IAI Accepts, published online ahead of print on 28 December 2006
Infect. Immun. doi:10.1128/IAI.01445-06

Experimental *Fasciola hepatica* infection alters responses to tests used for diagnosis of bovine tuberculosis

Flynn, R.J., Mannion, C., Golden, O., Hacariz, O., Mulcahy, G.*

Veterinary Sciences Centre, School of Agriculture, Food Science and Veterinary Medicine, College of Life Sciences, University College Dublin, Belfield, Dublin 4, Ireland.

Running Title: *Fasciola hepatica* and diagnosis of bovine tuberculosis

*Correspondent footnote:
Veterinary Sciences Centre, School of Agriculture Food Science and Veterinary Medicine, College of Life Sciences, University College Dublin, Belfield, Dublin 4, Ireland.
Email: grace.mulcahy@ucd.ie
Phone: 00 353 (01) 716 6180
Fax: 00 353 (01) 716 6185

Abstract

Fasciola hepatica is a prevalent helminth parasite of livestock. Infection results in polarization of the host's immune response and generation of type 2 helper (Th2) immune responses are generated, which are known to be inhibitory to Th1 responses. Bovine tuberculosis (BTB) is a bacterial disease of economic and zoonotic importance. Control polices for this disease rely on extensive annual testing and a "test and slaughter" policy. The correct diagnosis of BTB relies on cell-mediated immune responses. We established a model of co-infection of *F. hepatica* and *Mycobacterium bovis* BCG to examine the impact of helminth infection on correct diagnosis. We found the predictive capacity of tests to be compromised in co-infected animals, and that *F. hepatica* infection altered macrophage function. IL - 4 and IFN - γ expression in whole blood lymphocytes re-stimulated *in vitro* with *M. bovis* antigen were also altered in co-infected animals. These results raise the question of whether *F. hepatica* infection can affect the predictive capacity of tests for diagnosis of BTB and possibly, also influence susceptibility to BTB and other bacterial diseases. Further studies on the interplay between helminth infection and BTB are warranted.

47

48

49

50 1. Introduction

51 The helminth parasite *Fasciola hepatica* is the causative agent of fasciolosis, a
52 prevalent disease of livestock in temperate regions. Fasciolosis causes losses to agri-
53 business estimated at around US$2 billion per year (32). The parasite has a complex
54 lifecycle utilising the mud snail, *Galba truncatula*, as an intermediate host, while sheep
55 and cattle, as well as various wildlife species, serve as the definitive hosts. At present,
56 chemotherapy is the only treatment available (24), however much effort is being focused
57 on the production of a recombinant vaccine (12). The prevalence of fasciolosis is
58 increasing, due in part perhaps to climatic changes (30), and a recent abattoir study
59 indicates that 65% of cull cows in Ireland are infected (26),

60 The immune response to *F. hepatica* is skewed towards type 2 helper (Th2)
61 dominance, characterised by interleukin (IL) - 4 production, eosinophilia, and a specific
62 IgG1 response with little or no specific IgG2 (5, 8, 25). Studies on the response in cattle
63 suggest that animals experience a downregulation of Th1 responses, including interferon
64 gamma (IFN - γ) production and lymphocyte responsiveness by week four of infection (8,
65 9). These results are similar to those emerging from studies carried out using a murine
66 model. Here, a complete downregulation of Th1 responses was seen to occur with an
67 upregulation of the Th2 cytokines IL - 4 and IL - 5, with the magnitude of the effect
68 dependent on the parasite burden (27). In murine models of co-infection, *F. hepatica*
69 delays bacterial clearance and inhibits bacterial specific IFN - γ production (4). These

70 results suggest that *F. hepatica,* like many other helminths, modulates the host's immune
71 system in order to evade detection, damage, or ultimately expulsion (22). A study of *F.*
72 *hepatica* infection in the murine model has also revealed the presence of alternatively-
73 activated macrophages in infected animals (14). These macrophages were seen to
74 produce increased levels of IL - 10 and TGF - β, both of which are regulatory cytokines,
75 have anti-inflammatory properties and could be responsible for reduced lymphocyte
76 proliferation. Alternatively-activated macrophages also have reduced microbicidal
77 properties and could result in poor innate defence against bacterial infection (19). These
78 immunomodulatory effects may also be responsible for increased susceptibility to
79 secondary bacterial diseases. De-worming of a human study group has been shown to
80 lead to enhanced cellular responses to *Mycobacterium tuberculosis* antigens (17). In
81 another study, *Schistosoma mansoni* and hepatitis C virus (HCV) co-infection revealed
82 that cellular immunity and cytokine production directed at HCV was inhibited (18).
83 Vaccination against experimental *M. tuberculosis* infection in mice using BCG was found
84 to be less effective in cases where co-infection occurred with *S. mansoni* (15)
85 Bovine tuberculosis (BTB), caused by *M. bovis*, is one of the most intractable
86 diseases of livestock (13). Eradication schemes for BTB in the UK and Ireland rely on a
87 "test and slaughter" policy that uses the single comparative intradermal tuberculin test
88 (SCITT). This test detects animals that specifically react to intradermal inoculation of a
89 specific antigen - purified protein derivative B (PPD - B) - derived from *M. bovis* with a
90 response greater than that directed against its equivalent, PPD - A, derived from *M.*
91 *avium*. The development of a reaction to the SCITT test relies on the animal forming a
92 delayed-type hypersensitivity (DTH) response to PPD - B (23). Because of issues with

low sensitivity in some situations, in Ireland, a second-line test, the whole blood IFN - γ assay was introduced, to be used in problem areas. This test relies on measuring the differences in IFN - γ production *in vitro* in response to PPD - B and PPD - A (20). The influence of other intercurrent infections on the performance characteristics of these tests is largely unknown. One previous study has demonstrated that acute bovine viral diarrhoea virus infection could generate false negative responses to both tests in BTB - infected calves (6). The possible influence of *F. hepatica* or other common helminths on the outcome of the SCITT or the whole blood IFN - γ assay is relevant to eradication and testing policies for BTB and also to the wider question of co-infection.

The objective of this study was to examine the influence of *F. hepatica* infection on the outcome of the two BTB diagnostic assays routinely used in control schemes. We also sought to determine if the timing of helminth infection had an influence on the response to tests and to outline possible mechanisms that could be responsible for altered responses in co-infected animals. We established a co-infection model using *F. hepatica* and *M. bovis* BCG, an avirulent strain of *M. bovis* that is commonly used for vaccination in man. Vaccination with *M. bovis* BCG is known to induce a positive reaction on BTB diagnosis in cattle (34). Hence, it is useful model to use when examining the effect of co-infection on the predictive capacity of tests used in the diagnosis of BTB. However, it is also important to bear in mind when interpreting these results that the model does not replicate in full the host-pathogen relationship developing in BTB, which involves a virulent, persistent organism that continues to multiply within the host.

2. Materials and methods

2.1 Experimental design.

Experimental animals were male, castrated Friesian calves aged between 6 and 9 months of age, purchased from herds free of BTB as determined by a negative result on the annual SCITT carried out as part of the Irish Bovine Tuberculosis Eradication Scheme. The most recent SCITT on this herd, which included these experimental calves, was carried out three months prior to the start of the experiment. The calves were free from exposure to *F. hepatica* infection as determined by history, serum antibody levels, and faecal examination. They were group housed in slatted pens with good ventilation and fed good quality grass silage *ab libtum*. Animals were randomly assigned to one of four treatment groups as shown in Table 1. At week 0, animals in Group 1 and 2 were infected with 400 *F. hepatica* metacercariae, (obtained from Dr. G. Coles, Dept of Veterinary Clinical Sciences, University of Bristol), contained within a 5% gelatin bolus, as previously described (7). Animals in Groups 3 and 4 were inoculated subcutaneously in the right shoulder with 10^6 - 10^7 colony forming units (CFU), of *M. bovis* BCG, Danish strain 1331 (Statens Serum Institute, Denmark). Four weeks after the initial infection animals in Group 2 were inoculated with BCG as above, while animals in Group 3 were infected with *F. hepatica*. Animals were necropsied 23 weeks following initial infection or inoculation. All experiments were approved by the University ethics committee and were performed under Licence from the Irish Department of Health and Children.

2.2 Antigens.

Excretory - Secretory (ES) products were produced as previously described (14). To prepare endotoxin-free ES, phase separation was employed (1). Briefly proteins, adjusted to 1mg/ml in sterile endotoxin free PBS, were vortexed with 5% Triton X - 114, and incubated on ice for 5 min, then at 37ºC for 5 min. Following the last incubation solutions were centrifuged at 5000 x g for 7s at 37ºC. Following centrifugation, the upper phase of the solution, containing the endotoxin-free proteins, was collected. Protein concentration was quantified using the BCA assay (Pierce) using bovine serum albumin (BSA) as a standard. Proteins were determined to be endotoxin-free by testing with Cambrex QCL-1000 Chromogenic LAL Endpoint Assay as per manufacturer's instructions. Only ES found to contain no endotoxin was used for macrophage stimulation.

2.3 ELISA for *F. hepatica* specific antibodies

Blood was collected by venepuncture of the coccygeal vein and allowed to clot. Serum was removed and stored at -20°C prior to analysis. Specific antibodies to *F. hepatica* were measured by ELISA using recombinant *F. hepatica* cathepsin L1 (rFhCL1) as antigen. This is a recombinant version of a molecule secreted by the parasite, shown to be useful in detecting animals exposed to *F. hepatica* infection (10). Briefly, 96-well plates (Sartstedt) were coated overnight at 36°C with 1µg/ml of antigen in carbonate /bicarbonate coating buffer. Plates were washed in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST), and blocked with 1% BSA-PBS. Samples diluted 1/200 in 1% BSA-PBS were loaded onto the plate (100µl/well) with appropriate positive and negative controls and doubling serial dilutions performed. Following incubation, (30

min at 37°C) plates were washed 3 times in PBST and monoclonal mouse anti - bovine IgG1 (1/4000) (Cedi Diagnostics) was added. Following incubation and washing, polyclonal rabbit anti - mouse IgG:HRP (1/2000) (Dako) was added and incubated as above. Following a final wash, 100µl of TMB (Sigma-Aldrich) was added and the reaction stopped after 10 minutes by addition of 100µl of H_2SO_4. Plates were read at a wavelength of 450nm, on a Dynatec 4500 plate reader and results were expressed as log_{10} antibody titers.

2.4 BTB Diagnostic Tests.

Two tests used in the diagnosis of BTB were employed in this experiment, the SCITT and the whole blood IFN - γ assay. The SCITT was carried out and interpreted using standard procedures as outlined in EU directive 80/219 EU, by a qualified veterinary surgeon who was unaware of the treatment groups to which animals belonged. Hair was clipped from a site over the neck region. Using McClintock syringes, avian tuberculin (0.5mg/ml, Leystad) was injected intradermally at one point on the neck and bovine tuberculin (1mg/ml, Leystad) at a distinct site directly below. Skin thickness (mm) at both sites was measured before injection and again 72 hours later. For the whole blood IFN - γ assay, heparinised blood was collected and divided into 1.5ml aliquots in 12-well tissue culture plates. These aliquots were stimulated in duplicate with PPD - A and PPD - B both at 20µg/ml at 37°C, 5% CO_2. Twenty-four hours later plasma was collected and tested for the presence of IFN - γ using the Bovigam enzyme immunoassay (CSIRO) as per manufacturer's instructions. Results were interpreted as previously described (20). Further aliquots of the supernatant (plasma) from this assay were used for IL-4

measurement, as described below.

2.5 Macrophage isolation and phenotyping

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood taken 14 weeks post BCG-inoculation by centrifugation over Ficoll-Histopaque (Sigma). Monocytes were then further isolated from PBMC using anti-CD14 microbeads (Miltenyi Biotec) on a magnetic separation column (21). Adherent cells were incubated for 24 or 48 hours in tissue-culture flasks at 37°C, 5% CO_2, in D-MEM medium (Invitrogen) containing 10% FCS (Sigma), 200U/ml penicillin (Sigma) and 200µg/ml streptomycin (Sigma), after which they were tested for nitric oxide (NO) production and intracellular arginase levels. To generate macrophages from naïve monocytes, blood was collected in heparinised tubes and PBMC and CD14+ cells isolated as above. Adherent cells were cultured for 10 days, with media changes every 2 days until mature macrophages had formed. Cells were collected in Trypsin/EDTA without Mg^{+2} Ca^{+2} and plated at a density of 10^5 cells per well for stimulation. Cell lysates and supernatants were then examined as detailed below.

2.6 Nitric Oxide measurement.

Cell culture supernatants were tested in duplicate for NO using the Griess reagent system (Promega). Briefly, 50µl of supernatant was added to wells of a 96-well mircrotiter plate (Sarstedt). To this, 100µl of sulfanimide solution was added and the plate was incubated for 10 minutes in the dark at room temperature. Following this, 100µl of N - 1 - napthylethylenediamine dihydrochloride (NED) solution was added and the

plate incubated as before. Readings were taken at 570nm and NO concentration was determined by comparison with a standard curve using serial dilutions of a 100µM solution of nitrate.

2.7 Arginase activity.

Cell lysates were prepared by addition of 400µl of 1% Triton X-100 (Sigma), and incubation on a rocking platform for 40 minutes. Lysate (50µl) was added to 50µl of Tris-HCl buffer, pH 7.5, and incubated at 55°C for 10 minutes to allow for enzyme activation. Following this, 25µl of the activated lysate was added to 25µl of arginine substrate at a concentration of 0.5M (pH 9.7). This mixture was incubated at 37°C for 1 hour. The reaction was stopped by addition of 400µl of acid stop solution, comprising H_2SO_4 (96%), H_3PO_4(85%), and H_2O in a ratio of 1:3:7. Colour was developed by adding 25µl of 9% isonitrosopriopherone (Sigma) and heating to 100°C for 45 minutes (10). A 1:20 dilution of beef liver homogenate was used as a positive control.

2.8 IL - 4 detection.

IL - 4 produced in response to PPD - B, in plasma derived from the IFN - γ whole blood assays, was measured using a commercial ELISA (Endogen, USA) as per manufacturer's instructions. Briefly, recombinant IL - 4 capture antibody was coated onto 96 well plates overnight at 4°C. Samples were added in duplicate in 100µl volumes and incubated for 1.5 hours. IL - 4 detection antibody was added and incubated for a further hour. Strepadavin:HRP conjugate was added to the plate for 30 minutes, after which colour was developed by addition of substrate. The reaction was stopped and the plate

was read at 450nm with reference wavelength of 550nm. Results were quantified using a standard curve prepared from recombinant bovine IL - 4 and reported as pg/ml.

2.9 Statistical Analysis

SCITT and IFN - γ test results were expressed as percentages of animals in each group testing positive. Significant differences between groups were determined using the Chi - Squared test, as appropriate for categorical data. Measurement of arginase activity, IL - 4 levels, and antibody titers produced normal data, and differences between groups were accordingly tested using a one-tailed Students T - Test. All statistical analysis was carried out using MiniTab (Microsoft Inc.).

3. Results

3.1 *F. hepatica* burdens and specific immune response

Analysis of the serum antibody response of the 3 groups infected with *F. hepatica* showed that humoral immune responses specific for *F. hepatica* were unaffected by BCG immunisation in the two groups of co-infected animals (Figure 1). All infected groups seroconverted and continued to produce increasing levels of parasite-specific IgG1 throughout infection. There were no significant differences between mean IgG1 titers amongst groups. Parasite-specific serum IgG2 did not increase above background during the course of the experiment (data not shown). Livers were collected post-mortem and examined to detect parasites present. The average parasite burden (+/- S.E.M.) in Groups 1, 2, and 3 were 26±4, 15±3, and 8±5 respectively. These burdens were not statistically

254 different.

255

256 3.2 BTB test results

257 Thirteen weeks following immunisation with BCG, all animals were tested using
258 the SCITT, and on the same day blood was collected for use in the whole blood IFN - γ
259 assay. The results of both tests are shown in Table 2. Group 3 contained one animal that
260 responded to the IFN - γ assay, while the same animal was also a reactor on the SCITT
261 along with a second animal from this group. No animals from Group 2 were classed as
262 responders on either the IFN - γ assay or the SCITT. Group 2 animals had produced a
263 patent infection at the time of BTB diagnosis (faecal egg positive- data not shown), while
264 tanimals in Group 3 had a pre-patent infection at time of testing

265

266 3.3 Macrophage Phenotyping.

267 We sought to characterise the activation status (classically or alternatively
268 activated) of blood-monocyte derived macrophages from animals in each treatment group
269 We isolated CD14-positive cells using a microbead system from peripheral blood
270 mononuclear cells of animals in each treatment group, cultured adherent cells *in vitro*,
271 and measured their production of NO and arginase (Figure 2a). We found only trace
272 amounts of NO, (data not shown) while we found selective upregulation of arginase
273 production in the macrophages from animals infected with *F. hepatica*. These cells all
274 showed significantly greater levels of arginase than those taken from animals which were
275 inoculated with BCG only or from naïve control animals. We also examined the effect of
276 *in vitro* stimulation with PPD-B or F. hepatica ES on blood monocyte-derived

277 macrophages from uninfected animals. (two calves obtained from the same source as the
278 infected groups, and within the same age range). Mature macrophages were stimulated
279 with PPD - B or *F. hepatica* Excretory-Secretory (ES) products. Following incubation,
280 arginase and nitric oxide levels were measured and confirmed findings that ES induced
281 alternatively activated macrophages, while PPD - B stimulation favoured NO generation
282 indicative of classically-activated macrophages (Figure 2b & 2c).

283 3.4 Cytokine production in whole blood assays.

284 Levels of IL-4 and IFN-γ produced following incubation of whole blood with
285 PPD-B or *F. hepatica* ES are shown in Figures 3, 4 and 5 for Groups 2,3 and 4,
286 respectively. We found that Group 4 animals produced a peak response of IL - 4 at week
287 5. However Group 3 animals displayed a different pattern of IL - 4 expression. These
288 animals produced a peak IL - 4 response at week 13 while levels were depressed at week
289 5 (Figure 3). Group 2 animals in comparison to Group 4 animals had strong IL - 4
290 responses directed at PPD - B one week following immunisation. This group also showed
291 elevated IL - 4 responses at time of SCITT, however these levels were not significantly
292 greater than those found in Group 4.

293 The IFN - γ response of animals to PPD - B in the whole blood assay also showed
294 that some co-infected animals differed in respect of the kinetics and magnitude of this
295 response as compared with BCG-infected animals (Figure 4). Animals in Group 4 that
296 mounted an IFN - γ response, produced levels that were maximal between weeks 3 and 5
297 post-infection; these animals were reactors on the SCITT. Two animals produced poor

responses but one of these was still a reactor on SCITT. In Group 3, one animal produced a strong and continuous IFN - γ response and was a reactor on SCITT. The others produced weak IFN - γ responses and failed to become reactors on SCITT. One animal produced an early IFN - γ response similar to Group 4 animals but this response collapsed following *F. hepatica* infection, and this animal subsequently failed to react on SCITT. IL - 4 is known to be inhibitory to IFN - γ production and this relationship was evident in the co-infected groups. Co-infected animals had peak IL - 4 responses at time of SCITT where IFN - γ is essential for the production of a positive response. Co-infected animals whose levels of IL - 4 elevated above those of BCG-immunised animals were those whose IFN - γ levels were suppressed and consequently responded poorly on SCITT.

4. Discussion

The results presented here indicate that a secondary or concurrent helminth infection may influence the predictive capacity of tests used for diagnosis of BTB. The

finding that, following experimental infection, SCITT detection rates fell from 80% in Group 4 to 40% in Group 3, to 0% in Group 2 animals has grave implications for a control policy that relies on "test and slaughter". There is also evidence for a potential effect of parasite burden visible within co-infected groups. Group 3 animals had a mean parasite burden less than those in Group 2, while this difference did not reach significant levels (P = 0.0662), due to the relatively high variance within groups (commonly seen following experimental *F. hepatica* infection) it could indicate that higher parasite levels in Group 3 might have resulted in a further decrease in the number of animals that were reactors to SCITT and IFN - γ tests. It also raises the possibility that BCG immunisation had an "adjuvant" or non-specific effect on the ability of the animals to contain *F. hepatica* infection.

Group 2 animals were 17 weeks post *F. hepatica* infection while Group 3 animals were 9 weeks post *F. hepatica* infection at time of SCITT. This difference in numbers of reactors between these two groups of co-infected animals may be due to the difference in the relative timing of exposure to *F. hepatica* and BCG. This may indicate that the duration of helminth infection or the presence of helminth infection prior to BCG immunisation may have played some role in the e influence on response to BTB diagnostic tests.

Here we have shown that infection with a common helminth parasite can have a major effect on immune responses relevant to diagnosis of BTB. Positive responses in the SCITT and whole blood IFN - γ of BTB infected animals require sufficient cellular immune responses directed towards PPD - B (29). In general, dominance of humoral over

340 cellular immunity in cattle infected with BTB is correlated with a more progressive form
341 of the disease (35). Even if the effects of natural infection with *F. hepatica* on the
342 response to diagnostic tests for BTB are much less than those seen here following
343 experimental *F. hepatica* infection with BCG immunisation, the potential implications for
344 disease control programmes are grave. Only one previous study (6) has examined the
345 effect of an intercurrent infection on the performance of SCITT and IFN - γ tests. Acute
346 infection with bovine viral diarrhoea virus resulted in temporary suppression of
347 lymphocyte and IFN - γ responses.

348 The ability of helminths to modulate their host's immune system has been
349 extensively reviewed (22). A number of studies detailing the effects of co-infection with
350 helminths highlight the detrimental effect this modulation can have on immune responses
351 directed at a secondary or concurrent pathogen. In a murine model of *F. hepatica* and
352 *Bordetella pertussis* infection, cellular immunity directed towards *B. pertussis* was
353 abrogated resulting in delayed bacterial clearance of bacteria and poor *B. pertussis*
354 specific IFN - γ production (4). This effect could also be produced by injection of the *F.*
355 *hepatica* protease cathepsin L1 (28). This effect was also seen to be dependent on IL - 4,
356 as IL - 4 knockout mice did not suffer the same effects when cathepsin L1 was
357 administered (28). In our study we found IL - 4 levels to be elevated in co-infected
358 animals, corresponding to downregulated IFN - γ production and in negative responses on
359 SCITT. This suggests that IL - 4 plays a role in helminth immunomodulation in this
360 model. *S. mansoni* infection is known to increase susceptibility to BCG infection in mice
361 (16), with IFN - γ production in response to PPD - B downregulated in co-infected mice,
362 while the Th2 cytokines IL - 4 & 5 were found to be unchanged in co-infected animals in

response to stimulation by *S. mansoni* antigen. Co-infected animals were also found to have higher CFUs of BCG in their lungs, liver, and spleen at three different time points post infection, when compared to singly infected animals. A study conducted in cattle infected with *F. hepatica* found that co-infection with *F. hepatica* and *Salmonella dublin* resulted in increased excretion of *S. dublin* and higher mortalities (2), although no specific mechanisms were identified. In summary, suppression of Th1 responses is known to occur in a variety of helminth infections. This is likely to be one of the factors resulting in increased susceptibility and to bacterial infections, and in altered performance of diagnostic tests.

The macrophage plays a vital role in the outcome of mycobacterial infection, acting as a host cell and providing anti - mycobacterial defenses. The effective recruitment of macrophages is needed for the induction of a DTH response, which is vital in the SCITT. Macrophage activation can follow two pathways, classical or alternative, depending on the metabolism of L - arginine *via* inducible nitric oxide synthase (iNOS) or arginase, respectively (19). Phenotyping of macrophages can therefore be carried out by monitoring the production of NO and the levels of arginase activity. Alternatively-activated macrophages are typically associated with helminth infections and have been highlighted as a possible cause of cellular anergy (33). In some cases this has been linked to the direct upregulation of programmed death ligand 2, (PD - L2), on the macrophage surface (33). PD - L2 has been shown to be responsible for decreasing cellular responsiveness by direct contact with T-cells. In this study we examined the possible role of macrophages in co-infection and found that animals infected with *F. hepatica* and those co-infected with *F. hepatica* and BCG produced macrophages which were

386 alternatively -activated. It has been shown that helminth-induced alternatively-activated
387 macrophages have reduced IFN - γ and NO output (14). During the DTH response, Th1
388 cells are responsible for recruiting and activating macrophages via IFN - γ secretion. We
389 have shown in this study that cellular immune responses relevant to BTB diagnosis are
390 compromised in animals following experimental *F. hepatica* infection. This lack of Th1
391 effector mechanisms and presence of alternatively-activated macrophages may be
392 responsible for the poor SCITT responses elicited from co-infected animals. The "cause
393 and effect" relationship between alternatively-activated macrophages and failing Th1
394 effector mechanisms is still to some extent unclear.

395 Our data demonstrates the continuing presence of IL - 4, a typical Th2 cytokine,
396 at the time of SCITT in co-infected animals. Previous studies have shown that peak IL - 4
397 expression in BTB occurs between weeks 5 -8 post-infection, with the response fading to
398 background levels as infection proceeds (31). This is consistent with the results from
399 Group 4 animals in our study, with IL - 4 production peaking at week 5, and levels
400 declining to the week of SCITT. Levels of IL - 4 in Groups 2 and 3, however, indicate
401 that cytokine expression patterns deviate from the norm in co-infected animals. In Group
402 3, IL - 4 levels were significantly reduced at week 5 while being significantly higher
403 when compared to Group 4 at time of SCITT. In Group 2, levels were also higher at time
404 of SCITT but not significantly so. This suggests that a Th2 response was ongoing and
405 being specifically directed at PPD - B.

406 The differences in IFN - γ responses seen between BCG-only and co-infected
407 groups may also have been responsible for the poor SCITT test results. It appears that *F.*
408 *hepatica* infection can cause the antigen specific IFN - γ response to shut down.

Notably, animal 3.3, the only one positive on both SCITT and γ-INF tests from either co-infected groups, was also the only one not to have a detectable IL-4 response and whose γ-INF response remained elevated 13 weeks post-BTB immunization. The measurement of additional cytokines, such as IL-4, in helminth-infected animals in response to PPD-B restimulation of lymphocytes *in vitro*, may offer additional diagnostic potential.

The experiments described here deal with the possible influence of diagnosis of BTB in animals with *F. hepatica* infection. This experimental model, using *M. bovis* BCG, while useful, fails to take into consideration the complex interplay that may take place in active BTB where bacterial multiplication will have greater influence due to prolonged exposure to mycobacterial antigen. In view of the continuing spread of BTB, uncertainty in relation to transmission dynamics, and the impact of proposed control measures on wildlife populations, we consider it important to carry out further experimental and epidemiological studies on the effect of helminth parasitism on the diagnosis and control of this disease including a co-infection study involving an active BTB infection. Conclusions from this study may also have wider implications in the context of human health, where multiple co-infections are common and vaccination is seen as the most sensible route towards eradication of such diseases (3).

Acknowledgements: R.F was funded by IRCSET under the EMBARK Initiative. Funding for the study was also provided by the EU Commission under Framework 6, Project ref. FOOD-CT-2005 -02305-DELIVER. The authors would also like to thank E. Gormley in the School of Agriculture, Food Science and Veterinary Medicine, UCD and E. Costello at the Department of Agriculture and Food for providing *M. bovis* antigens

431 and J. McNair, AFBI, Belfast, for helpful discussions.

432

433

434

435 **References**

436 1. **Aida, Y., and M.J. Pabst.** 1990. Removal of endotoxin from protein solutions by
437 phase separation using Triton X-114. J. Immunol. Methods. **132:** 191-195.

438

439 2. **Aitken, M.M., P.W. Jones, G.A. Hall, D.L. Hughes, and K.A. Collis**. 1978. Effects
440 of experimental *Salmonella dublin* infection in cattle given *Fasciola hepatica* thirteen
441 weeks previously. J. Comp. Pathol. **88:** 75-84.

442

443 3. **Borkow, G. and Z. Bentwich.** 2000. Eradication of helminth infections may be
444 essential for successful vaccination against HIV and tuberculosis. Bull. World Health
445 Organ. **78:** 1368-1369.

446

447 4. **Brady, M.T., S.M. O'Neill, J.P. Dalton, and K.H. Mills.** 1999. *Fasciola hepatica*
448 suppresses a protective Th1 response against *Bordetella pertussis*. Infect. Immun. **67:**
449 5372-5378.

450

5. **Brown, W.C., W.C. Davis, D.A Dobbelaere, and A.C. Rice-Ficht.** 1994. CD4+ T-cell clones obtained from cattle chronically infected with *Fasciola hepatica* and specific for adult worm antigen express both unrestricted and Th2 cytokine profiles. Infect. Immun. **62:** 818-827.

6. **Charleston, B., J.C. Hope, B.V. Carr, and C.J. Howard.** 2001. Masking of two in vitro immunological assays for *Mycobacterium bovis* (BCG) in calves acutely infected with non-cytopathic bovine viral diarrhea virus. Vet. Rec. **149:** 481-484.

7. **Clery, D.G., P. O'Mahony, and G. Mulcahy.** 1995. A novel method for the administration of metacercariae of *Fasciola hepatica* to adult cattle. Res, Vet. Sci. **58:** 290-291.

8. **Clery, D.G., and G. Mulcahy.** 1998. Lymphocyte and cytokine responses of young cattle during primary infection with *Fasciola hepatica*. Res. Vet. Sci. **65:** 169-71.

9. **Clery, D., P. Torgerson, and G. Mulcahy.** 1996. Immune responses of chronically infected adult cattle to *Fasciola hepatica*. Vet. Parasitol. **62:** 71-82.

10. **Cornelissen, J.B., C.P. Gassenbeek, W. Boersma, F.H. Borgsteede, and F.J. van Milligen.** 1999. Use of a pre-selected epitope of cathepsin-L1 in a highly specific peptide-based immunoassay for the diagnosis of *Fasciola hepatica* infections in cattle. Int. J. Parasitol. **29:** 685 - 696.

11. **Corraliza, I.M., M.L. Campo, G. Soler, and M. Modolell.** 1994. Determination of arginase activity in macrophages: a micromethod. J. Immunol. Methods. **174:** 231-235.

12. **Dalton, J.P., S. O'Neill, C. Stack, P. Collins, A. Walshe, M. Sekiya, S. Doyle, G. Mulcahy, D. Hoyle, E. Khaznadji, N. Moire, G. Brennan, A. Mousley, N. Kreshchenko, A.G. Maule, and S.M. Donnelly.** 2003. *Fasciola hepatica* cathepsin L-like proteases: biology, function, and potential in the development of first generation liver fluke vaccines. Int. J. Parasitol. **33:** 1173-1181.

13. **Donnelly, C.A., R. Woodroffe, D.R. Cox, J. Bourne, G. Gettinby, A.M. Le Fevre, J.P. McInerney, and W.I. Morrison.** 2003. Impact of localized badger culling on tuberculosis incidence in British cattle. Nature **426:** 834-837.

14. **Donnelly, S., S.M. O'Neill, M. Sekiya, G. Mulcahy, and J.P. Dalton.** 2005. Thioredoxin peroxidase secreted by *Fasciola hepatica* induces the alternative activation of macrophages. Infect. Immun. **73:** 166-173.

15. **Elias, D., H. Akuffo, A. Pawlowski, M. Haile, T. Schon, and S. Britton.** 2005. *Schistosoma mansoni* infection reduces the protective efficacy of BCG vaccination against virulent *Mycobacterium tuberculosis*. Vaccine **23:** 1326-1334.

16. **Elias, D., H. Akuffo, C. Thors, A. Pawlowski, and S. Britton.** 2005. Low dose

497 chronic *Schistosoma mansoni* infection increases susceptibility to *Mycobacterium bovis*
498 BCG infection in mice. Clin. Exp. Immunol. **139:** 398-404.
499
500 17. **Elias, D., D. Wolday, H. Akuffo, B. Petros, U. Bronner, and S. Britton.** 2001.
501 Effect of deworming on human T cell responses to mycobacterial antigens in helminth-
502 exposed individuals before and after bacille Calmette-Guerin (BCG) vaccination. Clin.
503 Exp. Immunol. **123:** 219-225.
504
505 18. **Farid, A., M. Al-Sherbiny, A. Osman, N. Mohamed, A. Saad, M.T. Shata, D.H.**
506 **Lee, A.M. Prince, and G.T. Strickland. 2005.** *Schistosoma* infection inhibits cellular
507 immune responses to core HCV peptides. Parasite Immunol **27:** 189-196.
508
509 19. **Gordon, S.** 2002. Alternative activation of macrophages. Nat. Rev. Immunol. **3:** 23-
510 35.
511
512 20. **Gormley, E., M.B. Doyle, T. Fitzsimons, K. McGill, and J.D. Collins.** 2006.
513 Diagnosis of *Mycobacterium bovis* infection in cattle by use of the gamma-interferon
514 (Bovigam) assay. Vet. Microbiol. **112:** 171-179.
515
516 21. **Liebana, E., A. Aranaz, M. Welsh, S.D. Neill, and J.M. Pollock. 2000.** *In vitro* T-
517 cell activation of monocyte-derived macrophages by soluble messengers or cell-to-cell
518 contact in bovine tuberculosis. Immunol. **100:** 194-202.
519

22. **Maizels, R.M., A. Balic, N. Gomez-Escobar, M. Nair, M.D. Taylor, and J.E. Allen.** 2004. Helminth parasites--masters of regulation. Immunol. Rev. **201:** 89-116.

23. **Monaghan, M.L., M.L. Doherty, J.D. Collins, J.F. Kazda, and P.J. Quinn.** 1994.The tuberculin test. Vet. Microbiol. **40:** 111-124.

24. **Mas-Coma, S., M.D. Bergues, and J.G. Esteban.** 1999. Human Fasciolosis, p 411-434. *In* J.P Dalton (ed.), Fasciolosis. CAB International, Wallingford, UK.

25. **Mulcahy, G., P. Joyce, and J.P. Dalton.** 1999. Immunology of *Fasciola hepatica* infection, p 341-376. *In* J.P Dalton (ed.), Fasciolosis. CAB International, Wallingford, UK.

26. **Murphy, T.M., K.N. Fahy, A. McAuliffe, A.B. Forbes, T.A. Clegg, and D.J. O'Brien.** 2006. A study of helminth parasites in culled cows from Ireland. Prev. Vet. Med. **76:** 1-10.

27. **O'Neill, S.M., M.T. Brady, J.J. Callanan, G. Mulcahy, P. Joyce, K.H. Mills, and J.P. Dalton.** 2000. *Fasciola hepatica* infection downregulates Th1 responses in mice. Parasite Immunol. **22:**147-155.

28. **O'Neill, S.M., K.H. Mills, and J.P. Dalton.** 2001. *Fasciola hepatica* cathepsin L cysteine proteinase suppresses *Bordetella pertussis*-specific interferon-gamma production

543 in vivo. Parasite Immunol. **23:** 541-547.

544

545 29. **Pollock, J.M., J. McNair, M.D. Welsh, R.M. Girvin, H.E. Kennedy, D.P. Mackie,**
546 **and S.D. Neill.** 2001. Immune responses in bovine tuberculosis. Tuberculosis **81:** 103-
547 107.

548

549 30. **Pritchard, G.C., A.B. Forbes, D.J. Williams, M.R. Salimi-Bejestani, and R.G.**
550 **Daniel.** 2005. Emergence of fasciolosis in cattle in East Anglia. Vet Rec. **157:** 578-582.

551

552 31. **Rhodes S.G., N. Palmer, S.P. Graham, A.E. Bianco, R.G. Hewinson, and H.M.**
553 **Vordermeier.** 2000. Distinct response kinetics of gamma interferon and interleukin-4 in
554 bovine tuberculosis. Infect Immun. **68:** 5393-5400.

555

556 32. **Spithill, T.W., P.M. Smooker, J.L. Sexton, E. Bozas, C.A. Morrison, J. Creany,**
557 **and J.C. Parsons.** 1999. Development of vaccines against Fasciola hepatica, p 377-401.
558 *In* J.P Dalton (ed.), Fasciolosis. CAB International, Wallingford, UK.

559

560 33. **Terrazas, L.I., D. Montero, C.A. Terrazas, J.L. Reyes, and M. Rodriguez-Sosa.**
561 2005. Role of the programmed Death-1 pathway in the suppressive activity of
562 alternatively activated macrophages in experimental cysticercosis. Int. J. Parasitol. **35:**
563 1349-1358.

564

565 34. **Vordermeier, H.M., M.A. Chambers, B.M. Buddle, J.M. Pollock, and R.G.**

Hewinson. 2006. Progress in the development of vaccines and diagnostic reagents to control tuberculosis in cattle. Vet. J. **171:** 229-244.

35. **Welsh, M.D., R.T. Cunningham, D.M. Corbett, R.M. Girvin, J. McNair, R.A. Skuce, D.G. Bryson, and J.M. Pollock. 2005.** Influence of pathological progression on the balance between cellular and humoral immune responses in bovine tuberculosis. Immunology **114:** 101-111.

Figure Legends

Figure 1.

F. hepatica specific IgG1 responses (+/- S.E.M.) in Groups 1, 2, 3, and 4 All animals in Group 1, 2, and 3 seroconverted while Group 4 did not. No significant difference in titers between Groups 1, 2, and 3 were found. Parasite-specific IgG2 levels did not rise above background during the course of the experiment.

Figure 2

(a) Levels of arginase production in blood-monocyte derived macrophages (+/- S.E.M.) , 24 hours post-isolation, from each group. Measurements were made 14 weeks post BCG-inoculation and 18 weeks post *F. hepatica* infection, except in the case of Group 3, when the sampling was 12 weeks post *F. hepatica* infection. Arginase production was significantly greater ($P<0.05$) by cells from Group 1, 2 and 3 than Group 4. The latter did not produce levels significantly greater than those from negative control animals. (b)

589 Arginase production in blood-monocyte derived macrophages from naïve (uninfected)
590 calves stimulated with PPD - B and *F. hepatica* ES at 10µg/ml. Figure represents mean
591 (+/- S.E.M.) values from three experiments using cells from two animals. (c) NO
592 production by macrophages under conditions as described in (b).
593
594 **Figure 3.**
595 Il-4 (a) and γ-INF (b) production by individual animals (*F. hepatica*/BCG co-infected)
596 animals in Group 2. Cytokine levels were measured in plasma recovered from cultures of
597 whole blood taken at one week and thirteen weeks post-BCG immunisation and
598 stimulated *in vitro* with PPD - B. Results are presented as mean of triplicate wells _/-
599 S.E.M. The response of each animal to SCITT and whole blood γ-INF BTB test results is
600 also indicated on each panel as follows; N:N negative on both assays, N:P SCITT
601 negative whole blood positive, P:N SCITT positive whole-blood negative, and P:P
602 positive on both assays.
603
604 **Figure 4.**
605 Il-4 (a) and γ-INF (b) production by individual animals (BCG/*F. hepatica* co-infected)
606 animals in Group 3.). IL - 4 was measured in animals at week 1, 5, and 13 post-BCG
607 immunization and IFN - γ at weeks 1, 3, 5, and 13 post-BCG immunization, as described
608 for Figure 3. Response to BTB diagnostic tests is also indicated as for Figure 3.
609
610 **Figure 5.**
611 Il-4 (a) and γ-INF (b) production by individual animals (BCG-immunised) animals in

612 Group 4 Sampling times and other conditions were as described for Figure 4.

Table 1.

Treatment Group	Week 0	Week 4	Week 13	Week 17
1	*F. hepatica*	-	SCITT	-
2	*F. hepatica*	BCG	-	SCITT
3	BCG	*F. hepatica*	SCITT	-
4	BCG	-	SCITT	-

Table 1. Experimental Design. Calves were allocated to one of four treatment groups, as shown. In the case of groups 2, 3 and 4 SCITT was carried out 13 weeks post-BCG immunization. In addition to the groups shown above two additional calves, sourced from the same farm as the other animals, and within the same age range, were used as donors of blood monocytes for macrophage preparation and in vitro stimulation with PPD-B and *F. hepatica* ES.

Table 2:

Group	Treatment	Whole Blood IFN - γ Assay	SCITT		
		Positive	Negative	Positive	Negative
1	*F. hepatica* (n = 4)	-	4*	-	4*

2	*F. hepatica*/BCG (n = 4)	-	4*	-	4*
3	BCG/*F. hepatica* (n = 5)	1	4*	2	3*
4	BCG (n = 5)	4	1	4	1

Table 2: SCITT and IFN - γ assay results.

Whole blood IFN - γ assay was carried out on the same day as injection of PPD antigens for SCITT. Numbers of animals from each group that was classed as a positive or a reactor was expressed as a percentage for statistical analysis.

* Results were significantly different from those of the BCG-only group as tested by the χ^2 test, $p \leq 0.05$.

Figure 1

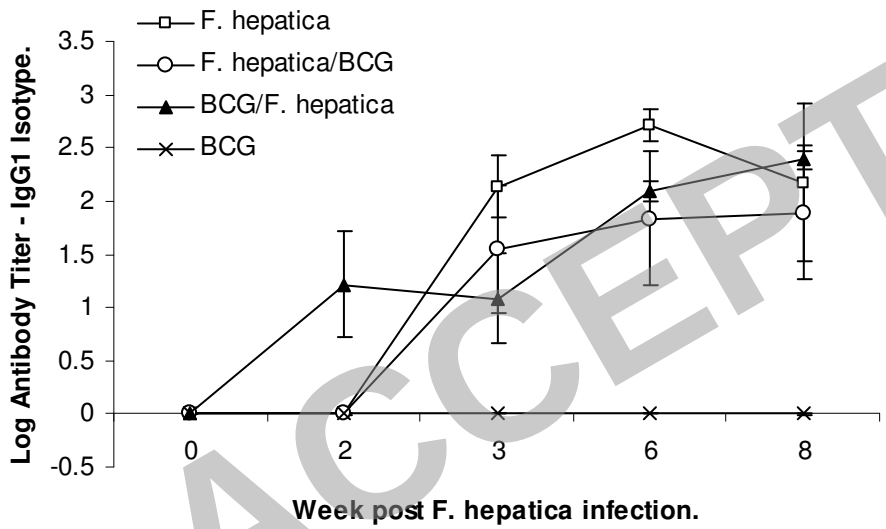


Figure 2

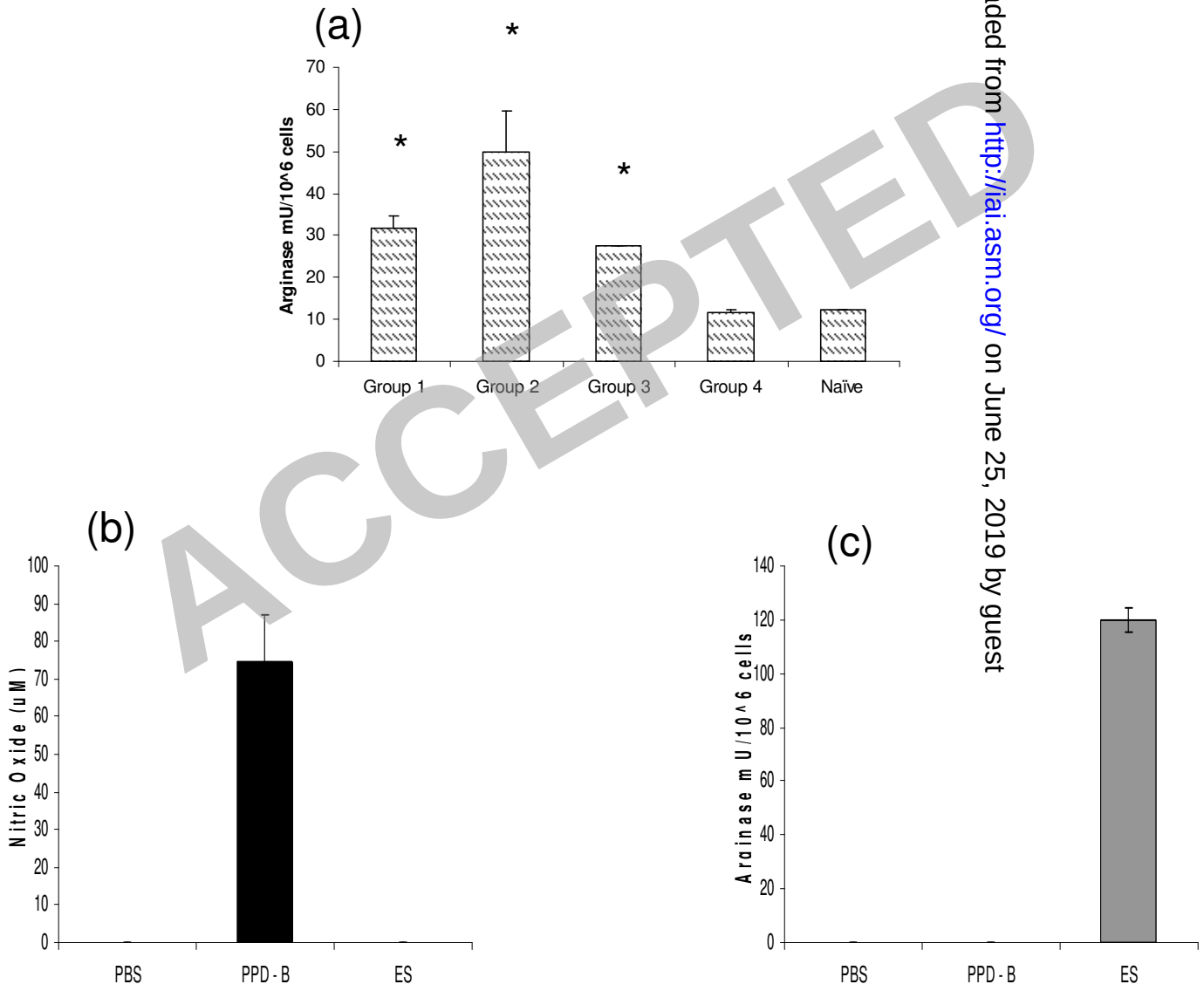


Figure 3

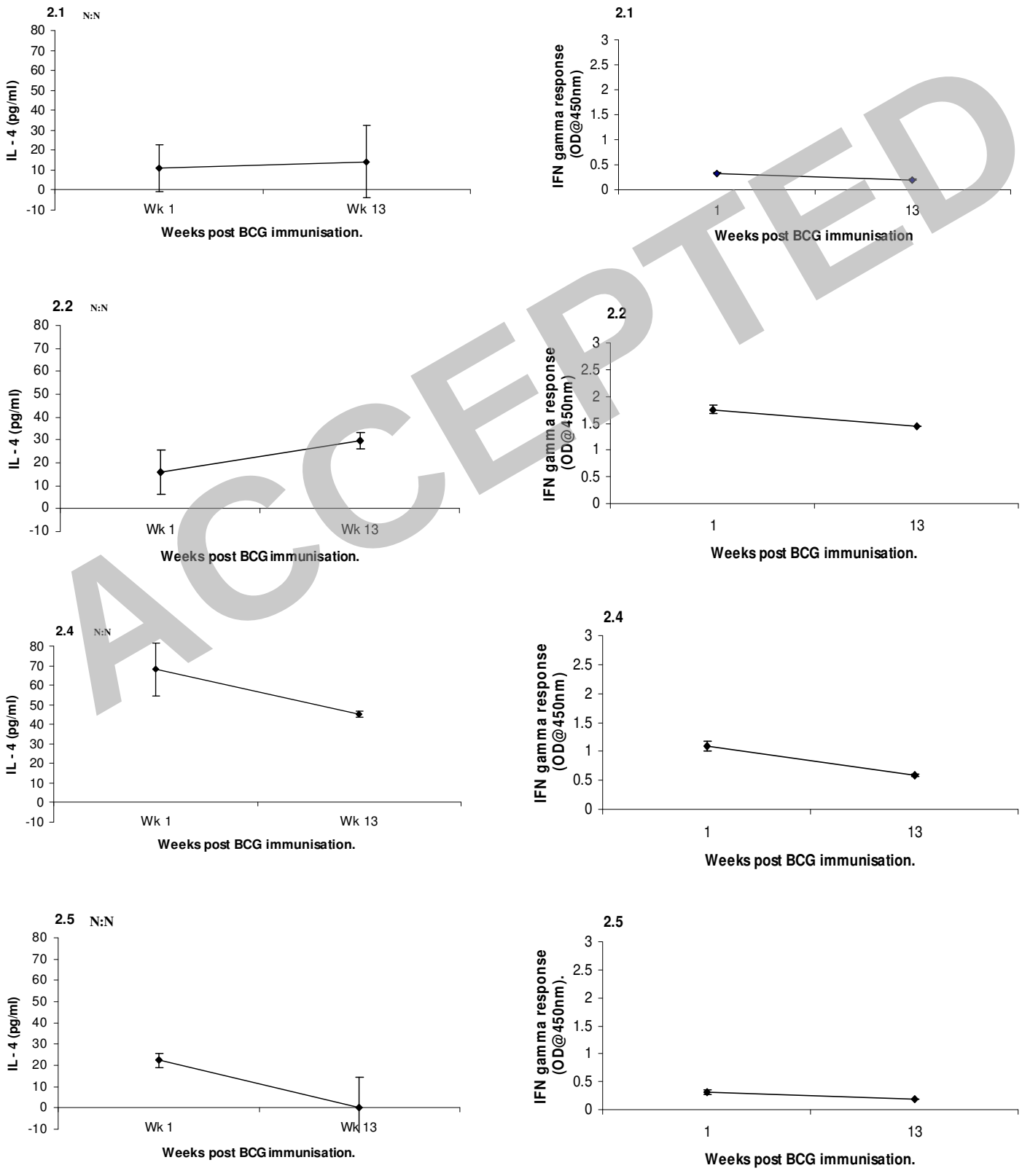


Figure 4:

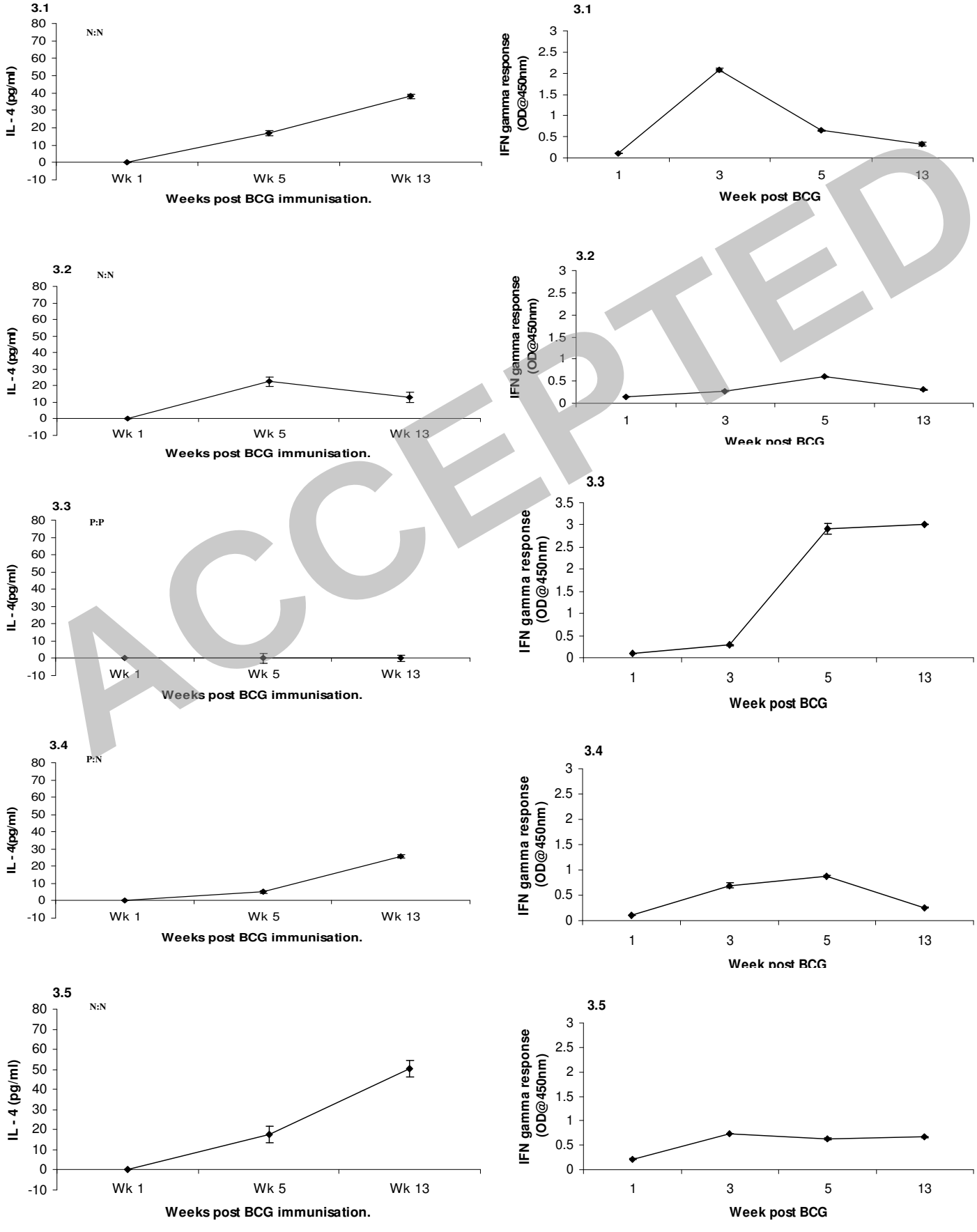
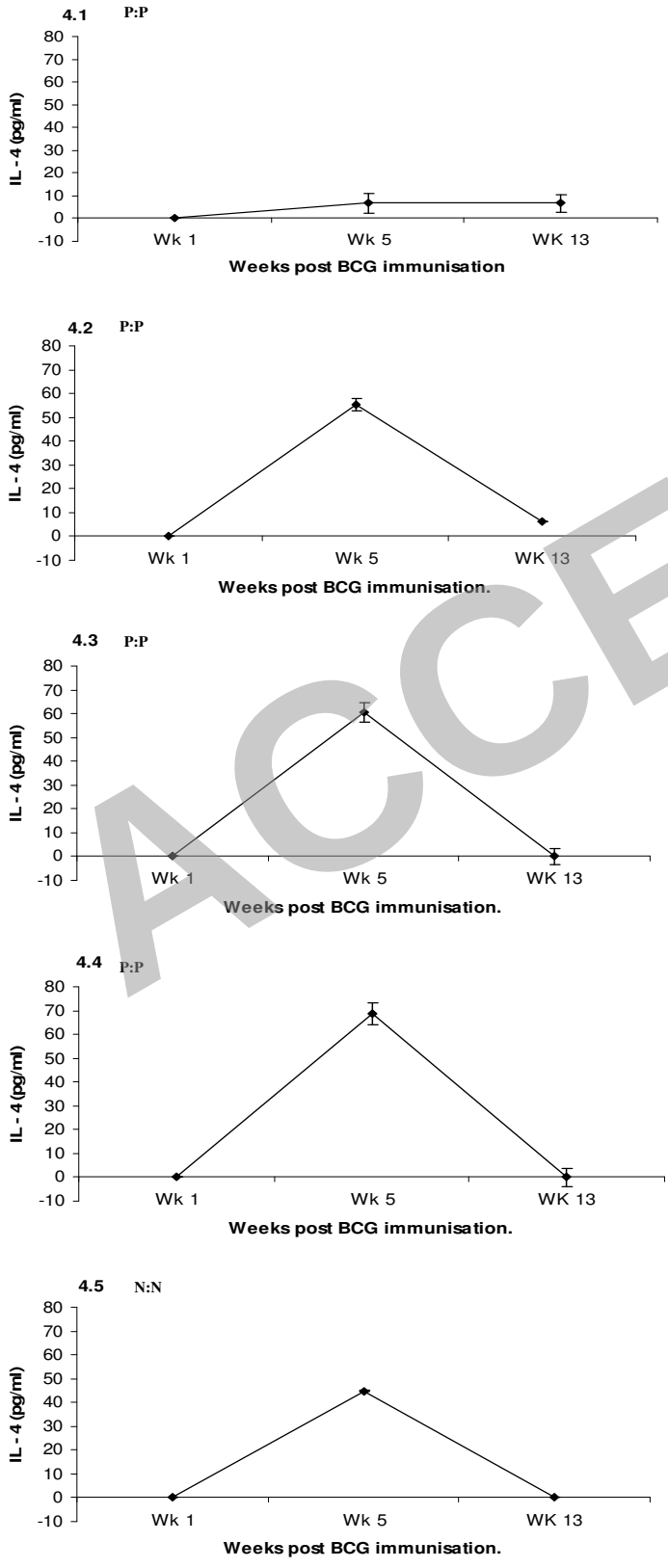


Figure 5:

(a)



(b)