Fasciola hepatica isolates induce different immune responses in unmaturated bovine macrophages

Piotr Bąska[1], Anna Zawistowska-Deniziak[2], Luke James Norbury[2],
Marcin Wiśniewski[3], Kamil Januszkiewicz[4]

[1]Division of Pharmacology and Toxicology, Department of Preclinical Sciences,
[3]Division of Parasitology and Parasitic Diseases, Department of Preclinical Sciences,
Faculty of Veterinary Medicine, Warsaw University of Life Sciences, 02-786 Warsaw, Poland
[2]Witold Stefański Institute of Parasitology, Polish Academy of Sciences, 00-818 Warsaw, Poland
[4]Central Forensic Laboratory of the Police (CFLP), 00-583 Warsaw, Poland
piotr_baska@sggw.pl

Abstract

Introduction: *Fasciola hepatica* (liver fluke) is a parasite of great socioeconomic importance. A number of fluke isolates have been identified; however, to date the differences between the immunomodulatory properties of different parasite isolates have not been sufficiently investigated. The aim of this study was to explore differences between the immunomodulatory properties of two *F. hepatica* isolates using unmaturated bovine macrophages. **Material and Methods:** A cell line of bovine macrophages was stimulated with excretory/secretory products released by adult flukes from either a laboratory (*Fh*-WeyES) or wild (*Fh*-WildES) strain and subsequently subjected to microarray and ELISA analyses. **Results:** Both *Fh*-WeyES and *Fh*-WildES dampened the release of interleukin-10 by bovine macrophages, but only *Fh*-WildES dampened the release of proinflammatory tumour necrosis factor-α. Microarray analysis revealed that *Fh*-WildES down- and upregulated 90 and 18 genes, respectively, when compared to *Fh*-WeyES. **Conclusion:** The results indicated different impacts of the isolates on macrophages. A number of researchers use flukes obtained from local slaughterhouses for experiments. Our findings may explain some discrepancies between published results arising from parasite strain choice. The findings indicate that consideration should be given to the use of different strains, and open new and currently unexplored avenues in parasitology for controlling the parasite.

Keywords: *Fasciola hepatica*, macrophages, gene expression, immunoregulation, immune modulation.

Introduction

Fasciola hepatica (liver fluke) is a parasite of great socioeconomic importance, with a worldwide distribution. The parasite infects a variety of mammalian hosts and is responsible for significant losses in animal production estimated at over US$ 3.2 billion per year (21). Adult parasites settle in the bile ducts where they mature and cause long-lasting infections inflicting liver damage.

F. hepatica, like other helminths, has developed sophisticated mechanisms to trick, evade, and shift the host immune response. The fluke inhibits dendritic cells' maturation, induces the M2 phenotype in macrophages, and promotes IL-10 secretion. Many of these powerful immunomodulators are antigens actively released by the parasite as components of *F. hepatica* excretory/secretory products (*Fh*-ES). Despite reports of intraspecies differences among *F. hepatica* isolates, immunomodulatory properties have not been as thoroughly investigated as drug resistance (8).

We previously investigated the influence of *Fh*-ES from a laboratory Weybridge (*Fh*-WeyES) isolate and a wild (*Fh*-WildES) isolate strain on LPS-activated macrophages. Both isolates were able to dampen TNF-α release, whereas only the wild isolate dampened release of IL-10 from LPS-activated macrophages (2). For further characterisation of the immunomodulatory differences between isolates, we

stimulated a bovine monocyte/macrophage (BOMA) cell line with *Fh*-ES obtained from a commercially available Weybridge isolate of *F. hepatica* and *Fh*-ES from a wild isolate. Bovine macrophages were used since ruminants, the most economically important definitive hosts, are of special interest for drug and vaccine development. The paradigm of the immune response following exposure to the parasite is a shift towards a regulatory phenotype with diminishing proinflammatory effects. Despite human or mouse models usually being first choice in hepatic inflammation research, bovine cells have also shown increased release of IL-10 during chronic liver fluke infection (11). IL-12 and TNF-α are typical proinflammatory cytokines released by bovine cells upon *Mycoplasma bovis* stimulation (17) and greater attention to their regulation during fasciolosis is required. To uncover the different effects *Fh*-WeyE*S* and *Fh*-WildES have on macrophages, analyses of macrophage transcriptomes and levels of released IL-10, IL-12, and TNF-α were undertaken. Microarray analysis of gene expression is the first step to comprehending the intracellular pathways of signalling. Analyses of gene networks are complicated and computational technology is still being developed; however, the identified genes may be classified as modulators of the immune response, and the data may be very helpful in future meta-analyses. Transcriptome analyses combined with measurements of cytokine levels were applied here to shed light on the interactions between bovine macrophages and *F. hepatica*.

Material and Methods

***Fasciola hepatica* excretory–secretory (*Fh*-ES) products collection.** The CVL Weybridge isolate has been maintained in our laboratory since 2003 through experimental infections of sheep and *Galba truncatula* snails. Sheep were euthanised to collect adult flukes of the Weybridge isolate. Wild isolate flukes were collected from bison which were culled at Bialowieża National Park (Poland) in order to maintain the population in good condition. *Fh*-ES were collected as described previously (1). Briefly, adult flukes were collected and rinsed in PBS and then incubated (2 h, 37°C). Media containing residues of host proteins and bile were discarded. Flukes were then incubated (2 h, 37°C, 5% CO_2) in Roswell Park Memorial Institute-1640 medium (supplemented with 100 U/mL of penicillin and 0.1 mg/mL of streptomycin) for 24 h, with 4-hourly changes. Six batches of collected media (containing *Fh*-WeyES or *Fh*-WildES) were combined, spun (4,500 × *g*, 20 min, 4°C), and concentrated using a Centricon device with a 3 kDa cut-off (EMD Millipore, Ireland). The samples containing *Fh*-WeyES or *Fh*-WildES were titrated and stored at −80°C. Before

cell stimulation, the samples were passed through a 0.2 µm filter. The protein concentrations of the samples were determined by Bradford assay.

BOMA cell stimulation with *Fasciola hepatica* excretory – secretory (*Fh*-ES) products. The bovine monocyte/macrophage cell line was propagated as previously described (3). Briefly, cells were cultured in Dulbecco's modified Eagle's medium (with 5% FCS, 2 mM of glutamine, 100 units/mL of penicillin, 100 µg/mL of streptomycin); during the experiment the cells were seeded into 24-well dishes at a concentration of 6.4×10^5 cells/mL and stimulated in quadruplicate with *Fh*-WeyES or *Fh*-WildES at a final concentration of 10 µg/mL for 24 h, scraped and centrifuged. Unstimulated cells were treated as controls. Cells and media were stored separately at −70°C and −20°C, respectively.

Microarray experiments. Total RNA was isolated from both stimulated and control cells using a total RNA isolation kit (A&A Biotechnology, Poland). The genomic DNA residues were removed using DNAseI (Thermo Scientific, Lithuania) according to the manufacturer`s protocol, followed by phenol-chloroform extraction. The concentration of the RNA was determined spectrophotometrically. RNA from control and stimulated cells was labelled using Cy3 and Cy5, respectively, using a Quick Amp labelling kit (Agilent Technologies, USA). The further steps of the microarray experiment were performed according to the manufacturer`s instructions (Agilent Technologies).

Data analyses. The statistical analysis was performed using GeneSpring software (Agilent Technologies). Eight microarrays were analysed together. A baseline was created using the median of all samples, followed by filtering on flags to identify mRNAs that changed expression after *Fh*-WildES stimulation (compared to cells stimulated with *Fh*-WeyES – the laboratory strain was adopted as the control). Moderated *t*-tests (P < 0.05) were applied and Westfall–Young permutative correction was undertaken. The results were filtered, and probes indicating expression changes over two-fold were used for further analyses.

The involvement of the identified genes in various biological processes was determined using PANTHER (21) and the DAVID classification system (16). Other genes that may be involved in the immune response were added by examination of gene lists that exhibited changed expression and that were not classified by PANTHER or DAVID as participants in the immune response.

ELISA. Collected media were tested for the presence of TNF-α (Bovine TNF-α DuoSet ELISA, R&D Systems, USA), IL-10 (BioTSZ, USA), and IL-12 (BioTSZ, USA). Statistical analyses were performed using Student's *t*-test with STATISTICA 10 software.

Combining genomic data with release of IL-10 and TNF-α. To assess whether transcriptomic changes affected cytokine expression, computational analyses were performed. Using GeneMANIA (22), potential functional interactions and coexpression between genes were identified. Due to the lack of appropriate databases for cattle, mouse, rat, or human databases were used.

Results

Fh-ES from reference and wild isolates of F. hepatica induce different gene expression profiles. After stimulation of cells with Fh-WildES, 30 probes identified mRNAs as upregulated and 131 as downregulated compared to gene expression after Fh-WeyES stimulation (full list in supplement 1). These probes identified 18 and 90 mRNAs, respectively, which were deemed to be involved in various biological processes (Fig. 1).

Upon further literature review 3 upregulated and 22 downregulated mRNAs were identified as genes involved in immune responses (Table 1). The genes involved in immune responses were comprehensively analysed, and their potential functions during fasciolosis are hypothesised. Each one's influence on the immune response (associated with their expression change) during an infection was hypothesised and classed as an „increase", „decrease", „unknown" or „complicated" (Table 1). Although these effects are only hypotheses, they indicate the potential influence and outcomes of Fh-ES from both strains on the immune system.

ES from laboratory and wild isolates of F. hepatica decreased IL-10 secretion, whereas only ES from the wild isolate decreased TNF-α secretion. After stimulation of BOMA cells with Fh-WildES and Fh-WeyES, IL-10 levels decreased by 38% and 41%, respectively (Fig. 2A). Stimulation with Fh-WildES resulted in a 19% reduction of TNF-α, whereas no effect was observed from Fh-WeyES stimulation (Fig. 2B). The basal level of IL-12 secreted by BOMA cells was below the detection threshold, and stimulation with Fh-WildES or Fh-WeyES did not induce release of the cytokine (data not shown).

Genes with altered expression show association with TNF-α and IL-10 levels. Upon analysis of genes engaged in immune responses, six gene products showed interactions with TNF-α or IL-10, whereas two gene products showed the same expression localisation as the cytokines (Fig. 3). Moreover, a number of interactions were detected among the gene products engaged in immune responses (Fig. 3), as well as with related genes (Fig. 4).

When analysing all genes with altered expression with TNF-α and IL-10, TNF-α was revealed to interact with ACTA1 (actin, alpha 1, skeletal muscle), CDH13 (cadherin 13), and PIWIL2 (piwi-like RNA-mediated gene silencing 2), and IL-10 with CNIH3 (cornichon family AMPA receptor auxiliary protein 3). All identified interactions are shown in supplement 2.
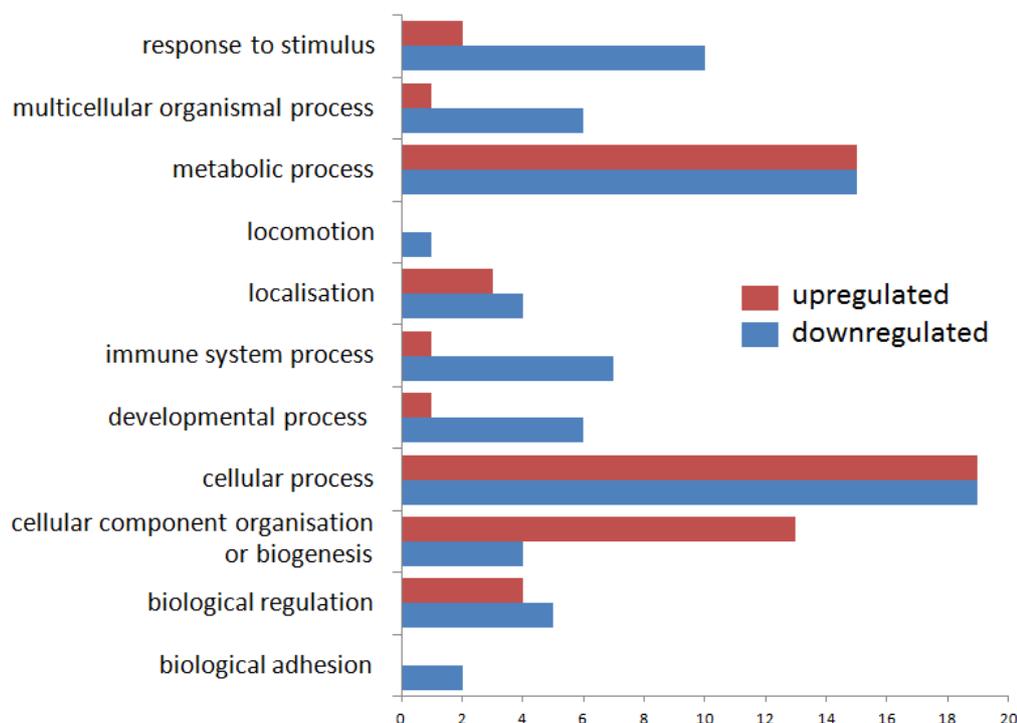

Fig. 1. Genes involved in biological processes identified by PANTHER

Table 1. Genes (involved in immune response) with changed expression level after macrophage stimulation with *Fh*-WildES (compared to *Fh*-WeyES)

Gene downregulated in macrophages stimulated with *Fh*-WildES compared to macrophages stimulated with *Fh*-WeyES	GenBank No.	Predicted influence on immune response after expression change
C-type lectin domain family 12 member A (CLEC12A)	NM_001105345	Complicated
Oligoadenylate synthetase 2 (OAS2)	NM_001024557	Decrease
C-type lectin domain family 4 member G (CLEC4G)	NM_001205606	Unknown
GTPase, IMAP family member 1 (GIMAP1)	NM_001083677	Decrease
Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, delta NFKBID)	NM_001193019	Increase
Cell division cycle 25A (CDC25A)$^{TNF-\alpha}$	NM_001101100	Decrease
Chemokine (C-X-C motif) ligand 2 (CXCL2)	NM_001048165	Decrease
Cytokine receptor-like factor 2 (TSLPR) $^{TNF-\alpha,\ IL-10}$	XM_010800619	Decrease
Glycerol-3-phosphate acyltransferase, mitochondrial (GPAM) $^{TNF-\alpha,\ IL-10}$	NM_001012282	Decrease
Coronin, actin binding protein 1A (CORO1A)	NM_174521	Decrease
Immunoglobulin superfamily member 8 (IGSF8)	NM_001082439	Complicated
Interferon regulatory factor 6 (IRF6)	NM_001076934	Increase
Inteferon omega 1 (IFN-ω1)	NM_174351	Decrease
Killer cell lectin-like receptor B1 (KLRB1)	NM_001206636	Complicated
Mitogen-activated protein kinase 13 (p38δ)	NM_001014947	Decrease
Thymus-specific serine protease (TSSP) $^{IL-10}$	BC148914	Decrease
Regulator of G-protein signalling 13 (RGS13)	NM_001080232	Increase
Transcription factor EC (TFEC) $^{TNF-\alpha,\ IL-10}$	NM_001083739	Complicated
T-cell receptor alpha chain V region (TRAδ)	JX101718	Decrease
CD63 molecule(CD63)	XM_002699657	Decrease
Complement component 3a receptor 1 (C3AR1)$^{TNF-\alpha,\ IL-10}$	NM_001083752	Decrease
Complement factor H (CFH)	NM_001033936	Increase
Gene upregulated in macrophages stimulated with *Fh*-WildES compared to macrophages stimulated with *Fh*-WeyES		
SAM and SH3 domain-containing 1 (SASH1)	XM_588302	Increase
Cathelicidin 2 (CATHL2)	NM_174826	Increase
Oncostatin M (OSM)	NM_175713	Complicated

Superscript shows genes which exhibited altered expression levels upon stimulation and directly interact with the cytokine TNF-α or IL-10
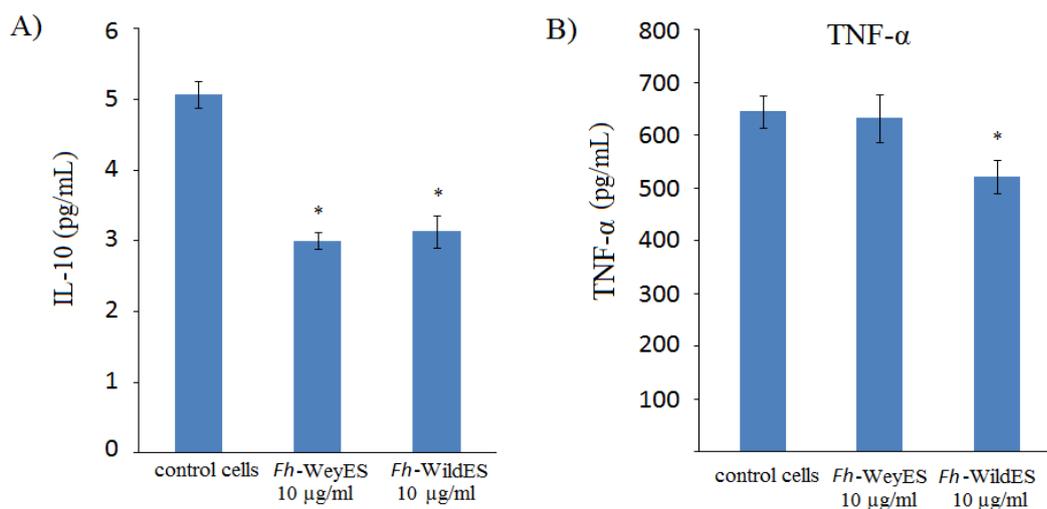

Fig. 2. Concentration of IL-10 (A) and TNF-α (B) after stimulation of bovine macrophages (BOMA) with *Fh*-WeyES or *Fh*-WildES. Each sample consisted of four replications. Mean and SEM are shown
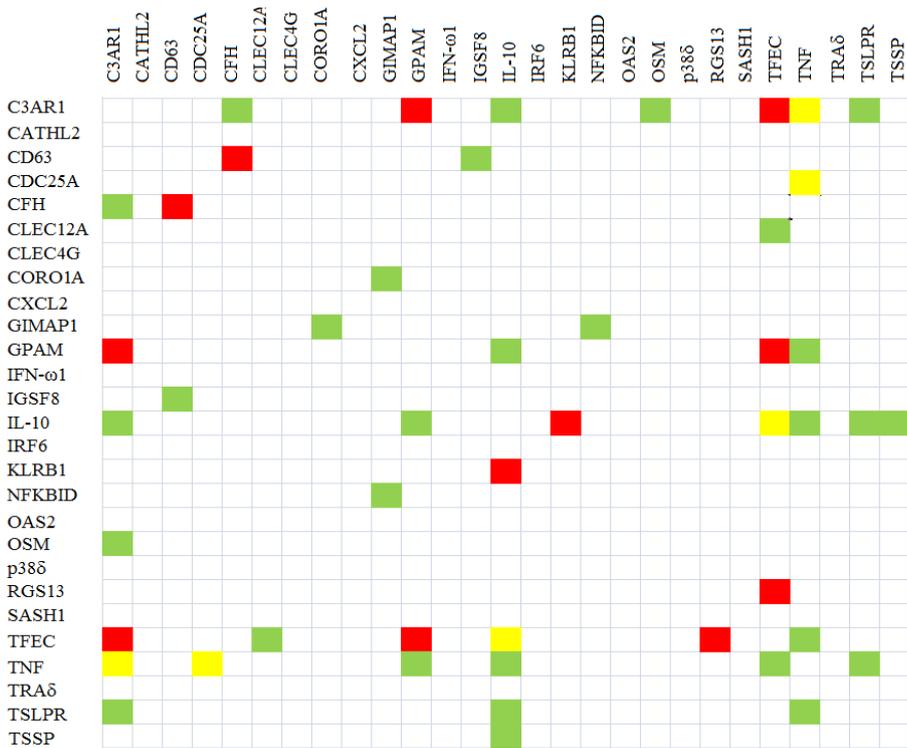* – significant difference compared to control cells (P < 0.05)

Fig. 3. Predicted interactions between TNF-α, IL-10, and products of genes with altered expression levels. Potential functional interactions (green) and joint expression location (red) were identified. If two genes were identified as having both functional and colocalised expression they were plotted with yellow
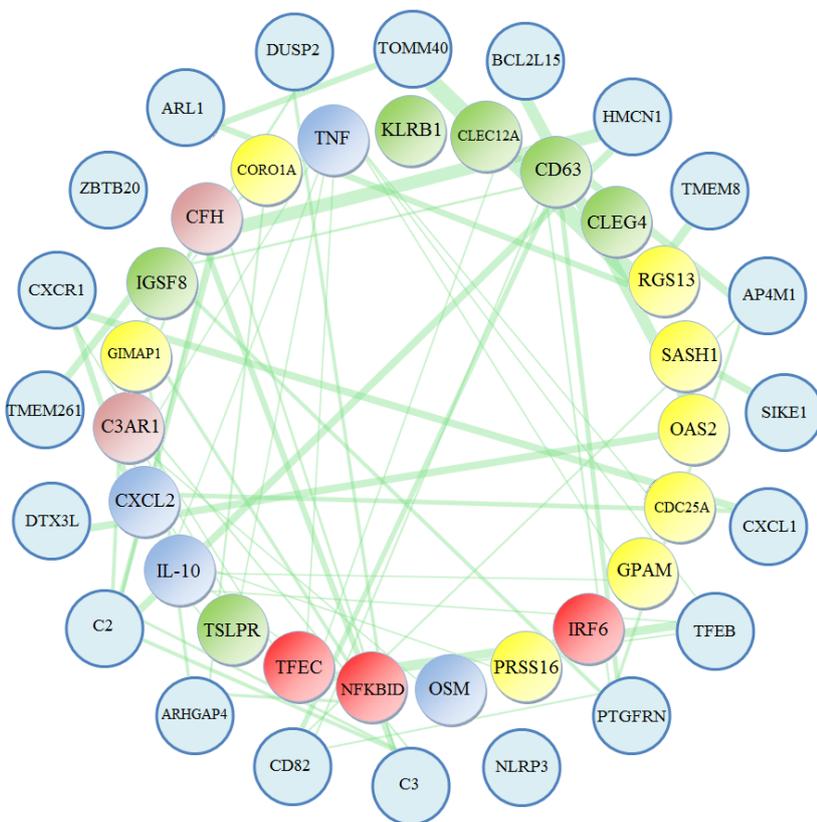

Fig. 4. Predicted functional interactions between genes engaged in immune responses and related genes. Green – membrane associated genes, violet – complement associated genes, blue – cytokines and chemokines, yellow – other. Outer genes (blue with dark outline) are related genes identified by GeneMania

Discussion

Fh-WildES up- and downregulated 18 and 90 mRNAs, respectively, compared to *Fh-Wey*ES (the *F. hepatica* laboratory strain considered as the control); these are comparable numbers to the 27 upregulated and 30 downregulated genes observed when LPS-activated BOMA cells were stimulated with *Fh*-WeyES and *Fh*-WildES (2). As it has been discussed previously (2), comparisons with other transcriptomic analyses of *F. hepatica* interactions with host cells are limited, as previous analyses only investigated the effects from one fluke isolate (12). However, similar findings come from analysis of the response to another helminth, *Trichuris muris* where two isolates induced differences in gene expression detected by 268 probes (7). Nevertheless, a direct comparison with these results is limited as we worked on a single cell population, while the *T. muris* experiments were performed on gut tissue which is comprised of a number of cell populations. A similar situation occurs when comparing isolates of protozoan parasites: *Toxoplasma gondii* and *Trypanosoma brucei* isolates have been shown to dysregulate 310 to 920 mRNAs (15, 23) in peritoneal cells (15) or splenocytes (23). Despite differences in study methodology, these findings when compared provide some clarity on the number of genes that may be dysregulated by distinct isolates of particular parasites.

Detailed microarray analysis showed that there were significant differences in gene expression levels in macrophages in response to *Fh*-WildES and *Fh*-WeyES, including to NF-κβ associated genes. Transcription factor NF-κβ stays inactive when coupled to its inhibitor, – IκB. Upon activation, IκB is phosphorylated, ubiquitinated, and targeted for proteasomal degradation, while free NF-κβ translocates into the nucleus where it modulates gene expression, orchestrating the immune response. Although *F. hepatica* tegumental antigens have been shown to decrease NF-κβ activity, these findings did not provide information about the strain of fluke (14). Our findings show that *Fh*-ES may significantly affect NF-κβ pathways. *Fh*-WildES boosts the immune response *via* NFKBID and IRF6 downregulation and SASH1 upregulation which may lead to improvement of NF-κβ signal transduction and induction of inflammation (6). Alternatively, CDC25 and CXCL2 were dampened by *Fh*-WildES, which skews the immune response towards suppression. While the overall effect on NF-κβ signalling is intricate, it indicates that different pathways are affected by the two isolates and in macrophages the induction of particular suppressive pathways may be compensated for by upregulation of certain proinflammatory pathways, *e.g.* through the dampened expression of other signalling-engaged genes. In regard to genes associated with other signalling pathways, *Fh*-WildES induced p38δ and downregulated GIMAP1, which clearly skews the

immune response towards a Th_reg/Th_2 phenotype through dampening of TNF-α release (26), whereas RGS13 downregulation by *Fh*-WildES favours the induction of inflammation. The effects of TFEC downregulation following *Fh*-WildES exposure are complicated since lower expression may result in impaired development of alternatively activated macrophages (30) which show a suppressive effect, but may, however, play a role in parasite expulsion. The results suggest that different pathways are affected to different degrees by various isolates. Different parasite isolates may influence different routes to achieve similar end results in immune regulation, while the degree to which different parasite isolates can depress certain protective immune responses, or induce non-protective/helpful immune responses also appears to differ.

Whereas dysregulation of genes involved in intracellular signalling pathways shows that both strains may skew the immune response towards either inflammation or suppression, changes to the expression of genes encoding membrane-associated and other intracellular proteins favour the hypothesis that the wild isolate induces stronger immunosuppression than the laboratory isolate. *Fh*-WildES diminished CD63, TSLPR, and TRAδ expression, which leads to impairment of mast cell degranulation (18) and antigen presentation (25). T cell functioning is also abrogated by TSSP, GPAM, and CORO1A downregulation, resulting in depleted T cell numbers, abrogation of receptor repertoire, and impaired IFN-γ release (5, 28). Similar effects may come from the dysregulation of the intercellular proteins TSSP and GPAM which regulate T cell function. The possibility of a stronger *Fh*-WildES suppressive effect also seems to be corroborated by OAS2 downregulation; its role in parasitic infection has not been confirmed, nevertheless its antiviral properties support the hypothesis that decreased expression would favour immune response inhibition. Other proteins associated with membranes (IGSF8, CLEC4G, KLRB1, and CLEC12A) were also expressed distinctly; however, their influence on the immune response against fluke is unresolved. Nevertheless, especially those containing C-type lectin domains (CLEC4G, KLRB1, and CLEC12A) may recognise *F. hepatica* antigens and affect the immune response (27). A complicated situation occurs also in regard to downregulation of OSM belonging to the IL-6 family. Downregulation of another cytokine, IFN-ω1, by *Fh*-WildES is likely to dampen the immune response. *Fh*-WildES also dampened expression of CFH and C3AR1, which may induce or suppress complement action by allowing its activation or reducing C3a signal transduction, respectively. Another secreted factor with altered expression was CATHL2, an antimicrobial peptide. Its immature form may restrain inflammation by inhibiting host cathepsin L engaged in elastinolytic and collagenolytic activities (29). This is significant not only in regard to the anti-

inflammatory effects, but also due to the abundance of cysteine proteases among antigens released by *F. hepatica* (24). The cysteine proteases of *F. hepatica* can cleave TLR3 and CD4, which inhibits the release of pro-inflammatory factors or affects T cell function and has also been shown to cleave host immunoglobulins (24). If CATHL2 inhibits liver fluke cysteine proteases, it may be an important as yet unexplored pathway of defence against fluke infection.

Computational analysis showed that six genes may have functional interactions with TNF-α and IL-10, influencing their expression levels and providing an insight into the mechanisms of change of cytokine levels. *Fh*-ES from both isolates dampened the release of immunosuppressive IL-10. However, a different expression pattern was observed when LPS-maturated macrophages were stimulated with *Fh*-ES from both isolates, with IL-10 levels inhibited more upon exposure to *Fh*-WildES (2). This difference may result from the different state of the macrophages (LPS-maturated or naive) which express different sets of receptors and show distinct responses to stimuli. Other findings show potentiated release of IL-10 during infection by peripheral blood mononuclear cells (11). However, macrophages alone from infected animals showed reduced ability to release IL-10; moreover, the reduction was most pronounced in the animals harbouring more flukes (13). This data may corroborate the hypothesis raised previously (2) that increased release of IL-10 by peripheral blood mononuclear cells is caused by cells other than monocytes/macrophages. Regarding TNF-α, *Fh*-WildES exposure led to diminished TNF-α release. The ability of *Fh*-ES to dampen TNF-α release is in concordance with our previous findings (2) and with the ability of fluke tegumental antigens to impair TNF-α release (14). It should be remembered that here cells were stimulated with *Fh*-ES from two isolates of one parasite species, which show a high degree of similarity, and only slight differences in ES composition or sequence variability among immunomodulatory proteins are likely to occur. Thus, while differences in the levels of the released cytokines were not large, nevertheless they did exist. The more potent immunosuppressive potential of the wild isolate may result from the greater genetic diversity of the population from which the *Fh*-ES was obtained, with the strain also under higher selection pressure in the field than the laboratory isolate which has been maintained in the laboratory for several generations. As such, the *Fh*-ES of the wild isolate would be expected to contain a more diverse milieu of proteins, possibly explaining its more profound effects. Regarding *Fh*-WeyES, it is expected to be produced from extremely genetically similar flukes, so it is expected that protein sequences and levels would be relatively consistent, while for *Fh*-WildES, greater protein expression variability would be expected.

Highlighting the various immunomodulatory abilities of liver fluke isolates has practical implications. Nowadays, parasites are not only considered foes but also future remedies for increasingly common autoimmune diseases and allergies. *F. hepatica* exposure has been shown to be promising in the mitigation of autoimmune and allergic diseases (9, 19). Our findings indicate that research should be performed with fully-typed laboratory isolates, or that when using flukes collected from abattoirs or the environment, it is worth preserving isolates that show abilities to mitigate symptoms of allergies or autoimmune diseases for future cultivation. This will allow future detailed insights and characterisation, which will be a boon when the results are promising and worthy of further exploration. It is also possible that some discrepancies in the literature may be attributable to differences between various isolates of the flukes investigated, *e.g.* differences in susceptibility to bacterial coinfections (4, 10).

Summing up, the findings show that distinct isolates of *F. hepatica* show different immunomodulatory potentials and suggest stronger immunosuppressive abilities for the wild isolate. This may shed some light on some discrepant literature data and underlines the importance of providing information on the source of *F. hepatica* isolates in publications. We believe that further exploration of intraspecies variability in *F. hepatica* immunomodulatory abilities will provide a deeper understanding of the interplay between the host and the liver fluke as well as lead to those isolates being identified, which exhibit the most suitable abilities to mitigate allergies and autoimmune diseases.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Availability of Data and Material: The datasets analysed during the current study are available from the corresponding author on reasonable request. The raw data of the experiment were deposited in GEO (no. GSE95019).

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