

ORIGINAL PAPER

Fasciola hepatica products can alter the response of bovine immune cells to *Mycobacterium avium* subsp. *paratuberculosis*

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Abstract

Background: *Fasciola hepatica* causes economically important disease in livestock worldwide. The relevance of this parasitic infection extends beyond its direct consequences due to its immunoregulatory properties.

Objectives: Given the importance of the T helper 1 (Th1) immune response in controlling infections with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in cattle, we aimed to establish the immunological consequences that co-infection with *F. hepatica* might have on the course of Johne's disease (JD).

Methods: This study compared the in vitro response of bovine immune cells to infection with MAP or exposure to MAP antigens following *F. hepatica* infection or stimulation with *F. hepatica* products.

Results: We found a decreased proliferation of peripheral blood mononuclear cells (PBMCs) after infection with *F. hepatica*. This reduction was inversely correlated with fluke burden. Pre-stimulation with *F. hepatica* molecules produced a significant reduction of ileocaecal lymph node leucocyte proliferation in response to MAP antigens. Additionally, *F. hepatica* products reduced expression of the CD14 receptor by macrophages and increased levels of apoptosis and bacterial (MAP) uptake.

Conclusions: Overall, *F. hepatica* infection had little impact on the in vitro response of immune cells to MAP, whereas in vitro co-stimulation with *F. hepatica* molecules had a measurable effect. Whether this is likely to affect JD progression during in vivo chronic conditions remains unclear.

KEYWORDS

co-infection, fasciolosis, ileocaecal lymphocytes, immunoregulation, Johne's disease, macrophages, peripheral blood mononuclear cell

1 | INTRODUCTION

Like many parasites, *Fasciola hepatica* produces immunoregulatory changes in its host. Cattle co-infected with *F. hepatica* and

Mycobacterium bovis display reduced responsiveness to skin and interferon gamma (IFN- γ) tests, reduced bovine tuberculosis (BTB) lesion scores and decreased bacterial recovery compared to cattle infected with *M. bovis* only.¹⁻³ Studies of co-infection with other

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bacteria have reported an increase in the severity of the concurrent infection or a suppression of a protective T helper 1 (Th1) immune response.⁴⁻⁷ A range of immunoregulatory molecules produced by parasitic helminths have been described.⁸ For example, protein 24 in the excretory/secretory (ES) products from *Haemonchus contortus* (HcES) reduces proliferation and IFN- γ and nitric oxide (NO) production by goat peripheral blood mononuclear cells (PBMCs).⁹ ES products from *Teladorsagia circumcincta* and *Ostertagia ostertagi* fourth-stage larvae have also been shown to modulate lymphocyte cytokine responses.^{10,11} Other molecules such as macrophage inhibitory factor and apyrases may influence monocyte movement and activation, or control inflammation within the gastric gland micro-environment, respectively.¹²⁻¹⁴ Important molecules identified from *F. hepatica* extracts with proven immunomodulatory potential include cathepsin L cysteine proteases (FhCL), peroxiredoxin (FhPrx) and cathelicidin-like helminth defence molecules (FhHDM-1).¹⁵⁻¹⁸

Infection of cattle, sheep and other ruminants with *Mycobacterium avium* subspecies *paratuberculosis*-(MAP) has a silent and long progression that can last for years in a subclinical stage. Ultimately, it can lead to Johne's disease (JD), a chronic wasting disease of global importance characterized by the development of granulomatous lesions in the intestine of ruminants and resulting in chronic diarrhoea, rapid weight loss and decreased appetite.¹⁹ JD represents a challenge to the farming industry because of the difficulty of detecting MAP-infected animals at an early stage of infection, and because of the lack of effective treatment methods. Infected animals can live for years without showing clinical signs, which occurs when the effective host immune response declines allowing the bacteria to spread. This decline is partly orchestrated by MAP, which relies on immune subversion mechanisms such as reduced macrophage responsiveness to IFN- γ , blocked phagolysosome fusion, induction of interleukin 10 (IL-10) production, inhibition of NO synthase or manipulation of cell apoptosis to survive within the intestinal tissue. Like other mycobacteria, MAP also induces a shift from a predominant Th1 to a Th2 or mixed (Th1/Th2) immune response.²⁰⁻²⁷ It has been suggested that co-infection with helminth parasites may facilitate or fast-forward this immunological shift, because parasitic worms also elicit a strong Th2 response.^{8,28} Furthermore, it has been shown that *Schistosoma mansoni* and *Nippostrongylus brasiliensis* enhance susceptibility to co-infections with *M. tuberculosis* and *M. bovis*-Bacille Calmette Guerin (BCG), respectively, leading to higher levels of bacilli in the organs and greater lung pathology in mice.²⁹⁻³¹

This study aimed to establish whether *F. hepatica* infection has a regulatory influence on the systemic and local cell-mediated immune (CMI) responses to MAP. PBMCs, monocyte-derived macrophages (MDMs) and ileocaecal lymph node (ILN) leucocytes from *F. hepatica*-infected and *F. hepatica*-uninfected cattle were stimulated with *F. hepatica* products that are known to have immunoregulatory properties (FhES and LFH) and MAP antigens (purified protein derivative johnin [PPDj] and MAP whole-cell sonicate [MPS]) or directly infected with MAP. The effects of prior *F. hepatica* infection or concurrent exposure to *F. hepatica* antigen on cell proliferation, bacterial uptake and cytokine production were analysed by flow cytometry and cell proliferation assays.

2 | MATERIALS AND METHODS

2.1 | Experimental design

All cells used in this study derived from animals in one of two separate experimental trials. For experimental trial 1, PBMCs were collected by jugular venepuncture from seven cattle 14 weeks after infection with 200 *F. hepatica* metacercariae, Italian strain (Ridgeway Research Ltd, UK).³² At the same time, PBMCs were collected from six uninfected control animals that had served as controls in the trial. Fluke burdens were determined by counting the number of adult and immature parasites in bile ducts and liver parenchyma at post-mortem. This was done 16 weeks after the challenge infection due to logistical reasons. Animals and procedures carried out were approved by the UCD Animal Research Committee (AREC-16-23-Mulcahy) and licensed by the Health Products Regulatory Authority (AE18982/P098). For experimental trial 2, ILN leucocytes were collected post-mortem from five unchallenged experimental cattle that had served as controls in an unrelated study. Following euthanasia by intra-jugular administration of pentobarbital sodium (Euthanal 200 mg/mL, Merial Animal Health), lymph nodes were extracted during post-mortem examination and suspended in ice-cold Hank's balanced salt solution (HBSS; without calcium or magnesium) supplemented with 1% antibiotic-antimycotic solution (Thermo Fisher Scientific). The protocols used in this study were approved by the UCD Animal Research Ethics Committee for exemption from full ethical review (AREC-E-16-38-Mulcahy), University College Dublin, Ireland. The experimental set-up and cell types employed in each experimental assay are summarized in Figure 1.

2.2 | Bacterial culture and preparation

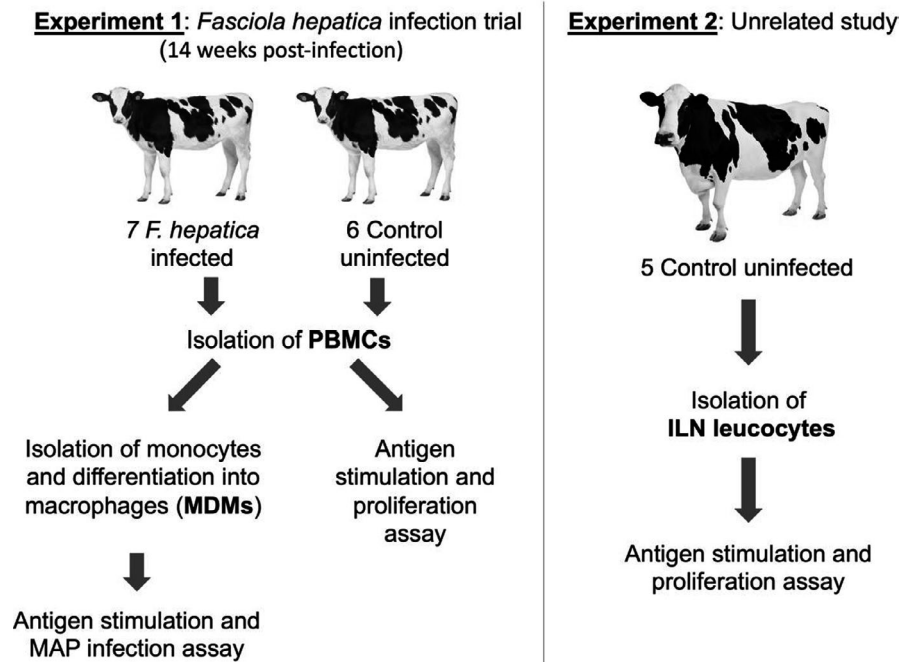
MAP BAA-968, designated as strain K-10 and isolated from animal faeces in 1990, was purchased from the American Type Culture Collection (ATCC) and used throughout. The bacteria were grown at 37°C in Middlebrook 7H9 medium (Difco™, Becton Dickinson) supplemented with 2 mg/mL mycobactin J (IDVet), 10% Middlebrook Oleic Albumin Dextrose Catalase (OADC) enrichment (Becton Dickinson) and 0.05% Tween 80 (Sigma-Aldrich). MAP cultures were maintained for 4 months prior to in vitro infection and antigen preparation.

2.3 | Antigen preparation

2.3.1 | MAP antigens

Two different MAP antigens were employed during cell culture experiments: PPD-j and MPS. PPD-j was kindly provided by Prof. Eamon Gormley (UCD School of Veterinary Medicine, Dublin, Ireland). MPS was prepared according to a protocol kindly provided by Judith R Stabel (United States Department of Agriculture-Agricultural

FIGURE 1 Experimental set-up and assays performed



Research Service). Briefly, 100 mL bacterial culture in log phase was aliquoted into two 50-mL conical flasks and centrifuged at 12 000 g for 30 minutes at 4°C. Pellets were washed, combined using cold Dulbecco's-phosphate-buffered saline (D-PBS, Sigma-Aldrich D5652) and centrifuged at 10 000 g for 30 min at 4°C. The pellet was washed again using cold D-PBS and centrifuged as before. The pellet was then resuspended in sonication buffer (250 mmol/L sucrose, 10 mmol/L 4-(2-hydroxyethyl) piperazine-1-ethanesulphonic acid (HEPES) sodium salt, 1:100 Protease Inhibitor Cocktail in dH₂O) and sonicated four times for 5 minutes at a final output of 25 W using a Branson Sonic Disruptor 150. After each sonication step, the preparation was left to cool for 10 minutes on ice. Finally, the sonicate was centrifuged at 10 000 g for 20 minutes at 4°C. The supernatant was collected, taking care not to disturb the cell debris pellet, filter-sterilized and frozen at -20°C until use.

2.3.2 | Liver fluke homogenate and excretory-secretory products

Liver fluke homogenate (LFH) and ES products were prepared according to the protocol described by Smith et al.³³ Briefly, for LFH, adult *F. hepatica* parasites were collected from bovine livers at an abattoir and placed in ice-cold endotoxin-free PBS (Sigma-Aldrich). Within 3 hours of collection, all samples were rinsed and frozen at -80°C. Subsequently, the frozen flukes were homogenized using a pestle and mortar. For ES, *F. hepatica* specimens were collected as described above. On return to the laboratory, all dead and damaged parasites were removed. Those remaining were rinsed and incubated at a density of 10 specimens per 5 mL of warm culture medium consisting of Roswell Park Memorial Institute-1640 (RPMI-1640; Invitrogen) supplemented with 25 mmol/L HEPES, 2% glucose

and 25 µg/mL gentamycin in T-25 tissue culture flasks for 4-8 hours at 37°C and 5% CO₂. The resulting supernatant was centrifuged at 600 g for 20 minutes at 4°C to remove debris. Finally, the centrifuged ES fraction was concentrated using 3 kDa centrifugal concentrators (Millipore). The resulting antigen suspensions were filtered through a 70-µm strainer and then filter-sterilized. Endotoxin was removed using Triton X-114 phase separation as described by Flynn et al.²

2.4 | Cell isolation and culture

Peripheral blood mononuclear cells were isolated from heparinized bovine blood by density gradient centrifugation over Ficoll-Paque PLUS (GE Healthcare). An aliquot of PBMCs was separated to perform direct experiments. These were resuspended in complete medium (RPMI-1640 medium supplemented as before with additional 10% foetal calf serum [FCS]; Bio-Sciences), seeded in 96-well plates at a concentration of 1×10^6 cells/mL and incubated at 37°C in 5% CO₂ for 24 hours before any stimulations were performed. The remaining PBMCs were dispensed into Nunc EasYFlasks (75-cm² surface, filter cap, Thermo Fisher Scientific) at a concentration of 1×10^8 per flask in RPMI-1640 medium (Invitrogen) supplemented with 1% glutamine (Thermo Fisher Scientific), 1% nonessential amino acids (Sigma-Aldrich) and 1% penicillin-streptomycin (incomplete medium). Following incubation at 37°C in 5% CO₂ for 2 hours, nonadherent cells were removed with the supernatant and complete medium containing 50 ng/mL recombinant ovine macrophage colony stimulation factor (rovM-CSF1, Immunotools) to stimulate differentiation into MDMs was added. Culture flasks were maintained at 37°C in 5% CO₂ for 7 days. Half of the medium volume was changed with fresh medium every 3 days. At day 8, macrophages were harvested

using TrypLE Express (Thermo Fisher Scientific) as suggested by the manufacturer. MDMs were then aliquoted into 24-well plates with complete medium (without antibiotics) at a concentration of 2×10^5 cells/mL and allowed to re-attach overnight.

Ileocaecal lymph node leucocytes were isolated by macerating the ILNs in a petri dish using two sterile scalpels and straining the resulting cell solution through a 70- μ m cell strainer (Thermo Fisher Scientific). Strained cells were then centrifuged at 300 g for 10 minutes and resuspended in complete medium. They were then aliquoted into 96-well plates at a concentration of 1×10^6 cells/mL and incubated at 37°C in 5% CO₂ for 24 hours before stimulation.

2.5 | MDM stimulation and infection

MAP bacteria for in vitro infection of cultured MDMs were prepared by centrifuging 10 mL of MAP culture in the exponential growth phase at 1000 g, vortexing using glass beads, sonicating at 25 W for 1 minute and staining with BacLight™ red bacterial stain (Thermo Fisher Scientific) following the manufacturer's instructions.

MDMs from six *F. hepatica*-infected and six uninfected animals were isolated. Three wells (per individual) were infected with MAP, and another three wells of MDMs (per animal) were overlaid with PBS alone as negative control. Where enough MDMs were available (five *F. hepatica*-infected and five uninfected animals), three further wells (per animal) were stimulated with lectin from *Phytolacca americana* (pokeweed mitogen [PWM]; Sigma-Aldrich) at a concentration of 2.5 μ g/mL. Finally, three wells of MDMs (per animal) from four *F. hepatica*-infected and four uninfected animals were stimulated with FhES at a concentration of 20 μ L/mL and incubated at 37°C in 5% CO₂ for 24 hours before being infected with MAP at 1:10 multiplicity of infection. This dose had previously been established as optimal by our laboratory by performing dose-response curves.³⁴ Overall, MDMs from all 12 animals were infected with MAP and stimulated with PBS, from 10 animals were stimulated with PWM and from eight animals were stimulated with FhES and infected with MAP. All preparations were incubated overnight at 37°C in 5% CO₂.

Due to the lack of available MDMs, control wells stimulated exclusively with FhES and left uninfected were not initially included. However, those were included in a separate experiment. Due to the

quality control measures employed in the flow cytometry protocol, it is safe to compare data obtained from different experiments.

2.5.1 | Harvesting of MDMs

Twenty-four hours after macrophage stimulation or infection, the supernatants were collected, filter-sterilized and stored at -20°C for future analysis. To remove extracellular bacteria, infected MDMs were washed three times with warm D-PBS. Subsequently, all MDMs (stimulated, infected and control) were harvested by exposure to TrypLE Express (Thermo Fisher Scientific) for 5 minutes at 37°C. The suspensions were then collected into polypropylene test tubes (Beckman Coulter) and centrifuged at 300 g for 5 minutes. Prior to staining, the cells were washed three times in 1 mL D-PBS.

2.6 | Flow cytometry for measurement of bacterial uptake and receptor expression

2.6.1 | Antibody staining

Prior to antibody staining, fragment crystallizable (Fc) region receptors on MDMs were blocked by incubating the cells in blocking buffer (D-PBS containing 20% normal goat serum; Bio-Rad) for 30 minutes at 4°C. The blocking buffer was removed by decanting after centrifuging the cells at 300 g for 5 minutes, and a master-mix containing all antibodies except for DRAQ7 (and the bacteria) was added to each pellet at a final volume of 50 μ L. The antibodies employed were as follows: Brilliant Violet 421 CD14 Clone M5E2 (BioLegend), BacLight red bacterial stain (Thermo Fisher Scientific), TO-PRO-3 Iodide (Thermo Fisher Scientific) and DRAQ7 Drop & Go (Biostatus). The concentrations for all antibodies, optimized by titration, are shown in Table 1. The staining buffer used to dilute the antibodies was D-PBS supplemented with 5% FCS. An equal volume of staining buffer was used for Fluorescence Minus One (FMO), unstained or single positive samples (controls). Following incubation at 4°C for 1 hour, the cells were washed in 0.5 mL of staining buffer (centrifugation at 300 g for 5 minutes) and resuspended in 0.5 mL of D-PBS. Two drops of

TABLE 1 Description of fluorescent antibodies panel used for flow cytometry

Antibody/Stain	Characteristic being measured	Reporter	Dilution	Laser and filter
Brilliant Violet 421 CD14 Clone M5E2 (BioLegend)	CD14 Surface receptor expression	V450-PB	1:100	405 nm: 450/45BP
BacLight red bacterial stain (Thermo Fisher Scientific)	Labelled bacteria	Y610-mCHERRY	0.1 μ mol/L	561 nm: 610/20BP
TO-PRO-3 Iodide (Thermo Fisher Scientific)	Viability staining (apoptosis)	R660-APC	100 η mol/L	638 nm: 660/10 BP
DRAQ7 Drop & Go (Biostatus)	Viability staining	R763-APCA750	2 drops to 0.5 mL cells	638 nm: 763/43 BP

DRAQ7 DROP & GO were added to each sample before proceeding with flow cytometry.

2.6.2 | Instrument details and gating

All analyses were carried out on a Beckman Coulter CytoFLEX LX flow cytometry machine using Software CytExpert version 2.3.0.84. Data files were kept as Flow Cytometry Standards (FCS). Compensation was not necessary, and gating was carried out by including FMO controls which were the same cells as those used in the experiment. Fluorescence signals were measured using Avalanche PhotoDiodes. Gating for fluorescence intensity of the bacteria (ie bacterial uptake) was applied by selecting target cells based on side scatter (SSC) area vs SSC height plot (singlets 1), forward scatter (FSC) height vs FSC area (singlets 2), SSC area vs V450-PB (cluster of differentiation 14 [CD14]) and R763-APCA750 (viability) vs Y610-mCHERRY (bacteria) (see Figures S1A,B,D). Median fluorescence intensity was used to quantify CD14 receptor expression and bacterial uptake.

For apoptosis, two viability dyes that enter apoptotic cells at different stages (TO-PRO3 enters earlier than DRAQ7), were employed.^{35,36} Double negative cells were considered to be alive, TO-PRO3-positive and DRAQ7-negative cells were considered apoptotic, and double positives were considered dead. This method has previously been described by using combinations of YO-PRO 1/7-AAD, YO-PRO-1/PI or TO-PRO3-A5 based on their different affinity for DNA and other molecules.^{37,38} The following gating was applied: SSC area vs SSC height plot (singlets 1), FSC height vs FSC area (singlets 2). Figure S1C shows the effect of each in vitro culture condition on MDM apoptosis by using DRAQ7 vs TO-PRO3 gating. Lasers and filters employed are shown in Table 1. Percentage of cells in each apoptotic stage was quantified using the gating strategy demonstrated in Figure S2.

2.6.3 | Quality control

Instrument quality control checks were carried out in parallel with each assay using QC CytoFLEX beads. These reads were also used to standardize fluorescence across samples analysed on different days. In addition, single stained controls and FMO controls were prepared and measured. Labels and antibodies were titrated to optimize the performance. Gains were adjusted so that the fluorescence intensity was identical for each antibody regardless of the date of acquisition. Automatic thresholding triggering the FSC-H signal was initially used. Samples were run at slow flow rate. Unstained samples, as well as stained controls, were included in each analysis.

2.7 | Proliferation assay

Peripheral blood mononuclear cells from experimental trial 1 animals (seven animals in the *F. hepatica*-infected group and six from

the uninfected group) and ILN leucocytes from experimental trial 2 ($n = 5$) were plated into 96-well plates at a concentration of 1×10^6 cells/mL in complete medium. ILN leucocytes were stimulated and incubated overnight with LFH or PBS, while no previous stimulation was performed on PBMCs. LFH was employed to follow the same procedures as previous work performed using lymphocyte and proliferation assays.^{1,39,40} After 24-hour incubation at 37°C, 5% CO₂, ILN leucocytes or PBMCs were stimulated with PWM (2.5 µg/mL), PPDj (10 µg/mL), MPS (10 µg/mL) or PBS. There were two replicate wells for each antigen per animal. Following incubation for 48 hours at 37°C, 5% CO₂, in vitro cell proliferation was measured using a colorimetric immunoassay based on 5-bromo-2'-deoxyuridine (BrdU) incorporation during DNA synthesis (Roche Cell Proliferation ELISA) according to the manufacturer's instructions.

2.8 | Nitric oxide and cytokine detection

IFN-γ was measured in supernatants of PBMCs and ILN leucocyte cultures using the bovine IFN-γ ELISA reagent kit (Invitrogen) as per the manufacturer's instructions.

NO concentration in the culture supernatants of MDMs was measured using the Griess reagent kit (Promega), according to the manufacturer's instructions. IL-10 in the supernatant of MDMs was measured using a sandwich ELISA and paired monoclonal antibodies as previously reported by others.^{41,42} Capture antibody (MCA2110, Bio-Rad) was diluted to 5 µg/mL in carbonate-bicarbonate buffer, and 100 µL was added to each well of a 96-well plate. Following incubation overnight at room temperature (RT), plates were washed three times with PBS-T (0.05% Tween-20), and 100 µL of blocking buffer was added (1% bovine serum albumin in PBS-T) for 1 hour at RT. Plates were washed as before and 100 µL of cells supernatants or fresh culture medium (as background control) was added and left 1 hour at RT. After washing the plates, 100 µL of detection antibody (MCA211B, Bio-Rad) was added to each well at a concentration of 2 µg/mL and incubated for 1 hour at RT. Following another PBS-T wash, 100 µL of streptavidin-horseradish peroxidase (HRP; diluted 1:5000 in PBS) was added and incubated for 45 minutes at RT. After a further three washes, 100 µL of tetramethylbenzidine (3,3',5,5'-TMB; Sigma) was added as substrate solution. Plates were incubated in the dark for 20 minutes at RT. The reaction was stopped by adding 100 µL of stop solution (1N H₂SO₄) and absorbance measured at 450 nm. Optical density (OD) index (ODI) was calculated by subtracting the background control reading (medium) and multiplying the OD by 1000.

3 | RESULTS – EXPERIMENT 1

3.1 | Effect of *F. hepatica* infection on proliferation and cytokine production by PBMCs

In animals that had previously been infected with *F. hepatica*, PBMC proliferation in response to PWM showed a significant

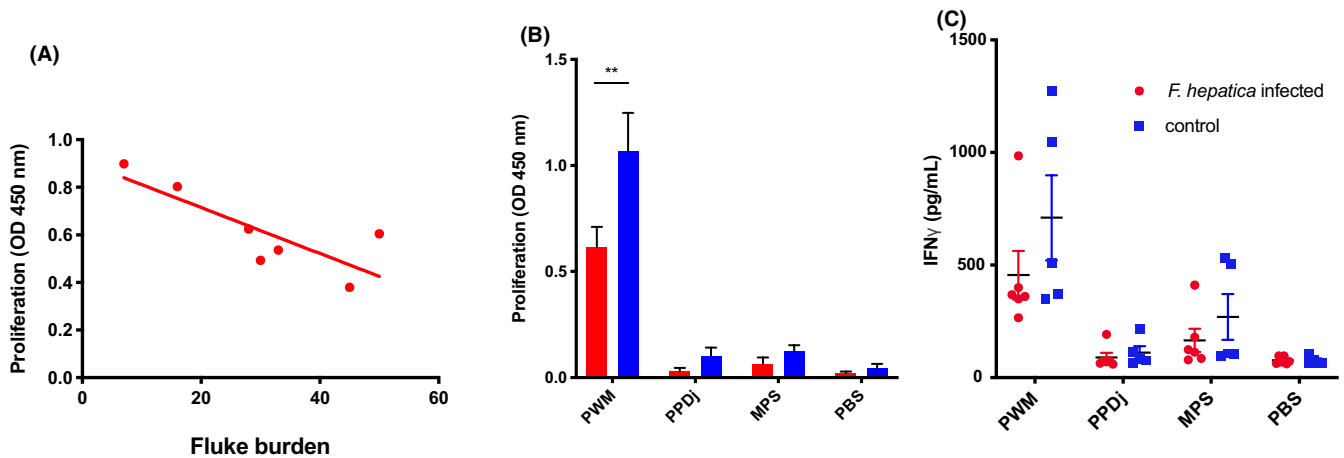


FIGURE 2 Response of PBMCs from experimental trial 1 (7 *Fasciola hepatica*-infected and 6 uninfected animals) to stimulation with PWM and various antigens (A) Proliferation of PBMCs in response to PWM against fluke burden in seven infected animals. Cell proliferation was measured using a BrdU cell proliferation ELISA (OD at 450 nm), and background values were subtracted from each experimental condition. The negative correlation was significant (Pearson $r = -.81$, $P < .05$). B, Proliferation of PBMCs in response to PWM, PPD-j and MPS. C, IFN- γ production by PBMCs after stimulation with PWM, PPD-j and MPS. IFN- γ concentration (pg/mL) was measured in the PBMC supernatants using a bovine IFN- γ ELISA (OD at 450 nm). Results are expressed as mean \pm SEM of the OD or concentration. ** $P < .01$

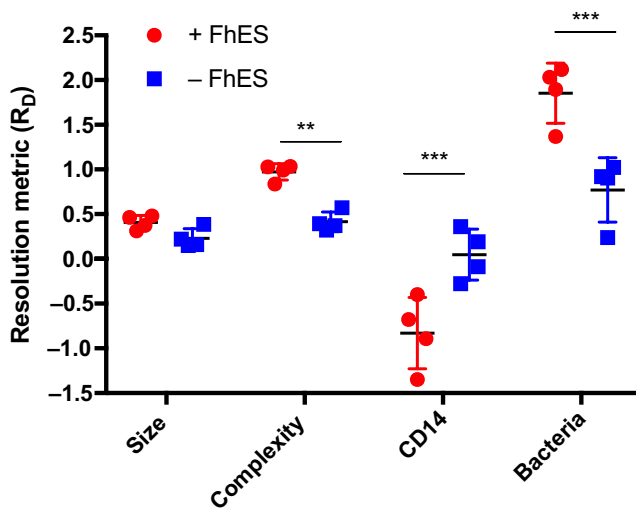


FIGURE 3 Cell size, internal complexity (ie granularity), CD14 receptor expression and MAP bacterial uptake in four uninfected animals from Experimental trial 1. Red dots indicate cells that were stimulated with FhES 24 hours prior to MAP infection, and blue square cells from the same animals without FhES stimulation prior to MAP infection. The y-axis indicates the median of fluorescence intensity normalized using control wells (PBS stimulated) from the same animal. Results are expressed as the mean $R_D \pm$ SEM. ** $P < .01$, *** $P < .001$

inverse correlation with fluke burden ($r = -.81$, $P < .05$; Figure 2A). This effect was not observed with regard to their IFN- γ production (data not shown). Proliferation in response to PWM was also significantly higher in PBMCs from uninfected than from *F. hepatica*-infected cattle ($P < .01$) (Figure 2B). Proliferation in response to MAP antigens, PPDj and MPS, showed a similar trend to that

following PWM stimulation, but differences between cells from *F. hepatica*-uninfected and infected animals were not significant. IFN- γ production by PBMCs stimulated with PWM, PPDj or MPS was on average higher in cells from uninfected than *F. hepatica*-infected animals (Figure 2C), but again these differences were not statistically significant.

3.2 | Effect of *F. hepatica* infection and stimulation with Fh products on the in vitro uptake of MAP

MDMs from *F. hepatica*-infected and uninfected animals showed similar bacterial uptake and expression of CD14 receptors (Figure S3A). When data from uninfected animals only were analysed, MAP uptake by MDMs was significantly higher in cells stimulated with FhES ($P < .001$; Figure 3). Complexity or granularity of the cells increased in this case ($P < .001$).

3.3 | Effect of *F. hepatica* infection or stimulation with Fh products on apoptosis of MDMs following infection with MAP (or PWM stimulation)

Apoptosis of MDMs after MAP infection with or without previous co-stimulation with FhES was investigated by flow cytometry. There was no difference in the rate of apoptosis (% of alive, early apoptosis, mid-apoptosis, advanced apoptosis and dead MDM) from *F. hepatica*-infected and uninfected animals following in vitro infection with MAP (Figure S3B) or stimulation with PWM (Figure S4B). The response of cells from uninfected animals revealed that stimulation with FhES accelerated the apoptotic process. Figure 4 shows that 24 hours after MAP infection in the absence of FhES,

FIGURE 4 Apoptosis stages.

Percentage of MDMs in each apoptosis stage in control (PBS, $n = 6$), MAP-infected ($n = 6$) and FhES-stimulated and MAP-infected ($n = 4$) in vitro cultures. Results are expressed as the mean percentage \pm SEM. **** $P < .0001$

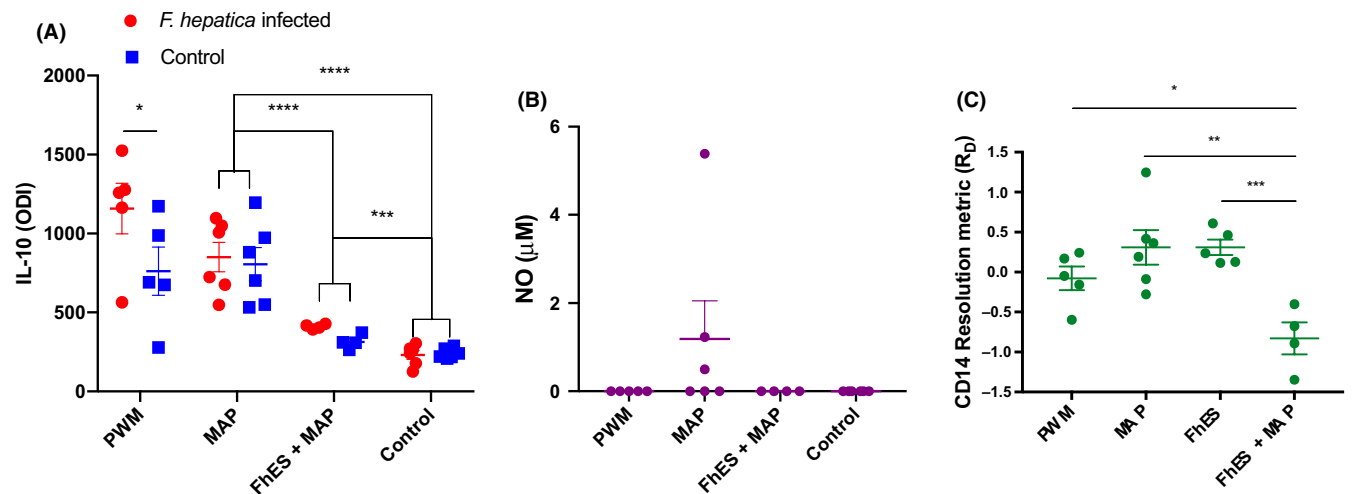
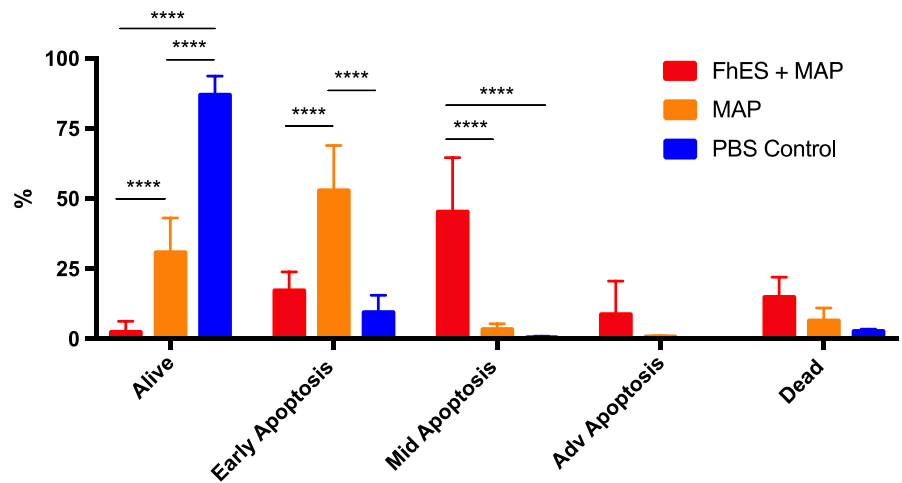


FIGURE 5 Production of cytokines and expression of CD14 by MDMs. A, IL-10 production (4, 5 or 6 *F. hepatica*-infected and 4, 5 or 6 control-uninfected animals in FhES + MAP, MAP and Control, and PWM conditions, respectively). B, NO production by MDMs from *F. hepatica*-uninfected animals (PWM $n = 5$, MAP $n = 6$, FhES + MAP $n = 4$, Control $n = 6$) (C) CD14 receptor expression by MDMs from *F. hepatica*-uninfected animals (PWM $n = 5$, MAP $n = 6$, FhES $n = 5$, FhES + MAP $n = 4$). Results are expressed as the mean ODI, concentration or $R_D \pm$ SEM. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$

about 60% of MDMs were at an early stage of apoptosis. By comparison, about 50% of cells previously exposed to FhES were at the mid-apoptotic stage. At this time point, over 80% of the control MDMs (PBS, MAP-uninfected) were alive.

Figure S5A shows there were no significant differences with regard to percentage of cells in each apoptotic stage between FhES-stimulated MDMs and control (PBS) MDMs from the five animals employed at a later experiment. Finally, stimulation of MDMs with PWM resulted in similar apoptosis stages to those control (PBS; Figure S5B).

3.4 | Effect of *F. hepatica* infection or stimulation with *F. hepatica* products on the production of NO and IL-10 by MDMs

Stimulation with PWM induced significantly increased production of IL-10 in cells from *F. hepatica*-infected animals as compared to those from uninfected cattle (Figure 5A). However,

infection with *F. hepatica* had no significant effect on the production of IL-10 in response to MAP infection with or without previous stimulation with FhES. Data from uninfected animals showed a significant increase of IL-10 production after MAP infection or FhES + MAP infection when compared to PBS control cells. On the other hand, pre-stimulation with FhES before MAP infection induced significantly lower levels of IL-10 as compared to MAP infection alone.

There was no difference between *F. hepatica*-infected and uninfected animals with respect to NO production by MDMs infected with MAP, stimulated with FhES + infected with MAP or stimulated with PWM (Figure S6). Data from uninfected animals showed that MAP infection alone induced more NO production than when cells were also exposed to FhES, although these changes were not significant (Figure 5B). Stimulation with PWM had no effect on NO production. Overall the CMI varied greatly between individuals with cells from only three (out of six) animals exhibiting raised NO levels in response to MAP infection.

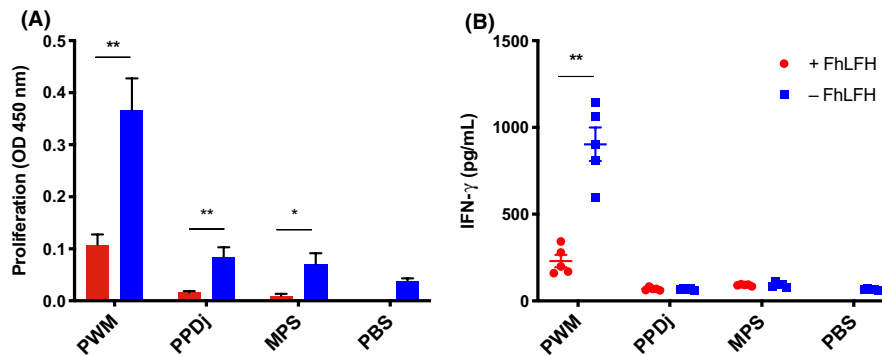


FIGURE 6 Response by ILN leucocytes from experimental trial 2 following exposure to PWM, PPDj or MPS (with or without previous stimulation with LFH) ($n = 5$). A, Cell proliferation was measured using a BrdU cell proliferation ELISA (OD at 450 nm) and background values were extracted from each experimental condition. B, IFN- γ concentration (pg/mL) was measured in the PBMC supernatants using a bovine IFN- γ ELISA (OD at 450 nm). Results are expressed as mean \pm SEM of the OD or concentration. ** $P < .01$, * $P < .05$

3.5 | Expression of CD14 receptor by MDM

CD14 expression in cells stimulated with FhES + MAP was significantly lower than in those infected with MAP, stimulated with PWM or with FhES only (Figure 5C). Although it should be remembered that stimulation of MDMs with FhES alone was performed on cells collected from different animals. However, flow cytometer quality control measures and individual animal calculation of the resolution metric (R_D) value with its own unexposed control wells allow for inter-experiment comparison of R_D .

4 | RESULTS - EXPERIMENT 2

4.1 | Effect of stimulation with *F. hepatica* products on the proliferation and IFN- γ production by ILN leucocytes

Proliferation of ILN leucocytes in response to stimulation with PWM and MAP antigens PPDj and MPS was significantly decreased under the influence of liver fluke homogenate (LFH) stimulation in vitro (Figure 6A). In addition, LFH stimulation significantly reduced IFN- γ production in response to PWM stimulation (Figure 6B). Production of IFN- γ in response to PPDj or MPS was low regardless (not different from the PBS control) and unaffected by exposure to LFH.

Table 2 shows a summary of significant results obtained from experimental trials 1 and 2.

5 | DISCUSSION

The clinical and economic significance of MAP infection has driven the need to better understand the immune response developed by the host. It is thought that the appearance of clinical signs is associated with a loss of a protective Th1 CMI response which may be triggered by stress or Th1-Th2 polarization.⁴³ This study aims to determine whether *F. hepatica* infection, which is known

to exert an immunoregulatory influence by down-regulating Th1 responses in cattle, may also influence the progression of MAP infection.^{44,45}

Our results showed that PBMCs isolated from cattle that were infected with *F. hepatica* had reduced proliferation in vitro in response to stimulation with PWM as compared to uninfected animals. Moreover, this proliferation was inversely related to fluke burden. It appears therefore that higher parasite burdens have a greater immunoregulatory effect. The same has previously been observed in sheep by Haçariz et al.,⁴⁶ who reported a significant inverse correlation between PBMC proliferation in response to Concanavalin A (ConA; another cell mitogen) and fluke burden.

Stimulation with MAP antigens also induced lower cell proliferation in PBMCs isolated from *F. hepatica*-infected cattle; however, in this case, the difference was not significant. IFN- γ production followed a similar pattern; it was reduced in PBMCs derived from *F. hepatica*-infected animals exposed to stimulation with PWM and MPS, although again the difference to *F. hepatica*-uninfected animals was not significant. Individual responses, as expected, were highly variable and it is possible that if a greater number of animals had been included in the study a statistically significant difference would have been recorded. PWM is a powerful mitogen and therefore an excellent inducer of proliferation which is why it is often used as a positive control in cell proliferation assays.⁴⁷ In fact, it has been shown that production of IFN- γ in response to PWM is about 80 times higher than in response to MPS stimulation.⁴⁸ In contrast, MAP antigens induce a much more subtle immune response. PBMCs from animals at the subclinical stage of infection usually produce more IFN- γ in response to MPS than uninfected control animals, which, in turn, secrete more IFN- γ than clinically affected animals.⁴⁸

Overall our results indicated that infection with *F. hepatica* reduced cell proliferation and IFN- γ production by bovine PBMCs in response to PWM. However, when MDMs isolated from these PBMCs were infected with MAP, there was no difference with regard to cell size, cell complexity, CD14 receptor expression, bacterial uptake or apoptosis between cells derived from

TABLE 2 Summary of significant results obtained in Experiments 1 and 2

	Experimental trial 1		Experimental trial 2
	PBMC	MDM	ILN leucocytes
<i>F. hepatica</i> infection	+ PWM stimulation Decreased proliferation to PWM (inversely correlated to fluke burden) + MAP antigens stimulation No significant results As compared to control uninfected	+ PWM stimulation Increased IL-10 production +MAP infection No significant results As compared to control uninfected	
FhES/LFH stimulation		+ MAP infection Decreased CD14 expression Increased bacterial uptake Increased cell granularity Acceleration of apoptotic process Decreased IL-10 As compared to MAP infection alone	+ PWM stimulation Decreased proliferation in response to PWM and MAP antigens Decreased IFN- γ production in response to PWM + MAP antigens stimulation Decreased proliferation As compared to no previous FhES/LFH stimulation

Abbreviations: FhES, *F. hepatica* excretory/secretory products; ILN, ileocaecal lymph node; LFH, liver fluke homogenate; MAP, *Mycobacterium avium* subspecies *paratuberculosis*; MDM, monocyte-derived macrophages; PBMC, peripheral blood mononuclear cells; PWM, pokeweed mitogen.

F. hepatica-infected and *F. hepatica*-uninfected animals. Analysis of culture supernatants also revealed that *F. hepatica* infection status had no impact on the production of NO. However, significantly increased production of IL-10 in *F. hepatica*-infected animals was observed in response to PWM stimulation confirming that infection with *F. hepatica* can affect the response of macrophages to powerful mitogenic stimuli. This effect was not observed in response to MAP infection which was surprising as previous work in our laboratory had shown significant differences in the response of MDMs to *M. bovis*-BCG before and after *F. hepatica* infection.³ An important difference is that in the previous study the effect of individual variability was reduced as cells from the same animals were used before and after *F. hepatica* infection. Moreover, MAP replicates much more slowly in the host than *M. bovis* and as a result might adapt differently to the immunoregulatory effects of *F. hepatica*. It is therefore possible that due to distinct interactions between mycobacterial microbes and bovine MDM, infection with *F. hepatica* or stimulation with *F. hepatica* products results in different outcomes.

As we found no significant differences in bacterial uptake by MDMs derived from *F. hepatica* -infected and uninfected animals, we stimulated MDMs from all animals with FhES before exposure to MAP in an attempt to assess its immunoregulatory activity in the context of infection with MAP. Cells stimulated with FhES had increased cell granularity, increased bacterial uptake and reduced CD14 receptor expression. Cell granularity or complexity is determined by the SCC of the flow cytometer, which detects the light that runs through the cell determining the density of organelles (or particles) within the cytoplasm. In our study, this could represent the increase in phagosomes in response to MAP infection.

CD14 is mainly expressed at the cell surface of macrophages and granulocytes.⁴⁹ Its involvement in the phagocytosis of mycobacterial pathogens was shown by Souza et al,⁵² who reported that addition

of anti-CD14 receptor antibody inhibited phagocytosis of MAP by monocytes and that a bovine macrophage cell line that lacked expression of the CD14 receptor (BoMac) was less able to phagocytose the organisms than normal bovine monocytes.⁵⁰⁻⁵² During infection of MDMs with *M. bovis*-BCG, previous stimulation with Fh products reduced CD14 receptor expression, but had no effect on bacterial uptake. In contrast, in the present study reduced expression of the CD14 receptor in response to FhES was associated with an increased uptake of bacteria. This may indicate that pre-stimulation with FhES led to different responses by MDMs to BCG or MAP infection. It is possible that *F. hepatica* ES molecules resulted in a partial change in the macrophage phenotype with an impairment of controlled phagocytosis, resulting in an even greater influence of MAP on this process. It is interesting to note that this effect of FhES on CD14 receptor expression was only observed when cells were subsequently infected with MAP. CD14 receptor expression after PWM stimulation was slightly lower than after infection with MAP, or stimulation with FhES alone, but FhES + MAP significantly reduced its expression (mean $R_D = -0.07, 0.3, 0.3$ and -0.8 to PWM, MAP, FhES and FhES + MAP, respectively).

It has previously been reported that in the absence of T-cell activation, macrophages are susceptible to aggressive bacterial invasion followed by bacterial proliferation and destruction of the macrophage.⁵³ Our results indicate that stimulation of MDMs with FhES may accelerate apoptotic events following infection with MAP. We also found that stimulation with FhES reduced NO production by MAP-infected MDMs although, as expected, significant individual differences were observed. A recent study reported that bovine MDMs stimulated with lipopolysaccharide (LPS) and IFN- γ responded with increased transcription of the *nos2* gene. However, these failed to translate into increased NO production.⁵⁴ Similarly, MDMs stimulated with PWM in the present study failed to produce NO. Instead, they responded by releasing the immunoregulatory

cytokine IL-10. In addition, and in line with previous studies, infection of MDMs with MAP induced an increased production of IL-10 (in fact, with similar levels to those induced by PWM), and very little NO.⁵⁵⁻⁵⁷ Pre-stimulation with FhES (before MAP infection) did not increase this effect. Previous studies have reported both increased and decreased IL-10 production by macrophages after FhES stimulation.^{34,58,59} We show here that FhES significantly decreases the production of IL-10 induced by MAP in MDMs. These results combined support our hypothesis that FhES may induce an impairment of the normal phagocytosis of MAP by bovine MDMs.

It is expected that immunological responses detected in PBMCs during *F. hepatica* infection are a reflection of local immune responses in the peritoneum and liver and that any Th1/Th2 polarization would be most pronounced in local tissues. However, systemic immune cells that are recruited to other sites (such as the lungs or gut) would also carry a Th2 bias affecting their immune responsiveness in those tissues. To determine whether intestinal lymphocytes are affected by immune modulation due to *F. hepatica*, we measured the effect of LFH on the proliferation and IFN- γ production of ILN leucocytes co-stimulated with PWM, PPDj and MPS (Experiment 2). Proliferation was significantly reduced by co-stimulation with LFH, although IFN- γ production was only significantly reduced in ILN leucocytes co-stimulated with LFH and PWM. These results suggest reduced responsiveness of local lymphocytes in response to LFH stimulation, consistent with previous studies using systemic lymphocyte preparations.^{1,34,39,40}

To conclude, there is abundant evidence, from both laboratory animal studies, and in farm animals, that the liver fluke, *F. hepatica*, alters the immune responsiveness of its host in multiple ways. This was shown in experimental co-infections, ex vivo studies involving cells and parasite extracts and transcriptomic studies.^{1,3,32} However, while some mechanistic clues are emerging, we do not yet have a comprehensive picture of the network of events that underlies these interactions.

With respect to interactions between *F. hepatica* and mycobacterial infections, a superficial analysis might lead to the premature conclusion that their interaction is relatively simple. Mycobacterial infections are controlled by Th1 response and INF- γ production. Hence, helminth-induced Th2/T regulatory (Treg) bias would be expected to increase susceptibility and/or the manifestation of clinical disease. However, because the immune response also has a part in the development of disease, the actual effect of co-infection is much more complicated. In the case of BTB, *F. hepatica* does indeed reduce Th1 responsiveness resulting in reduced lesion size and reduced bacterial numbers, consistent with an anti-inflammatory, pro-apoptotic response, and moving the disease towards the "latent" end of the disease spectrum.³ In bovine MAP infection, a key question is whether helminth infection, including fasciolosis, can affect the timing and probability of "silent" MAP infection becoming clinically manifest as JD. The evidence here indicates that in vitro stimulation with *F. hepatica* products can reduce the responsiveness of bovine MDMs or ILN leucocytes to MAP antigens or

infection with MAP. However, further in vivo work is needed to determine whether higher fluke burdens of *F. hepatica* infection or a more chronic stage of the disease is likely to affect the progression of JD.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

The *F. hepatica* infection trial (experimental trial 1) was carried out by AG-C, with the help of AN-L and LG-C who collected samples from animals and carried out PBMC isolation. Experimental trial 2 was carried out by LB, and AN-L and LG-C collected samples and isolated ileocaecal lymph node leucocytes. AN-L performed laboratory work and analysed data. AB supported and supervised flow cytometry work. AN-L, GM and AZ designed the study and participated in the writing and editing of the manuscript. All authors read and approved the final manuscript.


PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/pim.12779>.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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