



# *Fasciola hepatica* infection reduces *Mycobacterium bovis* burden and mycobacterial uptake and suppresses the pro-inflammatory response

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## SUMMARY

*Bovine tuberculosis (BTB), caused by Mycobacterium bovis, has an annual incidence in cattle of 0.5% in the Republic of Ireland and 4.7% in the UK, despite long-standing eradication programmes being in place. Failure to achieve complete eradication is multifactorial, but the limitations of diagnostic tests are significant complicating factors. Previously, we have demonstrated that Fasciola hepatica infection, highly prevalent in these areas, induced reduced sensitivity of the standard diagnostic tests for BTB in animals co-infected with F. hepatica and M. bovis. This was accompanied by a reduced M. bovis-specific Th1 immune response. We hypothesized that these changes in co-infected animals would be accompanied by enhanced growth of M. bovis. However, we show here that mycobacterial burden in cattle is reduced in animals co-infected with F. hepatica. Furthermore, we demonstrate a lower mycobacterial recovery and uptake in blood monocyte-derived macrophages (MDM) from F. hepatica-infected cattle which is associated with suppression of pro-inflammatory cytokines and a switch to alternative activation of macrophages. However, the cell surface expression of TLR2 and CD14 in MDM from F. hepatica-infected cattle is increased. These findings reflecting the bystander effect of helminth-induced downregulation of pro-inflammatory responses provide insights to understand host-pathogen interactions in co-infection.*

**Keywords** *co-infection, Fasciola hepatica, monocyte-derived macrophages, Mycobacterium bovis, uptake*

## INTRODUCTION

The control of infectious diseases within populations, whether through vaccination programmes and/or identification and removal of infected individuals, is dependent on immune responsiveness to the pathogens concerned. Immune responsiveness to a particular pathogen within a population is subject to modulation by a multitude of factors including host genetics (1–3), reproductive cycles and the presence of co-infections (4). Such factors are easily controlled in experimental studies, but they may have a significant impact in the real world of naturally occurring infection (5–8). Parasitic helminths, ubiquitous in grazing livestock and common in humans in many parts of the world, typically use immunoregulatory strategies to achieve persistent infections, while reducing inflammatory pathology in the host (9,10). They also have the potential, through bystander immunoregulation, to alter the pathology and course of other infections in the same host (11–16). Here, we model some aspects of the interaction between a helminth and a bacterial pathogen, both of which cause economically important diseases of livestock.

The liver fluke, *Fasciola hepatica*, is a highly prevalent helminth parasite of farmed ruminants (17), estimated to cause annual losses of about €2.5 billion annually to the livestock industry worldwide. *F. hepatica* also affects a wide range of other mammals, including humans, with approximately 170 million people at risk of infection (18). *Mycobacterium bovis*, the causative agent of bovine tuberculosis (BTB), also has zoonotic implications; as an

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estimate, 3.1% of human tuberculosis cases globally are caused by *M. bovis* (19). In some developed countries, eradication programmes have reduced or eliminated BTB. However, reservoirs of *M. bovis* in wildlife (20–22), together with the limitations of diagnostic tests (23), have made complete eradication difficult in others (24).

In previous studies, we have shown that two diagnostic tests used in BTB eradication schemes, the single intradermal comparative cervical tuberculin test (SICCT) and the *in vitro* interferon (IFN)- $\gamma$  assay, were between 50% and 80% less effective in detecting *M. bovis*-infected cattle when the animals were co-infected with *F. hepatica* (25,26), leading to a lower positive predictive value of the tests. This was also shown in a large-scale epidemiological study in England and Wales, where dairy herds with a high prevalence of *F. hepatica* infection had a lower number of BTB reactors (6). This reduced sensitivity of the diagnostic tests was hypothesized to be due to an immunosuppression induced by *F. hepatica* infection. A downregulation of the Th1 immune response in other co-infection models has been associated with an increase in bacterial loads (14–16,27). Thus, we proposed that these changes in *F. hepatica*-infected animals might also result in an increase in the *M. bovis* numbers in co-infected animals.

Previously, it has been shown that CD14 played a major role in phagocytosis of *M. bovis* BCG by macrophages, and optimal uptake required the presence of both CD14 and complement receptor 3 (CR3). In addition, co-expression of CD14 and TLR2 markedly enhanced uptake of BCG (28). Other studies have shown the importance of these receptors in mycobacterial phagocytosis (29,30) and immune responsiveness (31,32). Thus, given that expression of these receptors might vary after infection with helminths (33), we proposed that TLR2 and CD14 could be modified in macrophages from *F. hepatica*-infected cattle, leading to alterations in mycobacterial uptake and recovery from such macrophages. Next, we investigated the cytokine profile and the activation of macrophages *in vitro* after *F. hepatica* infection of cattle. Alternatively activated macrophages, known to develop in *F. hepatica* infections (34,35), are not efficient at bacterial phagocytosis (36). In addition, these alternatively activated macrophages are characterized by the production of Th2 and Treg cytokines and a reduced production of Th1 cytokines. Some of these pro-inflammatory cytokines such as IL-12, IL-1 $\beta$  and IL-6 directly affect IFN- $\gamma$  production (37–39) and the immune response in the SICCT (40), the two standard tests for the diagnosis of BTB in Europe. Thus, we hypothesized that an alteration in the pro-inflammatory cytokine profile in MDM from *F. hepatica*-infected cattle could relate to a modified bacterial uptake as well as explaining the lower response to the

diagnostic tests for BTB in animals co-infected with *M. bovis* and *F. hepatica*.

## MATERIALS AND METHODS

### Experimental design

For the co-infection experiments (experiments 1 and 2), male castrated Holstein–Friesian cattle aged between 3 and 7 weeks of age (12 animals for each experiment) were purchased from farms known to be TB-free, as determined by the official tuberculosis eradication programme in the UK, and where fasciolosis had not been reported. Animals were housed in Containment Level 3 (CL3) facilities at AFBI (Stormont, Belfast, UK) as described by Flynn *et al.* (26). Animals were then randomly divided into two groups and assigned to *M. bovis*-only-infected group ( $n = 6$ ) or the *F. hepatica*–*M. bovis*-infected group ( $n = 6$ ), in order to compare mycobacterial burden. Blood was collected weekly to measure IFN- $\gamma$  production until week 14 (Experiment 1) and week 22 (Experiment 2).

For the *in vitro* experiment (Experiment 3), six male castrated Holstein–Friesian calves aged between 6 and 8 months of age were purchased from herds free of BTB and where *F. hepatica* infection was not reported. Animals were maintained under uniform housing conditions at University College Dublin (UCD) Lyons Research Farm (Newcastle, County Kildare, Ireland). All six animals were infected with *F. hepatica*, and mycobacterial recovery and uptake, TLR2 and CD14 receptor expression and cytokine production from MDM, compared prior to and after *F. hepatica* infection.

Experiments 1 and 2 were approved/licensed by the Animal Scientific Procedures Act 1986 (DHSSPS) under the scrutiny of the Animal Welfare and Ethical Review Body (AWERB), PPL2728, Agri-Food and Biosciences Institute, Belfast, UK. Experiment 3 was approved/licensed by the UCD Animal Research Ethics Committee/Health Products Regulatory Agency (AE18982/P023), University College Dublin, Ireland.

### Experimental *F. hepatica* infection of cattle

In all experiments, to ensure that animals were free from *F. hepatica* infection before starting the study, animals were serologically screened by ELISA using recombinant mutant *F. hepatica* cathepsin L1 (rmFhCL1), as previously described (35), and by faecal egg examination. For *F. hepatica* infection, 150 *F. hepatica* metacercariae (Baldwin Aquatics, (Oregon)), dispersed in 10 mL of dH<sub>2</sub>O, were administered orally *via* a syringe onto the back of the tongue. For confirmation of infection, numbers of parasites

in the liver of each animal in Experiment 1 (26) and Experiment 2 (Table S1) were recorded. In addition, specific antibody responses to *F. hepatica* infection were measured and no effect of *M. bovis* infection on such antibody levels to *F. hepatica* was observed in Experiment 1 (26) or Experiment 2 (data not shown). For Experiment 3, post-mortem examinations were not carried out, but seroconversion for *F. hepatica* infection using recombinant cathepsin-like 1 (rmFhCL1) by ELISA (Figure S1) was monitored as previously described (35).

### Bacterial strains and bacterial stocks

In all three experiments, *Mycobacterium bovis* AF2122/97, a UK isolate, was used. For Experiment 3, in addition, the nonvirulent *M. bovis* BCG-GFP Pasteur was used. All the work involving virulent *M. bovis* was performed in a Biosafety Containment Level 3 (CL3) laboratory and conformed to guidelines on the use of Hazard Group 3 infectious organisms.

*M. bovis* was cultured in Middlebrook 7H9 medium (Difco™, Becton Dickinson) containing 10% Middlebrook albumin–dextrose–catalase (ADC) enrichment 5% BSA (Fisher Chemical), 111 mM glucose (GPR), 145.4 mM NaCl (Fluka BioChemika) and 10 mM sodium pyruvate (Sigma–Aldrich) at 37°C. Bacterial cultures were grown to logarithmic phase (O.D = 1). To achieve this, 1 mL of bacterial frozen aliquot was diluted in 4 mL of Middlebrook 7H9 medium in a 50-mL Falcon tube and incubated at 37°C in static conditions for 5 days. Then, cultures were scaled up by adding the 5 mL of bacterial culture to 45 mL of Middlebrook 7H9 medium into an Erlenmeyer flask (Corning) and incubated at 37°C for further 2 weeks.

### Experimental *M. bovis* infection of cattle

For co-infection experiments (experiments 1 and 2), all animals were prescreened for immunological responses to the *M. bovis* antigens PPDA, PPDB, ESAT-6 and CFP-10 to ensure that there was no pre-existing exposure. All animals were housed in Containment Level 3 facilities under negative air pressure at AFBI Veterinary Sciences Division, Stormont. At 4 weeks post-*F. hepatica* infection, animals were exposed to low-dose aerosol challenge with *M. bovis* using a Madison chamber modified for use with cattle as previously described (41). The intended target infection dose was  $5 \times 10^2$  colony-forming units (CFU) of *M. bovis* strain AF2122/99, which has been demonstrated to be infective in animals while mimicking the natural infection (42). Actual doses delivered were calculated by side-flow sampling from the Madison chambers, and there

were no statistically significant differences between delivered doses between groups (See Table S2 of Supporting Information).

### IFN- $\gamma$ test

For the co-infection experiments (experiments 1 and 2), IFN- $\gamma$  production was measured in whole blood cultures after stimulation with PPDB-PPDA and ESAT-6 antigens, using the Bovigam Kit as per manufacturer's instructions.

### Post-mortem examination

Fourteen (Experiment 1) or 22 (Experiment 2) weeks after *F. hepatica* infection, the animals were euthanized and samples from each lung lobe, right and left bronchial lymph nodes, along with hepatic and cervical lymph nodes, were collected. Lungs were palpated by hand and then sliced at 1-cm intervals to reveal any tuberculous tissue. Each tissue sample was dissected carefully to reveal lesions indicative of tuberculosis, which were counted and measured. Lesions or suspect lesions, together with the other tissues, were sliced into cubes measuring approximately 5 mm in diameter and sent for bacteriology for preparation of cultures (see details below) and for histology processing as previously described (43,44).

For confirmation of *F. hepatica* infection, each liver was carefully sliced at 1-cm intervals. The bile ducts were squeezed to extrude any contained fluke. After dissection, the cubes of liver tissue were placed inside two layers of muslin cloth, soaked in water and squeezed several times. The inside of the cloth was then rinsed in water to remove flukes. All flukes were fixed in formaldehyde for microscopic verification of fluke morphology and counting.

### Bacterial recovery from tissues

For experiments 1 and 2, the cubed tissue samples were placed into double-thickness stomacher bags with between 4 and 15 mL PBS and homogenized in a stomacher at maximum speed for 2 min. Tissue homogenates were decanted into sterile universal bottles for inoculation into media for qualitative and quantitative cultures.

### Qualitative culture

For qualitative culture, tissue homogenates were inoculated into the BACTEC MGIT 960 culture system and incubated for up to 56 days. Samples identified as positive by the BACTEC MGIT 960 system were stained using the Ziehl–Neelsen method for confirmation purposes. Samples containing typical acid-fast rods were presumed to be

positive for *M. bovis* and analysed further by VNTR to identify the infection strain (AF2122/97).

#### Quantitative culture

For quantitative culture, tissue homogenates were decontaminated in 0.075% hexadecylpyridinium chloride (HPC). Hundred microlitres of this was serially diluted and inoculated in quadruplicate onto 7H11-OADC agar plates and incubated at 37°C for up to 6 weeks. *M. bovis* colonies were counted weekly from 3 weeks onwards and the average colony counts at 6 weeks post-inoculation used to calculate the total number of CFU per gram of sample. *M. bovis* colonies were initially identified on the basis of colony morphology and a selection of colonies was prepared for VNTR typing to confirm isolation of AF2122/97.

#### Isolation of monocyte-derived macrophages (MDM)

For Experiment 3, 300 mL of blood was collected per each of the 6 animals, into lithium heparin vacutainers (Cruinn Diagnostics), pre-*F. hepatica* infection and at 4, 8 and 10 weeks post-*F. hepatica* infection. Then, peripheral blood mononuclear cells (PBMC) were isolated by the density gradient centrifugation method using Histopaque (Sigma–Aldrich). Next, anti-CD14+ microbeads (Miltenyi Biotec) were used to isolate monocytes as per manufacturer's instructions (detailed protocol in Supporting Information Page 17). Cell viability was checked with trypan blue (Sigma–Aldrich), and selected CD14+ cells were adjusted to  $1 \times 10^6$  cells/mL in complete medium (RPMI-1640 [Bio-Sciences], 1% glutamine [Sigma–Aldrich], 1% nonessential amino acids [Sigma–Aldrich], 15% heat-inactivated foetal calf serum [Bio-Sciences], 1% penicillin–streptomycin [Sigma–Aldrich]) and plated in 12-, 24- or 96-well plates (Greiner), depending on the experiment.

#### MDM culture, stimulation and infection

For Experiment 3, plates were incubated for 7 days at 37°C, 5% CO<sub>2</sub> to allow the maturation of monocytes into macrophages as previously described (45). The culture medium was changed every 2–3 days. From Day 3 onwards, antibiotic-free medium was used. At Day 6, selected wells containing MDM were stimulated with *F. hepatica* antigen by adding 20 µg/mL of FhES. For FhES preparation, see Method S2 in Supporting Information. At Day 7, *M. bovis* or *M. bovis* BCG was added at a ratio of 1 : 1 (45) to selected wells containing either FhES or PBS and then incubated for a further 24 h. PBS was used as a negative control.

For flow cytometry experiments, at Day 7 PPDB (1 µg/mL) (kindly provided by Prof. Eamon Gormley, UCD) was added to selected wells, TLR2 as a positive control (46). In all the cases, at least 3 wells were used for each *in vitro* condition, with 3 unstimulated controls (PBS), 3 mycobacteria, 3 mycobacteria plus FhES and 3 FhES wells, per animal.

#### Confirmation of bacterial infection of MDM

Ziehl–Neelsen staining was used to confirm intracellular macrophage infection with *M. bovis* or *M. bovis* BCG (see Method S2 and Figure S2 in Supporting Information).

#### Bacterial recovery from MDM (CFU)

In Experiment 3, for bacterial recovery from MDM, mycobacterial colony-forming units (CFU) were calculated. When culturing *M. bovis*, agar plates were made using Middlebrook 7H11 medium (Difco™, Becton Dickinson) containing 10 mM of sodium pyruvate (Sigma–Aldrich) and 10% ADC. For BCG-GFP culture in agar, glycerol 1% (Sigma) was added instead of sodium pyruvate.

After removal of medium and extracellular bacteria from the 96-well plates, cells were lysed by the addition of 65 µL of 0.1% Triton X-100 (Sigma–Aldrich) for 5 min. Next, the lysates were serially diluted in Middlebrook 7H9 medium at concentrations from  $10^{-2}$  to  $10^{-4}$  before plating out in triplicate on agar plates. In parallel, 3 wells for each condition and animal were used to count the number of total macrophages in each well. After incubation of agar plates for 4 weeks, colony counts were performed and colony-forming units (CFU)/macrophage were calculated using the formula:

$$\text{CFU per macrophage} = \frac{\text{average of CFU in 3 agar plates} \times 200 \mu\text{L (total volume of the dilution)}}{50 \mu\text{L (volume used to plate out bacteria)} \times 10^3 \text{ (dilution factor)} \times 65 \mu\text{L (volume of lysis buffer added to the well)}}{20 \mu\text{L (volume used to do the dilution)}/\text{number of macrophages in a well.}$$

#### Bacterial uptake and receptor expression in MDM by flow cytometry

##### Quality control measures

In Experiment 3, titration of antibodies for flow cytometry was carried out by performing serial dilutions (1/20 to 1/10 000) and selecting the optimal concentration in pilot experiments. Instrument quality control was carried out using Cytometer Setup and Tracking Beads (BD CS&T beads), which were also used to standardize fluorescence

across samples acquired on different days. Final voltage settings were set using single-stained cell controls. For the gating strategy and confirmation of consistency of the results, fluorescence minus one controls (FMO) (cells containing all fluorochromes except one) were used. Unstained samples, as well as stained controls noninfected and nonstimulated for each animal and each time point, were included.

#### Harvesting of macrophages and viability staining

At Day 8 post-MDM culture (24 h post-BCG-GFP stimulation), medium and extracellular bacteria were washed off and macrophages were harvested by incubating cells for 10 min at 37°C with Accutase® (Sigma). For the single positive control for viability dye, a mixture of dead/alive cells was prepared, as per manufacturer's instructions. Next, 50 µL of previously titrated viability dye (Fixable Viability Dye eFluor® 780, eBiosciences) (1/1000 in D-PBS) was added to 50 µL of D-PBS containing the cells. Then, tubes were incubated for 30 min at 2–8°C in the dark. After this, the excess dye was washed off (D-PBS [cell culture grade, Gibco], 3% heat-inactivated foetal calf serum [Gibco] and 0.09% sodium azide [Sigma–Aldrich], pH 7.5) and cell pellets were incubated in 50 µL of blocking buffer (0.05 M Tris-buffered saline, pH 7.4, containing 1% normal bovine serum, 0.1% azide [Sigma–Aldrich] and 2% heat-inactivated foetal calf serum [Gibco]) for 20 min at 4°C.

#### Antibody staining and cell fixation

TLR2 antibody CD282, a bivalent human recombinant Fab antibody (AF 647 CD282 (AbD Serotec)), was prepared at 1/10 dilution in staining buffer. CD14 antibody, a mouse anti-human antibody cross-reactive with bovine (PerCP-Cy5.5 CD14 (BioLegend)), was diluted 1/25 in staining buffer. Twenty-five microlitres from each diluted antibody was added to 50 µL of blocking buffer containing cells, to have a final concentration of 1/40 for TLR2 and 1/100 for CD14 antibody. For detailed antibody description, see Table S5 included in Document S1 of Supporting Information. Then, tubes were incubated at 4°C for 1 h. After washing cell pellets, those were fixed in 100 µL of 1% paraformaldehyde in D-PBS for 1 h. Then, 550 µL of staining buffer was added and tubes were left overnight at 4°C until flow cytometry processing of samples.

#### Instrument and data analysis details

A Beckman Coulter model Cyan ADP Lx flow cytometry machine was used. The 488-nm laser was selected for FL1 (530/40-GFP) and FL4 (680/30 – PerCP-Cy5.5). The 635-nm laser was used for FL8 (665/20 – Alexa Fluor 647) and FL9 (750LP – eFluor 780).

Data were analysed with Beckman Coulter Summit V4.03.02 Build 2451 software, and re-analysis was carried out with Summit and De Novo Software FCS Express 4 RUO. Data files were kept as FCS data files, compensation was not required, and the gating applied was carried out by including FMO controls which were the same cells as used in the experiment. Gating was applied by selecting target cells based on FSC vs. SSC plot, pulse width vs. FSC area, SSC line vs. SSC area and LD (live death cells) vs. FSC (Figure S3). The purity of monocyte-derived macrophages was 99% as indicated by CD14 labelling. The median of fluorescence intensities of each sample obtained was normalized to its own unstimulated control, resulting in a fold change value, so that BCG-stimulated samples were comparable with Pre-F.hep and Post-F.hep. For further information, see MIFlowCyt Standard in Document S1.

#### mRNA analysis of MDM

##### mRNA extraction and cDNA transcription

In Experiment 3, for RNA extraction, the EZNA Total RNA Kit 1 (OMEGA Bio-Tek) was used as per manufacturer's instructions. For reverse transcription into cDNA, 500 ng of RNA was added to 12 µL of RNase-free water (Sigma–Aldrich), containing 1 µL of oligo DT (0.5 µg/µL) and 1 µL of 10 mM dNTP mix (Promega). A master mix containing 1 µL of Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) (Biosciences), 4 µL of first-strand buffer (5×), 2 µL of 0.1 M DTT and 1 µL of RNase OUT (40 U/µL) (Biosciences), per sample, was prepared. Nonenzyme controls (no MMLV) and a nontemplate control (RNase-free water) were also prepared. Once samples were incubated at 65°C for 5 min and at 4°C for 2 min, 8 µL of the master mix per vial was added. Samples were then incubated at 37°C for 50 min followed by 15 min at 70°C, and from then until the cDNA was fully transcribed, kept at 4°C. cDNA samples were stored at –20°C until further processing.

qPCR. For real-time qPCR, all samples and controls were included in duplicate. Nontemplate control and nonenzyme control cDNA template were included for each set of primers. Five nanograms of cDNA from each sample was added to the qPCR plate containing 10 µL of SYBR Green PCR (2×) (Applied Biosystems), 0.3 µL of the forward primer and 0.3 µL of the reverse primer, and these were adjusted to a final volume of 20 µL with RNase-free water. A standard qPCR program was used to run the reactions: 95°C for 1 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A dissociation step was included for all reactions

to confirm the presence of a single PCR product of the expected size. Reference genes were established by carrying out a GeneNorm analysis of 10 potential candidates for internal controls. Reference target stability was assessed across a set of 12 samples selected at random. Results were analysed using the GeNorm qbase<sup>PLUS</sup> software package (Biogazelle NV, Belgium) as described by Hellemans *et al.* (47). Analysis determined peptidyl-prolyl isomerase A (PPIA) to be the most stable target gene for our samples with an M (gene expression stability value) <0.02, and PPIA was included as internal control on all plates analysed. Intron-spanning primer was designed for arginase I, iNOS, IL-6 and TGF- $\beta$  using the Primer3Plus package and synthesized commercially (Eurofins MWG Operon, Ebersberg, Germany) (48):

(Arg1\_fwd\_180: 5'-CCAAGGTGTGTGGGAAAAGC-3';  
Arg1\_rev\_180: 5'-GATGTCCGTGTGAGCATCCA-3'),  
(iNOS\_fwd: 5'-AGAGACGGGGAGATCGGAAA-3';  
iNOS\_rev: 5'-CATGCAGAGAACCTTGGGGT-3'),  
(IL-6\_fwd: 5'-ACGAAAGAGAGCTCCATCTGC-3';  
IL-6\_rev: 5'-AATGGAGTGAAGGCGCTTGT-3'),  
(TGF- $\beta$ \_fwd: 5'-ACAATTCCTGGCGCTACCTC-3';  
TGF- $\beta$ \_rev: 5'-ATTTCCTCTCTGCGGGTCAG-3')

### Nitric oxide and cytokine production

In Experiment 3, nitric oxide (NO) production in MDM supernatants was measured using the Griess reagent system (Promega), according to the manufacturer's instructions. IL-6, IL-1 $\beta$  and TGF- $\beta$  were measured using the ELISA kits for Bovine IL-6 (Cat: ESS0029, Thermo Scientific), IL-1 $\beta$  (Cat: ESS0027, Thermo Scientific) and TGF- $\beta$  (Cat: G7590, Promega Corporation), according to the manufacturer's instructions. IL-12 was measured using paired antibodies. A standard curve (Cat: RP0077B, Kingfisher Biotech) was included starting from 5000 ng/mL. The working volumes were 100  $\mu$ L/well, washing steps were carried out with PBS-Tween three washes, and incubation times were 1 h at room temperature unless otherwise indicated. The capture antibody for IL-12 (MCA1782EL, AbD Serotec) was coated onto a 96-well plate (8  $\mu$ g/mL) and incubated at RT overnight. After this, plates were washed and blocked using 1% BSA (Sigma-Aldrich) in PBS-T. After the addition of the samples and incubation, the detection antibody (MCA2173B, AbD Serotec) was added (5  $\mu$ g/mL). For the next step, streptavidin-HRP (1 : 5000 dilution) (Invitrogen) was added and incubated for 45 min and subsequently washed 5 times. TMB was used to develop the colour under dark conditions for 15 min. Finally, the reaction was stopped by the addition of 1 N H<sub>2</sub>SO<sub>4</sub> (Fisher Chemical), and the absorbance was read at 450 nm.

### Statistical analysis

For co-infection studies (experiments 1 and 2), the response variable Y for each animal (CFU) was defined as  $Y = \log(1 + \text{total CFU})$  where total CFU denotes the sum of bacterial CFU recovered from the left bronchial lymph node, the right bronchial lymph node, the caudal mediastinal lymph node, the cranial mediastinal lymph node and the cervical lymph node. The treatment effect is the difference between the mean of Y in animals co-infected vs. infected with *M. bovis* only. Data from experiments 1 and 2 were combined, and analysis was carried out by randomized block ANOVA, with 'experiment' as the blocking factor on two levels. Firstly, the hypothesis that fluke infection reduces mycobacterial load was tested. Secondly, we tested the hypothesis that in co-infected animals, fluke count was negatively associated with intensity of *M. bovis* infection. A simple linear regression analysis adjusted for 'experiment' as a blocking factor on two levels was used to determine the association between fluke count and *M. bovis* load and the number of *M. bovis*-positive tissues.

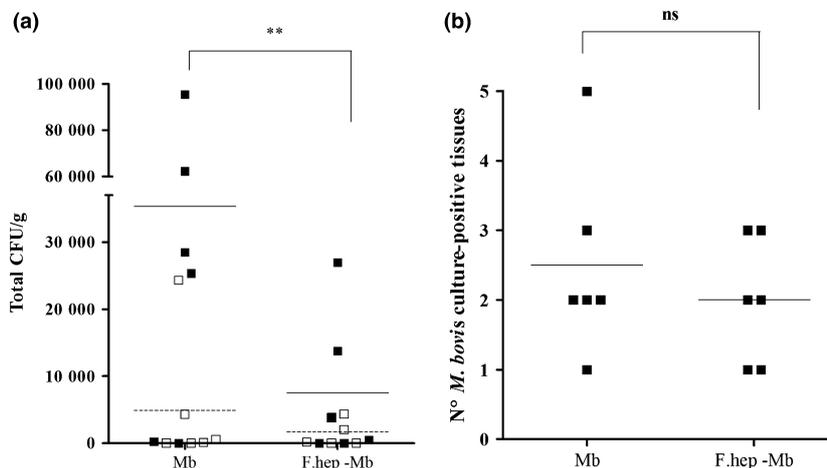
For Experiment 3, data were analysed using one-way ANOVA for comparison of more than 2 groups and *t*-test for comparison of 2 groups, using the software GraphPad Prism 5. For qPCR data, relative gene expression fold changes for each sample were calculated according to calibrated normalized relative quantities (CNRQ) utilizing the qbase<sup>PLUS</sup> package (Eurofins MWG Operon).

## RESULTS

### Effect of *F. hepatica* infection on bacterial load in cattle following aerosol challenge with *M. bovis*

For experiments 1 and 2, statistical analysis of bacterial burden revealed that although the numbers of bacteria recovered differed between the two experiments, there was no interaction between treatment (co-infected or *M. bovis* only infected) and experiment ( $P = 0.657$ ). Combining the results of both experiments, the total *M. bovis* bacterial load was significantly lower in co-infected animals ( $P = 0.0031$ ), allowing for a significant difference ( $P = 0.017$ ) between experiments (Figure 1a). In Experiment 1, all animals were confirmed positive for *M. bovis* infection with either the qualitative or quantitative technique, with the exception of 1 animal in the co-infected group (26). In Experiment 2, all animals were confirmed positive in at least one tissue. The number of culture-positive tissues was lower in the co-infected group; however, this was not statistically significant (Figure 1b). We also compared the number of liver flukes and bacterial load between animals; however, there was no correlation (data not shown).

The distribution of infection identified through bacterial culture was similar in both the *M. bovis* and the co-



**Figure 1** Effect of *F. hepatica* Infection on Bacterial Recovery in *M. bovis*-infected Cattle. For experiments 1 and 2, one group of 6 animals in each was infected with  $5 \times 10^2$  CFU *M. bovis* and the other 6 animals with  $5 \times 10^2$  CFU *M. bovis* and 150 metacercariae from *F. hepatica*. Tissues were recovered, homogenates were cultured onto 7H11-OADC agar plates, and CFU were counted per gram of tissue, to obtain the quantitative value. CFU were transformed to  $Y = (1 + \text{total CFU})$ . Data from experiments 1 and 2 were combined, and analysis was carried out by randomized block ANOVA, with 'experiment' as the blocking factor on two levels. Co-infected animals (*F. hep-Mb*) showed a reduced total bacterial recovery in comparison with those infected with *M. bovis* only (Mb). \*\* means  $P = 0.0031$  with a 95% confidence interval  $-9.083 \pm 5.446$  (a). For qualitative culture, tissue homogenates were cultured into the BACTEC MGIT 960 system, which gave a positive or negative result. In Experiment 1, all animals were culture positive with the exception of 1 in the *M. bovis-F. hepatica* group (Flynn *et al.* (2009)). In Experiment 2, all animals were confirmed infected with *M. bovis* with either the qualitative or quantitative technique (b). Co-infected animals showed a reduction in the number of *M. bovis* culture-positive tissues; however, the differences were not statistically significant (ns) (b). White or black squares correspond to each animal of Experiment 1 or Experiment 2, respectively. Dashed lines (Experiment 1) or black lines (Experiment 2) indicate the mean CFU per gram of tissue (a) or mean number of *M. bovis* culture-positive tissues (b). See Table S3 and Table S4 for detailed information in the bacterial counts.

infected groups with the majority of bacteria recovered from the bronchial, mediastinal and cervical lymph nodes (Tables S3 and S4). A small number of additional isolates were recovered from lung tissues (Experiment 2) in which small (<1 cm in diameter) early-stage lesions were visible projecting from pleural surfaces with central cores of caseous necrosis. Overall, no qualitative or quantitative differences were observed in tuberculous lesions between the *M. bovis*-infected and *F. hepatica-M. bovis*-infected animals. In both treatment groups, after microscopical examination, lesions exhibited varying degrees of central caseation necrosis with contained foci of dystrophic mineralization. Necrotic cores were bounded by layers of closely apposed macrophages, macrophage giant cells and interspersed lymphocytes. This layer in turn was surrounded by bands of fibrosis. While there was some minor variation in the extent of these lesion components, there was no major difference in these features between the two groups (data not shown).

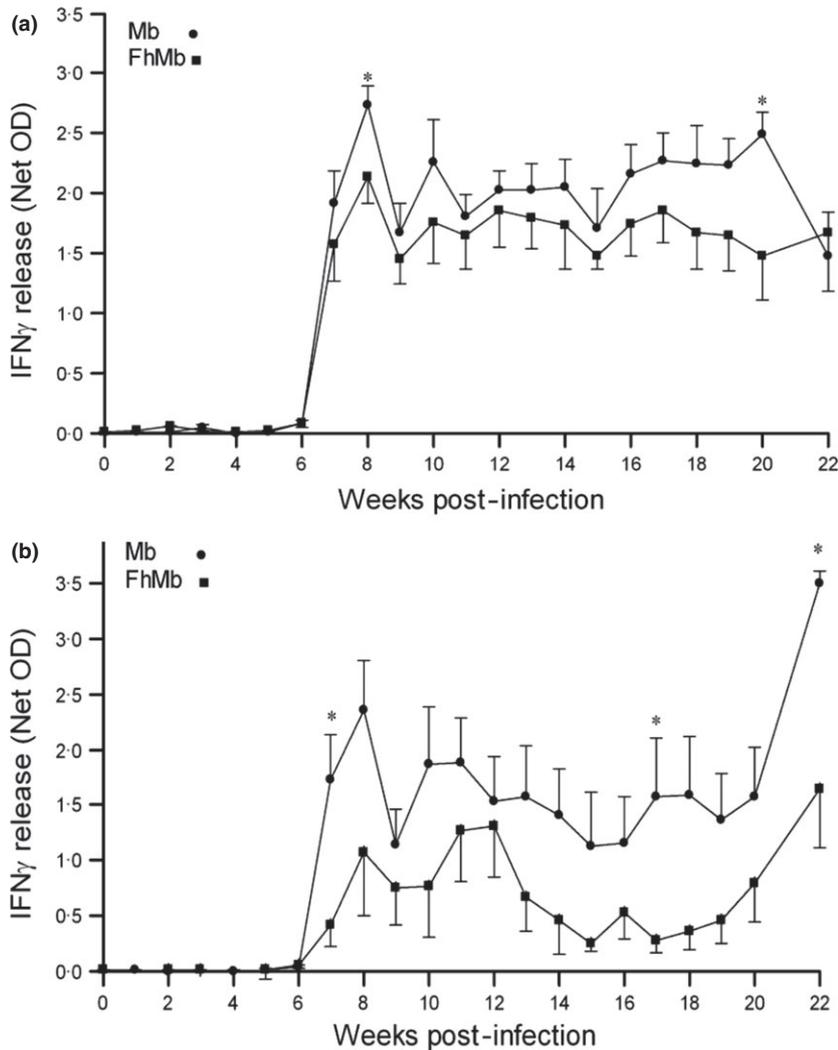
#### Effect of *F. hepatica* infection on IFN- $\gamma$ production in animals co-infected with *M. bovis*

In co-infection experiments (experiments 1 and 2), IFN- $\gamma$  production was measured in whole blood cultures after stimulation *in vitro* with PPDB-PPDA and ESAT-6. In

Experiment 2, both groups responded with distinct increases in IFN- $\gamma$  release after week 6 (2 weeks post-*M. bovis* infection) in response to PPDB-PPDA stimulation and these increases were sustained throughout the experimental period. In the group co-infected with *F. hepatica* and *M. bovis*, there was a noticeably reduced IFN- $\gamma$  response being statistically significant at 7 and 20 weeks post-infection ( $P < 0.05$ ) (Figure 2a). Similarly, co-infected animals showed a reduced IFN- $\gamma$  production in response to ESAT-6 which was statistically significant at 7, 17 and 22 weeks post-infection ( $P < 0.05$ ) (Figure 2b). These results are similar to those of Flynn *et al.* (26) (Experiment 1).

#### The effect of *F. hepatica* infection on mycobacterial recovery in monocyte-derived macrophages infected with *M. bovis* and *M. bovis* BCG *in vitro*

In parallel, we used an *in vitro* study (Experiment 3) to examine the effect of *F. hepatica* infection on bovine monocyte-derived macrophages (MDM) in response to *M. bovis*, *M. bovis* BCG-GFP and *F. hepatica* excretory/secretory antigen (FhES). First, acid-fast (ZN) staining was used to confirm mycobacterial infection of MDM (Figure S2). Next, cultures of mycobacteria in agar plates, obtained from lysed stimulated MDM were analysed. The



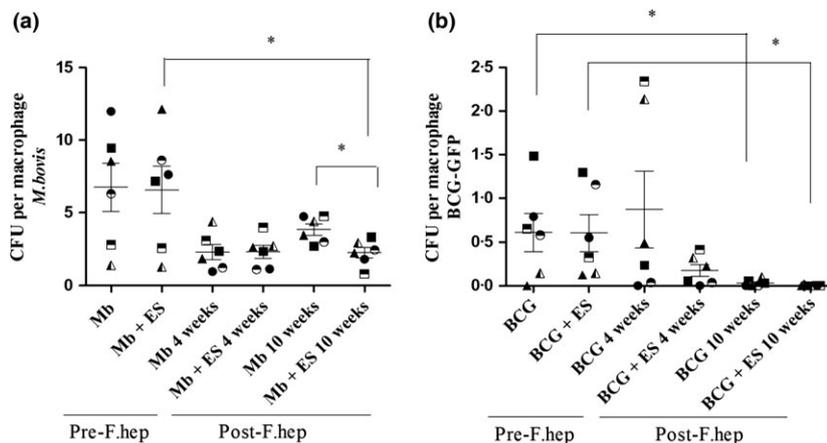
**Figure 2** Effect of *F. hepatica* infection on IFN- $\gamma$  production in *M. bovis*-infected cattle. For co-infection experiments IFN- $\gamma$  release was measured from whole blood cultures following stimulation with PPDB-PPDA or ESAT-6. Results (Net OD) were calculated from PPDB minus PPBA, with positives indicated by the value for PPDB greater than 0.1 OD and PPDB-PPDA greater or equal to 0.05 OD (a). In (b), Net OD was calculated from ESAT-6 minus PBS, with positives indicated by a value >0.05 OD. Co-infected animals with *F. hepatica* and *M. bovis* (squares) showed a reduced response to the mycobacterial antigens in comparison with only *M. bovis*-infected animals (circles). \* =  $P < 0.05$ .

mean number of *M. bovis* recovered from cultures prior to *F. hepatica* infection was 6 colony-forming units (CFU) per macrophage, which decreased to 4 CFU per macrophage 10 weeks post-*F. hepatica* infection. At this time point, the numbers of *M. bovis* recovered from MDM co-stimulated with FhES decreased further to 2 CFU per macrophage, which was significantly different in comparison with before *F. hepatica* infection ( $P < 0.05$ ) (Figure 3a). Four weeks post-*F. hepatica* infection, bacterial recovery tended to be lower than prior to *F. hepatica* infection also; however, differences were not statistically significant. In the same way as described for *M. bovis*, the nonvirulent BCG-GFP was cultured in agar plates. A reduction in the numbers of BCG-GFP recovered (CFU) was seen 10 weeks post-*F. hepatica* infection ( $P < 0.05$ ); in this case, the addition of FhES did not alter these numbers (Figure 3b).

#### The Effect of *F. hepatica* Infection on Bacterial Uptake and TLR2-CD14 Expression in Macrophages Infected with BCG-GFP *in vitro*

Next, we used flow cytometry to investigate whether bacterial uptake and surface receptor expression in MDM would be modified by *F. hepatica* infection. At 8 weeks post-*F. hepatica* infection, the fluorescence intensity of BCG-GFP-infected MDM was shifted towards the left, indicating a reduction in fluorescence intensity (Figure 4a). However, the fluorescence intensity of TLR2 and CD14 receptors from BCG-GFP-infected MDM moved to the right after *F. hepatica* infection, indicating an increase in fluorescence intensity (Figure 4b, c).

Statistical analysis of the data from all animals showed that after *F. hepatica* infection, bacterial uptake was lower than before *F. hepatica* infection ( $P < 0.05$ ) (Figure 4d).



**Figure 3** Effect of *F. hepatica* Infection on Recovery of *M. bovis* and *M. bovis* BCG-GFP from MDM. For Experiment 3, MDM were isolated from cattle before, and 4 week and 10 week after *F. hepatica* infection and then incubated with *M. bovis* (Mb) (a) or BCG-GFP (b) (MOI 1 : 1) *in vitro* for 24 h or added to MDM previously stimulated with FhES (ES) (20  $\mu\text{g}/\text{mL}$ ) for 24 h. Cultures in 7H11-OADC agar plates of mycobacteria show that 10 weeks post-*F. hepatica* infection, *M. bovis* recovery per macrophage was lower than before *F. hepatica* infection, although this was not statistically significant until after co-stimulation with FhES ( $*=P < 0.05$ ) (a). Similarly, lower numbers of BCG-GFP were recovered 10 weeks post-*F. hepatica* infection either with or without FhES restimulation ( $*=P < 0.05$ ) (b). The CFU per macrophage were calculated, with three replicates per animal (represented as different symbols). Horizontal lines represent the mean CFU per macrophage  $\pm$  SEM,  $n = 6$ .

On the contrary, TLR2 expression following *F. hepatica* infection was increased and statistically significant when MDM were restimulated with FhES *in vitro* (Figure 4e). In the case of CD14, its increased expression after *F. hepatica* infection was statistically significant ( $P < 0.05$ ) regardless of the addition of FhES (Figure 4f). Nevertheless, these surface receptors and bacterial uptake did not show a statistically significant inverse correlation (data not shown).

#### The effect of *F. hepatica* infection on macrophage activation and cytokine profile

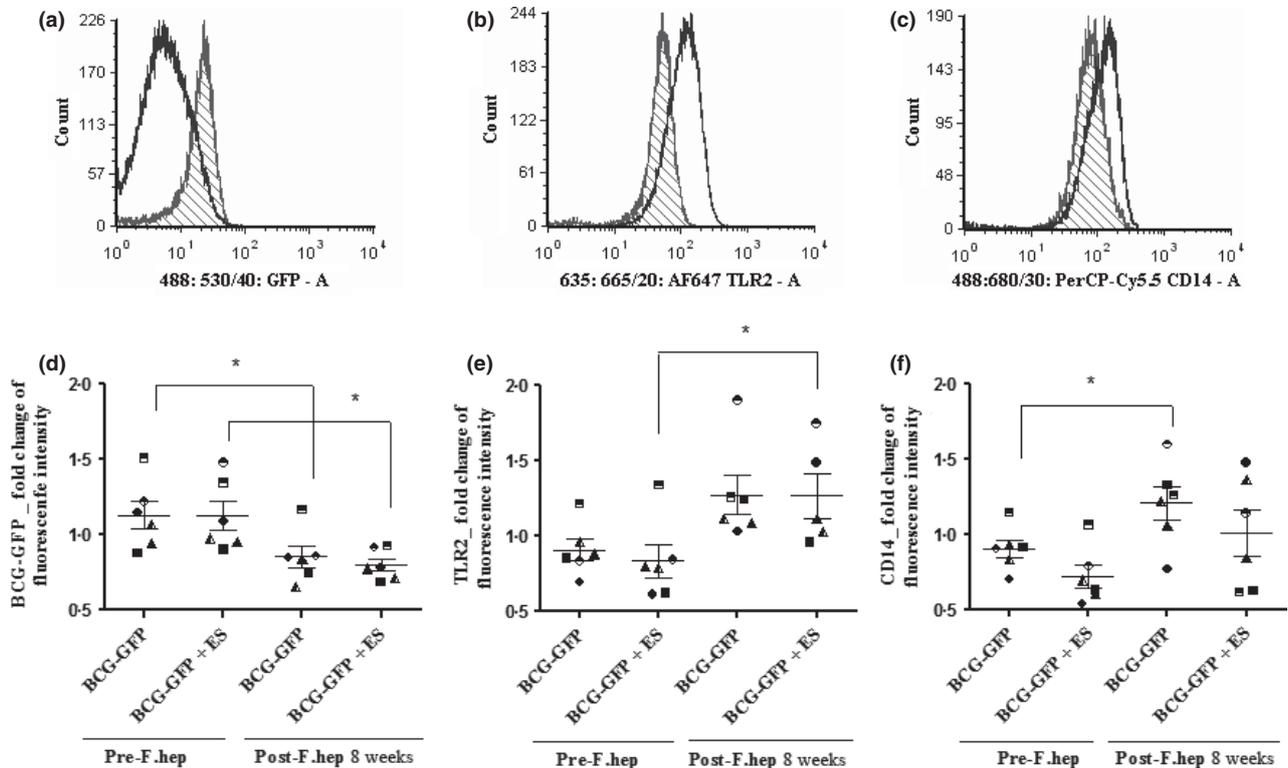
We hypothesized that reduced mycobacterial recovery after *F. hepatica* infection of cattle could be associated with an alteration in the activation of macrophages and their cytokine profile. To study such modification in macrophage phenotype, we measured arginase and nitric oxide production, markers of alternative and classical activation, respectively. MDM showed upregulated arginase I mRNA expression at 10 week after *F. hepatica* infection ( $P < 0.05$ ) (Figure 5a). However, as expected, there was a downregulation in iNOS mRNA expression (fold change of 5500) ( $P < 0.05$ ) and nitric oxide production ( $P < 0.01$ ) in *M. bovis*-stimulated MDM after *F. hepatica* infection (Figure 5b, c). Next, we investigated the levels of a panel of cytokines. The levels of pro-inflammatory cytokines IL-1b, IL-12 and IL-6 were reduced after *F. hepatica* infection ( $P < 0.001$ ,  $P < 0.01$  and  $P < 0.05$ , respectively),

whereas TGF- $\beta$  was increased after *F. hepatica* infection ( $P < 0.05$ ) (Figure 6).

#### DISCUSSION

We have previously described reduced responsiveness to *M. bovis* diagnostic tests (SICCT test and the IFN- $\gamma$  test) in cattle co-infected with *F. hepatica* (26). However, to date, there has been no evidence to link this effect to bacterial load or disease dynamics. Given the substantial resources expended in national control programmes for bovine tuberculosis, which rely on those immunodiagnostic tests, any such interactions warrant further study.

Thus, one of our objectives was to study the effect of *F. hepatica* on bacterial load from *M. bovis*-infected animals. Helminth-mediated immunoregulation is a multifactorial phenomenon, with macrophage-mediated effects one of the principal facets involved. As macrophages are also key elements in the progress of and response to mycobacterial infections, we chose also to study changes in macrophages. Previous co-infection studies have shown that *Schistosoma mansoni* (27) and *Nippostrongylus brasiliensis* (14) co-infection in mice increased the bacterial burden and growth of *M. tuberculosis*, respectively. *F. hepatica* infection in cattle induced higher excretion rates in concurrent infection with *Salmonella Dublin* and more disseminated bacterial infection in the organs (15). Similarly, another study in mice showed that co-infection with *F. hepatica* and *Bordetella pertussis* delayed bacterial

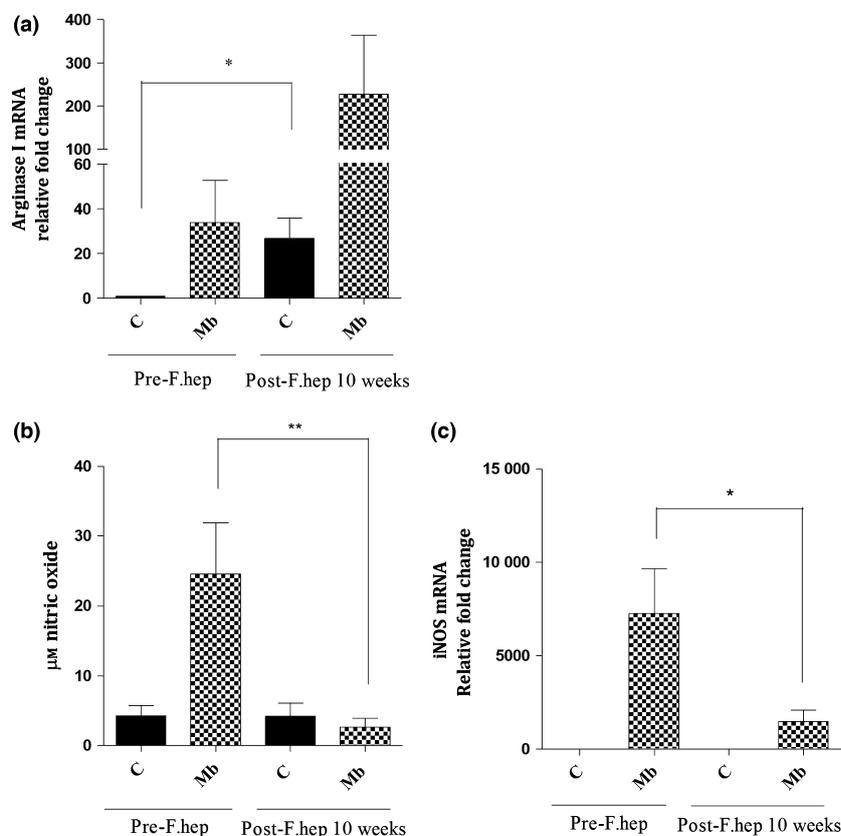


**Figure 4** Effect of *F. hepatica* Infection on Bacterial Uptake, TLR2 and CD14 Receptor Expression by MDM. In Experiment 3, MDM isolated from cattle prior to and 8 weeks post-*F. hepatica* infection were stimulated *in vitro* with BCG-GFP (MOI 1 : 1) for 24 h or FhES (20  $\mu$ g/mL) (ES) for 48 h. Then, MDM were collected and stained with the antibodies Alexa Fluor 643 TLR2 and with PerCP-Cy5.5 CD14 and analysed with flow cytometry. In the overlaid univariate histograms, a representative sample stimulated with BCG-GFP shows a shift towards the left in fluorescence intensity after *F. hepatica* infection of cattle (a). However, there is a shift towards the right when the CD14 and TLR2 receptors were measured after *F. hepatica* infection (b, c). Y-axis indicates cell count and the x-axis indicates median fluorescence intensity. Nonfilled histogram = Post-*F. hepatica* infection; filled line histogram = Pre-*F. hepatica* infection. A minimum of  $6 \times 10^4$  single cells have been recorded and analysed per sample (sample = biological replicate) (a–c). Statistical analysis of all samples from the 6 animals shows that at 8 week post-*F. hepatica* infection, bacterial uptake is reduced in MDM (d); however, TLR2 (e) and CD14 (f) receptor expression was maintained or increased. Each symbol with the same shape represents the average of 3 biological replicates of each animal. The median of fluorescence intensity obtained from the histograms was normalized to the unstimulated controls, and results were expressed as the mean  $\pm$  S.E.M (d–f). \* $P < 0.05$ ,  $n = 6$ . For gating strategy, see Figure S3.

clearance in the co-infected groups (16). Thus, based on our previous (26) results demonstrating reduced Th1 responses, with reduction in *M. bovis*-induced IFN- $\gamma$  production in animals co-infected with *F. hepatica* (Figure 2), we hypothesized that mycobacterial recovery and uptake would be enhanced in the presence of the helminth infection. In contrast, however, the opposite was observed, with reduced mycobacterial burden directly recorded from the tissues of co-infected animals (Figure 1). Our data also show that bacterial recovery from MDM infected with *M. bovis* *in vitro* was reduced after helminth infection (Figure 3). Additionally, given that *F. hepatica* ES antigen (FhES) is able to induce the production of Th2 and Treg cytokines as well as arginase (25,49), we suggested that FhES could further influence such mycobacterial uptake and survival. In this case, the addition of FhES to

macrophages further decreased *M. bovis* survival (Figure 3a). However, when the nonvirulent *M. bovis* BCG was used for the experiments (Figure 3b and Figure 4d), the addition of FhES did not result in any further differences in bacterial uptake or recovery in comparison with the BCG-only-stimulated cells. This could be explained because *M. bovis* BCG uptake and growth is much lower than when *M. bovis* is used (45), so a subtle further difference added with FhES may not have been detected.

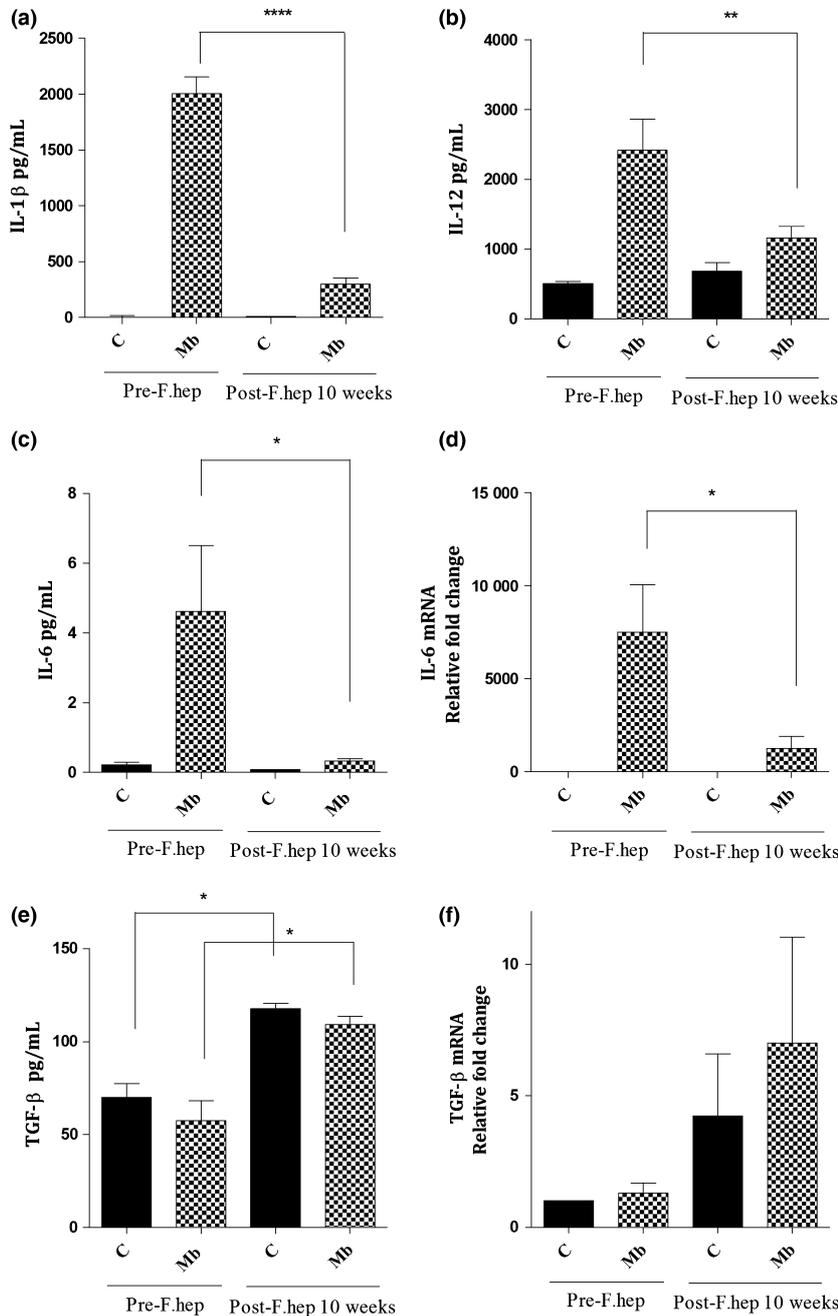
Nevertheless, the finding of lower mycobacterial uptake and recovery after *F. hepatica* infection of cattle is in line with some previous studies. It has been shown that rats co-infected with the nematode *Litomosoides sigmodontis* and *M. tuberculosis* had lower bacterial loads than rats with *M. tuberculosis* infection only (50). Likewise, another study demonstrated that acute *Nippostrongylus brasiliensis*



**Figure 5** Effect of *F. hepatica* Infection on Macrophage Activation. For Experiment 3, MDM were isolated from cattle before and 10 week after *F. hepatica* infection and then incubated with *M. bovis* (Mb) (MOI 1 : 1) *in vitro* for 24 h or left unstimulated. qPCR results show that arginase I mRNA in MDM was upregulated after *F. hepatica* infection of cattle, in both unstimulated MDM and after Mb stimulation. However, there was a downregulation in iNOS mRNA expression in Mb-MDM after *F. hepatica* infection (c) which coincided with reduction in nitric oxide production (b). \* =  $P < 0.05$ , \*\* =  $P < 0.01$ . Results express mean  $\pm$  SEM of relative fold change of mRNA (a,c) or  $\mu\text{M}$  nitric oxide (b),  $n = 6$ .

(Nb) infection in mice reduced early pulmonary mycobacterial colonization (11). Additionally, other studies have shown decreased *M. tuberculosis*-GFP uptake in human DC when they were pre-exposed to microfilaria (51). Next, we investigated a potential cause of this lowered bacterial uptake, the receptors TLR2 and CD14. TLR2 is one of the pathogen-recognition receptors (PRRs) on the surface of macrophages which recognize pathogen-associated molecular patterns (PAMPs) from mycobacteria (52,53). TLR2 together with its co-receptor CD14 has been associated with recognizing and triggering mycobacterial immune responses (31,32). Sendide *et al.* (2005) showed that in THP-1 and CHO cell lines, optimal *M. bovis* BCG uptake required the presence of both CD14 and complement receptor 3 (CR3) and co-expression of CD14 and TLR2 markedly enhanced such uptake (28). In bovine macrophages, Souza *et al.* (2007) demonstrated that mannose receptors and CD14 contributed to *Mycobacterium avium* phagocytosis (29). Blocking TLR2 receptors in bovine macrophages enhanced killing of *Mycobacterium avium paratuberculosis* by enabling phagosome maturation within the cell (30). Hence, we might have expected that lower bacterial uptake would be associated with reduced expression of TLR2 and CD14 after *F. hepatica* infection

of animals. However, expression of TLR2 and CD14 was conserved or enhanced in *F. hepatica*-infected animals (Figure 4b, c, e, f). In agreement with those findings, however, other studies have also shown enhancement of TLR2 expression following helminth infection (33,54). For example, schistosome-specific phosphatidylserine (PS) activated TLR2 in dendritic cells (55). In another study, dendritic cells and B cells from *Schistosoma mansoni* infected patients with multiple sclerosis, stimulated with soluble egg Ag (SEA), resulted in significant TLR2 upregulation (33). Given this inverse correlation between TLR2-CD14 expression and bacterial uptake, we examined other mechanisms involved in lower mycobacterial uptake by macrophages in the context of *F. hepatica* infection. For this, we studied the activation phenotype of macrophages after *F. hepatica* infection. Our results show that *M. bovis*-stimulated MDM had upregulated mRNA expression of arginase I but downregulated mRNA expression of iNOS, which was associated with lowered production of nitric oxide (Figure 5). Previously, it has been demonstrated that *F. hepatica* infection and *F. hepatica* products elicit alternative activation of peritoneal macrophages (56) and of a ruminant macrophage cell line (34). These alternatively activated macrophages, known to develop in *F. hepatica*



**Figure 6** Effect of *F. hepatica* Infection on Macrophage Cytokine Profile. For Experiment 3, MDM were isolated from cattle before and 10 week after *F. hepatica* infection of cattle and then incubated with *M. bovis* (Mb) (MOI 1 : 1) *in vitro* for 24 h or left unstimulated. The levels of cytokines IL-1 $\beta$ , IL-12, IL-6 and TGF- $\beta$  in MDM cultures were measured by ELISA (a–c, e) or qPCR (d, f). There was a decrease in the production of IL-1 $\beta$  (a), IL-12 (b) and IL-6 in Mb-MDM after *F. hepatica* infection (c). Similarly, IL-6 mRNA expression in Mb-MDM was downregulated (d). However, TGF- $\beta$  production (e) and TGF- $\beta$  mRNA expression (f) in MDM were increased after *F. hepatica* infection. \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\*\* =  $P < 0.001$ . Results express mean  $\pm$  SEM of pg/mL (a–c, e) or relative fold change of mRNA (d, f).  $n = 6$ .

infections (34,35), are not as efficient in phagocytosing bacteria as classically activated macrophages. For example, Varin *et al.* (2010) showed low uptake of *Neisseria meningitidis* after IL-4 and IL-13 stimulation of murine macrophages which was related to alternative activation of macrophages (36). As our study shows such phenotype switching after *F. hepatica* infection (Figure 5), alternative activation of macrophages could be the reason for a lower bacterial uptake. Additionally, another group found that

*F. hepatica* fatty acid binding protein (FABP) induced alternative activation of human macrophages and reduced production of the pro-inflammatory cytokine IL-1 $\beta$  by MDM after LPS stimulation (57). Likewise, we have demonstrated a diminution in IL-1 $\beta$  in MDM stimulated with *M. bovis* after *F. hepatica* infection (Figure 6a). In mycobacterial infections, IL-1 $\beta$  has been described as essential for recruiting immune cells to the site of infection (58). In addition, IL-1 $\beta$  is involved in direct activation of

humoral immune responses (59), antigen presentation, and expansion and recruitment of T cells to the skin, which in consequence promotes local inflammation in delayed-type hypersensitivity responses (40). As our data show that IL-1 $\beta$  production by *M. bovis*-infected MDM is reduced when animals are infected with *F. hepatica*, this could indicate that in co-infected animals, the responsiveness to the SICCT, which is based on a delayed-type hypersensitivity reaction, would be reduced. Moreover, IL-12 together with IL-1 $\beta$  is able to induce IFN- $\gamma$  production in Th1 lymphocytes (37,38). As we have found that *M. bovis*-stimulated MDM produced less IL-12 after *F. hepatica* infection (Figure 6b), this could explain why blood cultures from co-infected animals with *F. hepatica* had lower IFN- $\gamma$  production than those infected with *M. bovis* only (Figure 2) (25,26). Our results also show a reduction in IL-6 production by MDM after *F. hepatica* infection of cattle. Among the multiple functions of IL-6, it has been described to be a marker for *M. tuberculosis* infection in mouse peritoneal macrophages (60). In addition, IL-6 has been associated with inhibition of type I interferon, which is involved in disease progression (61) and with differentiation of T cells to the Th17 phenotype (62,63), which contributes to Th1 response expansion (64–66). A reduction in IL-6 production after *F. hepatica* infection, as shown in our study (Figure 6c, d), would potentially affect bacterial burden, and granuloma formation in *M. bovis* and BCG infections (64–66). On the other hand, our results show an increase of TGF- $\beta$  in MDM after *F. hepatica* infection. This regulatory cytokine has been found to be produced in *F. hepatica*-infected cattle, in PBMC stimulated with *F. hepatica* LFH (liver fluke homogenate) (67). In another study, peritoneal macrophages from naïve mice produced TGF- $\beta$ , IL-10 and arginase I after *in vitro* stimulation with *F. hepatica* ES as well as after *F. hepatica* infection of mice (68). In the context of *F. hepatica* and mycobacterial co-infection, TGF- $\beta$  would downregulate the type 1 responses elicited against the bacterial infection. Indeed, a study with humans immunized with *M. bovis* BCG showed that concurrent helminth infection produced greater levels of TGF- $\beta$ , which was associated with reduced response to the immunization, affecting vaccine efficacy (69).

In our studies, in summary, bovine MDM infected *ex vivo* with *M. bovis* showed strikingly reduced production of IL-6, IL-1 $\beta$ , IL-12 and NO after donor animals were infected with *F. hepatica* infection. As it has been shown that INF- $\gamma$  production in response to mycobacterial infection is dependent on IL-6, IL-1 $\beta$  and IL-12 (37,38,70,71), this could explain the reduced IFN- $\gamma$  production observed when animals were infected with *F. hepatica* in the co-infection experiments (Figure 2) (25,26). Additionally, the fact that IL-1 $\beta$  promotes local inflammation in delayed-

type hypersensitivity responses (40) indicates that a reduced production of IL-1 $\beta$  in *F. hepatica*-infected animals could cause a lowered SICCT test reaction in those animals (25,26). Based on these results, we propose that early in the course of *M. bovis* infection, alternatively activated macrophages limit the uptake of mycobacteria, setting the stage for a slower rate of bacterial proliferation and lesion development, leading to a reduced pro-inflammatory response. In fact, Atkinson *et al.* (2000) showed that production of TNF- $\alpha$ , IL-12, IL-6 and IL-10 by human MDM was dependent on the *M. bovis* BCG dose used, so that lowered bacterial dose reduced cytokine production (72). As macrophages produce cytokines upon stimulation (73), a reduced stimulus elicited by an inefficient phagocytosis (in our case lowered *M. bovis* uptake) could decrease a pro-inflammatory response (74). Therefore, we hypothesize that this immune downregulation may induce a latency-like state in co-infected animals, which would explain why cattle herds with high prevalence of fasciolosis would have lower reactivity in tests for BTB. This hypothesis is consistent with the data on an epidemiological scale shown by Claridge *et al.* (2012) who showed the under-ascertainment rate of BTB was about one-third in herds infected with *F. hepatica* (6).

Latent infections with *M. tuberculosis* in humans are extremely common, with some estimates indicating that up to one-third of the world's population are latently infected (19), representing a reservoir from which clinically apparent infections can emerge, given appropriate environmental circumstances. Latent infection is characterized by asymptomatic, chronic infection, with lowered responsiveness to skin testing (75). Latent *M. bovis* infections in cattle have not been described. However, the presence of such infections in cattle is suggested by findings such as IFN- $\gamma$  test (23,76) and skin test positivity (77,78) in animals that lack lesions at post-mortem; the positive culture of bacteria in animals without lesions (44,79); and IFN- $\gamma$  positive and skin test negative animals, which later convert to positive, that have lesions at post-mortem examination (78). Studies on the influence of concurrent helminth infections on latent/active tuberculosis in humans have given contradictory results. Babu *et al.* (2009) observed a reduction in mycobacterial-specific TLR2 and TLR4 expression as well as a decrease in pro-inflammatory production in individuals with filariasis/TB in comparison with patients with TB only (8). However, subsequent studies by this group observed no alteration in the incidence of progression from latent to active tuberculosis in populations co-infected with gastrointestinal and/or filarial nematodes (80).

Such apparent paradoxes can be resolved by considering that helminth infection limits the pro-inflammatory environment, favouring a slower development of

mycobacteria, with reduced bacterial loads, but a higher diagnostic barrier for immune-based detection assays. This may ensure a strategy to maintain *M. bovis* in the population by increasing the reservoir of undetected infection. Therefore, these findings have significant implications for the control of bovine TB and especially for eradication programmes based on detection and slaughter of infected animals.

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## AUTHOR CONTRIBUTIONS

LG carried out the design of the experiments and the experimental work and wrote the manuscript. JOS contributed to the design of experimental methodology and carried out the experimental work. AB designed and supervised the flow cytometry experiments. JMN and MW designed and carried out the co-infection studies. RF contributed to interpretation of the data and revision of the manuscript. DW and PD suggested and carried out the statistical analysis of bacterial recovery following *in vivo* experiments. JC analysed the pathology of the lesions. GM conceived the study, edited the manuscript and managed the collaborations. All authors contributed to the intellectual discussion and structure of the manuscript.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

**Table S1.** *Fasciola hepatica* numbers. At post-mortem examination of cattle (Experiment 2), livers were processed and liver flukes counted, confirming *F. hepatica* infection in all 6 animals.

**Table S2.** Mycobacterial dose obtained by side-flow sampling in Madison Chambers. During infection with *M. bovis*, side-stream samples of air were taken from the Madison Chamber, and then *M. bovis* was cultured to determine the actual dose (CFU) delivered to each individual animal. There were no statistically significant differences between the bacterial doses delivered to the calves between groups. In brackets is represented the standard deviation.

**Table S3.** Experiment 1\_Isolation of *M. bovis* from tissues recovered from calves infected with *M. bovis* only or *M. bovis* and *F. hepatica*. There was a decrease in the total bacterial load and total number of positive tissues recovered in the *M. bovis*-*F. hepatica* co-infected group in comparison with the *M. bovis* infected group only. Results are expressed as  $10^1 \times$  CFU/g (quantitative counting).

**Table S4:** Experiment 2\_Isolation of *M. bovis* from tissues recovered from calves infected with *M. bovis* and *F. hepatica* (a) or *M. bovis* only (b). There was a reduced bacterial load recovery and number of positive tissues (by quantitative or qualitative bacteriology) in the *M. bovis*-*F. hepatica* co-infected group vs. the only infected with *M. bovis*. When the same

tissue was positive in both techniques, it was counted as positive only once. <sup>a</sup>QL – Qualitative bacteriology (BAC-TEC MGIT system). <sup>b</sup>QN – Quantitative bacteriology (7H11-OADC agar plates). <sup>c</sup>Results expressed as  $\times 10^1$  CFU/g

**Figure S1.** Seroconversion of *F. hepatica* Infected Cattle.

**Figure S2.** Confirmation of MDM infected with mycobacteria *in vitro*

**Figure S3.** Gating strategy.

**Method S1.** *F. hepatica* Excretory/Secretory Products (FhES)

**Method S2.** Acid Fast Staining

**Document S1.** Experiment Annotation Using MIFlowCyt Standard

**Table S5.** Description of antibodies and lasers used.