

Excretory/secretory products from two *Fasciola hepatica* isolates induce different transcriptional changes and IL-10 release in LPS-activated bovine “BOMA” macrophages

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Abstract *Fasciola hepatica* are trematodes that reside in the bile ducts of mammals. Infection causes US\$3 billion in losses annually in animal production and is considered a zoonosis of growing importance. An under-represented area in *F. hepatica* research has been the examination of the different immunomodulatory abilities of various parasite isolates on the host immune system. In this paper, this issue was explored, with the bovine macrophage cell line “BOMA”. The cells were matured by LPS treatment and stimulated with excretory/secretory antigens (ES) from two *Fasciola hepatica* isolates: a laboratory isolate “Weybridge” (*Fh*-WeyES) and a wild isolate (*Fh*-WildES). As expected, stimulation with antigen mixtures with highly similar compositions resulted in mild transcriptomic differences. However, there were significant differences in cytokine levels. Compared to *Fh*-WeyES, exposure to *Fh*-WildES upregulated 27 and downregulated 30

genes. *Fh*-ES from both isolates diminished the release of TNF- α , whereas only *Fh*-WildES decreased IL-10 secretion. Neither *Fh*-WeyES nor *Fh*-WildES had an impact on IL-12 release. Our results indicate that various isolates can have different immunomodulatory abilities and impacts on the bovine immune system.

Keywords *Fasciola hepatica* · Liver fluke · Parasite isolate · Immune response · Helminths

Introduction

Fasciola hepatica (liver fluke) is a parasite that infects humans and other mammals. Adult flukes live in the bile ducts where they feed on host blood. Released eggs hatch in the environment and undergo multiple stages inside and outside the intermediate snail host, before infecting mammals to complete the parasites’ life cycle. Infection results in liver damage, and decreased weight gain, milk production and fertility which causes loss in animal production estimated at over US\$3 billion annually (Cwiklinski et al. 2016). Despite numerous trials, an effective vaccine has not yet been developed (Toet et al. 2014). To date, infection is controlled pharmacologically mainly through the use of triclabendazole (TCBZ); however, the development of drug resistance among fluke isolates indicates new approaches may be necessary in the future (Fairweather, 2011). Besides drug resistance, other phenotypic differences have been described between *F. hepatica* isolates, including in the dynamics of development outside the definitive host, with the number of cercariae released by snails and metacercariae viability differing between compared isolates (Januszkiewicz et al. 2015; Walker et al. 2006). Taking into account these differences, it is

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reasonable to hypothesize that other phenotypes between isolates may also be distinct, including those important for parasite survival such as immunomodulatory abilities.

F. hepatica actively releases a number of antigens into the surrounding environment. Among *F. hepatica* excretory/secretory (*Fh*-ES) products are antigens that act as potent immunomodulatory molecules which are accountable for suppressing the host immune system. These molecules may shift dendritic cells towards a tolerogenic phenotype (Falcón et al. 2010), induce apoptosis in macrophages (Guasconi et al. 2012) and eosinophils (Serradell et al. 2007), and stimulate macrophages to produce regulatory cytokines (Guasconi et al. 2011). Although the immunomodulatory actions of *Fh*-ES have been explored, work needs to be extended to examine immunomodulatory differences between various fluke isolates; there is a paucity of such data not only regarding liver fluke but also other helminths. The only robust results come from mouse whipworm (*Trichuris muris*) and show that various parasite isolates manipulate Th₁ and Th₂ responses in different manners, which affects clinical signs of infection (Klementowicz et al. 2012). Taking into account the diversity of *F. hepatica* isolates (Fairweather, 2011), it is reasonable to hypothesize that immunomodulatory differences occur among the isolates. The application of new techniques in the *-omics* era allows for the identification of isolate phenotypes and a deeper comprehension of host–parasite interactions.

The objective of the present study was to investigate if different *F. hepatica* isolates are able to differentially influence the immune response generated by the host. This was achieved by stimulating lipopolysaccharide (LPS)-activated bovine monocyte/macrophage “BOMA” cells with *Fh*-ES from the *F. hepatica* Weybridge isolate (*Fh*-WeyES) and a wild isolate (*Fh*-WildES). Upon stimulation with *Fh*-ES from both isolates, genes expressed distinctly between treatment groups and differences in the release of certain cytokines (TNF- α , IL-10, and IL-12) were determined. The results clearly showed that the macrophages responded distinctly upon stimulation with antigens released by the two *F. hepatica* isolates. Noticeable differences in gene expression profiles and levels of released cytokines between groups indicate different immunomodulatory properties for the two *F. hepatica* isolates.

Materials and methods

Collection of *F. hepatica* excretory/secretory products

F. hepatica adult ES proteins were collected from living parasites freshly isolated from the main bile ducts of sheep (*Fh*-WeyES) or bison (*Fh*-WildES). The CVL Weybridge isolate has been maintained in our laboratory since 2003 through experimental infections of sheep and *Galba truncatula* snails.

For this study, flukes were collected after experimental infection of Merino sheep. A number of bison have been culled at Białowieża National Park (Poland) in order to maintain the population at an appropriate level, as well as to remove old and sick animals from the group. After necropsy of several of these animals, adult *F. hepatica* parasites were obtained.

The flukes were washed in PBS, and incubated for 2 h at 37 °C, 5% CO₂, to ensure host proteins were expelled, and then the media were discarded. Flukes were then incubated in RPMI-1640 with 100 units/ml of penicillin and 0.1 mg/ml streptomycin for a further 24 h, with media changed every 4 h. After each incubation, medium was collected, centrifuged at 4500×g for 20 min at 4 °C to remove any insoluble material, then concentrated using a Centricon device with a 3-kDa cut-off (EMD Millipore). The samples were aliquoted and stored at –70 °C, and passed through a 0.2- μ m filter prior to use. Protein concentrations of samples were determined by Bradford assay.

Stimulation of LPS-activated BOMA cells with *F. hepatica* excretory/secretory products

A previously established laboratory model using a bovine monocyte/macrophage cell line (BOMA) was used (Başka et al. 2013). The cells were cultivated in DMEM supplemented with 5% FBS, 2 mM L-glutamine and penicillin/streptomycin (100 units/ml and 100 μ g/ml, respectively). Media, FBS, and antibiotics were purchased from Sigma-Aldrich. During cell passage, trypsin (Sigma-Aldrich) was used to detach cells from the flask surface. When cells reached an appropriate number they were seeded into 24-well plates at a concentration of 6.4×10^5 cells/ml, activated with 8.9 μ g/ml of LPS (from *Escherichia coli* O127:B8, Sigma-Aldrich) and left for 60 min at 37 °C, 5% CO₂ to adhere. Cells were stimulated in quadruplicate with *Fh*-WeyES or *Fh*-WildES at a final concentration of 10 μ g/ml. Four wells of cells were not stimulated and were treated as controls. To minimize differences in conditions between stimulations, stimulated and control cells were seeded on one plate. The cells were stimulated for 26 h, followed by cell scraping and centrifugation. Cells and media were stored separately at –70 and –20 °C, respectively.

Microarrays and data analyses

Total RNA was isolated from cells using a total RNA isolation kit (A&A Biotechnology), followed by removal of contaminating genomic DNA using DNaseI (Thermo Scientific) according to the manufacturer’s protocol. RNA was purified using phenol-chloroform extraction. The concentration of the RNA was determined spectrophotometrically, followed by assessing its quality using a bioanalyzer (Agilent Technologies), then labeling with

Cy3 (control cells) and Cy5 (stimulated cells) using Quick Amp Labeling (Agilent Technologies). Further steps of the microarray experiment were performed according to the manufacturer's instructions (Agilent Technologies).

The statistical analyses were performed using GeneSpring (Agilent Technologies). All eight microarrays were analyzed collectively. Samples were filtered on flags following creation of a baseline based on the median of all samples. A moderated *T* test followed by Westfall-Young permutative correction was applied to determine messenger RNAs (mRNAs) that changed expression upon *Fh*-WildES treatment (compared to *Fh*-WeyES treated cells—where exposure to ES from the Weybridge laboratory strain was considered as the control). mRNA expression changes of at least twofold ($p < 0.05$) were considered as significant. Biological processes affected by the identified genes were identified using PANTHER (Mi et al. 2016) and the DAVID Classification System (Huang da et al. 2009). Other genes likely to be engaged in the immune response and not identified by PANTHER or DAVID were identified by examination of gene lists and a literature review.

ELISA

The concentrations of cytokines in collected media were determined using kits: TNF- α (R&D Systems), IL-10 (TSZ ELISA), and IL-12 (TSZ ELISA). Statistical analyses were performed using Student's *t* test with STATISTICA 10 software.

Results

Microarrays

Fh-WildES induced changes in expression at 80 probes; 38 and 42 probes detected up- and downregulation (compared to *Fh*-WeyES), respectively, identifying 27 upregulated and 30 downregulated genes (supplementary material). Analysis with PANTHER revealed that the identified genes are engaged in various biological processes (Fig. 1). Further inspection of the microarray results revealed that 10 upregulated and 11 downregulated genes are involved in immune responses. The genes were further analyzed by literature review to discover their potential functions during fasciolosis. Potential effects on the induced bovine immune response by the *Fh*-ES of both isolates were hypothesized (Table 1). Although these effects were not confirmed experimentally, the data shed light on the molecular effects and immune response outcomes induced by both liver fluke isolates.

ELISA

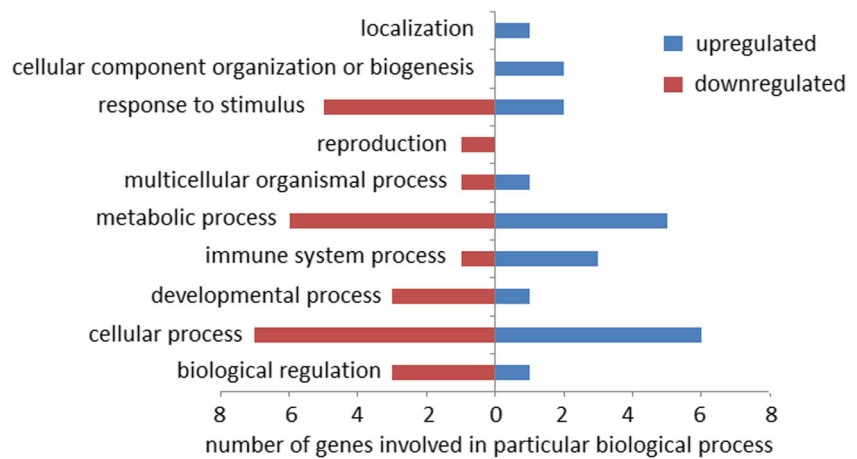
Both *Fh*-WeyES and *Fh*-WildES induced diminished release of TNF- α (31 and 19%, respectively) (Fig. 2a). *Fh*-WildES dampened the release of IL-10 by 42%, whereas no influence on IL-10 secretion was noted after stimulation with *Fh*-WeyES (Fig. 2b). The basal level of IL-12 released by LPS-activated BOMA cells was undetectable and neither *Fh*-WeyES nor *Fh*-WildES increased the release of this cytokine (data not shown).

Discussion

Investigations into the differences among *F. hepatica* isolates have previously focused on diagnosis and drug resistance, aspects of significance for clinical practice (Fairweather, 2011). A number of isolates have developed resistance to anthelmintics, and it is reasonable to assume other features of flukes have also differentiated. *F. hepatica* isolates live in various hosts (e.g., cattle, sheep, bison, and humans), so we hypothesized that the immunomodulatory abilities of isolates may have evolved and that the *Fh*-ES from various isolates could affect immune systems distinctly. Here, we chose to investigate the interaction of *Fh*-ES with bovine macrophages, since infection of cattle causes significant economic loss. Transcriptomic analysis and measurement of the release of certain cytokines corroborate the raised hypothesis and provide insight into the interactions between *F. hepatica* isolates and bovine macrophages.

Upon stimulation with *Fh*-WildES or *Fh*-WeyES, LPS-activated bovine macrophages differ in the expression of 57 genes (of which 21 are engaged in immune responses). Whether 57 and 21 represent a large or small number of genes remains unclear due to a paucity of appropriate data for comparison among helminths. Some previous analyses of transcriptomic responses to *F. hepatica* have been performed (Fu et al. 2016; Rojas-Caraballo et al. 2015; Wesolowska et al. 2013); however, the use of different hosts (mice, rats, or sheep), cell types (hepatocytes, lymph nodes, or PBMC), parasite isolates, or previous exposure to vaccine antigens complicates and limits comparison with our results. This means there is no identified “reference number” of genes that differ in expression upon stimulation with antigens released by distinct *F. hepatica* isolates. However, findings from the protozoan parasites *Toxoplasma gondii* and *Trypanosoma brucei* may be helpful; two distinct isolates may affect expression of 310 to 920 mRNAs (Hill et al. 2012; Morrison et al. 2010). Unfortunately, direct comparison with our results are hindered since the aforementioned experiments were performed with peritoneal cells (Hill et al. 2012) or splenocytes (Morrison et al. 2010) which both consist of

Fig. 1 Number of genes involved in particular biological processes revealed by PANTHER



a number of cell populations (e.g., dendritic cells, T cells, B cells, and other immune cells) with each population possibly reacting distinctly. Thus, the lower number of genes—57—with altered regulation following exposure to ES from different *F. hepatica* isolates may result from either the fact that we investigated only one cell population—macrophages, or that the examined liver fluke isolates are relatively similar and do not affect the gene expression pattern in immune cells as much as protozoans.

F. hepatica infection typically results in modulation towards Th₂/T_{reg} host immune response phenotypes, which includes alternative activation of macrophages and downregulation of certain inflammation pathways. Analysis of the genomic data confirms the hypothesis that different *F. hepatica* isolates have different immunomodulatory abilities. The two fluke isolates affected in a different manner the expression of genes associated with potent orchestrators of the immune response—NF- κ B and MAPKs, as well as mRNAs encoding cytokines, chemokines, and other proteins associated with immune responses. NF- κ B is a profound enhancer of the immune response. This transcription factor is present in the cytoplasm attached to its inhibitor—I κ B. Upon I κ B phosphorylation (through activated IKK) the inhibitor is ubiquitinated and then degrades in the proteasome; released NF- κ B enters the nucleus, binds to DNA, and regulates transcription. Four out of five of the identified genes associated with NF- κ B favor its inhibition, indicating a more substantial dampening of the immune response by *Fh*-WildES. Upregulation of both *Lrrc14* and *TEK* and downregulation of both *NME9* and *KCNMA1* would therefore be expected to lead to a reduced inflammation response (Lu et al., 2015; Wu et al., 2016), along with reduced macrophage activity (Gu et al., 2010), reduced TNF- α levels (Papavlassopoulos et al. 2006), and cell migration inhibition (Gu et al., 2010). Alternatively, upregulation of *IKK2*, which encodes a kinase that activates NF- κ B through inhibitor phosphorylation, is likely to promote NF- κ B activity and the immune response (Pannicke et al., 2013). The other intracellular signaling pathway distinctly affected by *Fh*-WeyES and *Fh*-

WildES is associated with MAP kinases. *Fh*-WildES upregulates *DUSP13*, which inactivates profound mediators of cell signaling: JNK, p38, and ERK kinases as well as transcription factor AP-1, which may lead to a dampened inflammation process (Yang et al. 2014). On the other hand, inactivation of p38 and ERK by increased *DUSP13* expression would explain the lower IL-10 release upon cell stimulation with *Fh*-WildES compared to *Fh*-WeyES (Risco et al. 2012). Treatment with *Fh*-WildES leads to comparatively reduced complement activity by decreasing *C3* expression, while also increasing aspects of the *C3a* pathway through upregulation of *KLK14* and *C3AR1* (involved in *C3a* synthesis and its signal transduction, respectively). These results indicate certain isolates may be more effective at directing macrophage activation as well as modulating certain inflammatory responses, which would have implications for both innate and adaptive immunity against *F. hepatica* infection and responses to bystander antigens. Isolate dependent differences in complement activity may also explain the discrepancies in infection susceptibility observed with certain bacteria and liver fluke coinfections (Brady et al. 1999; Flynn et al. 2009).

In contrast to suppression of the immune response through negative regulation of NF- κ B, TGF- β 3 downregulation by *Fh*-WildES suggests a stronger proinflammatory potential for this isolate. TGF- β is upregulated during the first weeks of infection followed by downregulation during the chronic phase (Flynn and Mulcahy 2008a). Moreover, animals harboring heavy *F. hepatica* infections express less TGF- β 1 compared to those suffering from mild infections (Haçariz et al. 2009), suggesting adult flukes may reduce TGF- β expression. It should be noted that the mentioned studies (Flynn and Mulcahy 2008a; Haçariz et al. 2009) measured TGF- β 1 expression and our results showed decreased TGF- β 3, so some discrepancies may be physiological. Nevertheless, the discussed data indicate that adult flukes have a lower potential to induce TGF- β than immature ones. The other two genes with distinct expression levels are *IL36RN* and *GM-CSF*. *IL36RN* downregulation amplifies the biological effects of

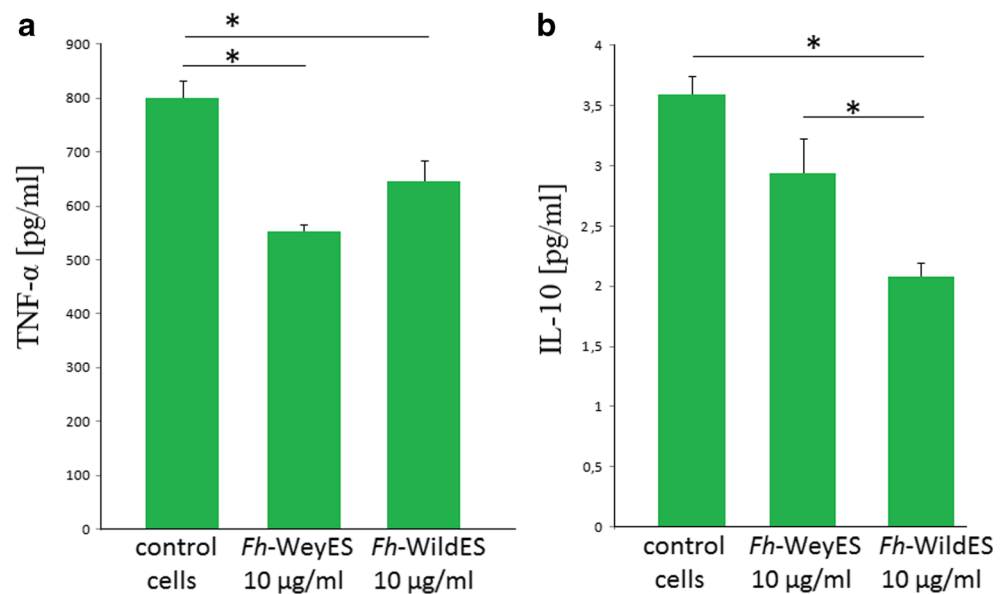
Table 1 Genes which expression changed upon *Fh*-WildES stimulation compared to *Fh*-WeyES in BOMA macrophages

Gene upregulated	GenBank no.	Predicted influence on immune response after expression change
Dual specificity phosphatase 13 (DUSP13)	DV931475	Decrease
Folate receptor 2 (fetal) (FOLR2)	NM_001075325	Increase
Complement component 3a receptor 1 (C3AR1)	NM_001083752	Increase
Leucine-rich repeat-containing 14 (LRRC14)	NM_001102379	Decrease
Inhibitor of kappa light polypeptide gene enhancer in B cells, kinase beta (IKK2)	NM_174353	Increase
Kallikrein-related peptidase 14 (KLK14)	XM_010815249	Increase
TEK receptor tyrosine kinase (TEK)	NM_173964	Decrease
Granzyme B (granzyme 2, cytotoxic T lymphocyte-associated serine esterase 1) (GZMB)	NM_174296	Increase
Colony-stimulating factor 2 (GM-CSF)	NM_174027	Increase
T cell activation inhibitor, mitochondrial (TCAIM)	NM_001102016	Decrease
Gene downregulated	GenBank No.	Predicted influence on immune response after expression change
Recombination activating gene 1 (RAG1)	XM_010812795	Decrease
Interleukin 36 receptor antagonist (IL-36RN)	NM_001075775	Increase
Beta-1,3-glucuronyltransferase 1 (B3GAT1)	NM_001014393	Complicated
Transforming growth factor beta 3 (TGF- β 3)	NM_001101183	Increase
NME/NM23 family member 9 (NME9)	NM_001075615	Decrease
Glia maturation factor, gamma (GMFG)	CK772108	Complicated
Potassium channel, calcium-activated large conductance subfamily M alpha, member 1 (KCNMA1)	NM_174680	Decrease
Complement component 3 (C3)	NM_001040469	Decrease
Olfactory receptor 1030 (LOC539064)	XM_002693111	Complicated
Olfactory receptor, family 10, subfamily J, member 5 (OR10J5)	XM_002685900	Complicated
Olfactory receptor 51V1-like (LOC789134)	XM_003583137	Complicated

proinflammatory IL-36 (Towne et al. 2011) while GM-CSF upregulation enhances other arms of the proinflammatory

response, i.e., TLR-4 (Bozinovski et al. 2004) expression and TNF- α release (Scian et al. 2011). These effects are in

Fig. 2 Level of TNF- α (a) and IL-10 (b) after stimulation of LPS-activated BOMA cells with *Fh*-WeyES and *Fh*-WildES. $p < 0.05$ is indicated with asterisk. Control cells—BOMA macrophages stimulated with LPS. *Fh*-WeyES and *Fh*-WildES—BOMA macrophages activated with LPS and stimulated with Weybridge and wild strain ES, respectively



concordance with TGF- β downregulation by *Fh*-WildES, corroborating its greater proinflammatory properties compared to *Fh*-WeyES.

A similar conclusion, that of the stronger proinflammatory potential of the wild isolate, can be drawn from the lower levels of IL-10 released by the cells upon *Fh*-WildES stimulation, whereas *Fh*-WeyES did not affect the cytokine's release. Contrary to our results, Flynn and Mulcahy (2008b) showed increased IL-10 levels in infected cattle; however, the authors investigated PBMCs (containing monocytes and various lymphocyte populations), so cells other than monocytes/macrophages may have released increased levels of IL-10 (Brown et al. 1994), swamping any decreases in IL-10 production that may have been induced in monocytes or macrophages. Moreover, the results we achieved (diminished IL-10 release) are in accordance with the fact that dendritic (Hamilton et al. 2009) and mast cells (Vukman et al. 2013) treated with *F. hepatica* proteins and LPS also show decreased levels of released IL-10. Our findings combined with those of others give insights into previously raised question about the source of IL-10 in PBMC from infected cattle (Flynn and Mulcahy (2008a), as with the exclusion of monocytes/macrophages, dendritic, and mast cells the most probable IL-10 producers are lymphocytes. Both *Fh*-WeyES and *Fh*-WildES dampen the release of TNF- α , consistent with other reports of lower TNF- α release by *F. hepatica* exposed monocytes (Garza-Cuartero et al. (2016). While TNF- α levels were lower following stimulation with *Fh*-WeyES compared to *Fh*-WildES, levels were not significantly different between populations ($p = 0.74$). *F. hepatica* skews the host immune response towards a Th₂ phenotype, and while they suppress resistance to *Bordetella pertusis* (Brady et al. 1999), this does not affect susceptibility to certain other bacteria susceptible to a Th₁ response, such as *Mycobacterium bovis* (Flynn et al. 2009) or *Salmonella dublin* (Naranjo Lucena et al. 2017). Our findings revealing *F. hepatica* intraspecies differences in immunomodulatory properties may bring some insights into this mentioned discrepancy. It raises the possibility that the different responses to *B. pertusis*, *S. dublin*, and *M. bovis* in *F. hepatica* co-infected animals may have developed (at least to some degree) due to infection with fluke isolates showing distinct impacts on the immune system. However, a full explanation requires further research. The findings may also be relevant for future *F. hepatica* investigations, in particular, those investigating fluke interference with the host immune system. While experiments with flukes collected from animals from slaughterhouses still provide valuable and significant sources of data, in the future, they may require validation using characterized fluke isolates. This is extremely important especially during experiments with practical implications where *F. hepatica* is tested as a cure for autoimmune diseases like type 1 diabetes (Lund et al. 2014) or autoimmune encephalomyelitis (Walsh et al. 2009). Based on our findings such

experiments demand the use of a characterized *F. hepatica* isolate, or maintenance of field isolates allowing for future characterization. Not preserving fluke strains used for studies risks the loss of isolates that show desirable immunomodulatory properties, e.g., mitigating allergy or autoimmune disease symptoms.

Conclusions

The findings show that different *F. hepatica* isolates have various immunomodulatory abilities. Taking into account the potential diversity of isolates in the environment, this has implications for the interpretation of certain published results. Moreover, it is almost certain that the impact of intraspecies differences on host immune systems have not been adequately considered not only for liver fluke, but also other helminths. Our next major task is to combine the outlined findings with proteomic analysis of *Fh*-ES from different isolates to identify which antigens are most likely to be responsible for any differences observed. With the publication of these findings and the techniques now available, the authors feel that isolate differences and their effects on host immunity will be further explored, leading to a better understanding of host–parasite interactions, with benefits for the treatment of allergies and autoimmune diseases through the identification of suitable antigens not only among helminth species but also among isolates with the most desirable immunomodulatory abilities.

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Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

Ethics statement All experiments with animals followed institutional ethical guidelines and were approved by the Third Local Ethical Committee of Warsaw University of Life Sciences – SGGW (Permission no. 82/2010).

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