1 **Distribution of Foxp3+ T cells in the Liver and Hepatic Lymph Nodes of Goats and**
2 **Sheep Experimentally Infected with *Fasciola hepatica***
3
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22 **Abstract**
23 Foxp3 regulatory T cells (Tregs) are now considered to play a key role in modulation of
24 immune responses during parasitic helminth infections. Immunomodulation is a key
25 factor in *Fasciola hepatica* infection; however, the distribution and role of Foxp3^{+}

26 Tregs cells have not been investigated in *F. hepatica* infected ruminants. The aim of this
27 study was to evaluate the presence of Foxp3$^+$ Tregs in the liver and hepatic lymph nodes
28 from experimentally infected sheep and goats during acute and chronic stages of
29 infection. Three groups of goats (n=6) and three groups of sheep (n=6) were used in this
30 study. Goats in groups 1-2 and sheep in groups 4-5 were orally infected with
31 metacercarie of ovine origin. Groups 1 and 4 were killed during the acute stage of the
32 infection, at nine days post infection (dpi); groups 2 and 5 were killed during the
33 chronic stage, at 15 and19 weeks post infection respectively (wpi). Groups 3 (goats) and
34 6 (sheep) were left as uninfected controls. Fluke burdens and liver damage were
35 assessed and the avidin–biotin–complex method was used for the immunohistochemical
36 study. At nine dpi in acute hepatic lesions, the number of both Foxp3$^+$ and CD3$^+$ T
37 lymphocytes increased significantly in goats and sheep. In the chronic stages of
38 infection (15-19 wpi), the number of Foxp3$^+$ and CD3$^+$ T lymphocytes were also
39 significantly increased with respect to control livers, particularly in portal spaces with
40 severely enlarged bile ducts (response to adult flukes) while the increase was lower in
41 granulomas, chronic tracts and smaller portal spaces (response to tissue damage).
42 Foxp3$^+$ Tregs were increased in the cortex of hepatic lymph nodes of sheep (chronic
43 infection) and goats (acute and chronic infection). The estimated proportion of T cells
44 which were Foxp3+ was significantly increased in the large bile ducts and hepatic
45 lymph node cortex of chronically infected goats but not sheep. This first report of the
46 expansion of Foxp3$^+$ Tregs in acute and chronic hepatic lesions in ruminants suggests
47 that these cells may be involved in both parasite survival and modulation of hepatic
48 damage, and that expansion of these cells may be more pronounced in goats compared
49 to sheep. Future studies should be focused on the investigation of parasite molecules
50 and cytokines involved in this process.

Keywords: *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).

76 To date, the distribution and role of $Foxp3^+$ Tregs has not been investigated in *F.*

77 *hepatica* infected ruminants, although it has been suggested that they may play a role in

78 immunomodulation caused by *F. hepatica* (Dalton et al, 2013). The aim of this study

79 was to evaluate the presence of $Foxp3^+$ Tregs in liver and hepatic lymph nodes (HLN)

80 from experimentally infected sheep and goats during acute and chronic stages of

81 infection.

82

83 **2. Materials and methods**

84 *2.1. Experimental design*

85 Eighteen six-month-old male Malagueña goats and 18 six-month-old Merino sheep

86 were used in this study. Animals were obtained from a liver fluke-free farm: they were

87 housed indoors and faecal sedimentation tests were conducted to ensure that animals

88 were free of internal parasites. Before the experiment, an ELISA was carried out to

89 detect antibodies specific for *F. hepatica* cathepsin L1, and the results were negative for

90 all animals. Animals were distributed into treatment groups as shown in Table 1. The

91 experiment was approved by the Bioethical Committee of the University of Cordoba

92 (No. 7119 and No. 1118), and it was carried out taking into account European

93 (86/609/CEE) and Spanish (RD 223/1988) directives for animal experimentation.

94 *2.2. Fluke burdens and histopathology*

95 All animals were necropsied, the duodenum was tied proximally and distally to the bile

96 duct (a length of 8 to 10 cm), the liver was removed and the visceral and diaphragmatic

97 aspects were photographed for gross evaluation. Hepatic lymph nodes (HLN) were

98 weighed and results expressed in g±standard deviation (SD) per group. Samples were

99 collected from HLN and affected areas of the liver. Four samples were collected from

100 the left liver lobe and one from the right lobe as the left lobe consistently had more

101 lesions, presumably due to its close proximity to the duodenum. All the samples were
102 fixed in 10% buffered formalin for 24 hours and routinely processed and embedded in
103 paraffin wax for histopathology. Four micrometre thick tissue sections were stained
104 with haematoxylin and eosin for histopathology. A quantitative estimation of liver
105 damage was carried out: in the acute stages of infection, the total number of gross
106 hepatic lesions was counted in each animal using Image Pro- plus 6.0 software (Media
107 Cybernetics, Silver Spring, Maryland, USA) and results expressed as mean ± SD per
108 group. In the chronic stages of infection, the percentage of affected liver surface was
109 calculated as described previously (Zafra et al., 2013).
110 In groups 1 and 4 (chronic stages of infection) fluke burdens were assessed. The
111 gallbladder and major biliary ducts were opened and flukes were recovered. Then, the
112 bile ducts were opened and flukes were removed with blunt forceps. Finally, the livers
113 were cut into small pieces (1 cm^2) and washed in hot water to collect the remaining
114 flukes.
115 *2.3. Immmunohistochemistry*
116 The avidin–biotin–complex method described by Zafra et al. (2013) was used for the
117 immunohistochemical study. Four-µm serial sections were used for CD3 and Foxp3
118 antibodies. The anti-mouse/rat Foxp3 monoclonal antibody (clone FJK-16s, rat IgG2a,
119 eBioscience Inc. San Diego, CA, USA) diluted 1:100 in PBS containing 10% normal
120 goat serum, and the rabbit anti-human CD3 (Dako, Glostrup, Denmark) diluted 1:200 in
121 PBS containing 10% normal goat serum, were applied overnight at 4 ºC. The Foxp3
122 mAb has been shown to cross react with Foxp3 in sections of formalin-fixed sheep
123 tissues (McNeilly et al 2013). Serial sections were used for Foxp3 and CD3 antibodies.
124 A biotinylated goat anti-rabbit immunoglobulin serum (Dako) diluted 1:200 was applied
125 as the secondary antibody for the CD3 slides, whereas a biotinylated goat anti-rat

126 immunoglobulin (Dako) diluted 1:200 in PBS was applied as the secondary antibody for

127 the Foxp3 slides. The avidin–biotin–peroxidase complex (Vector Laboratories) diluted

128 1:50 was finally applied and after washed, tissue sections were incubated with

129 NovaREDTM substrate kit (Vector Laboratories, Burlingame, USA), rinsed in tap

130 water, lightly counterstained with Mayer's haematoxylin and mounted with DPX

131 (Shandon, Pittsburgh, Pennsylvania, USA). Specific primary antibodies were

132 substituted with PBS or non-immune isotype-matched sera as the negative control.

133 Lymph node sections from uninfected goats and sheep were used as positive controls.

134 *2.4. Cell counting*

135 Immunoreactive cells were counted using Image Pro-plus 6.0 software (Media

136 Cybernetics). The software was calibrated for labelling intensity and cell size to include

137 all immunolabelled cells. Photomicrographs (0.16 mm^2 each) from each animal were

138 used for cell counting. In negative control livers (groups 3 and 6), 10 photomicrographs

139 were selected randomly from portal areas for cell counting. In chronic infection stages

140 (groups 2 and 5), cell counting was carried out to evaluate: (1) response to tissue

141 damage (10 photomicrographs selected randomly from inflammatory infiltrates

142 associated with chronic tracts and granulomas) and (2) response to adult flukes (10

143 photomicrographs selected randomly from inflammatory infiltrates associated with

144 portal areas with severe bile duct hyperplasia). In early infection stages (groups 1 and

145 4), 10 photomicrographs randomly selected from damaged areas were used for cell

146 counting. In HLN, 10 photomicrographs randomly selected from cortical areas, and 10

147 pictures selected from medullary areas, were used for cell counting. Results were

148 expressed as mean ± SD per group. The percentage of CD3 T cells expressing Foxp3

149 was calculated for each animal and results expressed as mean ± SD per group.

150 *2.5. Statistical analysis*

151 Statistical analysis was carried out with PRISM 6.0 software (Graphpad Software Inc.,
152 San Diego, California, USA). The Kolmogorov-Smirnov test was applied to evaluate if
153 data were normally distributed and according to the results, data were analysed with the
154 non-parametric Kruskall-Wallis with Dunn´s multiple comparisons tests. Statistical
155 significance was set at $P < 0.05$.
156
157 **3. Results**
158 *3.1. Fluke burdens*
159 Goats from group 1 (chronic stages of infection) showed 64, 42, 51, 73, 59 and 42
160 flukes, respectively (55.4 ± 12.2), whereas sheep from group 4 (chronic stages of
161 infection) showed 57, 40, 54, 58, 41 and 52 flukes, respectively (50.3 ± 7.9). Percentage
162 of implantation was 27.7 % and 25.1% in goats and sheep, respectively.
163 *3.2. Gross and histopathological study*
164 *3.2.1. Liver*
165 In the early stages of infection (9 dpi), all goats (group 2) and sheep (group 5) showed
166 reddish spots and small tortuous whitish tracts mainly involving the diaphragmatic
167 aspect of the left liver lobe. Quantification of those lesions resulted in mean of
168 113.4±54.6 in goats and 111.8±35.7 in sheep. Microscopic hepatic changes in the early
169 stages post infection consisted of necrotic foci and tracts within the hepatic parenchyma,
170 often associated with focal haemorrhage, mainly involving subcapsular areas.
171 Inflammatory infiltrates associated with these necrotic areas consisted mainly of
172 eosinophils with fewer lymphocytes and macrophages. Adjacent portal spaces showed
173 severe infiltration of lymphocytes, eosinophils and macrophages. These inflammatory
174 cells migrated from portal areas through hepatic sinusoids to necrotic areas of the
175 hepatic parenchyma.

176 In the late stages of infection, both goats (group 1) and sheep (group 4) demonstrated
177 scars and tortuous white tracts, particularly involving the left liver lobe. Percentage of
178 affected areas in goats was 36.02±9.4% and 33±17.6% in sheep. The gallbladder and
179 major biliary ducts were whitish and enlarged, and they contained brownish fluid
180 admixed with adult flukes. Microscopically, hepatic lesions were composed of marked
181 fibrosis in portal spaces containing large bile ducts and severe infiltration of
182 lymphocytes and plasma cells, either in a diffuse or lymphoid follicle pattern (response
183 to adult flukes, observed within enlarged bile ducts which often showed epithelial
184 erosion). Additionally, chronic tracts with macrophages containing abundant
185 hemosiderin pigment, granulomas with necrotic centres, macrophages and giant
186 multinucleate cells and variable infiltrates of lymphocytes, plasma cells and eosinophils
187 were found in the hepatic parenchyma (response to tissue damage).

188 *3.2.2 Hepatic lymph nodes*

189 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
190 and 3, respectively. In sheep HLN weight was 1.4 ± 0.3, 6.3 ± 0.8 and 1.0 ± 0.5 in
191 groups 4, 5 and 6, respectively. Significant HLN weight increase ($P<0.01$) was found in
192 chronic infection (groups 2 and 5) with respect to negative controls and acute infection
193 in goats and sheep. The histological study revealed that the HLN weight increase in
194 chronic infections was due to a marked hyperplasia of lymphoid follicles, interfollicular
195 areas and medullary cords.

196 *3.3. CD3 and Foxp3 expression in the liver*

197 Results of cell counting for CD3 and Foxp3 in livers of goats and sheep during acute
198 and chronic infections and negative controls are shown in Table 1. Uninfected control
199 goats and sheep showed occasional $CD3^+$ T lymphocytes mainly located in portal areas.
200 $Foxp3^+$ cells were also occasionally noted and also located in portal areas. The

201 percentage of Foxp3/CD3 T cells was 20.3% and 18.8% in goats and sheep,
202 respectively (Table 1).
203 At 9 dpi (acute infection stage), $CD3^+$ and $Foxp3^+$ T cells were found at the periphery of
204 necrotic foci and adjacent portal spaces (Fig. 1). The number of both $Foxp3^+$ and $CD3^+$
205 T lymphocytes increased significantly in goats and sheep with respect to negative
206 controls (Table 1). However, the percentage of $Foxp3^+/CD3^+$ did not show a significant
207 change in either goats (P=0.55) or sheep (P=0.48) with respect to negative controls.
208 In the chronic stages of infection (15-19 wpi), the number of $CD3^+$ T lymphocytes and
209 $Foxp3^+$ T cells was significantly increased in both goats and sheep with respect to
210 control livers, particularly in portal spaces with severely enlarged bile ducts (Figs. 2 and
211 3; location B in Table 1), whereas in granulomas, chronic tracts and smaller portal
212 spaces, the number of $CD3^+$ and $Foxp3^+$ T cells was also increased with respect to
213 negative controls but lower than in the vicinity of enlarged bile ducts (Location A in
214 Table 1). The percentage of $Foxp3^+/CD3^+$ T cells in areas of tissue damage in goats was
215 significantly reduced with respect to both the negative controls and acute infections,
216 while it did not change significantly in sheep. In areas of response to adult flukes
217 (periphery of enlarged bile ducts), the percentage of $Foxp3^+/CD3^+$ was not significantly
218 modified with respect to uninfected controls.
219 There was no statistical correlation between number of $Foxp3^+$ T cells and fluke burden
220 or gross pathology in any of the studied groups.
221 *3.3.2. Hepatic lymph nodes*
222 Results of cell counting for $CD3^+$ and $Foxp3^+$ in HLN of goats and sheep during acute
223 and chronic stages of infection and in negative controls are shown in Table 2.
224 Uninfected control goats and sheep showed abundant infiltrates of $CD3^+$ T lymphocytes
225 and $Foxp3^+$ T cells in the cortex, particularly in interfollicular areas (Fig. 4), whereas

226 the number of both cell populations was lower in the medulla (medullary cords and
227 medullary sinuses).
228 During acute infections, $CD3^+$ T lymphocytes were significantly increased with respect
229 to negative controls in sheep and goats (cortex) and sheep (medulla) (Table 2). $Foxp3^+$
230 T cells were mainly found in interfollicular areas of the cortex (Fig. 5). The number of
231 $Foxp3^+$ T cells and ratio $Foxp3^+/CD3^+$ increased significantly only in the cortex of
232 acutely infected goats with respect to negative controls (Table 2). The number of
233 $Foxp3^+$ Tregs and the ratio of $Foxp3^+/CD3^+$ in the medulla were very similar in negative
234 controls and acutely infected goats and sheep (Table 2).
235 During chronic infections, the number of $CD3^+$ T cells was significantly increased in the
236 cortex and medulla of goats and sheep with respect to negative controls (Table 2).
237 $Foxp3^+$ Tregs were mainly found in interfollicular areas of cortex (Fig. 6), where a
238 significant increase was found in chronically infected goats and sheep with respect to
239 uninfected controls. The percentage of $Foxp3^+/CD3^+$ did not change significantly with
240 respect to the uninfected controls in the cortex and medulla of either goats or sheep
241 (Table 2).
242

243 **4. Discussion**

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

250 In the present study, we have found a significant increase of Foxp3$^+$ Tregs in the hepatic
251 lesions of both goats and sheep, in the acute and the chronic stages of the infection. This
252 increase of Foxp3$^+$ Tregs was generally correlated to an increase in the number of CD3$^+$
253 T cells, so the percentage of Foxp3$^+$/CD3$^+$ cells did not change.
254 The rapid expansion of Foxp3$^+$ Tregs in the acute phase of the infection (9 dpi) seems to
255 be related to the larval migration in the hepatic parenchyma, since most of those cells
256 were found around necrotic foci and tracts and in the adjacent portal spaces. However,
257 no correlation was found between number of Foxp3$^+$ Tregs and the number of necrotic
258 lesions. The early presence of Foxp3$^+$ Tregs in the initial stages of parasitic infections
259 has been shown in gastrointestinal nematode mouse model (Finney et al., 2007) and it
260 has been explained as an immunomodulatory mechanisms facilitating the survival of the
261 parasite. In sheep, McNeilly et al., (2013) described an increase of Foxp3$^+$ Tregs at 10
262 dpi in the abomasal mucosa of sheep infected with *Teladorsagia circumcincta*, that may
263 reflect a homeostatic regulatory mechanism within the abomasal cellular immune
264 response to minimize immune-mediated abomasal pathology. Our data suggest Foxp3$^+$
265 Tregs may play a role in modulating the initial host immune response, contributing to
266 the survival of *F. hepatica* during the migratory stage. However, further studies are
267 required to clarify the relationship between the initial Foxp3$^+$ Tregs expansion and the
268 inability of the immune effector mechanisms to kill the newly excysted juveniles of *F.*
269 *hepatica* in the early peritoneal and hepatic migration, as occurred in the protective
270 responses observed in the *F. gigantica* sheep model (Piedrafita et al., 2007).
271 In the chronic hepatic lesions, the increase of Foxp3$^+$ Tregs was more pronounced in the
272 inflammatory infiltrates adjacent to large bile ducts than in the periphery of granulomas,
273 chronic tracts and small portal areas. Since the adult parasites in these stage are located
274 within the bile ducts and gallbladder, it seems that Foxp3$^+$ Tregs are specifically

275 recruited to the vicinity of *F. hepatica* adults or are actively induced by the adult
276 parasites and this may be related to the chronicity of the infection and the long survival
277 of the parasite in the host (Escamilla et al., 2016).
278 A dual role has been described for Foxp3$^+$ Tregs in hepatic helminth infections:
279 minimising tissue pathology and modulating the host immune response to facilitate
280 parasite survival, as reported in the case of *Schistosoma japonicum* infection in mice
281 (Zhu et al., 2015). In the present study, the different proportion of Foxp3$^+$ T cells in the
282 periphery of granulomas and chronic tracts compared to those of large bile ducts and the
283 lack of correlation between Foxp3$^+$ T cells and fluke burden or gross pathology may
284 also suggest this dual role for Tregs in *F. hepatica* infections. The evaluation of
285 cytokines such as IL-10 and TGF-β produced by Foxp3$^+$ T cells in each of these hepatic
286 lesions is of foremost interest to elucidate this point.
287 The number of Foxp3$^+$ Tregs was also significantly increased in the cortex of the HLN,
288 as well as the number of CD3$^+$ T lymphocytes in both the cortex and the medulla. This
289 data agrees with the increase of Tregs in mesenteric lymph nodes found in helminth
290 infected mice (Smith et al., 2016). Some differences were observed between sheep and
291 goats, since goats showed higher number of Foxp3$^+$ Tregs in both the acute and the
292 chronic stages, with a significant elevation of the percentage of Foxp3$^+$/CD3$^+$ in the
293 acute phase of the infection. However, we found no correlation between this higher
294 number of Foxp3$^+$ Tregs in HLN in goats and any other parasitological or pathological
295 data. Sheep and goats seems to have a different immune mechanism in response to
296 gastrointestinal nematodes (Hoste et al., 2010), but no such difference seems to appears
297 in the case of *F. hepatica* infection.
298 In conclusion, this is the first report describing the distribution of Foxp3$^+$ Tregs in acute
299 and chronic hepatic lesions and HLN of *F. hepatica* infected goats and sheep. The

300 expansion of Tregs in acute and chronic hepatic lesions may be involved in parasite
301 survival. Future studies should focus on the investigation of parasite molecules,
302 particularly from newly excysted juveniles, involved in the expansion of Foxp3 Tregs,
303 as well as the cytokines produced by this cell type in the different hepatic lesions to
304 elucidate their roles in *F. hepatica* infection.
305
306 **Acknowledgments**
307 This work was supported by EU grants (H2020-635408-PARAGONE) and the Spanish
308 Ministry of Science grant AGL2009-08726. TM receives funding from the Scottish
309 Government.
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374 **Figure Legends**

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

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

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

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

385 **Figure 6.** Acute infection goat hepatic lymph node showing numerous Foxp3^{+} T cells
386 (brown-red colour) in interfollicular (IF) areas. ABC method, x200.

387 **Figure 7.** Chronic infection goat hepatic lymph node showing a lymphoid follicle (LF)
388 and interfollicular areas (IF) containing numerous Foxp3^{+} T cells (brown-red colour) in
389 interfollicular (IF) areas. ABC method, x200.

390

391

392 **Table 1**

393 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

394 All infected animals were infected orally with metacercariae of ovine origin (Ridgeway

395 Research Ltd.), administered in gelatine capsules with a dosing gun. All animals were

396 killed by intravenous injection of T61 (Intervet International GMBH,

397 Unterschleissheim, Germany).

398

399 **Table 2**

400 Number and percentage of Foxp3$^+$ and CD3$^+$ T lymphocytes in livers of acute and

401 chronic stage of infection, and in uninfected controls. Results expressed as mean ± SD

402 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			

403 **Location A**: Uninfected control (UC): randomly selected portal areas. Acute infection

404 (AI): portal areas, necrotic foci. Chronic infection (CI): granulomas, chronic tracts and

405 small portal areas.

406 **Location B**: Chronic infection: Periphery of large bile ducts.

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

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

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

410

411 **Table 3**

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

415

	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

416

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

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

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

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423