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Title:

Dairy heifers naturally exposed to *Fasciola hepatica* develop a type-2 immune response and concomitant suppression of leukocyte proliferation

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Bovine Immune Responses to *Fasciola hepatica*

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Abstract:

Fasciola hepatica is a parasitic trematode of global importance in livestock. Control strategies reliant on anthelmintics are unsustainable due to the emergence of drug resistance. Vaccines are under development, but efficacy is variable. Evidence from experimental infection suggest vaccine efficacy may be affected by parasite-induced immunomodulation. Little is known about the immune response to *F. hepatica* following natural exposure. Hence we analysed the immune responses over time in calves naturally exposed to *F. hepatica* infection.

Cohorts of replacement dairy heifer calves (n=42) with no prior exposure to *F. hepatica,* on three commercial dairy farms, were sampled over the course of a grazing season. Exposure was determined through *F. hepatica*-specific serum antibody ELISA and fluke egg counts. Concurrent changes in peripheral blood leukocyte sub-populations, lymphocyte proliferation and cytokine responses were measured. Relationships between fluke infection and immune responses were analysed using multivariable linear mixed effect models.

All calves from one farm showed evidence of exposure, whilst cohorts from the remaining two farms remained negative over the grazing season. A type-2 immune response was associated with exposure, with increased interleukin (IL)-4 production, IL-5 transcription and eosinophilia. Suppression of parasite-specific PBMC proliferation was evident; while decreased mitogen stimulated IFN-γ production suggested immunomodulation, which was not restricted to parasite-specific responses. Our findings show that the global immune response is modulated towards a non-proliferative type-2 state following natural challenge with *F. hepatica*. This has implications for vaccination programmes in terms of the timing of administration of vaccination programmes, and for host susceptibility to co-infecting pathogens.

Introduction

Liver fluke (*Fasciola hepatica*) is a parasitic trematode of global importance capable of infecting a wide range of vertebrate hosts, including humans. Fasciolosis is considered a major issue for global

51 food security, with over 600 million sheep and cattle thought to be infected worldwide (1). In cattle,
52 clinical presentation ranges from severe, acute to chronic disease depending on intensity and stage
53 of infection, although both result in significant morbidity and/or production losses. Sub-clinical
54 infections reduce weight gain, fertility and milk yield, which impacts on the economic viability of
55 farm production systems (2, 3) and may last for months or years if untreated (4).
56 Both incidence and prevalence of infection has increased across Europe over the last decade. This is
57 largely attributed to climatic changes, namely increases in ambient temperatures and rainfall,
58 favouring the development of both *F. hepatica* and its intermediate snail host, *Galba truncatula.* As
59 a result the range and prevalence of infection is increasing spatially and temporally, with changes in
60 climate projected to further increase prevalence across Europe in the coming decades (5).
61 Control of fasciolosis in livestock is currently based on a limited number of anthelmintics, of which
62 triclabendazole (TCBZ) has been the most heavily used due to its efficacy against both adult and
63 migratory juvenile stages of the parasite (6). TCBZ resistant *F. hepatica* infections in livestock are
64 now widely reported (7-12). There is therefore a need to develop novel approaches to control
65 fasciolosis, with vaccination proposed as a potential adjunctive measure.
66 Current vaccine trials are focussed on several immunodominant parasite antigens including
67 cathepsin L proteases, glutathione S-transferase (GST), fatty acid binding proteins (FABP) and leucine
68 aminopeptidase (LAP), which have been tried in a number of formulations and host species (cattle,
69 sheep and goats) in both native and recombinant forms with levels of protection ranging from 0-72%
70 protection (13, 14). In cattle, as with other ruminant species, protection is often only partial with
71 reductions in fluke burden, egg output and viability observed in vaccinated animals compared to
72 unvaccinated controls (15, 16). In these circumstances, mathematical modelling has shown that such
73 partial protection must be induced in at least 90% of the herd to have a meaningful impact on
74 disease control (17).
75 Where vaccine-induced protection is demonstrated, it has been shown to correlate with parasite
76 specific IgG2 isotype antibody titre and avidity and a reduction in arginase activity in $CD14^{+}$ blood

77 monocyte derived macrophages, suggesting presence of a cell-mediated type-1 response is an
78 important component of vaccine induced protection to *F. hepatica* (15, 18). Such responses,
79 however, are not typically associated with infection in unvaccinated animals.
80 Epidemiological evidence shows prevalence of *F. hepatica* infection increases with age (19) whilst
81 chronically infected cattle remain susceptible to super-imposed experimental infections (20),
82 suggesting an absence of any protective immunity. Studies investigating the immune responses of
83 cattle experimentally infected with *F. hepatica* initially show a pro-inflammatory response
84 progressing over the course of infection towards a polarised, non-proliferative state. Parasite-
85 specific IL-2, IL-4 and IFN-γ production is described in hepatic lymph nodes at 10-14 days post
86 infection (21), and parasite-specific IL-2 and IFN-γ production is also detected in peripheral blood
87 mononuclear cells (PBMCs) from 1-3 weeks post infection (wpi), becoming absent by 5 wpi (22, 23).
88 Similarly, mitogen and parasite-specific stimulated PBMC proliferation peaks around 2 wpi before
89 returning to pre-infection levels (22-25). Thereafter, responses progress towards a non-proliferative
90 state associated with type-2 cytokines, an IgG1 isotype antibody response and an eosinophilia (20,
91 24, 26). By 10-12 wpi, when the parasite has reached the bile ducts and matured, there is a lack of
92 mitogen or parasite-specific lymphocyte proliferation and an upregulation in IL-10 and TGF-β (27,
93 28). It has been suggested that this fluke-induced modulation of the immune response facilitates its
94 long term survival within the host (29), and may also impact on the host's susceptibility to other, co-
95 infecting pathogens such as Salmonella Dublin (30) and *Mycobacterium bovis*, the causative agent of
96 bovine tuberculosis (31, 32).
97 Current vaccine development programmes are based on the assumption that immune responses
98 observed in experimentally infected cattle are representative of those in naturally infected cattle.
99 Little however is known about the immune responses associated with naturally acquired *F. hepatica*
100 infections, specifically the early stages of infection, and the extent to which parasite-induced
101 immunomodulation is induced. This may have implications for how vaccines are delivered in the
102 field, and for understanding how the parasite may affect host susceptibility to co-infecting

pathogens. The aim of this study was therefore to define the immune responses in cohorts of dairy heifers on UK farms with typical management conditions and naturally exposed to challenge with *F. hepatica.*

Materials and Methods

Three commercial dairy farms were recruited into the study. They were identified as *F. hepatica* positive through bulk milk tank (BMT) antibody ELISA results (33) and positive composite faecal egg counts in adult cattle. On each farm, cohorts of replacement dairy heifer calves were recruited (n=42; 17, 17 and 8 animals from farms A, B and C, respectively) aged 90-377 days (mean=218.5, SD ±62.0). These animals had been housed since birth so had not been exposed to *F. hepatica*. This was confirmed prior to turn out by faecal fluke egg counts and anti-*F. hepatica* IgG serum antibody ELISA (34). Negative ELISA results indicated no maternally derived antibodies were detectable at turnout. Animals were sampled monthly over the course of their first grazing season from turn-out in spring (April-May) through to housing in autumn (October-November) 2013. On each occasion blood was collected via jugular venepuncture into plain and EDTA coated vacutainers and faecal samples were collected rectally. BMT samples were also taken at each visit to assess changes in level of exposure within the milking herd.

All the procedures used in this study were approved by the University of Liverpool's Veterinary Research ethics committee (VREC100) and adhered to the conditions of the project license granted by the UK Home Office (HOL PPL40/3621). All farm data was stored in accordance with the UK Data Protection Act (1998).

F. hepatica serum and BMT IgG antibody ELISA

Tubes containing clotted blood were centrifuged at 2,000g for 5 min, serum collected, stored at 4^{o}C and tested for fluke specific antibody within 5 days. A positive cut off of 20 Percent Positivity (PP) was used, giving a diagnostic sensitivity of 95% and specificity of 99% (34). BMT samples were

analysed using a positive cut-off value of ≥27PP, giving a diagnostic sensitivity of 96% and specificity of 80% as described previously (33).

Fluke egg counts

Faecal samples were stored at 4°C prior to analysis and examined for evidence of *F. hepatica* eggs following a standard sedimentation technique using 10g of faeces (35). This was performed on individual samples from the point of sero-conversion onwards, with counts done for every animal at the final time point irrespective of whether they had sero-converted. Nematode infections for all animals at each time point were assessed by faecal egg counts using the McMaster method with a sensitivity of 50 eggs per gram (epg) (36).

Preparation of *F. hepatica* antigens

Adult *F. hepatica* tegument and somatic antigen fractions for use in *in vitro* PBMC stimulation assays were prepared using previously described methods (23, 37); live adult fluke were collected from infected livers and incubated overnight to purge caecal contents, then washed three times in D-PBS (Sigma-Aldrich, St. Louis USA).

Tegument antigen (TegAg) was prepared by placing fluke in D-PBS with 1% Nonidet P-40 (BDH Chemicals, Poole UK), 1ml per fluke, and agitating for 1 hour at 4°C. Pierce® Detergent Removal spin columns (Thermo Fisher Scientific, Waltham USA) were used to remove the Nonidet P-40 detergent. Somatic antigen (SomAg) was prepared from tegument-depleted fluke. These were washed in ice cold D-PBS and snap frozen overnight at -80°C. Fluke were then homogenised and diluted in D-PBS, 0.5ml per fluke, and left to settle overnight at 4°C. The supernatant was collected, centrifuged at 12,000g for 30 min at 4°C.

Both antigens were filter sterilised and shown to contain negligible levels of endotoxin at tissue culture concentrations (Thermo-scientific) (38). Protein concentrations were estimated using a Bradford assay (Thermo-scientific) and aliquots stored at -80°C.

155

156 Haematology, PBMC purification and Flow cytometry

157 Total and differential leukocyte counts were performed on EDTA-treated whole blood using a
158 haemocytometer and thin blood smears, respectively, and used to calculate absolute counts for
159 each leukocyte phenotype per ml of blood.

160 PBMCs were isolated from whole blood in a lateral flow hood using Optiprep™ (Sigma-Aldrich, St.
161 Louis USA) following manufacturer's recommendations. Optiprep™ was added to, and mixed with,
162 EDTA treated whole blood, 1.3ml of optiprep per 10ml of blood, in a 50ml falcon tube onto which
163 1ml 20mM tricine-buffered saline was layered. Samples were centrifuged at 1,000g for 35 min at
164 20°C with the brake off following which the middle aqueous layer containing PBMCs was harvested
165 and washed in PBS with 0.1% EDTA (Lonza, Bazel Switzerland) to a maximum volume of 20ml.
166 Samples were centrifuged at 350g for 8 min at 20°C. Resulting supernatants were discarded and cell
167 pellets re-suspended in 2ml of 0.9% NH_4Cl haemolysis buffer and gently agitated for 1 min at room
168 temperature. Samples were then washed in 20ml PBS EDTA and centrifuged at 150g for 8 min at
169 20°C twice, with purified PBMCs then re-suspended and prepared for flow cytometry, proliferation
170 and cytokine assays as described previously (67).
171 One colour indirect immunofluorescence labelling was performed on PBMCs as previously described
172 (39), with lineage specific monoclonal antibodies to identify bovine $CD4^+$ and $CD8^+$ (40), $WC1^+$ (41)
173 and $CD14^+$ subsets (42). Sub-populations of leukocytes were analysed using a MACSQuant® analyser
174 (Miltenyi Biotech Ltd.). PBMCs were isolated through gating of forward and side scatter channels,
175 with adjustments made using a post-hoc analysis template (MACSQuantify v.2.4.1221.1, Miltenyi
176 Biotech Ltd.) to ensure appropriate fit for all samples. Leukocyte sub-populations were identified
177 and quantified through FITC-positive fluorescence emission and used to calculate absolute counts
178 per ml of blood.

179

180 Proliferation and cytokine measurements

PBMCs were adjusted to a concentration of 2 x 10^6 per ml in RPMI with 10% FCS and 100µg/ml penicillin and streptomycin and incubated *in vitro* with either ConA (5µg/ml), *F. hepatica* SomAg or TegAg (both 25µg/ml) or as unstimulated medium controls to assess proliferative and cytokine responses. For proliferation assays, 2 x 10^5 cells per well were cultured in triplicate in 96-well U-bottomed plates (Corning Life Sciences, Corning USA) for 5 days at 37°C, 5% CO_2. On the 5th day cultures were pulsed with 1µCi of [H^3] tritiated thymidine (Perkin Elmer, Boston USA) for 5 hours then harvested onto glass filter mats and embedded in scintillation wax (Perkin Elmer, Boston USA). Beta-particle counts were measured with a MicroBeta^2 plate counter (Perkin Elmer, Boston USA). The stimulation index (SI) of mitogen/antigen stimulated cultures was calculated as the fold increase in emission counts compared to medium controls. If SI for the ConA positive control was <2, proliferation values for both mitogen and antigen stimulated cultures were excluded from further analysis.

For cytokine assays, PBMCs were incubated with ConA (5µg/ml), SomAg (25µg/ml) or as unstimulated medium controls for 48 hours at 37°C, 5% CO_2 in flat bottomed 24 well cell culture plates (VWR, Radnor USA). Upon completion, culture supernatants were removed and stored at -20°C with PBMCs stored separately at -20°C in RNAlater (Sigma-Aldrich, St. Louis USA).

Cytokine production and transcription assays

Cytokine production and transcription was measured in animals that sero-converted (farm A). Interferon (IFN)-γ and IL-4 concentrations were measured in supernatants using commercially available ELISAs following manufacturer's protocols (MCA5638KZZ & MCA5892KZZ respectively, AbD Serotec, Raleigh USA). IL-10 production was measured in SomAg-stimulated and medium control cultures using a previously validated sandwich ELISA (43).

Paired samples were used to investigate difference in TGF-β production in early versus chronic infection; samples for each individual animal were selected at the closest available time-point to sero-conversion to represent early infection, and from the last available time point to represent

chronic stages of infection. Bio-active bovine TGF-β was measured in SomAg-stimulated and medium control cultures using a commercially available ELISA kit according to manufacturer's recommendations (Promega, Madison USA) (44).

Quantitative real-time (q)PCR was used to measure IL-2 and IL-5 mRNA against 28s housekeeper gene transcription in cultured PBMCs using previously published primers (NCBI accession No. AF154866, M12791 and EU915048.1, respectively). RNA extraction was performed using the RNeasy Mini kit (QIAGEN, Limburg Netherlands), and mRNA quantified with RiboGreen® (Invitrogen Life Technologies, Grand Island USA). Genomic DNA digestion and cDNA synthesis was then performed on 1µg of mRNA template for each sample using Quantitect Reverse transcriptase kit (QIAGEN, Limburg Netherlands). qPCR analysis was performed using a standard protocol with SYBR Green (Bioline reagents Ltd., London, UK) as described previously (45).

Samples were assayed alongside known concentration standards diluted in a 10-fold series in 100ng/µl yeast tRNA (Invitrogen Life Technologies, Grand Island USA) to prevent aggregation. Reactions were performed using a DNA Engine opticon 2 continuous Fluorescence detector. A final melting curve analysis was performed from 50-95°C to confirm the specificity of the amplification products. Sample copy numbers were determined using linear regression of standard concentrations following adjustment of Ct cut-off values to the log-linear phase of amplification. Results for IL-2 and IL-5 transcription are shown as relative expression against the 28s housekeeper gene (per million copies).

Statistical Analysis

Data analysis was performed using multivariable linear mixed effects models in the "nlme" package (46) in the R statistical software environment (47). Response variables (Y) chosen as indicators of exposure to *F. hepatica* were antibody PP value (Ab) to assess responses over the course of infection, and change in PP values (ΔAb) used to examine immunological parameters associated with the early stages of infection. This approach was taken as whilst PP values (Ab) increased over the

course of the study (Figure 1), the greatest changes in PP values (ΔAb) were seen at or soon after sero-conversion (Figure 2).

Details of the structure of each statistical model are shown in (Table 1). For all models, leukocyte counts, PBMC proliferative responses, farm location, animal age, weight and days exposure (days at pasture) were modelled as fixed effects (Table 1), with individual animal identity modelled as a random effect to account for the increased relatedness of responses measured in the same animal. Models containing data from the fluke infected cohort (farm A) only (Table 1; lme_2 & 4) were analysed to allow the inclusion of the *ex vivo* PBMC cytokine responses measured as explanatory variables, thereby giving a more in-depth analysis of the immune responses present in these animals. Raw immunological data are summarized in supplemental material (Figures S1-6).

Three models were produced using Ab as the response variable (Table 1; lme_1, 2 & 3). The first analysis (lme_1) included all data for all three farms over the 7 month sampling period. Model lme_2 included data from the fluke infected cohort (farm A) only. Model lme_3 showed the analysis of data from fluke negative cohorts (farms B & C) only.

When analysing immune responses associated with ΔAb, data collected pre- and post-seroconversion was considered separately to allow the small changes in PP value associated with pre-seroconversion and late stage infection to be considered separately. Consequently, two models were produced using (ΔAb) as the response variable (Table 1; lme_4 & 5). The model lme_4 used post sero-conversion data from the fluke positive cohort (farm A) to measure changes in immune response associated with early exposure, whilst the model lme_5 used sero-negative data from farms A, B & C.

Models containing only data from sero-negative measurements (lme_4 & 5) served to investigate potential physiological and/or age related changes, thus avoiding incorrectly attributing such observations to *F. hepatica* exposure.

Prior to the linear mixed model analysis, the relationship between response and fixed effect explanatory variables was assessed, using a Box-Cox transformation (48) with power λ determined

by maximum likelihood analysis used to transform the response variable to ensure an appropriate linear relationship (Table 1) (49).

For models with Ab as the response variable (Y), an autoregressive correlation matrix of order 1 was also used to account for the relatedness of measurements resulting from repeated sampling, and in particular the fact that measurements taken close together in time are likely to be more similar than those taken further apart.

Model refinement was then carried out using a stepwise Akaike information criterion (AIC) selection method (50) using complete case data for all models being compared with maximum likelihoods (ML) using the "MASS" package. The criterion of a reduction in AIC of 2 or greater was deemed indicative of model superiority (Table 1). The selected model was then fitted to all data for which complete observations were available using restricted maximum likelihoods (REML). This final model was checked for goodness of fit and normality using residual analysis and qq-plots, respectively (Figures S7-8). Missing (NA) values were considered to be "dropouts completely at random" (DCAR) since animals were moved between management groups throughout the grazing season for unrelated reasons (eg. stocking density, pasture management etc.) (51).

Model outputs were interpreted by inspecting the coefficient (β) and associated standard error for each explanatory variable (x) included in the final model. The estimated coefficients indicated whether a positive or negative relationship was present between each explanatory variable (x) and the (transformed) response variable (Y), once all other explanatory variables present in the analysis had been taken into account.

To assess TGF-β expression in early versus late stage infection, results were analysed using two-tailed paired T-tests. Differences in measurement between medium control and SomAg stimulated PBMC cultures for each time point were analysed using unpaired two-tailed T-tests.

Results

1. Faecal egg counts and antibody responses

All calves remained healthy throughout the study, with no clinical signs observed as a result of *F. hepatica* infection, nematode burden or other disease. On each farm the adult milking herd was monitored monthly using a sample of milk from the bulk tank. All three herds tested positive on every occasion with BMT antibody values ranging from 33-50PP, 54-61PP and 79-114PP for farms A, B and C, respectively.

Sero-conversion was observed in all 17 calves on farm A; PP values increased over the course of the study, reaching 37-98 PP at the final time point (Figure 1). Fluke eggs were detected in 10 of the 17 animals from farm A by the final time point, with counts in all cases less than 1 egg per gram of faeces. Paramphistome eggs were also observed in low numbers in seven calves at the final time point. Six of these were positive for both *F. hepatica* and paramphistome eggs.

PP values for the cohorts from farms B and C remained negative throughout the study except for two animals from farm B that had positive PP values at a single time point. These PP values were close to the cut off (20.3 & 24.7) and samples taken from these two calves on every other occasion were negative suggesting these results were false positives. All calves from farms B and C remained negative for fluke eggs for the duration of the study.

Low numbers of nematode eggs were detected in calves on all three farms. Only two positive samples, with counts of 50 epg were detected on farm A at a single time point. Both trichostrongyle and *Nematodirus* spp. eggs were observed intermittently in 15/17 calves from farm B from August onwards, and trichostrongyle eggs were observed intermittently in 4/8 calves from farm C from July onwards.

2. Immunological correlates of infection

Twenty three immune and four signalment parameters were measured monthly for each calf, yielding over 1100 separate data points. To analyse these data we used multivariable linear mixed effect modelling. Two response variables were used: 1) antibody PP value (Ab) was used to indicate infection progression and 2) change in antibody (ΔAb) after sero-conversion was used to indicate the

311 point of first exposure and early stage of infection. ΔAb was used because the increase in PP values
312 were greatest at the point of sero-conversion (Figure 2).
313
314 i. Association between Ab and immune responses
315 The output from the three models using Ab as the response variable (lme_1, 2 & 3) are shown in
316 Table 2. Statistically significant positive associations were found in all three models between Ab
317 (response variable) and days of exposure, showing that the longer the calves were grazing, the
318 greater the likelihood of infection. A significant negative relationship between Ab and SomAg-
319 specific PBMC proliferation was also observed in all three models.
320 When data from all three farms were used (lme_1), statistically significant negative associations
321 were found for farms B and C relative to farm A. Statistically significant positive associations were
322 detected for eosinophil and $CD8^+$ PBMC counts and a negative coefficient was estimated for
323 peripheral blood $WC1^+$ counts.
324 When the immune response data from farm A were used (lme_2), there was a negative association
325 between Ab and ConA-induced IFN-γ production, and a positive association with ConA-induced IL-4
326 and SomAg-induced IL-5 transcription.
327 The model with data from fluke-negative cohorts on farms B & C (lme_3) showed a significant
328 increase in peripheral $CD8^+$ T-cell counts and corresponding decrease in $CD4^+$ T-cell counts.
329
330 ii. Association between ΔAb and immune responses
331 When change in PP value (ΔAb) was used to indicate time of exposure (Table 3; lme_4), there was a
332 significant negative association between ΔAb and days of exposure, suggesting infection occurred
333 early after turnout and that ΔAb is a good measure of early infection. A negative association was also
334 observed for SomAg-specific PBMC proliferation, whilst a positive associations were observed for
335 TegAg-specific PBMC proliferation, ConA stimulated IL-4 production and SomAg stimulated IL-5
336 transcription.

When using sero-negative data from all farms (Table 3; lme_5), there was a significant negative association with SomAg-specific PBMC proliferation, and a positive association with peripheral blood $CD8^+$ counts.

3. Cellular, proliferation and cytokine responses

Eosinophil counts increased in all the animals on all three farms over the course of the study (Figure 3). PBMC proliferation responses were variable both between animals and for individual animals at different time points. The highest parasite-specific proliferation responses were observed in animals from farm A.

Similar variation was observed in the cytokine responses of animals from farm A. An increase in ConA-stimulated IFN-γ production was observed following sero-conversion and there was evidence of ConA stimulated IL-4 production also. No IFN-γ production was detected in response to stimulation with SomAg, whilst only low levels of IL-4 production were observed. IL-2 and IL-5 transcription was similarly variable between and within individuals over the course of the study in response to stimulation with both ConA and SomAg.

No significant difference was found between levels of TGF-β expression in early versus late stage infection in either medium control or SomAg stimulated PBMC cultures (p=0.791 & 0.828, respectively), nor was any significant difference found between TGF-β levels in medium control or SomAg stimulated cultures at either time point (p=0.291 & 0.306, respectively).

Discussion

In this study, we have demonstrated that dairy calves develop a polarised, non-proliferative type-2 response following primary natural challenge with *F. hepatica*. Studies using experimentally infected calves have described an initial inflammatory response comprised of mixed cytokine (IFN-γ and IL-4) production and antigen specific proliferation in PBMC cultures, which subside from 4-6 weeks post-infection. Thereafter parasite-specific IgG1 and IL-4 responses are detected indicating a polarisation

363 towards a type-2 immune response as the infection progresses (20, 22, 23, 52). Our analysis of
364 naturally infected calves show an association with increased eosinophil counts, IL-4 production, IL-5
365 transcription and decreased IFN-γ production, indicating polarisation towards a type-2 response as
366 infection progresses. Furthermore, the increased IL-4 and decreased IFN-γ production by ConA-
367 stimulated PBMCs suggests polarisation of the global T-cell response is present. This is consistent
368 with findings in experimentally infected cattle (32, 53). The presence of a generalised type-2 immune
369 environment may help explain why calves are less able to respond effectively to co-infecting
370 pathogens. For example, cattle infected with *F. hepatica* are more susceptible to Salmonella Dublin
371 infection (30, 32).
372 Our findings differ from those obtained from experimentally infected calves however, in the rate and
373 stage at which immune modulation is observed. Experimentally infected cattle have increased IL-2
374 and IFN-γ production in the first 2-3 weeks post infection (22, 23), whilst our study suggests the early
375 stages of natural infection are associated with increased IL-4 production and IL-5 transcription.
376 Similarly, D. G. Clery and G. Mulcahy (22) showed parasite specific proliferation within 2 to 3 weeks
377 post infection in experimentally infected calves, whilst our study detected a significant negative
378 association between SomAg specific PBMC proliferation at all stages of infection. Early proliferative
379 responses were observed in only 3/17 animals. For this study, samples were taken monthly, hence
380 some transient early pro-inflammatory responses may have been missed. Nonetheless, our findings
381 suggests that, unlike experimental infections, polarisation of the immune response in naturally-
382 acquired infection is present from very early in infection.
383 These differences may relate to the infectious challenge administered in experimental infections
384 compared to those encountered under field conditions. Most experimental infections have used
385 either a single dose, or 'trickle' type infection, where boluses of several hundred, up to 1000
386 metacercariae are administered per dose (26, 54). The epidemiology of *F. hepatica* in temperate
387 regions typically results in small numbers of metacercariae present on pasture early in the spring
388 followed by increasing numbers of metacercariae appearing on pasture towards the end of the

grazing season (55). Hence administering a large infectious challenge to immunologically naïve cattle may not be representative of normal field conditions and may result in a more profound innate cellular response during the early stages of experimental infection.

Our results suggest that challenge at the start of the grazing season was not associated with detectable pro-inflammatory or proliferative responses. The immune-modulation induced during this primary challenge also may prevent any subsequent pro-inflammatory, proliferative responses developing later in the season when animals are exposed to a more substantial infectious challenge as pasture contamination increases. These findings have implications for vaccine programmes, since current efforts are focussed on enhancing cell-mediated type-1 responses (15, 18). Our results suggest the efficacy of such vaccines would be negatively impacted if administered following natural exposure to *F. hepatica* under field conditions. These vaccines would therefore need to be administered and be fully effective prior to any exposure to infection.

In experimentally infected calves, a systemic regulatory response develops during the chronic stages of infection characterised by increased parasite specific IL-10 and TGF-β production by PBMC (27). In this study, we found no significant association between infection status and parasite-specific IL-10 or TGF-β production by PBMCs. Again, this may relate to differences in the way in which the calves were exposed to infection, although it is possible that such regulatory responses were present in local and regional lymph nodes, as this has been observed previously in cattle and sheep harbouring naturally acquired, chronic *F. hepatica* infections (28, 56).

Eosinophilia is a feature of many helminth infections. Calves on farm A were treated with three doses of ivermectin over the course of the grazing season, and nematode egg counts remained at or close to zero throughout the study period. The eosinophilia observed in this cohort was most likely to be in response to *F. hepatica* rather than nematode infection, particularly since there was a significant positive relationship between fluke Ab PP value and both peripheral eosinophil counts and SomAg-stimulated IL-5 transcription. This is consistent with previous findings of eosinophilia in *F. hepatica* infected cattle (26, 57).

Both fluke infection and specific fluke antigens are known to have immuno-suppressive and/or modulatory effects on both the innate and adaptive immune responses (20, 27, 52, 58). Differences between the relationships of SomAg and TegAg PBMC proliferation and the early stages *F. hepatica* exposure (ΔAb) may be the result of differences in composition of these two antigen fractions and their functions *in vivo*. Overall, however, our results show that in naturally infected calves, there is an absence and/or suppression of parasite-specific T cell proliferation.

The negative relationship between Ab and $WC1^+$ PBMC counts is most likely to be due to age and maturation of the immune system. $WC1^+$ γδT-cells are known to decrease in number in the peripheral blood as cattle mature. $WC1^+$ cell populations constitute approximately 15% of PBMCs in calves aged 3-12 months in age, decreasing to around 5% by 3 years of age (59). In our study, animals ranged in age from 90-377 days at the beginning and were 310-587 days of age by the end. The respective mean $WC1^+$ PBMC counts were 20% (6-32%) and 6% (0.5-24%). Similarly, the positive relationship between Ab and $CD8^+$ PBMC counts may also signify the normal maturation of the immune system rather than a direct impact of infection since this was also observed in uninfected animals.

In spite of evidence from the adult milking herd that *F. hepatica* infection was present on all three farms, the calves on farms B and C showed no evidence of infection. In both cases these calves were kept in different parts of the farm and did not share pastures with the adult cows. In contrast the cohort on farm A was managed in a rotational grazing system that included pastures grazed by adult cattle. This suggests the fields used for calves on farms B and C had no habitat suitable for the intermediate host, *Galba truncatula* and were not contaminated with metacercariae.

In this study, multivariable linear mixed-effect regression models were used to analyse a longitudinal set of data from cattle naturally exposed to *F. hepatica*. This analysis was useful since it allowed the simultaneous assessment of multiple immunological variables to identify the key parameters associated with infection. To the best of our knowledge this is the first time such techniques have

been used to analyse complex immunological responses to natural infection in livestock and may represent a potentially useful template for future studies.

In conclusion, through analysis of a longitudinal dataset using multivariable linear mixed-effect regression analysis we have demonstrated that natural exposure to *F. hepatica*, dairy heifers results in a generalised type-2 immune state with concomittent suppression of proliferation responses. This has significance for both vaccine administration programmes and susceptibility to other common co-infecting pathogens of livestock.

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614 **Table 1: Linear mixed effects model structures.** Response variable (Y) indicates the subject of each respective model. Box-Cox value (λ) is the optimal
615 transformation factor for the response variable to ensure linear fit to the proposed fixed-effect variables. Where *nil* is quoted, transformation was not
616 required. For initial fixed-effect explanatory variables "Leuk. counts" refers collectively to leukocyte count data (eosinophils (Eo), neutrophils (No) and $CD4^+$,
617 $CD8^+$, $WC1^+$ & $CD14^+$ PBMCs) per ml of peripheral blood, "PBMC prolifn." to PBMC proliferation data (ConA, SomAg and TegAg stimulated cultures), whilst
618 "PBMC cytokines" refers to all available PBMC cytokine production and transcription data (IFN-γ, IL-4 and IL-10 production, IL-2 & IL-5 relative gene
619 transcription). For cytokine data present as final explanatory variables square parentheses [] indicate the culture conditions present; medium control (Med),
620 ConA, SomAg or TegAg. Time series plots of the immunological data included in these models are included as supplementary material.

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Linear mixed effect model	Response variable (Y)	Box-Cox value (λ)	Data analysed	Initial fixed-effect explanatory variables (x); *[AIC value]*	Final explanatory variables (x) following stepwise AIC selection; *[AIC value]*
lme_1	Ab	-0.2	All data	Farm + Days exposure + Age + Weight + Leuk. counts + CD4:CD8 ratio + PBMC prolifn.; *[AIC = 44.98]*	Farm + Days exposure + Eo + CD4 + CD8 + WC1 + SomAg prolifn. + TegAg prolifn.; *[AIC = 37.99]*
lme_2	Ab	0.2	Farm A	Days Exposure + Age + Weight + Leuk. counts + CD4:CD8 ratio + PBMC prolifn. + PBMC cytokines; *[AIC = 166.04]*	Days Exposure + Eo + SomAg prolifn. + IFNγ [ConA] + IFNγ [SomAg] + IL-4 [ConA] + IL-10 [Med] + IL-10 [SomAg] + IL-2 [ConA] + IL-5 [SomAg]; *[AIC = 145.57]*
lme_3	ΔAb	*nil*	Sero-positive values; Farm A	*(see lme_2)*; *[AIC = 354.80]*	Days exposure + CD4 + WC1 + SomAg prolifn. + TegAg prolifn. +IL-4 [ConA] + IL-5 [SomAg]; *[AIC = 329.74]*
lme_4	Ab	-0.2	Farms B & C	*(see lme_1)*; *[AIC = 38.29]*	Farm + Days exposure + SomAg prolifn + Eo + CD4 + CD8 + WC1; *[AIC = 26.06]*
lme_5	ΔAb	*nil*	Sero-negative values; Farms A, B & C	*(see lme_1)*; *[AIC = 653.12]*	Farm + CD4 + CD8 + CD4:CD8 ratio + SomAg prolifn. + TegAg prolifn.; *[AIC = 640.33]*
lme_6	ΔAb	*nil*	Farms B & C	*(see lme_1)*; *[AIC = 580.03]*	Eo + CD8; *[AIC = 561.09]*

Table 2: Multivariable linear mixed effect model outputs for anti-*F. hepatica* antibody PP value (Ab) as response variable (Y). Statistically significant ($p<0.05$) explanatory variables (x) are shown with corresponding coefficient (β) and standard error (SE) values to describe their respective relationships with Ab. Values rounded to 3 decimal places, or 1 significant figure.

Model ID *Data used*	**Explanatory variable (x)**	**Coefficient value (β)**	**Standard error (SE)**	**P-value**
lme_1 *All data*	Farm B	-0.687	0.077	<0.001
	Farm C	-0.874	0.087	<0.001
	Days exposure	0.005	<0.001	<0.001
	Eϕ/ml	0.0001	0.00004	0.024
	CD8 /ml	0.0002	<0.0001	0.016
	WC1/ml	-0.0001	0.00004	0.028
	SomAg	-0.006	<0.003	0.024
lme_2 *Farm A* *(positive cohort)*	Days exposure	0.027	0.003	<0.001
	SomAg	-0.012	0.005	0.039
	[ConA] IFNγ	-0.0001	<0.00006	0.026
	[ConA] IL-4	0.0005	0.0002	0.039
	[SomAg] IL-5	0.003	0.001	0.012
lme_3 *Farms B & C* *(negative cohorts)*	Farm C	-0.201	0.086	0.029
	Days exposure	0.004	<0.001	<0.001
	CD4/ml	-0.0001	0.00004	0.010
	CD8/ml	0.0004	0.0001	<0.001
	SomAg	-0.007	0.003	0.042

Table 3: Multivariable linear mixed effect model outputs for change in antibody PP value (ΔAb) as response variable (Y). Statistically significant ($p<0.05$) explanatory variables (x) are shown with the coefficient (β) and standard error (SE) values to describe their respective relationships with ΔAb. Values rounded to 3 decimal places.

Model ID *Data used*	**Explanatory variable (x)**	**Coefficient value (β)**	**Standard error (SE)**	**P-value**
lme_4 *Farm A (post sero-conversion)*	Days exposure	-0.354	0.107	0.004
	SomAg	-2.794	1.211	0.034
	TegAg	2.254	1.064	0.049
	[ConA] IL-4	0.012	0.050	0.033
	[SomAg] IL-5	0.071	0.029	0.026
lme_5 *Farms A, B & C (Sero-negative values)*	Farm B	-12.009	4.675	0.015
	Farm C	-14.760	5.096	0.006
	CD8/ml	0.027	0.009	0.008
	SomAg	-0.621	0.276	0.027
	TegAg	0.712	0.248	0.005

Figure 1: Antibody PP values of individual animals over the study period for the three dairy farms (A, B & C). Diagnostic positive cut-off value (PP=20) is denoted by horizontal line. Month of sampling denotes the time point for each sequential visit to each farm.

Figure 2: Change in antibody PP value (ΔAb) over the course of infection for farm A. Positive values indicate an increase in Ab PP value from one time point to the next, and negative values denote a decrease in Ab PP value compared to the previous month. Values from 1 month prior to sero-conversion (-1) are considered separately from other sero-negative values (-ve) as infection may have been present at this time point but the animals had not seroconverted. In experimentally infected animals, seroconversion occurs 2-4 weeks post-infection (34).

Figure 3: Peripheral blood eosinophil counts for individual animals over the study period for the three dairy farms (A, B & C). Month of sampling denotes the time point for each sequential sampling visit.