DOI: 10.2478/s11686-013-0120-3

Acta Parasitologica, 2013, 58(2), 198–206; ISSN 1230-2821

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Evaluation of the immune response of male and female rats vaccinated with cDNA encoding a cysteine proteinase of *Fasciola hepatica* (FhPcW1)

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Abstract

Not only do males and females of many species vary in their responses to certain parasitic infections, but also to treatments such as vaccines. However, there are very few studies investigating differences among sexes following vaccination and infection. Here we demonstrate that female Sprague-Dawley rats vaccinated with cDNA encoding a recently discovered cysteine proteinase of *Fasciola hepatica* (FhPcW1) develop considerably lower liver fluke burdens after *F. hepatica* infection than their male counterparts. This is accompanied by differences in the course of their immune responses which involve different eosinophil and monocyte responses throughout the study as well as humoral responses. It is evident that host gender influences the outcome of parasitic infections after vaccination and research on both sexes should be considered when developing new treatments against parasites.

Keywords

Fasciola hepatica, sex differences, DNA vaccination, immunology, cysteine proteases

Introduction

Fasciola hepatica infections are a vital problem in veterinary medicine since the parasite is distributed worldwide and infects a wide range of mammalian hosts. Fasciolosis causes considerable economic losses of over US$ 3 billion per year in ruminant farming – mainly affecting cattle and sheep. During the last few years a pronounced increase in the number of infected animals has been observed in some European Union Member States, a trend that is predicted to continue into the future. Disease control is currently based almost exclusively on drug treatment, however, this approach is not satisfactory as drug resistant parasites have appeared. Recent years have seen an emphasis placed on research aimed at anti-*F. hepatica* vaccine development.

Vaccination trials have revealed that *F. hepatica* cathepsin L (catL) proteinases are effective targets of protective immunity. These enzymes are required for parasite feeding, tissue penetration, immune evasion and modulation, egg production and excystment (Dalton *et al.* 2003, 2006). Vaccination with catLs contributes to reductions in worm burdens which results in less extensive tissue damage, and also induces anti-fecundity effects in liver flukes that survive immune attack, greatly reducing the risk of infection of other pasture-raised animals and hence disease transmission. Vaccination with native catL in cattle has resulted in worm reductions up to 69%, or up to 72% when in combination with haem-binding protein (Dalton *et al.* 1996), while a combination of catL and leucine aminopeptidase induced up to 79% protection in sheep (Piacenza *et al.* 1999). More recently Jayaraj *et al.* (2009) demonstrated a 76% reduction in worm burdens in rats following immunisation with recombinant catB and catL5.

DNA vaccination represents an attractive approach to obtain therapeutic and commercial products. To date there are four DNA vaccines that have been licensed for use in the veterinary field (Davidson *et al*. 2005, Garver *et al*. 2005,

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Bergman *et al*. 2006, Person *et al*. 2008) and numerous are undergoing clinical trials. Since the DNA vaccine concept only arose from initial experiments by Wolff *et al*. (1990), the introduction of these products to market in the first decade of the millennium is a great success, and highlights the promise with this form of vaccination. In the area of anti-*F. hepatica* vaccine research there have been several attempts to develop DNA vaccines encoding selected antigens of the parasite. The greatest success was achieved by immunisation with cDNA encoding a cysteine proteinase, where male rats exhibited 100% protection against infection (Kofta *et al*. 2000). In the same study females responded with a protection level of 74%.

There are numerous studies demonstrating that host gender may influence the outcome of parasitic infection. In general, it is understood that male mammals are more susceptible to parasitic infection than females due to gender-associated differences in exposure and the immunosuppressive properties of androgens. It is widely held that sex hormones act on immunocompetent cells and thereby influence the immune system. The role of sex steroids has been well documented in affecting clonal expansion, phagocytosis, apoptosis, antigen presentation and physiological responses to cytokines/chemokines (Morales-Montor *et al*. 2004). Females appear to generally mount higher innate, cell-mediated and humoral responses than males (Marriott and Huet-Hudson 2006, Cook 2008, Hewagama *et al*. 2009). Moreover, female rodents exhibit increased mitogen-induced lymphocyte proliferation, faster wound healing and higher immunological intolerance to foreign substances (Morales-Montor *et al*. 2004). However, the female host supremacy paradigm has recently come under criticism for representing too simplistic a scenario since many notable exceptions to the paradigm exist (Morales-Montor *et al*. 2004).

Parasites themselves can also alter hormone concentration/signaling and as a result complex immunoneuroendocrine host-parasite interactions are present (Klein 2004). Indeed, in *F. hepatica* infected rats hepatic metabolism of progesterone and testosterone is impaired (Biro-Sauveur *et al*. 1994). Further, in Fresian heifers infected with liver fluke considerably lower levels of progesterone and higher levels of estrogen were reported as compared with uninfected animals (López-Diaz *et al*. 1998). These findings imply that the normal metabolism of sex hormones is influenced by the parasite which consequently may affect the ultimate result of infection. While males and females vary in their responses to parasitic infections, they also differ in their responses to treatments such as vaccination. Nevertheless, there are very few studies investigating differences among sexes following vaccination and infection.

Previously, we performed molecular and bioinformatic characterisation on a recently discovered catL isoform, *F. hepatica* cysteine proteinase Warsaw1 (FhPcW1); the data obtained suggested that the protein may have immunogenic potential (Jaros *et al*. 2010b). The present paper outlines the results of a vaccine trial using cDNA encoding FhPcW1 as a vaccine and the characterisation of the immune responses of both male and female rats after vaccination and infection.

Materials and Methods

Vaccine construct. Obtaining cDNA encoding FhPcW1 (GenBank accession: EF407948) was described previously (Jaros *et al*. 2010b). The sequence for FhPcW1 was then amplified using the primers FhPcW1pCMV-L (5' GTGCTGCAGATGTGGTTCTTCGTATTAGCCGT) and FhPcW1pCMV-R (5' GAAGCGGCCGCTTACACGGAAATCGTGCCAAT) which contained sequences recognised by the restriction enzymes *Pst*I and *Not*I, respectively. Additionally, FhPcW1p CMV-R also contained a native stop codon for reasons explained elsewhere (Jaros *et al*. 2010a). PCR conditions were as follows: 94°C for 45 s, 61°C for 45 s, 72°C for 100 s, 35 cycles (MJ Research Thermocycler). After purification from an agarose gel (NucleoSpin® Extract II, Macherey-Nagel) the PCR product was cloned into the pGEM®-T Easy Vector (Promega) using ligation conditions as described previously (Jaros *et al*. 2010b). Subsequently transformation of electrocompetent *Escherichia coli* DH5α was carried out (1.38 kV, Electro Cell Manipulator ECM 600 BTX). Bacteria were cultured and plasmids from transformed colonies were isolated and subjected to restriction analysis using *Pst*I and *Not*I. After restriction analysis the nucleotide sequence of the insert was determined (AbiPrism 3100 Genetic Analyzer). Correct inserts were then subcloned into pCMV (Invitrogen) through digestion with *Pst*I and *Not*I, and ligation with T4 ligase (MBI Fermentas). Electrotransformation of *E. coli* DH5α was performed as above. Plasmids from transformed colonies were isolated and subjected to restriction analysis and sequencing to confirm construct integrity prior to vaccination.

Vaccination trial. The experiment was carried out on Sprague-Dawley rats, which at the beginning of the trial were 3-months-old. The trial involved three experimental groups containing 8 males and 8 females each. Animals from the first group were immunised intramuscularly with the cDNA-FhPcW1/pCMV construct 4 weeks apart (first immunisation – 25 μg of construct per rat, second immunisation – 50 μg of construct per rat). Alternatively, animals from the second group received empty vector (pCMV) injections at the same time points (at the same doses as mentioned above). Both cDNA-FhPcW1/pCMV and pCMV were administered to rats in 0.05% bupivacaine. The bupivacaine is understood to cause damage to muscle fibers, which is followed by cell regeneration and increased transfection rates. Animals from the third group remained unvaccinated but were infected (challenge control group). All rats were infected intragastrically 4 weeks after the second immunisation with 35 two-month-old metacercariae. Nine weeks post infection (wpi), rats were euthanised and after autopsy worm burdens in livers were calculated. All procedures were approved by the 3rd Local Ethical Committee, Warsaw.

Haematology analysis. Rats were bled by tail vein on the day of infection and at 2, 4, 6 and 9 wpi and subsequently blood samples were subjected to analysis in an automated analyser (Abacus JunVet). The following blood parameters

were investigated: total white blood cells (WBC), total red blood cells, platelet count, haemoglobin, haematocrit, red blood cell indices – mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, and red blood cell distribution width.

Flow cytometry. Blood samples were subjected to flow cytometric analysis using immunofluorescence staining and a side scatter versus forward scatter profile. Blood samples at a final cell count of 5×10^5/ml were incubated with appropriate antibody combinations at dilutions recommended by the manufacturer. Antibody combinations (Pharmigen Inc) were the following: (1) CD45 fluorescein isothiocyanate (FITC)-conjugated and CD14 phycoerythrin (PE)-conjugated antibodies to define the optimal lymphocyte gate (leucogate), (2) CD4 FITC-conjugated and CD8 PE-conjugated antibodies to distinguish T helper and T cytotoxic lymphocytes, and (3) appropriate isotypic controls. A 12 min treatment with FACS lysing buffer (Becton Dickinson) was necessary to avoid erythrocyte contamination. After centrifugation (5 min, RT, 1000 × *g*) the resultant cell pellet was washed with phosphate buffered saline (PBS). Following centrifugation, all samples were fixed with 0.5% formaldehyde and subjected to flow cytometry analysis. Ten thousand gated events were counted in a FACS Calibur flow cytometer and analysed by the use of CellQuest software. Leucocyte populations such as lymphocytes, neutrophils, eosinophils, and monocytes were identified according to their characteristic appearance on scatter dot plots.

ELISA. Excretory-secretory (ES) products were obtained from adult liver flukes collected from infected cattle according to Şimşek *et al*. (2006). Microtiter plates were coated with 15 µg/ml of ES and incubated overnight at 4°C. After blocking with PBS containing 5% skimmed milk (1 h, 37°C) plates were washed thrice with PBS containing 0.05% Tween 20 (PBST). Sera from individual rats were serially diluted along the plates (starting from a 1:100 dilution) and were subsequently incubated for 1 h at 37°C and washed. For the detection of antibody isotypes, goat horseradish peroxidase (HRP)-conjugated anti-rat IgG1, IgG2a or IgG2b (1:100,000; Bethyl) were added and incubated at 37°C for a further 1 h. Following six washes with PBST, plates were developed using a solution containing tetramethylbenzidine (Sigma). After 15 min at RT the enzymatic reaction was stopped by adding 50 µl of 2 M H_2SO_4 per well. Reciprocal titres were defined as the highest dilutions yielding an OD_{450} of 0.2.

Necropsy. At necropsy, livers were collected and a score of gross hepatic damage was assigned according to the scale proposed by Raadsma *et al*. (2007), where 0 represents no signs of liver damage, 1 corresponds with a slight damage confined to less than 5% of the liver, 2 represents slight damage up to 15%, 3 moderate damage up to 30%, 4 heavy damage up to 50%, and 5 extensive damage of more than 50% of the liver.

Statistical analysis. Statistical analyses were performed using STATISTICA 6.1 software. Non-parametric Kruskal-Wallis test was used and if results were statistically significant Kruskal-Wallis ANOVA by ranks test was chosen to determine differences between groups. The level of significance was taken as $p<0.05$.

Results

Effectiveness of vaccination. The liver fluke burdens recovered from individual rats are presented in Table I. Only in the male group immunised with pCMV was there observed a statistically significant difference. The percentage of protection was calculated according to the formula: P = (1-d/k) × 100% (where d stands for the mean number of flukes found in cDNA-FhPcW1/pCMV or pCMV vaccinated rats; and k stands for the mean number of flukes in unvaccinated rats). In males vaccinated with cDNA-FhPcW1/pCMV or pCMV we recovered more flukes than compared to the non-immunised control group, a 26.8% and 121.4% increase, respectively. However, in females the number of isolated flukes decreased by 19.4% in the cDNA vaccinated group and by 9.7% in the group immunised with an empty vector.

Table I. Analysis of liver flukes numbers recovered from rats

Group	Counts in individual rats	Total	Mean±SD
♂cDNA-FhPcW1/pCMV	7,14,15,5,11,8,7,4	71	8.88±4.05
♀cDNA-FhPcW1/pCMV	5,7,2,10,10,8,9,7	58	7.25±2.72
♂pCMV	19,19,14,9,12,19,14,18	124	15.5±3.82*
♀pCMV	5,8,10,9,11,7,6,9	65	8.13±2.03
♂Challenge control	3,3,9,13,10,2,8,8	56	7±3.93
♀Challenge control	5,12,9,5,17,9,6,9	72	9±4.04

*Result statistically significant as compared to appropriate challenge control group ($p<0.05$).

Liver damage score. Mean liver damage scores were comparable between female and male challenge control groups (Table II). In females vaccinated with cDNA-FhPcW1/pCMV or an empty vector we observed a statistically significant reduction in liver damage. Alternatively, liver injury in males immunised with cDNA-FhPcW1/pCMV was comparable to the male challenge control group, while males vaccinated with pCMV exhibited significantly more extensive liver damage.

Table II. Mean liver damage scores in infected rats

Group	Mean liver damage score
♂cDNA-FhPcW1/pCMV	3.13±0.64
♀cDNA-FhPcW1/pCMV	2.38±0.52*
♂pCMV	4.25±0.71*
♀pCMV	2.50±0.53*
♂Challenge control	3.00±0.75
♀Challenge control	3.25±0.46

*Result statistically significant as compared to appropriate challenge control group ($p<0.05$).

Table III. White blood cell count throughout the study

Group	White blood cell count [G/l]				
	0 wpi	2 wpi	4 wpi	6 wpi	9 wpi
♂cDNA-FhPcW1/pCMV	9.29±3.29	13.48±1.75	13.77±1.17	19.34±4.71*	14.93±2.57
♀cDNA-FhPcW1/pCMV	6.30±1.18	8.95±1.79	11.55±1.70*	13.12±2.87*	5.52±1.57
♂pCMV	8.32±1.30	10.15±2.09	17.77±7.19*	18.2±3.31*	17.9±2.77*
♀pCMV	6.44±0.77	11.00±3.52	13.05±1.96*	16.37±0.82*	5.24±1.66
♂Challenge control	10.84±1.78	9.56±1.87	11.89±1.18	10.97±4.87	8.86±1.81
♀Challenge control	6.67±1.99	9.47±1.78	12.36±1.31*	11.40±1.72	3.80±1.88

*Result statistically significant ($p<0.05$).

Table IV. Eosinophil count throughout the study

Group	Eosinophil count [G/l]				
	0 wpi	2 wpi	4 wpi	6 wpi	9 wpi
♂cDNA-FhPcW1/pCMV	0.35±0.17	2.39±0.95*	2.49±0.46*	2.06±0.25*	2.33±0.31*
♀cDNA-FhPcW1/pCMV	0.16±0.05	1.97±0.55*	1.66±0.49*	1.10±0.14*	0.48±0.24
♂pCMV	0.18±0.06	2.23±0.51*	3.04±1.66*	3.09±0.54*	2.58±0.50*
♀pCMV	0.16±0.06	2.84±0.99*	2.45±0.61*	1.64±0.60*	1.13±0.28*
♂Challenge control	0.30±0.12	1.68±0.76*	3.22±0.60*	1.77±0.47*	1.26±0.69*
♀Challenge control	0.20±0.09	1.88±0.27*	1.61±0.49*	1.30±0.23*	0.61±0.33

*Result statistically significant ($p<0.05$).

Table V. Monocyte count throughout the study

Group	Monocyte count [G/l]				
	0 wpi	2 wpi	4 wpi	6 wpi	9 wpi
♂cDNA-FhPcW1/pCMV	0.23±0,03	0.89±0.42*	1.37±0.29*	0.03±0.01	0.03±0.01
♀cDNA-FhPcW1/pCMV	0.002±0.001	0.20±0.12*	0.16±0.01*	0.21±0.15*	0.33±0.18*
♂pCMV	0.28±0.13	0.47±0.17	0.61±0.26*	0.18±0.07	0.11±0.02
♀pCMV	0.007±0.003	0.43±0.16*	0.30±0.02*	0.46±0.29*	0.83±0.30*
♂Challenge control	0.33±0.07	0.45±0.27	1.75±0.60*	0.88±0.19	0.02±0.01
♀Challenge control	0.004±0.001	0.009±0.002	0.23±0.01*	0.28±0.08*	0.27±0.11*

*Result statistically significant ($p<0.05$).

Haematology parameters. In the case of females all groups exhibited marked leukocytosis by 4 wpi, with both immunised groups showing peak levels at 6 wpi, although values for the pCMV group were higher than for the cDNA-FhPcW1/pCMV group (summarised in Table III). At 9 wpi a rapid drop in WBC count was noted for all groups. Interestingly, no statistically significant change in WBC count was observed for the male challenge control group throughout the study, resulting in significant differences compared to vaccinated males, which exhibited a steady increase in WBC count and peaks at 6 wpi.

We did not observe any significant differences in the erythrocyte related parameters mentioned in the Materials and Methods section among investigated groups across the experimental period (data not shown).

Table VI. Neutrophil count throughout the study

Group	Neutrophil count [G/l]				
	0 wpi	2 wpi	4 wpi	6 wpi	9 wpi
♂cDNA-FhPcW1/pCMV	2.17±0.88	3.36±1.10	4.02±0.78*	5.18±0.22*	9.73±2.63*
♀cDNA-FhPcW1/pCMV	1.04±0.28	2.89±0.80	4.53±0.85*	5.07±1.16*	4.76±0.54*
♂pCMV	1.05±0.30	1.67±0.30	6.02±1.72*	5.53±0.84*	9.84±1.31*
♀pCMV	1.02±0.12	4.41±1.52*	5.36±1.20*	6.69±1.23*	8.08±1.49*
♂Challenge control	1.53±0.42	1.29±0.29	3.99±0.98*	4.00±0.76*	4.90±2.80*
♀Challenge control	1.35±0.72	3.90±0.70*	4.51±0.92*	4.64±.0.74*	4.90±1.54*

*Result statistically significant ($p<0.05$).

Cytometric analysis. We did not detect any statistically significant changes among either CD4+ or CD8+ lymphocyte counts in any experimental groups during the study (data not shown). However, we reported elevated eosinophil numbers in blood samples from as early as 2 wpi in all investigated groups (Table IV). Among female rats peak eosinophil values were noted at 2 wpi, whereas peak eosinophil counts were delayed to 4 or 6 wpi in male rats and greater eosinophilia was observed. Further, male and female rats differed in their monocyte responses (Table V). Considerably higher monocyte counts in male rats were evident. Males groups had peak monocyte responses at 4 wpi followed by a significant decline, with the pCMV group showing a significantly diminished response. Alternatively, all female groups showed a gradual increase across the experimental period, the immunised groups showing increased monocyte counts earlier than their control group counterpart, and the pCMV group maintaining higher monocyte levels across the study period. The trend in neutrophil responses was similar in both sexes, with a gradual increase throughout the study (Table VI). In both sexes the highest counts were observed in the pCMV groups, and in the case of rats vaccinated with cDNA-FhPcW1/pCMV the neutrophil counts among the females were almost half those of their male counterparts immediately prior to autopsy.

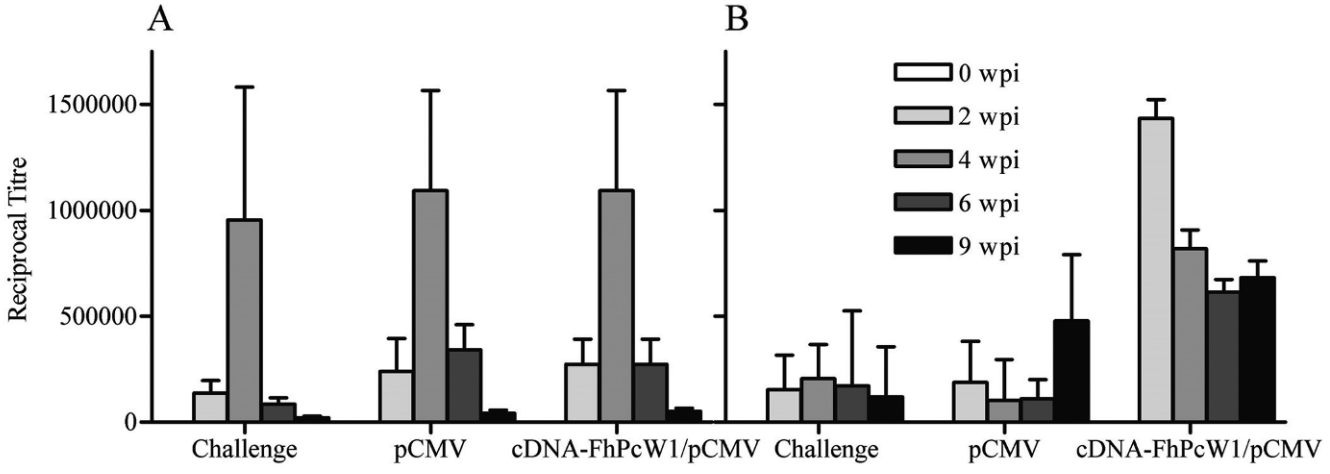

Fig. 1. IgG1 responses among (A) male and (B) female Sprague-Dawley rats to *F. hepatica* excretory-secretory products following infection. Results are means +SD, n = 8

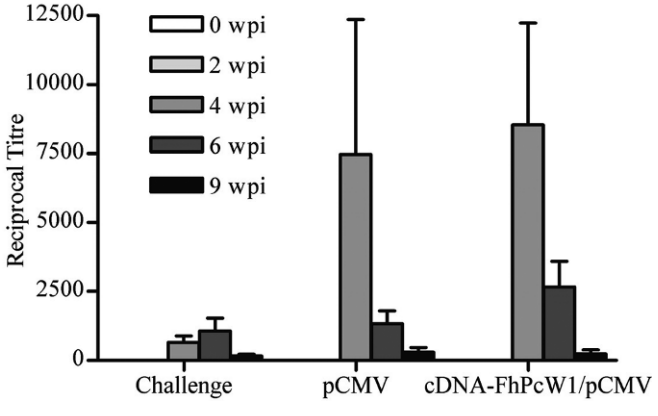

Fig. 2. IgG2b responses among male Sprague-Dawley rats to *F. hepatica* excretory-secretory products following infection. Results are means +SD, n = 8

Humoral responses. The serum IgG1 titres were measured throughout the study in male and female rats and are presented in Fig. 1. The IgG1 profiles in serum samples collected from experimental males follow the same trend in all groups; the highest values were observed at 4 wpi, followed by a substantial drop. Female rats vaccinated with cDNA-FhPcW1/

pCMV had the highest IgG1 titre at 2 wpi, which was followed by a decline, however, the titre still remained significantly elevated. Titres for pCMV and challenge control groups were relatively low, with an incidental increase at 9 wpi in the pCMV group. Further, we did not detect any ES-specific IgG2a antibodies in serum samples from any groups (data not shown). In females there was also no IgG2b production detected (data not shown), nevertheless, in males we observed detectable IgG2b titres from 4 wpi (Fig. 2).

Discussion

Host gender influence in regard to *F. hepatica* vaccine efficiency has been little studied so far, however, it should be taken into account when designing vaccine trials. Typically, female rats are more resistant to liver fluke infection than their male counterparts. Research performed by Cho *et al*. (1997) demonstrated higher fluke burdens in male Sprague-Dawley, Wistar and spontaneously hypersensitive rats. Only in the Fisher 344 rat strain was the number of fluke recovered slightly higher in females. In the present study the fluke burdens and subsequent liver damage were higher in male rats vaccinated with an empty vector or a vaccine construct than compared to their female counterparts. It appears that female immune responses after cDNA-FhPcW1/pCMV vaccination are better suited to combat the infection, however, the level of protection was not high or significant (19.4%). The overall fluke burden (and liver damage) was the highest in the male pCMV immunised group – the number of isolated flukes was more than double that of the challenge control rats. The presence of cytosine-phosphate-guanosine (CpG) immunostimulatory sequences within the pCMV vector can induce antigen-independent immune response enhancement (reviewed by Vollmer 2006). In the case of the male pCMV immunised group a strong inflammatory response was induced since dramatic peripheral leucocytosis was observed. Although cellular components associated with inflammation are involved in protection, a strong inflammatory response can be disadvantageous for the host as it may lead to severe immunopathological liver damage. Consequently, vaccines which provoke massive inflammation are not acceptable for use. Further, in cDNA-FhPcW1/pCMV vaccinated male rats 26.8% more liver flukes were recovered than in the challenge control male group. The vaccine construct also induced a potent inflammatory response with maximal WBC count at 6 wpi, however, WBC count decreased at 9 wpi whereas in the vector group high counts still persisted. Whether the increased delayed inflammatory responses contributed to increased fluke survival, or whether the increased number of flukes prolonged the inflammatory response is unclear. Interestingly, in challenge control male rats the WBC count hovered around the value reported on the day of infection. Thus, male rats immunised with cDNA-FhPcW1/pCMV or pCMV were unable to effectively combat *F. hepatica* invasion. Their immune response was not conducive to controlling the parasite's growth, migration and survival, but rather created an environment that favoured the survival of a greater number of parasites.

For females we observed a low protective effect of vaccination, with fluke burdens recoveries only slightly lower than those noted in the challenge control female group. The highest WBC counts were observed at 6 wpi for cDNA-FhPcW1/pCMV and pCMV immunised rats. It is at approximately this time during infection that the parenchymal stage of the disease ends and liver flukes start passing into the bile ducts, signifying the beginning of the chronic phase of the disease. When flukes are found in the bile ducts the parenchyma can start to regenerate and consequently the inflammatory process in the liver is attenuated, though is still present in epithelia of the bile ducts and directly adjacent liver tissue (Behm and Sangster 1999). This may explain why a diminution in the WBC count at 9 wpi was reported among all the female groups.

The peritoneal areas of rats are infiltrated with neutrophils, eosinophils and lymphocytes during the early stages of infection (Jedlina *et al*. 2011), while similar cell types also infiltrate the liver when flukes are tunneling through liver parenchyma (Keegan and Trudgett 1992). Here, we did not detect any statistically important changes in either CD4+ or CD8+ lymphocyte counts in peripheral blood among any experimental groups during the study. However, we observed a substantial rise in eosinophil and neutrophil levels. While the neutrophil count increased steadily in both sexes, eosinophilic response varied among female and male rats. While an early significant response was observed in all groups, in females peak values were reported at 2 wpi, followed by a gradual decline, whereas eosinophil counts continued to rise in male groups until 4 to 6 wpi, and were still elevated at the end of the experiment.

Several experiments have suggested that eosinophils are pivotal in conveying protection during early stages of *F. hepatica* infection, with a correlation between eosinophil levels at the gut wall and protection in rats (reviewed by Piedrafita *et al*. 2004). However, the role of eosinophils in mediating protection is not clear as *in vitro* studies have not confirmed participation of these cells in parasite killing. It appears that eosinophils are stimulated during the infection, however, they are not necessarily components of effective immunity against *F. hepatica* while it migrates through the liver. The maintenance of high eosinophil counts in males in the present study are likely to confirm this assumption. Eosinophilia may actually contribute to the severe tissue damage that was observed in male rats as eosinophils and neutrophils are major sources of reactive oxygen species. It appears that high eosinophil counts at a later stage of invasion do not favour removing *F. hepatica* infection in rats.

Eosinophils and neutrophils are known to exert their biological activities through antibody binding, and the protective immune response in immune rats, including ADCC, predominantly targets juvenile fluke (van Milligen *et al*. 1998a, b;

Piedrafita *et al*. 2004). Here we observed high IgG1 titres among investigated groups with the highest value reported as early as 2 wpi in female rats vaccinated with cDNA-FhPcW1/pCMV. The combination of the early eosinophil response observed in the female vaccinated rats combined with the early specific antibody response likely contributed to the limited protection observed amongst this group. Alternatively, other groups which did not have reduced fluke burdens either did not generate equivalently high IgG1 titres, or the response was delayed as in the case of male rats vaccinated with cDNA-FhPcW1/pCMV which possessed a maximum titre at 4 wpi. We did not detect IgG2a production, which is understood to favour protective responses during fasciolosis (Mulcahy *et al*. 1998). However, we demonstrated a small but significant elevation of IgG2b production in males vaccinated with either the construct or empty vector. Previously Paz *et al*. (1998) suggested that IgG2b production is stimulated by the parasite while it migrates through the liver and when *F. hepatica* is found in the bile ducts the antibody titre declines. Here, the highest IgG2b titres were found in groups with the highest fluke recoveries, further highlighting a correlation between fluke numbers and IgG2b titres.

It is worth noting that the profiles of monocyte responses also differed among male and female rats investigated during our study. A marked increase in monocyte numbers was observed in males by 4 wpi, followed by an equally marked decrease by 9 wpi, whereas females display a more moderate but still significant increase in monocyte counts throughout the experimental period. Monocytes are important players in the inflammatory cascade. They are capable of producing both pro- and anti-inflammatory cytokines during infection that may be involved in tissue damage or repair, respectively. An imbalance of these processes may have serious detrimental effects on the host. It is evident that in male Sprague-Dawley rats elevated monocyte counts are not associated with protection. Once again it is proposed that male's immune response promotes development of an immunopathological condition in the liver.

Although cysteine proteases have already proven their vaccine potential in many trials (for ref. see introduction) we did not observe marked protectivity after cDNA-FhPcW1/pCMV immunisation in our study. This was unexpected as FhPcW1 shows high identity to catL2 which has previously been shown to be a promising vaccine candidate through vaccine trials undertaken in cattle and sheep using native purified protein (Dalton *et al*. 1996; Piacenza *et al*. 1999). Protective immunity observed in previously mentioned experiments seem to be antibody dependent. It has been proposed that specific antibodies produced by immunised hosts neutralise cysteine proteases, activate cellular effector mechanisms and have a direct detrimental effect on the parasite (Jayaraj *et al*. 2009). Here, although we noticed high antibody titres and pronounced cellular immunity only a slight reduction in fluke burden was observed among cDNA-FhPcW1/pCMV vaccinated females.

Fasciola hepatica possess a number of immune evasion mechanisms, and it is possible that one or more of these played a role in limiting the efficacy of the vaccine under study, including the ability to cleave immunoglobulin, thus neutralising protective antibodies. *F. hepatica* is known to secrete a number of different cysteine proteases during their development, some of which may act as smoke screen antigens distracting/interfering with host immune responses to critical epitopes on other proteases, while redundancy through overlapping specificities between proteases may also confer some degree of protection. The expression of catL2-type cathepsins only once the fluke starts to mature (Robinson *et al*. 2009) may also limit the effectiveness of the vaccine, as an early, strong immune response, predominantly focused towards juvenile flukes has been shown to confer protection in rats. While some cross-reactivity of antibodies to closely related secreted cathepsins would be expected, it is possible that the immune response provoked by the FhPcW1 vaccine does not target the parasite at a suitably susceptible period of its development within the host. The importance of the generation of a rapid response is highlighted in the results of this study, with the cDNA-FhPcW1/pCMV vaccinated female rats the only group shown to mount a protective response, possessing maximal eosinophil counts and significantly elevated antibody titres within 2 wpi, as opposed to 4 wpi among their male counterparts.

This report highlights the importance of gender differences in immune responses following vaccination with cDNA-FhPcW1/pCMV and liver fluke infection. It is demonstrated that host gender contributes to the ultimate outcome of infection with female Sprague-Dawley rats better protected by vaccination in this study. It becomes apparent that the differences between the sexes must be taken into account when developing not only new immunoprophylactic strategies but also drugs directed against *F. hepatica*. Currently the majority of *F. hepatica* research is carried out using male rats as they lack periodic fluctuations of hormonal cycle. Nevertheless, the effectiveness of an animal treatment can be influenced by the hosts gender and may not be successful in both sexes. Further, farmed females are often of greater economic interest in animal husbandry than males, e.g. dairy cattle, and research should also focus on them. Taken together, data presented here highlights the necessity of research on both sexes in experiments when developing control methods against parasitic infection.

Acknowledgements. This work was supported by a grant from the European Union – the DELIVER project, no. FOOD-CT-200X-023025.

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Accepted November 29, 2012