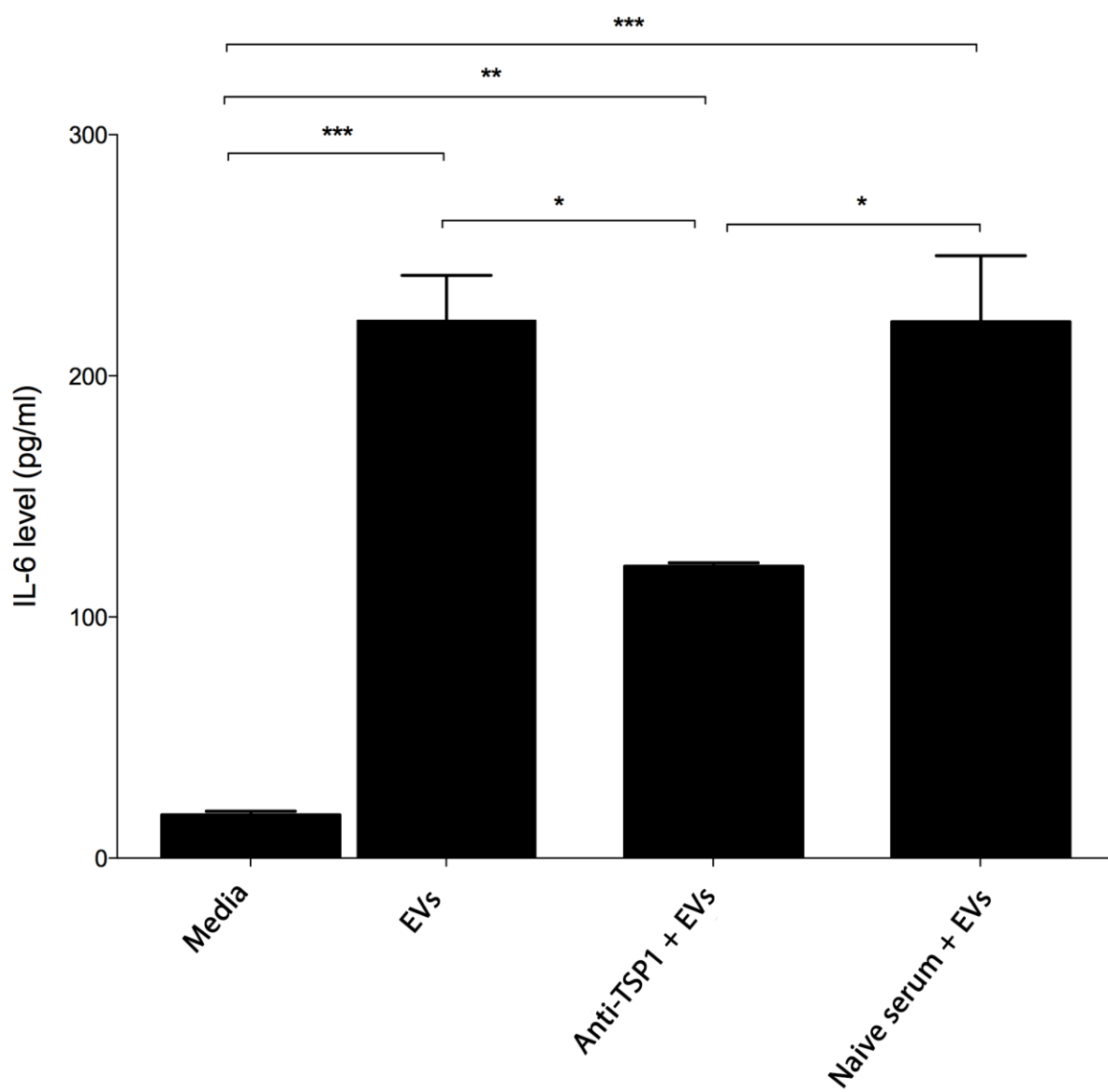
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