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Early hepatic immune response in rats infected with *Fasciola hepatica*

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Abstract – We investigated the phenotype of the T cells (CD4⁺ and CD8⁺) that produced Th1 (IFN- γ) and Th2 cytokines (IL-4 and IL-10) during the first two weeks of experimental fasciolosis in rats. We also followed the kinetics of the cytokine and proliferative responses of hepatic mononuclear cells (HMNC) over the same period. We found that HMNC were more numerous in the infected animals than in the controls. The percentage of CD4⁺ cells increased significantly after infection, whereas the percentage of CD8⁺ cells did not change. Moreover, the frequency of the cells producing (CP) cytokine changed after infection. The frequency of CP IFN- γ on 7 days postinfection (pi) was similar to that in control animals. However, the frequency of CP IFN- γ was clearly lower on day 14 pi, whereas the frequency of CP IL-4 and CP IL-10 had increased. The CP IL-10-were mostly CD4⁺. Mitogenic stimulation (phorbol myristate acetate/ionomycin) of HMNC led to an increase in the amounts of the Th2 cytokines in the supernatant on days 7 and 14 pi, with the increase more pronounced on day 14. In contrast, IFN- γ levels also increased by day 7 pi but then decreased to below control levels by day 14. In addition, HMNC proliferation in response to mitogen followed a similar pattern to IFN- γ production. These findings suggested that, during the first 2 weeks of infection, *F. hepatica* induced a transient Th0 cytokine profile followed by downregulation of the cellular response and the induction of a Th2 cytokine profile.

Fasciola hepatica / rat / cytokine / liver / lymphocytes

Résumé – Réponse immunitaire hépatique précoce chez le rat infesté par *Fasciola hepatica*.

Nous avons caractérisé le phénotype des sous-populations lymphocytaires T (CD4⁺, CD8⁺) qui produisent des cytokines de type Th1 (IFN- γ) et Th2 (IL-4, IL-10) au cours de l'infestation expérimentale par *F. hepatica* chez le rat (phase précoce). Nous avons aussi suivi la production des cytokines et

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la réponse proliférative des cellules mononucléaires hépatiques (CMNH) après une stimulation *in vitro* par des mitogènes (phorbol myristate acetate) et par des antigènes spécifiques (les produits d'excrétion-sécrétion de *F. hepatica*). Le pourcentage des cellules CD4⁺ augmente après infestation tandis que celui des cellules CD8⁺ demeure inchangé. Après infestation, la fréquence des cellules productrices (CP) de cytokine change. Tandis qu'au 7^e jour après infestation (JAI) la fréquence des CP d'IFN- γ reste identique à celles des animaux témoins, elle est nettement plus basse au 14^e JAI ; la fréquence des CP d'IL-4 et des CP d'IL-10 augmente (surtout les CD4⁺ IL-10⁺, notamment au 14^e JAI). En outre, dans les surnageants de culture des CMNH, la production de cytokines de type Th2 est accrue au 7^e JAI et plus importante encore au 14^e JAI. Le niveau de production d'IFN- γ augmente au 7^e JAI mais décroît jusqu'à devenir inférieure à celle du témoin au 14^e JAI. De plus, la réponse proliférative est plus importante au 7^e JAI mais est réduite au 14^e JAI. L'ensemble de ces résultats montre que durant la période précoce de l'infestation, le parasite induit une réponse transitoire de type Th0 (au 7^e JAI), suivie d'une réduction de la réponse cellulaire et d'une induction d'une réponse de type Th2 (au 14^e JAI).

Fasciola hepatica / rat / cytokine / foie / lymphocyte

1. INTRODUCTION

Fasciola hepatica is a trematode parasite that induces both acute and chronic fasciolosis in livestock, resulting in considerable financial losses to farmers. The main pathogenic event in fasciolosis is the migration of juvenile flukes in the liver. During this migration, the migratory tracts are surrounded by an extensive local inflammatory reaction. This inflammation persists for the first six weeks of infection and involves the massive recruitment of eosinophils [26]. Hepatic lymphocytes are uniformly distributed throughout uninfected livers but are recruited to the infected areas and portal spaces following infection.

It is well accepted that the pattern of T-cell cytokine production determines the type and magnitude of the immune response [20]. The release of cytokines by lymphocytes is triggered by antigen recognition, which in turn initiates and regulates cell-to-cell communication. Granuloma formation, Ig isotype and the massive eosinophilia observed after 14 days of infection with *F. hepatica* indicate an oriented Th2 immune response [4, 17, 26]. However, before day 14 postinfection (pi), such data are unavailable and it is difficult to analyse the

immune response by these means. Other tools are therefore required. Consequently, we studied the profile of cytokine production in the first two weeks of experimental fasciolosis in rats. We investigated the phenotype of the T cells (CD4⁺ and CD8⁺) that produced Th1 (IFN- γ) and Th2 (IL-4, IL-10) cytokines. We also monitored cytokine production in the culture supernatant and the proliferation response of hepatic mononuclear cells (HMNC).

2. MATERIALS AND METHODS

2.1. Animals and experimental infection

Inbred Wistar rats, aged about ten weeks and of both sexes (reared in our laboratory) were used. Two groups of four rats were orally infected with 60 metacercariae 7 or 14 days before sacrifice (by chloroform inhalation) [24]. One group of four healthy rats was not infected. The experiment was set up so that all the animals were killed on the same day. All animal experiments were carried out in accordance with French national guidelines for the care and use of laboratory animals.

2.2. Media and reagents

2.2.1. Media

Hanks balanced salt solution without phenol red (HBSS), RPMI 1640 medium, foetal calf serum (FCS), penicillin-streptomycin, sodium pyruvate, HEPES and L-glutamine were purchased from Life Technologies (Cergy Pontoise, France); β -mercaptoethanol was obtained from Merck (Darmstadt, Germany); 7.5% sodium bicarbonate solution, phorbol myristate acetate (PMA), ionomycin and brefeldin A were purchased from Sigma (Saint Louis, USA); Lymphoprep density gradient medium was purchased from Nycomed (Oslo, Norway). Before use, RPMI 1640 medium was supplemented with 2 mM L-glutamine, 20 mM HEPES, 100 μ g penicillin-streptomycin, 20 mM sodium pyruvate, 5×10^{-5} M β -mercaptoethanol and 10% heat-inactivated FCS; HBSS was also buffered with bicarbonate (0.05%).

2.2.2. Reagents

Recombinant rat IL-10, IFN- γ and IL-4 were purchased from Becton Dickinson (Le Pont de Claix, France), Life Technologies (Cergy Pontoise, France) and R&D (Oxon, UK), respectively. Mouse anti-rat CD4⁺(W3/25), mouse anti-rat CD8⁺(OX8), rabbit anti-rat IFN- γ (AAR19), mouse anti-rat IFN- γ (DB-1), mouse anti-rat IFN γ fluorescein isothiocyanate (FITC)-conjugated (DB-1F) and mouse IgG1-FITC (F8-11-13) antibodies were from Serotec (Argènè Varhiles, France). Mouse biotinylated anti-rat IL-4 (B11-3B), mouse anti-rat IL-10 (A5-7) and mouse biotinylated anti-rat IL-10 (A5-6B) antibodies were obtained from Becton Dickinson. Mouse anti-rat IL-4 (56567,111) antibody was obtained from R&D. Phycoerythrin-conjugated donkey anti-mouse Ig (DAM-PE) and peroxidase-conjugated goat anti-mouse (GAM-PO) antibodies were obtained from Interchim (Montluçon, France). Streptavidin-FITC, Streptavidin-peroxidase

and 3, 3', 5'- tetramethylbenzidine (TMB) were obtained from Dako (Denmark), Sigma and KPL (Realef, France), respectively.

2.3. Excretory-secretory *F. hepatica* (ESFh) preparation

Live *F. hepatica* adults were recovered from the livers of slaughtered cattle. These flukes were washed five times and incubated for 3 hours at room temperature in 0.9% NaCl (1 mL/fluke). The medium was then recovered and centrifuged at 10 000 g for 15 min. Finally, the supernatant was filter sterilised (pore size = 0.22 μ m) and stored at -20 °C.

2.4. Preparation of HMNC suspensions

Liver perfusion and cell preparation were performed as previously described [30]. Briefly, the rats were killed by chloroform inhalation and the livers were perfused with HBSS to wash out peripheral blood cells. The livers were then immediately removed. HMNC were collected by the Ficoll Hypaque method and resuspended in 5% FCS in RPMI (10⁶ cells/mL). The viability of the recovered cells was > 90%.

2.5. Flow cytometry

Freshly isolated HMNC were stimulated for 18 h at 37 °C in a 5% CO₂ atmosphere in 6-well plates (Nunc, Kamstrup, Denmark) (3 mL/well) with either 10 ng/mL PMA plus 0.5 μ g/mL ionomycin and 2 μ g/mL Brefeldin A or in the absence of activators. After culture, the cells (400 μ L per tube) were washed (5% FCS in PBS) and collected by centrifugation at 250 g for 7 min. The pellet was resuspended in 5% FCS in PBS and 100 μ L of cell suspension was incubated with anti-rat CD4⁺ antibody (mAb) (W3/25, 1/100) or with anti-CD8⁺ mAb (OX-8, 1/100) on ice for 30 min. Then, the cells were washed again and stained with 100 μ L of DAM-PE mAb

(1/200 dilution). For intracellular cytokine staining, the cells were fixed with 2% paraformaldehyde (w/v), permeabilised with 0.5% saponin (w/v). IL-4 and IL-10 staining was performed by incubating cells with B11-3B and A5-6 (1/300 and 1/50 respectively) for 45 min on ice followed by Streptavidin-FITC incubation for 15 min. IFN- γ positive cells were directly stained with DB-1F (Serotec, 1/20 dilution) for 30 min on ice. Finally, cytokine production was determined by two-colour flow cytometry of gated CD4⁺ and CD8⁺ cells in a FACStar Plus machine (Becton Dickinson). For each sample, 15 000 events were recorded.

2.6. Cytokine production and proliferation assay

Based on the results of preliminary experiments to optimise cytokine production and proliferation in response to PMA/ionomycin, a culture time of 48 h for subsequent assays was selected.

Freshly isolated HMNC were stimulated with 10 ng/mL PMA plus 0.5 μ g/mL ionomycin or with 25 μ g/mL ESFh, or left unstimulated. Cultures were set up in a final volume of 200 μ L in 96-well round-bottom plates (10^5 cells/well). The cells were incubated for 48 h at 37 °C under a 5% CO₂ atmosphere. We then collected 100 μ L of each supernatant, which was stored at -70 °C until cytokine determination. The rest of the cell suspension was used for the lymphoproliferative assay.

The amounts of IFN- γ , IL-4 and IL-10 in the supernatant were analysed by standard-capture enzyme-linked immunosorbent assays (ELISA) in Nunc microplates. IL-10 and IFN- γ levels were determined according to the kit manufacturer's recommendations (Becton Dickinson and R&D, respectively). IL-4 levels were determined with a Duo Set Kit (R&D) used according to the manufacturer's protocol. The results are representative of three experiments.

After 48 h of culture, 1 μ Ci of [³H]-thymidine (ICN, France) was added to the cell suspensions. The cells were then incubated for an additional 6 h before harvesting on glass fibre disks. Cell proliferation was expressed as mean counts per minute (cpm) for triplicate experiments and as stimulation indices (SI) = cpm of stimulated HMNC/cpm of unstimulated HMNC. Three independent experiments were carried out.

2.7. Statistical analysis

Differences between experimental groups were compared using the Kruskal-Wallis test and the level of significance was set at $P < 0.05$.

3. RESULTS

3.1. Frequency of CD4⁺ and CD8⁺ T cells among HMNC in *F. hepatica*-infected rats

The total numbers of cells harvested on days 7 and 14 pi were significantly higher than those in the control animals (Tab. I). The total number of cells decreased thereafter, with a smaller number of cells harvested on days 21 and 28 pi ($< 4 \times 10^5$ cells recovered per liver).

The proportion of CD4⁺ cells was significantly lower ($P < 0.05$) than that of CD8⁺ cells in uninfected rat livers. The proportion of CD4⁺ T cells was significantly higher ($P < 0.05$) in infected animals than in controls while the proportion of CD8⁺ cells did not change significantly during the observation period (Tab. I). There was no significant difference between the mean fluorescence intensity values of the CD8⁺ and CD4⁺ cell populations of infected and control animals, indicating that the surface expression of CD4⁺ and CD8⁺ was not modified (data not shown).

Table I. Percentages and numbers of CD4⁺ and CD8⁺ hepatic cells in *F. hepatica* infected and control rats.

	Total recovered HMNC ($\times 10^6$)	CD4 ⁺		CD8 ⁺	
		($\times 10^6$)	(%)	($\times 10^6$)	(%)
Control	5.7 \pm 1.5	1.3 \pm 0.3	22.4 \pm 1.7	3.2 \pm 0.3	55.9 \pm 2.8
Infected:					
7 days pi	*9.5 \pm 0.7	*2.5 \pm 0.2	*26.0 \pm 2.2	*4.8 \pm 0.3	50.3 \pm 2.2
14 days pi	*9.9 \pm 1.8	*3.2 \pm 0.6	*33.5 \pm 1.3	*4.9 \pm 0.9	49.5 \pm 1.3

*Indicates $P < 0.05$ compared to values in uninfected animals.

Unstimulated cells and cells stimulated with *ESFh* did not produce detectable levels of cytokines. After mitogenic stimulation, the frequency of IFN- γ ⁺ cells within the CD4⁺ and CD8⁺ subsets had not significantly changed on day 7 pi, but was significantly lower ($P < 0.05$) than that of controls on day 14 pi (Tab. II). On day 7 pi, the frequencies of IL-4- and IL-10-producing CD4⁺ cells were significantly higher than those in the controls ($P < 0.05$). The proportions of IL-4-producing CD4⁺ cells did not change significantly between 7 and 14 days pi. Conversely, the frequency of IL-10-producing CD4⁺ cells continued to increase.

The frequency of IL-4-producing-cells within the CD8⁺ subset was significantly higher ($P < 0.05$) in all infected animals than in the controls. However, IL-10-producing cells were absent from this subset (CD8⁺) in all infected animals (Tab. II).

3.2. Patterns of cytokine secretion into HMNC supernatants (Fig. 1)

Unstimulated cells produced no detectable cytokines in culture supernatants. Stimulation with *ESFh* resulted in a non-significant

Figure 1. In vitro cytokine production by HMNC stimulated with PMA/ionomycin in control and *F. hepatica*-infected rats ($n = 4$ per group). Values represent the means \pm SD. The results are representative of 3 separate experiments. *Indicates $P < 0.05$ compared to values in uninfected animals.

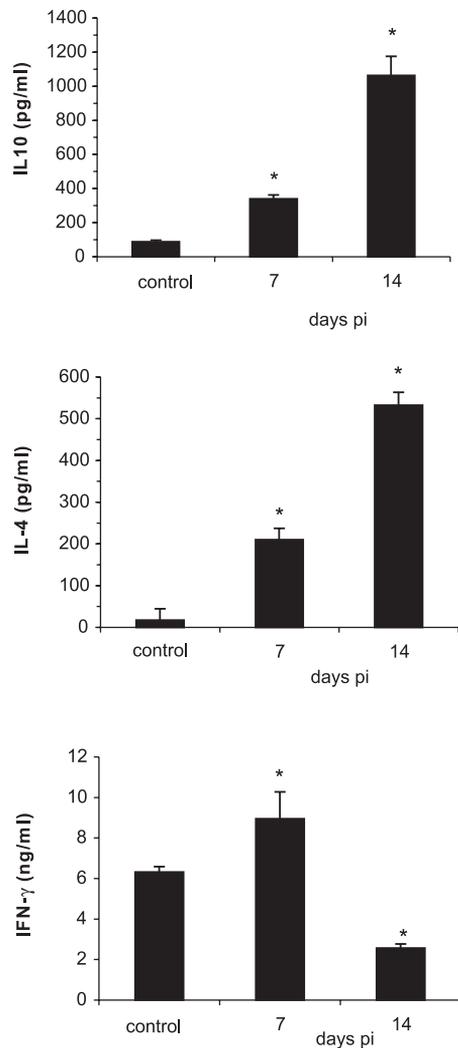


Table II. Percentage of cytokine-producing CD4⁺ or CD8⁺ hepatic cells in *F. hepatica* infected and control rats.

Cytokine	Control		Infected			
	CD4 ⁺	CD8 ⁺	7 days pi		14 days pi	
			CD4 ⁺	CD8 ⁺	CD4 ⁺	CD8 ⁺
IL-4	6.2 ± 0.1	11.0 ± 0.3	*9.1 ± 0.1	*14.3 ± 0.3	*10.7 ± 0.1	*15.8 ± 0.2
IL-10	2 ± 0.1	1.5 ± 0.4	*9.8 ± 0.1	0	*14.0 ± 0.1	0
IFN-γ	2.2 ± 0.1	35.6 ± 0.4	2.4 ± 0.5	38.9 ± 0.2	*1.8 ± 0.5	*18.5 ± 6.3

*Indicates $P < 0.05$ compared to values in uninfected animals.

increase in cytokine levels and was unaffected by infection at the tested doses of the *ESFh* antigen.

The levels of IFN-γ production by HMNC following in vitro stimulation with PMA/ionomycin were significantly higher on day 7 pi than those in control animals ($P < 0.05$). As infection progressed, IFN-γ levels decreased significantly in infected animals by day 14 pi ($P < 0.05$). The levels of production of both IL-4 and IL-10 were significantly higher in infected rats on days 7 and 14 pi (for IL-4: 12.3 and 31.2 times higher respectively; for IL-10: 4 and 12.2 times higher respectively, $P < 0.05$) (Fig. 1).

3.3. Proliferative response of HMNC to mitogens and to *ESFh* (Fig. 2)

On day 7 pi, the HMNC of *F. hepatica*-infected rats showed significantly stronger proliferative responses than did the controls following stimulation with PMA/ionomycin ($P < 0.05$). In contrast, on day 14 pi, the proliferative responses of these cells were significantly weaker than those of the controls ($P < 0.05$) (Fig. 2).

A significantly stronger proliferative response to *ESFh* was observed in the infected cells than in the control cells on day 7 pi ($P < 0.05$), but not on day 14. Various doses of *ESFh* antigens (25, 50, 100 μg) were tested and all gave similar results (data not shown).

4. DISCUSSION

Control over the early immune response is crucial for the establishment of the fluke in its host. Experiments in rats have shown that juvenile flukes modulate the immune response by inhibiting the early peripheral inflammatory response [2] and by delaying the hepatic inflammatory response during the first 2 weeks after infection [26]. In this study, we compared the hepatic cellular immune response in uninfected rats and rats infected with *F. hepatica* for 7 and 14 days. We investigated the production of T-cell cytokines in response to a mitogenic stimulus by flow cytometry [1, 14]. The advantage of this method over cytokine RNA monitoring [27], is its ability to examine the cytokine production of individual cells within mixed populations.

Our results indicate that a significant proportion of CD4⁺ and CD8⁺ HMNC from healthy rats produce IL4, IL10 and IFN-γ upon stimulation with PMA/ionomycin. Moreover, these cells proliferate in response to mitogens, such as concanavalin A and PMA/ionomycin, and specific *F. hepatica* antigens, but the levels of proliferation observed are lower than those observed with splenocytes or PBL (data not shown). To our knowledge, this is the first study focusing on cytokine production and cell proliferation in the rat liver.

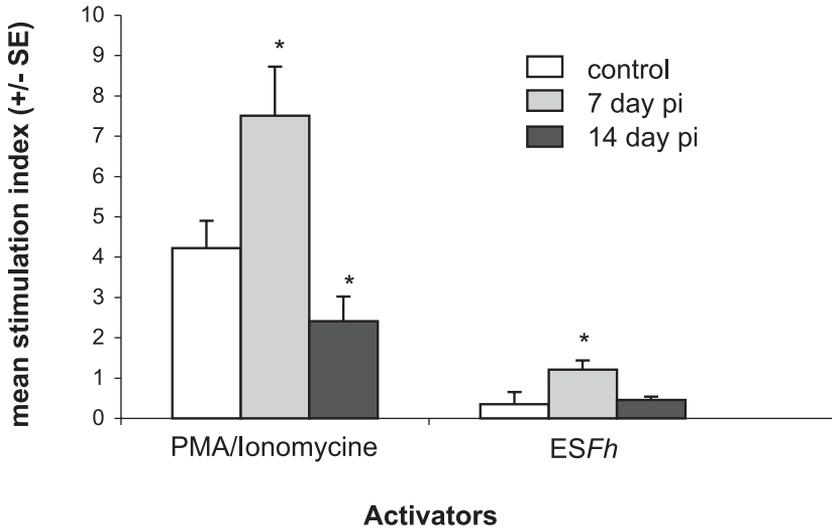


Figure 2. Proliferative responses of HMNC from *F. hepatica*-infected rats ($n = 4$ per group) to mitogens: PMA (10 ng/mL)/ionomycin (500 ng/mL) and the specific antigen, and to *ESFh* (25 μ g/mL). The values for unstimulated cells were 7200 ± 2300 cpm. The cells were harvested at the indicated time after infection (0, 7 and 14 days postinfection) and stimulated for 48 hours. Proliferation was measured by incorporation of ^3H -thymidine. The results are presented as the stimulation index (SI). The results are representative of 3 separate experiments. *Indicates $P < 0.05$ compared to values for uninfected animals.

After 7 days of infection, rat HMNC displayed higher levels of cytokine production than the controls. These results are consistent with RT-PCR analysis [27], which showed that levels of IL-4, IL-10 and IFN- γ mRNA increase on day 7 pi. The production by HMNC of both Th1 and Th2 cytokines suggests that these cells have a Th0 profile. Similar results were obtained in a previous study [31], which showed that the co-production of these cytokines was induced by IL-12. We have previously detected IL-12 mRNA on day 7 pi [27], which may account for the production of both Th1 and Th2 cytokines by HMNC. This is consistent with other studies on the peripheral response of mammals infected with *F. hepatica*, which was found to involve an early Th1/Th0 cytokine profile [5, 6, 18, 21, 22].

However, our results concerning IFN- γ levels are of particular interest. The concentration of this cytokine in the culture supernatants of HMNC collected from infected animals was higher on day 7 pi than that in control rats, although the frequency of IFN- γ^+ cells was similar in the two groups of rats. This suggests that other non-CD4 $^+$ non-CD8 $^+$ T cells may be the source of this increase. NK cells, which are abundant in the liver [16], may constitute the alternative source of IFN- γ [20] and this possibility is currently under investigation in our laboratory.

The cytokine production capacity of HMNC was modified in rats infected for 14 days. The percentage of IFN- γ -producing cells was significantly lower than that of controls on day 14 pi. This down-regulation

of IFN- γ production in the culture supernatant may result from a decrease in the frequency of IFN- γ -producing T lymphocytes (CD4⁺ and CD8⁺). ELISA analysis showed that more IL-4 was present on day 14 pi than on day 7 pi, despite similar frequencies and fluorescence intensities of IL4⁺ cells on these two days. This result suggests that other non-CD4⁺ non-CD8⁺ T cells may be the source of this increase (e.g. contamination with granulocytes, detected even on day 14 pi by flow cytometry [15, 32]). As on day 7 pi, the CD8⁺ IL10⁺ population was absent on day 14 pi, whereas the frequency of CD4⁺ IL10⁺ cells had increased further. This indicates that CD4⁺ cells are the major source of increases in IL-10 production during fasciolosis.

There are several possible reasons for the observed down-regulation of the Th1 response during the first two weeks following *F. hepatica* infection in rats. The decrease in IL-1 and IL-12 mRNA levels (produced by macrophages or dendritic cells and involved in Th1 differentiation [25, 28]) in rats on day 14 pi [27] may account for the observed decrease in IFN- γ production. In addition, IL-10 produced in massive amounts early in infestation may inhibit IL-12 production [8, 9, 12], resulting in the inhibition of IFN- γ production. Moreover, TGF β (the mRNA for which is widely produced in early fasciolosis [27]) has been reported to inhibit IFN- γ production in an IL-10-independent manner [10, 11]. If, as suggested by the murine *S. mansoni* and *Trichinella spiralis* models, Th1 cell-based effector mechanisms limit fluke survival, the suppression of these responses may be of crucial biological importance to helminth parasites. The suppression of the Th1 response by *F. hepatica* has been reported in several previous studies [3, 23]. Induction of the "wrong" set of cytokines may inhibit other responses that are potentially more damaging for the parasite [19].

Infected animals displayed a weaker proliferative response than control animals

only on day 14 pi; this may be related to changes in the distribution of leukocyte infiltrates in the livers of infected rats for 7 or 14 days [26]. On day 7 pi, the recruited immune cells were generally spread throughout the liver. In contrast, on day 14 pi, these cells were mostly localised around the migratory tracts and flukes. This is probably due to the chemoattractive molecules produced by *F. hepatica* [13], which facilitate direct contact between fluke molecules and immune cells, and may reduce overall responsiveness to mitogen. Indeed, it has been demonstrated that biologically active substances isolated from *F. hepatica* tissue may inhibit the proliferation of tumour cells in vitro [29]. The high levels of IL-10 production may also account for the decrease in proliferative response observed in HMNC on day 14 pi, similar to that observed in humans infected with *Onchocerca volvulus* [7].

In conclusion, our results indicate an early shift (7 dpi) from the Th1/Th2 response to a more pronounced Th2-regulated immune response, which may be mediated by IL10-producing CD4⁺ cells. They also demonstrate that CD4⁺ T lymphocytes are numerically and functionally the most important lymphoid cell population in the early immune response to fasciolosis. Further work is required to determine the location of NK cells in the liver and their pattern of cytokine production. These non-specific cells may play a crucial role in the early immune response of rats infected with *F. hepatica*.

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