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Th1/Th2 Balance in the Liver and Hepatic Lymph Nodes of Vaccinated and Unvaccinated Sheep During Acute Stages of Infection with *Fasciola hepatica*

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Highlights

- *F. hepatica* induces upregulation of Th2 and downregulation of Th1 responses in the liver and hepatic lymph nodes at 9 and 18 days post-infection in CL1-vaccinated and unvaccinated sheep.
- CL1-vaccinated sheep showed higher increase of liver IFN-γ expression than unvaccinated sheep at 9 dpi coinciding with lower hepatic lesions in the vaccinated group.

Abstract

The expression of IFNγ and IL4 was quantified using q-PCR in the liver and hepatic lymph nodes (HLN) of sheep during early stages of infection with *Fasciola hepatica* (1, 3, 9 and 18 days post-infection, dpi). A group of animals (Group 1) were vaccinated with *Fasciola hepatica* recombinant cathepsin L1 (FhCL1) in montanide 70 VG prior to infection, a second group (group 2) was used as infected control and a third (group 3) was used as uninfected control. To study vaccine efficacy three additional groups were sacrificed 19 weeks post-infection (group 4 immunized with CL1, group 5 with the adjuvant and group 6 was used as infected control). The vaccinated group did not show significant fluke reduction compared to the adjuvant group and infected control group. IL4 expression was observed to increase at 9 dpi and and was further elevated at 18 dpi in the liver and HLN of vaccinated and infected control groups compared to the uninfected group. IFNγ expression exhibited different dynamics in the liver and HLN compared to IL4; thus, in the liver this cytokine increased at 9 dpi in the vaccinated and at 18 dpi in vaccinated and infected control groups, while in the HLN it decreased

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gradually and significantly from 1 dpi onwards. These results suggest that a marked Th2 polarization is present from 9 dpi in HLN and from 18 dpi in the liver. The increase of IFNγ in the liver may correspond with tissue damage response with granuloma formation. The FhCL1 vaccine did not alter the Th1/Th2 balance when compared to unvaccinated and infected sheep. The study of IFNγ and IL4 in the various tissue compartments in sheep could facilitate selection of new adjuvants inducing a strong Th1 response for a more rationale vaccine formulation.

Keywords: *Fasciola hepatica*, Cytokines, Sheep, Lymph Node, Liver, Th1/Th2 immune response.

1. Introduction

The importance of economic losses caused by *Fasciola hepatica* in production animals, the emergent increase of anthelmintic resistance and the risk of chemical residues in milk and meat have increased the interest in developing immunoprophylactic control strategies for fasciolosis in ruminants (Cwiklinski et al., 2016). Nowadays, no commercial vaccines against *F. hepatica* has been obtained mainly due to the high immunomodulatory capacity of this parasite (Toet et al., 2014; Molina Hernández et al., 2015). It has been reported that peripheral T cells (PBMCs) and hepatic lymph node (HLN) lymphocytes of sheep chronically infected, cattle at acute and chronic stages of infection and liver of naturally infected cattle with *F. hepatica* showed downregulated IFNγ expression and upregulated IL4 expression suggesting a predominant Th2 response (Mulcahy et al., 1999; Waldvogel et al., 2004; Haçariz et al., 2009; Mendes et al., 2013). Similar results were reported in HLN lymphocytes of Indonesian thin-tail (ITT) sheep at 3 and 10 weeks after *F. hepatica* infection, whereas IL4/IFNγ ratio did not change in resistant ITT sheep after *F. gigantica* infection (Pleasance et al., 2011).

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These results suggest that a down regulated Th1 and upregulated Th2 responses allow the parasite to survive in the host. Since the protective response against *F. hepatica* occurs during early stages of infection, the knowledge of local host immune response mechanisms, including cytokine expression during early stages of *F. hepatica* infection in ruminants is of interest for the design of optimized vaccine formulation (Molina-Hernandez et al., 2015, Toet et al., 2014).

The aim of this study was to evaluate the expression of IL4 and IFNγ using qRT-PCR in sheep vaccinated with recombinant *F. hepatica* cathepsin L1 before parasite challenge, in unvaccinated-infected and in negative control sheep. The study was focused in the liver and hepatic lymph nodes during early stages of the infection.

2. Materials and methods

2.1. Experimental design

Forty-four 7-month old female Merino-breed sheep obtained from a liver fluke-free farm were used to study early stages of infection. Animals were purchased at one month of age and housed indoors in the experimental farm of the University of Córdoba until they reached the appropriate age for challenge. All animals were tested monthly for parasite eggs by fecal sedimentation and the results were negative in all cases. In addition, all animals were tested for serum IgG specific for *F. hepatica* cathepsin L1 (FhCL1) by ELISA prior to challenge and all of them were negative. The sheep were distributed into two groups of twenty animals each: group 1 was immunized subcutaneously with two doses four weeks apart with 100µg of recombinant CL1 from *F. hepatica* in 1 mg of Montanide ISA 70 VG (Seppic, Puteaux, France). Recombinant FhCL1 was generously provided by Prof. John Dalton (QUB, Belfast, UK). Group 2 was used as infected control. Group 3 (n=4) was used as the uninfected negative

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control. Groups 1 and 2 were orally infected with 200 metarcercariae of *F. hepatica* (Ridgeway, Gloustershire, UK) and divided into four subgroups each (n=5) which were sacrificed in pairs at one day post infection (dpi), 3 dpi, 9 dpi and 18 dpi. To study vaccine efficacy another 34 sheep of the same age were divided into three groups. Group 4 (n=13) were immunized as group 1; group 5 (n=12) was immunized only with the adjuvant and group 6 (n=9) was used as infected control. These 3 groups were orally infected with 150 metacercariae and sacrificed at 19 weeks post-infection for fluke burdens. The experiment was approved by the Bioethics Committee of the University of Cordoba (No.1118) and it was conducted in accordance with European (2010/63/UE) and Spanish (RD 1201/2005) directives on animal experimentation.

2.2. Fluke burdens and pathology

Sheep were subjected to necropsy and the liver was removed, photographed on the visceral and diaphragmatic aspects for gross evaluation. Liver tissue samples showing hepatic lesions were collected and samples frozen for qPCR examination while the remaining were fixed in 10% neutral buffered formalin for 24 h, then routinely processed and embedded in paraffin wax. Four micron-thick tissue sections were stained with hematoxylin and eosin (H&E) for histopathology. Fluke burden was conducted in animals from groups 4-6 opening gallbladders and bile ducts and flukes were removed. Finally, the livers were cut into small pieces (1 cm^3) and washed in hot water to collect the remaining flukes.

Gross hepatic lesions of early stages of infection (groups 1 and 2) were counted using the Image Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD, USA). Results were given as number of lesions ± SD. Hepatic lymph nodes (HLN) samples were collected for histopathology and q-PCR.

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2.3. RNA extraction and cDNA synthesis

Hepatic lymph node and a samples from left liver lobe were collected, washed in DEPC biomolecular water, frozen in liquid nitrogen and after that were individually disrupted with a mortar and pestle in liquid nitrogen and stored at −80°C. Total RNA was isolated from 300 mg of grounded tissue (liver or HLN) homogenized in 1.5 ml of TRIzol® reagent (Ambion, life technologies, Carlsbad, CA, USA) using a sterilized IKA®T10 basic disperser and RNA was extracted with the RNeasy ®mini kit (Qiagen, Hilden, Germany) according to manufacturer's guidelines. The protocol included a 15 min incubation with RNase-free DNase (Qiagen, Hilden, Germany), and a final 10 min incubation at 65°C to denature the RNA. RNA purity and concentrations were determined by spectrophotometry. The Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used to determine the RNA integrity number (RIN, whose values range from 0 for degraded RNA to 10 for intact RNA (Schroeder et al. 2006). Only RNAs with RIN values >8.5, ratios 260/280 about 2 and free of gDNA were used in qRT-PCR experiments.

The cDNAs were generated from the 1 µg of total RNA from each sample individually using the iScript™ cDNA Synthesis kit (BioRad, Hércules, CA, USA).

2.4. Primers design

The primer pairs for gene expression analysis of the target IFNγ and IL4 genes in the liver and HLN tissues, and those to quantify the transcript amounts of *Pgk1, B2m, Rplp0, SdhA* and *G6pd*, used as reference genes, were designed with the Oligo 7 software (Colorado Springs, USA) over sequences obtained from the GenBank database. In all cases, primers sized between 25 and 30 bp and generated amplicons with lengths ranged from 99 and 192 bp. The sense and antisense primers were located

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in different close exons to secure primer specificity for target cDNA. To obtain high specificity and better performance, primers devoid of hairpin and duplex structure, were required to have a high Tm (≥70°C) and optimal 3'-ΔG (≤ -6 kcal/mol) values for use in two-step 94/68°C PCR reactions. All primer pairs produced amplicons of the predicted size (Supplemental Information **Table 1**). All PCR products were further verified by nucleotide sequencing.

2.5. Identification of valid reference genes for the normalization of the qRT-PCR expression studies

The mRNA expression stability of five putative reference genes (Pgk1, B2m, Rplp0, SdhA, and G6pd) in our samples were measured by real-time RT-PCR. Suppl. Inf. **Fig. 1** shows the box plot of the threshold cycles (Ct) values obtained after amplifying in triplicate the five individuals in each experimental groups (n=159 for each gene). The RefFinder software package(http://fulxie.0fees.us), which integrates the currently available major computational programs (geNorm, Normfinder, BestKeeper, and the comparative ΔΔCt method) was further used to compare, rank and assign an appropriate weight to each individual gene and to calculate the geometric mean of their weights for the overall final ranking. The recommended comprehensive rankings are shown in Suppl. Inf. **Table 2.**

2.6. Absolute quantitation of cytokine transcript by real-time PCR

Real-time PCR reactions were performed in triplicate by using 50 ng of cDNA template, 0.3 µM of each primer and the SsoAdvanced ™ Universal SYBR® Green Supmermix (BioRad) kit, according to manufacturer's guidelines. A MyiQ™2 Two Color Real-Time PCR Detection System (BioRad). Cycling conditions consisted of 2 min at 95°C for Platinum Taq activation followed by 40 cycles for melting (15 s, 95°C),

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annealing and extension (30 s, 70°C). After 40 cycles, a melting curve analysis was performed (60–95°C) to verify the specificity of amplicons. Replicate PCR reactions generated highly reproducible results with SEM <10% of the mean (<1% for threshold cycle data). All targets amplified with the same optimal PCR efficiency (100%) and high linearity (r >0.99) in the range of 20 − 2×105 pg of total RNA input. An inter-run calibrator (IRC) RNA sample, with a known amount of transcripts of the A170 gene, was introduced in each experiment to guarantee the quality of the retro-transcription and to detect and remove inter-run variation. An absolute calibration curve was constructed, and the number of transcript molecules was calculated from the linear regression of the standard curve.

2.7. Statistical analysis

The number of gross hepatic lesions and number of mRNA molecules per µg RNA total are shown with averages and standard error of the mean (SEM). Comparisons of variables between control and infected groups were carried out by using a Student´s t-test followed by the Bonferroni correction for multiple comparisons. The $p<0.05$ value was established as statistically significant. The statistics program SigmaStat 5.1 (San Jose, CA, USA) was used throughout the study.

3. Results and Discussion

3.1 Fluke burdens, liver pathology and cytokine expression in the liver

Mean number of fluke burdens of groups 4, 5 and 6 was 43.4±10.8, 40.8±11.7 and 49.0±9.7, respectively, without significant differences between the three groups.

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This non-protective result coincides with the lack of protection reported in sheep immunized with the same vaccine (Fu et al., 2016).

Gross and histopathological hepatic changes were absent in the control group 3 and in groups 1 and 2 at 1 dpi and 3 dpi. However, at 9 dpi and 18 dpi, groups 1 and 2 showed tortuous whitish tracts and hemorrhagic spots, mainly involving the left hepatic lobe. At 9 dpi mean number of gross hepatic lesions was 58.6±27.9 and 111.8±37.6 in groups 1 and 2, respectively ($P = 0.03$), whereas at 18 dpi 425.4±58.0 and 412.4±135.5 gross hepatic lesions were found in groups 1 and 2, respectively. Histopathological changes at 9 dpi in both infected groups consisted of necrotic foci with variable infiltrate of eosinophils, macrophages and lymphocytes. Some of these necrotic foci were surrounded by a palisade of large macrophages and an outer layer of lymphocytes conforming granulomas. At 18 dpi, necrotic foci and tracts and granulomas were larger and more abundant than that found at 9 dpi.

During the course of infection, IFNγ expression did not change significantly at 1 and 3 dpi in both infected controls and immunized groups respect to the negative control group (**Fig. 2**). At 18 dpi, both the infected control and immunized groups showed marked increase ($P<0.001$) respect to the negative control group without statistical differences between the infected control and immunized groups. The increased expression of IFNγ at 9 and 18 dpi contrasts with the marked decreased expression of this cytokine at 7 dpi in PBMCs from sheep vaccinated with the same vaccine used in the present study and in infected controls (Fu et al., 2016). Since it is known that IFNγ plays an important role in granuloma formation in several parasitic diseases such as schistosomiasis (Attia et al., 2014) and cysticercosis (Freitas et al., 2016), the increase of IFNγ in the liver of the sheep from the two infected groups may reflect a response to tissue damage and granuloma formation rather than a response to

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the parasitic larvae. At 9 dpi lower hepatic lesions were found in the vaccinated group compared to the infected control coinciding with higher gene expression for IFNγ in the liver; however, since the increase of this cytokine was transient and only found in the liver, we cannot associate it with the lower hepatic damage found at 9 dpi in the vaccinated group.

IL4 gene expression did not change significantly at 1 and 3 dpi in the two infected groups respect to the control group, but it increased at 9 dpi and particularly at 18 dpi (**Fig. 2**). This finding agree with the increased IL4 gene expression found in PBMCs from infected and vaccinated sheep at 7 dpi (Fu et al., 2016). The ratio IL4/IFNγ gene expression in the liver increased significantly at 9 and particularly at 18 dpi (**Fig. 2**), suggesting parasite infection up-regulates Th2 and down-regulates Th1 responses.

3.2 Cytokine gene expression levels in hepatic lymph nodes

Gene expression levels for IFNγ and IL4 in HLN is shown in **Fig. 2**. In the negative control group (0 dpi) IFNγ was overexpressed compared to IL4 (1000 molecules/ng total RNA for IFNγ versus 200 molecules/ng total RNA for IL4). The levels for IFNγ mRNA decreased ($P< 0.01$) from 1 dpi onwards both in the infected control and immunized groups respect to the negative control group (0 dpi). This decrease was more pronounced at 9 and 18 dpi in the vaccinated group and at 18 dpi in the infected group (**Fig. 2**). According to Moreau et al. (1998) who quantified IFNγ expression levels in PBMCs and HLN in sheep, higher levels were observed in the first 2 weeks post-infection (wpi), however, Fu et al. (2016) found higher IFNγ gene expression in PBMCs from sheep at 20 weeks post-infection than at one week post-infection. Comparison between the infected control and vaccinated groups revealed that

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at 3 dpi IFNγ expression was higher in the immunized group while at 1, 9 and 18 dpi IFNγ gene expression was higher in the infected group (**Fig. 2**). On the contrary, IL4 gene transcripts remained almost constant during the first days after the infection (1 and 3 dpi), but suffered a marked and very significant ($P = 0.0001$) increase in both infected control and immunized groups at 9 and 18 dpi (**Fig. 2**).

It is noteworthy to mention a study carry out by Pleasance (2011) which compared IFNγ and IL4 expression in Indonesian thin-tail sheep (ITT) infected with *F. gigantica* and *F. hepatica* separately at 3 wpi and 10 wpi; sheep infected with *F. hepatica* had a lower expression of IFNγ than IL4, particularly at 10 wpi. Both studies agree with the results of the present study in which downregulation of Th1 immune response and upregulation Th2 immune response in the HLN started at 9 dpi and was more marked at 18 dpi. No significant differences were found between the infected control and the immunized groups at any of the sampling times for IL4 mRNA in HLN. The ratio IL4/ IFNγ was 0.22 in group 3 (negative control) and it did not change significantly at 1 and 3 dpi in groups 1 and 3, but it increased significantly at 9 dpi and particularly at 18 dpi (**Fig. 2**). Having said that, we had a greater expression of IFNγ than IL4 in the first 3 dpi which agree with a high level of that cytokine in the first two weeks after infection with mature and immature forms of *F. hepatica* in cattle vaccinated with CL1 and CL2 (Mulcahy et al. 1999). This may indicate the ease with which the parasite modulates immune response to establish a Th2 environment which allows it to survive in the host.

In summary, this study suggests that *F. hepatica* modulates the expression of IL4 and IFNγ, polarizing the immune response in the HLN to a non-protective Th2 response from 9 dpi onwards, and to a mixed Th1/Th2 response in liver, due to the production of hepatic granulomas against parasite infection from 9 dpi onwards. In

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order to improve the protection against *F. hepatica* it would be of interest to know which molecules and mechanisms the parasite use to induce this early immune response modulation, thus it can be prevent using appropriate adjuvants stimulating Th1 response and antigens trying to block parasite molecules involved in immune modulation.

Acknowledgments

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Figure legends

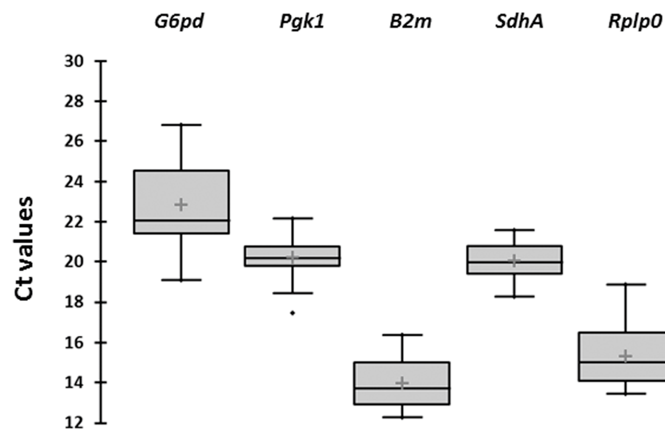

Fig. 1. Distribution of the threshold cycle (Ct) values for candidate reference genes obtained using qRT-PCR in sheep. Boxes show the range of Ct values within each candidate gene; the black center line indicates the median Ct; the cross represents the mean score of the group; the extended upper and lower hinges indicate 75 and 25 percentiles; the whiskers show the largest/smallest Ct values that falls within a distance of 1.5 times IQR (Interquartile range) from the upper and lower hinges.

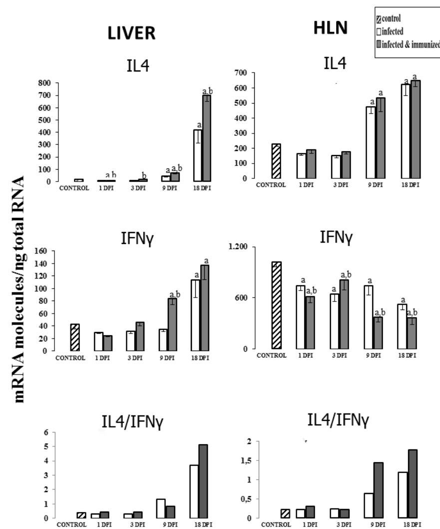

Fig. 2. Expression levels of IFNγ and IL4 genes in hepatic lymph (HLN) nodes and liver. Absolute transcript levels of IFNγ and IL4 genes and IL4/IFNγ ratio in the HLN and liver of control and treated sheep. Each bar represents the mean ± SD of the mRNA molecules/ng of total RNA quantified individually in each of the five animals per experimental condition and sampling time, after four real-time PCR reactions per individual. Statistical significances ($p<0.05$) were determined by a Student's two-tailed *t*-test followed by the Bonferroni correction for multiple comparisons and are expressed as ***a*** for comparison of each treatment with the negative control, and ***b*** for comparisons between the infected and infected and immunized groups.

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Table 1. Description and sequences of the oligonucleotides designed to quantify specific ovine genes using real-time PCR.

Genes	Sequences	Amplified product size (bp)	Accession number
IL4	F 5’-CATGTGCTTGAACAAATTCCTGGGCGGAC-3’ R 5’-TAGCCTTTCCAAGAGGTCTCTCAGCGTAC-3’	124	NM_001009313.2
IFNγ	F 5’-ACCGATTTCAACTACTCCGGCCTAACTC-3’ R 5’-CAGAAAAACCCAAAAGCACACAGAGCAG-3’	97	NM_001009803.1
Pgk1	F 5’-GTGAAGGGGAAGCGGGTCGTCATGAGAG-3’ R 5’-GCTTGGAACAGCAGCCTTGATCCTCTGG-3’	99	NM_001142516.1
B2m	F 5’-CAAGACACCCGCCAGAAGATGGAAAGC-3’ R 5’-GGAGTGAACTCAGCGTGGGACAGAAGG-3’	180	NM_001009284.2
Rplp0	F 5’-CGGCTGCTGCCCGTGCTGGTGCCAT-3’ R 5’-TTCGCTGGCGCCCACCTTGTCTCCGGTC-3’	191	XM_004017413.2
G6pd	F 5’-CGGGCGAGAGCAACGAAGCACAGAGAGC -3’ R 5’-CCAGGTCCCCCGATGCACCCATGATG-3’	161	NM_001093780.1
SdhA	F 5’-CCATGAGTTTGATGCCGTGGTGGTCGGTGC-3’ R 5’-CCGCCAGTTGTCCTCCTCCATGTTCCCCA -3’	184	XM_012097183.1

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Table 2. Reference genes.

Dlta Ct		**BestKeeper**		**NormFinder**		**geNorm**		**RefFinder**	
Genes in ranking order	Av. of STDEV	Genes in ranking order	Av. of STDEV	Gene name	Stability value	Gene name	Stability value	Genes	Geomean of ranking values
Pgk1	1.03	***Pgk1***	0.61	***Pgk1***	0.25	***Pgk1***	0.735	***Pgk1***	**1.00**
B2m	1.06	***SdhA***	0.71	***B2m***	0.45	***B2m***	0.735	***B2m***	**1.86**
Rplp0	1.11	***B2m***	1.01	***Rplp0***	0.64	***Rplp0***	0.829	***Rplp0***	**3.22**
G6pd	1.4	***Rplp0***	1.23	***G6pd***	1.19	***G6pd***	0.998	***SdhA***	**3.98**
SdhA	1.61	***G6pd***	1.66	***SdhA***	1.45	***SdhA***	1.243	***G6pd***	**4.23**

Stability ranking of five reference genes analyzed by five algorithms across all cell lines. The expression levels of six candidate references genes (Pgk1, B2m, Rplp0, G6pd and SdhA) were evaluated as threshold cycle (Ct) values from the samples with five biological and three technical replicates. Data were analyzed with the RefFinder tool (http://fulxie.0fees.us) that integrates the comparative ΔCt, the BestKeeper, the Normfinder and the geNorm methods and finally gives a comprehensive ranking of the studied genes.