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Fasciola hepatica infection downregulates Th1 responses in mice

SANDRA M.O'NEILL[1,2], MIRIAM T.BRADY[2], JOHN J.CALLANAN[3], GRACE MULCAHY[4], PATRICK JOYCE[5], KINGSTON H.G.MILLS[2] & JOHN P.DALTON[1]

[1]Molecular Parasitology Laboratory, School of Biotechnology, Dublin City University, Glasnevin Dublin, [2]Infection and Immunity Group, Department of Biology, National University of Ireland, Maynooth, Co. Kildare, [3]Department of Veterinary Pathology, University College Dublin, Ballsbridge, Dublin, [4]Department of Microbiology and Parasitology, University College Dublin, Ballsbridge, Dublin and [5]Department of Zoology, University College Dublin, Belfield, Dublin, Ireland

SUMMARY

Immune responses induced with helminth parasites have been extensively studied, but there is limited information on those to Fasciola hepatica, *especially on the subtype of T cell induced with this parasite. We investigated the local and systemic T cell responses of different strains of mice following oral infection with doses of metacercariae from* F. hepatica. *Spleen cells from BALB/c and 129Sv/Ev mice given a low-dose (5 metacercariae) infection exhibited a Th2 response, producing high levels of the cytokines IL-4 and IL-5, and low levels of IFN-γ and IL-2. In contrast, C57BL/6 mice showed a mixed Th1/Th2 response. A more marked polarization to a Th2 response was observed in BALB/c, 129Sv/Ev exposed to a high-dose (15 metacercariae) infection and the C57BL/6 mice also exhibited a clear Th2 response. IL-4 defective (IL-4$^{-/-}$) C57BL/6 mice infected with 5 metacercariae produced less IFN-γ and more IL-5 compared to their wild-type C57BL/6 counterparts, suggesting that IL-4 is important in establishing the Th2 type response in murine fasciolosis. However, the secretion of IFN-γ and IL-2 was completely suppressed in the high-dose infection and this was also observed in IL-4$^{-/-}$ mice. Thus, liver flukes may secrete molecules that downregulate Th1 responses. T cell responses in the mesenteric (MLN) and hepatic lymph nodes (HLN) were also examined since newly excysted juveniles infect through the intestinal wall of their host before migrating to the hepatic tissue. Cells from both MLN and HLN secreted higher levels of IL-4 and IL-5 compared to spleen cells. We also observed a difference in cytokine profiles secreted by the MLN and HLN, which may reflect responses to antigens liberated by newly excysted juveniles and hepatic stage parasites, respectively.*

Keywords Fasciola hepatica, *helminth, immune responses, cytokines, Th cells*

Correspondence: John P.Dalton, School of Biotechnology, Dublin City University, Glasnevin Dublin 9, Ireland
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INTRODUCTION

The helminth parasite *Fasciola hepatica* is the causative agent of liver fluke disease, or fasciolosis. The disease, which primarily infects sheep and cattle, results in a global annual economic loss to the agricultural community of approximately US$ 2000 million. Recent reports indicate that fasciolosis is also an important emerging pathogen of humans, with approximately 2·5 million people infected worldwide (Spithill & Dalton 1998). Infection is acquired following the ingestion of encysted larvae that contaminate vegetation or water. The parasites excyst in the intestine, migrate through the intestinal wall into the peritoneal cavity and then into the liver parenchyma where they cause extensive tissue damage and haemorrhaging. After approximately 8 weeks, the parasites move into the biliary passages, become sexually mature and commence egg production.

It is well accepted that CD4$^+$ T cells can be separated into two major subsets, Th1 and Th2, on the basis of their cytokine secretion patterns and function (Mosmann *et al*. 1986, Mosmann & Coffman 1989). Th1 cells produce IFN-γ, IL-2 and TNF-β and promote the activation of macrophages, the production of opsonizing antibodies and mediate delayed type hypersensitivity reactions (DTH) and inflammatory responses. In contrast, Th2 cells produce IL-4, IL-5, IL-6 and IL-10 and promote immediate type hypersensitivity reactions involving IgE, eosinophils and mast cells. The cytokines of each T cell subtype are mutually inhibitory for the differentiation and effector functions of the reciprocal subset resulting in the polarization of the immune response to either type 1 or type 2.

Recent studies have shown that helminth parasites, such as Schistosoma mansoni, induce Th2 responses (Sher & Coffman 1992). However, information regarding the immune responses generated during liver fluke infections is limited (Mulcahy *et al*. 1999). Several studies have observed a marked eosinophilia in both peripheral blood and bone marrow cells of *F. hepatica*-infected mice, rats and sheep (Doy *et al*. 1978, Milbourne & Howell 1990, Keegan

& Trudgett 1992, Chauvin *et al*. 1995). Histological investigations on the local immunological and inflammatory reactions in the liver of infected sheep showed that in the early stages of infection there was an infiltration of eosinophils and $CD4^+$ cells, although as infection progressed into the chronic stages there was also an infiltration of $CD8^+$ and $\gamma\delta$ TCR^+ cells (Meeusen & Brandon 1994, Chauvin *et al*. 1995). In cattle, mRNA for both the Th1 cytokine IFN-γ and the Th2 cytokine IL-4 was detected in the early stages of infection (Clery *et al*. 1996, Clery & Mulcahy 1998) and Th2/Th0 clones, but not Th1 clones, have been isolated from chronically infected cattle (Brown *et al*. 1994). Finally, antibody responses in cattle and sheep show a marked predominance of IgG1 over IgG2 isotypes (Chauvin *et al*. 1995, Clery *et al*. 1996, Mulcahy *et al*. 1999). Collectively, these studies indicate that Th2 responses may predominate in fasciolosis.

In the present study, we investigated the T cell and antibody responses of three strains of mice (BALB/c, C57BL/6 and 129Sv/Ev) exposed to a low (5 metacercariae) and high (15 metacercariae) infection of *F. hepatica*. The responses in two knockout strains of mice was also examined; one of these lacked the IFN-γ receptor (IFN-$\gamma R^{-/-}$) while the other is defective in IL-4 (IL-$4^{-/-}$). In addition, we compared T cell responses in hepatic (HLN) and mesenteric lymph nodes (MLN) with those in the spleen to determine if different cytokine profiles can be observed at different lymphoid organs. Our data demonstrate that infection with *F. hepatica* induces strong local and systemic Th2 responses in mice.

MATERIALS AND METHODS

Parasite material

Metacercariae were obtained from Compton Paddock Laboratories (Berkshire, UK). Mature liver flukes were removed from the bile ducts of infected cattle and washed in sterile phosphate buffered saline, pH 7·3 (PBS). Liver fluke homogenate (LFH) and excretory/secretory (ES) products were prepared as previously described (Smith *et al*. 1993). All antigens were stored at −20 °C and protein concentration quantified using a bicinchoninic protein assay kit (Pierce & Warriner, Chester, England).

Infection of mice

Female BALB/c mice, were purchased from Harlan Olac Ltd (Blackthorn, UK). Breeding pairs of C57BL/6, 129Sv/Ev, IL-$4^{-/-}$(C57BL/6 background) and IFN-$\gamma R^{-/-}$ (129Sv/Ev background) were purchased from B+K Universal Ltd (Hull, UK). All mice were bred and maintained under the guidelines of the Irish Department of Health and were 6–8 weeks old at the commencement of each experiment. Groups of four to 13 BALB/c, C57BL/6, 129Sv/Ev, IFN-$\gamma R^{-/-}$ and IL-$4^{-/-}$ mice were infected orally with 5, 10 or 15 metacercariae of *F. hepatica*. The mice were sacrificed by cervical dislocation 3 weeks after infection, serum was obtained via cardiac puncture and the spleens, HLN and MLN removed.

Th1/Th2 responses

In-vitro culturing of spleen and lymph node cells was performed as previously described (Mills 1996). Briefly, spleens and lymph node cell suspension was prepared in RPMI 1640 medium containing 8% heat inactivated FCS (foetal calf serum), penicillin (100 U/ml), streptomycin (100 μg/ml) glutamine (2 nM) and 2-mercaptoethanol (5×10^5 M). Spleen cells (2×10^6/ml) and lymph node cells (1×10^6/ml) were stimulated in triplicate in 96-well culture plates with varying concentrations of LFH antigen (20–100 μg/ml). Cells were stimulated with PMA (20 ng/ml) and anti-CD3 (2 μg/ml) and RPMI alone served as positive and negative controls, respectively. After 24 h, 50 μl of supernatant was removed to measure IL-2 production and after 72 h supernatant was removed to measure IL-4, IL-5 and IFN-γ. IL-2 concentrations were quantified by measuring the growth of an IL-2 dependent cell line, CTLL, as previously described (Mills 1996). The concentration of IL-2 was expressed as international units per ml. The cytokines IL-5, IL-4, and IFN-γ were measured by immunoassay using matched pairs of anticytokine antibodies purchased from Pharmingen (San Diego, CA, USA), as previously described (Mills 1996). Recombinant cytokines of known concentration were used to generate standard curves.

IgG subtype analysis

The titre of antifluke IgG subclasses was determined using liver fluke excretory/secretory as antigen in ELISA as previously described (O'Neill *et al*. 1998). Alkaline phosphatase conjugated antimouse IgG1 and IgG2a (Pharmigen) were used at a dilution of 1 : 1000 and 1 : 500, respectively, to detect the bound antibody.

Statistical analysis

Cytokine and antibody responses were compared by the non-parametric Mann Whitney test and significance was assumed for P-values $< 0{\cdot}05$. All experiments were repeated two or more times with similar results being obtained in each case.

RESULTS

Cytokine production by spleen cells of mice infected with 5 metacercariae of *F. hepatica*

Thirteen C57BL/6, 129Sv/Ev, BALB/c, IFN-$\gamma R^{-/-}$ and IL-$4^{-/-}$ mice were infected with five *F. hepatica*

metacercariae. Three weeks later, all mice were sacrificed and the number of infected animals estimated by examining the livers for tissue damage due to the migratory tracts of the parasite. The number of mice infected in each group and the extent of liver damage is shown in Table 1. BALB/c and IFN-$\gamma R^{-/-}$ mice exhibited the highest level of tissue damage, whereas IL-$4^{-/-}$ mice and C57BL/6 showed the lowest level of damage.

Spleens cells from four infected mice of each strain were isolated and stimulated with LFH *in vitro* 3 weeks after infection. Supernatants, removed after 24 and 72 h, were tested for IL-4 and IL-5, IFN-γ and IL-2 (Figure 1). Spleen cells from C57BL/6 mice produced IL-5, IFN-γ, IL-2 and low levels of IL-4. Spleen cells from 129Sv/Ev mice produced higher levels of IL-4, IL-5 and IL-2, but no detectable IFN-γ. BALB/c mice produced relatively high levels of IL-4, medium levels of IL-5, low levels of IL-2 and no significant IFN-γ (Figure 1). Therefore, 129Sv/Ev and BALB/c exhibit more Th2-like immune responses (Table 1). Spleen cells from IFN-$\gamma R^{-/-}$ mice secreted moderate to high levels of IL-4 and IL-5, but low levels of IL-2 and no detectable IFN-γ. In contrast, spleen cells from IL-$4^{-/-}$ mice produced IFN-γ and IL-2 but low levels of IL-5 and no IL-4 (Figure 1). Stimulation of spleen cells from the C57BL/6, 129Sv/Ev and BALB/c *in vitro* with PMA and anti-CD3 demonstrated that all cells were capable of secreting both Th1 and Th2 cytokines. However, spleen cells from IFN-$\gamma R^{-/-}$ mice secreted IL-5, IFN-γ, IL-2 and low IL-4, whereas spleens cells from IL-$4^{-/-}$ knockout mice secreted high levels of IFN-γ and IL-2, low levels of IL-5 and, as expected, no IL-4 in response to PMA and anti-CD3. Spleen cells obtained from noninfected mice of each strain did not produce any cytokines in response to stimulation with LFH (data not shown).

Cytokine production by spleen cells of mice infected with 15 metacercariae of *F. hepatica*

Eight C57BL/6, 129Sv/Ev, BALB/c, IFN-$\gamma R^{-/-}$ and IL-$4^{-/-}$ mice were infected with 15 *F. hepatica* metacercariae.

Table 1 Outcome of infection in C57BL/6, 129Sv/Ev, BALB/c, IFN-$\gamma R^{-/-}$ and IL-$4^{-/-}$ mice given 5 metacercariae of *F. hepatica*

Mouse strain	No. infected	Pathology*	T cell response
IL-$4^{-/-}$	6/13	+	Th1
C57BL/6	6/13	++	Th1/Th2
129Sv/Ev	10/13	++++	Th2
IFN-$\gamma R^{-/-}$	11/13	+++++	Th2
BALB/c	11/13	+++++	Th2

* An arbitrary measurement of damage to liver tissue due to migrating parasites was used to estimate pathology.

All mice of each strain became infected. The level of pathology observed in each mouse strain was similar and histological examination of the infected livers did not reveal differences in the cellular makeup of the inflammatory lesions caused by infection.

The most striking difference observed in the cytokine production by spleen cells obtained from mice infected with 15 metacercariae, as compared to cells from those infected with 5 metacercariae, was the almost complete lack of secretion of the type 1 cytokines, IL-2 or IFN-γ (Figure 2). This pattern was apparent in all mouse strains but was particularly dramatic in IL-$4^{-/-}$ knockout mice. Moreover, the suppression of type 1 cytokine production was observed even when the spleen cells were stimulated with the polyclonal activators PMA and anti-CD3. While the higher infection dose also suppressed the production of IL-4, this suppression was less marked than that observed for IL-2 or IFN-γ. The production of the type 2 cytokine, IL-5, by spleen cells of BALB/c, C57BL/6, 129Sv/Ev infected with 5 metacercariae was not significantly different from those given 15 metacercaraie. However, IL-5 production in the IL-$4^{-/-}$ and IFN-$\gamma R^{-/-}$ mice was significant reduced ($P < 0{\cdot}05$) in mice given the higher dose.

The production of IL-10 by spleen cells of the various infected mice was also measured in this experiment (Figure 3). C57BL/6, 129Sv/Ev, BALB/c and IFN-$\gamma R^{-/-}$, but not IL-$4^{-/-}$ mice produced high levels of IL-10 in response to LFH.

Comparison of lymph node with spleen cell cytokine production in BALB/c mice infected with *F. hepatica*

In order to compare the local and systemic T cell responses to infection, cytokine production was assessed in cells isolated from the spleens, HLN and MLN of BALB/c mice 3 weeks after an infection with 10 metacercariae of *F. hepatica*. Spleen, HLN and MLN cells secreted a predominant Th2 cytokine profile, i.e. IL-4 and IL-5 and no detectable IFN-γ (Figure 4). Furthermore spleen and lymph node cells secreted predominant Th2 cytokine profile in responses to PMA and anti-CD3 (Figure 4). The production of IL-4 was significantly higher ($P > 0{\cdot}01$) in the HLN and MLN compared to the spleen cells. However, the HLN produced an almost nine-fold greater amount of IL-4 than the MLN. In contrast, cells from the MLN secreted significantly greater amounts of IL-5 compared to that secreted by cells from the HLN and spleen.

IgG1 and IgG2a responses in *F. hepatica*-infected mice

The titres of IgG1 and IgG2a antibodies against LFH were measured in all mice 3 weeks after infection with five or 15 metacercariae of *F. hepatica*. Antibodies of the IgG1 subtypes

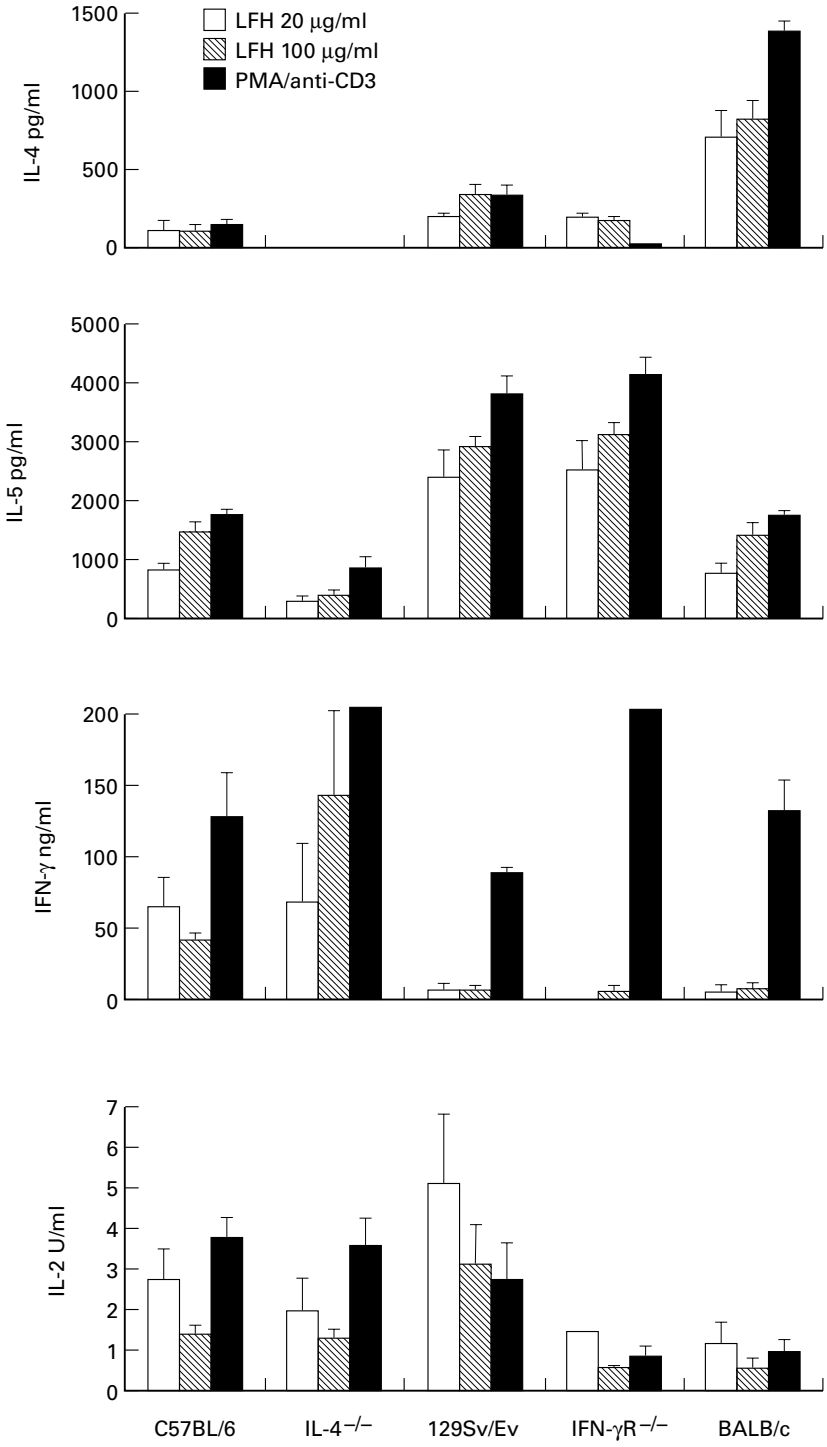

Figure 1 Cytokine production by spleen cells of mice infected with five metacercariae of *F. hepatica*. Spleen cells derived C57BL/6, IL-4$^{-/-}$, 129Sv/Ev, IFN-γR$^{-/-}$ and BALB/c mice obtained 3 weeks after infection were stimulated *in vitro* with 20 μg/ml and 100 μg/ml of LFH and, as a positive control, PMA and anti-CD3. The cytokines IL-4, IL-5, IFN-γ and IL-2 released into the cultured supernatant were measured by bioassay or immunoassay. Results are mean ± SE for four mice per group.

were detected in all mouse strains with the exception of the IL-4$^{-/-}$ mice, which normally have reduced IgG1 responses, because they are incapable of producing IL-4. The IgG1 titres observed in the groups infected with 5 metacercariae (Figure 5a) were similar to those observed in mice infected with 15 metacercariae (Figure 5b). There was no LFH-specific IgG2a antibodies detected in the serum of *F. hepatica* infected mice of any strain examined (Figure 5).

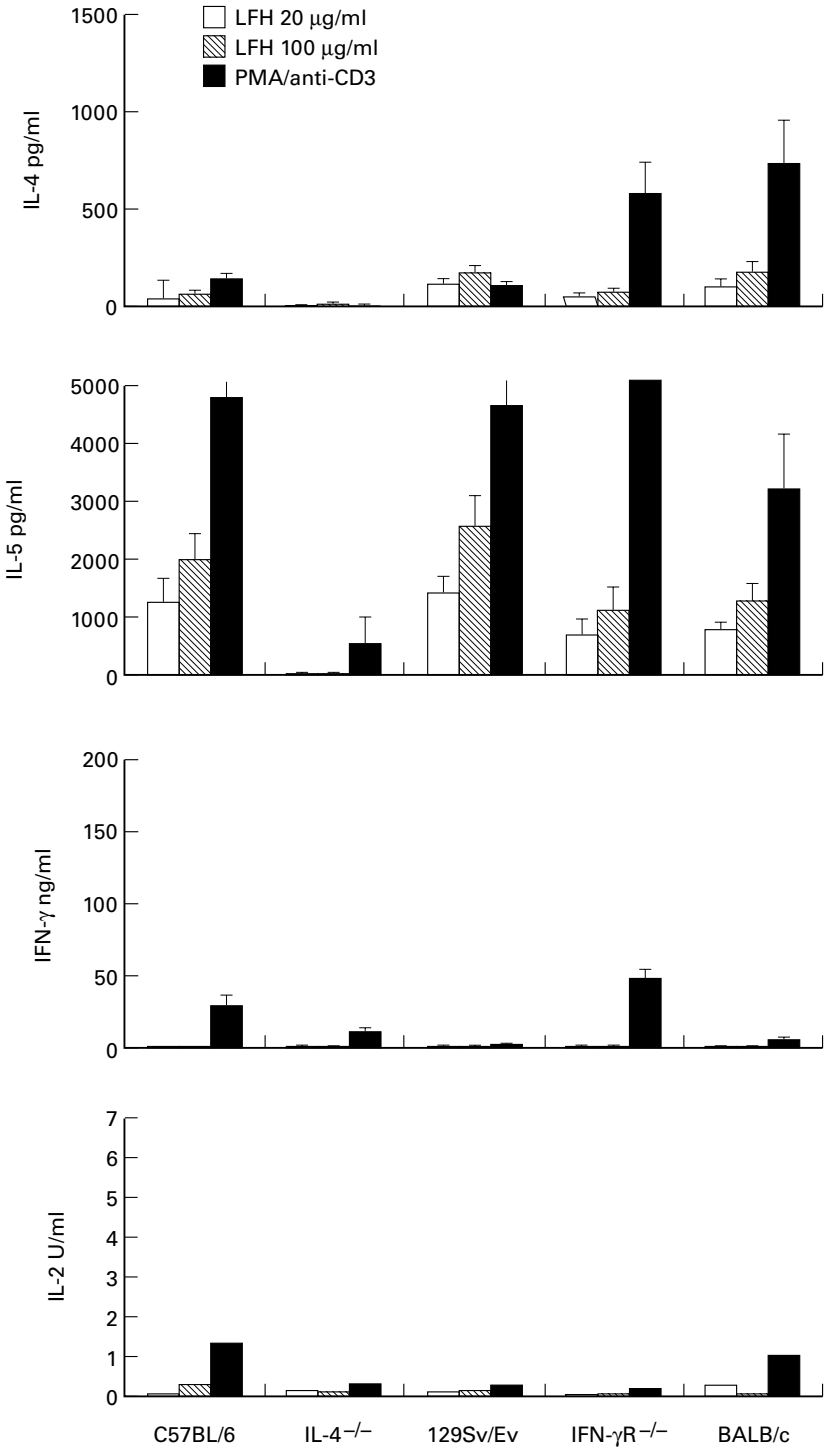

Figure 2 Cytokine production by spleen cells of mice infected with 15 metacercariae of *F. hepatica*. Responses were assessed as described in the legend to Figure 1.

DISCUSSION

In the present study, we examined antigen-specific cytokine production by spleen cells and lymph node cells in murine fasciolosis and demonstrated that a type 2 immune response predominates. However, the extent to which the immune responses of mice are polarized to the type 2 immune responses depends on the level of the infecting dose. While BALB/c and 129Sv/Ev mice exposed to a low dose infection elicited a Th2-like response, producing predominantly IL-4

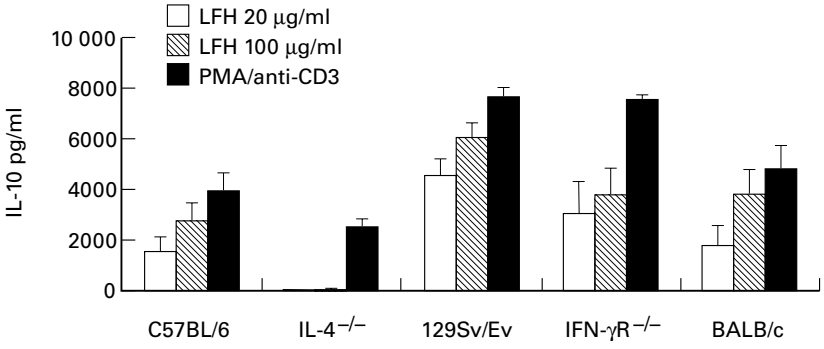

Figure 3 IL-10 production by spleen cells of mice infected with 15 metacercariae of *F. hepatica*. Mice were infected and cells cultured as described in the legend to Figure 1. IL-10 production was detected by immunoassay.

and IL-5, C57BL/6 mice, which are predisposed towards the production of type 1 cytokines (O'Garra *et al*. 1998) showed a mixed Th1/Th2 response. Moreover, a generalized shift of the immune response towards a Th2 cytokine profile was not observed when the spleen cells from these three strains of mice were stimulated *in vitro* with the ploy clonal activators PMA and anti-CD3. A large infecting dose of 15 metacercariae, however, did result in the complete downregulation of the Th1 cytokines IL-2 and IFN-γ cytokines in BALB/c and 129Sv/Ev mice, and even C57BL/6 mice produced a predominant Th2 cytokine profile. Furthermore, PMA and polyclonally stimulated spleen cells from mice given the high dose infection exhibited a polarization of the cytokine production towards a Th2-type profile.

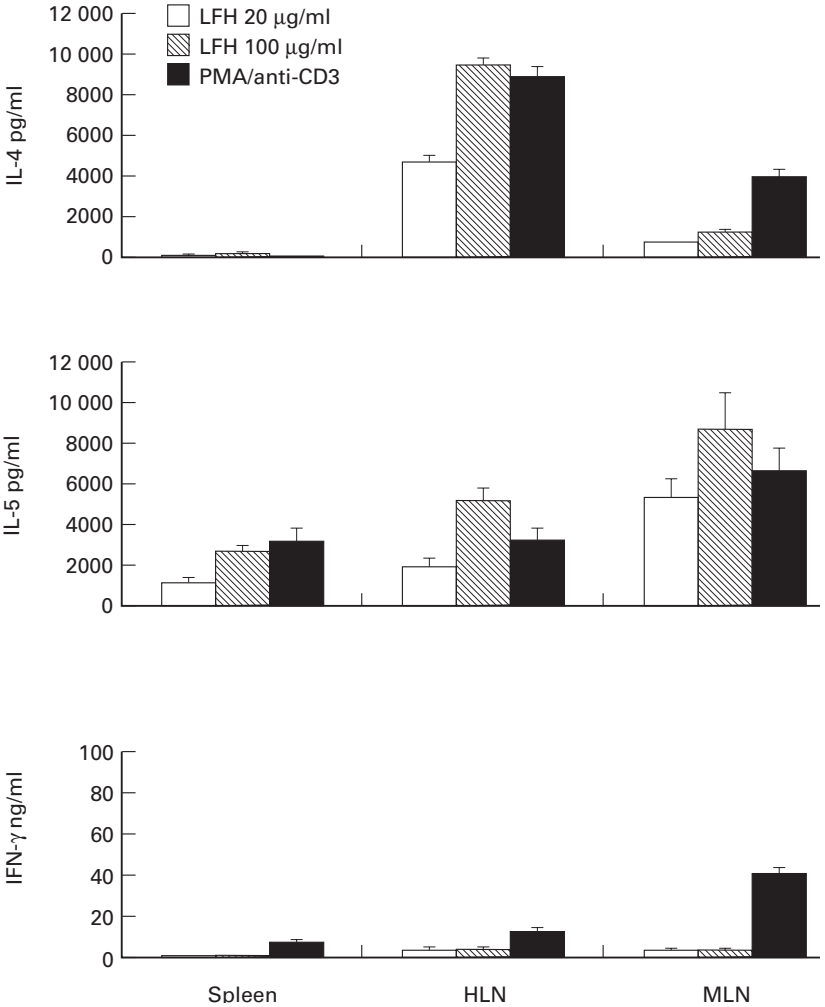

Figure 4 Comparison of T cell responses in spleen and lymph nodes of mice infected with *F. hepatica*. Spleen, hepatic lymph nodes (HLN) and mesenteric lymph nodes (MLN) obtained from BALB/c mice infected with 10 metacercariae of *F. hepatica*, were stimulated *in vitro* with 20 μg/ml and 100 μg/ml of LFH and with PMA/anti-CD3 and the production of the IL-4, IL-5, and IFN-γ was determined after 3 days of culture. Results are mean ± SE values for four mice per group assayed in triplicate using individual spleens or pooled lymph node cells.

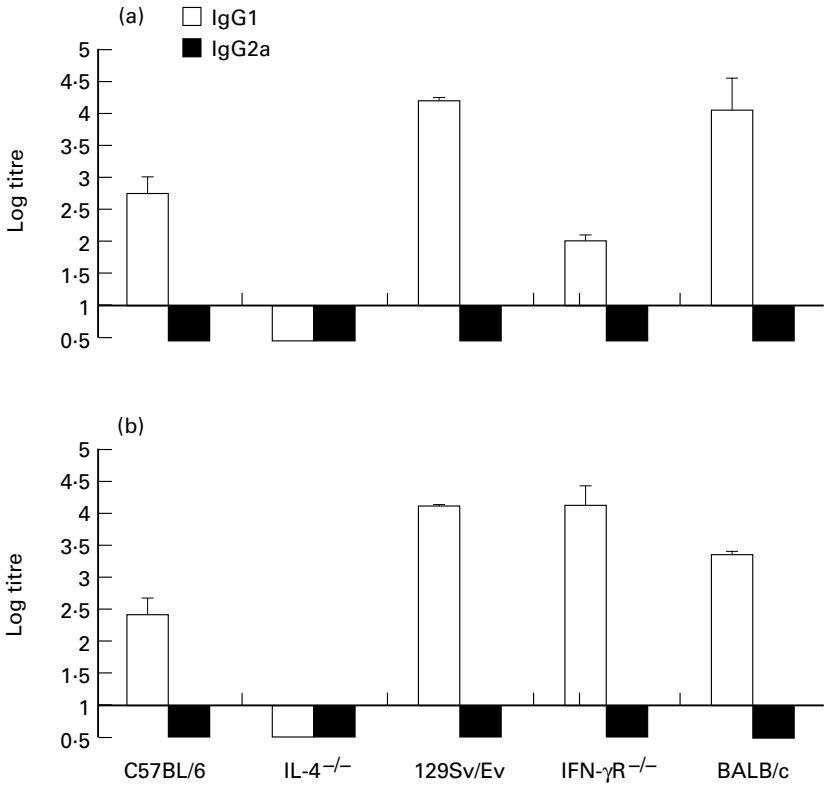

Figure 5 Serum IgG1 and IgG2a antibodies specific for liver fluke excretory/secretory products in mice infected with *F. hepatica*. Antibodies were measured 3 weeks after infection of BALB/c, C57BL/6 and 129Sv/Ev, IFN-$\gamma R^{-/-}$ and IL-$4^{-/-}$ mice strains with five (a) or 15 (b) *F. hepatica* metacercariae. Results are the mean ± SE of three separate experiments with each group containing six mice. The background titre calculated using serum from uninfected mice was < 1 : 25.

The production of IL-4 is believed to be important in the activation of precursor $CD4^+$ Th cells and promoting their differentiation into Th2 cells (Pearce *et al*. 1996). Indeed, in schistosomiasis the development of type 2 responses to parasite antigens is inhibited in the absence of IL-4 (Cheever *et al*. 1994, Pearce *et al*. 1996). In this study, we found that spleen cells from all mouse strains produce IL-4 and that, at least in BALB/c mice, this cytokine was produced in high levels by both MLN and HLN (see below). It is notable, however, that spleen cells of IL-$4^{-/-}$ mice infected with 5 metacercariae produced significantly higher levels of IFN-γ and significantly lower levels of IL-5 than their wild-type C57BL/6 counterparts, suggesting that the downregulation of Th1 responses and development of the Th2 response in fasciolosis is dependent upon IL-4.

The regulation of immunoglobulin subclasses by subsets of antigen specific helper T-cells has been well established (Stevens *et al*. 1988). Studies performed in mice infected with helminth parasites have demonstrated that IL-4 is associated with the secretion of IgG1 and IgE whereas IFN-γ is associated with secretion of IgG2a (Cauladа-Benedetti *et al*. 1991, Lawrence *et al*. 1994). In agreement with these studies, we found that all mice exhibiting a predominant Th2 cytokine response and had antifluke IgG1 antibodies present in their serum; only the IL-$4^{-/-}$ knockout mice did not produce antibody. There was no significant difference in the antibody production when mice received five or 15 *F. hepatica* metacercariae.

All mice infected with 15 metacercariae exhibited a complete suppression of the Th1 cytokines IFN-γ and IL-2 compared to those mice that received 5 metacercariae. In addition, we also observed some suppression of the Th2 cytokine IL-4 in the higher infection, while the production of IL-5 was only significantly reduced in the knockout strains of mice. It appears that the parasite is capable of suppressing the production of cytokines, most particularly cytokines of the Th1 type. This suppression may not only be mediated through secretion of cytokines by antigen-stimulated T-cells but also by immunomodulatory molecules secreted by the parasite. Immunosuppression during *F. hepatica* infection has been observed in several studies published over the last two decades. Sandman & Howell (1981) first suggested that the lack of antibody responses in infected sheep may indicate that liver fluke secrete immunosupressive factors. Zimmerman *et al*. (1983) and Chauvin *et al*. (1995) also noted a reduction in the proliferative responses of lymphocytes to Concanavalin A (Con A) in fluke-infected sheep. Similarly, in infected cattle Oldham & Williams (1985) observed a suppression of T-cell proliferation and IL-2 production while McCole *et al*.

(1998) found a nonresponsiveness of lymphocytes to Con A. Finally, Baeza *et al*. (1994a,b) demonstrated reduced inflammatory responses in liver fluke-infected rats.

The liver fluke molecules that may induce immunosuppression have yet to be characterized but they are most likely actively secreted by the parasite. Milbourne & Howell (1990, 1993) observed a systemic eosinophilia in rats following an intraperitoneal injection of excretory/secretory (ES) antigen and suggested that an IL-5-like substance is produced by flukes or a molecule released by the parasite stimulates T lymphocytes to secrete this cytokine. Moreover, Cervi *et al*. (1996) showed that ES antigens of *F. hepatica* suppressed the delayed hypersensitivity responses, which are regulated by Th1 cytokines, both specifically and nonspecifically. Candidate immunosuppressive molecules include glycoconjugates sloughed from the parasite surface glycocalyx (Mulcahy *et al*. 1999), phosphorylcholine-rich antigens (Sloan *et al*. 1991), Kunitz-type serine proteinase inhibitors (Bozas *et al*. 1995) and/or cysteine proteinases (Dalton & Brindley 1997).

Cytokine responses at MLN and HLN were examined since newly excysted juvenile liver flukes burrow through the gut wall (which is drained by the MLN) before migrating across the liver parenchyma (which is drained by the HLN). Both the local and peripheral immune responses elicit a Th2 cytokine profile, although cells of the MLN and HLN secreted significantly higher amounts of IL-4 and IL-5 compared to those of the spleen. In addition, we found that the quantity of cytokines produced by the draining lymph nodes differed; the HLN produced a nine-fold greater amount of IL-4 compared to the MLN, while cells from the MLN produced twice the amount of IL-5 cytokine compared to the HLN. The difference in the quantity of cytokine produced may reflect the responses elicited to different antigens liberated by the parasite as it migrates from the intestine to the liver. Rapid morphological and antigenic changes take place in the surface tegument of the flukes during this period (Fairweather 1999). In addition, Meeusen & Brandon (1994) showed that different antibody isotypic responses occurred at the MLN and HLN of infected rats that correlated with the parasites' migration from the intestine to the hepatic tissue. Because the present study only examined cytokine responses in the lymph nodes at 3 weeks after infection, we have no information regarding the dynamics of cytokine secretion in the local lymph nodes during parasite migration. However, studies are in progress to examine this and to determined at which point of infection precursor Th cells are induced to differentiate into the Th2 direction.

The induction of type 2 responses in mice are in agreement with earlier studies which collectively suggest that type 2 responses predominate in *F. hepatica*-infected rats, sheep and cattle (Mulcahy *et al*. 1999). Studies in our laboratory have shown that susceptibility of cattle to infection with *F. hepatica* is correlated with the induction of type 2 responses, while vaccine-induced protection is mediated by type 1 responses (Mulcahy *et al*. 1999). In our low-dose experiment, we observed that C57BL/6 and IL-4$^{-/-}$ mice, which were predisposed towards a Th1 immune response, were less susceptible to infection and exhibited less liver damage compared to the 129Sv/Ev, BALB/c and IFN-γR$^{-/-}$ mice that elicited type 2 responses. Larger infection studies are required to determine conclusively whether type 1 and 2 immune responses are associated with resistance and susceptibility, respectively, of mice to liver fluke infection. It is also possible that differences in the resistance level in the various strains examined was due to nonimmune related factors. Nevertheless, the murine model of fasciolosis could prove useful for studies aimed at the elucidation of the mechanism by which the parasite modulates immune responses toward the Th2 type and at the development of Th1-inducing vaccines.

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