Research Square

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# Immunological Signature of Fractionated Excretory–Secretory Products from Adult Fasciola hepatica

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## Abstract

# Background

The liver fluke *Fasciola hepatica* is a major parasitic pathogen of grazing ruminants and an important food-borne zoonosis, yet effective control remains challenging. Chronic infection is sustained by complex host-parasite interactions that skew the immune response towards non-protective type-2 and regulatory pathways while suppressing inflammatory effector mechanisms. This immune modulation is mediated, in part, by the collective activity of parasite excretory-secretory products (ESPs), which comprise multiple bioactive components. To expand current knowledge of host-interacting secreted molecules, adult *F. hepatica* ESPs were fractionated and analysed to identify fraction-specific immune signatures through the characterisation of humoral and cellular responses and subsequent proteomic profiling.

# Results

Adult *F. hepatica* ESP was separated by gel filtration into seven protein peaks and five intermediate plateau fractions. Sera from cattle during the migratory stage of infection displayed stronger reactivity than sera from chronically infected animals, with responses concentrated in high-molecular-weight fractions, whereas uninfected controls showed only background reactivity in some fractions. Isotype-specific ELISAs revealed a consistent type-2-biased humoral profile across all fractions and infection stages, dominated by IgG1 and IgM, while IgG2 responses remained low. In ovine peripheral blood mononuclear cells, whole ESP induced a polarized type-2/tolerogenic response. Fractionation resolved this activity into three distinct immunological sub-signatures: Peak 1 broadly stimulated cytokine expression and was the only fraction containing extracellular vesicles; Peak 6 (dominated by Cathepsin L1 and Kunitz-type protein 8) selectively induced IL-10 expression; and the remaining fractions caused widespread cytokine suppression, largely overriding LPS-induced co-stimulation. Proteomic analysis identified 172 proteins, including several known immunomodulators, such as cathepsin L1, cathepsins B and B5, fatty acid-binding protein, cystatin, serpin 4, glutathione transferase, Kunitz-type protein 8, myoglobin 2, and kininogen-1/bradykinin.

# Conclusions

Fraction-level profiling resolved the composite immunomodulatory activity of adult *F. hepatica* ESP into distinct molecular and functional signatures associated with specific host immune phenotypes. The findings support a layered model of immune evasion in which soluble suppressive factors, a discrete IL-10-inducing activity, and extracellular vesicle-mediated signaling operate in parallel. This immunological map of fractionated adult *F. hepatica* ESPs provides a framework for the identification of improved diagnostic antigen combinations, the prioritisation of vulnerable candidate proteins in vaccine design, and novel insights into helminth-host immune interactions.

# Introduction

The common liver fluke *Fasciola hepatica* is a globally distributed trematode and one of the most economically damaging helminths of grazing livestock. It infects millions of cattle and sheep worldwide and is estimated to cause annual agricultural losses in excess of US$3 billion [1], with a recent global meta-analysis placing the pooled prevalence at around 17% in cattle and 13% in sheep [2]. The burden extends well beyond overt clinical disease: subclinical infection reduces carcass weight, milk yield, and reduced fertility in modern production systems [3, 4]. *F. hepatica* can modulate the response to co-infecting pathogens, compounding its impact on herd health [4]. Fasciolosis is also an important food-borne zoonosis, classified by the World Health Organization as a neglected tropical disease, with an estimated 17 million people infected and a further 180 million at risk [5]. These converging animal-health, economic and public-health dimensions make fasciolosis a paradigmatic One Health problem.

Disease severity is shaped by the parasite's life cycle. Following ingestion of metacercariae, newly excysted juveniles (NEJ) emerge in the intestine and migrate through the peritoneal cavity to the liver, tunnelling through the parenchyma for several weeks before the maturing flukes settle in the bile ducts. Tissue destruction during this migratory phase is driven in large part by a petard of excreted and secreted products [6]. The clinical outcome is host-dependent: sheep may succumb to fatal acute fasciolosis when the metacercarial challenge is high, whereas in cattle the infection typically becomes chronic, with parasites persisting for years [6, 7].

Control still relies almost entirely on chemotherapy, and this cornerstone is failing. Triclabendazole is the only flukicide active against both early immature and adult stages, but resistance is now widespread and increasingly compromises fluke control in both livestock and humans [8, 9], while the small remaining drug arsenal is limited by poor activity against juvenile stages and by restrictions on use in dairy animals [4]. In this setting, vaccination is widely regarded as the most sustainable long-term alternative for fasciolosis control.

Despite decades of effort, no commercial vaccine against *F. hepatica* exists [10]. Progress has been hampered by an incomplete understanding of the naturally acquired immune mechanisms that confer resistance and by a lack of reliable immune correlates of protection. In this sense, several studies have demonstrated the importance of a link between vaccine protection and a Th1 response for an efficient cellular immunity [11–14]. This particular challenge is inseparable from the parasite's capacity to actively reshape host immunity. Like other successful helminths, *F. hepatica* releases a complex mixture of excretory–secretory products (ESP) that redirects the host response towards a non-protective type-2/regulatory phenotype and dampens inflammatory effector arms, allowing the fluke to persist. The secretome is a rich source of candidate immunomodulators, proteases, antioxidants, mucin-like peptides and the *F. hepatica*-derived helminth defence molecules.

Most functional analyses to date have examined either total ESP or single recombinant proteins, leaving a gap between the bulk immunomodulatory activity of the secretome and the contributions of its individual constituents. However, based on the hypothesis that the composite immunomodulatory

activity of whole ESP is the emergent sum of discrete, fraction-level immune signatures, a fractionation would resolve separable stimulatory, regulatory and suppressive activities and localise them to defined protein families. The aim of this study was thus to generate a catalogue of parasite–host immune interactions for adult *F. hepatica* ESP based on ESP fractionation, immune-readouts and an underlying protein-identity map. This complements the available data to prioritise adversely reactive, and therefore potentially vulnerable, parasite proteins for future diagnostics, vaccine and immunotherapeutic development.

# Methods

This study describes the humoral and cellular response against the complete and gel-filtration separated excretory-secretory products (ESP) from adult *Fasciola hepatica* by their isotype-specific antibody response in bovines and their expressed cytokine profiles in ovine peripheral blood mononuclear cells (PBMCs). Additionally, the top abundant proteins of each fraction were identified by a label-free quantification (LFQ) proteomics approach.

### F. hepatica adult ESP and fractions

Adult *F. hepatica* liver flukes and their excretory-secretory products (ESPs) were collected and cultured according to Rechsteiner et al. [15]. For further fractionation, ESP was concentrated and buffer exchanged with 25 mM ammonium acetate using an Amicon Ultra-15 filter unit with a MWCO of 3 kDa (UFC900308, Sigma-Aldrich) at 4°C. Protein concentration of the ESP was measured using the Pierce™ BCA Protein Assay Kit (#23225, Thermo Fisher Scientific). ESP was fractionated by gel filtration on a FPLC machine (Äkta™ Pure, Cytiva) using a HiPrep 16/60 Sephacryl S-300 HR column in 25 mM ammonium acetate (flow rate 1 mL/min for 1.5 column volume) and fractions of 3 mL were collected.

Protein concentration of each fraction was estimated by absorption at OD280 nm from the readout of the FPLC machine. Seven peak-, and 5 intermediated plateau peak fractions were collected (see Fig. 1) and illustrated by SDS-PAGE and silver staining (Pierce™ Silver Stain kit, #24612, Thermo Fisher Scientific). The gel filtration was repeated reproducible with different batches of ESP (figure in “Additional file 1”). The protein concentration of the fractions was determined by using the Pierce™ Micro BCA Protein Assay Kit (#23235, Thermo Fisher Scientific). All fractions were kept at -80°C until further use.

# Serum samples

Serum samples from 60 well characterized cattle (18 months to 10 years of age) previously collected at Swiss abattoirs were selected [16]. These samples were divided into 5 groups based on their infection status. Group 1 consisted of 15 cattle negative for *F. hepatica* by slaughterhouse inspection of the liver, by analysing of the bile for liver flukes eggs, by a f2-ELISA (Fasciolosis Verification Test, IDEXX) for the detection of early acute infections > 2–4 weeks post infection [17, 18], and an *in house* ELISA based on adult *F. hepatica* ESP [19]. Group 2 to 4 consist of animals with chronic *F. hepatica* infections based on

positivity by all previously mentioned tests, whereas cattle in Group 2 presented 1–10 *F. hepatica* eggs/mL bile (N = 14), Group 3 with 11–50 eggs/mL bile (N = 15) and Group 4 with > 50 eggs/mL bile (N = 9). Group 5 consisted of animals acutely infected with *F. hepatica* liver migratory larvae (N = 7). These animals were collected by the absence of adult worms and parasite eggs, positive reaction in the f2- and ESP-ELISA. These samples were selected by the detection of circulating *F. hepatica* antigen in the serum based on a previously published sandwich-ELISA with a monoclonal antibody (coating) and a rabbit polyclonal serum (detection) against *F. hepatica* ESP [15]. The Group 1 animals all did not show any signal (OD > 0.1) in this test. Further, in 2 of the Group 5 animals, migratory larvae could be observed during liver inspection. All cattle were considered negative for other infections by slaughterhouse inspection and antibodies against *Neospora caninum* (Neospora Ab Test, IDEXX) and serology for bovine cysticercosis [16].

## Determination of the humoral immune response by ELISA

Antibody response against adult *F. hepatica* ESP and peak fractions from the gel filtration was analysed by ELISA performed according to Kronenberg et al. [20]. In brief, whole ESP and peak fractions were coated overnight (4°C in a wet chamber) on Maxisorp 96-well microtiter plates (Nunc®, Sigma-Aldrich) normalized by protein concentration of 0.25 µg/well. Following blocking with PBS containing 0.3% Tween20, 200mg/L $NaN_3$ and 0.05% bovine haemoglobin (H2625, Sigma-Aldrich), bovine serum samples from the 5 groups (see above) were used in a dilution of 1:200 in the blocking buffer.

To determine the overall humoral reaction to the different protein fractions, an unspecific goat anti-bovine immunoglobulin conjugated to alkaline phosphatase (#5220 – 0383, KPL) was applied and developed with 1 mg pNPP / mL 0.05M Na-carbonat-bicarbonat with 1mM $MgCl_2$ at pH 9.8. Absorbance at 405 nm was measured after 15 minutes with a Multiskan RC ELISA reader (Thermo Labsystems). To account for plate-to-plate and day-to-day variations, values were normalized to standard positive and negative control samples.

Next, specific isotypes were quantified in selected peak fractions by ELISA coated with 0.25 µg/well. Standard curves were generated on each plate from isotype series (1µg/ml to 0.125µg/ml) with purified bovine IgG1 (P10-116, Bethyl), IgG2 (P10-117, Bethyl) and IgM (001-0107, Rockland). Six samples from group 1 (uninfected), group 2 to 4 (infected with adult worms), and group 5 (infected with migratory larvae) were applied. As secondary antibody unspecific anti-bovine immunoglobulin (see above), sheep anti-bovine IgG1 (1:5000; A10-116AP, Bethyl), sheep anti-bovine IgG2 (1:5000; A10-117AP, Bethyl), and sheep anti-bovine IgM (1:5000; A10-101AP, Bethyl) were used. Isotype concentrations were quantified based on the standard curve and expressed as relative antibody reaction compared to the overall humoral response per applied protein fraction. Mean values of the replicates were used.

## Determination of the cellular immune response by stimulation of ovine PBMCs and RT-qPCR

Peripheral blood mononuclear cells (PBMCs) were isolated from blood by jugular venepuncture using lithium heparin-coated vacutainers from healthy sheep owned by the Institute of Parasitology from the University of Zurich. The PBMC isolation was done by density gradient centrifugation according to Chibssa et al. [21] with modifications. Briefly, fresh blood was mixed with PBS (1:2) and carefully layered over Histopaque® at a density 1.077 g/mL (10771, Sigma-Aldrich) and centrifuged at 400 x g for 30 minutes at room temperature with soft acceleration and deceleration. The buffy coat was collected and washed 3 times with PBS including a lysis of red blood cells with distilled water for 10 seconds. Subsequent, the cells were centrifuged at 300 x g for 10 min. at room temperature. The cells were manually counted, and viability was checked with Türk's solution before being frozen in freezing medium (90% FCS + 10% DMSO) at -80°C until further use.

PBMC stimulation assay was performed similar as described in Puech et al. [22]. In a total volume of 200 µL/well, 200,000 cells were cultured in RPMI 1640 (21875034, GibcoTM) containing 1% Penicillin/Streptomycin (Sigma-Aldrich, P0781) and 10% native foetal calf serum (2-01F10-I, Bioconcept). Following an overnight adaptation of the cells at 37°C and 5% $CO_2$, the cells were incubated with fresh medium containing 5 µg/mL of the peak fractions (previously determined to result in optimal cytokine secretion from PBMCs). Absence of significant endotoxin-levels (below detection level) was confirmed by Pierce™ Chromogenic Endotoxin Quant Kit (Thermo Fisher Scientific). As controls (viability and different cytokine profiles), PBMCs were incubated in parallel with 5µg/well Lipopolysaccharin (LPS; 00-4976, Thermo Fisher), 100ng/well Ionomycin (Sigma-Aldrich, 10634) and 10ng/well phorbol-12-myristate-13-acetate (PMA; 19–144 Sigma-Aldrich) or left unstimulated as a control. Cells were harvested after 24 hours (pools of 4-wells per condition) and performed in triplicates.

Total RNA isolation was carried out with Trizol (T9424, Sigma-Aldrich) according to the user's manual, with additional UltraPureTM Glykogen (1µg/µl, 10814010, Thermo Scientific) used as a carrier after elution. The RNA concentration was estimated by a Nanodrop™ 1000 Spectrophotometer (Thermo Scientific). The total RNA from each condition was reverse transcribed with Revert Aid H Minus Reverse Transcriptase (EP0452, Thermo Scientific) using the protocol for first strand cDNA synthesis and according to the manufacturer's instructions with slight modifications. 2µl of random nonamers (100µM; Microsynth), 6µl of nuclease free water (10977, Fisher Scientific) was added to 19µl of eluted total RNA, and samples were denatured at 65° for 5 minutes and subsequently chilled on ice. For each sample, the synthesis of cDNA was accomplished by adding a 8µl of 5x RT buffer (Super ScriptTM Ⅳ; Thermo Fisher), 4µl of 10mM dNTP (100µM; 02-21-00100, Solis BioDyne) and 1 µl of the transcriptase (see above). The samples were heated up to 25°C for 10 minutes, followed by 42°C for 60 minutes, 70°C for 10 minutes, and cooling to 4°C. cDNA samples were stored as multiple aliquots at -80°C until further use.

RT-qPCR was performed with predesigned primers based on available literature (Table 1)[21–24]. Cytokine profiling targeting a core panel for different immune response profiles with known importance in helminth infections were selected, with IFN-γ and IL-12p40 indicative for a type 1 and pro-inflammatory response, combination of IL-4, IL-5, IL-10 for a type 2 reaction, whereas IL-6 is a pleotropic

cytokine involved in inflammation and acute phase response, and IL-10 as a key regulatory / suppressive cytokine. PPIA gene was used as reference (housekeeping) gene.

Table 1
Primer sequences and annealing temperatures used for the qPCR.

| Gene | Primer sequence (5'–3') | Reference |
|---|---|---|
| IL-4 | F: CAGCATGGAGCTGCCT | [22] |
| | R: ACAGAACAGGTCTTGCTTGC | |
| IL-5 | F: CACTGCTCTCCACGCATCAA | [23] |
| | R: TCATCAAGTTCCCATCACCTATCA | |
| IL-10 | F: CTTTAAGGGTTACCTGGGTTGC | [22] |
| | R: CTCACTCATGGCTTTGTAGACAC | |
| IFN-γ | F: CAGAGCCAAATTGTCTCCTTC | [22] |
| | R: ATCCACCGGAATTTGAATCAG | |
| IL-12p40 | F: CAGCAGAGGCTCCTCTGAC | [22] |
| | R: GTCTGGTTTGATGATGTCCCTG | |
| IL-6 | F: CCGCTTCACAAGCGCCTTC | [21, 24] |
| | R: CCAGTGTCTCCTTGCTGCTT | |
| PPIA | F: TGACTTCACACGCCATAAT | [22] |
| | R: CTTGCCATCCAACCACTC | |
| F: forward, R: reverse | | |

The selection of primers allowed using a single cycling program for all the included genes with the annealing temperature in the range of 59–60°C. The experiments were run on QuantStudioTM 7 Flex Real-Time PCR System (4485701, Applied BiosystemsTM). Each reaction was carried out in a final volume of 15µl containing 3.5µl of undiluted cDNA, 1µl of each forward and reverse primer at the optimal concentration (300nM to 600nM), 2µl of water and 7.5µl of SYBR-Green (04913914001, Roche Diagnostics). The qPCR program consisted of an initial denaturation step at 95°C for 5 min., followed by 50 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 45 seconds at 72°C. The data was analysed using the relative expression of the gene of interest (average Ct-value) normalized with the expression of our reference housekeeping gene (PPIA), using the $2^{-\Delta\Delta Ct}$ indicated as the fold gene expression values for all individual samples according to Pfaffl [25].

## Enrichment and measurement of extracellular vesicles (EVs)

Similar as described above, ESP was concentrated in PBS, and the fractions were enriched for EVs after gel filtration. The peak fractions were centrifuged sequentially at 15,000 x g for 45 minutes to remove potential micro-vesicles in the pellet, followed by ultracentrifugation at 115,000 x g for 3 hours to obtain the EVs in the pellet. The size and concentration of the particles in the different fractions were measured by tunable resistive pulse sensing (TRPS; qNano, Izon) according to Eichenberger et al. [26] with a few adjustments. The calibration was performed using CPC100 carboxylated polystyrene calibration particles (Izon) at a 1:2,000 dilution and sample dilution of 1:20 in a 2xPBS solution.

## Identification of peak proteins by a quantitative proteomics approach

Peak protein fractions were further analysed by mass spectrometry. Efficient enzyme digestion of the protein prior to the LC-MS analysis was supported by an initial deglycosylation step using the Protein Deglycosylation Mix II (New England Biolabs, Ipswich, USA) according to the manufacturer's protocol. For mass spectrometry analysis, protein digestion was performed by SP3- assisted digest with carbamidomethylation [27] with an initial binding on carboxylated magnetic beads in 100% ethanol (v/v) for 30 min at 800 rpm in a ThermoMixer and tryptic digestion (Trypsin/Lys-C mix; Promega) in 10 mM Tris/2 mM CaCl2 (pH 8.2) in a 1:50 enzyme to protein ratio. 50 µg protein per fraction was applied in triplicates. Peptides were cleaned by inhouse produced C18 stage tip columns using C18 extraction disks (CDS Empore™; Thermo Fisher). Data-dependent analysis (DDA) was performed on an Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher) operated in line with a nanoACQUITY UHPLC M-class system (Waters).

Per sample, 2 µL was loaded and eluted by running a linear gradient from 5% to 32% solvent B over 90 min (solvent A:0.1% FA in water, solvent B:0.1% FA in ACN) at 300 nl/min. Peptides were ionised utilising a nano-electrospray ionisation (ESI) source (Digital PicoView 565, O/N: DPV-550-565, New Objective, Woburn, MA) and a 10-mm fused-silica spray tip emitter (New Objective, PN). MS1 scans covering 300-2,000 m/z were recorded in profile mode with a resolution of 120,000 using positive polarity and automated gain control (AGC) with a target value of 500,000 and a maximum injection time (maxIT) of 50 ms. Every full MS1 scan was followed by DDA scans recorded in centroid mode with a resolution of 30,000. The AGC target was set to 10,000 and the maxIT to 80 ms. Isolated precursors were fragmented with higher-energy collisional dissociation (HCD) at a normalised collision energy (NCE) of 35%. Fixed first mass was set to 140 m/z.

For protein identification and label-free quantification (LFQ) MaxQuant software (version 2.3.1.0 [28]) was used. The protein database was built with the *F. hepatica* gene models PRJNA179522 and PRJEB25283 retrieved from WormBase ParaSite (version: WBPS16 (WS279)) supplemented with common contaminants from an *in house* database substituted with the common repository of adventitious proteins (cRAP; https://www.thegpm.org/crap/). Carbamidomethylation of cysteines was set as fixed modification and methionine oxidation and deamidation of asparagine (N) was included as variable modification.

Only proteins identified with a minimum of two unique peptides and in all three replicates were included in the final list. Protein quantity values were based on mean LFQ intensities across the three replicates, represented as relative abundance within the corresponding peak fraction. Gene ontology mapping for functional annotation of proteins was done by Blast2GO in OmicsBox 3.5.3 [29]. A potential involvement of the *F. hepatica* protein hits in host-immune interaction was ranked by AI search using MS Copilot v.2.20260708.19.0 on GPT-5 model. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD083072 and doi 10.6019/PXD083072.

# Results

### Humoral immune reaction to adult F. hepatica ESP gel-filtration fractions

Adult *F. hepatica* ESP separated by gel-filtration show 7 protein peaks and 5 intermediated plateau areas (Fig. 1; “Additional file 1”). The gel filtration separation usually elutes a gradient from high to low molecular weight (MW) proteins, beginning with the high MW fractions. The humoral response to the different fractionated parasite proteins was explored with serum from different groups of cattle (Fig. 1). Overall, highest antibody reaction was seen in cows acutely infected with migrating *F. hepatica* larvae. Cows chronically infected with adult parasites, indicated by different numbers of eggs in the bile, showed similar responses, which are lower than in the acute infections. Uninfected animals show background signals, mainly against “high molecular weight” (MW) fractions. In general, it is noticeable that the reactions are more reactive in the “high MW” fractions, with the reactions flatten towards the “low MW” fractions. There is also little ELISA reaction to these “low MW” fractions, despite showing protein peaks.

By isotype-specific ELISAs, a type 2 reaction indicated by IgG1 and IgM was observed, independently of the protein fraction and the infection status – infections with larvae and adult parasites (Fig. 2). Uninfected animals react to certain protein fractions, mainly via IgM immunoglobulins. IgG2 reactions across all fractions are on average below 3.35% in cattle infected with adult parasites or larvae, but even in negative animals, the values never reached more than 8.9% of the observed isotype. The low MW fractions, especially those starting from fraction 34, show increased reactivity by IgM isotypes and less by IgG1, regardless of the infection status.

### Cytokine expression in ovine PBMCs following stimulation with different adult F. hepatica ESP gel-filtration fractions.

The mRNA expression levels of a Th1-type (IFN-γ and IL12p40) and a Th2-type (IL-4, IL-5, IL-6, IL-10) cytokines are presented in Fig. 3. Control reactions with LPS stimulation and Iono/PMA suppression display expected cytokine profiles. Stimulation of PBMCs with the entire adult *F. hepatica* ESP shows a typical immune reaction with helminths, representing a polarized Th2-dominant response, with tolerogenic immune cells (no or slight reaction compared to unstimulated cells) and IL-10 expression. By fractionating the ESP proteins, starting from Peak 2, a general immune cell suppression is presented indicated by a global downregulation of cytokine production. Proteins in Peak 6 seem to induce the

immune-suppressive IL-10 expression, while other cytokines are not affected or slightly downregulated. Only Peak 1 proteins show a distinct profile by stimulating the expression of various cytokines.

Co-stimulation experiments with the peak-fractions and LPS or Ionomycin/PMA are summarized in “Additional file 2”. Adult *F. hepatica* ESP and Peak 1 proteins do not show any distinct cytokine profile compared to LPS alone. However, most of the other fractions can overrule and downregulate all cytokines but IL12-p40. Interestingly, peak 5 together with LPS induce IL-4 expression. On the other side, all fractions show different cytokine profiles by co-stimulation with Ionomycin/PMA.

## Analysis of the presence of extracellular vesicles (EVs)

The presence of EVs were analysed due to a distinct immune activation profile of the Peak 1 fraction (see before), and “high-MW” fractions are expected to contain *F. hepatica* EVs [30]. The analysis of the fractions by TRPS revealed that only Peak 1 contained EVs and bigger particles (“Additional file 3”). The median particle size was 89 nm (mean size: 111 nm; SD ± 43.3) with a maximum particle diameter of 528 nm and minimum of 65nm.

## Proteomics characterisation of peak protein fractions

Across all fractions (peak and intermediate plateau fractions) 172 proteins could be identified (table in “Additional file 4”). The identified proteins were analysed for known involvement in parasite-host immune interactions. Many proteins are housekeeping, transport, cytoskeletal, or metabolic proteins for which there is currently no convincing evidence of a direct role in host immune modulation. However, key proteins with such previously reported involvement in host-immune modulation are Cathepsin L1, Cathepsin B/B5, Fatty acid binding protein, Cystatin A (Stefin A), Serpin 4 (serine protease inhibitors), Glutathione transferase, Kunitz protein 8, Myoglobin 2, and Kininogen-1/Bradykinin. Further, many common extracellular vesicle proteins could be identified in the Peak 1 fraction, such as proteins involved in EV-biogenesis (e.g. tetraspanin, rab/ras), structural proteins (tubulin), and known EV-cargo proteins (e.g. annexin, serpin, HSPs, GST, SAP3, enolase). Top abundant hits per peak fraction are shown in Table 2.

Table 2

List of proteins identified by a label-free quantitative (LFQ) proteomics approach in the 7 peak fractions. Only top 5 protein hits (if available) are noted. The entire list is provided in “Additional file 4”.

| Functional description (B2GO) | Relative abundance [%] | Mean LFQ signal intensity | Known immunomodulator |
|---|---|---|---|
| **Peak 1 fraction** | | | |
| Tetraspanin | 8.30 | 1.794.E + 08 | |
| SAP-3 | 7.20 | 1.555.E + 08 | YES |
| Multidrug resistance protein 1B (ABCB1 / P-glycoprotein 1 / CD243) | 5.33 | 1.151.E + 08 | |
| Acid sphingomyelinase phosphodiesterase 3a | 5.16 | 1.114.E + 08 | |
| AQP-1 | 4.59 | 9.909.E + 07 | |
| **Peak 2 fraction** | | | |
| Lysosomal Pro-X carboxypeptidase | 51.04 | 2.926.E + 08 | |
| Dipeptidyl peptidase 2 | 19.81 | 1.135.E + 08 | |
| Fructose-bisphosphate aldolase | 8.19 | 4.693.E + 07 | |
| MF6p protein | 6.82 | 3.907.E + 07 | YES |
| Secreted Saposin-like Protein (SAP-3) | 2.12 | 1.214.E + 07 | YES |
| **Peak 3 fraction** | | | |
| Fatty acid binding protein (FABP) | 86.29 | 3.01E + 08 | YES |
| Myoglobin 2 | 6.47 | 2.255.E + 07 | YES |
| Glutathione transferase sigma class | 5.07 | 1.767.E + 07 | YES |
| Cystatin A (Stefin A) | 0.87 | 3.043.E + 06 | YES |
| Serpin 4 | 0.85 | 2.973.E + 06 | YES |
| **Peak 4 fraction** | | | |
| Cathepsin L1 | 98.99 | 1.564.E + 08 | YES |
| Thioredoxin | 1.01 | 1.588.E + 06 | YES |
| **Peak 5 fraction** | | | |
| Cathepsin L1 (L1D) | 78.56 | 1.76E + 08 | YES |
| Kunitz-type protein 8 | 19.37 | 4.330.E + 07 | YES |

| Functional description (B2GO) | Relative abundance [%] | Mean LFQ signal intensity | Known immunomodulator |
|---|---|---|---|
| **Peak 1 fraction** | | | |
| Heat Shock Protein (HSP) 75 (TNFR-associated protein) | 2.08 | 4.645.E + 06 | |
| **Peak 6 fraction** | | | |
| Cathepsin L1 | 90.97 | 3.22E + 08 | YES |
| Kunitz protein 8 | 5.37 | 1.904.E + 07 | YES |
| Heat Shock Protein (HSP) 75 (TNFR-associated protein) | 1.00 | 3.549.E + 06 | |
| Secreted Saposin-like Protein (SAP-3) | 0.87 | 3.096.E + 06 | YES |
| Actin alpha cardiac muscle 1 | 0.70 | 2.490.E + 06 | |
| **Peak 7 fraction** | | | |
| YTH domain-containing protein 1 | 36.28 | 1.897.E + 07 | |
| NAD(P) transhydrogenase | 13.92 | 7.279.E + 06 | |
| U4/U6.U5 tri-snRNP-associated protein 2 | 11.35 | 5.936.E + 06 | |
| Fras1 extracellular matrix protein | 10.33 | 5.401.E + 06 | |
| Nipped-B-like protein (Delangin / SCC2 homolog) | 7.41 | 3.876.E + 06 | |

# Discussion

Central to *F. hepatica*'s ability to infect and sustain a chronic infection is the release of a complex mixture of excretory–secretory products (ESP) that redirects the ruminant immune response towards a non-protective type-2/regulatory phenotype and dampens innate and adaptive effector arms [7, 11]. Most functional studies to date have addressed either total ESP or individual recombinant proteins, leaving a gap between the bulk activity of the secretome and its constituent parts. This work bridged this gap by fractionating adult *F. hepatica* ESP by gel filtration and characterising each fraction with three complementary readouts: bovine humoral responses across defined infection stages, ovine peripheral blood mononuclear cell (PBMC) cytokine expression, and label-free quantitative proteomics. Thereby, a catalogue of parasite–host immune interactions have been generated in which adversely reactive parasite proteins can be prioritised for further functional work (diagnostics, vaccine and immunotherapeutics).

By gel filtration, adult ESP resolved into seven protein peaks and five intermediate plateaus. Sera from cattle harbouring migrating larvae reacted more strongly than sera from chronically infected animals and

were strongly concentrated on high-molecular-weight (MW) fractions, with only background responses in uninfected controls. Isotype ELISAs showed a uniform pattern across fractions and infection stages: dominant IgG1 and IgM and consistently low IgG2. Whole ESP elicited the expected polarised type-2/tolerogenic cytokine profile in ovine PBMCs, but fractionation resolved this composite response into three sub-signatures: Peak 1 broadly stimulated cytokine expression and was the only fraction containing extracellular vesicles (EVs); Peak 6 selectively induced IL-10; and the remaining peaks produced a global cytokine suppression that in most cases overrode LPS co-stimulation. Proteomics identified 172 proteins including previously described immunomodulators, including cathepsin L1, cathepsin B/B5, fatty acid binding protein (FABP), cystatin (stefin A), serpin 4, glutathione transferase (GST), Kunitz-type protein 8, myoglobin 2 and kininogen-1/bradykinin [7, 31, 32].

Chronicity reflects long host–parasite co-evolution, in which the fluke actively triggers non-protective responses that permit survival for years [6, 7]. Consistent with this, the total humoral response was already attenuated in animals with adult flukes relative to those with migrating larvae, and previous cattle trials show no correlation between antibody titre and protection, underscoring the need to distinguish immuno-dominant from subdominant or cryptic antigens [33, 34]. The high-MW-biased high reaction seen in uninfected controls is consistent with helminth-ESP-induced hypergammaglobulinaemia and helminth cross-reactive glycan epitopes rather than parasite-specific memory [6, 35].

The uniform IgG1/IgM-dominated, IgG2-poor isotype pattern indicates that the type-2-biased humoral environment is a global property of adult *F. hepatica* ESP, in agreement with kinetic studies where IgG1 was dominant, IgM earliest and IgG2 weak or delayed [35–38]. Importantly, Mulcahy et al. [34] reported that cathepsin L2/haemoglobin immunisation induced a qualitatively different, IgG2-associated response correlating with high protection, indicating that a Th1-type IgG2 arm should be actively promoted by a rational fluke vaccine. The stronger acute-phase reactivity against high-MW fractions is compatible with evidence that immature liver-stage flukes secrete immunomodulatory FABPs, helminth defence molecules and cathepsin peptidases involved in tissue destruction and immune evasion [32, 39].

At the cellular level, whole ESP produced a canonical polarised type-2/regulatory response in ovine PBMCs, consistent with the established view of *F. hepatica* ESP as tolerogenic [11, 40–42] and with reports that *F. hepatica* glycoconjugates modulate DC maturation and suppress IFN-γ [43]. An active inhibition of a Th1 immune response and thus suppression of IFN-γ cytokine production via MAPK signalling in dendritic cells by tegumental proteins, which can be shed in the ESP, was previously demonstrated [44]. We did not, however, see induction of the classical Th2 cytokines but rather broad downregulation, matching reports that *F. hepatica* establishes a tolerogenic state within hours of infection, with newly excysted juveniles (NEJ) inducing this state in under 24 h [39, 45]. Species differences matter: in sheep, IL-10 signalling and Th2 activation are upregulated only in the chronic phase whereas in cattle they are already active in the acute phase, contributing to the more severe acute signs of ovine fasciolosis [46, 47]. Both species share eosinophilia and IgG1 dominance [35, 48, 49], and

within hours of infection the parasite drives alternative macrophage activation, attributed to secreted peroxiredoxin [50].

Fractionation showed that the polarised whole-ESP signal is the emergent output of at least three arms of the secretome. Peak 1 was the only fraction that broadly stimulated cytokine expression, consistent with the capacity of parasite EVs to deliver a multiplexed cargo of glycoproteins, enzymes and non-coding RNAs [30, 51, 52]. Peak 6 selectively induced IL-10, indicative of a discrete regulatory activity that biases the response towards tolerance. Peaks 2–5 produced broad cytokine downregulation that persisted with LPS co-stimulation, indicating dominant suppression. The proteomics catalogue provides molecular correlates: cathepsin L1 and GST activate a suppressive DC phenotype attenuating Th17 but not inducing Th2 [53]; Fh12/FABP induces alternative macrophage activation, elevates IL-10 and suppresses TNF-α, IL-12 and IL-1β via TLR4 [54]; recombinant FhCystatin suppresses NO, IL-6 and TNF-α while promoting TGF-β and IL-10 [40, 55]; and a Kunitz-type molecule prints tolerogenic features onto DCs following LPS activation [42]. The distribution of these molecules across the low- and mid-MW peaks provides a coherent molecular basis for the observed phenotypes. Limiting, gel filtration separates by hydrodynamic size, not purity, so each peak may contain co-eluting proteins, having an impact on the outcome of the immunological experiments. The 172 proteins identified is fewer than in deep EV proteomes of *F. hepatica* [32, 56], so low-abundance or membrane-embedded EV components are likely under-represented.

EV recovery in the high-MW fraction aligns with the *F. hepatica* EV literature. Cwiklinski et al. [30] showed that adult *F. hepatica* releases at least two EV subpopulations differing in size, cargo and site of release, with cargo developmentally regulated across life-cycle stages. The size range observed here (median 89 nm; 65–528 nm) is consistent with a mixture of these subpopulations, and the stimulatory profile of Peak 1 matches the phenotype reported by Murphy et al. [56] for FhEVs, which suppress T-cell IL-2. Hookworm EVs show a closely analogous effect, suppressing colitogenic cytokines and driving marked IL-10 in inducible colitis [26]. *F. hepatica* EVs are also enriched in immuno-regulatory microRNAs not captured by proteomics [57], and helminth EVs can confer protective immunity when used as vaccines [58, 59]. Our findings diverge from the literature only in the magnitude of Th2 induction attributable to individual soluble molecules: recombinant cathepsin L1 and GST do not drive Th2 in isolation but suppress Th17 via DCs [53]. No single peak reproduced the polarised type-2 profile of whole ESP; the "Th2 signature" is better described as a composite of soluble-molecule suppression, IL-10 induction and EV-mediated multiplex stimulation. The unique contribution of this work is the catalogue itself; a fraction-based immune-readout followed by mapping the protein-identity, that separates soluble from EV-mediated immunomodulation within one dataset.

The data support a layered model of *F. hepatica* immune evasion in which soluble suppressors (Peaks 2–5), a discrete IL-10 activity (Peak 6) and vesicle-mediated multiplex signalling (Peak 1) operate in parallel [30], reconciling chronic infection with a vigorous but non-protective humoral response [33, 34]. For diagnostics and vaccine development, the fraction-level view argues against single-antigen approaches, as naturally infected animals often poorly recognise recombinant cathepsin L1 alone [60].

The catalogue overlaps with lead vaccine candidates: rFhLAP achieves up to ~ 87% reduction in fluke burden in sheep [61]; FhSAP-2 confers ~ 81% reduction in fluke burden and > 83% reduction in faecal eggs in rabbits [62]; combined cathepsin/haemoglobin vaccination reaches > 70% protection in cattle with an atypical IgG2/IgG1 profile [33, 34]. A commercially viable vaccine requires sustained efficacy of ≈ 80% with the NEJ as the priority target [8]. The catalogue also offers rational candidates for helminth-derived immunotherapeutics in allergic and inflammatory disease, already validated for whole ESP in allergic asthma [63].

# Conclusions

Fractionation of adult *F. hepatica* ESP with parallel bovine humoral profiling, ovine PBMC cytokine analysis and label-free proteomics converted the bulk immunomodulatory activity of the secretome into a resolved catalogue linking specific molecular families to specific host effector phenotypes. The type-2-biased, IgG1/IgM-dominated, IgG2-poor humoral response was a global property of adult ESP and strongest in animals with migrating larvae, consistent with the non-protective type-2/regulatory bias previously reported for *F. hepatica* infection [33–35]. The composite tolerogenic profile of whole ESP resolved into three arms: vesicle-associated multiplex stimulation confined to the EV-containing Peak 1, a discrete IL-10-inducing activity in Peak 6, and dominant, LPS-overriding cytokine suppression across Peaks 2–5, each with plausible molecular correlates in the proteomic catalogue [30, 42, 54, 55]. These findings support a layered model of *F. hepatica* immune evasion, argues against single-antigen strategies, and argue that future vaccine efforts should focus on migration- and immune-evasion-related NEJ proteins and on adjuvant/route combinations that elicit a protection-associated mixed IgG1/IgG2 response [8, 34]. The catalogue provides a rational basis for the selection of diagnostic antigen combinations and vaccine and immunotherapeutic candidates in fasciolosis and offers a transferable methodological framework for other veterinary and human helminth pathogens.

# Abbreviations

ELISA
enzyme-linked immunosorbent assay
ESP
excretory secretory products
EV
extracellular vesicle
FABP
fatty acid binding protein:FPLC:fast protein liquid chromatography
GST
glutathione S-transferase:LC-MS:liquid chromatography-mass spectrometry
LFQ
label-free quantification
MW

molecular weight
PBMC
Peripheral blood mononuclear cells
PBS
phosphate-buffered saline
RT-qPCR
reverse transcription quantitative polymerase chain reaction
SDS-PAGE
sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TRPS
tunable resistive pulse sensing.

# Declarations

### Ethics approval and consent to participate

The collection of blood from healthy sheep was approved by Cantonal Veterinary Office of Zurich (permission number ZH150/18). The sampling was conducted in accordance with the Swiss animal rights and regulation standards.

### Consent for publication

Not applicable.

### Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. Mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD083072 and doi 10.6019/PXD083072.

### Competing interests

The authors declare no competing interests.

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### Authors’ contributions

Conceptualisation: PD, RME; Methodology: SB, RME; Validation: SB, RME; Formal analysis: SB, RME; Investigation: SB, SH, RME; Resources: PD, RME; Data Curation: SB, RME; Visualisation: SB, RME; Writing – original draft: SB, RME; Writing –Review and Editing: SB, SH, PD, RME; Supervision: RME; Project Administration: RME; Funding acquisition: PD, RME.

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# Figures

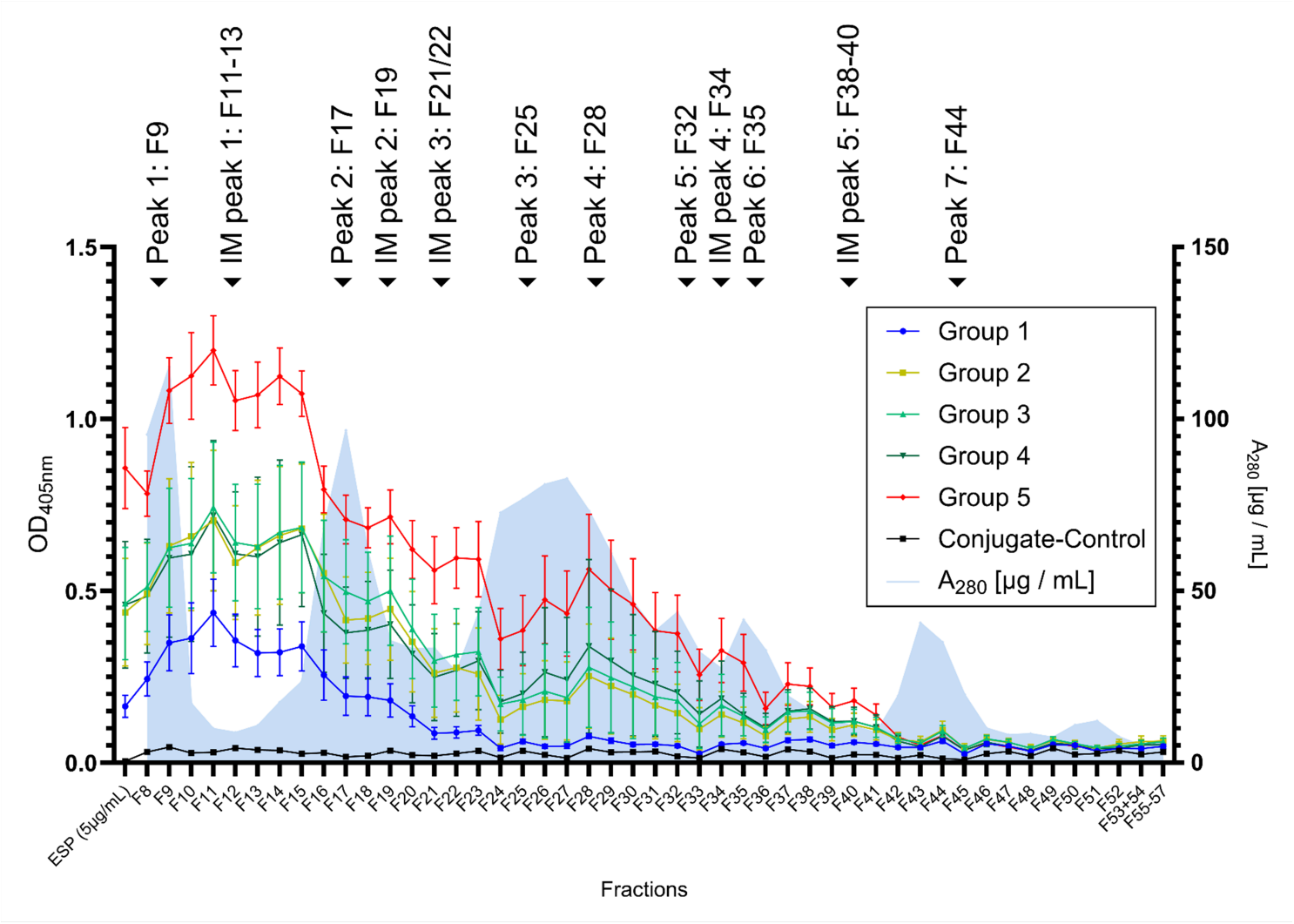



**Figure 1**

**Gel-filtration of adult *F. hepatica* ESP.**Chromatogram and different collected fractions are presented. Protein concentration of the different fractions by absorption at 280nm is shown in light blue. The reactivity (optical density; OD) to all fractions (F) have been evaluated by ELISA using sera from different groups of slaughtered cattle: Group 1: uninfected animals; Group 2-4: cows with adult *F. hepatica*worms as indicated by liver inspection and various numbers of parasite eggs in the bile; group 5: cows with liver-migratory larvae. A secondary antibody (conjugate) control was included (black line). Various protein peaks and intermediate (IM) plateau peaks have been identified and used in further approaches. Dots and error bars represent mean values and 95% confident intervals.

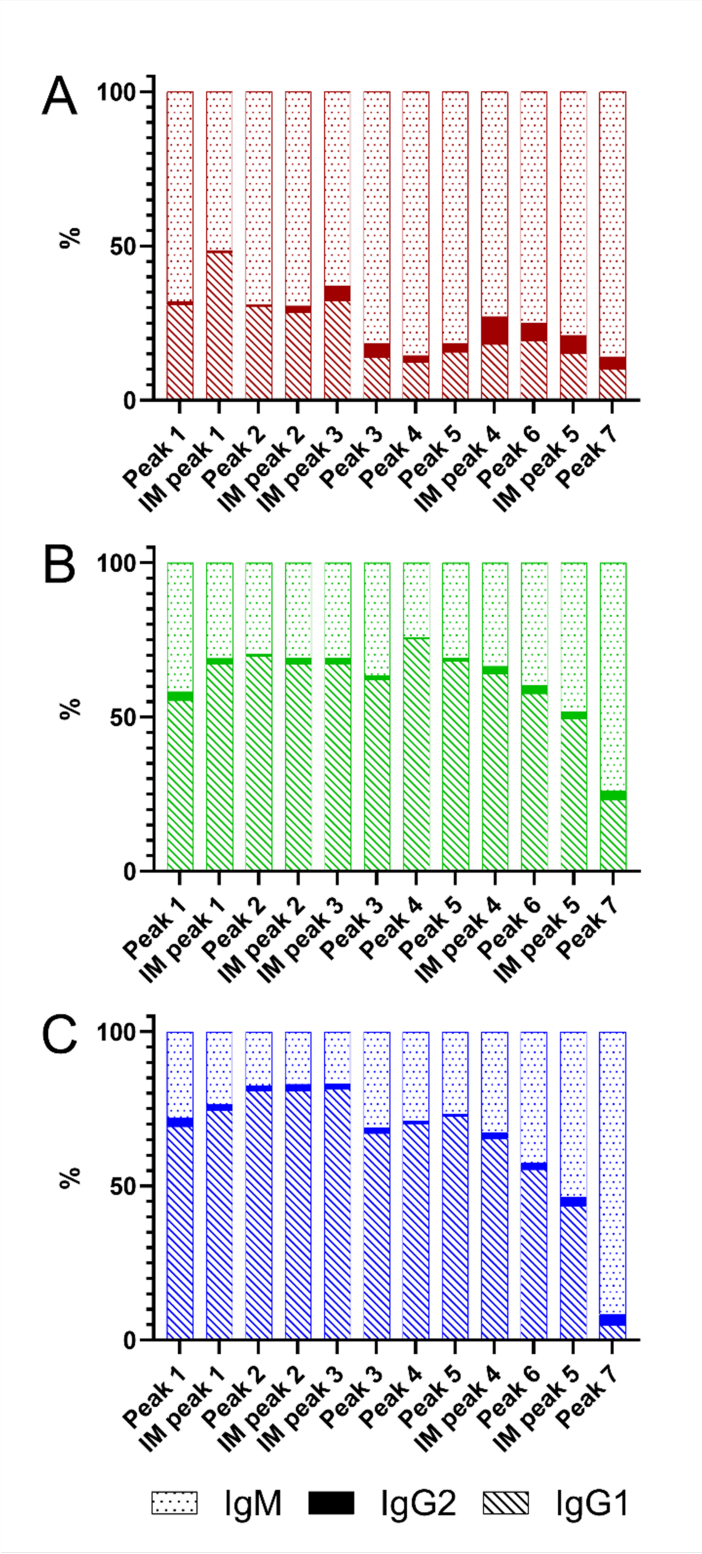



**Figure 2**

**Specific bovine antibody isotypes against *F. hepatica* fractionated proteins.**Relative abundance of specific bovine isotypes (IgM, IgG1 and IgG2) against gel-filtrated ESP peak and intermediated (IM) plateau peak fractions. Reaction of (A) uninfected animals (Group 1), (B) animals with adult *F. hepatica*worms (Group 2 to 4), and (C) animals with *F. hepatica* liver migratory larvae (Group 5).

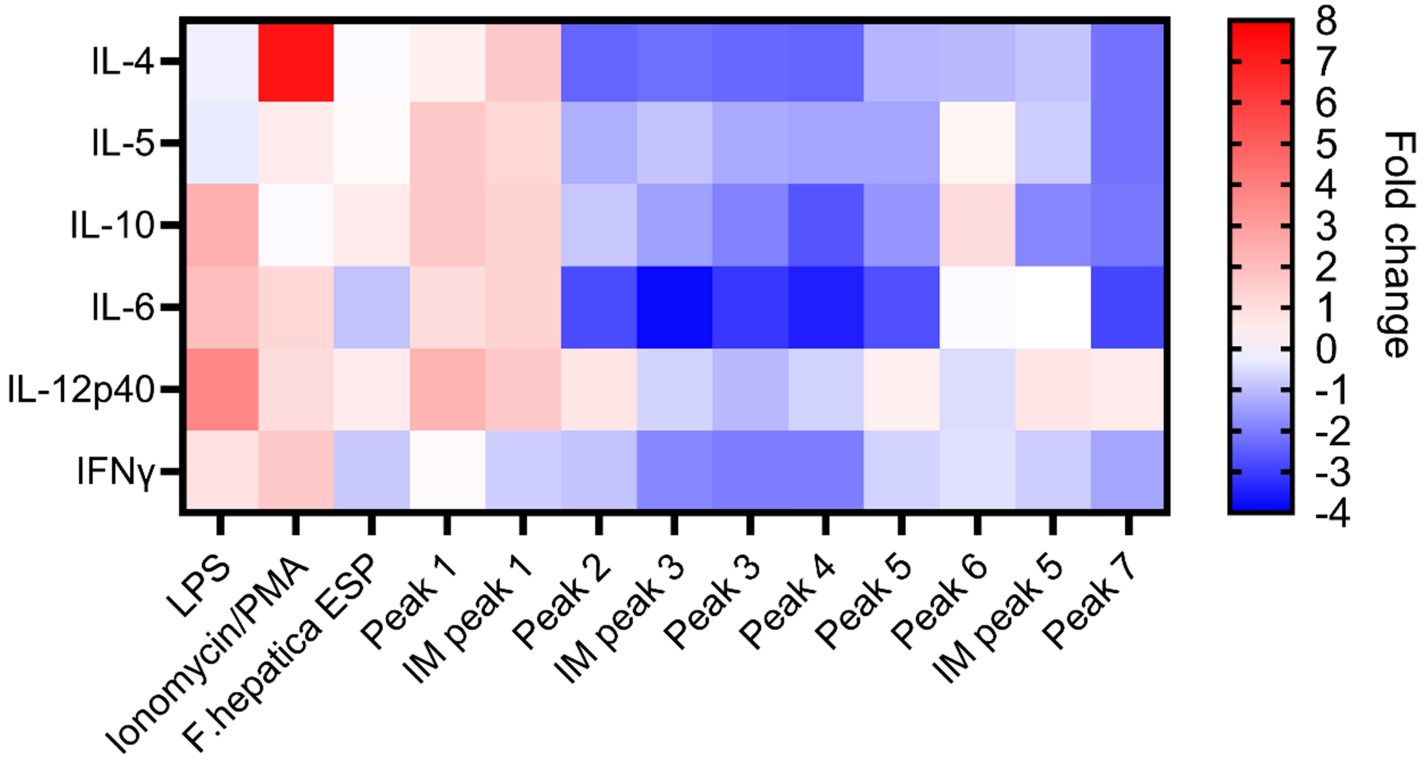



**Figure 3**

**Cytokine gene expression in ovine peripheral blood mononuclear cells (PBMCs) after exposure to adult *F. hepatica* ESP and peak fractions.** Fold change in expression levels of selected cytokines (IL-4, IL-5 and IL-10 indicative for a type 2-, IL-12p40 and IFN-γ for a type 1 response, and IL-6 as a general marker inflammation and an acute phase response). mRNA expression was normalized to the housekeeping reference gene PPIA and in comparison with unstimulated cells.

## Supplementary Files

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