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Distribution of Foxp3+ T cells in the Liver and Hepatic Lymph Nodes of Goats and Sheep Experimentally Infected with *Fasciola hepatica*

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HIGHLIGHTS

- The involvement of Foxp3$^+$ Tregs in acute and chronic hepatic lesions in small ruminants is studied for the first time.
- The rapid expansion of Foxp3$^+$ Tregs in the acute phase of the infection seems to be related to the larval migration in the hepatic parenchyma.
- In the chronic hepatic lesions, Foxp3$^+$ Tregs increase was more pronounced in the large bile ducts in which adult parasites are located.
- The expansion of Foxp3$^+$ Tregs in acute and chronic hepatic lesions in sheep and goats may be involved in parasite survival as well as in minimising tissue pathology.

Abstract

Foxp3 regulatory T cells (Tregs) are now considered to play a key role in modulation of immune responses during parasitic helminth infections. Immunomodulation is a key factor in *Fasciola hepatica* infection; however, the distribution and role of Foxp3$^+$ Tregs cells have not been investigated in *F. hepatica* infected ruminants. The aim of this study was to evaluate the presence of Foxp3$^+$ Tregs in the liver and hepatic lymph nodes from experimentally infected sheep and goats during acute and chronic stages of infection. Three groups of goats (n=6) and three groups of sheep (n=6) were used in this study. Goats in groups 1-2 and sheep in groups 4-5 were orally infected with metacercarie of ovine origin. Groups 1 and 4 were killed during the acute stage of the infection, at nine days post infection (dpi); groups 2 and 5 were killed during the chronic stage, at 15 and19 weeks post infection respectively (wpi). Groups 3 (goats) and 6 (sheep) were left as uninfected controls. Fluke burdens and liver damage were assessed and the avidin–biotin–complex method was used for the immunohistochemical

study. At nine dpi in acute hepatic lesions, the number of both Foxp3$^+$ and CD3$^+$ T lymphocytes increased significantly in goats and sheep. In the chronic stages of infection (15-19 wpi), the number of Foxp3$^+$ and CD3$^+$ T lymphocytes were also significantly increased with respect to control livers, particularly in portal spaces with severely enlarged bile ducts (response to adult flukes) while the increase was lower in granulomas, chronic tracts and smaller portal spaces (response to tissue damage). Foxp3$^+$ Tregs were increased in the cortex of hepatic lymph nodes of sheep (chronic infection) and goats (acute and chronic infection). The estimated proportion of T cells which were Foxp3+ was significantly increased in the large bile ducts and hepatic lymph node cortex of chronically infected goats but not sheep. This first report of the expansion of Foxp3$^+$ Tregs in acute and chronic hepatic lesions in ruminants suggests that these cells may be involved in both parasite survival and modulation of hepatic damage. Future studies should be focused on the investigation of parasite molecules and cytokines involved in this process.

Keywords: *Sheep; goat; Foxp3; immunohistochemistry; Fasciola hepatica*

1. Introduction

Fasciolosis caused by *Fasciola hepatica* is an economically important disease of ruminants in temperate climates. *Fasciola hepatica* has developed a variety of mechanisms to modulate or suppress the host response making it ineffective, which allows the parasite to survive in the host for years (Dalton et al., 2013) and became a serious obstacle in creating protective vaccines for ruminants (Toet et al., 2014; Molina-Hernández et al., 2015).

Several cell types, such as B cells (Bregs), macrophages and T cells (Tregs) can induce immune suppression in helminth infections; however, Foxp3$^+$ Tregs are considered the most prominent immunoregulatory cells during infection (Taylor et al., 2012).

Foxp3+ regulatory T cells represent a lymphocyte subset with an important role in the maintenance of immune system homeostasis (Belkaid, 2007). They can suppress the immune response to self-antigens and prevent autoimmune diseases, but they can also control the immune responses to parasites and fungi (Adalid-Peralta, 2011). Therefore, Tregs have a crucial role in immune responses by limiting immunopathology associated with anti-pathogen immune responses, but they can also be beneficial to the pathogen through subversion of the host protective immune response (Belkaid, 2007; Adalid-Peralta, 2011).

A variety of helminths (Finney et al., 2007; McNeilly et al., 2013) induce Foxp3$^+$ Treg cell expansion to suppress or modulate immune responses allowing them to survive for long periods in the host. Parasite-induced Foxp3$^+$ Tregs cells also play a role in controlling immune pathology; thus, in infections with the trematode *Schistosoma mansoni*, the severity of egg-induced liver pathology was negatively correlated with the number of Foxp3$^+$ Tregs in the liver (Watanabe et al., 2009).

To date, the distribution and role of Foxp3$^+$ Tregs has not been investigated in *F. hepatica* infected ruminants, although it has been suggested that they may play a role in immunomodulation caused by *F. hepatica* (Dalton et al, 2013). The aim of this study was to evaluate the presence of Foxp3$^+$ Tregs in liver and hepatic lymph nodes (HLN) from experimentally infected sheep and goats during acute and chronic stages of infection.

2. Materials and methods

2.1. Experimental design

Eighteen six-month-old male Malagueña goats and 18 six-month-old Merino sheep were used in this study. Animals were obtained from a liver fluke-free farm: they were housed indoors and faecal sedimentation tests were conducted to ensure that animals were free of internal parasites. Before the experiment, an ELISA was carried out to detect antibodies specific for *F. hepatica* cathepsin L1, and the results were negative for all animals. Animals were distributed into treatment groups as shown in Table 1. The experiment was approved by the Bioethical Committee of the University of Cordoba (No. 7119 and No. 1118), and it was carried out taking into account European (86/609/CEE) and Spanish (RD 223/1988) directives for animal experimentation.

2.2. Fluke burdens and histopathology

All animals were necropsied, the duodenum was tied proximally and distally to the bile duct (a length of 8 to 10 cm), the liver was removed and the visceral and diaphragmatic aspects were photographed for gross evaluation. Hepatic lymph nodes (HLN) were weighed and results expressed in g±standard deviation (SD) per group. Samples were collected from HLN and affected areas of the liver. Four samples were collected from the left liver lobe and one from the right lobe as the left lobe consistently had more lesions, presumably due to its close proximity to the duodenum. All the samples were fixed in 10% buffered formalin for 24 hours and routinely processed and embedded in paraffin wax for histopathology. Four micrometre thick tissue sections were stained with haematoxylin and eosin for histopathology. A quantitative estimation of liver damage was carried out: in the acute stages of infection, the total number of gross hepatic lesions was counted in each animal using Image Pro- plus 6.0 software (Media Cybernetics, Silver Spring, Maryland, USA) and results expressed as mean ± SD per

group. In the chronic stages of infection, the percentage of affected liver surface was calculated as described previously (Zafra et al., 2013).

In groups 1 and 4 (chronic stages of infection) fluke burdens were assessed. The gallbladder and major biliary ducts were opened and flukes were recovered. Then, the bile ducts were opened and flukes were removed with blunt forceps. Finally, the livers were cut into small pieces (1 cm^2) and washed in hot water to collect the remaining flukes.

2.3. Immmunohistochemistry

The avidin–biotin–complex method described by Zafra et al. (2013) was used for the immunohistochemical study. Four-µm serial sections were used for CD3 and Foxp3 antibodies. The anti-mouse/rat Foxp3 monoclonal antibody (clone FJK-16s, rat IgG2a, eBioscience Inc. San Diego, CA, USA) diluted 1:100 in PBS containing 10% normal goat serum, and the rabbit anti-human CD3 (Dako, Glostrup, Denmark) diluted 1:200 in PBS containing 10% normal goat serum, were applied overnight at 4 ºC. The Foxp3 mAb has been shown to cross react with Foxp3 in sections of formalin-fixed sheep tissues (McNeilly et al 2013). Serial sections were used for Foxp3 and CD3 antibodies. A biotinylated goat anti-rabbit immunoglobulin serum (Dako) diluted 1:200 was applied as the secondary antibody for the CD3 slides, whereas a biotinylated goat anti-rat immunoglobulin (Dako) diluted 1:200 in PBS was applied as the secondary antibody for the Foxp3 slides. The avidin–biotin–peroxidase complex (Vector Laboratories) diluted 1:50 was finally applied and after washed, tissue sections were incubated with NovaREDTM substrate kit (Vector Laboratories, Burlingame, USA), rinsed in tap water, lightly counterstained with Mayer's haematoxylin and mounted with DPX (Shandon, Pittsburgh, Pennsylvania, USA). Specific primary antibodies were

substituted with PBS or non-immune isotype-matched sera as the negative control. Lymph node sections from uninfected goats and sheep were used as positive controls.

2.4. Cell counting

Immunoreactive cells were counted using Image Pro-plus 6.0 software (Media Cybernetics). The software was calibrated for labelling intensity and cell size to include all immunolabelled cells. Photomicrographs (0.16 mm^2 each) from each animal were used for cell counting. In negative control livers (groups 3 and 6), 10 photomicrographs were selected randomly from portal areas for cell counting. In chronic infection stages (groups 2 and 5), cell counting was carried out to evaluate: (1) response to tissue damage (10 photomicrographs selected randomly from inflammatory infiltrates associated with chronic tracts and granulomas) and (2) response to adult flukes (10 photomicrographs selected randomly from inflammatory infiltrates associated with portal areas with severe bile duct hyperplasia). In early infection stages (groups 1 and 4), 10 photomicrographs randomly selected from damaged areas were used for cell counting. In HLN, 10 photomicrographs randomly selected from cortical areas, and 10 pictures selected from medullary areas, were used for cell counting. $CD3^+$ T cells and $Foxp3^+$ cells were counted in adjacent areas of serial sections and the percentage of $Foxp3^+/CD3^+$ T cells was estimated from by dividing numbers of Foxp3+ cells by the number of CD3+ cells in those adjacent serial sections. Results were expressed as mean ± SD per group.

2.5. Statistical analysis

Statistical analysis was carried out with PRISM 6.0 software (Graphpad Software Inc., San Diego, California, USA). The Kolmogorov-Smirnov test was applied to evaluate if data were normally distributed and according to the results, data were analysed with the non-parametric Kruskall-Wallis with Dunn´s multiple comparisons tests. Correlations

between parasitological, pathological and immunohistochemical data were estimated using the Spearman's rank non-parametric correlation test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Fluke burdens

Goats from group 1 (chronic stages of infection) showed 64, 42, 51, 73, 59 and 42 flukes, respectively (55.4 ± 12.2), whereas sheep from group 4 (chronic stages of infection) showed 57, 40, 54, 58, 41 and 52 flukes, respectively (50.3 ± 7.9). Percentage of implantation was 27.7 % and 25.1% in goats and sheep, respectively.

3.2. Gross and histopathological study

3.2.1. Liver

In the early stages of infection (9 dpi), all goats (group 2) and sheep (group 5) showed reddish spots and small tortuous whitish tracts mainly involving the diaphragmatic aspect of the left liver lobe. Quantification of those lesions resulted in mean of 113.4±54.6 in goats and 111.8±35.7 in sheep. Microscopic hepatic changes in the early stages post infection consisted of necrotic foci and tracts within the hepatic parenchyma, often associated with focal haemorrhage, mainly involving subcapsular areas. Inflammatory infiltrates associated with these necrotic areas consisted mainly of eosinophils with fewer lymphocytes and macrophages. Adjacent portal spaces showed severe infiltration of lymphocytes, eosinophils and macrophages. These inflammatory cells migrated from portal areas through hepatic sinusoids to necrotic areas of the hepatic parenchyma.

In the late stages of infection, both goats (group 1) and sheep (group 4) demonstrated scars and tortuous white tracts, particularly involving the left liver lobe. Percentage of

affected areas in goats was 36.02±9.4% and 33±17.6% in sheep. The gallbladder and major biliary ducts were whitish and enlarged, and they contained brownish fluid admixed with adult flukes. Microscopically, hepatic lesions were composed of marked fibrosis in portal spaces containing large bile ducts and severe infiltration of lymphocytes and plasma cells, either in a diffuse or lymphoid follicle pattern (response to adult flukes, observed within enlarged bile ducts which often showed epithelial erosion). Additionally, chronic tracts with macrophages containing abundant hemosiderin pigment, granulomas with necrotic centres, macrophages and giant multinucleate cells and variable infiltrates of lymphocytes, plasma cells and eosinophils were found in the hepatic parenchyma (response to tissue damage).

3.2.2 Hepatic lymph nodes

In goats weight of HLN was 2.2 gr ± 0.9, 18.2 gr ±3.4 and 1.0 gr ± 0.5 in groups 1, 2 and 3, respectively. In sheep HLN weight was 1.4 ± 0.3, 6.3 ± 0.8 and 1.0 ± 0.5 in groups 4, 5 and 6, respectively. Significant HLN weight increase ($P<0.01$) was found in chronic infection (groups 2 and 5) with respect to negative controls and acute infection in goats and sheep. The histological study revealed that the HLN weight increase in chronic infections was due to a marked hyperplasia of lymphoid follicles, interfollicular areas and medullary cords.

3.3. CD3 and Foxp3 expression in the liver

Results of cell counting for CD3 and Foxp3 in livers of goats and sheep during acute and chronic infections and negative controls are shown in Table 1. Uninfected control goats and sheep showed occasional $CD3^+$ T lymphocytes mainly located in portal areas. $Foxp3^+$ cells were also occasionally noted and also located in portal areas. The percentage of Foxp3/CD3 T cells was 20.3% and 18.8% in goats and sheep, respectively (Table 1).

At 9 dpi (acute infection stage), $CD3^+$ and $Foxp3^+$ T cells were found at the periphery of necrotic foci and adjacent portal spaces (Fig. 1). The number of both $Foxp3^+$ and $CD3^+$ T lymphocytes increased significantly in goats and sheep with respect to negative controls (Table 1). However, the percentage of $Foxp3^+/CD3^+$ did not show a significant change in either goats (P=0.55) or sheep (P=0.48) with respect to negative controls.

In the chronic stages of infection (15-19 wpi), the number of $CD3^+$ T lymphocytes and $Foxp3^+$ T cells was significantly increased in both goats and sheep with respect to control livers, particularly in portal spaces with severely enlarged bile ducts (Figs. 2 and 3; location B in Table 1), whereas in granulomas, chronic tracts and smaller portal spaces, the number of $CD3^+$ and $Foxp3^+$ T cells was also increased with respect to negative controls but lower than in the vicinity of enlarged bile ducts (Location A in Table 1). The percentage of $Foxp3^+/CD3^+$ T cells in areas of tissue damage in goats was significantly reduced with respect to both the negative controls and acute infections, while it did not change significantly in sheep. In areas of response to adult flukes (periphery of enlarged bile ducts), the percentage of $Foxp3^+/CD3^+$ was not significantly modified with respect to uninfected controls.

There was no statistical correlation between number of $Foxp3^+$ T cells and fluke burden or gross pathology in any of the studied groups.

3.3.2. Hepatic lymph nodes

Results of cell counting for $CD3^+$ and $Foxp3^+$ in HLN of goats and sheep during acute and chronic stages of infection and in negative controls are shown in Table 2. Uninfected control goats and sheep showed abundant infiltrates of $CD3^+$ T lymphocytes and $Foxp3^+$ T cells in the cortex, particularly in interfollicular areas (Fig. 4), whereas the number of both cell populations was lower in the medulla (medullary cords and medullary sinuses).

During acute infections, $CD3^+$ T lymphocytes were significantly increased with respect to negative controls in sheep and goats (cortex) and sheep (medulla) (Table 2). $Foxp3^+$ T cells were mainly found in interfollicular areas of the cortex (Fig. 5). The number of $Foxp3^+$ T cells and ratio $Foxp3^+/CD3^+$ increased significantly only in the cortex of acutely infected goats with respect to negative controls (Table 2). The number of $Foxp3^+$ Tregs and the ratio of $Foxp3^+/CD3^+$ in the medulla were very similar in negative controls and acutely infected goats and sheep (Table 2).

During chronic infections, the number of $CD3^+$ T cells was significantly increased in the cortex and medulla of goats and sheep with respect to negative controls (Table 2). $Foxp3^+$ were mainly found in interfollicular areas of cortex (Fig. 6), where a significant increase was found in chronically infected goats and sheep with respect to uninfected controls. The percentage of $Foxp3^+/CD3^+$ did not change significantly with respect to the uninfected controls in the cortex and medulla of either goats or sheep (Table 2).

4. Discussion

Chronic parasitic infections are facilitated by the modulation and/or suppression of the host immune response caused by these parasites, and $Foxp3^+$ T cells are the main cell population mediating such modulation of the host immune response (Adalid-Peralta et al., 2011; Taylor et al., 2012). While *F. hepatica* has shown a potent capacity to modulate the host immune response (Dalton et al., 2013), the distribution of $Foxp3^+$ T cells has not been investigated in *F. hepatica* infected cattle or sheep.

In the present study, we have found a significant increase of $Foxp3^+$ T cells in the hepatic lesions of both goats and sheep, in the acute and the chronic stages of the infection. This increase of $Foxp3^+$ T cells was generally correlated to an increase in the number of $CD3^+$ T cells, so the percentage of $Foxp3^+/CD3^+$ cells did not change. The

increase in the total number of Foxp3$^+$ T cells without modification of the Foxp3$^+$/CD3$^+$ percentage has been described in different parasitic infections in sheep, as in *Psoroptes ovis* infection (McNeilly et al, 2010) and *Teladorsagia circumcinta* infection (McNeilly et al., 2013). In our work, it is noteworthy the differences between the total number of Foxp3$^+$ cells in different stages and locations of the infection, with the higher number of cells found in correlation with the presence of adult flukes in the bile ducts (chronic stage).

The rapid expansion of Foxp3$^+$ T cells in the acute phase of the infection (9 dpi) seems to be related to the larval migration in the hepatic parenchyma, since most of those cells were found around necrotic foci and tracts and in the adjacent portal spaces. However, no correlation was found between number of Foxp3$^+$ T cells and the number of necrotic lesions. The early presence of Foxp3$^+$ T cells in the initial stages of parasitic infections has been shown in gastrointestinal nematode mouse model (Finney et al., 2007) and it has been explained as an immunomodulatory mechanisms facilitating the survival of the parasite. In sheep, McNeilly et al., (2013) described an increase of Foxp3$^+$ T cells at 10 dpi in the abomasal mucosa of sheep infected with *Teladorsagia circumcincta*, that may reflect a homeostatic regulatory mechanism within the abomasal cellular immune response to minimize immune-mediated abomasal pathology. Our data suggest Foxp3$^+$ T cells may play a role in modulating the initial host immune response, contributing to the survival of *F. hepatica* during the migratory stage. However, further studies are required to clarify the relationship between the initial Foxp3$^+$ T cells expansion and the inability of the immune effector mechanisms to kill the newly excysted juveniles of *F. hepatica* in the early peritoneal and hepatic migration, as occurred in the protective responses observed in the *F. gigantica* sheep model (Piedrafita et al., 2007).

In the chronic hepatic lesions, the increase of Foxp3$^+$ T cells was more pronounced in the inflammatory infiltrates adjacent to large bile ducts than in the periphery of granulomas, chronic tracts and small portal areas. Since the adult parasites in these stage are located within the bile ducts and gallbladder, it seems that Foxp3$^+$ T cells are specifically recruited to the vicinity of *F. hepatica* adults or are actively induced by the adult parasites and this may be related to the chronicity of the infection and the long survival of the parasite in the host (Escamilla et al., 2016).

A dual role has been described for Foxp3$^+$ T cells in hepatic helminth infections: minimising tissue pathology and modulating the host immune response to facilitate parasite survival, as reported in the case of *Schistosoma japonicum* infection in mice (Zhu et al., 2015). In the present study, the different proportion of Foxp3$^+$ T cells in the periphery of granulomas and chronic tracts compared to those of large bile ducts and the lack of correlation between Foxp3$^+$ T cells and fluke burden or gross pathology may also suggest this dual role for these cells in *F. hepatica* infections. The evaluation of cytokines such as IL-10 and TGF-β produced by Foxp3$^+$ T cells in each of these hepatic lesions is of foremost interest to elucidate this point.

The number of Foxp3$^+$ T cells was also significantly increased in the cortex of the HLN, as well as the number of CD3$^+$ T lymphocytes in both the cortex and the medulla. This data agrees with the increase of Foxp3$^+$ T cells in mesenteric lymph nodes found in helminth infected mice (Smith et al., 2016). Some differences were observed between sheep and goats, since goats showed higher number of Foxp3$^+$ cells in both the acute and the chronic stages, with a significant elevation of the percentage of Foxp3$^+$/CD3$^+$ in the acute phase of the infection. However, we found no correlation between this higher number of Foxp3$^+$ T cells in HLN in goats and any other parasitological or pathological data. Sheep and goats seems to have a different immune mechanism in response to

gastrointestinal nematodes (Hoste et al., 2010), but no such difference seems to appears in the case of *F. hepatica* infection.

In conclusion, this is the first report describing the distribution of $Foxp3^+$ T cells in acute and chronic hepatic lesions and HLN of *F. hepatica* infected goats and sheep. The expansion of Tregs in acute and chronic hepatic lesions may be involved in parasite survival as well as in minimising tissue pathology. Future studies should focus on the investigation of parasite molecules, particularly from newly excysted juveniles, involved in the expansion of Foxp3 T cells, as well as the cytokines produced by this cell type in the different hepatic lesions to elucidate their roles in *F. hepatica* infection.

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Figure Legends

Figure 1. Acute infection sheep liver showing a portal space with a bile duct (B) surrounded by severe inflammatory infiltrate (I) with several Foxp3^{+} T cells (red-brown colour). ABC method, x200.

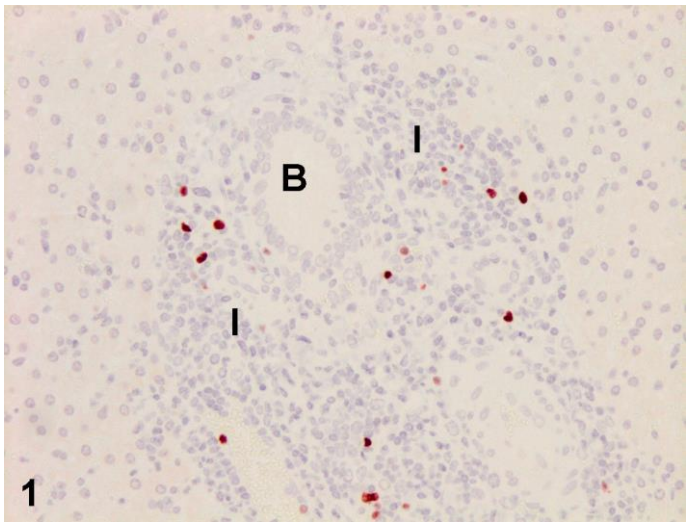

Figure 2. Chronic infection sheep liver showing severe inflammatory infiltrate surrounding large bile ducts (arrows) showing numerous $CD3^+$ T lymphocytes (brown colour). ABC method, x200.

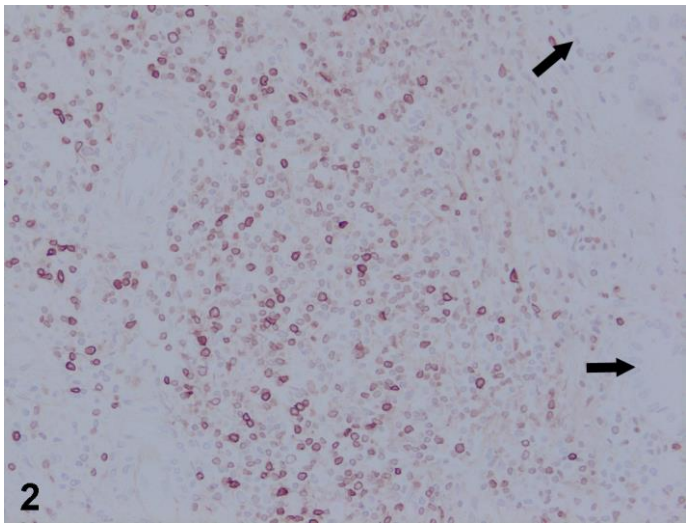

Figure 3. Serial section of that shown in Fig. 3, showing numerous Foxp3^{+} T cells in the inflammatory infiltrate surrounding bile ducts (arrows). ABC method, x200.

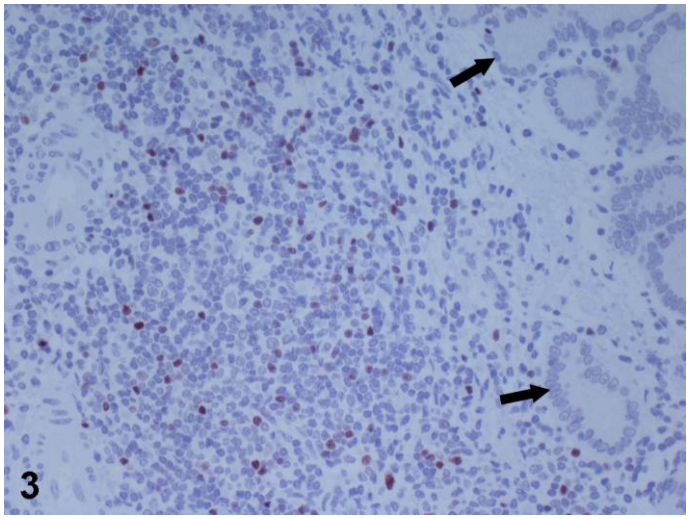

Figure 4. Negative control goat hepatic lymph node showing moderate number of Foxp3^{+} T cells (brown-red colour) in interfollicular (IF) areas. ABC method, x200.

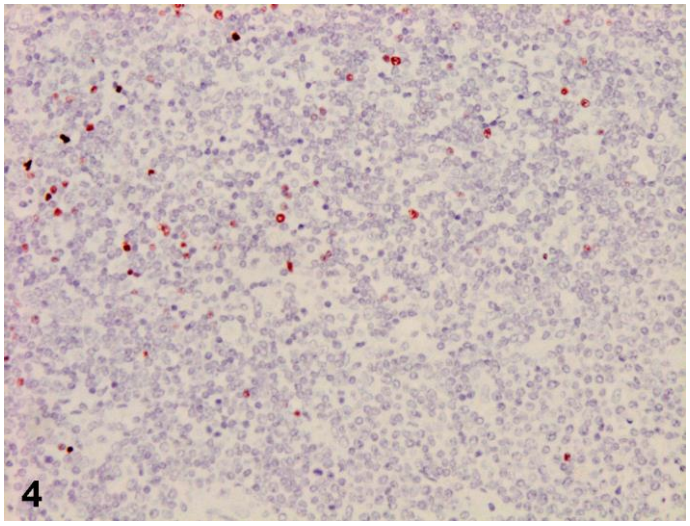

Figure 5. Acute infection goat hepatic lymph node showing numerous Foxp3^{+} T cells (brown-red colour) in interfollicular (IF) areas. ABC method, x200.

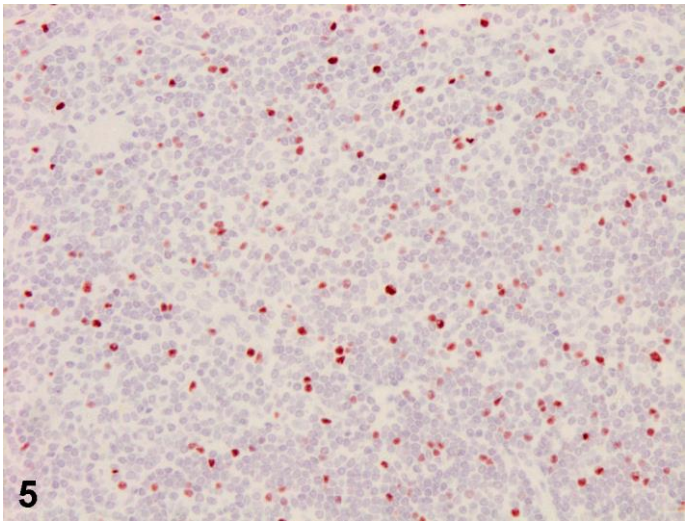

Figure 6. Chronic infection goat hepatic lymph node showing a lymphoid follicle (LF) and interfollicular areas (IF) containing numerous Foxp3^{+} T cells (brown-red colour) in interfollicular (IF) areas. ABC method, x200.

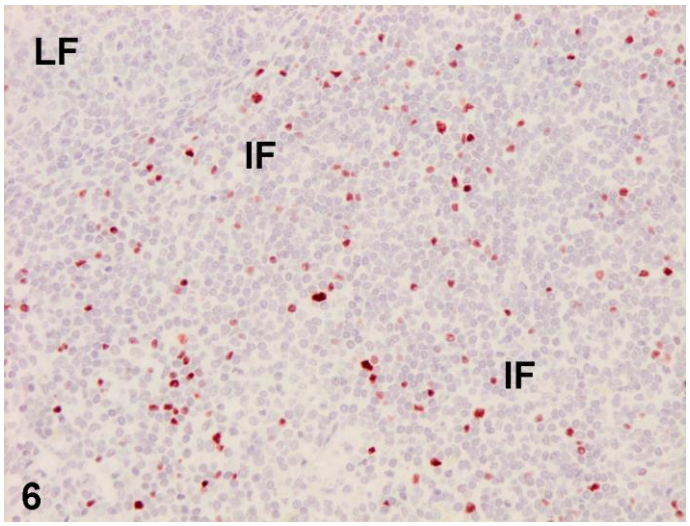

Table 1

Experimental design: groups distribution and infection.

Group	**Hosts**	**n**	**Infection**	**Killing**	
1	Goat	6	100 mtc	9 dpi	Acute stage
2	Goat	6	200 mtc	15 wpi	Chronic stage
3	Goat	6	-	9 dpi/15 wpi	Uninfected controls
4	Sheep	6	150 mtc	9 dpi	Acute stage
5	Sheep	6	200 mtc	19 wpi	Chronic stage
6	Sheep	6	-	9 dpi/19 wpi	Uninfected controls

All infected animals were infected orally with metacercariae of ovine origin (Ridgeway Research Ltd.), administered in gelatine capsules with a dosing gun. All animals were killed by intravenous injection of T61 (Intervet International GMBH, Unterschleissheim, Germany).

Table 2

Number and percentage of $Foxp3^+$ and $CD3^+$ T lymphocytes in livers of acute and chronic stage of infection, and in uninfected controls. Results expressed as mean ± SD of cells per 0.2 mm^2 per group.

	Location A			Location B		
Group (Species-stage of infection)	**Foxp3**	**CD3**	**%Foxp3/ CD3[a]**	**Foxp3**	**CD3**	**%Foxp3/ CD3[a]**
1 (goats-AI)	5.5±2.2*	25.4±3.6*	21.8±9.2			
2 (goats-CI)	6.1±2.0*	60.9±11.6*	10.5±3.9*	31.0±5.1*§	123.7±21.1*§	25.1±1.3
3 (goats-UC)	0.7±0.3	3.7±0.2	20.3±10.0			
4 (sheep-AI)	6.2±2.9*	30.2± 4.4*	19.9±7.4			
5 (sheep-CI)	6.8±2.8*	52.9±15.1*	12.8±3.9	30.8±6.0*§	129.2±19.3*§	24.1±4.6
6 (sheep-UC)	0.6±0.3	3.2±0.5	18.8±8.1			

Location A: Uninfected control (UC): randomly selected portal areas. Acute infection (AI): portal areas, necrotic foci. Chronic infection (CI): portal areas, granulomas and chronic tracts.

Location B: Chronic infection: Periphery of enlarged bile ducts. This location is studied only in the chronic stage of the infection, when adult liver flukes are in the bile ducts and marked histopathological alterations occurred in the surrounding tissue.

[a] Estimated percentage of $CD3^+$ T cells which are $Foxp3^+$

* Significant difference ($P < .05$) compared to the uninfected control group.

§ Significant difference ($P < .05$) respect to the acute infection stages.

Table 3

Number and percentage of $Foxp3^+$ and $CD3^+$ T lymphocytes in hepatic lymph nodes of acute and chronic stage of infection and negative controls goats and sheep. Results expressed as mean ± SD per group.

	Cortex			**Medulla**		
Group (Species-stage of infection)	**Foxp3**	**CD3**	**%Foxp3/ CD3**	**Foxp3**	**CD3**	**%Foxp3/ CD3**
1 (goats-AI)	46.0±5.3*	299.0±15.8§	15.4±1.8*	7.6±1.5	107.0±6.1	7.1±1.1
2 (goats-CI)	45.4±16.3*	389.8±18.2*	11.7±5.9	8.0±2.1	117.1±4.6*	6.8±1.0
3 (goats-UC)	22.2±4.3	287.6±3.3	9.5±2.3	7.3±0.9	94.2±3.7	7.7±1.2
4 (sheep-AI)	22.6±3.0	303.7±37.8*	7.4±1.4	8.4±3.3	122.2±16.8*	6.9±2.3
5 (sheep-CI)	36.3±6.9*§	392.9±6.3*§	9.2±2.6	9.7±3.1	115.2±6.7*	8.4±2.1
6 (sheep-UC)	21.0±6.6	246.0±18.4	8.9±4.1	7.6±1.5	82.8±6.5	9.2±1.5

AI: acute infection (9 days post-infection, dpi); CI: chronic infection (15 -19 weeks post-infection, wpi); UC: uninfected controls.

* Significant difference ($P < .05$) compared to the uninfected control group.

§ Significant difference ($P < .05$) respect to the chronic infection stage.