Studies on ~~experimental~~ co-infections with *Schistosoma mansoni* and *Leishmania donovani*. ~~in mice~~

MOHAMED F. HASSAN
(MB.B.CH, MSc Inf. Trop. Dis.)

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IMMUNOLOGY UNIT
DEPARTMENT OF INFECTIOUS AND TROPICAL DISEASES
LONDON SCHOOL OF HYGIENE & TROPICAL MEDICINE,
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ABSTRACT

Schistosomiasis and visceral leishmaniasis, each responsible for serious morbidity and mortality, also have overlapping endemicities e.g. in the Sudan. Both cause granulomatous responses in the liver. *Leishmania donovani* amastigotes are killed by Th1-dependent macrophage activation whereas schistosome eggs induce Th2 dependent granulomas which protect the liver tissue from toxic products and from Th1 dependent inflammatory responses. In view of the reciprocal counter-regulation of Th1 and Th2 responses it was hypothesized that co-infection would lead to altered immune responses and exacerbation of disease. This was modelled in the mouse using *Schistosoma mansoni* and *L. donovani*. When C57BL/6 mice were infected with 25 cercariae of *S. mansoni* and superinfected, 8 weeks later with *L. donovani* the co-infected mice (CO-INFECT) suffered increased morbidity and mortality and elevation of serum liver enzymes, ALT/AST (aspartate/alanine aminotransaminase) compared with mice given the single infections (LEISH or SCHISTO). Schistosome worm and egg burdens and egg granulomatous responses were comparable in CO-INFECT and SCHISTO mice. In contrast, CO-INFECT and LEISH mice showed comparable *L. donovani* infection (Leishman Donovan units [LDUs]) in both liver and spleen at +2 weeks but the CO-INFECT mice showed progressively increased LDU up to +8weeks post *Leishmania* infection compared to declining LDUs in the LEISH group.

The *S. mansoni* infection induced significant IL-4 and IL-10 but not IFN-γ splenocyte and liver lymphocyte recall responses to schistosome antigens and mitogen whereas the LEISH infection induced specific IFN-γ production but not IL-4 to *Leishmania* antigen (Formalin fixed Leishmania amastigote antigen, FLAA). This IFN-γ response was markedly lower at +2 and +8 weeks post infection in the CO-INFECT mice but FLAA-specific IL-10 responses were higher. Since B-cells have been reported to be a major source of IL-10 in *S. mansoni* infected mice, co-infection experiments were carried out in μMT mice which lack B cells. Indeed there was lower IL-10 production in the CO-INFECT μMT mice and also a lower fold increase in LDU in the liver of CO-INFECT vs. LEISH mice compared with the wild-type (WT, C57BL/6) mice. However, the μMT mice were also much less susceptible to infection with *L. donovani* alone than the WT mice so it is difficult to interpret the significance of these results. Administration of anti-IL-10 receptor antibody

(anti-IL-10R) between weeks 6-8 after super-infection reduced the high *L. donovani* LDU in the CO-INFECT mice but the significance of this was uncertain since anti-IL-10R treated LEISH mice also had lower LDUs.

Morphologically mature *L. donovani* granulomas are associated with leishmanicidal activity. Histology and immuno-histology showed an increasing proportion of morphologically mature granulomas in the hepatic parenchyma of LEISH mice as the infection resolved (i.e. between weeks +2 and +8) but in the CO-INFECT mice this proportion did not change. Higher mean amastigote numbers in *L. donovani* granulomas in the parenchyma of CO-INFECT mice suggested reduced leishmanicidal activity. Throughout the time course 60% of the foci of *L. donovani* infection were seen in various locations within the egg granulomatous response, most commonly in a ring around the outside of the granulomas but occasionally within giant cells in egg shells at the centre of the granulomas. Typical hepatic *L. donovani* granulomas did not form around such foci and the amastigotes appeared partly contained.

The data suggests that in LEISH mice mature granulomas effectively kill the contained amastigotes limiting new infections so that by +8 weeks the granulomas are all mature or fully resolved. In contrast although morphologically normal mature *L. donovani* granulomas form in the parenchyma of CO-INFECT mice these show reduced leishmanicidal activity resulting in spread and establishment of new infections. It is concluded that raised Th2 responses to *S. mansoni* results in lowered *Leishmania* specific IFN-γ responses and raised levels of IL-10, both of which would reduce Th1 mediated macrophage activation required for killing of *L. donovani* amastigotes. The effects of IL-10 plus the failure of *Leishmania* granulomas to develop around foci within the egg granulomas suggests very poor leishmanicidal activity in such foci.

The final section of these studies was concerned with the effects of an established *L. donovani* infection on a *S. mansoni* infection superimposed 2 weeks later. At 8 weeks post super-infection, there was no effect on the schistosome worm or egg burdens or the immunological and pathological response to the eggs i.e. a strong Th2 response still developed. However, lower *L. donovani* LDUs were seen in the CO-INFECT mice than in the LEISH alone and it is suggested that the early phase of the schistosome infection which is characterised by Th1 responses may have promoted the protective anti-leishmanial response.

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ABBREVIATIONS

M	Molar
Mm	Millimolar
cm	Centimetre
mm	Millimetre
nm	Nanometre
mg	Milligram
ng	Nanogram
µg	Microgram
L	Litre
ml	Millilitre
µl	Microlitre
w/v	Wave length
s.c	Subcutaneously
i.p	Intraperitoneally
i.n.	Intranasally
h	Hour
x	Times
p.i.	Post-infection
+d	Superinfection in days
+wks	Superinfection in weeks
spp	Species
co2	Carbon dioxide
SD	Standard deviation
SE	Standard error
ELISA	Enzyme linked immuno-sorbent assay
PCR	Polymerase chain reaction
mRNA	Messenger ribonucleic acid
DNA	Deoxyribonucleic acid
ICAM-1	Intracellular adhesion molecule
PGE2	Prostaglandin E2
PGD2	Prostaglandin D2

IFN-γ	Interferon gamma
IFN-α/β	Interferon alpha/beta
IL-	Interleukin-
Gm-CSF	Granulocyte-macrophage stimulating factor
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-α
TNFR1	Tumour necrosis factor-α receptor I
TNFRII	Tumour necrosis factor-α receptor II
iNOS	Inducible nitric oxide synthase
NO	Nitric oxide
ROI	Reactive oxygen intermediates
COX	Cyclooxygenase
cAMP	Cyclic adenosine monophosphate
PKC	Protein kinase C
kD	Kilodalton
PHA	Phytohaemagglutinin
PPD	Protein purified protein
BCG	Bacille Calmette Guerin
TT	Tetanus Toxoid
CTB	Cholera toxin β subunit
MOG	Myelin oligodendrocyte glycoprotein
ConA	Concanavalin A
SEA	Soluble egg antigen
SWAP	Soluble worm antigen preparation
FLAA	Formalin fixed Leishmania amastigote antigen
Ags	Antigens
LNFPIII	Lacto-*N*-fucopentose III (schistosome egg glycoconjugate)
LNT	Lacto-*N*-neotetraose (schistosome egg glycoconjugate)
CTLA4	Cytotoxic T lymphocyte-associated antigen 4
GST28	glutathione S-transferase
Sm-p40	Schistosoma mansoni-protein 40 (epitope)
Pa	Proprionibacterium acnes

IgG	Immunoglobulin G
IgE	Immunoglobulin E
Abs	Antibodies
mAb	Monoclonal antibody
Rat IgG	Rat-immunoglobin G
Anti-IL-10R	Anti-interleukin 10 receptor monoclonal antibody
Anti-LV9	Hamster anti-leishmania donovani-strain 9
TLR	Toll-like receptor
aaMø	Alternatively activated macrophages
CaMØ	Classically activated macrophages
DC	Dendritic cells
APC	Antigen presenting cells
GM	Granuloma macrophage
Grn	Granuloma
KCs	Küpffer cells
Tr	T regulatory cells
Th1	T helper cell-type1
Th2	T helper cell-type2
Th3	T helper cell-type3
CTLs	Cytotoxic T cells
γ/δ T cells	Gamma delta T cells
CMI	Cell mediated immunity
MLN	Mesenteric Lymph node
TCC	T cell clone
ADCC	Antibody dependent cytotoxic cells
LDU	Leishman Donovan Units
U/L	Unit/Litre
ALT	Alanine Aminotransaminase
AST	Aspartate aminotransaminase
SM2	major codominant gene responsible for familial hepatic fibrosis
Ym1/ECF	Eosinophil chemotactic factor
MHC-II	Major histocompatability-type 2

TCR	T cell receptor
FCγR	Fc gamma receptor
MR	Macrophage mannose receptor
FOXP3	Treg transcription factor forkhead box P3
STAT4	Signal transduction and activator of transcription 4-
STAT6	Signal transduction and activator of transcription 6-
SCID	Severe combined immuno-deficient mice
RAG	Recombination activated genes knockouts (lacking B and T cells)
μMT	B-cell deficient mice
T	Transgenic mice
KO	Knock out mice
BDC2.5	Genetically predisposed non-obese diabetic mice
NOD	Non-obese diabetic mice
Nramp1	Natural resistant associated protein 1
INT	Intestinal form of schistosomiasis
HS	Hepatosplenic form of schistosomiasis
SCHISTO	*Schistosoma mansoni* infected mice
CO-INFECT	Co-infected mice
LEISH	*Leishmania donovani* infected mice
Ld	*Leishmania donovani*
VL	Visceral leishmaniasis
PBMC	Peripheral blood mononuclear culture
HIV	Human immunodeficiency virus
HCV	Hepatitis C virus
HBV	Hepatitis B virus
GBV-C/ HGV	GB virus type C /Hepatitis G virus
LPS	Lipopolysaccharide
NS	Non specific

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Chapter 1

INTRODUCTION

1.1 Introduction to *Schistosoma mansoni*:

1.1.1 General Introduction:

Schistosomiasis is considered one of the most important public health problems in the tropics and subtropics, affecting 4% of the world's population, an estimated 200 million people in 74 countries (WHO, 1985; Doumenge and Mott, 1984; Bergquist, 2002). The most affected areas are: Africa and the Middle East especially the Nile delta (Egypt); parts of the Far East and South-East Asia; and in the New World, notably the Amazon Basin. For example, a total of 2.5 million individuals are estimated to be infected by *Schistosoma mansoni* in Brazil despite years of attempted control (Passos and Amaral, 1998). Globally, schistosomiasis is recognised as being the most important human helminth infection, ranking second to malaria among parasitic causes of human ill-health. The estimated mortality due to *Schistosoma mansoni* and *Schistosoma haematobium* in sub-Saharan Africa is 280,000 per year (van der Werf *et al.,* 2003). Schistosomiasis is an important issue for travel medicine with an increased incidence of prolonged unexplained pyrexia among tourists travelling to endemic areas thought to be due to acute Katayama fever (van Lieshout *et al.,* 1997; Elliott, 1996). The infection is transmitted by water snails and surface waters such as irrigation canals, rivers, streams, ponds or lakes are the usual source of infection but water piped or carried from rural areas to towns may be a source of infection too.

The outcome of schistosome infection is influenced by many factors, such as the species of infecting parasite (there are three major human species, *S. mansoni*, *S. haematobium* and *S. japonicum*), the intensity and duration of infection, and genetic makeup of the human host. In many endemic areas, the prevalence of infection is often very high among young individuals but even so, most infections are light, and only in the case of a heavily infected minority does significant disease occur (Salam *et al.*, 1979; Kojima *et al.,* 1984; Kamel *et al.,* 1984; Ohta *et al.,* 1990; Hafez *et al.,* 1991; Marquet *et al.,* 1996; Abel *et al.,* 2000).

Schistosomiasis is curable but, if left untreated, can persist for 30 years even though the parasite cannot reproduce in the human host (Lucey and Maguire, 1993). In longstanding *S. mansoni* and *S. japonicum* infections constant egg deposition causes extensive liver fibrosis and may give rise to irreversible calcification of the portal vessels, leading to increased portal blood pressure and periportal fibrosis (Symmer's clay pipe-stem fibrosis). However, fibrosis and collateral circulation development may progress insidiously and fatal haematemesis may occur without warning. Also some patients develop liver failure, perhaps caused by concomitant infection with hepatitis viruses (Dean *et al.,* 1987). Chronic *S. haematobium* infections cause fibrosis and dysfunction of the bladder and urinary tract. The infection has also been associated with bladder cancer mostly in endemic areas (Dean *et al.,* 1987; Wilkins *et al.,* 1987; Farid *et al.,* 1990) and *S. haematobium* has now been officially classified as a carcinogen (Vainio and Kleihues, 1994). Schistosomiasis has serious health consequences ranging from reversible growth faltering, permanent growth retardation, clinically overt symptoms (e.g. nausea, diarrhoea, dysentery, and fever). Malnutrition caused by loss of appetite, nutritional loss, malabsorption, and decreased nutrient utilization also occur (Crompton *et al.,* 2000; Stephenson *et al.,* 2000; O'Lorcain *et al.,* 2000). In severe intense infection, schistosomiasis can lead to a decrease in working capacity (Parker, 1992; 1993), and there is increasing evidence that *S. japonicum* (McGarvey *et al.,* 1993), *S. haematobium* (Stephenson *et al.,* 1986; 1989) and *S. mansoni* (Jordan and Randall. 1962; Costa *et al.*, 1988; Corbett *et al.,* 1992) can each adversely affect child growth and nutritional status. It has also been reported (Kimura *et al.*, 1992) that *S. haematobium* infection depresses cognitive function in children.

There is at present only one widely available schistosomicide, praziquantel. This is safe, effective and now relatively cheap. However, there are concerns about the possible development of drug resistance. Recently, Cioli *et al.* (2000) demonstrated that schistosomiasis cure rates using praziquantel are worse than those a decade ago and other treatment failures have been reported (Ismail *et al.*, 1994a; 1994b; 1999). Also praziquantel-resistant strains of *S. mansoni* have been generated experimentally (Fallon and Doenhoff, 1994) although this does not appear to be widespread as yet in the field.

Given its distribution in the developing world, which is burdened by many other disease agents, schistosome infection is frequently found together with other infectious organisms,

viruses, bacteria, protozoa and other worms. The significance of the interplay between these different organisms has only recently been appreciated and attention focussed on the interplay between microparasites, which tend to induce Th1 type responses, and the worms which tend to induce Th2 responses (Scott, 1991a). The influence that the immune response induced by infectious agents may have on the Th2-dominated allergic reactions including asthma is also the subject of great interest (Yazdanbakhsh *et al.*, 2001)

1.1.2 Life cycle:

Adult schistosomes are about 1cm long and the male has a deep ventral groove or *'Schist'* in which the female worm resides *in copulo*. The adult parasites reside in the smaller venules of the gastrointestinal or genitourinary tract where they ingest red blood cells and break down haemoglobin enzymatically to obtain amino acids for protein synthesis but elicit no significant pathology. The female deposits eggs in the smallest venules of the intestine or the bladder wall, near the superficial layer of the mucosa. The eggs mature after about one week in the tissues to contain the large ciliated 'miracidium' larvae, which infect the snail host.

Eggs are the main cause of clinical disease (Warren, 1978) and when trapped in tissue elicit a marked inflammatory reaction with both mononuclear and polymorphonuclear cellular infiltrates and formation of a microabscess or granuloma. Granulomas in the gut or bladder wall usually rupture into the lumen allowing the eggs to be excreted in faeces or urine. The eggs move through the tissues perhaps aided by lytic enzymes which are secreted by the miracidium and diffuse through the rough microspores in the egg shell. After reaching fresh water, eggs hatch and the swimming miracidium larvae must find an appropriate freshwater snail to act as intermediate host. In the snail, they divide asexually into cercariae which are released into the water. If they contact susceptible hosts, they actively penetrate through the skin losing their tails and becoming schistosomula which undergo morphological and biochemical alterations of their membrane. After 2-4 days in the skin, schistosomula penetrate into blood and lymphatic vessels and reach the lungs. Crossing the capillary bed into the systemic circulation they eventually reach the liver and develop further into male and female worms before pairing and migrating to the mesenteric or vesical veins (Jordan and Webbe, 1993).

1.1.3 Pathology

A proportion of the eggs pass through the wall of the intestine or bladder and so find their way into the environment to complete the life cycle but a significant proportion are retained in the intestine or bladder wall or in the case of *S. mansoni*, the species used in this study, are swept by the blood stream to the liver. Here leakage of soluble egg antigens from microspores in the egg shell stimulate a host response to contain the organism but this results in immuno-pathology notably in longstanding infections with constant egg deposition. Extensive fibrosis occurs and may give rise to irreversible calcification of the portal vessels, with major complications being upper digestive tract haemorrhage secondary to portal hypertension (Bina, 1987; Prata, 1982; Andrade and Azevedo, 1987; Raia *et al.*, 1994; Strickland, 1994). The immuno-pathological basis of the schistosome granuloma will be considered below in the section on murine schistosomiasis (1.1.9).

1.1.4 The general immune response, schistosome development, and immune evasion.

All stages of schistosome infection induce varying degrees of immune response, however, the schistosome eggs induces the strongest response. In the course of an infection, the immune response progresses through at least three phases (Pearce and Macdonald, 2002). In the first phase (at 3-5 weeks), during which the host is exposed to migrating immature parasites, the dominant response is T helper 1 (Th1)-like. In phase two the schistosome worms mature, mate and begin to produce eggs at weeks 5-6 (300 to 5000 eggs per day for *S. mansoni*, and *S. haematobium* respectively) and these result in the emergence of a strong Th2 response with a parallel decrease of the Th1 response. During the chronic phase of infection (at 8-16 weeks) the Th2 response is modulated and granulomas that form around newly deposited eggs are smaller than at earlier times during infection.

The adult worms survive for many years in the blood stream in spite of strong humoral and cell-mediated immune responses induced by the infected host to adult worm antigens. Within minutes of penetrating the skin of the host, a series of adaptations are initiated which enable the developing schistosomula to survive attack first by components of the innate immune system and then by components of acquired immunity. The trilaminate plasma membrane of the cercariae loses its outer glycocalyx coating followed by the formation of a double outer plasma membrane which appears heptalaminate (from the two

apposed trilaminate plasma membrane plus the space between them). This is due to the deployment of preformed membranous bodies at the larval surface and these persist in the adult tegument allowing constant repair (Smithers *et al.,* 1977; Ramalho-Pinto *et al.,* 1978). Complement activation and action is inhibited by anti-complement factors notably decay accelerating factor (DAF) which is derived from the cells of the host and which inhibits C3 deposition at the surface and SCIP-1 (schistosome C-inhibitory protein), which is expressed by the worms and which inhibits the formation of the membrane attack complex (Pearce and Sher, 1987). The uptake of cholesterol and triglycerides increases membrane rigidity and the surface acquisition of host-derived antigens is believed to cover the foreign antigens of the worm, so preventing antibody binding to the worm surface. These host antigens include blood group glycolipids, MHC glycoprotein, fibronectin and immunoglobulin (Smithers *et al.,* 1969).

The intrinsic tegumental resistance to attack varies between the different parasite stages. So the skin stage schistosomula are highly susceptible to membrane attack and killing *in vitro* by eosinophil degranulation and the adults are also susceptible (Racoosin *et al.,* 1999; Pearce and MacDonald, 1986) but the lung stage is highly resistant to this attack (Bickle and Ford, 1982). Even if the lung stage parasite is made antigenic by conjugation of the hapten TNP (trinitrophenyl) to its surface and incubated in anti-TNP antibody plus eosinophils, these will degranulate onto the larval surface but the larvae are completely unaffected by the toxic products released (Bickle and Ford, 1982; Moser *et al.,* 1980).

Other immune evasion strategies are directed at subverting the immune response. Schistosomula have been shown to produce prostaglandin D2 (PGD2) which inhibits the migration of epidermal antigen-presenting Langerhans cells from the site of infection (Angeli *et al.*, 2001), thereby downregulating the innate response leading to Th1 activation and inflammation which might otherwise interfere with larval migration. Significant quantities of PGE(2) were also produced by cercariae of *Schistosoma mansoni* following incubation with linoleic acid. Cyclooxygenase (COX) 2 inhibitors failed to block this PGE(2) production, suggesting that a different biochemical pathway may be involved in the production of PGE(2) by the parasite. In addition, the parasites were also able to induce PGE(2) and IL-10 from human and mouse keratinocytes. COX2 inhibitors blocked the parasite-induced PGE(2) and IL-10 from keratinocytes. This effect could be blocked by

anti-IL-10 treatment. The significance of IL-10 in host immuno-regulation by skin stage schistosomula of *S. mansoni* was further confirmed by using IL-10-deficient mice. Compared with the wild type (WT) mice, a prominent cellular reaction occurred around the parasites, and there was considerable delay in parasitic migration through the skin. Thus these results suggest a key role for parasite-induced PGE(2) in IL-10-dependent down-regulation of host immune responses in the skin. (Ramaswamy *et al.*, 2000).

Not only is the worm able to inhibit immune activation but it also seems to have evolved to respond to immune components of the host. Certain studies have provided evidence that TNF-alpha induces worm fecundity and initiates granuloma formation around the parasites eggs (Amiri *et al.*, 1992; Cheever *et al.*, 1999; Davies and McKerrow, 2001). Davies *et al.* (2001) reported that parasite development was delayed in $RAG^{-/-}$ mice (which lack both T cell and B cells) and detailed analyses have shown that a previously unrecognised subset of CD4+ T cells lacking both MHC class I and II molecules and localized primarily within the liver is likely to have an important in promoting schistosome maturation. It is possible that the hepatic T cells produce, or are dependent on, IL-7, because the phenotype that is described for schistosomes in $Rag^{-/-}$ mice is similar to that described for parasites that grow in IL-$7^{-/-}$ mice (Wolowczuk *et al.*, 1999). A recent study Beall and Pearce, (2001), demonstrated that schistosomes express a receptor, SmRK1 (*S. mansoni* receptor kinase-1), on their surface that can bind the cytokine TGF-β, which indicate that host cytokines can have effects on these parasites.

1.1.5. **Human immunity**

In areas where schistosomiasis is endemic, there is an obvious pattern of age-dependent intensity of infection; individuals who are below the age of puberty carry most of the parasites, and those in older age brackets are generally less heavily infected (Butterworth *et al.*, 1994). For many years the high intensity in children was attributed to their increased water contact (Warren, 1973) but studies of reinfection following chemotherapy in which rates of water contact were monitored reached a different conclusion. Such studies on *S. mansoni* in Kenya (Butterworth *et al.*, 1984; 1985) identified a group of children classed as "resistant" because they had high water contact but low rates of reinfection and a group of susceptible children which become heavily reinfected following treatment. Comparison of the data for these two cohorts demonstrated that the average age of the resistant group was 13 and

that of the susceptible group 11 which indicated the operation of age-dependent factors that prevent reinfection. Demeure *et al.* (1993) took a similar approach and concluded that although water contact was a major factor in intensity of infection it, alone, could not explain the patterns adequately.

More recent studies of populations in which the infection had been recently introduced (reviewed by Gryseels, 1994) demonstrate that similar age-intensity profiles develop relatively soon after the start of community exposure indicating that it is age *per se* more than duration of exposure that is responsible for the lower infection intensities in the adults (Polman *et al.*, 1995; van Dam *et al.*, 1996). Fulford *et al.* (1996) looked at data from a number of studies and showed that the peak in intensity of re-infection occurred at a very consistent age followed by a sharp decline. The data on water contact for several areas showed generally higher contact for the younger age groups but the distribution was much more spread and clearly did not mirror the age intensity pattern. Furthermore, in a study of a fishing community in Uganda, the pattern of observed contact with water known to contain infected snails was greater in adults than in children (Fulford *et al.,* 1996). Despite this, six months after treatment, the Ugandan community showed a peak of reinfection intensity at about ten years of age, which fell away to a much lower level by the age of twenty (Kabateriene *et al.*, 1999). As infection levels begin to decline around the early teen years, it has been considered that this may be due to anatomical and physiological changes associated with puberty e.g. increased skin thickness or fat deposition which may affect skin penetration, or to hormonal changes associated with the onset of puberty (e.g. adrenal androgen; dihydroepiandrostrone [DHEA]) (Fallon *et al.,* 1998; Abebe *et al.,* 2003).

It should be appreciated that these various studies on human immunity have looked at reinfection after treatment and the finding that multiple reinfection episodes (and cures) correspond with increased time to subsequent reinfections for some people led to the hypothesis that the treatment itself and the consequent worm death and antigen release may result in priming and boosting of the immune response to resist reinfection (Woolhouse and Hagan, 1999).

Hagan *et al.* (1991) provided the first insight into the possible immune mechanisms responsible for the human immunity. They found a correlation between high specific anti-

worm immunoglobulin E (IgE) antibody levels which increased up to the teenage years and apparent resistance to reinfection following chemotherapy but there was also a correlation with lowered specific anti-worm IgG4 which also increased up to early teenage and then declined. The positive influence of specific IgE and the negative reciprocal effect of IgG4 in immunity to *S. mansoni* reinfection have also been reported in several independent investigations carried out in Africa and Brazil (Dunne *et al.*, 1992a and 1992b; Demeure *et al.*, 1993; Rihet *et al.*, 1991; Caldas *et al.*, 2000). The slow development of appropriate immune responses to worm antigens might be linked to the fact that schistosomes are very long-lived-parasites and the host becomes exposed to these antigens only after the parasites die (Woolhouse and Hagan, 1999), either as a result of ageing or drug intervention. The implication of high IgE and lowered IgG4 led to the notion that IgE was an effector isotype and IgG4 a blocking isotype. Despite the current acceptance that immunity does operate in humans Mwanakasale *et al.* (2003) demonstrated that resistance to reinfection with *S. haematobium* after treatment with praziquantel is not altered in subjects co-infected with HIV but this was prior to the development of symptoms and signs of AIDS/HIV disease and so the level of immuno-compromise was not severe.

Other studies have implicated eosinophils in human immunity. Hagan *et al.* (1985) reported that resistance to *S. haematobium* in humans was related to high eosinophil counts. A longitudinal study of cellular responses of *S. mansoni*-infected individuals in Kenya before and after treatment showed a negative association between the proliferative responses to adult worm- and schistosomulum-stage antigens and subsequent reinfection intensity in older individuals (the resistant group), who also had significantly higher levels of IL-5 in tissue culture supernatants of *in vitro*-stimulated cells than did younger individuals (the susceptible group) (Roberts *et al.*, 1993). IL-5 is produced by Th2 cells and as it is responsible for eosinophil production/action this also suggested a role for eosinophils in human immunity *in vivo*.

So IgE and eosinophils have been implicated but it is not known if or how this might operate *in vivo*. *In vitro*, however, human effector cells, eosinophils, macrophages and platelets are able to kill freshly transformed schistosomula in the presence of complement and/or antibody (Butterworth *et al.*, 1975; Joseph *et al.*, 1985). Regarding antibody-mediated cellular cytoxicity both the IgG (Anwar *et al.*, 1979) and IgE (Capron *et al.*, 1984) fractions

of human infection sera have been shown to be capable of opsonising the schistosomula for killing but IgE/eosinophils is a particularly potent combination (Dunne *et al.*, 1993).

The ability of antibodies to block *in vitro* ADCC was initially described by Grzych *et al.*, (1982, 1984) using rat monoclonal antibodies. Mouse monoclonal antibodies of the IgM isotype directed at larval surface antigens were later shown to block *in vitro* killing of schistosomula by immune human serum and eosinophils (Dunne *et al.*, 1987). Evidence that blocking antibodies are raised during a natural infection of humans was provided by Khalife *et al*. (1986) who demonstrated that the *in vitro* killing of schistosomula by the IgG fraction of sera taken from infected individuals was abolished by prior incubation of the larvae with the IgM fraction which unlike IgG does not have Fc receptors on eosinophils but can compete with the IgG for opsonization of the larvae. In regard to the IgE/IgG4 relation with human immunity it is suggested that IgG4 which also does not have Fc receptors on eosinophils may similarly block IgE mediated larval killing (Nutten *et al.*, 1997; Capron *et al.*, 2001).

The above implication of specific IgE and eosinophils suggested the importance of Th2 responses in human immunity (Hagan *et al.*, 1991; Roberts *et al.*, 1993; Grogan *et al.*, 1997; Medhat *et al.*, 1998). Supporting evidence comes from a number of studies. Karanja *et al.* (2002) demonstrated an increased risk of reinfection among HIV-1 positive individuals with decreased CD4+ T cell count and a marked decrease in levels of Th2 immune response in HIV-1 positive schistosomiasis patients. Immuno-epidemiological studies in Brazil demonstrated that the increased intensity of infection is influenced by a major gene (Sm1) that maps to a region of chromosome 5 (5q31-q33) that encodes the Th2 cytokines (Marquet *et al.*, 1996). Cells from homozygous individuals for the resistant allele generated Th2 skewed T-cell clones (TCC) which secreted 10-1000 fold more IL-4 and IL-5 and a much higher IL-4/IFN or IL-5/IFN ratio than TCC from homozygous sensitive individuals when stimulated with parasite antigens (Rodrigues *et al.*, 1999). This indicates that the S1 locus controls the differentiation of Th2 lymphocytes and further implicates Th2 mediated effector mechanisms in resistance to schistosomes.

1.1.6 **Evidence for resistance to reinfection in experimental animals–"concomitant immunity".**

There are no good animal models for the slow build up of infection which occurs in humans, but rodents, such as mice and rats and, to a lesser extent, primates such as the rhesus monkey and the baboon have all been used in experimental infections with *S. mansoni*. Studies in rhesus monkeys (Smithers and Terry, 1969) showed that primary light infections could be established giving rise to low level egg production in the faeces, but that following challenge infection with large numbers of cercariae, egg output was unaltered although in challenge control monkeys faecal output rose dramatically. This was termed "concomitant immunity" as the primary worm burden was apparently unaffected by the response which eliminated the challenge infection. It has been suggested that a similar phenomenon may operate in naturally acquired immunity in humans. Unfortunately nothing is known of the mechanism of the resistance in rhesus monkeys.

In mice, resistance to challenge arises within 8 weeks of a single unattenuated infection (Dean, 1983) and resembles concomitant immunity in that the primary infection persists but the incoming larvae appear to succumb. However, it now accepted that the apparent resistance seen in this model is due to the pathological response of the host to parasite eggs deposited within the tissues (Wilson *et al.*, 1983; McHugh *et al.*, 1987) or with factors related to egg-associated pathology, such as the degree of portal hypertension (Harrison *et al.*, 1982) or formation of portal shunts which allow the migrating larvae to bypass the liver and die in other organs (Wilson *et al.*, 1983). This has limited the use of the mouse to mimic human immunity despite the widespread use of mice for most schistosome research.

1.1.7 Prevention and control

The most practical way of controlling schistosomiasis at present is through eradication of the adult worms using chemotherapy; eliminating the snail intermediate hosts by habitat modification or chemical attack; by changing human behaviour through health education and by providing safe water supplies and sanitation so that excreta containing live eggs do not reach water containing snails (Webbe and Jordan, 1993). There are problems with all of these approaches notably rapid re-infection after treatment and recurrent costs of chemotherapy and the costs provision and maintenance of sanitation and safe water.

1.1.8 Prospects for vaccination

There has been intensive research effort to develop a schistosome vaccine (Bergquist, 1995). High level of resistance can be induced in a range of experimental hosts ranging from rodents to baboons by exposure to relatively large number of schistosome larvae which have been irradiated with γ-, X- or ultra-violet- radiation in order to make them die out before the egg-laying adult stage (Taylor, 1994). The live attenuated vaccines are not suitable for human use because of the logistical difficulties of producing and preserving such a vaccine and for safety reasons. However, analysis of the immunity they induce may contribute to development of defined antigen vaccines although it is unknown if the immunity induced is in any way comparable to naturally acquired resistance in man.

The mechanism of live attenuated vaccine immunity in mice and rats operates during larval migration through the lungs where there is an accelerated and enhanced cellular reaction around the migrating lung schistosomula which seems to trap the parasites in immune animals (Coulson, 1997). In rats this reaction can be transferred with antibody but in the .protection is dependent on CD4+ve T cells (Vignali *et al.,* 1989) and IFN-γ (Smythies et al., 1992; 1999). Consistent with this Th1 requirement following single vaccination, IL-12 and bacterial CpG motifs (which can induce the production of IL-12 by dendritic cells and macrophages) can be used as adjuvants to boost attenuated vaccine immunity (Wynn *et al.*, 1996; Chiaramonte *et al.,* 2000). However, when mice are repeatedly exposed to attenuated larvae Th2 cytokines increase (Caulada-Benedetti *et al.,* 1991), the requirement for CD4 +ve Tcells disappears (Kelly and Colley, 1988) and antibody assumes a major role as shown by the ability of the serum to transfer protection (Mangold and Dean, 1992). In the once vaccinated mouse the macrophages in the inflammatory lung reactions are in the activated state and show NO synthase activity but immunity is not dependent on NO production as iNOS-knockout mice show comparable protection to wild-type mice (Coulson *et al.,* 1998). Vaccinated IL-$10^{-/-}$ mice develop exaggerated Th1 and Th2 responses and are almost entirely resistant to infection (Hoffmann *et al.,* 1999), which indicates that a high-magnitude immune response involving both antibody and Th1 responses might be the best option for induced resistance to schistosome infection.

With the advent of recombinant DNA technology numerous schistosome protein genes have been cloned. Possible candidates for recombinant antigen vaccines includes: (i) Larval

surface antigens which might mediate ADCC or (ii) antigens secreted from the lung stage larvae which might mediate antibody or T cell mediated inflammatory reactions in the lung as seen with the irradiated vaccine. Numerous recombinant antigens representing both soluble and larval surface antigens have been tested and some reported to induce protection but none have proved reliable in independent testing (Bergquist, 1998). Only one such antigen, the 28kDa glutathione S-transferase of *S. haematobium* has entered phase 1 human clinical trials (BILVAX rP28), and the main protective effect of this vaccine may be to reduce egg production and thus reducing the pathology (anti-pathology vaccine) (Hagan *et al.*, 2000).

1.1.9 The Murine model of *S. mansoni* infection

The murine response to *S. mansoni* mimics what happens in man in terms of the full development of the infection and the pathological conditions. The egg stage is the most immunogenic and immuno-dominant phase of the infection and the immune response to the eggs causes the pathology in *S. mansoni* infection and involves granulomatous reactions around the eggs in the liver and gut (Pearce *et al.*, 1991; Grzych *et al.*, 1991). By contrast, the worms themselves do not induce significant pathology. At the 5th wk post-infection, the mature female worms begin to produce large numbers of eggs many of which lodge in the liver and intestinal tissue. The nature of the granulomatous response has been studied in the livers of infected mice and also in the "synchronous pulmonary granuloma" model (Wynn *et al.*, 1993; 1997) in which eggs which had been purified are injected into the lungs of recipient mice.

In both mice and humans the granuloma is composed of lymphocytes, macrophages, epithelioid cells, giant cells, eosinophils and fibroblasts although the relative proportion of these cell types changes as the miracidium dies and the granuloma ages such that eventually the empty egg shell surrounded by a small fibrotic reaction is left (Dunne and Pearce, 1999; Davies and McKerrow, 2001). During the course of infection of mice granulomas reach a maximum size at 8-10 weeks post-infection but are then markedly modulated or reduced in size by 12 weeks and thereafter (von Lichtenberg *et al.*, 1962, Boros *et al.*, 1975).

The granulomatous reaction is considered to be a T-cell mediated immune response (Domingo and Warren, 1968, Boros *et al.*, 1975), as evidenced by the suppression of

hepatic granuloma formation in anti-CD4 treated mice (Mathew and Boros, 1987; Phillips *et al.*, 1987) and the formation of small granulomas in athymic mice (Phillips *et al.*, 1977). In humans, a study of CD4+ T cell-deficient humans (HIV co-infected) also suggested that CD4+ve T cells are involved in granuloma formation Karanja *et al.* (1997). Thus it had previously been shown that T-cell deprived mice showed reduced faecal egg output and it was reasoned that the granuloma formation was necessary for this excretion (Doenhoff *et al.*, 1981; Cheever *et al.*, 1999). Similarly the HIV and schistosome co-infected individuals had a significant defect in egg excretion in comparison to HIV negative, schistosome positive individuals suggesting that they also had impaired granuloma formation (Karanja *et al.*, 1997). In T-cell deprived mice (Dunne and Doenhoff, 1983) or mice which are tolerized against *S. mansoni* egg antigen, granuloma development does not occur during infection and the animals have severe hepatotoxic liver damage, which was evident as microvesicular lipid accumulations (or steatoses) with hepatocytes (Fallon and Dunne, 1999). This is thought to be mediated by hepatotoxins that are secreted from the eggs, and the granulomas, together with egg-antigen-specific antibodies which might act in a neutralizing capacity, are envisaged as sequestering these toxins away from hepatocytes (Dunne and Doenhoff, 1983). So, the granulomatous response around schistosome ova is seen as a host protective tissue response which plays an integral role in host defence against this parasite eggs (von Lichtenberg, 1964, Dunne *et al.*, 1981) and facilitates their destruction (Warren, 1978). From the parasite perspective the granuloma seems to facilitates egg excretion and so represents a balanced response of some benefit to both the host and the parasite

1.1.9.1. The cytokine response

A number of studies in mice have demonstrated that down-regulation of the production of Th1 type cytokines e.g. IFN-γ and IL-2 and an up-regulation in Th2 cytokines such as IL-4, IL-5 and IL-10 (although not exclusively a Th2 cytokine), occurs at the onset of egg production (Pearce *et al.*, 1991; Sher *et al.*, 1991; Grzych *et al.*, 1991). A Th2 biased response similarly develops in human infections (Parra *et al.*, 1992; Williams *et al.*, 1994; El Ridi *et al.*, 1997), although Montenegro *et al.* (1999) reported that in chronically infected individuals a mixed type-1/type-2 anti-egg cytokine response can be observed.

That the Th2 response is central to granuloma formation is shown by numerous studies. IL-4 is crucial for the development of Th2 responses and for the counter-regulation of Th1

responses. Thus IL-4 depletion with anti-IL-4 monoclonal antibody in schistosome-infected mice or intravenously egg-injected mice reduced egg-induced granuloma inflammation by 50%, reduced the production of the type-2 cytokines IL-5 and IL-13 and reduced fibrosis (Yamashita and Boros, 1992; Wynn *et al.*, 1993; Cheever *et al.*, 1994). Conversely, administration of recombinant IL-4 to chronically infected animals reversed the down-regulated granulomatous response typically observed in later stages of infection (Yamashita and Boros, 1992). These studies show that IL-4 is, at least in part, responsible for the generation and maintenance of the type-2 immune responses.

Various studies show, however, that IL-13 also plays a role in granuloma formation. Granuloma size is diminished in IL-$4^{-/-}$ mice but not as much as in STAT6 [signal transduction and activator of transcription 6-] deficient mice (Kaplan *et al.,* 1998). Such infected mice, which lack Th2 cells as STAT-6 is responsible for signalling by both IL-4 and IL-13, produced high levels of the Th1 cytokines, IFN-γ and IL-2, minimal IL-4 and greatly reduced IL-5 and IL-10. Furthermore, IL-4/IL-13 double knockout (KO) mice barely develop granulomas at all (McKenzie *et al.*, 1999). The contribution of IL-13 to granuloma formation was confirmed by conducting IL-13 blocking experiments using the soluble decoy receptor, IL-13Rα2, in IL-4-deficient mice, which resulted in a more significant (almost complete) abrogation of granuloma formation than in the IL-4- deficient mice alone (Chiaramonte *et al.,* 1999a). These findings demonstrate that IL-4 and IL-13 are both necessary and sufficient to mediate granuloma development, and formally explain the maintenance of granulomatous inflammation in infected IL-4-deficient mice.

Although Th2 cytokines predominate, both Th2 and Th1 cells are involved in the formation of granuloma as discussed below (section 1.1.11).

1.1.9.2. Production of profibrogenic cytokines, IL-4 and IL-13

Several studies have now demonstrated that IL-13 rather than IL-4, plays the major role in the development of egg-induced liver fibrosis. In a study by Fallon *et al.* (2000) IL-13 KO mice did not show a reduced granulomatous response but did show a dramatic reduction in collagen deposition whereas this was unaffected in IL-$4^{-/-}$ mice but was ablated along with granuloma formation in IL-4/IL-13 KO (Fallon *et al.*, 1999). Chiaramonte *et al.* (2001) reported that administration of the IL-13 decoy receptor, IL-13Rα2-Fc protein, to infected

mice led to significantly reduced collagen mRNA expression and so this could become a useful therapy for fibrotic disease. This receptor, which is present in a soluble form, will bind IL-13, preventing its binding to IL-4Rα/IL-13Rα1 which is the functional IL-13 receptor responsible for signal transduction leading to fibrosis. The level of soluble serum IL-13Rα was recently demonstrated to correlate with infection intensity in infected humans which suggested that it may have important anti-pathology effects *in vivo* (Mentink-Kane *et al.*, 2004).

The fibrogenic role of interleukin-13 (IL-13) seems to stem from its ability, together with IL-4, to induce the expression of arginase which has been demonstrated in macrophages (Hesse *et al.*, 2001). Arginase uses L-arginine as a substrate to make L-ornithine, which is converted to proline by ornithine aminotransferase. Proline is an essential amino acid that is involved in collagen production and therefore, in the development of fibrosis. Arginase activity is abundant within the *S. mansoni* egg granulomas (Hesse *et al.*, 2001). It was initially suggested that the granuloma macrophages were likely to be responsible for the collagen production and fibrosis in schistosomiasis but recent studies have shown that mice with a selective deletion of IL-4Rα expression in macrophages (and neutrophils) display normal levels of liver fibrosis (Herbert *et al.*, 2004). This led these authors to suggest that the likely source are the abundant granuloma fibroblasts. These have receptors for IL-13 (Murata *et al.*, 1998) and both IL-13 and IL-4 are capable of promoting collagen production in fibroblasts (Chiaramonte *et al.*, 1999; Murata *et al.*, 1999). However, the fact that cultured lymph node cells from *S. mansoni* infected mice produce nearly 100-fold more IL-13 than IL-4 would explain the greater role that IL-13 has in schistosome fibrosis (Chiaramonte *et al.*, 1999).

IL-13 plays other important roles in the immune response to schistosomiasis. IL-13 exhibits chemotactic activity for human eosinophils and may play a role in their survival by stimulating the production of IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Horie *et al.*, 1997). In addition, a novel IL-4-independent pathway for IgE switching in the mouse has been described (Morawetz *et al.*, 1996) which appears to be controlled by IL-13 (Emson *et al.*, 1998) explaining the ability of IL-13 to induce IgG4 and IgE synthesis and direct IgE isotypes switching in human B cells (Punnonen *et al.*, 1993). Also, IL-13 has been shown to regulate adhesion molecule expression on human

endothelial cells (Bochner *et al.*, 1995), and the expression of ICAM-1 in particular is important to granuloma development (Ritter and McKerrow, 1996).

1.1.9.3. Origin of the Th2 response to schistosome eggs.

Using the ELISPOT technique King *et al.* (2001) reported that the dynamics of IL-4, IL-5 and IFN-γ secreting splenocytes preceded by two weeks the cytokine production in the hepatic granulomas which peaked at 10 weeks and exceeded that observed in splenocytes by 5-10-fold. At 8 week post infection, the principle source of IL-4, IL-5 and IFN-γ secreting splenocytes were CD4+ T cells whereas only approximately half of the IL-4 and IL-5 secreting granuloma cells were CD4+ve T cells. These observations are consistent with the production of IL-4 and IL-5 by cells other than CD4+ T cells present in the granulomas, including NK cells (Mountford *et al.*, 1996), CD8+ T cells (Pedras-Vasconcelos and Pearce, 1996), γδ T- cells cells (Raziuddin *et al.*, 1992), activated eosinophils (Rumbley *et al.*, 1999), basophils (Gibbs *et al.*, 1996) and non-B, non-T cells (Sabin and Pearce, 1995). Eosinophils represent a dominant source of IL-4 in the granulomas (Rumbley *et al.*, 1999) and show greatest activation at the time of maximum granuloma formation, i.e. 10-12wk after initial cercarial exposure.

This suggests that the initial development and activation of SEA-reactive lymphocytes occur in peripheral lymphoid tissues. The earlier occurrence but lower frequency of egg antigen-reactive splenocytes compared to granuloma cells and their increased cytokine production in response to exogenous SEA is consistent with the presence in the spleen of low concentrations of SEA which partially activate, but do not terminally differentiate, SEA reactive CD4+ memory T cells. King *et al.* (2001) speculate that low concentrations of egg antigen reach the spleen and other peripheral tissues and there induce SEA-specific cells which migrate into the granuloma where they become fully activated by high local concentrations of endogenous antigen released by the viable ova. There they secrete specific cytokines such as IL-4, IL-5 and IFN-γ that mediate specific antibody responses, induce tissue eosinophilia and activate macrophages and multinucleated giant cells, respectively, that are thought to participate in ova destruction and local containment of parasite products. This explanation is supported by findings that activation antigens, such as CD69 and IL-2R, are more highly expressed in granuloma than splenic lymphocytes (Rumbley *et al.*, 1998).

1.1.10.3 Which egg components stimulate the Th2 response?

An area of current intense research interest is focused on how Th2 responses are induced. As for certain other helminth products, it is generally accepted that schistosome eggs induce an intense Th2 response without the need for additional adjuvant and that this is principally responsible for the shift from a Th1 response in the prepatent period to a strong Th2 response as the eggs are produced (Pearce *et al.*, 1991; Vella and Pearce., 1992). Much of this analysis has been based on splenocytes responses and Hayashi *et al.* (1999) reported that hepatic T cells deviated into type2 cytokine production without showing any type1 shift in the prepatent period. Unlike splenocytes, hepatic lymphocytes from infected mice during the prepatent period already produced a huge amount of IL-4, IL-13, and IL-5 and a lesser amount of IFN-γ in response to soluble worm Ag preparation (SWAP). These results suggests the prompt type 2 deviation in the liver after the infection might be due to the alteration of Küpffer cells that induces soluble worm antigen preparation (SWAP)-mediated type2-development of hepatic T cells (Hayashi *et al.*, 1999).

Nevertheless it is now clear that egg products promote a marked Th2 skewed response and that this largely controls the nature of the granulomatous response around the eggs. Various soluble egg antigen components e.g. proteins, glycans and lipoconjugates can all induce Th2 cells. One recombinant protein, IPSE, which is a prominent secreted egg product, can stimulate the release of IL-4 by human basophils (Schramm *et al.,* 2003). IPSE seems to bind to IgE present on the cell surface of basophils, irrespective of antigen specificity, and activates cells by cross-linking. A second recombinant protein, TCTP, also activates basophils in terms of histamine release (Rao *et al.,* 2002).

Recent work has shown that carbohydrates on egg antigens are integral to this process (Okano *et al.*, 1999; 2001; Williams *et al.,* 2001) and, specifically, that a polylactosamine sugar (lacto-*N*-fucopentaose III) acts as a Th2 adjuvant (Okano *et al.*, 2001) eliciting strong Th2-cell recall responses (IL-4, IL-5, IL-10, and IgE) in mice when conjugated to human serum albumin (Okano *et al.*, 2001). The most immunoreactive components are glycoproteins found in the eggs and schistosomula. Egg glycoproteins were found to stimulate production of B-1 cells (CD5+ B220+) which appeared in the peritoneal cavity of mice at the time of egg production (Harn *et*

al., 1989). B-1 cells, distinct from the conventional B cells, are characterized as a self-replenishing lineage of B cells which produce autoreactive immunoglobulin M (IgM) antibodies and are capable of producing large amounts of IL-10 (O'Garra *et al.*, 1992; Velupillai *et al.*, 1997; Palanivel *et al.*, 1996). Two immunoreactive oligosaccharides from the egg antigens were found to be involved (Harn *et al.*, 1989): (i) Lacto-*N*-fucopentaose III (LNFP-III) which contains the Lewis-X trisaccharide. Lewis-X is a weak ligand for P-selectin (Larsen *et al.*, 1992; Stoolman, 1989) and is also found on lymphocyte function-associated molecule-1, which is a ligand for intercellular adhesion molecules 1 (Larsen *et al.*, 1992). (ii) the non-fucosylated homologue, lacto-*N*-neotetraose (LNT). LNFP-III and related sugars (LNT) were found to be lympho-stimulatory and induced proliferation of splenic non-T cells, B220+, CD4-, CD8- cells (B cells) of schistosome infected and naïve mice. LNFP-III induced spleen cells to produce large amounts of IL-10 and prostaglandin E2, two molecules known to downregulate Th1 CD4+ T cells and so contribute to Th2-subset dominance. Interestingly, LNFP-III did not induce the production of IL-4 (Velupillai and Harn, 1994). Thus, a specific carbohydrate ligand has been identified that stimulates B cells to proliferate and produce factors that downregulate Th1 T cells (Velupillai and Harn, 1994). The emerging role of carbohydrates as factors that are important for the induction of the immune response during schistosomiasis opens up the possibility that innate pattern recognition receptors that identify carbohydrates might have a crucial role in the induction of a Th2 response. Notably, the host-like glycans, such as the Lewis-x related carbohydrate lacto-N-fucopentose III, which when conjugated to human serum albumin, elicits strong Th2-cell recall responses (IL-4, IL-5, IL-10, and IgE) in mice (Okano *et al.*, 2001).

Recently, lipid fractions from the eggs have been shown to induce Th2-cell differentiation, in a manner that is distinct from the stimulation of Treg cells, as only the latter depends on TLR2 (van der Kleij *et al.*, 2002; 2004).

1.1.10.4 The role of DC in inducing Th2 responses.

Recent elegant studies have focused on determining how SEA interacts with dendritic cells (DCs) to induce Th2 responses (MacDonald *et al.*, 2001; 1982). There is general acceptance that DCs are able to activate Th1 cells during primary antigen exposure by

delivering (at least) three signals (Banchereau *et al.*, 2003; Moser, 2001): antigen presentation via MHC-class II (major histocompatibility complex molecules-II); costimulatory signals by upregulation of costimulatory molecules such as CD80 and CD86; and a third signal, candidates for which include DC produced IL-12 (Macatonia *et al.*, 1989) and CD40 (on DC) and CD154 (on T cell) interactions (Cella *et al.*, 1996). Triggering of the DC for such functions is believed to arise from PAMPS (pathogen associated molecular pattern molecules) interaction with Toll-like receptors (TLR) on the DCs (Trinchieri, 2003). MacDonald *et al.* (2001) showed that DC exposed to antigen of the bacterium *Proprionibacterium acnes* (Pa), show this typical pro-Th1 activation and when transferred *in vivo* into naïve mice induce Th1 responses. In contrast, DC exposed to SEA failed to show upregulation of costimulatory molecules or detectable cytokine production (McDonald *et al.*, 2001) but were able to induce Th2 responses (IL-4, IL-5, IL-13) but not IFN-γ when transferred into naïve mice and this was shown to depend on CD40 expression by the DC. One suggestion is that Th2 responses reflect a default which occurs when antigen fails to induce DC maturation. It has also been suggested that there are different populations of DC able to respond to either Th1 or Th2 stimuli. These possibilities were investigated by stimulating DC with both Pa and SEA (Cervi *et al.*, 2004) and these studies argued against there being DC1 and DC2 by showing that the same DCs took up both antigens but could direct discrete Th1 or Th2 responses to Pa and SEA respectively when transferred to naïve mice. The Pa and SEA however, entered different intracellular compartments in the DC with different predicted antigen processing capabilities. This led the authors to suggest that molecular motifs on the different antigens led to them being directed into different compartments resulting in differential processing perhaps resulting in a higher density expression of Pa compared with SEA and that this may ultimately be a key trigger to Th1 or Th2 signalling. Carbohydrate components of the SEA have been implicated in DC priming because LN cells of mice that received periodate-treated SEA-pulsed DCs produced 6-to 10-fold less Th2 cytokines (IL-4, IL-5 and IL-10) upon *in vitro* stimulation with SEA (Faveeuw *et al.*, 2002).

Studies with human myeloid DCs have shown the upregulation of OX40L expression (a factor known to be expressed by the peripheral blood DC and involved in Th2 cell development [Brocker *et al.*, 1999]), and production of poor levels of IL-12p70, TNF-α

and IL-6 following interaction with SEA upon CD40 ligation (Kalinski *et al.*, 2000). Interestingly, glycan moieties present on egg glycoproteins or glycolipids have also been implicated in this DC priming for Th2 response.

1.1.11 Counter regulation between the Th1 and Th2 responses

In spite of this focus on Th2 responses it is clear that Th1 responses are induced in schistosomiasis and are crucial for immunoregulation of disease. The underlying Th1 response is revealed, for example, following treatment with anti-IL-4 monoclonal antibody (mAb) when antigen specific IFN-γ and IL-2 responses are increased (Yamashita and Boros, 1992). Treatment with recombinant IL-12 (rIL-12) alone or combined with neutralization of Th2 type cytokine production also suppressed Th2 cell development and profoundly inhibited pulmonary and early hepatic granuloma formation during infection (Wynn *et al.*, 1994; Boros and Whitfield, 1999). Conversely, administration of anti-IFN-γ (Luckacs and Boros, 1993) or anti-IL-12 mAb (Wynn *et al.*, 1994) promoted pulmonary granuloma formation due to enhanced Th2 cell expansion. Similar findings were reported in gene targeted KO mice models. IL-$12^{-/-}$ or IFN-$\gamma^{-/-}$ mice also developed severe disease, with excessive Th2 responses, and marked mortality associated with increased granuloma size and fibrosis (Hoffmann *et al.*, 2000). Conversely, STAT6-deficient mice (Kaplan *et al.*, 1998) produced high levels of Th1 cytokines IFN-γ and IL-2, minimal IL-4 and greatly reduced IL-5 and IL-10. They developed decreased size of (smaller) both pulmonary and hepatic granulomas with very sparse eosinophils, and decreased amounts of liver hydroxyproline content as a measure of collagen deposition. The balanced nature of the response in normal mice was shown by the fact that several of these treatments to promote Th1 or Th2 responses led to increased mortality, on the one hand due to reduced granuloma formation and on the other to increased responses.

The influence of IL-4 has been studied in several experiments in IL-$4^{-/-}$ mice. These show greatly impaired granuloma formation and impaired Th2 cytokine responses but somewhat different levels of mortality (Pearce *et al.*, 1996; Metwali *et al.*, 1996; Kaplan *et al.*, 1998). In the studies of Brunet *et al.* (1997) and Fallon *et al.* (2000) the *S. mansoni* infection led to cachexia, with evidence of hepatotoxicity and significant mortality. This process was accompanied by detectable levels of lipopolysaccharide in

the plasma, perhaps owing to the translocation of intestinal bacteria (Fallon *et al.*, 2000). This severe intestinal inflammation resulted in endotoxemia and death (Fallon and Dunne, 1999; Fallon, 2000, Patton *et al.*, 2001). Analyses of the immune system of the infected IL-$4^{-/-}$ mice showed there were an increase in production of pro-inflammatory cytokines i.e. IFN-γ and TNF-α (Fallon and Dunne, 1999; Hoffmann *et al.*, 2000), and there was a correlation between elevated levels of nitric oxide (NO) and disease severity (Brunet *et al.*, 1997). Treatment with uric acid, which is a peroxyradical scavenger, had marked ameliorative effects (La Flamme *et al.*, 2001).

However, Metwali *et al.* (1996) conclude that the increase in IFN-γ expression was only slight and suggested that IL-4-deficient mice failed to default to a robust Th1 response because they maintained a significant non-T-cell-derived immuno-suppressive IL-10 response. Several studies have shown that the Th2 cytokine responses can develop *in vivo* in the absence of IL-4 or IL-4 receptor alpha, which is consistent with the findings in the schistosomiasis model (Jankovic *et al.*, 1999; 2000).

Consistent with this, animals deficient in both IL-4 and IL-10 did develop highly polarized Th1 responses with dramatic increases in IFN-γ and simultaneous reductions in Th2-type cytokines both *in vitro* and *in vivo* (Wynn *et al.*, 1997; Hoffmann *et al.*, 2000). The IL-10/IL-4-deficient mice, developed an acute wasting condition at the onset of egg-laying leading to 100% mortality, and showing strong evidence of increased hepatotoxicity that seemed to be an exaggerated form of the disease that is observed in infected IL-$4^{-/-}$ animals (Hoffmann *et al.*, 2000). This acute mortality was linked to the over-expression of the pro-inflammatory mediators IFN-γ, TNF-α, both of which increase the expression of inducible nitric oxide synthase (iNOS) which converts the L-arginine to L-hydroxy-arginine eventually leading to excessive production of NO and citrulline but diverting L-arginine from the collagen pathway leading to non-fibrotic granulomas (Hesse *et al.*, 2001).

Together these results demonstrate that the early production of IL-4 and IL-10 are equally important to Th2 response polarization *in vivo*, with IL-4 driving Th2 development and IL-10 polarizing the response by downregulating IL-12 and Th1-type cytokine expression. IL-10 KO mice develop large granulomas with elevated Th1 and Th2 cytokines but with no significant increase in hepatic fibrosis compared to WT mice

showing that IL-10 modulates both the Th1 and Th2 components of the granulomatous response (Metwali *et al.*, 1996; Wynn *et al.*, 1997; Hoffmann *et al.*, 2000). IL-10 modulation of the developing Th1 response would explain the marked reduction which occurs in IL-12 production soon after egg injection in the pulmonary granulomatous model (Wynn *et al.,* 1994) further indicating the importance of rapidly inducing IL-10 response against *S. mansoni* eggs.

Studies in humans demonstrate a role for inflammatory cytokines and their regulation by IL-10 in urinary tract morbidity during *S. haematobium* infection (King *et al.*, 2001). IL-10 (and TNF-α) release from egg-stimulated PBMC cultures was compared in age- and sex-matched infected children and adolescents suffering from moderate to severe bladder wall pathology. A significantly lower ratio of egg-specific IL-10: TNF-α production from PBMC cultures obtained from the patients with severe bladder wall pathology, suggesting that low IL-10 and high TNF-α can correlate with an increased risk of developing severe disease during schistosomiasis. A recent study examining the correlates of developing severe fibrosis in adult male populations living in Uganda who were chronically infected with *S. mansoni*, also demonstrated that a deficiency in IL-10 was partly responsible (Hoffmann *et al.,* 2002).

Despite the serious inflammatory disease associated with uncontrolled Th1 responses to the eggs it is clear that the Th1 response serves an important function in modulating the Th2 response. Th2 polarized, IL-10/IL-12-deficient, mice developed increased hepatic fibrosis, formation of large eosinophil-rich granulomas, a 10-fold increase in IL-4 and IL-13, and significant mortality during the chronic stages of infection (Hoffmann *et al.*, 1999; 2000). It is thus important that the Th2 response to eggs is controlled and this is fulfilled by the counterbalancing Th1 response. Egg-injected IL-10-deficient mice treated with anti-IL-12 mAb showed more than a 10-fold increase in lymph node or spleen cell production of IL-4 and IL-5 upon *in vitro* activation (Wynn *et al.,* 1997). The highly polarized and exacerbated Th2 response in these mice was not seen in wild type mice treated with anti-IL-12mAb, which suggests that endogenous IL-10 in these animals serves as a potent inhibitory signal not only for Th1 cytokine expression as was previously demonstrated, but for Th2 responses as well. Thus, two main pathways for suppressing Th2 responses have been demonstrated; the counteregulatory Th1-type

IFN-γ response and IL-10, with the latter mechanism being better demonstrated in animals only after elimination of both inhibitory pathways.

Although a highly polarized Th1 response is particularly damaging, studies in inbred mice show that there is some scope to amplify the Th2-regulating Th1 response and so reduce fibrosis. This is accomplished by sensitizing mice to soluble egg-antigens in the presence of Th1 adjuvants, like IL-12 (Wynn *et al.,* 1995) or CpG oligonucleotides (Chiaramonte *et al.,* 2000), before schistosome infection to provoke the development of an egg-antigen-specific Th1 response (Wynn *et al.,* 1995), which was characterized by reduced granuloma size and fibrosis and reduced mortality. This was contrary to the effects of immunizing with egg antigen in complete Freund's adjuvant (CFA) which also induced a strong Th1 response but led to a acute lethal hepatotoxicity on subsequent infection (Rutitzky *et al.,* 2001). One possible explanation is that IL-12, but not CFA, promotes the production of high levels of IL-10 (Wynn *et al.,* 1994; Morris *et al.,* 1994), which, in turn, is needed for protection against potentially lethal pro-inflammatory mechanisms.

These findings show that the anti-egg response which has evolved represents a finely regulated balance between Th1 and Th2 responses with IL-10 playing a central regulatory role and induction of excessive type1 and type 2 cytokine responses triggering distinct, but equally detrimental, forms of pathology following infection.

1.1.11.1 Anti-fibrotic effects of Th1 in macrophages.

Therefore, Th2-driven production of arginase might be a critical component in the development of schistosome-egg induced liver pathology (Hesse *et al.,* 2001). Blocking the arginase activity in aaMø with L-hydroxy-arginine (LOHA), a product of iNOS activity, impairs the production of proline. It has been proposed that aaMø, by promoting the synthesis of polyamines through the arginase, increased collagen deposit and fibrotic reaction in the liver (Hesse *et al.,* 2001). In addition, the increased synthesis of polyamines through arginase activity in aaMø might favour the growth of helminths that are thought to relay on the host for the uptake of polyamines (Abdallahi *et al.,* 2001).

Arginase uses L-arginine as a substrate to make L-ornithine, which is converted to proline by ornithine aminotransferase. Proline is an essential amino acid that is involved in collagen production and therefore, in the development of fibrosis. Fibrosis is inhibited in mice that are immunized with egg antigens plus IL-12; cytokines are produced a components of the induced Th1 response (such as IFN-γ, and TNF-α) that prevents Th2 response development (and, so, IL-13 production) and also activate the macrophages to express inducible nitric oxide synthase (iNOS), rather than arginase (Modolell *et al.,* 1995). Concomitantly, arginase-positive aaMø and the liver fibrosis is strongly decrease. However, this immunization protocol is ineffective in iNOS-knockout mice, despite the induction of an excellent Th1 response in these animals (Hesse *et al.,* 2000). This seems to be due to the fact that iNOS uses arginine to make nitric oxide (NO) and citrulline—an intermediate pathway is the L-hydroxyarginine, which inhibits arginase effectively reducing the amount of proline that is available for collagen synthesis. This phenomenon depends on the NO secretion because $iNOS^{-/-}$ mice induced inflammation despite developing a Th1 cytokine response. Thus, Th1 mediators such as IFN-γ, IL-12, TNF-α, TGF-β (Lee *et al.,* 2001), and NO can prevent IL-13 mediated fibrosis in the schistosome granuloma (Hesse *et al.,* 2001). Taken together, these data indicate that arginase-positive aaMø contribute to the granuloma pathology during schistosoma infection.

1.1.11.2 Immuno-regulation - Regulation of fibrosis

In schistosomiasis, several studies have shown that IFN-γ can directly down-regulate the synthesis of extracellular matrix components (Stephenson *et al.,* 1985). The mice which developed the most severe fibrosis displayed the highest IL-13 to IFN-γ ratio (Chiaramonte *et al.,* 2001). Using a model of carbon tetrachloride-induced liver injury, Thompson *et al.* (1998) demonstrated increased fibrosis in the IL-10-deficient mice. A marked increased fibrosis among *S. mansoni* infected mice that were deficient for both IL-10 and IL-12 (Hoffmann *et al.,* 2000). These mice, in contrast to single knockout IL-10-deficient animals fail to develop an IFN-γ response and consequently developed much more fibrosis. The production of IL-13 was also increased more than 10-fold in these mice, providing an additional mechanistic explanation for their altered fibrotic response. Together, these data strongly suggest that the magnitude of fibrosis is

controlled, at least in part, by the pro-fibrogenic activities and anti-fibrotic effects of IFN-γ and IL-10.

1.1.11.3 Regulatory cells induced in schistosomiasis- Modulated macrophage function in schistosomiasis

Macrophages count among the most pleiotropic cells of the both innate and acquired immune system, exhibiting a wide range of biological functions. They act as effector cells by their specialized lysosomal enzymes and cytotoxic molecules, such as nitric oxide, oxygen radicals, and TNF-γ, and also play an important role as APCs in the acquired immune system (MacMicking *et al.,* 1997; Sprent and Schaefer, 1990). The degree of activation of macrophages defines the fate of the infection, either to control the infection (remission) or persistence of the inflammatory status and progression to chronic infection. Thus, macrophages play a crucial role as mediators of many chronic inflammatory diseases (Allen and Loke, 2001; Kiefer *et al.,* 2001; Kinne *et al.,* 2000).

Depending on the cytokine environment, macrophages can differentiate into distinct subsets that perform specific immunological roles (Gordon, 2002). Macrophages activated by interferon-γ, referred to as "classically activated macrophages" (caMø), have been extensively studied and shown to play an essential role in the protection against intracellular pathogens by means of increased oxidative burst and NO release, and to exert anti-proliferative and cytotoxic activities, and consequent production of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6). Such caMø are responsible for control of many micro-organisms which live inside cells including *L. donovani.* Macrophages activated in the presence of IL-4 and IL-13 are known as "alternatively activated" (aaMø) and have powerful immuno-suppressive properties (Stein *et al.,* 1992; Goerdt and Orfanos, 1999). Most recently, a new phenotype of macrophages exerting anti-inflammatory activity were called type 2-activated macrophages, which exhibit functional similarity to both caMø and aaMø. Like caMø, they produce pro-inflammatory cytokines TNF-α, IL-1β and IL-6. However, type2 activated macrophages do not produce IL-12, but secrete IL-10 and drive preferentially a Th2-like immune response. This phenotype is reminiscent of alternative activation. Yet in contrast to aaMø, type2-activated macrophages do not exert arginase activity (Mosser, 2003).

There is increasing evidence that aamØ are abundant in helminth infections. This was first demonstrated in mice in response to implanting the filarial worm *Brugia malayii* in mice peritoneal cavity (Allen *et al.,* 1996; MacDonald *et al.,* 1998) and these cells have been extensively studied. The implantation induced profound Th2 responses with recruitment of high numbers of eosinophils and macrophages to the site of parasite infection (Falcone *et al.,* 2001), paralleled with suppression in the proliferative response (Loke *et al.,* 2000). These alternatively activated macrophages actively blocked proliferation *in vitro* of peripheral blood lymphocytes, CD4+ T cells and many other cell types by cell-to-cell contact, implicating a receptor mediated mechanism (Loke *et al.,* 2000; Schebesch *et al.,* 1997, Allen *et al.,* 1996). Furthermore, the proliferative block was reversible and not a result of apoptosis. The suppressed cells accumulate in the G1 and G2/M phase of the cell cycle, suggesting a mechanism that affects degradation of cell cycle proteins. The suppressive function is dependent on IL-4, since macrophages recruited in IL-4-deficient mice are not suppressive (Loke *et al.,* 2000; MacDonald *et al.,* 1998) and not IL-10 (MacDonald *et al.,* 1998). Interestingly, when these macrophages are used as antigen presenting cells to stimulate naïve T cells from TCR transgenic mice, they induce the differentiation of IL-4 producing Th2 cells (Loke *et al.,* 2000). Recent studies have focused on differential gene expression in aaMø activated with IL-4 (Welch *et al.,* 2002; Loke *et al.,* 2002). As expected arginase is highly upregulated but 10% of the transcripts, not previously associated with macrophage function, encoded a recently recognized eosinophil chemotactic factor termed Ym1/ECF-L, which explains why the recruitment of the aaMØ in the Brugia model is accompanied by a localized infiltration of eosinophils and this may also play a role in the eosinophil recruitment to the schistosome egg granuloma (see below).

It is clear that there is also profoundly altered macrophage function in schistosomiasis. Splenocytes from *S. mansoni* infected mice when stimulated with SEA were able to produce a factor in supernatants which would suppress APC mediated proliferation of SEA specific Th1 cell clones (Flores Villanueva *et al.,* 1993). This activity was first seen at 5 weeks post infection peaked at 8 weeks and then increased (a little) but was still significant at 23 weeks. The ability of the supernatant to suppress was prevented by addition of anti-IL-10 antibody to cultures.

There is clear evidence that *S. mansoni* egg granuloma macrophages (GM) are alternatively activated, that they display modified function and that they play a key role immuno-modulating the disease. The GM arise from blood monocytes recruited to granulomata from the periphery (King *et al.,* 2001). In: Mahmoud AAF, Editor. Schistosomiasis. London. Imperial College Press, 213-264). GM were unable to stimulate SEA Th1 cell clones to proliferate in contrast to splenic APCs from infected mice and, in addition, culture of the Th1 clones with SEA and GM made the Th1 cells unresponsive to subsequent re-stimulation with splenic APCs (Stadecker *et al.,* 1990; 1999). The inhibitory effect of GM on proliferation of Th1 clone cells in the presence of antigen and APC was inhibited by anti-IL-10 but not anti-TGF-β (Flores Villanueva *et al.,* 1994). GM even when stimulated by LPS and IFN-γ expressed little if any B7-1, B7-2 or MHC-II but MHC-II and B7-2 expression could be upregulated by anti-IL-10 treatment. The molecules B7-1 (Reiser *et al.,* 1992) and B7-2 (Inaba *et al.,* 1994) are critical costimulators for the activation of Th cells; as their absence can lead to T cell anergy (Harding *et al.,* 1992; Wu *et al.,* 1993; Zheng *et al.,* 2004). On the other hand the cytokine IL-10 (Moore *et al.,* 1993) has variously shown to down-regulation of APC function, apparently through down-regulation of MHC class II (Kast *et al.,* 1984) as well as B7 molecule expression (Ding *et al.,* 1993).

Furthermore, when tested *in vivo* by injecting eggs iv into infected mice GM led to a reduction of 26% in granuloma size and a marked reduction (~3 fold) in SEA induced proliferation of MLN cells and a striking loss of IL-2 production with an associated increased in IL-4 and IL-10 (Flores Villanueva *et al.*, 1994). These GM were also able to inhibit expression of costimulatory molecules (B7-1) and MHC-II on APC when injected *in vivo* into mice (Flores Villanueva *et al.,* 1994).

Recent studies have formally shown that GM conform to the characteristics of aaMø (Gordon, 2003). Arginase-1 (Arg-1) is highly abundant in the granuloma of WT mice but in IL-4/IL-10 KO mice which make a highly skewed Th1 cytokine response Arg-1 was undetectable (Hesse *et al.,* 2001). Linehan *et al.* (2003) showed that GM expressed high levels of the prototype phenotypic marker of alternatively activated macrophages, the mannose receptor, and they confirmed the IL-4 dependence of this expression by showing a lack of MR expression in IL-4R$\alpha^{-/-}$ mice. However, KCs and endothelial

cells were also MR positive in both WT and IL-4R$^{-/-}$ mice as there is constitutive expression (Linehan *et al.*, 1999; Takahashi *et al.*, 1998). This means that it is not possible to say from the study of Linehan *et al.* (2003) if the KCs were also "alternatively activated" although this was not revealed by the staining for arginase-1 (Hesse *et al.*, 2001). There is also evidence for aaMø in *S. japonicum* infections as these induce the synthesis of YM, a molecule expressed by aaMø (Nio *et al.*, 2004).

Thus the alternatively activated GM seems to have potent immuno-suppressive properties which are particularly effective against Th1 cells leading to persistent immunological unresponsiveness. Although the schistosome granuloma is a predominant Th2 and to a lesser extent Th1 response, the underlying Th1 response clearly regulates the level of Th2 activity (section 1.1.11). Although this Th1 response seems to regulate the level of Th2 mediated pathology (as seen in highly Th2 polarized IL-12/IL-10$^{-/-}$ mice). Hoffmann *et al.* (2001) it clearly needs to be kept in tight control as Th1 polarized (IL-4/IL-10 KO mice) show high and early death. Recent studies show that the GM exert a crucial anti-inflammatory role. Herbert *et al.* (2004) engineered mice in which only the macrophages (and also neutrophils) lacked the IL-4Rα chain which is required for both IL-4 and IL-13 signalling and so were unable to generate alternatively activated GM. These mice showed essentially normal granuloma size and cellular composition but suffered high mortality apparently due to uncontrolled inflammatory damage to the liver and gut i.e. the sites of granuloma formation. The evidence from these studies is that GM are aaMø and that they have profound inhibitory effects on the Th1 component of the response to the egg antigens.

An apparently different population of suppressor macrophages has been reported in *S. mansoni* infected mice. Injection of Lacto-*N*-fucopentose III (LNFP-III) or Lacto-*N*-neontetraose (LNnt) coupled with dextran (LNFPIII-dex) induces the expansion of a GR1^{+}/D11b^{+}/F4/80^{+}/CD11c^{-}/IL-10R^{-} macrophage population in a T cell-independent manner, and expressing low levels of MHC class II, CD40 and CD86 molecules (Terrazas *et al.*, 2001; Atochina *et al.*, 2001). They spontaneously produce low levels of pro-inflammatory cytokines (IL-1β, IL-12, IL-18, IFN-γ), do not secrete NO or PGE2, but released IL-10 and TGF-β. Interestingly, LNFP-III and LNnt primed macrophages suppress the proliferation of CD4+ T cells induced by CD3 and CD28 costimulation. Moreover, the inhibition of T-cell proliferation requires cell contact with suppressive

cells, and the process of inhibition was found to be NO- and IFN-γ-dependent which is classically taken as a marker of the 'classically activated' suppressor population, in contrast, for example, to that of the typical *B. malayi*-elicited aaMø (Loke *et al.,* 2000).

It interesting to note that LNFP-III also induces the release of IL-10 by PBMC from *Schistosome*-infected individuals and of IL-10 and TGF-β by B cells from *Schistosoma*-infected mice (Velupillai *et al.,* 1994). Moreover, injection of structurally related glycoconjugate lacto-*N*-neotetraose-dextran (LNnT-dex) has also been shown to expand such suppressor cells and these were able to induce a Th2 phenotype on naïve CD4+ T cells, characterized by lower IFN-γ, but increased IL-13 production (Terrazas *et al.,* 2001).

Overall there is a body of evidence that excreted/secreted oligosaccharides from helminth parasites in general and schistosomes in particular play an important role in inducing both Th2 responses (Okano *et al.,* 1999) and suppressor macrophages. This results in the modulation of the host response and induction of anergy as the infection progresses (Atochina *et al.,* 2001) effects which will profoundly affect the outcome of encounter with other infectious organisms.

1.1.11.4 Modulation of the granulomatous response in chronic infections.

In mice following the peak granuloma size which occurs at the acute phase approximately 8-10 wk post infection there is a decrease in mean granuloma size (Warren *et al.,* 1967; Colley *et al.,* 1975) and a characteristic decrease of the immune response to egg derived antigens (Colley, 1981; Nash *et al.,* 1981; Grzych *et al.,* 1991). This regulatory response has been named modulation (Boros *et al.,* 1975) and coincides with the decrease of the symptoms observed during the acute phase of the infection. Studies in experimental models (Vella and Pearce, 1992; Wynn *et al.,* 1993), have demonstrated that cytokines are important factors in the formation as well as the modulation of the granulomatous immune response to the egg. Various mechanisms have been suggested to explain the downregulation of granulomatous hypersensitivity. Henderson *et al.* (1992) studied the cytokine responses in the process of modulation as the schistosome infection progresses from the acute to the chronic phase, peak tissue

levels of both IL-4 and IFN-γ mRNA were seen in acutely infected mice, and levels of both messages declined as infection became chronic.

Numerous mechanisms have been postulated for this down-modulation, including a role for CD8+ T cells (Pedras-Vasconcelos and Pearce, 1996), anti-idiotypic Abs (Olds *et al.,* 1989), B cells (Cheever *et al.,* 1985), and IL-10 (Stadecker and Flores Villanueva, 1994). To examine the role of endogenous IL-10 in the downmodulation of hepatic granuloma formation and lymphocyte responses that occurs in chronic schistosomiasis. Wynn *et al.* (1998) demonstrated that IL-10 gene knockout mice (IL-10T) displayed a significant increase in hepatic granuloma size at the acute stage of infection, which is associated with increased IFN-γ, IL-2, IL-1β and TNF-α mRNA expression in the liver and elevated Th1-type cytokine production by lymphoid cells. Sadler *et al.* (2003) also showed that the proliferative response to SEA and granuloma volume were downregulated in normal mice by 15 weeks (chronic infection) but not in IL-$10^{-/-}$ mice implicating IL-10 in this important modulatory process in mice.

In humans, evidence of the granuloma modulation was first reported by Neves and Raso, (1965) who demonstrated from granulomas derived from liver biopsies of acute patients and from hepatosplenic cases that all granulomas evaluated were significantly larger in the acute patients when compared to the individuals in the chronic phase. IL-10 has also been implicated in this down-modulation. Thus Malaquias *et al.* (1997) demonstrated that anti-IL-10 increased *in vitro* proliferative responses by PMBC from chronically infected humans in the presence of SEA and adult worm antigen whereas this did not happen with cells from acute patients.

1.1.11.4 The balance of Cytokine responses in humans in relation to disease

1.1.11.4.1 Acute schistosomiasis in humans

In humans schistosome infection causes a range of morbidities but there two main clinical syndromes are recognized, acute schistosomiasis (Katayama fever) and chronic schistosomiasis which takes different forms (chronic Intestinal form, Hepatosplenomegly syndrome), the development of which seems to be influenced to a large extent by the nature of the induced immune response and its effects on the

granuloma formation and associated pathogenesis in target organs (Dunne and Pearce, 1999; Cheever *et al*., 2000).

Acute schistosomiasis in humans is a debilitating febrile illness (Katayama fever) that can occur before the appearance of eggs in the stool and which is thought generally to peak between 6 and 8weeks after infection (Rabello, 1995). During acute illness, there is a measurable level of tumour-necrosis factor (TNF-α) in the plasma, and peripheral–blood mononuclear cells (PBMCs) produce large quantities of TNF-α, interleukin-1β (IL-1β) and IL-6 (de Jesus *et al.,* 2002). Notably, cytokine production by PBMCs after stimulation with parasite antigen reflects a dominant T helper 1 (Th1), rather than Th2 response (de Jesus *et al*., 2002). Field studies in endemic areas, combined with animal experiments, have led to the view that host genetics, infection intensity, *in utero* sensitization to schistosome antigen all influence the development of the immune response and so, disease severity.

This acute febrile illness that is associated with the initial stages of schistosome infection seems to be uncommon in individuals who live in areas that are endemic for schistosomiasis. It occurs, instead, in individuals who have no previous history of exposure who become infected after travelling into endemic area. One explanation for this difference is that individuals can become sensitized to schistosome *in utero* as a result of maternal infection, which subsequently allows them to respond differently from 'naïve' individuals when themselves become infected.

Data from analyses of cord-blood lymphocytes taken from the babies of infected and uninfected mothers support the view that *in utero* sensitization does occur and moreover, indicate that the fetal response is phenotypically similar to the response of the mother (King *et al.,* 1998; Malhotra *et al.,* 1997). The pre-existing Th2 response in such children might make them less likely to develop a pro-inflammatory response on first infection with schistosomes. An examination of disease in mice has shown that an inability to develop a Th2 response to regulate the initial pro-inflammatory response that is associated with acute schistosomiasis is lethal. This first became apparent when C57BL/6 IL-$4^{-/-}$ mice were infected with *S. mansoni*. Coincident with the onset of parasite egg production in these animals, a condition that was characterized by cachexia and significant mortality (Brunet *et al.,* 1997; 1999; Fallon *et al.,* 2000). These mice

developed normal hepatic granuloma (lacking the eosinophilic component) but pathological changes in the intestine were more evident in the absence of IL-4; non-haemorrhagic lesions on the mucosal surface (Brunet *et al.,* 1997; 1999) were associated with the inefficient passage of eggs into the lumen (Fallon *et al.,* 2000). This process was accompanied by detectable levels of lipopolysaccharide in the plasma, perhaps owing to the translocation of intestinal bacteria (Fallon *et al.*, 2000). Analysis of the immune responses of infected IL-$4^{-/-}$ mice showed that there was a correlation between elevated levels of nitric oxide (NO) and disease severity (Brunet *et al.,* 1997; 1999).

1.1.11.4.2 Chronic schistosomaisis in humans

Chronic disease is graded according to severity. The most serious form is a life-threating hepatosplenic disease, which is usually accompanied by severe hepatic and periportal fibrosis, portal hypertension and portosystemic shunting of venous blood (Dunne and Pearce, 1999), and a lesser severe form chronic intestinal schistosomiasis. Although Th2 response seem to have a crucial role in modulating potentially life-threating disease during the initial stages of schistosomiasis, prolonged Th2 responses contribute to the development of hepatic fibrosis and chronic morbidity (Cheever *et al.,* 2000).

The main Th2 cytokine that is responsible for fibrosis is IL-13. So, schistosome-infected mice in which IL-13 is either absent (IL-$13^{-/-}$) is ineffective (Fallon *et al.,* 2000), (IL-4Rα-chain KO) (Jankovic *et al.,* 1999) or neutrilizing by treatment with soluble IL-13Rα2-Fc (Chiaramonte *et al.,* 1999), fail to develop the severe hepatic fibrosis that normally occurs during infection, which leads to prolonged survival of these mice (Fallon *et al.,* 2000). Mediators that are associated with Th1 responses, such as IFN-γ, IL-12, TNF-α and NO can prevent IL-13-mediated fibrosis (Hesse *et al.,* 2001).

Infection intensity is one factor that can affect the severity of chronic schistosomatic disease, particularly in children (Mohamed-Ali *et al.,* 1999). However, it seems to be more important whether infected individual is genetically predisposed to disease (Mohamed-Ali *et al.,* 1999; Dessein *et al.,* 1999).

Other anti-inflammatory cytokines are known to limit the degree of disease progression and severity by modulating the schistosome granuloma. In a recent study, Correa-Oliveira *et al.* (1998) demonstrated that IL-10 is an important cytokine regulating the *in vitro* granulomatous reactivity of PBMC from intestinal (INT) patients. This was evidenced by blockage of this cytokine using anti-IL-10 mAb *in vitro* granuloma assay lead to a significant increase in granuloma size with cells from chronic intestinal schistosomiasis (INT) patients but not with individuals in the acute phase or with the hepatosplenic (HS) form of schistosomiasis. Furthermore, analysis of soluble IL-10 levels present in the *in vitro* granuloma culture demonstrated that high levels of IL-10 secretion occurred only in the cultures when PBMC from INT patients were tested. Moreover addition of recombinant IL-10 readily decreased the reactivity to SEA in PBMCs from patients with the three clinical syndromes (Acute, chronic intestinal schistosomiasis, and ambulatory hepatosplenic disease). Furthermore, the addition of anti-IL-4 mAb to the *in vitro* granuloma cultures with PBMC from individuals with either acute or hepatosplenic schistosomiasis, lead to decrease in granuloma size, suggesting that IL-4 plays an important role in the granuloma formation and in maintenance of cellular response in the severe form of the disease.

Serum IL-10 as well as *in vitro* cellular proliferation was found to be lower in HS patients followed by individuals of acute schistosomiasis further supporting the role of IL-10 on the regulation of the immune response to *S. mansoni* infection as well as on the control of the development of the severe form of schistosomiasis. These results demonstrate that lack of an effect of the blocking antibodies to IL-10 on the *in vitro* granuloma cultures is not due to the inability of these cells to respond to this cytokine, but again to lack of IL-10 secretion by PBMC from acute and hepatosplenic patients. Similar results were also obtained by other investigators demonstrating that in chronic asymptomatic schistosomiasis IL-10 plays a major role in regulating *in vitro* cellular proliferation (Araujo *et al.,* 1996, Sadler *et al.,* 2003).

No IFN-γ production was observed in PBMC cultures from 45 patients living in endemic areas of schistosomiasis in Brazil, following *in vitro* stimulation with schistosomula and adult worm antigens and was independent on the intensity of infection (Araujo *et al.*, 1994). This study was confirmed by another study on 22-

infected patients living in the same endemic area (Araujo *et al.,* 1996), showing that the majority (86%) showed no lymphocyte proliferative responses and none of them showed IFN-γ production, following *in vitro* stimulation of PBMCs with soluble adult worm antigen preparation (SWAP). This absence of IFN-γ production was antigen specific since high levels of this cytokine were detected in same patients when their cells were stimulated with PPD or PHA (Araujo *et al.,* 1994). In contrast, IL-4, IL-5 and IL-10 were detected. Restoration of lymphoproliferative response and IFN-γ production resulted from addition of anti-IL-10 mAb to PBMC culture.

There is also evidence of Th2 polarised responses and of IL-10 suppressed Th1 responses in infected humans. Infected individuals generally show raised Th2–type responses, i.e. raised IgE and eosinophilia (Snyman *et al.*, 1997; Souza-Atta *et al.,* 1999; Hagan, 1996). However, this is not always the case. On the basis of the amount of IFN-γ or IL-5 (or other Th2 cytokines) that is produced by PBMCs in response to antigen, some individuals do seem to have a more Th1-like response. In one of the few studies that have attempted systemically to correlate the immune response with disease severity, patients with hepatosplenomegly owing to *S. mansoni* infection were found to have a Th1-like response and high plasma levels of TNF-receptor 1 (TNFRI) and TNFRII, whereas individuals who had less severe disease but similarly intense infections (as assessed by counting the number of eggs in faecal samples) had Th2 responses and low plasma levels of soluble TNFR (Mwatha *et al.,* 1998).

In another study conducted of pedigrees in Sudan, in an area where *S. mansoni* is endemic, Dessein and collagues found that severe hepatic fibrosis (as identified by ultrasound) was more likely to occur in certain families (Dessein *et al.*, 1999). A segregation analysis showed that a codominant major gene, known as *SM2*, is responsible for the familial distribution of hepatic fibrosis and portal hypertension. 'Informative' families, which had multiple cases of severe fibrosis, were used to map *SM2* to chromosome 6q22-q23, a region that contains the gene for IFN-γ receptor 1 (IFNγ-R1) (Dessein *et al.*, 1999). One interpretation of these data is that mutations in the IFNγ-R1 that led to loss of function of the receptor are associated with a lack of effectiveness of IFN-γ in suppressing fibrogenesis. So in human studies, there is some

evidence that severe chronic disease is associated with Th1 rather than Th2 responses in at least a proportion of the population.

1.1.11.4.3 Role of T regulatory in immunity to infectious disease

Recent studies have suggested that activation of DCs that secretes IL-10, but not IL-12, can direct naïve T cell to a Tr1 subtype (termed DCr) (McGuirk and Mills, 2002; Mills and McGuirk, 2004).

Most recently, DCs can under certain conditions, differentiates into strong inducers of T reg cells (McGuirk and Mills, 2002). For example, if lipid fractions from *S. mansoni* are present during the maturation of human DCs, the DCs become conditioned to induce regulatory activity (such as IL-10 production) by naïve T cell populations (van der Kleij *et al.,* 2002). So regulatory accessory cells can develop under the influence of defined parasite molecules, and it seems that helminths might induce T reg cells through specific APC subsets.

A further subtype of T cells with immuno-suppressive function and cytokine profiles distinct from either Th1 or Th2 cells have been proposed and termed regulatory T (Treg) cells. Several subsets of regulatory T (Treg) cells with distinct phenotypes and distinct mechanisms of action have been described. These include type1 Tr cells (Tr1) (Groux *et al.,* 1997; McGuirk and Mills, 2002; MacDonald and Pearce, 2002; Levings *et al.,* 2001), which secretes high levels of IL-10, IL-5 and low to moderate levels of transforming growth factor (TGF-β), little or no IL-2, IL-4, or IFN-γ production and proliferate poorly following polyclonal TCR-mediated activation (Groux *et al.,* 1997). Functional studies on Tr1 cells have suggested that these cells have immuno-suppressive properties and have been shown to prevent the development of Th1-mediated autoimmune disease (Groux *et al.,* 1997). The suppressive effect of Tr1 cells clones are reversed by neutralizing IL-10 suggesting that Tr1 cell suppression is a bystander effect mediated through the production of IL-10.

Type3 T (Th3) cells (Chen *et al.*, 1994; Fukaura *et al.,* 1996) are also CD4+ Treg cells, but primarily secrete TGF-β, which disrupts early IL-12 signalling events in T cells (Bright and Sriram, 1998; Gorham *et al.,* 1998; Pardoux *et al.,* 1999) and alters either

IL-12R1 or R2 mRNA expression consequently suppressing IFN-γ production, proliferation of T lymphocytes and thus suppressing Th1 immune response. Similarly, TGF-β has multiple suppressive actions on T and B cells, macrophages, and other cell types (Letterio and Roberts, 1998; McCartney *et al.,* 1998).

$CD4^+CD25^+$ T cells, is a population of CD4+ T cells that constitutively express the IL-2Rα (CD25) which is regarded as a robust marker of Tregs (Hori *et al.,* 2002). $CD4^+CD25^+$ T cells comprise ~5-10% of peripheral T-cell pool which exhibit potent immuno-suppressive properties both *in vivo* and *in vitro* (Shevach, 2000). They inhibit IL-2 production by a mechanism dependent on cell-cell contact (Shevach *et al.,* 2000) and also expression of the inhibitory costimulatory molecule CTLA-4 (Thornton and Shevach, 2000; Read *et al.,* 2000). Cell surface TGF-β might be involved in cell-contact suppression by $CD4^+CD25^+$ Tr cells (Nakamura *et al.,* 2001). However, the presence of fully functional $CD4^+CD25^+$ Tr cells in both Smad3, TGF-β and TGF-β receptor-II defective mice have cast considerable doubt over the involvement of TGF-β in $CD4^+CD25^+$ Tr cells mediated suppression (Shevach, 2002). The suppression by $CD4^+CD25^+$ cells has been explained as being independent of membrane bound TGF-β, but these cells transferred suppressor activity to conventional CD4+ T cells, which functioned partially through soluble TGF-β (Jonuleit *et al.,* 2002).

In addition to the various CD4+ Tr populations, recent studies have identified CD8+ Tr cells, which secretes either IL-10 or TGF-β (Garba *et al.,* 2002; Gilliet and Liu, 2002).

Tregs have been shown to suppress activation and proliferation of naïve T cells (Annacker *et al.,* 2001), and suggested to suppress protective Th1 responses against an infectious pathogen (McGuirk *et al.,* 2002). However, certain Tr1 cells can also suppress inflammatory Th2 responses (Cottrez *et al.,* 2000). The suppression of the protective immune response by anti-inflammatory cytokines or cells induced directly or indirectly by the pathogen might provide a plausible explanation for the chronic infections caused by certain parasites, bacteria, and viruses.

There is strong recent evidence for the involvement of Treg in schistosomiasis. The demonstration that IL-10 is crucial for preventing schistosome eggs from inducing IL-12 dependent Th1 responses (as happens in IL-$10^{-/-}$ mice [Hoffmann *et al.,* 2000] led

McKee and Pearce, (2004) to investigate if Treg producing IL-10 were involved in schistosomiasis. They identified $CD4^+CD25^+$ and $CD4^-CD25^+$ cells in infected mice which produced IL-10 in response to SEA and which could inhibit IL-12 production by DC in response to CD40 ligation (with anti-CD40 antibody). Unlike the $CD4^+CD25^+$ population, the $CD4^-CD25^+$ also produced Th2 cytokines and were considered SEA specific Th2 cells. In the presence of SEA both populations from infected mice but not uninfected mice were able to inhibit IL-12 production by DCs. The $CD4^+CD25^+$ cells were found to express the the Treg transcription factor forkhead box P3 (Foxp3) which has been shown to program the development and function of Treg cells (Hori *et al.*, 2002). The $CD4^+CD25^+$ cells from infected mice were more effective than cells from uninfected mice in suppressing T cell proliferation (following anti-CD3 stimulation) and when transferred to IL-$10^{-/-}$ mice injected with eggs they inhibited the egg specific Th1 response as judged by IFN-γ production by 50% but had no effect on the Th2 response. The $CD4^-CD25^+$ (Th2) population also inhibited the Th1 response but also enhanced Th2 cytokines as expected. However, $CD4^+CD25^+$ cells from naive mice were equally effective as cells from infected mice and transfer of $CD4^+CD25^+$ cells from naïve mice into acutely infected IL-$10^{-/-}$ mice was also able to suppress Th1 IFN-γ production. These authors conclude that the production of IL-10 by both antigen specific Treg and Th2 cells suppresses the development of Th1 responses to schistosome egg antigens.

1.2 The Th2 response stimulated by eggs influences the nature of immune responses to concomitant antigens/vaccines/infections

The induction by schistosome infections of IL-4, IL-13 and IL-10 from Th2 cells and IL-10 from Treg cells has profound effects on the generation of Th1 responses and macrophage function as is characteristic of these cytokines (O'Garra, 1998; Gordon, 2003). Most people who live in areas that are endemic for schistosomiasis are also exposed to many other infectious diseases and there is growing interest in whether this influences an individual's immune response to other ongoing immune responses whether they be to vaccines, allergens, autoantigens or concurrent infections. Various of the studies on this and the modulation by other helminths has recently been reviewed by Cox, (2001) and some of these are reviewed below.

The realization that morbidity during schistosomiasis is dependent on a finely balanced Th2-Th1 response to egg antigens also raises interesting questions about the potential for Th1 polarizing responses induced by co-infecting organisms on the pathological response to the eggs.

1.2.1 Antigens

The principle that the Th2 response to *S. mansoni* could downregulate generation of a Th1 response was first demonstrated when *S. mansoni*-infected mice immunized with sperm whale myoglobin (SwMb) which normally induces a Th1 cytokine profile showed a down-regulation of antigen-specific splenocytes responses (IL-2 and IFN-γ production per CD4+ cell was reduced by 45% and 59% respectively) and elevation of Th2 responses (IL-4 per CD4+ cell was increased threefold) (Kullberg *et al.,* 1992; 1996). Addition of neutralising mAb specific for IL-10 to the spleen cell cultures, restored the suppressed IFN-γ response seen in infected mice, demonstrating the role of IL-10.

In addition, altered autoimmune responses to self antigens induced by *S. mansoni* infection is indicated by reduced spontaneous incidence of insulin dependent diabetes mellitus, a Th1-mediated disease, in infected non-obese diabetic mice (Cooke *et al.,* 1999). The absence of TCR-α-chain rearrangements of a diabetogenic CD4+ T cell clone, BDC2.5 transgenic mouse lead to development of diabetes (Haskins and McDuffie, 1990). Further studies have shown that IL-10 has been implicated in regulation of inflammation and autoimmunity (Asseman *et al.,* 1999). A regulatory population of T cells which produces IL-10, involved in regulatory process that prevent diabetes BDC2.5/NOD mice has been identified in both humans and mice (Groux *et al.,* 1997; Levings *et al.,* 2001). Th1 cells, dendritic cells and macrophages play a role in the development of diabetes (Parish *et al.,* 1995). IL-10 is known to be able to inhibit both DC and macrophage function and IL-10 is known to be an autocrine growth factor for DC, and neutralization of IL-10, together with a DC maturation signal, has been shown to increase expression of CD80, CD86, and MHC, as well as production of IL-12 and TNF-α. IL-10 inhibit the production of a range of inflammatory mediators (IL-1β, IL-1α, and TNF-α) by macrophages, together with IFN-γ have been shown to be particularly damaging to pancreatic β cells (Ablamunits *et al.,* 1994). Recently, Phillips *et al.* (2001) demonstrated that treatment with anti-IL-10R

facilitates the onset of diabetes in BDC2.5/TCR-α-transgenic NOD mice by increasing serum IL-12 levels and permitting the expression of Th1 immune response against islets.

Regarding responses to allergens von den Biggelaar *et al.* (2000) have reported a correlation between *S. haematobium* infection and reduced levels of immediate skin test responsiveness to house dust mite allergen. This reduced level of skin test responsiveness did not correlate with polyclonal IgE levels showing that this effect is not just due to swamping of IgE Fc receptors and infection did not affect the levels of mite specific IgE. Rather responsiveness correlated with levels of worm specific IL-10 produced *in vitro*. It was suggested that IL-10 may have local effects on mast cell function and these data support the notion that worms may play a role in the low levels of allergies which are reported to occur in developing countries.

Regarding modulation of the response to the schistosome infection T cell unresponsiveness *in vivo* can be induced by various means; one of these involves the administration of specific antigen conjugated with B subunit of cholera toxin (CTB). This strategy has been successful in curtailing harmful immune response in models of autoimmunity (Kim *et al.*, 2001), allergy (Rask *et al.*, 2000), and infectious disease (Xu *et al.*, 1998). More specifically, significant inhibition of egg antigen-specific T cell responses and amelioration of experimental murine schistosomiasis was observed following intranasal (i.n.) instillation of conjugates of CTB with GST28 and with the Sm-p40 antigen itself (Sun *et al.*, 2001). Hernandez *et al.* (2002) found that the intranasal treatment of infected CBA mice with a fusion of protein of cholera toxin B subunit (CTB) and the peptide 234-246 (CTB::peptide 234-246) resulted in significant down-modulation of hepatic granuloma inflammation and fibrosis. This was associated with upregulated production of transforming growth factor-β by egg antigen stimulated hepatic granuloma cells as well as mesenteric LN CD4+ T cells. One possible approach to reduce the morbidity in schistosomiasis is the amelioration of the egg-induced immuno-pathology by means of downregulating the underlying pathogenic T cell responses.

1.2.2 Decreased efficacy of Th1 vaccines in schistosomal patients

There have been a few studies conducted in schistosome endemic areas showing that schistosome induced Th2-response in infected individuals affected vaccine efficacy. IFN-γ

production by PBMC to Tetanus-Toxoid (TT) following primary TT immunization was shown to be dramatically reduced in *S. mansoni* infected individuals and inversely related to infection intensity (Sabin *et al.,* 1996). In contrast IL-4 levels did not show this dramatic difference.

Similarly, Malhotra *et al.* (1997) demonstrated that infants become sensitised *in utero* to worm antigens from infected mothers and they subsequently showed (Malhotra *et al.,* 1999) that children born of worm-infected (*S. haematobium or Wuchereria bancrofti*), mothers and vaccinated with BCG at birth make significantly lower IFN-γ responses to PPD (purified protein derivative of *Mycobacterium tuberculosis*) at 10-14 months after birth compared to offspring of uninfected mothers. The lower the helminth-driven Th1:Th2 cytokine ratio (i.e. the more Th2-like) at birth the lower the PPD response. Similar results have been reported in individuals with other helminth infections (Cooper *et al.,* 1999). In addition, Muniz-Junqueira *et al.* (1996) showed that *S. mansoni* patients particularly those with hepatointestinal disease, showed a decreased response after typhoid vaccine and they suggest that a diminished ability to mount an immune response towards typhoid antigens may interfere with the clearance of typhoid bacteria from the blood stream (chronic salmonellosis associated with schistosomiasis).

However, Bassily *et al.* (1997) showed that the immunogenicity of recombinant hepatitis B vaccine among infants of mothers with active schistosomiasis given the vaccine at 2, 4 and 6 months of age was not affected as judged by antibody levels to the recombinant Hepatitis B vaccine although cellular responses were not considered.

1.2.3 Viral infections

The influence on viral infections was first modelled in mice using *Vaccinia* virus. Virus-specific IFN-γ production and cytotoxic T cell responses (particularly CD8+ T cells) were markedly decreased and virus clearance was delayed in *S. mansoni*-infected mice exposed to recombinant *Vaccinia* virus. This was associated with a marked decline in IFN-γ production and a slight increase in IL-4 and IL-5 to virus antigen. Immunohistochemical staining of liver sections from vaccinia/*S. mansoni* co-infected mice with polyclonal anti-vaccinia antibodies revealed that viral epitopes are localized primarily within granulomas (Actor *et al.,* 1994). These experiments suggest that egg granulomas, by providing a

microenvironment for viral expression, in combination with the cytokine imbalance present during schistosome infection, can promote the expansion of *Vaccinia* virus and possibly other viral agents (Actor *et al.,* 1993; 1994).

HBV and HCV are both non-cytopathic hepatotropic viruses that can cause acute or chronic infections in humans which can result in severe hepatitis, cirrhosis and hepatocellular carcinoma. Both show overlap in their distribution with worm infections including *S. mansoni*. With HBV, approximately 10% of acutely infected adults and 90% of acutely infected neonates become chronically infected; and more than 80% of adults infected with HBV develop a chronic infection (Rehermann *et al.,* 1996). There is increasing evidence to suggest that the outcome of infection is highly influenced by the early immune response, with strong, sustained virus-specific CD4+ and CD8+ T cell responses being associated with virus clearance (Rehermann and Chisari, 2000; Maini and Bertoletti, 2000; Sing *et al.,* 2001; Gruener *et al.,* 2001). Control of virus replication is predominantly mediated by antiviral cytokines such as IFN-γ and TNF-α (Guidotti and Chisari, 1996; Heise *et al.,* 1999; Guidotti and Chisari, 2001). However, the immune response also mediates much of the liver damage associated with HBV and HCV infections (Chisari and Ferrari, 1995; Koziel, 1999; Bertoletti and Maini, 2000). Both infections are found together with schistosomiasis specifically and worms in general in many several parts of the world. Co-infection of hepatitis B virus (HBV) and *S. mansoni* is a frequent event in humans but little is known about the interactions between two pathogens. The confluence of these viral and helminth infections in the liver, in which the dominant Th2 response during schistosomiasis might downmodulate the Th1-like anti-viral immunity, offers a possible explanation for the increased occurrence of chronic hepatitis-virus infection in schistosomiasis patients.

Studies were later conducted in Brazil and in other regions, especially Egypt, where the prevalence of schistosomiasis and viral hepatitis is very high. The results were not uniform, some of them showing exacerbation of disease in co-infections between hepatitis B virus and schistosomiasis (Conceicao *et al.,* 1998; Hammad *et al.,* 1990; McClary *et al.*, 2000a; Hamadto *et al.,* 1989; Pereira *et al.,* 1994; Andrade and Cheever, 1995), and a higher rate of chronicity of hepatitis (Serufo *et al.*, 1998; Andrade and Cheever, 1995). Recent studies in HBV transgenic mice showed, surprisingly, that infection with *S. mansoni* inhibited virus replication, with concomitant increased NO and Th1-type cytokines (IFN-γ, IFN-α/β) and

in the absence of Th2-type cytokines at 5weeks p.i.. As judged by cytokine mRNA levels (by quantitative RNA protection assay) they noted upregulation of both Th1 and Th2 responses which both peaked at 8weeks and concluded that schistosome-induced IFN-γ overcomes a quantitative deficiency in HBV-induced IFN-γ and mediates most of this antiviral activity (probably via NO). The *S. mansoni*-dependent antiviral effect was partially blocked by genetically deleting IFN-γ, although it was unaffected by deletion of IFN-α/β. These results indicate that most of IFN-γ produced by cytotoxic T cells (CTLs)(probably via NO) mediates most of antiviral activity and that IFN-α/β inhibits HBV viral replication via NO-independent pathways (McClary *et al.,* 2000b; Guidotti *et al.,* 1996; 2000). Taken together, the data indicate that during *S. mansoni* infection the intrahepatic cytokine profile is initially of a Th1-type and this associated with the non-cytopathic inhibition of HBV replication. Surprisingly, Th2-type cytokines do not counteract the antiviral effect of IFN-γ. Similar events may suppress HBV replication during human *S. mansoni*, irrespective of the presence of Th2 cytokines in the liver (McClary *et al.,* 2000; Andrade *et al.*, 2001). Therefore, it is possible that persistence of HBV may primarily result from quantitative deficiency of Th1-type responses rather than qualitative differences in the intrahepatic cytokine profile. Accordingly, it has been shown recently that the number of IFN-γ-producing antigen T cells isolated from the peripheral blood of people chronically infected with HBV is much lower than that observed in the peripheral blood of people acutely infected with HBV (Jung and Pape, 2002).

However, in contrast with the earlier study, Ghaffar *et al.* (1991) demonstrated in his study that the increased HBsAg carrier rate was 25% at 12 months in those with schistosomiasis compared with 9% in those with on acute HBV infection, and patients with concomitant schistosomiasis had higher liver function test results compared to the AVH only. Concomitant schistosomiasis with the AVH increased the prevalence of splenomegly and prolonged HBsAg carrier state and frequently converts uncomplicated intestinal schistosomiasis to hepatosplenic schistosomiasis (Ghaffar *et al.,* 1991).

Moreover, it now seems clear that in Egypt at least, where HCV and schistosomiasis are two of the most important public-health problems and have geographically overlapping distributions, the coincidence of infection is a result of the unfortunate initial widespread transmission of HCV by mass parenteral antischistosomal therapy, which continued into

the 1980's (Frank *et al.*, 2000). Several studies conducted in Egypt (Abdel Wahab *et al.*, 1994; Angelico *et al.*, 1997; El-Zayadi *et al.*, 1997; Hibbs *et al.*, 1993; Koshy *et al.*, 1993; 1995; Waked *et al.*,1995; Kamal *et al.*, 2000, Gad *et al.*, 2001) and in Brazil (Yoshida *et al.*, 1993; and 1993; Pereira *et al.*, 1995) demonstrated a higher prevalence of HCV virus infection in schistosome patients and defined its importance in the decompensation of hepatic disease.

Mabrouk, (1997) demonstrated that HCV and schistosomiasis play a major role in the development of hepatocellular carcinoma in Egypt. In addition, Kamal *et al.* (2001) showed that patients with acute HCV and schistosomiasis co-infection cannot clear viremia and show rapid progression to histological proven chronic active hepatitis and accelerated fibrosis. This rapid progression in the co-infected individuals is associated with a strong Th2 response in peripheral immune responses, suggesting that development of vigorous Th1 responses facilitates clearance and delays disease progression. HCV-specific CD4+ proliferative response to HCV Ag was detected in 73% of patients infected with HCV alone which produced type1 cytokines, compared with 8.6% of patients co-infected with HCV and *S. mansoni* which produced type2 cytokine production. In contrast, there was no difference in response to schistosomal antigens in patients infected *S. mansoni* alone, compared to those co-infected with HCV and *S. mansoni*. These findings suggest that the inability to generate an HCV-specific CD4+/Th1 T cell response plays a role in the persistence and severity of HCV infection in patients with *S. mansoni* co-infection. In an earlier study, Kamal *et al.* (2000) demonstrated that patients with concomitant HCV and schistosomiasis infection were characterized by more advanced liver disease, higher HCV RNA titers, predominance of HCV genotype 4a, higher histological activity, higher incidence of cirrhosis and hepatocellular carcinoma as well as higher mortality rate. Indeed, there is evidence that schistosomiasis prevents the development of Th1 response to HCV (Kamal *et al.*, 2001a; 2001b). Similarly, Gad *et al.* (2001) demonstrated that concurrent HCV infection and schistosomal liver disease result in much more severe liver disease than that seen with either disease alone. However, the activity of HCV infection in terms [increased viral loads (viral titer) and/or replication] seems partially suppressed in patients with schistosomal liver disease.

Some other studies have not supported the exacerbation of disease in this co-infection. By multivariant analysis Angelico *et al.* (1997) showed that 55% of Anti-HCV positive sera had HCV-RNA of genotype 4a, which was associated with more severe liver disease but this occurred less frequently in patients with history of schistosomiasis. Similarly, Helal *et al.* (1998) reported a lack of enhancement of HCV pathology in schistosome patients and no significant difference between the schistosomal and non-schistosomal groups regarding the semi-quantitative histological score. However, Elrefaei *et al.* (2004) showed that Egyptian patients infected with HCV genotype 4a can mount HCV-specific CD4+ and CD8+ T cell responses despite the prevalence of concomitant schistosomiasis.

On the other hand, there is some evidence that HBV and HCV infections are a factor in the development of hepatosplenic schistosomiasis (Dunne and Pearce, 1999).

In a descriptive study of the prevalence of GB virus type C /Hepatitis G virus (GBV-C/ HGV) infection in a rural population of Northeastern Brazil, in which the prevalence of schistosomiasis is 80-90% Gallian *et al.* (1998) showed that despite the absence of parenteral risk exposure, the prevalence of GBV-C/HGV markers of infection (viremia, 16.4%; specific antibody, 18.3%) were unusually increased. It was concluded that helminth infection influenced the immune response to GBV-C/HGV infection by shifting the balance of cytokine responses from Th1 to Th2, resulting in a delayed viral clearance.

The abundance of chronic helminth infections in sub-Saharan Africa has been proposed to influence the high susceptibility to HIV infection and apparent faster progression to AIDS observed in the region (Bentwich *et al.,* 1995; Bentwich, 2000). It has shown that CD4+ T cells that produce type 2 cytokines (Th2 cells) provide a more conducive environment for the replication of certain strains of HIV-1 than do Th1 cells (Maggi *et al.,* 1994; Wang *et al.,* 1998; Galli *et al.,* 1998). For example, cells from persons who have active filariasis and associated Th2-like immune responses are more susceptible to HIV-1 infection than cells from the same individuals after antifilarial chemotherapy and diminution of the Th2-like phenotype (Gopinath *et al.,* 2000). In addition, PBMC from HIV-1 positive schistosomiasis patients demonstrated decreased proliferation and decreased Th2-like cytokine production, less IL-4 and IL-10 but similar (low) amounts of IFN-γ than did HIV-1 negative individuals with active schistosomiasis. Furthermore, in patients with HIV-1, a decrease in CD4+ T cells was correlated with an increased Th1:Th2 cytokine production ratio, which indicates

that there were a swing in the overall balance of the response from Th2 and Th1 (Mwinzi *et al.*, 2001). The reduced production of Th2 cytokines by PBMC from the schistosome-HIV co-infected patients is consistent with the hypothesis that HIV-1 has a predilection for antigen activated Th2 cells in an actual infection (Weissman *et al.*, 1996), and that schistosome-antigen-responsive cells are then eliminated by the virus. Mechanistically, this could be occuring through IL-4-mediated up-regulation of CXCR4 and the switch to the syncytium-inducing phenotype of HIV-1 that is associated with higher viral replication and an increased rate of CD4+ T cell decline (Wang *et al.*, 1998; Galli *et al.*, 1998; Suzuki *et al.*, 1999; Nakayama *et al.*, 2000). Although, treatment of schistosome infections in HIV-1 positive patients does not lead an immediate drop in plasma HIV-1 loads (Lawn *et al.*, 2000), prompt diagnosis and treatment to prevent pathology in chronic schistosomiasis, which further lowers CD4+ T cells levels (Colley *et al.*, 1983; Kalinkovich *et al.*, 1998), may help HIV-1 infected persons with respect to overall immuno-competence. Mwanakasale *et al.* (2003) demonstrated that treatment with praziquantel is still very effective in control of *S. haematobium* even when there is co-infection with HIV. Interestingly, individuals with co-infection excreted fewer eggs and complained less of hematuria than those without HIV infection, and the sensitivity and positive predictive value of reported hematuria were lower in the group co-infected with HIV (Mwanakasale *et al.*, 2003). Similarly, in a comparison of *S. mansoni*-infected HIV+ and HIV- patients, a correlation between diminished egg excretion and decreased CD4+T cell counts was apparent (Karanja *et al.*, 1997). Similarly in animal models, the egg excretion is minimal in immuno-compromised mice, but can be increased by the transfer of sera or lymphocytes from infected animals (Doenhoff, 1997).

Genital schistosomiasis has been proposed as a possible cofactor in the genesis of malignant disease that might contribute to its high incidence in regions where bilharziasis is endemic. Cervical cancer is the most common malignant tumour among women in Tanzania and other countries in Tropical Africa. Petry *et al.* (2003) demonstrated that infection with *Schistosoma haematobium* seems to favour persistent genital human papillomavirus (HPV) infection either by traumatizing the genital epithelium and/or by local immuno-suppression. As most cases show a history suggestive of bilharzias and/or active schistosomiasis there is evidence for a significantly increased risk of infection with high risk HPV types.

1.2.4 Bacterial infections

A relationship between persistent or recurrent *Salmonella* species bloodstream infection (chronic salmonellosis) and schistosomiasis has been described in both adults and children (Gendrel *et al.*, 1994; Omer, 1978; Mikhail *et al.,* 1981; Becquet *et al.,* 1982, Hennequin *et al.,* 1991; Lambertucci *et al.,* 1987; 1998) and concurrent schistosomal infection has also been proposed to explain persistence of non-typhoid *Salmonella* (NTS) infection and poor response to antibiotic therapy. Both immunological and non-immunological reasons have been proposed to explain this. Murine studies of S*almonella typhi* in concurrent infection with *S. mansoni*, revealed that *S. typhi* was more frequently cultured, bacterial growth rate was more rapid, and bacteria persisted much longer in mice infected with adult *S. mansoni* compared to the controls (Njunda and Oyerinde, 1996). Studies have shown that *Salmonella* bacterial pili adhere to a mannose receptor-like surface glycoprotein on the tegument of *Schistosome mansoni*, making it possible for the adult worm to provide a site of intravascular persistence leading to recurrent infection. Muniz-Junqueira *et al.* (1996) suggested that suppressed macrophage activity in schistosome-infected mice was involved as there was no increase in phagocytosis with increasing bacterial load.

In the murine experimental model, acute and chronic *S. mansoni* infection can predispose to pyogenic liver abscess following concurrent infection with *S. aureus* (Lambertucci *et al.,* 1998; Mahmoud and Awad, 2000; Teixeira *et al.,* 2001).

1.2.5 Protozoal infections

Experimental studies in particular have shown that schistosome infections interact with a variety of protozoal species (Chieffi, 1992, Cox, 2001).

In experimental malaria infection of C57BL/6 mice infected 8 weeks earlier with *S. mansoni* and superinfected with blood-stage *Plasmodium chabaudi* (which initially induces a Th1 response) resulted in increased malaria parasitaemia (Susceptibility), attributed to a defect in TNF-α but not in IFN-γ production in the co-infected mice. Reciprocally, spleen cell proliferative and Th2 responses to SEA were reduced for a month following the malaria infection but the *S. mansoni* pathology was not examined (Helmby *et al.,* 1998). However, *P. yoelii* superinfection was actually shown to inhibit the development of

schistosome granulomas in experimentally infected mice by Abdel-Wahab *et al.* (1974). In contrast to C57/BL mice, A/J mice, which normally show high mortality associated with a Th2 response to *P. chabaudi* malaria, prior infection with *S. mansoni* surprisingly promotes a Th1 response characterised by enhanced malaria specific IFN-γ and mice are protected from death (Yoshida *et al.*, 2000). Reciprocal interaction was reported in humans by Mutapi *et al.* (2000), who showed that malaria positive *S. mansoni* infected children produce significantly more anti-schistosome egg IgE and IgG3 than schistosome infected children who are negative to malaria.

Toxoplasma gondii infection normally kills C57BL/6 mice due to intestinal inflammation associated with high IFN-γ production. Marshall *et al.* (1999), reported that *S. mansoni*-infected mice super-infected with *Toxoplasma gondii* showed markedly reduced serum IFN-γ and NO levels which were presumed to be due to inhibition of development of Th1 responses by worm induced Th2 cytokines. However, *T. gondii* cyst burdens were unaffected even though the animals showed accelerated mortality associated with hepatotoxicity. This was hypothesised to be due to high levels of systemic TNF-α since it was shown that *S. mansoni* can prime mice for TNF-α production after endotoxin challenge (Marshall *et al.*, 1999) and suggested that *T. gondii* may act like LPS in *S. mansoni* primed mice to produce high levels of TNF-α. Araujo *et al.* (2001), showed that this TNF-α production was IL-12 dependent. Conversely, the *T. gondii* superinfection induced a considerable reduction in schistosome granuloma size (Marshall *et al.*, 1999).

An early study of co-infection with *T. cruzi* by (Kloetzel *et al.*, 1971) showed a much higher and persistent *T. cruzi* parasitaemia in co-infected animals but there was also slowed schistosome maturation, reduced worm burden and retarded ovideposition.

There is some indication from epidemiological studies in Egypt that schistosome infection interferes with the acquisition of immunity to *Entamoeba histolytica* and/or *E. dispar* (Mansour *et al.*, 1997). *S. mansoni*-infected mice are also reported to develop more intense infections with intestinal protozoa *Entamoeba muris*, *Trichomonas muris* and *Spironucleus muris* (Higgins-Opitz *et al.*, 1990).

A limited number of studies have investigated schistosome/ *Leishmania* spp co-infections.

Two studies have recently been carried out using the cutaneous infection *L. major*. Yoshida *et al.* (1999) reported that *S. mansoni* infection did not affect the outcome of *L. major* infection in either susceptible BALB/c and resistant C57BL/6 mice. Yoshida *et al.* (1999) demonstrated that Th2-dominant immune responses alter susceptibility of the host to other concomitant parasitic infections. Similarly, La flamme *et al.* (2002), initially infected mice with 35-70 Sm (peurto Rican strain) Sm cercaria and 2 weeks later infected with *L. major*). Also there was no major skewing of the immune response, popliteal lymph node cells of BALB/c mice expressed mRNA IL-10 rather than IL-4 but C57BL/6 mice expressed IFN-γ mRNA upon *L. major* antigen stimulation, even in *S. mansoni*-infected mice. In contrast La Flamme *et al.* (2002), demonstrated that infection with *S. mansoni* significantly delays the resolution of cutaneous lesions and parasitaemia following *L. major* infection given at +2 weeks after *S. mansoni* so that the time of generation of the resolving immune response to the *L. major* (4 weeks) coincided with the peak Th2 response in the *S. mansoni* infection. The co-infection with *S. mansoni* resulted in decreased *Leishmania*-antigen induced IFN-γ, TNF-α and nitric oxide production, but increased levels of IL-4 by popliteal LN cells 4 weeks after *L. major* infection. The *L. major* infection did not appear to alter the course of the *S. mansoni* infection.

Coelho *et al.* (1980) assessed the susceptibility of mice infected with *S. mansoni* to the cutaneous infection *L. mexicana*. 12 days after inoculation with *Leishmania* lesions appeared in all of the seven mice with the double infection but in only one of the 10 control animals. The immuno-depressive effect of *S. mansoni* infection was implicated but not demonstrated.

The only study on visceral leishmaniasis demonstrated that *L. infantum* infections are more severe in hamsters infected with *S. mansoni* (Mangoud *et al.*, 1998) whereas the *L. infantum* in hamsters delayed the *S. mansoni* granuloma formation.

1.2.6 Worm infections

Infections with *Schistosoma mansoni* and other helminths are also widespread and overalapping so that co-infections are common. Recent schistosome co-infection studies showed that resistance to challenge with the intestinal nematode, *Strongyloides venezuelensis* is increased. The recovery of *S. venezuelensis* adult worms from the small

intestine was significantly decreased by *S. mansoni* co-infection, and the protection to *S. venezuelensis* appeared to act on migrating larvae, this a result of boosting of Th2 immune response due to both helminth parasites in *S. japonicum*-infected mice (Maruyama *et al.*, 2000) or *S. mansoni*-infected mice (Yoshida *et al.*, 1999). This resistance (protection) to *Strongyloides venezuelensis* appeared to act on the migrating larvae and antibodies elicited by *S. mansoni* infection showed cross reactivity to third-stage larvae. Histology revealed that resistance was associated with eosinophilic infiltration in the lungs or mucosal mastocytosis depending on the route of challenge and that activation of eosinophils and intestinal mast cells was correlated with expression of mRNA for IL-13, IL-4, and IL-5 in *S. japonicum* infected mice (Maruyama *et al.*, 2000).

Trichuris muris superinfection profoundly altered the response to in a strain of mouse that normally made a non-protective Th1 response to *T. muris* and developed chronic infection (susceptible mice). Instead a protective Th2 response was made and the worms rejected (Curry *et al.*, 1995). This was believed to be due to the Th2 cytokine environment at the time of development of the *T. muris* response. In humans Keiser *et al.* (2002) did not find evidence of reciprocal protective effects in that a study among school children in Côte d'Ivore, demonstrated that increased intensity of *S. mansoni* was significantly correlated with an increased likehood of concomitant hookworm infections.

1.2.7 Autoimmune diseases

For many years it has been suggested that autoimmune disease may result in part from a deficit in regulatory T cells (Cooke *et al.*, 1978). A variety of markers to define these regulatory T cells include CTLA-4 and CD25 (Takahashi *et al.*, 2000).

An ongoing schistosomiasis-induced Th2 responses might be host protective, and in preventing the onset of Th1-mediated autoimmunity (for example, co-infection with schistosomiasis prevented diabetes mellitus in genetically predisposed non-obese diabetic mice) (Cooke *et al.*, 1999). Also, Cooke *et al.* (2001), demonstrated that this observation was due to endogenous IL-10, as these BDC2.5/TCR-α transgenic NOD mice developed following anti-IL-10R treatment, and similarly, Zaccone *et al.* (2003) demonstrated that *S. mansoni* antigens modulate the activity of the innate immune response and prevent the onset of type1 diabetes and decreased incidence of allergy.

A most recent study confirming that pre-established infection with the parasitic helminth, *Schistosoma mansoni*, significantly reduced the incidence and delayed the onset of experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice immunized with myelin oligodendrocyte glycoprotein (MOG)(35-55) peptide (La Flamme *et al.,* 2003). This altered disease progression was not solely due to the induction of a strong Th2 response, since intraperitoneal injection of schistosome eggs did not affect disease development. MOG-specific gamma interferon (IFN-gamma), nitric oxide, and tumour necrosis factor alpha production by splenocytes was significantly reduced in schistosome-infected mice compared to uninfected mice. However, similar levels of interleukin-10 (IL-10) were produced in an antigen-specific manner, suggesting that the induction of antigen-specific responses was not inhibited. Analysis of *in vivo* cytokine production by real-time PCR indicated that IL-12p40, but not IFN-gamma, transcript levels were dramatically reduced in the spinal cords of schistosome-infected, MOG-immunized mice. Furthermore, analysis of the cellular composition of the spinal cords and brains revealed that a pre-established infection with *S. mansoni* decreased central nervous system (CNS) inflammation, particularly of macrophages and CD4 T cells, by downregulating the production of proinflammatory cytokines and altering CNS inflammation.

Clear parallels exists between the immunology of allergy and of helminth infections in that both are associated with Th2-dominated immune response. Paradoxically, allergic disease seems to be less frequent in the developing countries that still have widespread helminth infection (Asher and Weiland, 1998), and evidence is accumulating that regulatory processes associated with helminth infection might be responsible (Yazdanbakhsh *et al.,* 2002; Wills-Karp *et al.,* 1996). Recent studies in human populations with *S. mansoni* (Araujo *et al.,* 2000) and *S. haematobium* (Van den Biggelaar *et al.,* 2000) infections have shown inverse relationships between allergen responsiveness (skin prick test to house dust mite allergen) and schistosome infection. There was no correlation between this reduced level of skin test responsiveness and polyclonal IgE levels indicating that the effect was unlikely to be due to non-specific blockage of mast cell receptors by excess non-allergen IgE, which has been suggested as a mechanism of interference. However, there was an inverse correlation of skin test response with levels of IL-10 produced *in vitro* to schistosome antigen. It has been proposed that the regulatory mechanisms that are induced

as a component of the immune response to chronic helminth infection, such as the production of IL-10 and possibly TGF-β, can non-specifically suppress inflammatory consequences of allergic reactivity (Yazdanbakhsh *et al.*, 2002).

These findings have led to reappraisal of the "hygiene hypothesis", the basis of which was that the Th2-cell mediated allergies would be counteracted by microorganisms that induce Th1 cells. The association of Th2-inducing helminths with inhibition of Th2-mediated inflammatory disease, has led to the proposal that mechanisms involving non-Th1 cell populations, such as regulatory T (Treg) cells, are involved (Yazdanbakhsh *et al.*, 2001; 2002).

1.3 *Leishmania donovani* Introduction, Life cycle and Disease

All parasites of the genus Leishmania are obligate, intracellular parasites that infect cells of the mononuclear phagocyte lineage of their vertebrate hosts, in which they exists as amastigotes (reviewed by Alexander and Russell, 1992). There are two developmental forms of Leishmania: the extracellular motile promastigotes and non-motile intracellular amastigotes. The extracellular motile promastigotes are introduced into the mammalian host when an infected female sandfly of the genus *Lutzomyia* in the new world and of genus *Phlebotomus* in the old world takes a bloodmeal. Shortly after inoculation in the dermis by the bite of sandfly, the metacyclic promastigotes are thought to infect macrophages and/or dendritic cells of the skin (Langerhans cells)(Titus *et al.*, 1994; Moll *et al.*, 1995); and the salivary gland material from the sand fly Lutzomyia longipalpis has an inhibitory effect on macrophage function *in vitro* (Theodos and Titus, 1993). Where they transform into amastigotes (Moore *et al.*, 1994). Visceral leishmaniasis (VL) occurs when members of *Leishmania donovani* complex (*L. donovani, L. infantum, L. chagasi*) are inoculated in the skin by the sandfly vector during a bloodmeal. However, cutaneous leishmaniasis is the most common form and can be caused by *L. major* in the old world and *L. mexicana* or *L. amazonenesis* in the new world (Bosque *et al.*, 1995)

In the case of visceral leishmaniasis (VL), due to *L. donovani, L. infantum* and *L. chagasi*, amastigotes spread via the blood stream to the bone marrow, spleen and lymph nodes but in advanced disease all organs may be involved (Ridley, 1980; Bittencourt and Barral, 1991). In humans and genetically susceptible mice, the liver, spleen and bone marrow become the

major sites of parasite growth and pathology (Rosbotham *et al.,* 1996; Smelt *et al.*, 1997). Intracellular amastigotes grow rapidly in the liver for the first 28 days of infection and, depending on the mouse strain, are then cleared over the following month (Bradley *et al.*, 1977). Replication also occurs in the spleen and bone marrow and these become sites of chronic infection, with parasites surviving for the lifetime of the animal (Wilson *et al.*, 1996; Bradley and Kirkle, 1977). Dendritic cells (Moll *et al.*, 1995) and especially fibroblasts (Bogdan *et al.,* 2000) seem to be important sites of chronic infections suggesting that such cells are 'safe targets' possibly lacking the immune-potentiated killing mechanisms of macrophages.

Regarding the various clinical forms of leishmaniasis, low numbers of viable organisms persist within lymphoid tissue and/or the site of former skin lesion after selfcure or successful chemotherapy (Ramirez and Guevara, 1997; Schubach *et al.,* 1998). Such latent infection often quite frequently gives rise to severe reactivation of disease, including visceral leishmaniasis (VL) associated with, e.g. HIV co-infection (Alvar *et al.,* 1997); the development of post-kala azar dermal leishmaniasis (PKDL) after cure of VL (el-Hassan *et al.*, 1992); reactivation of former skin lesions, termed recidivans type (Momeni and Aminjavaheri, 1994); and the development of mucosal leishmaniasis months or years after healing of localized cutaneous ulcer (Saravia *et al.,* 1985).

Modification of splenic function seems to be an important aspect of *L. donovani* induced disease sequelae. In chronic VL, the spleen show many pathological features including germinal center involution and loss of follicular dendritic cells (Zijlstra and el-Hassan, 2001; Smelt *et al.,* 1997). Marginal zone macrophages (MZMs) are heavily infected soon after infection but then disappear (Smelt *et al.,* 1997; Engwerda *et al.,* 2002). Since MZMs are also specialized for the uptake of polysaccharide (Humphrey and Grennan, 1981), loss of this activity may allow increased systemic spread of encapsulated bacteria in humans infected with *L. donovani*. Indeed, secondary bacterial infections are responsible for significant mortality in such patients (Bryceson *et al.,* 1995). Also, the disruption to lymphocyte migration is likely to contribute to the immuno-compromised status of patients with VL. The disease in humans is characterised by fever (100%), splenomegly (96-100%) hepatomegaly (50-100%), and enlarged lymph nodes (36-84%) (Siddig *et al.,* 1990; Zijlstra *et al.,* 1991; Hashim *et al.,* 1994). Case fatality in VL ranges from 10-14% in those

diagnosed and treated, 100% in those who received no treatment (Hashim *et al.,* 1994). Visceral leishmaniasis is an important problem in various parts of the world. Notably in recent years it has been the most important health problem in Sudan, particularly in the main endemic areas in the eastern and central regions and there have also been several major epidemics occurred in Western upper Nile province in southern Sudan claiming over 100,000 lives in 1988 (Zijlstra and el-Hassan, 2001; Khalil *et al.,* 2002). PKDL was much more common than expected (56% of patients with VL developed PKDL) which served as a human reservoir of infection in the heavily populated and affected villages (Zijlstra and el-Hassan, 2001; Musa *et al.,* 2002).

1.3.1 Human immune response

The majority of individuals remain subclinically infected (Badaro *et al.,* 1986a) even though parasites may be detected in the visceral organs (Badaro *et al.,* 1986b). Recent epidemiological studies of human visceral leishmaniasis suggest that up to 85% of infected individuals may also spontaneously control infection (Badaro *et al.,* 1986b; Carvalho *et al.,* 1992; Evan *et al.,* 1992; Holaday *et al.,* 1993a). These individuals either remain asymptomatic or develop oligosymptomatic infection which eventually resolves without treatment (Badaro *et al.,* 1986b; Carvalho *et al.* 1992; Evan *et al.,* 1992; Holaday *et al.,* 1993a). However, subclinical infection can (even years later) progress to active disease if cellular immune function is suppressed, such as in the setting of human immunodeficiency virus infection (Alvar *et al.,* 1997) indicating that there is a protective adaptive immune response controlling the disease.

1.3.1.1 Subclinical form of VL:

There has been interest in the immunological characterization of the subclinical form of VL in relation to active disease in order to define whether and which immune components control the infection. Carvalho *et al.* (1992) observed high levels of IFN-γ in cultures supernatant of 100% of oligosymptomatic individuals (subclinical cases) who evolved to spontaneous cure, supporting the fact that resistance is related to an efficient cellular immune response. IL-4 plays an important role in the progression to disease and is also involved in the downregulation of IFN-γ, so the fact that it was not detected in the subclinical to spontaneous resolution cases maybe relevant (Carvalho *et al.,* 1994; Uyemura

et al., 1993). A recent prospective cohort study, to identify the immunological markers for the subclinical form of VL compared to the acute form was conducted on 784 children aged 0 to 5 years from *Leishmania* endemic area in Brazil (Gama *et al.,* 2004). In the subclinical form of VL, variable levels of IL-2 were detected in 52.3% of children, IL-12 in 85.2%, IFN-γ in 48.1%, IL-10 in 88.9%, and TNF-α in 100%, with the last two cytokines showing significantly lower levels than in the acute form. IL-4 was not detected in oligosymptomatic individuals. Multiple discriminant analysis used to determine the profile or combination of cytokines predominating in the subclinical form revealed both a *Leishmania* resistance (Th1) and susceptibility (Th2) cytokine profile (Gama *et al.,* 2004).

The subclinically infected individuals show evidence of parasite-specific T-cell reactivity (Carvalho *et al.,* 1992), but active VL is associated with the absence of a parasite-specific cell mediated immune response (Carvalho *et al.,* 1981; 1985).

1.3.1.2 Clinical active VL:

Only a small percentage of infected people develop active disease that is characterized by fever, wasting, hepatomegaly, blood cytopenia, and ultimately death.

In infected humans, Ghalib *et al.* (1993) demonstrated both TH1 (IFN-γ and IL-2) and Th2 (IL-10) cytokine mRNA in lymph nodes during acute disease and the ability of the infection to induce both Th1 and Th2 responses was also shown by the demonstration of both Th1 and Th2 CD4+ve clones in cured individuals (Kemp *et al.,* 1993). However, Ghalib *et al.* (1995) reported that PBMC from patients with active disease did not produce *Leishmania* antigen specific IL-12 p40 or IFN-γ lymphokine which induces macrophages to express leishmanicidal activity (Murray, 1990; Murray *et al.,* 1983). The deficient production of IFN-γ is associated with an increase in CD4+ Th cells expressing a Th2 cytokine profile (susceptibility), and with the production of IL-4, IL-10 and TNF-α (Carvalho *et al.,* 1994; Mattner *et al.,* 1997; Bogdan and Rollinghoff, 1998). Also PBMCs produce IL-10 mRNA the level of which correlates with pathology (Ghalib *et al.*,1995). The lack of IFN-γ production by PBMC during initial stages of infection predicts progression of disease, whereas individuals producing large amounts of IFN-γ remain asymptomatic (Reiner and Locksley, 1995). The IL-10 appears to inhibit Th1 cytokine production during active disease as treatment with anti-IL-10 resulted in raised IFN-γ and

proliferation (Ghalib *et al.*, 1993). Cure of VL is associated with a restoration of the ability to make IFN-γ and IL-12p40 (Bacellar *et al.*, 2000) and of lymphoproliferation but this was blocked by addition of recombinant IL-10 but not by IL-4 or TGF-β (Bacellar *et al.*, 2000).

So there is strong evidence that the presence of IL-10, a Th2 cell-associated cytokine (Mosmann and Coffman, 1989), is crucially involved in disease progression through its suppression of the secretion and/or host defence effects of activating cytokines, notably IFN-γ (Howard and O'Garra, 1992; Lehn *et al.*, 1989; Mosmann and Coffman, 1989; Silva *et al.*, 1992).

1.3.2 Murine model of visceral leishmaniasis

Many of the clinical features of the spectrum of human VL occur in murine models in which parasites target resident macrophages in the liver, spleen, and bone marrow but multiply predominantly in the liver and spleen. Different mouse strains vary in their ability to control infection due to genetic differences in innate and adaptive immune responses.

1.3.2.1 Infection in the liver

L. donovani multiplies in Küpffer cells, the resident macrophages of the liver and these provide the focus of small granulomas consisting predominantly of monocytes and T cells (Murray *et al.*, 1987, McElrath *et al.*, 1988). The parasite load and duration of infection vary markedly between different mouse strains but in the most commonly studied BALB/c mouse (regarded as a susceptible mouse) mature granulomas develop in the liver 21-28 days. Liver parasitic burdens in naïve BALB/c mice progressively increase, peak at 4weeks as resistance is acquired, and then decline to low levels at 8-9 weeks (Murray *et al.*, 1982; Murray *et al.*, 1987; Wilson *et al.*, 1996). This stage of disease is referred to as the chronic or recovery phase (Bradley, 1987). During this phase, parasites continue to proliferate in the spleen as described below (Smelt *et al.*, 1997; Rousseau *et al.*, 1997; Wilson *et al.*, 1996).

Nude and SCID mice cannot control *L. major* infection unless they are reconstituted with CD4+ T cells (Mitchell, 1983; Holaday *et al.*, 1991) and Belkaid *et al.* (2001) demonstrated the importance of both CD4+ and CD8+ for resolution and clearance of parasites using anti-CD4 and CD8 mAb. CD4+ T cells predominate in the formation of mature granulomas

but CD8+ T cells are more abundant later and both are necessary for granuloma development (Stern *et al.,* 1988). IFN-γ and it's principal inducer, IL-12, are necessary to direct the assembly of T cells and blood monocytes into granulomas that encircle infected macrophages; granuloma assembly and maturation (Murray, 2001a; Engwerda *et al.,* 1998; Taylor and Murray, 1997; Murray *et al.,* 2000; Satoskar *et al.,* 2000; Murray, 2001b).

1.3.2.2 Infection in the spleen

Systemic *L. donovani* infection induces major changes to the architecture and the splenic cellular immune response. At 1 week post infection *L. donovani* parasites are primarily localized and phagocytosed by marginal and metallophillic macrophages surrounding the red pulp and the marginal zone (Gorak *et al.,* 1998). There is an initial increase in the numbers of T cells and dendritic cells in the periarteriolar lymphoid sheath and marginal zone, but the red pulp where the abundant parasitized macrophages occur is not infiltrated by inflammatory cells until later in the course of infection. During this early phase, IL-10 production has been shown within the marginal zone and TGF-β production by macrophages in the red pulp (Melby *et al.,* 2001). It has been suggested that these macrophage-inhibitory cytokines may suppress the development of protective, macrophage activating cytokine responses and allow the infection to establish.

By day 28 of infection, when the visceral parasite burden starts to drop, the number of IL-10-producing spleen cells returns to the baseline level, but IFN-γ production was higher and the number of IL-12-producing cells was increased dramatically (Melby *et al.,* 2001; Engwerda *et al.,* 1998). It is believed that it is the interaction of T cells with the antigen presenting cells (dendritic cells) from the splenic PALS and marginal zone (Gorak *et al.,* 1998; de Smedt *et al.,* 1996) rather than the abundant parasitized macrophages from the red pulp which initiates protective T cell response (Banchereau and Steinman, 1998). T cells and dendritic cells then move out of the lymphoid follicle and marginal zone into the red pulp where they can reduce the splenic parasite burden (Melby *et al.,* 2001). Parasites proliferate in the spleen without inducing an inflammatory focus but splenomegly does occur with accumulation of fibrous tissue and mononuclear cell infiltrate (Saha *et al.,* 1991). Although some control is exerted over the spleen parasitaemia parasites do persist giving a chronic infection (Engwerda *et al.,* 1998; Gorak *et al.,* 1998; Smelt *et al.,* 1997; Wilson *et al.,* 1996).

Later on, breakdown of the white pulp structure, destruction of follicular dendritic cells and germinal centres (Zijlstra and el-Hassan, 2001; Smelt *et al.,* 1997). Engwerda and Kaye, (2000) suggests multiple mechanisms may be involved in the failure to eliminate the infection from the spleen: lack of inflammatory foci in the spleen; failure in recruitment of lymphocytes to infected cells due to lack of chemoattractants, lack of cytokines (IL-12); increased production of TGF-β leading to reduced capacity to generate (ROI, NO); and low levels of expression of MHC class II and competition between receptor-ligand pairs such as CTLA-4 for B7-1 and B7-2.

1.3.2.3 The murine immune response to visceral leishmaniasis:

1.3.2.3.1 Innate factors

Inbred strains were found to segregate into two groups (*Lshr* or *Lshs*) depending on whether they are resistant (C3H/HeJ, C57BL/6) or susceptible (BALB/c, and B10D2/n) to early replication of the parasite (Kaye *et al.,* 1988; Davies *et al.,* 1988; Lehmann *et al.,* 2000).

Gene encoding Nramp1 (natural resistant associated protein 1) which is present as two allelic forms, a dominant resistant phenotype and a recessive susceptible phenotype which control susceptibility to a variety of organisms including *L. donovani* (Blackwell *et al.,* 1996). Nramp1 has recently been shown to be a transporter of bivalent cations (Goswami *et al.,* 2001) which may have direct anti-microbial effects within the phagolysosome. MHC (H-2) genes also affect the outcome of infection by influencing the development of the adaptive immune response. H-2 d, f, q alleles confer late or non-curing phenotype, H-2 b an early curing and H-2 r, s rapid curing pheontypes (Blackwell *et al.,* 1980). Disruption of MHC class II-genes renders otherwise resistant C57BL/6 mice susceptible (Erb *et al.,* 1996; Chakkalath *et al.,* 1995). It is known that an otherwise susceptible host can be rendered resistant to *L. major* infection by virtue of strong Th1 induction through vaccination (Soong *et al.,* 1995) or direct transfer of Th1 cells (Reiner and Locksley, 1995).

1.3.2.3.2 Adaptive response

The key role of the adaptive immune response is shown by the fact that Nude (T cell deficient mice) and athymic mice (Miralles *et al.,* 1994) are unable to control the spread of

the infection and ultimately die unless reconstituted with CD4+ T cells (Murray *et al.*, 1987).

Inbred strains of mice show a range of patterns of infection kinetics and cytokine production. *L. donovani* infection in the liver induces mRNA expression of both Th1 (IFN-γ, IL-2, IL-12, TNF-α) and Th2 (IL-4 and IL-10) cytokines in the liver over the first 28days of infection (Engwerda *et al.*, 1996; Miralles *et al.*, 1994). However, the balance of these cytokines varies markedly between different inbred strains of mice and it is the capacity to produce IFN-γ which is correlated with resistance (Chatelain *et al.*, 1992). C3H/HeN or C57BL/6 mice, for example, mount a Th1 response with high levels of antigen specific IFN-γ and are able to cure the infection within weeks and are then completely resistant to reinfection. IL-12, which stimulates T cells and NK cells to produce IFN-γ (Trinchieri, 1993), plays a key role in the control of infection in this model (Engwerda *et al.*, 1998; Murray *et al.*, 1997). In contrast susceptible mice e.g. BALB/c mice produce moderate amounts of both Th1 (IFN-γ) and Th2 (IL-4 and IL-10) cytokines and show a delay in recovery (Locksley and Scott, 1991). However, with time, visceral parasite burdens are reduced by >85%, and thereafter these chronically infected animals are resistant to rechallenge (Miralles *et al.*, 1994). Although initially susceptible to infection, BALB/c mice proceed to develop a T cell-dependent immune response which results in control over visceral parasite replication (Murray *et al.*, 1987; Miralles *et al.*, 1994).

In the liver parenchymal Küpffer cells (KCs) become infected and as the immune response develops there is the formation of small lymphocyte and macrophage-rich granulomas around these amastigotes filled KCs. The Th1 cytokines, notably IFN-γ activate inflammatory macrophages and infected KC secrete TNF-α throughout the infection (Engwerda *et al.*, 1996). IFN-γ induces ROI production by granuloma macrophages and IFN-γ and TNF-α act together to induce expression of the type 2, inducible nitric oxide synthase (NOS2), expression of which can be demonstrated in *L. donovani*-infected tissues (Melby *et al.*, 1998; Taylor and Murray, 1997; Engwerda *et al.*, 1996). Use of gene knock out (KO) mice has shown that both ROI and RNI are necessary for the control of infection, ROI KOs showing delayed clearance but RNI KOs failing to control liver infection at all (Murray and Nathan, 1999). *In vitro* studies have also shown that Th1 cytokines notably IFN-γ can activate macrophages to kill ingested parasites via the production of reactive

oxygen intermediated (ROI), especially H_2O_2 (Murray, 1982; Murray *et al.*, 1983) or reactive nitrogen intermediates (RNI) (Roach *et al.,* 1993). TNF-α production also contributes to parasite killing by augmenting the production of reactive nitrogen intermediates by IFN-γ-primed macrophages (Theodos *et al.,* 1991). The essential role of TNF-α in the expression of macrophage anti-leishmanial activity is demonstrated by the fact that TNF-α deficient mice eventually die after *L. donovani* infection (Wilhelm *et al.,* 2001). Neutralisation of TNF-α also prevents control and resolution of infection although this does not affect the granuloma inflammation (Tumang *et al.,* 1994). IFN-γ also leads to increase in MHC class I and II expression and contributes to efficient antigen presentation to lymphocytes.

1.3.2.3.2 Modulation of the response by Th2 cytokines

The association of protracted infections with Th2 responses characterized by low IFN-γ and high IL-4, IL-5, IL-10 (Scott *et al.*, 1988, Heinzel *et al.,* 1989, 1991; Mosser and Karp, 1999) and TGF-β production (Barral-Netto *et al.,* 1992; Mosser and Karp, 1999) raises the question as to which are the key cytokines in this modulation.

1.3.2.3.2.1 IL-4

Confirming to the earlier data in human VL, neutralization of endogenous IL-4 *in vivo* by treatment with soluble murine IL-4 receptor did not result in significant decreases in the parasitic burdens in the spleen and liver but did cause a decrease in the serum IgE level of *L. donovani*-infected BALB/c mice (Miralles *et al.,* 1994; Satoskar *et al.,* 1995; Belkaid *et al.,* 2001). It was concluded that the capacity to produce IFN-γ rather than the presence of IL-4 determined the efficacy of the immune response in susceptible mice. This is in contrast to the role of IL-4 in *L. major* infections in which high levels of IL-4 induced by *L. major* is clearly a co-determinant of non-healing infections e.g. in BALB/c mice (Sadick *et al.*, 1990).

In agreement with its important role in human visceral leishmaniasis (Kaye *et al.,* 1991; Karp *et al.,* 1993, Ghalib *et al.,* 1993, Holaday *et al.,* 1993), IL-10 can also have a major influence on control of infection in mice. IL-10 is a immuno-modulatory cytokine, produced by a wide variety of cells, including activated Th2 cells, monocytes and

macrophages, B cells, thymocytes, and keratinocytes (De Waal Malefyt *et al.,* 1991; Fiorentino *et al.,* 1991; Go *et al.,* 1990; Mizuno *et al.*, 1994; Thompson-Snipes *et al.,* 1991). It plays a key role in deactivating macrophages by suppressing the afferent and/or efferent arms of the Th1 cell activation mechanism (Moore *et al.,* 2001; Groux *et al.,* 1999). The effects on the afferent arm include its ability to downregulate MHC class II molecules (Chang *et al.,* 1994) and costimultory molecules such as B7.1, B7.2, and ICAM-1 (Chang *et al.,* 1994; Willems *et al.,* 1994). Its effects on the efferent arm are also directed principally at inducing a state of macrophage deactivation (Bogdan *et al.,* 1991) either by making the effector macrophages hyporesponsive to activating stimuli, including cytokines, notaby IFN-γ, and/or by directly impairing the intracellular microbicidal effects (Bogdan *et al.,* 1991). So IL-10 inhibits O_2- generation (Bogdan *et al.,* 1991), TNF-α, NO generation in the parasitized macrophage (Bhattacharyya *et al.,* 1998; 2001b). Since the Th1-associated proinflammatory cytokine TNF-α acts as a triggering signal for NO generation (Green *et al.,* 1982) this further inhibits NO activity. Apart from its effects on macrophages it has recently been reported that repeated antigen-specific activation of both human and mouse CD4+ T cells in the presence of IL-10 results in the differentiation T regulatory cells (Tr1) (Ruedl *et al.,* 2000).

1.3.2.3.2.2 <u>IL-10</u>

IL-10 can effectively disable the afferent arm of the Th1 cell-associated responses that are required for induction and expression of cell mediated immunity involved in the control of various intracellular pathogens such as *Toxoplasma gondii* and *Trypanosoma cruzi* (Neyer *et al.,* 1997; Ghalib *et al.,* 1993; Gasim *et al.,* 1998; Hunter *et al.,* 1997). It is now firmly established that IL-10 has an important role in various *Leishmania* species i.e. *L. major* (Vieth *et al.,* 1994), *L. amazonesis* and *L. mexicana* (Padigel *et al.,* 2003) and also *L. donovani* (Bhattacharyya *et al.,* 2000c; Murray *et al.,* 2002).

Normal mice express IL-10 mRNA as part of the initial response to *L. donovani* (Miralles *et al.,* 1994) and recent studies of *L. donovani*-infected mice, firmly point to IL-10 as an important regulator of outcome. IL-10 KO mice showed enhanced anti-microbial action towards a variety of Leishmania species (e.g. *L. donovani*, *L. major*, and *L. amazonensis* (Moore *et al.,* 2001; Murphy *et al.,* 2001; Murray *et al.,* 2002; Belkaid *et al.,* 2001; Kane and Mosser, 2001; Padigel *et al.,* 2003), and is typically accompanied by increased

expression of activating cytokines (e.g. IL-12, IFN-γ, and/or TNF-α), induction of iNOS, and generalized inflammation (Moore *et al.,* 2001). The leishmanicidal activity against *L. donovani* in IL-10 KO animals clearly requires both endogenous IL-12 and IFN-γ, because treatment of KO mice with neutralizing anti-IL-12 and anti-IFN-γ monoclonal antibody did not fully restore parasite replication (Murphy *et al.,* 2001; Murray *et al.*, 2002). Similarly normal mice treated prophylactically with anti-IL-10R showed accelerated granuloma assembly and rapid parasite killing without untoward inflammation. There was increased IL-12 and IFN-γ mRNA expression, inducible nitric oxide synthase reactivity (Murray *et al.*, 2002). In a more recent publication, Murray *et al.* (2003) further demonstrated that IL-10R blockade maintained IL-12 protein 40, markedly increased IFN-γ serum levels, and enhanced tissue inducible nitric oxide synthase (iNOS) expression and granuloma assembly. Treatment of *in vitro L. donovani* infected macrophages with anti-IL-10R mAb restored the PKC activity in the parasitized macrophages, and caused direct reduction of parasitic burden (Bhattacharyya *et al.,* 2001a). Furthermore, preincubation of parasitized macrophages with neutralizing anti-IL-10R mAb significantly blocked the inhibition of nitric oxide, RIO and murine TNF-α release by the infected macrophages (Bhattacharyya *et al.,* 2001b).

It has been suggested that IL-10R blockade might be a potential immuno- and/or immuno-chemotherapeutic approach for this disease. Thus anti-IL-10R treatment in conjunction with antimony, allowed a substantial reduction (~10-fold) in the dose of antimony required to kill the majority of liver amastigotes (Murray *et al.,* 2000; 2001). In human leishmaniasis pentavalent antimony is administered daily over 28 days with significant toxic effects (Murray, 2001). Given its toxicity, reduction in either dose or duration of antimony therapy by co-administration of anti-IL-10R, might improve treatment.

Conversely, in IL-10 transgenic mice *L. donovani* parasite replication was unrestrained and the Th1 associated cytokines, IL-12 and IFN-γ were initially impaired and despite subsequent granuloma assembly, high levels of infection persisted (Murray *et al.,* 2002). A high level of splenic IL-10 expression was observed in the murine model of visceral leishmaniasis, which in turn contributed to the suppression of splenic T-cell function and was associated with multiplication of the visceral parasites (Wilson *et al.,* 1996).

In conclusion, production of IL-10 during *L. donovani* infection reduces effective clearance of the parasite whereas a deficiency of IL-10 allows the Th1 response to be highly effective in clearing the infection.

1.3.2.3.3 *Role of regulatory T cells*

There is growing evidence that regulatory T cells and, in particular, the endogenous $CD4^+CD25^+$ T cells (T reg cells) play fundamental role on control of *Leishmania* infections. Endogenous $CD4^+CD25^+$ T cells comprise 5-10% of peripheral CD4+ T cells in naïve mice and humans, and are believe to play in important role in suppressing potentially pathogenic responses *in vivo*, particularly T-cell responses directed against self-antigens (Shevach *et al.,* 2002). T reg functions reflect a number of features of the immune response in *Leishmania* infections e.g. high levels of IL-10 and transforming growth factor (TGF-β) and immuno-suppression (Thornton and Shevach, 1998; Papiernik *et al.,* 1998). Apart from their proposed role in autoimmunity there is now a realization that $CD4^+CD25^+$ T cells operate in infectious disease suppressing excessive Th2 responses controlling protective Th1 responses, allowing for parasite survival and perhaps as a consequence maintaining memory response in susceptible mouse strains.

A recent study showed the involvement of Treg in *L. major* infection. (Belkaid *et al.,* 2002). During *L. major* infection $CD4^+CD25^+$ T cells accumulate in the dermis where they apparently suppress the ability of $CD4^+CD25^-$ effector T cells to eliminate the parasite from the site due to both IL-10-dependent and IL-10-independent mechanisms. Despite this most parasites are eliminated but a few remain at the site and in the draining lymph nodes.

Most recent study, Mendez *et al.* (2004) demonstrated that depletion of CD25(+) cells at the time of secondary challenge prevented disease reactivation at the site of persistent infection while strengthening the expression of immunity at the site of secondary challenge. Finally, transfer of T reg cells purified from infected mice into chronically infected mice was sufficient to trigger disease reactivation and prevent the expression of an effector memory response.

However, large number of CD4+ve lymphocytes remain at the skin site and 40-50% show Treg phenotype expressing CD25 and CTLA-4 and low for CD45RB (Sakaguchi *et al.,*

1996; Thornton and Shevach, 1998; Piccirillo *et al.,* 2002). Such lesion-derived $CD4^+$ $CD25^-$ T cells inhibited IFN-γ production by antigen stimulated $CD4^+CD25^+$ T cells in the presence of *L. major*-infected macrophages but this was not reversed by the addition of anti-IL-10R although $CD4^+CD25^+$ T cells did release large amounts of IL-10. *In vivo*, transfer of $CD4^+CD25^+$ T cells plus $CD4^+CD25^-$ T cells to $RAG^{-/-}$ mice (lacking T and B cells) was able to prevent the resolution of *L. major* infection which was induced by $CD4^+CD25^-$ T cells transfer on its own. However, transfer of IL-$10^{-/-}$ $CD4^+CD25^-$ T cells healed faster and completely showing that IL-10 production by the Treg is essential for maintaining the infection. It was particularly interesting that induction of resolution of infection by treatment with IL-10R antibody during the chronic phase of infection were unable to resist reinfection showing that IL-10 induced maintenance of residual infection was necessary for maintenance of acquired immunity.

In humans, the more severe and non-healing forms of leishmaniasis are associated with IL-10 (Karp *et al.,* 1993; Gasim *et al.,* 1998), might be due to an imbalance in the number and activity of parasite-driven regulatory T cells. Consequent to the chronic infection and the potential for disease reactivation, parasite persistence itself provides a major benefit to the host by maintaining life long immunity to reinfection. In addition, the regulatory T cells seem to produce the paradoxical effects of suppressing effector T-cells functions locally, while maintaining a recirculating pool of tissue-seeking memory cells that confer powerful immunity to reinfection. This equilibrium that is established between effector and regulatory T cells in sites of chronic infection reflects the co-evolution of the host and parasite survival strategies.

1.3.2.3.4 Role of Th2 cytokines in polyamine production

Recently a non-immunological consequence of Th2 cytokine action on proliferation of *Leishmania* parasites was described (Iniesta *et al.,* 2001; 2002). This work was prompted by the knowledge that *Leishmania* infections induce both Th1 and Th2 responses; that these two differing cytokine groups induce alternate macrophage activation leading to iNOS or arginase 1 activation respectively; that arginase 1 leads to polyamine synthesis and that polyamines are required for the growth of all trypanosomatidae. It was shown that N(omega)-hydroxy-nor-L-arginine (nor-LOHA), a physiological inhibitor of arginase limits cellular infection in macrophages *in vitro* by *L. major* and *L. infantum* (Iniesta *et al.*, 2001).

It was subsequently shown that proliferation of *L. major* amastigotes in macrophage cultures could be triggered by IL-4, IL-10 and TGF-β and that this depends on arginase I induction which increased the pool of L-ornithine required for the production of polyamines needed for parasite replication.

Iniesta *et al.* (2001; 2002), demonstrated that arginase I induction in macrophages, triggered by Th2-type cytokines supports the growth of intracellular *Leishmania* amastigotes. This enzyme which synthesis *L-ornithine* which can be used by the parasite to generate polyamines and proliferate. This was done by treating *L. major* infected BALB/c macrophages with IL-4, IL-10 and TGF-β, which are all inducers of Arginase I in murine macrophages, led to increase in the number of intracellular amastigotes. Moreover, parasite proliferation and arginase activity levels in macrophages from the susceptible BALB/c mice were significantly higher than these from infected C57BL/6 cells when treated with identical doses of these cytokines, indicating that a strong correlation exist between the permissibility of the host cells to *L. major* infection and the induction of arginase I in macrophages. Therefore, arginase induction in the context of a Th2 predominant response might be a contributor to susceptibility in leishmaniasis (Iniesta *et al.*, 2002).

1.3.2.3.5 Latency is a direct result of imbalance of Th1/Th2 immune response

An imbalance in the Th1- and Th2-type cytokine responses may allow certain pathogenic organisms to modify the host immune response to favour their own persistence and replicate within the host to cause disease (Mosmann and Sad, 1996; Reiner and Seder, 1995; Trinchieri, 1997). Accordingly, live intracellular pathogens such as *Leishmania* (Weinheber *et al.*, 1998; Kane and Mosser, 2001), *Toxoplasma* (Bliss *et al.*, 2000), and *Mycobacterium* (Balcewicz-Sablinska *et al.*, 1999) modulate macrophage phenotype as effective immune evasion strategies. Moreover, Ahuja *et al.* (1998) demonstrated that infection/pulsing of human CD34+ peripheral blood hemopoietic progenitor cell-derived dendritic cells (DCs) with *L. donovani* promastigotes, *H. capsulatum*, and *M. kansasii* impairs the constitutive production of IL-12 from these cells.

The spleen and bone marrow can become sites of chronic infection, where parasites might survive for the life of the animal (Wilson *et al.*, 1996; Bradley and Kirkley, 1977). Nonetheless, the observations that dendritic cells (Moll *et al.*, 1995) and especially

fibroblasts (Bogdan *et al.*, 2000), harbour low numbers of amastigotes during latency have provided strong support for the concept of 'safe targets'. Most recently, the ability of pathogens to establish latency involve Treg cells, Belkaid *et al.* (2002) showed that the persistence of *L. major* in the skin after healing in resistant C57BL/6 mice is controlled by an endogenous population of $CD4^+CD25^+$ regulatory T cells. During infection by *L. major*, $CD4^+CD25^+$ T cells accumulate in the dermis, where they suppress-by both IL-10-dependent and IL-10-independent mechanisms-the ability of $CD4^+$ $CD25^-$ effector T cells to eliminate the parasite from the site.

Self cure is associated with the killing of more than 99% of the parasites in the skin, and the animals develop long lasting immunity to secondary challenge. With respect to the various clinical forms of leishmaniasis, low number of viable organisms persist within the lymphoid tissue and/or the site of former skin lesion after selfcure or successful chemotherapy (Ramirez and Guevara, 1997; Schubach *et al.,* 1998).

Such latent infection often give rise to severe forms of reactivation disease, including visceral leishmaniasis (VL) associated with, e.g. HIV co-infection (Alvar *et al.,* 1997); the development of post-kala azar dermal leishmaniasis (PKDL) after cure of VL (el-Hassan *et al.,* 1992); reactivation of former skin lesions, termed recidivans type (Momeni and Aminjavaheri, 1994); and the development of mucosal leishmaniasis months or years after healing of localized cutaneous ulcer (Saravia *et al.,* 1985).

An imbalance in the Th1-and Th2-cytokine responses may allow certain microbes to modify the host response to favor their persistence. Therapeutic strategies aimed at modulating a dysregulated Th1/Th2 response to infection, to secrete biologically active IL-12 and IFN-γ. Based on these observations, there has been significant interest in restoring and/or modulating the imbalance in the Th1 and Th2 responses by using cytokine/anticytokine therapies for recruiting pathogen-specific protective Th cell response (Badaro *et al.,* 1990; DiPiro, 1997).

A therapeutic strategy using genetically modified, microbial Ag-pulsed DCs, Ahuja *et al.* (1998) demonstrated in her study using retrovirus-mediated somatic gene transfer techniques to engineer human DCs to secrete biologically active IL-12 and IFN-γ. DCs pulsed with microbial antigens (*e.g.* leishmania and histoplasma Ags) were capable of

inducing proliferative responses in autologous CD4+ lymphocytes. CD4+ lymphocytes cocultured with IL-12-transduced autologous DCs had enhanced Ag-specific proliferative responses compared to CD4+ lymphocytes cocultured with nontransduced or IFN-γ-transduced DCs. These results indicate that IL-12-transduced DCs may be specifically suited in inducing or down-modulating Ag-specific Th1 or Th2 responses, and thus may be used as adjunctive therapy in those intracellular infections in which a dominant Th1 response is critical for the resolution of infection.

1.3.2.3.6 Leishmanial evasion of host immune response:

Recent studies, demonstrated that the sandfly saliva which contain salivary peptide maxadilan suppresses macrophage leishmanicidal activity, which inhibits tumour necrosis factor-α (TNF-α), nitric oxide (NO) production (Hall and Titus, 1995) leading to acceleration of lesion development (Lima and Titus, 1996). It has been demonstrated that the epidermal Langerhans cells *in vitro* due to the absence of inducible nitric oxide synthase (NOS-2, iNOS) in these cells neither allow the replication of intracellular parasites nor cause rapid parasite killing (Blank *et al.,* 1996).

The interaction between the parasite ligands and the host cell receptors can be both via direct binding or indirectly via parasite-associated host derived serum molecules (e.g. complement C3b/C3bi), fibronectin, C-reactive protein (Mosser and Rosenthal, 1993; Brittingham *et al.,* 1995; Culley *et al.,* 1996). These multiple receptor systems allow the parasite easy access into macrophages; these also facilitate access into Langerhans cells in the epidermis, where the parasites transform into amastigotes (Moll *et al.,* 1993; 1996).

Recently, Sutterwala *et al.* (1997) have showed that ligation of the FcγR by immune complexes not only inhibited the production of IL-12, but unlike complement receptor ligation, FcγR ligation also induced the synthesis and secretion of IL-10 only in cells containing a functional FcRγ-chain (Sutterwala *et al.,* 1998). Additionally, the *Leishmania* amastigotes have host-derived IgG on their surface (Guy and Belosevic, 1993, Pearson and Roberts, 1990), which act as opsonin for enhanced parasite adhesion for the macrophages was proposed (Peters *et al.,* 1995). Most recently, Kane and Mosser, (2001) demonstrated that host-derived surface IgG on *Leishmania* amastigotes allows them to ligate Fcγ receptors on the inflammatory macrophages to preferentially induce the production of high

amounts of IL-10, diminished their production of IL-12, and TNF-α and contributes to parasites growth in lesions (enhancement in parasite intracellular survival).

1.3.2.3.7 Leishmanial co-infections:

1.3.2.3.7.1 Viral infections

The prevalence of human immunodeficiency virus type 1 (HIV-1) and visceral leishmaniasis (VL) coinfection ranges between 2 and 9% in patients living in Mediterranean countries (Alvar *et al.,* 1997). Sicily in Italy has the highest number of cases of HIV and *L. infantum* coinfection in Europe after Spain, and with highest variety of zymodymes (Moreno-Camacho *et al.,* 1998). In this area, 25 to 75% of visceral leishmaniasis cases occur in HIV-infected patients (Gradoni *et al.,* 1996; Pintado *et al.* 2001; Alvar *et al.,* 1992).

Both subclinical and visceral leishmaniasis cases are frequent in HIV-1-seropositive individuals (Pineda *et al.,* 1996), and is often a consequence of a reactivation of a latent infection, and the majority of studies have been carried out with symptomatic patients. Because subclinical cases of this disease the previous studies could underestimate the true extent of *L. infantum* and HIV-1 coinfection in Europe. Pineda *et al.* (1998) carried out bone marrow aspiration to 291 HIV-1 carriers in south Spain regardless of their symptoms and, results showed that 11% of patients showed visceral leishmaniasis and 41% of patients had subclinical cases of infection.

Amastigotes multiply slowly in cutaneous and LN macrophages and starts invading multiple visceral sites due to progressive T cell immuno-suppression (Alvar, 1994). Dissemination of the parasites that are commonly dermotropic has occasionally reported in severely immuno-suppressed HIV-1 infected patients (Pineda *et al.,* 1998; Pratlong *et al.,* 1995). In contrast, Villanueva *et al.* (2000) and Kubar *et al.* (1998) demonstrated that many patients with *L. infantum* and HIV coinfection not receiving antiretroviral HAART therapy, do not develop VL despite severe and immuno-suppression. The clinical spectrum of VL, ranges from paucisymptomatic infections to disseminated forms with a typical locations of the parasite and frequent relapses despite appropriate treatment (Pineda *et al.,* 1998; Villanueva *et al.,* 1994; Rosenthal *et al.,* 1995; Lozano *et al.*, 1996).

The HIV coinfection can induce changes in clinical symptoms, response to treatment is lower in HIV-infected patients than immuno-competent patients (Piarroux *et al.,* 1994; Pintado *et al.,* 2001; Rosenthal *et al.,* 2000), increased high rates of visceral leishmaniasis relapse (between 25 to 61%) and mortality. However, Sanchez *et al.* (1999) ruled out any major difference in clinical manifestations or laboratory data between the two groups.

The incidence of concomitant opportunistic infections ranged from 42 to 68% of patients during any VL episode in the pre-HAART era (Rosenthal *et al.,* 1995; Medrano *et al.,* 1992), but the percentage of overall concomitant opportunistic infections was only 34% in the present series and was 17% in patients who had received HAART. It has been recently pointed out that active VL is associated with increased viral replication and that the pre-treatment HIV viral load influences the response to antileishmanial therapy (Berhe *et al.,* 1999; Preiser *et al.,* 1996), which impairs the patient's condition by further immuno-suppression (Cacopardo *et al.,* 1996). This might be partly due to Th2 immune activation, as demonstrated by high plasma levels (Cacopardo *et al.,* 1996) and from *in vitro* of PBMCs supernatant studies (Preiser *et al.,* 1996; Nigro *et al.,* 1999) demonstrated an increase in Th2 cytokines, IL-4, IL-6, and IL-10 in HIV-Leishmania co-infected patients than in HIV-alone infected individuals and the shift from Th1 to Th2 cytokine response in the coinfected individuals is irreversible (Cacopardo *et al.,* 1996). Multiple studies demonstrated the degree of the immuno-compromised state of *L. donovani*/HIV coinfection (Clerici and Shearer, 1993) that the shift from a Th1 to a Th2 cytokine pattern has been previously hypothesized to parallel or even precede the progressive CD4+ impairment that accompanies the transition towards the final stage of acquired immunodeficiency syndrome (AIDS).

The reason for this change in cytokine profile is due to impaired production of IL-12 (Chehemi *et al.,* 1994; 1995). Moreover, levels of IFN-γ production from PHA-stimulated PBMCs was significantly higher in the HIV-positive group compared to that of the HIV-*Leishmania* coinfected subjects. Multiple studies stressed on the importance of IL-4 plays a more prominent role in HIV/Leishmania coinfection than IL-10 (Nigro *et al.,* 1999; Kemp *et al.,* 1993). However, many other investigators demonstrated the importance of IL-4 in both *in vivo* and *in vitro* studies in VL without HIV coinfection (Sundar *et al.,* 1997;

Raziuddin *et al.,* 1994). Furthermore, during the natural course of HIV infection, IL-4 also appears to play a primary role (Clerici *et al.,* 1993; Navikas *et al.*, 1994).

It is also well known that highly active antiretroviral therapy (HAART) can modify the incidence of visceral leishmaniasis (Kaplan *et al.,* 2000; Palella *et al.,* 2003), clinical picture and response to specific drugs of some opportunistic infections. After long term HAART treatment causes improvement in monocyte-macrophage function and also in NK cells suggesting that their amelioration is needed to completely clear *Leishmania spp*. and prevent further relapses (Villaneuva *et al.,* 2000; Mastroianni *et al.,* 1999).

The impact of antiretroviral therapy (HAART) the evolution of visceral leishmaniasis (VL) in HIV-1 infected patients was disputed (Villanueva *et al.,* 2000), as the HAART neither prevents the incidence of VL relapse nor modifies the clinical picture described in the pre-HAART era despite the therapy decreased the viral load and decreased the incidence of opportunistic infections and dramatically increased the median of CD4+ T cell count/µl (Villanueva *et al.,* 2000). However, in another prospective study for 180 days, *Leishmania*-HIV co-infected patients not treated with HAART, the relapse rate was 80% (Laguna *et al.,* 1999).

1.3.2.3.7.2 Worm infections

Multiple studies demonstrated that leishmaniasis induces immuno-suppression and other superinfections is common. A study on human visceral leishmaniasis in area where *L. chagasi* is endemic (Zwingenberger *et al.,* 1990) disclosed that over 50% of the population was also infected by different helminthiases.

Rousseau *et al.* (1997) examined the influence of pre-infection with *T. spiralis* 7 days earlier, on the course of visceral leishmaniasis due to *L. infantum*, presented an increase in IFN-γ, IL-4, and IL-5 mRNA levels locally (in the liver and spleen) and systemically (in the blood). Eighteen days after *Leishmania* inoculation corresponding to the acute phase of leishmaniasis, the hepatic amastigotes burden in mice coinfection with *L. infantum* and *T. spiralis* (LT) was significantly lower than that in mice infected with *L. infantum* only (L mice). On day 70, corresponding to the chronic phase, the splenic amastigotes load was significantly lower in LT mice than it in the L mice. IFN-γ transcripts were over-expressed

in both L and LT mice. After *Leishmania*- specific *in vitro* stimulation, cytokine production was enhanced in both groups, but spleen cells from L mice produced significantly more IFN-γ than did spleen cells from LT mice. Rousseau *et al.* (1997) concluded that there is: (i) a lack of a clear-cut correlation between the outcome of murine visceral leishmaniasis and the type of cytokine pattern; as L mice showed high spleen parasitic burden in spite of high IFN-γ. (ii) demonstrate that in LT mice, leishmaniasis takes a milder course than it does in L mice, providing information on the potential consequence of coinfection in a mammalian host.

The previous study was in contrast to, experimentally induced Th2-associated response, in which BALB/c mice were presensitized with injections of heat-killed *L. major* promastigotes, which exacerbated visceral leishmaniasis (Murray *et al.*, 1997). Also, Sadick *et al.* (1991) found out that there was little effect on the course of *L. major* infection with coinfection with *Nippostrongylus brasiliensis*, despite the marked elevation of endogenous IL-4 levels.

1.3.2.3.7.3 Protozoal infections

However, in another model of coinfection (Oliveira *et al.,* 2000), *L. tropica*-infected BALB/c mice reportedly had the ability to partially inhibit *Toxoplasma gondii* replication *in vivo*. Santiago *et al.* (1999) showed that susceptible BALB/c mice previously infected with *T. gondii* 5days prior to challenge with *L. major* become protected against lesion development in the footpad. The basis of this *T. gondii*-induced highly polarized Th1 response independent of the host genetic background (e.g. BALB/c and C57BL/6 mice). The ability of *T. gondii* to trigger a Th1 response, the IL-12, IFN-γ synthesis (initially by NK cells and later by T lymphocytes) (Hayashi *et al.,* 1996), instead of Th2 response, in the BALB/c mice to control the Leishmanial infection. These results were similar to the ones observed in C57BL/6-resistant mice.

Moreover, Santiago *et al.* (1999) showed that even at 16 weeks postinfection, the dually infected animals showed no major lesions in the footpads infected with *L. major*. In contrast, in chronic toxoplasmosis showed normal skin lesions development when superinfected with *L. major*. However, PCR-specific for *L. major* GP63 DNA showed a

small difference of tissue parasitism in footpads from mice infected only with *L. major* and mice chronically infected *T. gondii* and coinfected with *L. major*.

Focus of this thesis

The above review describes the both *S. mansoni* and *L. donovani* induce Th1, Th2 and Treg responses which normally result in a finely balanced immune response which serves to contain/kill the parasite and minimize immuno-pathology. *S. mansoni* is characterized by a Th1>Th2 response and *L. donovani* by Th1>Th2 response and imbalances in both infections can cause severe disease. The liver is a focus of intense infection in both diseases with granulomatous responses around eggs or amastigote-infected Kupffer cells serving to contain/kill the parasites.

The overlaping geographic distribution of *S. mansoni* and *L. donovani* e.g. Sudan means that both infections are found in the same areas (el-Hassan and Zijrich, 2001) and co-infections are known to occur El-Hassan (personal communication) and this may result in compromised immunological control and exacerbated disease.

2.0 Aim of the study:

To investigate in a murine model the consequences for infection, pathology, and immune responsiveness of concomitant infections with *S. mansoni* and *L. donovani*. Studies focused on superinfection by *L. donovani* on established *S. mansoni* infections and *vice versa*.

Chapter 2

2.0 MATERIAL & METHODS

2.1 Mice

Outbred, CD-1 strain, Swiss albino mice Charles River, UK were used to maintain the Puerto Rican strain of *Schistosoma mansoni* life cycle. Five to eight-week-old C57BL/6 wild type mice were used in most of the experiments and either bred and maintained at the London School of Hygiene and Tropical Medicine (LSHTM). Biological Services Facilities according to the UK Home Office guidelines, or purchased from Charles River, UK. B-cell knock out mice on the same background (B6/μMT) were bred and maintained in the Biological Services Facilities of LSHTM. The animals were housed under specific pathogen-free conditions in the Category 3 containment section at LSHTM, London, UK and had free access to food and water. All experiments were performed under the regulations of the Home Office Scientific Procedures Act (1986).

2.2 Production of Cercariae and infection of mice

The Puerto Rican strain of *Schistosoma mansoni* was maintained in laboratory-reared *Biomphalaria glabrata* snails. The snails were kept in clean tap water at 24-26°C. Cercariae were shed in a minimum volume of clean tap water at 28-30°C under direct illumination for 1-2 hours. *S. mansoni* cercariae were counted and used for infecting mice within two hours of collection.

Mice were anaesthetized with sodium pentobarbitone (0.06 mg/g body weight *Sagatal*; May and Baker Ltd., Dagenham, England) and then percutaneously infected on the shaved belly by the 'Ring Method' of Smithers and Terry, (1965) as adapted by Doenhoff *et al.* (1978a). A suspension (0.2-0.5ml) of normal or irradiated *S. mansoni* cercariae was placed in a plastic ring retained on the skin of the belly region with the aid of transparent self-adhesive tape. This was left on the skin for about 20 minutes, at which time fluid and rings were removed and the animals allowed to recover under gentle warmth.

2.3. *L. donovani* infection

An Egyptian strain of *L. donovani* (LV9) maintained in Syrian hamsters was used and amastigotes for infection were prepared as previously described (Smelt *et al.*, 1997). Such parasites for infection were kindly prepared by Dr Chris Engwerda at LSHTM. For mouse infection $2x10^7$ amastigotes were injected into the lateral tail vein in 100μl of medium.

2.4 Techniques used in sample recovery

Because the organs were to be used for cell culture care was taken to carry out all procedures using aseptic techniques and sterile equipment. Mice were sacrificed with an intraperitoneal injection of 100μl pentobarbitone sodium solution (Sagatal) containing 10u/ml of sodium heparin [Sigma, UK]/mouse). At first, the animals were weighed and then the ventral body wall covering the abdomen and thorax removed. Blood was recovered from the heart using a 21g needle and syringe. The mouse was then suspended over a conical funnel leading into a 30ml Universal tube the hepatic portal vein cut and the portal system was perfused using sterile ice-cold perfusion buffer (10ml PBSx1, 25µl Heparin sulphate(5u/ml), 50µl 5mM Glucose, 10µl 0.5 M EDTA) injected into the left ventricle of the heart. The liver and spleen were removed and weighed.

Tissues specimens recovered and stored were as follows:

1- Blood collected from the heart (for Liver transaminase tests).

2- Perfusion fluid obtained through the cut portal vein for counting of adult *S. mansoni* worms.

3- A portion of liver, spleen and small and large intestines was stored in 10% formal saline for histology.

4- A portion of liver and spleen was stored in 100µl *RNA later* (Ambion, Inc) at -20°C for RNase protection assays.

5- A lobe of liver was stored at -20°C for determining the schistosome tissue egg count.

6- A piece of liver was fixed with OCT fluid (Raymond Lamb, UK) into labelled cork blocks, covered with OCT and dropped into isopropanol in a glass bottle in contact with liquid nitrogen. These were subsequently stored at -70°C and used for cryosectioning for immuno-histochemical staining

7- A small slice of liver and spleen was used to make impression smears for determining the *L. donovani* LDU count.

8- The remaining liver and spleen tissue was placed in complete culture medium on ice for use in i*n vitro* cell culture.

2.5. *L. donovani* impression smears

The freshly cut specimens of liver and spleen were held in forceps and pressed lightly onto filter paper (Whatman number 1) a few times to remove excess blood and then pressed lightly onto a glass slide several times making several impression smears. Tissue imprints were fixed with 95% methanol alcohol for one minute and stained with *Giemsa stain* (BDH Laboratory supplies, England, UK).

2.6 Parasitology assessments

2.6.1 Schistosome adult worm counts:

Perfusion fluid collected from each animal was allowed to settle and the supernatant removed, leaving around 1ml fluid. A few drops of saponin solution (BDH) were added to lyse red blood cells and this fluid was poured intoa girded 6cm Petri dishes and screened for the presence of adult schistosome worms using a dissecting microscope.

2.6.2 Liver egg counts

Liver tissues were weighed and digested in 10ml of 4% Potassium hydroxide overnight in a water bath at 37°C. After shaking 50µl samples were removed placed on a glass slide and the eggs were counted using the x40 objective of a compound microscope. Three separate counts were made for each specimen and then expressed as the number of eggs per whole liver.

2.6.3 Leishman Donovani Units (LDU)

The Geimsa stained liver and spleen imprints were examined microscopically using the x40 objective and the number of amastigotes counted relative to (1000) cell nuclei. Leishman Donovan units (LDU) for the organ were then calculated as the number of amastigotes per 1000 hepatic or spleen nuclei multiplied by the weight of the organ.

2.7 Antigen preparation and reagents used

2.7.1 Schistosome Antigens:

Soluble egg antigen (SEA) was kindly supplied by Dr. M. Doenhoff (University of Bangor). The lyophilised material was made up to 0.5mg/ml with PBS, centrifuged at 15,000 g, filter sterilized (0.22μm membrane filter, Sartorius) and aliquoted in 50μl aliquots which were stored at -20°C

SWAP was prepared from a frozen pellet of adult *S. mansoni* worms recovered from mice (provided by Dr Q. Bickle). This was homogenized in PBS using a glass tissue grinder and then centrifuged, filtered and aliquoted as described for the SEA. The schistosome antigen solutions were employed at concentrations of 20µg/ml for SEA and 10µg/ml for SWAP based on previous experience in the laboratory.

2.7.2 Formalin fixed Leishmanial amastigote antigen (FLAA):

Purified amastigote preparations (10^9 parasites/ml) were diluted one to one (v/v) with 4% neutral formalin and incubated for 30 min on ice, washed 3 times with PBS and resuspended in a small volume of PBS. After counting on a haemocytometer the concentration was adjusted to $2x10^8$/ml, an equal volume of sterile glycerol added and the parasites stored at -20°C. Before using the parasites were washed 3 times (3000 rpm x10 min) in RPMI and recounted. The parasites were kindly supplied by Dr. C. Engwerda; ITD, LSHTM, London, UK).

2.7.3 Mitogen:

Concanavalin A (Con A) (SigmaDiagnostic INC, St Louis, Mo, USA) was used as a mitogen at a final concentration of 5µg/ml in cRPMI.

2.8 *In vitro* cell cultures

2.8.1 Spleen cell cultures

Single spleen cell suspensions were prepared by gently pushing the spleen through a 100µm cell strainer (Becton Dickinson-Falcon, UK) into a petri dish containing

complete medium (cRPMI: - RPMI-1640 medium [Gibco, UK] supplemented with 10% heat-inactivated foetal calf serum [FCS, Sigma, UK), 1mM Sodium Pyruvate [Sigma, UK], 2mM L-glutamine [Sigma, UK], 50μM 2-Mercaptoethanol [Gibco, UK], 100U/ml penicillin and 100μg/ml streptomycin [Gibco, UK]). A 5ml syringe plunger to disrupt the tissue. The cell suspension was centrifuged at 1200 revolutions per minute (rpm), for 10 minutes at 4°C, and the supernatant discarded. The cells were suspended in 1ml of cRPMI and treated with 9 ml Gey's solution (Tris-buffered ammonium chloride solution) for lysis of splenic RBCs at room temperature for 7 minutes. The cells were then washed twice with cRPMI. Between the washes the cells were pelleted by centrifugation for 10 minutes at 1200 rpm. After counting the cells using haemocytometer, spleen cells were cultured at 37°C with 5% CO_2 in flat-bottomed 96 well microtiter plates (Nunc, Denmark) at $5x10^5$ cells in a final volume of 200µl per well, in the presence of medium alone, Con A (5µg/ml), SEA (20µg/ml), SWAP (100µg/ml) and FLAA (10^7/ml). All cultures are performed in triplicate. The cell culture supernatants were harvested at 24h for (IL-4) and 72h for (IFN-γ, IL-10 and TNF-α) (Rumbley *et al.*, 1999).

2.8.2 Liver lymphocyte preparation

Owing to the low recovery of lymphocytes, five livers were pooled per group. The liver was aseptically chopped into small pieces using a scalpel and digested in a 0.1% collagenase D solution (Sigma, UK) in cRPMI for 60 minutes in a polypropylene tube. The tube was placed on an rotatory wheel and mixed end over end mixer in an incubator at 37°C. Digested tissue were forced through a beaker covered over the top with sieve, to remove the large liver particles (mostly fibrose tissue). The cell suspension was centrifuged at 300rpm for 5minutes at RT to remove hepatocytes and undigested tissue. The retained supernatant was carefully collected, avoiding material from the pellet. The supernatant was pelleted and the cells washed three times in complete RPMI 1640 at 1200rpm for 10min. The pelleted cells were resuspended in 5ml cRPMI and carefully layered over 7ml histopaque-70 (Sigma, UK) supplemented with 5mM EDTA. This was spin at 1700 rpm for 15 minutes at RT with the brake off the centrifuge. The suspended cell layer which compromised as fussy ring layer above the heavy histopaque layer were carefully and completely collected with a pastette, diluted with cRPMI, washed three times in cRPMI and resuspended in 1ml cRPMI. Cells were counted using a

haemocytometer, the concentration adjusted and the cells cultured as for spleen cells at $5x10^5$/well in the presence of medium alone, Con A (5μg/ml), SEA (20μg/ml), SWAP (100μg/ml) and FLAA (10^7/ml).

2.9 Measurement of cytokine levels

Cytokine levels in culture supernatants were measured for IFN-γ, IL-4, and IL-10 using anti-cytokine antibodies in commercially available enzyme linked immuno-sorbant assay (ELISA) kits (OptEIA™ cytokine detection kits [PharMingen, San Diego, CA]) according to the manufacturers recommended protocols. Briefly, 96-well Nunc Maxisorp plates were coated with 50μl/well of the primary antibodies diluted in freshly prepared coating buffer (0.1 M Carbonate, pH 9.5), covered, and incubated overnight at 4°C. Plates were washed three times with PBS plus 0.05% Tween 20 (Fisher Scientific), blocked with assay diluent (PBS, with FCS, pH7.0) at 200μL/well at room temperature for 2h and then washed again. Standard curves were generated using recombinant cytokines, serially diluted over a range of 3-2000 pg/ml, and samples were added at 50μl/well. Plates were covered and incubated for 1 hours at room temperature. The secondary antibodies were diluted in assay diluent, added at 50μl/well and incubated for 1 hour at room temperature. Following washing with PBS plus 0.05% Tween 20 100μl/well of substrate solution (Tetramethylbenzidine [TMB] and Hydrogen peroxide [PharMingen, San Diego, CA]) was added to each well, and incubated for 30 minutes at room temperature in the dark. The reaction was stopped with 50μl/well with 1N sulphuric acid and the optical density read at an absorbance of 450 nm using an ELISA reader (MRX microplate reader, Dynex Technologies, UK) within 30 minutes of stopping the reaction. The cytokine levels in the samples were calculated according to the standard curve obtained for each ELISA plate.

2.10. Measurement of nitrite levels by the Greiss assay (Nitric oxide estimation)

Griess reagent (1% sulphonamide, 0.1% napthylethylenediamine HCL, 2.5% orthophosphoric acid) was prepared in milli-Q water and dissolved for approximately 1hour at 37°C. Sodium nitrite (Sigma) standards were prepared in cRPMI (starting at 10μg/ml-5μg/ml-2.5μg/ml…*etc.*). 50μl samples of test supernatants and of nitrite standards were added to wells of 96well plates (Nunc, Denmark) and 50 μl of Greiss

reagent added. After 5 minutes at room temperature the optical density was read at 540nm, using a microplate reader (DYNEX) and the nitrite concentration determined relative to the nitrite standard curve.

2.11. Preparation of anti-IL-10R monoclonal antibody (mAb).

2.11.1 Culture of the 1B1.3A cell line

The anti-IL-10R producing ATCC cell line (1B1.3A) (Murray *et al.*, 2002) was cultured at 37°C with 5% CO_2 in cRPMI. Tissue culture supernatants were harvested in log phase growth, cells removed by centrifugation, and the supernatant collected. 0.01% sodium azide (NaN_3) was added to the supernatants which were then stored in 4°C. Three litres of the culture fluid were accumulated.

Supernatants are kept on ice in sterile flasks then Set the tubing into Persista pump sterilizing the tubing by running 70% ethenol into and outside the tubing. Attached to the (single path monitor UV-1 optical unit),

2.11.2 Purification using Protein G affinity Chromatography:

Cell culture supernatant was filtered using Whatman number 1 filter paper and then filter sterilized using 0.2μm filters (Nalgene). The supernatant was kept on ice.

Protein G Sepharose Fast Flow gel (Pharmacia Biotech, UK) was packed in a plastic column (Sigma, UK) which was connected via a Persista pump through a UV detection unit set at 280nm. The columns was equilibrated by running 5 column volumes of Running Buffer (0.02 M phosphate buffer, pH 7.0) through it. The supernatant was then slowly passed through the column at 4°C. The column was washed with Running buffer until the OD_{280} readings had returned to baseline. At this point Elution Buffer was run through the column (0.1 M glycine-HCl, pH 3.0-2.7) to dissociate specific IgG. The recovery was monitored and elute containing significant concentration of protein was collected. To preserve the activity of acid labile IgG 50μl of 1M Tris-HCl, pH 9.0 (Neutralizing Buffer) was added to every 1ml of elute collected to bring the pH to 7.0. The antibody fractions were pooled and dialysed against 0.09% NaCl for 72 hours at 4°C. The dialysis buffer was changed daily. The sample was then freeze-dried and resuspended in 1/10th the original volume with sterile distilled water. This was mixed

well and filter sterilized using 0.2μm filter (Pall Gelman Lab., UK). The concentration of the antibody was calculated by diluting antibody in distilled water, measuring the optical density at 280nm using a spectrophotometer (UV-1201, spectrophotometer, Shimadzu, UK) and then using the following formula:

$$\text{Concentration of sample} = \frac{\text{Optical Density at 280nm}}{\text{Extinction Coefficient at 280nm}} \times 10 \text{ mg/ml} \times \text{dilution factor.}$$

The antibody was kept at -20°C, until required.

In this way 54mg of 1.B.1.3A was obtained for use in the *in vivo* depletion experiments.

2.12. *In vivo* treatment with anti-IL-10R antibody.

To inhibit IL-10R function mice were injected intraperitoneally with 1mg 1B1.3A antibody/day on day +42 and 0.5mg on days +46, +49,+ 52 and +55. The likely efficacy of this regimen was based on the protocol used by Phillips *et al.*, (2001).

2.13. Plasma transminase assay

Mouse serum samples had been stored at -20°C. Sera from 5 mice per group were tested for liver function test at various time-points, +2, +4, +8wk post-infection with *L. donovani*. The presence of the liver-associated enzymes, aspartate aminotransaminase (AST) and Alanine Aminotransaminase (ALT) were determined using commercial kit EC 2.6.1.2 UV-TEST (procedure NO. DG159-UV) for ALT, (procedure NO. DG158-UV) for AST (Sigma Diagnostic INC, St Louis, USA) as previously described (Winefield *et al.*, 1995). Briefly, 20µl of sera were added to 100µl of 0.2 M DL-aspartate and 1.8 mM α-ketoglutaric acid in phosphate buffered saline (pH 7.5) (in measuring AST) and in measuring ALT (ALT reagent A) L-Alanine 1040 mmol/L, and 70.4µl (ALT Reagent B) 2-oxoglutarate. The solutions were mixed and incubated at 37°C for 1h and then 100µl of 2,4-diphenylhydrazine was added and the mixture was incubated for 20 min at room temperature. The reaction was stopped with 1ml of 0.4 N NaOH and the optical density was measured at 340nm using a Lambda 5 spectrophotometer.

2.14. Histopathology

Specimens from liver, spleen and large intestines, fixed and stored in 10% formalin saline, were embedded in paraffin wax blocks, sectioned at 6μm thickness and stained with hematoxylin and eosin.

2.14.1 Schistosome granuloma observations

For comparison of the *S. mansoni* egg granulomas between *S. mansoni* infected mice and mice with *S. mansoni* and *L. donovani* co-infections liver sections were prepared from mice with comparable egg burdens. The stained sections were systematically scanned field by adjacent field and each granuloma that met the criteria were measured. The granulomas selected were those which contained an egg with a morphologically distinguishable miracidia inside. These criteria meant that, on average, the granulomas would have been sectioned close to its centre and would not have been an old resolving granulomas in which the miracidia had been killed and the antigenic stimulus removed. Twenty one such schistosome granulomas per infected mouse were assessed. The longest diameter was measured using an ocular micrometer and the diameter at 90° to this was also recorded. The mean of these two measures was taken and used to calculate the granulomas volume assuming a spherical shape. The proportion of eosinophils, mononuclear cells and fibroblasts making up each granuloma were estimated and recorded.

2.14.2 L. donovani granuloma observations

L. donovani granulomas were characterised in haematoxylin and eosin stained sections as described by Murray. (2001), Murray *et al.* (2002). As the amastigotes multiply within the Küpffer cell (KC) so the granuloma develops through identifiable stages which have been characterized as:

Type of *L. donovani* granulomas	Cellular reactions around the granuloma
Infected Küpffer cell:	one or more than one adjacent infected cell with no associated inflammatory cells
Fused Küpffer cell:	Fusion of two or more infected KCs without adjacent inflammatory cells
Immature granuloma:	Infected KCs incompletely surrounded with more than 3 and less than 10 cells adjacent cells
Mature granuloma:	Infected KCs completely surrounded with more than 10 cells
Sterile granuloma:	Collection of inflammatory cells with no amastigotes

Fields of sectioned tissue were examined and any foci containing amastigotes were recorded according to the above categorization of granulomas. The numbers of visible amastigotes present was also recorded. In this way data was collected from 30 fields/mouse in the parenchyma and from similar numbers of fields inside the schistosome granuloma. In analysis of *L. donovani* granulomas an important population are those surrounded by inflammatory cells but in which the amastigotes have been killed *i.e.* "empty" granulomas. Unfortunately it was clear from sectioning of mice infected with only *S. mansoni* that morphologically similar inflammatory foci were also formed around Küpffer cells containing schistosome pigment. So in the Co-infected mice it was not possible to be certain what was an empty *L. donovani* granuloma or a "pigment reaction".

2.15. Immuno-histochemical staining of amastigotes with chromogenic substrates

Initial studies used chromogenic substrates to try to stain amastigotes to distinguish them from schistosome pigment which caused a problem in the slides initially stained with haematoxylin and eosin.. Cryosections were cut from frozen liver specimens at 6μm using a cryostat and placed on polylysine slides (BDH) which were then frozen at -20°C until use. For staining sections were fixed with ice-cold acetone for 10 minutes and then hydrated for 5 minutes in PBS in a coplin jar on a shaker. Fix with paraformaldehyde 2% for 10 minutes. They were then blocked with Ammonium chloride at 50μM for 10 minutes. They were washed again with PBS for 5 minutes and dried. The sections were circled with a silicone pen to prevent liquid spreading from one section to another and the sections blocked with 50μl per section of 1.5% normal goat serum (Sigma, UK) in PBS for 20 minutes. The slides were dried and incubated with 50μl per section of primary antibody (Hamster anti-LV9 serum or control normal hamster serum at 1:100 dilution and obtained by Prof. P. Kaye, LSHTM, London, UK) for 1 hour at room temperature. The slides were then washed with PBS for 5 minutes, dried and the secondary antibody added. For the immuno-histochemistry this was a peroxidase labelled goat anti-hamster antibody (Kirkegaard and Perry Ltd, USA) at a dilution of 1:500 for 1hour at room temperature. After staining the slides were washed 3x in PBS. To obtain a colour that could be distinguished from the schistosome pigment various substrates were tried. *i.e.* pink colour with Vector V.I.P. peroxidase substrate kit (Vector laboratories, Peterborough,UK), Red colour using peroxidase substrate kit AEC

SK-4200 (Vector laboratories, Peterborough,UK), and/or brown colour using peroxidase substrate kit DAB SK (Vector laboratories, Peterborough,UK). Although amastigotes which were free in the clear cytoplasm could be distinguished with these substrates the staining was either not strong enough or sufficiently different in colour from the haematin pigment to be of use.

2.16. Immuno-fluorescent staining of amastigotes

This was essentially similar to the method described above up to the incubation in primary antibody. After this the slides were washed with PBS for 5 minutes and the secondary antibody was added (Goat anti-hamster IgG; ALEXA-488-FITC; 1:250 dilution) (pharMingen, San Diego, CA) and incubated for 1 hour at room temperature in the dark in a humidified chamber. The slides were washed again in PBS for 5 minutes in the dark. The slides were dried and 10μl propodium iodide (Molecular Probes, Inc, USA) added per section and incubated in the dark for 20 minutes. After washing again with PBS for 5 minutes the slides were dried and 4μl per section of aqueous mounting medium with DAPI [Vectasheild mounting medium for fluorescence (Vector laboratories, Inc. Burlingame, CA 94010] added. A cover slide was placed over each the sections (avoiding air bubbles), the edges sealed with nail varnish and the slides left to dry in the dark after which they were further stored at 4°C. Sections were viewed using a Zeiss Axioplan immuno-fluorescence microscope at x400 magnification power in the dark. Images were recorded using a digital computerised camera (Optronics). Amastigotes were visualized as sharp green florescent spots and propidium iodide localized to the nuclei of surrounding inflammatory cells appeared red under UV light. This protocol was optimised using sections from mice with separate *S. mansoni* and *L. donovani* infections to avoid auto-fluorescence of haematin granules and to give clear distinction between amastigotes stained with immune hamster serum compared with control sera. The propidium iodide staining allowed the type of *L. donovani* granuloma to be characterised, according to the number of surrounding cells and whether they were completely surrounding the amastigotes, as infected Küpffer cells (KCs), fused KCs, immature granulomas, and mature granulomas (Murray, 2001; Murray *et al.*, 2002). For quantitation thirty adjacent fields were examined for each mouse, and the percentage of reactions of each type were determined for each mouse. The number of amastigotes/focus in the different inflammatory foci was also recorded.

Chapter 3.

Interaction between an established *S. mansoni* infection and a superimposed *L. donovani* infection – *Parasitological and Immunological observations.*

3.1. Introduction

Worm infections affect over a third of the world's population mainly in developing countries and infected individuals commonly harbour other infectious agents ranging from viruses to other species of worms. With an estimated 200 million (WHO, 1985; Doumenge and Mott, 1984; Bergquist. 2002) people infected with schistosomes a large number of people would be harbouring co-infections. Apart from the pathological burden imposed by multiple infections it is increasingly clear that worm infections in particular can have deleterious effects on the pathogenesis and control of certain concurrent infections. In recent years emphasis has focussed on the modulatory effects schistosome infections have on the development of immunity to concurrent infections and other ongoing immune responses (Cox, 2001). The present study was concerned with the effects of *Schistosoma mansoni* and *Leishmania donovani* co-infections. These two infections are found together in certain area of the world *e.g.* southern eastern borders of Sudan (el-Hassan 'personal communication') and the likelihood of co-infections in humans is increased with the displacement and movement of people between areas endemic for these infections (Zijrich and el-Hassan, 2001). A further reason for interest is that both infections develop in the liver, *L. donovani* requiring formation of a Th1 dependent granulomatous response around amastigote-infected macrophages and *S. mansoni* being characterised by Th2 dependent egg granulomas. In this chapter, two repeat co-infection studies in mice focused on the effect an established (8 week) *S. mansoni* infection has on a super-imposed *L. donovani* infection and specifically on the effects on parasite loads and on the immune responses stimulated. In susceptible strains of mice such as C57BL/6 used in these studies, control of the *L. donovani* infection and subsequent resistance to re-infection is mediated via a Th1-cell dependent, macrophage-activating mechanism involving IL-12 induction of IFN-γ leading to the production of

parasite killing by reactive oxygen and nitrogen intermediates (Murray *et al.*, 2001; Engwerda *et al.*, 1998; Satoskar *et al.*, 2000; Wilson *et al.*, 1996; Melby *et al.*, 1998; Taylor and Murray, 1997; Murray and Nathan, 1999). In contrast, along with other worm infections, *S. mansoni* infection is characterised by induction of a Th2 biased response (Grzych *et al.*, 1991) which is associated with a number of immuno-modulatory processes which could influence the development of the protective Th1 response to leishmaniasis in a variety of way.

The key cells are the Th (T helper cells) lymphocytes. Such cells are initially uncommitted but they gradually differentiate into Th1 and Th2 cells. Th1 and Th2 subsets of T helper cells were initially defined on the basis of differential cytokine production by Th cell clones, IL-2 and IFN-γ for Th1 clones and IL-4 and IL-5 for Th2 clones (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989; Swain *et al.*, 1991). It is known that the different subsets develop from the same T cell precursor (Hseih *et al.*, 1993; Rocken *et al.*, 1992; Paul and Seder, 1994) and the best understood influence on Th1 commitment are cytokines present at the time of antigen presentation during APC/T precursor cell interaction *i.e.* IL-12 (from DC and macrophages) and IFN-γ (from NK cells) (Hseih *et al.*, 1993; Manetti *et al.*, 1993; Macatonia *et al.*, 1995). Thus, Th1 responses are markedly reduced in IL-$12^{-/-}$ mice (Magram *et al.*, 1996) and in mice depleted of STAT4 (signal transducer and activator of transcription 4) (Kaplan *et al.*, 1996) which is activated by IL-12 signalling (Jacobson *et al.*, 1995). The influences on the development of Th2 responses are hotly debated but recent studies on *S. mansoni* demonstrate that egg antigen which is able to prime DCs for production of Th2 responses does not activate DCs to produce known cytokines (MacDonald *et al.*, 2001) and it has recently been suggested that the Th1/Th2 commitment could be related to differential uptake of antigen into separate antigen processing compartments leading to differential antigen presentation of Th1 and Th2 inducing antigens (Cervi *et al.*, 2004). The committed Th1 and Th2 populations themselves then preferentially produce IFN-γ and IL-4 respectively and these cytokines are also able promote the further development and function of the same subset but inhibit the other subset. Thus, IFN-γ promotes Th1 development by up-regulating IL-12 production by macrophages (Trinchieri, 1995) and

maintaining functional IL-12 receptor expression on Th1 cells (Szabo *et al.,* 1997) but inhibits development of Th2 cells (Fitch *et al.*, 1993; Maggi *et al.*, 1992). Conversely IL-4 is a potent inducer of differentiation of Th precursor cells to Th2 effectors (Maggi *et al.,* 1992) which act as growth factors to expand the Th2 response and can down-regulate IL-12Rβ2 expression on Th2 cells making them unresponsive to IL-12 (Rogge *et al.*, 1997; Szabo *et al.,* 1997). Also IL-10 which is associated with Th2 responses and worm infections inhibits Th1 development and activation (Sher *et al.,* 1992, Moore *et al.*, 1993; 2001). Both IL-10 and IL-4 can inhibit dendritic cell function by inhibiting IL-12 production in immature DCs (Kalinski *et al.*, 1998) and also their development so reducing their stimulatory potential and inducing a tolerogenic type of DC (Steinbrink *et al.*, 1997).

The onset of patency in *Schistosoma mansoni*-infected mice at 5-6 weeks post infection induces a down-regulation in parasite antigen- and mitogen- induced Th1 cytokine secretion (IL-2 and IFN-γ) with a simultaneous increase in the production of Th2 cytokines (IL-4, IL-5) and also IL-10 (Grzych *et al.*, 1991). That this was due to the eggs themselves was shown by injection of eggs in the lung of recipient mice leading development of "synchronous pulmonary granuloma" model (Wynn *et al.,* 1993; 1997). Th2 responses are also common in infected humans (Snyman *et al.*, 1997; Souza-Atta *et al.,* 1999; Hagan, 1996), although the level of Th2 responsiveness is influenced by other factors such as host genetics. For example the Sm2 gene, which is responsible for familial hepatic fibrosis and portal hypertension, is located in 6q22-q23, a region that contains the gene that encodes IFN-γ receptor 1 (IFN-γ R1) (Dessein *et al.*, 1999). Although the Th2 cytokines, IL-4 and IL-13, result in collagen synthesis around the parasite eggs lodged in the tissues (Fallon *et al.,* 2000), which underlies the pathogenesis of the disease, the Th2 response is regarded as protective as its absence *e.g.* in IL-4$^{-/-}$ IL-4/IL-10$^{-/-}$ or IL-4/IL-13$^{-/-}$ mice (Brunet *et al.*, 1999, La Flamme *et al.*, 2001; Hoffmann *et al.*, 2000; Fallon *et al.*, 2000) results in a switch to Th1 inflammatory responses to the persistent egg antigen stimulation during acute infection inducing damaging inflammatory disease characterised by high levels of IFN-γ, TNF-α and nitric oxide (Cheever *et al.*, 2000; Fallon, 2000).

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Similarly, severe hepatosplenic schistosomiasis in humans is associated with highly elevated IFN-γ, NO and TNF-α (Dunne and Pearce, 1999). So the generation of a Th2 bias to the egg is preferable to pro-inflammatory Th1 responsiveness although Th1 responses to egg antigens persist in a balanced state and exert a regulatory effect on the Th2 response as shown by the increase in granuloma size, severe fibrosis and marked mortality in mice treated with anti-IFN-γ (Luckacs and Boros, 1993).

Whether the concentrations of IL-4 and IL-10 would be sufficient to influence DC activation at the time of presentation of antigen from a co-infecting organism would be influenced by various factors such at the level of infection and the specific location of the IL-4 production in schistosome infection in relation to the prime site(s) of antigen presentation in induction of Th1 responses to co-infecting organisms. In the case of *S. mansoni*, egg granulomas are formed predominantly in the liver and the gut but also in the spleen and lungs. It is clear that Th2 cells able to produce Th2 cytokines in response to specific antigen re-stimulation are found in the spleen (Grzych *et al.*, 1991) but also within the egg granulomas themselves as has been shown from studies on cells isolated from granulomas (Kaplan *et al.*, 1998; Hayashi *et al.*, 1999; King *et al.*, 2001). Apart from the Th2 cells recruited to embolised eggs, the high percentage of eosinophils present in the granulomas are a source of IL-4 and Rumbley *et al.* (1999) reported that activated eosinophils comprise the majority of cytokine producing cells in the granuloma and are the dominant source of IL-4. Thus in the liver there would be high concentrations of Th2 cytokines associated with the granulomas and this could also have direct effects on the generation/function of the granulomas which develop around macrophages infected with *L. donovani.* It is conceivable also that IL-4 production by schistosome granulomas formed in the spleen could affect Th1 cell priming to antigens presented predominantly in the spleen inducing a Th1 to Th2 bias in the response.

S. mansoni infections themselves are subject to immune modulation. Thus after the eighth week of infection in mice there is a down-modulation of the granulomatous response around newly produced eggs (Andrade and Warren, 1964; Chensue and Boros, 1979) such that in chronic infections (variously defined as 15 to 20 weeks post infection) Th2

responses are down-regulated (Grzych *et al.*, 1991; Henderson *et al.*, 1992: Chensue *et al.*, 1992). Numerous mechanisms have been proposed for this immuno-modulation but the reciprocal inhibitory effects of the dual but Th2 dominated (Th2>Th1) response mediated by IL-4 and IFN-γ together with inhibition of both responses by IL-10 (Sadler *et al.*, 2003) are believed to be important in mediating a generally anergic state with down-regulation of both Th2 and Th1 responses (Chensue *et al.*, 1994; Jankovic and Sher, 1996; Wynn *et al.*, 1997; Boros and Whitfield, 1998). Apoptosis of sensitized lymphocytes in the granuloma has also been reported and suggested to account for modulation (Lundy *et al.*, 2001; Rumbley *et al.*, 2001) and IL-4 and IL-10 induction of Fas ligand expressing B-1 a lymphocytes have been reported to mediate CD4 apoptosis (Lundy and Boros, 2002). Whatever the mechanism of this down-modulation of immune responsiveness to the schistosome egg antigen, there could be bystander consequences for *L. donovani* infections especially in the liver. A key function of IL-10 is the down-regulation of macrophage function e.g. reducing antigen presentation, inhibiting expression of co-stimulatory molecules and also reducing microbicidal functions (Marshall *et al.*, 2001; MacDonald *et al.*, 2001; Bogdan and Nathan, 1991; Moore and O'Garra, 2001).

Macrophages with suppressive properties rather than stimulatory properties have recently been described and appear to be of two distinct types 'classically activated' macrophages which are IFN-γ dependent and generally described in relation to cancers and viral infections (Young *et al.*, 1996) and those activated by IL-4 and IL-13 to produce arginase and so are diverted from the production of NO (Murata *et al.*, 2002; Rodriguez-sosa 2002). Such cells have been demonstrated in mice in response to implanting the filarial worm *Brugia malayi*i in the peritoneal cavity (Allen *et al.*, 1996; MacDonald *et al.*, 1998) These develop profound Th2 responses with recruitment of high numbers of eosinophils and macrophages to the site of parasite infection (Falcone *et et al.*, 2001), parallelled with suppression in the proliferative response (Loke *et al.*, 2000).

Both types of suppressor macrophages have been implicated in schistosome infections (Classically activated - Atochina *et al.*, [2001] and aaMø – Linehan *et al.*, 2003). Herbert

et al. (2004) showed that the anti-inflammatory phenotype of aaMø is crucial to modulating severe inflammatory disease in the liver. The role these cells may have in the course of *S. mansoni/L. donovani* co-infections is considered more extensively in Chapter 4 but from the aspect of immuno-modulation they are likely to suppress generation of protective responses by suppressing cellular proliferation (Loke *et al.*, 2000) and by showing reduced ability to present antigen and to express co-stimulatory molecules (Jankovic *et al.*, 2004; MacDonald *et al.*, 2001).

The operation of CD8+ suppressor T cells has been suggested as a mechanism of endogenous desensitization in schistosomiasis (Chensue and Boros, 1979) but recently attention in the area of suppressor T cells has been focused on naturally occurring regulatory CD4+ cells. These cells, originally defined as $CD4^+CD25^+$ have since been defined by the additional markers (GITR, CTLA-4 and FoxP3 [Gavin and Rudensky, 2003]). Although their importance in regulating auto-immunity has been emphasized they have also been shown to influence the development of immunity to infection as in *L. major* infection. Almost half of the CD4+ T cells in *L. major* lesions were $CD4^+CD25^+$ Tr cells some of which were *L. major* antigen specific (presumed to be cross reacting with host tissue epitopes against which these Tr cells are targeted). The cells are believed to derive from the natural pre-immune population pool of $CD4^+CD25^+$ Tr cells and to be able to reduce tissue damage but promote parasite persistence due to IL-10 dependent cellular suppression (Belkaid *et al.*, 2002). In doing so, they may suppress sterilizing immunity but help to maintain long term T-cell memory and functional immunity. In studies of auto-immunity suppression of immune activation has been shown to be mediated by IL-10 and TGF-β (Gavin and Rudensky, 2003). Such cells have not been described in egg-induced schistosome granuloma described as expressing $CD4^+CD25^+$ T cells and demonstrates the major source of IL-10 production and exhibited potent suppressive activity *in vitro* (Hesse *et al.*, 2004) but they could make a significant contribution, possibly along with Th2 cells to the IL-10 seen in the infection and if they were similarly recruited to sites of granulomatous responses could also mediate the contact-dependent suppression which they have also been shown to mediate at least *in vitro* (Wood and Sakaguchi, 2003).

In addition to systemic immuno-modulatory effects of schistosome infection, the sites of infection provide an altered cellular environment for co-infecting organisms which may be particularly relevant for intracellular organisms. In the case of *S. mansoni* infection this is particularly relevant for organisms co-infecting the liver which is the prime site for the egg granulomas which result in the recruitment/generation of large numbers of T cells, eosinophils, fibroblasts and macrophages into the organ. For example, the granuloma comprises around 30% macrophages (Oswald *et al.,* 1993; Andrade and Cheever, 1995) providing alternative sites for the invasion of organisms which live inside macrophages *e.g. Leishmania* species.

The immuno-modulatory effect of *S. mansoni* infection was first investigated using a single antigen. Acutely *S. mansoni* infected mice were injected with sperm whale myoglobin (SwMb), an antigen which typically induces strong Th1 responses in normal uninfected mice (Kullberg *et al.*, 1992; 1996). It was found that splenocytes from schistosome-infected SwMb-immunized animals produced significantly lower Th1 cytokines (IL-2 and IFN-γ) and three fold higher IL-4 per CD4+ cell compared with immunized uninfected mice. Although this could be due simply to IL-4 produced by the schistosome infection suppressing the development of Th1 cells and promoting Th2 cells it was found that addition to the cultures of neutralizing mAb to IL-10 partly restored the suppressed IFN-γ response to SwMb seen in infected mice, suggesting a role for IL-10 in the observed down-regulation and this could come from Treg cells. An essentially similar pattern of skewed response was seen in Tetanus-Toxoid (TT) immunization *i.e.* following primary TT immunization specific IFN-γ production was shown to be reduced in *S. mansoni*-infected individuals and inversely related to infection intensity (Sabin *et al.*, 1996). In contrast IL-4 levels did not show this inhibition.

Altered responses have also been observed to viruses, bacteria, protozoa and other worms and the results attributed principally to the counter-regulatory effects of Th2 cytokines (notably IL-4) on the induction of the usual Th1 responses. Thus, *S. mansoni*-infected mice showed delayed clearance in *S. mansoni*-infected mice exposed to recombinant *Vaccinia* virus. They showed decreased virus-specific IFN-γ production and cytotoxic T

cell responses (particularly CD8+ T cells) and a slight increase in IL-4 and IL-5 to virus antigen. It was suggested that egg granulomas, by providing a micro-environment for viral expression, in combination with the cytokine imbalance present during schistosome infection, can promote the expansion of *vaccinia virus* and possibly other viral agents (Actor *et al.,* 1993; 1994).

An important example of modulation is the interaction between schistosome infection and Hepatitis C in Egypt, which is considered to be the most important public-health problem due to the geographically overlapping distributions. Several studies conducted in Egypt (Abdel Wahab *et al.*, 1994; Angelico *et al.*, 1997; El-Zayadi *et al.*, 1997; Hibbs *et al.*, 1993; Koshy *et al.*, 1993; Waked *et al.*, 1995; Kamal *et al.*, 2000; Gad *et al.*, 2001) and in Brazil (Yoshida *et al.*, 1993; Pereira *et al.*, 1995) demonstrated a higher prevalence of HCV virus infection in schistosome patients and defined its importance as seen by higher HCV RNA titers, decompensation of hepatic disease (higher histological activity, higher incidence of cirrhosis and hepatocellular carcinoma), poor response to combination anti-viral-therapy, as well as higher mortality rate. Multiple mechanisms have been postulated: (i) Chronic helminth infections, such as schistosomiasis, tip the Th1-like/Th2-like balance towards a Th2-like immune response (Sher *et al.,* 1992), evidenced by a strong Th2 response in peripheral immune responses (Kamal *et al.*, 2001) and are accompanied by a decreased ability to mount specific antiviral immune responses (ii) Due to the fact that chronic schistosomiasis causes T-cell hypo-responsiveness (Roberts *et al.*, 1993; Grogan *et al.*, 1996; Yazdanbankhsh, 1999) and decreases the total T cell count (Colley *et al.,* 1983; Kalinkovich *et al.,* 1998), there will be decreased cytotoxic effector T cells for elimination of HCV virus (iii) Endogenous IL-10 produced by Treg cells in response to schistosome infection may cause deactivation of macrophages and, together with alternative activation of macrophage functions, result in decreased microbicidal functions *e.g.* NO synthesis. IL-10 was proposed to account for the low percentage of HCV-specific CD4+ proliferative responses to HCV antigen (8.6%) in patients co-infected with HCV and *S. mansoni* (who tended to produce type2 cytokine production) compared to 73% of patients infected with HCV alone (who produced type1 cytokines) (Kamal *et al.*, 2001). These findings suggest that the inability

to generate an HCV-specific CD4+/Th1 T cell response plays a role in the persistence and severity of HCV infection in patients with *S. mansoni*.

A relationship between persistent or recurrent bloodstream infections with *Salmonella spp.* (chronic salmonellosis) and schistosomiasis has been described in both adults and children (Gendrel *et al.,* 1994; 1984; Hennequin *et al.*, 1991; Lambertucci *et al.*, 1998). Moreover, Hennequin *et al.* (1991) suggested that schistosomes behave as reservoirs of *S. typhi* bacteria and *Salmonella* bacterial pili adhere to a mannose receptor-like surface glycoprotein on the tegument of *Schistosome mansoni* (Mei *et al.*, 1996) providing a site of intravascular persistence and causing periodic bacterial discharges.

In mice infected with *S. mansoni* increased susceptibility has been shown to the parasites *Entamoeba histolytica* (Knight and Warren, 1973), *Trypanosoma cruzi* (Kloetzel *et al.,* 1973), and *Leishmania major* (La Flamme *et al.*, 2002). Minimal increases in parasitaemia were seen with *Plasmodium chabudii spp* (Helmby *et al.*, 1998) although not *P. yoelli* or *P. berghei* (Lwin *et al.*, 1982). However, a strain of mouse which is normally susceptible to persistent infection with *Trichuris muris* becomes resistant (Curry *et al.*, 1995). There is evidence that schistosome infections can alter the host's response to concurrent infection with *Toxoplasma gondii* (Marshall *et al.*, 1999). Superinfection of *S. mansoni* infected mice with *T. gondii* resulted in reduced *T. gondii*-Th1 induced gut pathology associated with markedly reduced serum IFN-γ and NO levels presumably due to inhibition of development of Th1 responses by schistosome induced Th2 cytokines (Marshall *et al.* 1999).

Like Hepatitis C, *Leishmania donovani* also infects the liver although it also has serious effects on the spleen, also. *L. donovani* and schistosomes are found in similar areas in certain countries *e.g.* Sudan and are likely to occur in the same patients but to date nothing is known about the possible interaction between these infections. However, Zijlstra *et al.* (1991a) and Zijlstra and el-Hassan, (2001), demonstrated a high incidence of Kala Azar in displaced people from southern Sudan along the river Nile areas, decribing the poor immunological (suppressed or absent IFN-γ production from PBMCs

Zijlstra *et al.* (1991a) and Zijlstra and el-Hassan, (2001), demonstrated a high incidence of Kala Azar in displaced people from southern Sudan along the river Nile areas, decribing the poor immunological (suppressed or absent IFN-γ production from PBMCs of infected Kala Azar patients) and poor therapeutic response. Such latent infections often give rise to severe forms of reactivation disease, including visceral leishmaniasis (VL) associated with, *e.g.* HIV co-infection (Alvar *et al.,* 1997) and the development of post-Kala Azar dermal leishmaniasis (PKDL) after cure of VL (el-Hassan *et al.,* 1992).

The possible involvement of worm infections in general and schistosomiasis in particular to this apparently exacerbated disease remains to be determined. In view of this it is of interest to determine if there is exacerbation of disease in experimental infections with these two parasites. This is of particular interest since they both induce granulomatous responses in the liver, in the case of *S. mansoni*, strong Th2 dominated reactions to the egg and in contrast with *L. donovani,* Th1 dominated responses.

Aim of research

To study the reciprocal effects of an established patent *S. mansoni* infection and a super-imposed *L. donovani* infection in terms of parasitology and immunology.

3.3. Results

Two essentially similar experiments are described and referred to as Experiment 1 and Experiment 2.

3.3.1 Experimental design – Experiment 1

Sixty six female C57BL/6 mice were divided into 4 groups. Initially thirty mice were each infected with 25 *Schistosoma mansoni* cercariae via the percutaneous route. This relatively low dose of cercariae was chosen in order to produce an infection that was not lethal during the acute phase but which meant that most mice would have a bi-sexual, egg-producing infection. Eight weeks after the schistosome infection 15 of the mice were co-infected, via the lateral tail vein, with $2x10^7$ amastigotes of *Leishmania donovani* strain LV9. The remaining 15 were left with the schistosome infection only. A further group of 15 mice received the *Leishmania* infection alone. Finally a group of 21 mice were untreated (CONTROLS).

Groups of mice were sacrificed at three time-points: 10, 12, and 16 weeks post *S. mansoni* infection (+2, +4, and +8 wks post infection with LV9). Throughout the thesis these time points are cited with reference to the duration of the superimposed infection *i.e.* +2, +4 and +8 wk rather than with reference to the duration of the schistosome infection. Five mice were sacrificed from the infected animal groups and seven mice from the control group. The use of more mice in the control group was to try to get sufficient lymphocytes from the livers of the control group. In contrast the livers of the infected mice were expected to have abundant cells. Throughout the thesis the groups of mice infected with *L. donovani* alone are referred to as LEISH, the *S. mansoni* alone as SCHISTO and the co-infected mice as CO-INFECT.

3.3.1.1 Animal and organ weights - Experiment 1

The weights of the mice sacrificed at each of the time points is shown in Figure 3.3.1. Over the time of the experiment the SCHISTO and CO-INFECT mice showed a loss of condition with signs of wasting but as can be seen in Figure 3.3.1 there was no significant difference in the weights of mice from the different groups at any of the time points.

Figure 3.3.1 Mean weights of mice [Experiment 1].

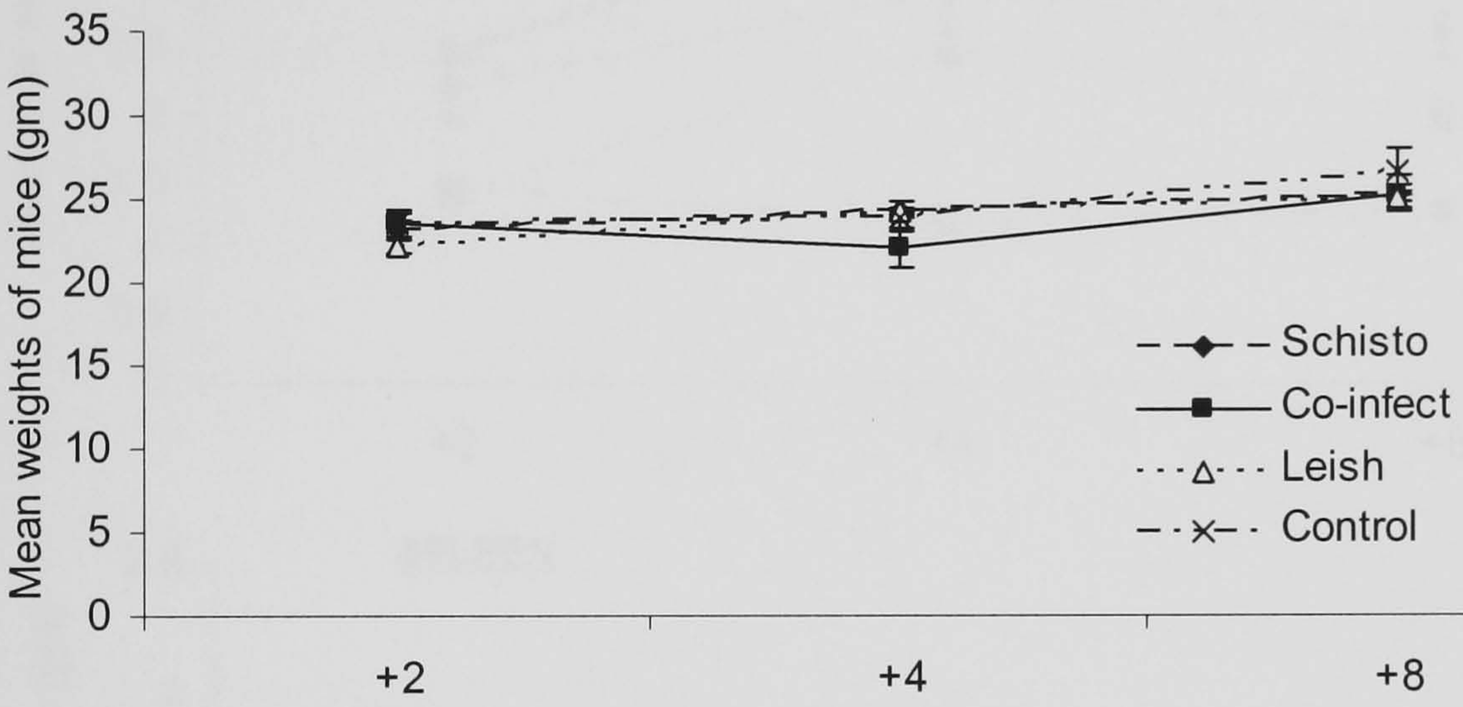


Figure 3.3.1. Mean weights of mice (±S.E.) at time of sacrifice based on 4 or 5 mice/time point. SCHISTO and CO-INFECT mice received 25 *S. mansoni* cercariae percutaneously and 8 weeks later the CO-INFECT mice received $2x10^7$ amastigotes of *L. donovani* intravenously along with the LEISH group (*i.e.* at week 0 post *L. donovani* infection). Control mice were uninfected naïve mice.

The weights of the livers and spleens were also recorded and the data is shown in Figure 3.3.2. At +2 weeks the liver and spleen weights were increased significantly compared to CONTROLS for the SCHISTO and CO-INFECT groups ($p=0.013$, <0.0001 for the liver and 0.0002, 0.0003 for the spleen) but not the LEISH group. The livers and spleens of the SCHISTO and CO-INFECT groups were also significantly heavier than the LEISH group ($p=0.0086$, <0.0001 for the liver and $p=0.0002$, 0.0003 for the spleen). By +4 and +8 weeks all infected groups showed significant hepatomegaly and splenomegaly compared with the CONTROLS (Liver: at +4 weeks, $p<0.0001$ for all groups; at week +8, $p< 0.0001$, 0.0019 and 0.0007 for SCHISTO, CO-INFECT and LEISH respectively; spleen: at +4 weeks,

p=0.0003, 0,0004, 0.0002 for SCHISTO, CO-INFECT and LEISH respectively and at +8 weeks, <0.0001 for all groups).

Figure 3.3.2: Mean weights of livers and spleens

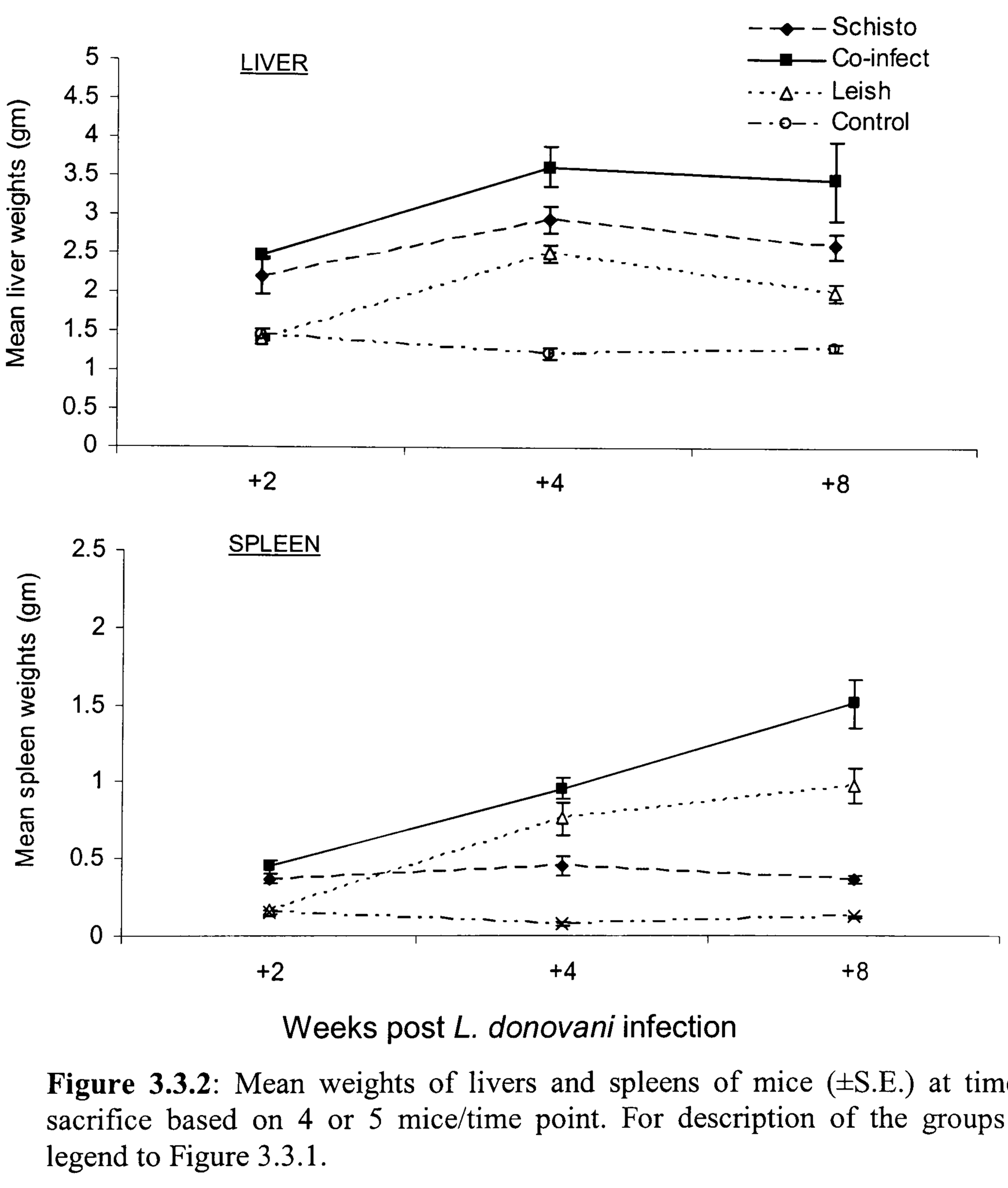


Figure 3.3.2: Mean weights of livers and spleens of mice (±S.E.) at time of sacrifice based on 4 or 5 mice/time point. For description of the groups see legend to Figure 3.3.1.

The CO-INFECT group showed increased liver and spleen weights compared to the SCHISTO and LEISH groups at +4 weeks but this was only statistically significant relative to the LEISH group (p=0.004) in the liver and the SCHISTO group in the spleen (p=0.04). At +8 weeks the CO-INFECT group showed significantly elevated liver weight compared

to the LEISH group ($p=0.017$) but not the SCHISTO group. Spleen weights were significantly higher in the CO-INFECT group than either the LEISH ($p=0.024$) or the SCHISTO ($p<0.0001$) groups but significantly higher in the LEISH than the SCHISTO ($p=0.0006$).

The percentage differences in liver and spleen weight are of interest as organ size could contribute to parasite proliferation notably of *L. donovani*. The CO-INFECT liver weights at +2, +4 and +8 weeks were 75, 44 and 74% higher in CO-INFECT compared to LEISH mice and the spleen weights, 173, 20, 54% higher. So early during the *L. donovani* infection, the livers and spleens of the CO-INFECT mice represent potentially more liver and spleen tissue for *L. donovani* infection. Thereafter, at +4 and +8 weeks both single infections were associated with hepatosplenomegaly, the *S. mansoni* infection contributing relatively more to the hepatomegaly and the *L. donovani* more to the splemomegaly as shown by the persistently greater liver weight in the SCHISTO compared with the LEISH groups and *vice versa* for the spleen.

3.3.1.2 Parasitological results - Experiment 1

3.3.1.2.1 Effect of *L. donovani* superinfection on the established *S. mansoni* worm burden and egg production – (Experiment 1)

Worm and egg burdens from the Schisto and Co-infect groups are shown in Table 3.3.1.

Not all mice which had been exposed to schistosome cercariae were found to have bisexual adult worm infections. At +2 and +4 weeks, 4 out of the 5 mice in the SCHISTO and CO-INFECT groups had bisexual infections and at +8 weeks 5 out of 5 SCHISTO mice and 4 out of 5 CO-INFECT mice had bisexual infections. There were no significant differences between the mean worm recoveries from the two groups at any of the time points although worm pair numbers were marginally higher in the CO-INFECT groups at each of the time points.

Table 3.3.1: Mean adult worm and geometric mean liver egg burdens (±SD) in SCHISTO and CO-INFECT groups at +2, +4 and +8wk after *L. donovani* super-infection.

Group	Time (wk)	Mean worm count		Geometric mean egg count (±SD)[#]	Geometric mean eggs/ worm pair* (±SD)
		Total (±SD)	Pairs (±SD)		
SCHISTO	+2	8.0 (±4.1)	2.8 (±1.5)	15127 (±9116)	5402 (±8447)
	+4	8.4 (±2.9)	2.8 (±1.1)	18782 (±8629)	6708 (±3274)
	+8	8.2 (±1.8)	2.6 (±0.9)	22141 (±6922)	8516 (±3914)
CO-INFECT	+2	8.3 (±1.5)	3.5 (±0.6)	15132 (±6858)	4323 (±2064)
	+4	7.4 (±3.3)	3.2 (±1.6)	15092 (±4747)	4716 (±1747)
	+8	9.3 (±2.4)	3.8 (±1.3)	16273 (±3658)	4282 (±1197)

Table 3.3.1: C57BL/6 mice were infected with 25 *S. mansoni* cercariae and super-infected with *L. donovani* 8 weeks later. #Egg count expressed as total for the whole liver. *Estimated using the number of mature female and male worms recovered in each animal. n = 4 or 5 mice.

The mice with bisexual worm infections were seen at autopsy to have granulomatous livers demonstrating the presence of patent infections. The geometric mean egg counts (GMECs), which were carried out on a weighed section of liver, were adjusted, based on the weight of the liver, to represent the number of eggs per liver. There were no significant differences between the GMECs of the SCHISTO and CO-INFECT groups at any of the time points. The GMEC/worm pair were lower at each of the time points in the CO-INFECT group compared with the SCHISTO alone group although none of these values were significantly different.

It is clear from the above that the superimposed *L. donovani* infection had no significant effect on the established worm burden or the numbers of eggs in the liver, which would be influenced by the rate of egg deposition and the rate of resolution of the egg granulomas.

3.3.1.2.2 Effect of prior *S. mansoni* infection on the superimposed *L. donovani* infection (Experiment 1).

Mice were sacrificed at +2, +4, and +8 weeks post-infection with *L. donovani* infection, and liver and spleen imprints were prepared and stained with Giemsa. The slides were examined under x40 magnification and the number of amastigotes counted relative to 1000 cell nuclei. The intensity of infection in the whole organ (liver or spleen) was expressed as Leishman Donovan units (LDU) *i.e.* the number of amastigotes per 1000 hepatic or spleen nuclei multiplied by the weight of the organ in grams.

The results are shown in Figure 3.3.3. Overall, the LEISH animals showed an initial infection in both liver and spleen but were able to control the disease, showing a progressive reduction in the LDU in the liver from +2 to +8 wk and an initial increase followed by a decline between +4 wk and +8 wk in the spleen. In contrast the CO-INFECT mice showed steadily increasing LDU in both liver and spleen. The failure to resolve the parasite burden in the CO-INFECT mice compared to the LEISH alone mice became particularly apparent between +4 and + 8 wk post infection with *L. donovani.*

At +2 weeks the mean LDU value for the liver was actually 40% lower in the CO-INFECT group than in the LEISH group ($p=0.02$). However, in the spleen, where the overall LDU values were very much lower than in the liver throughout, LDU values were twice as high in the CO-INFECT than in the LEISH animals ($p=0.02$).

At +4 weeks there were no significant differences between either the liver or the spleen LDU values in the LEISH and CO-INFECT groups. The levels of infection were comparable to the +2 week values in the liver but increased compared to the +2 week values in the spleen.

By +8 week the mean liver LDU values had dropped markedly (13.4 fold) in the LEISH group ($p<0.0098$) but had increased in the CO-INFECT group (1.7 fold) although not statistically significantly, such that the mean LDU values for LEISH and CO-INFECT groups were significantly different (27-fold, $P=0.0035$).

Figure 3.3.3.: Comparison of Leishman Donovan Units (LDU) from Liver and Spleen imprints at +2, +4 and +8 weeks post *L. donovani*

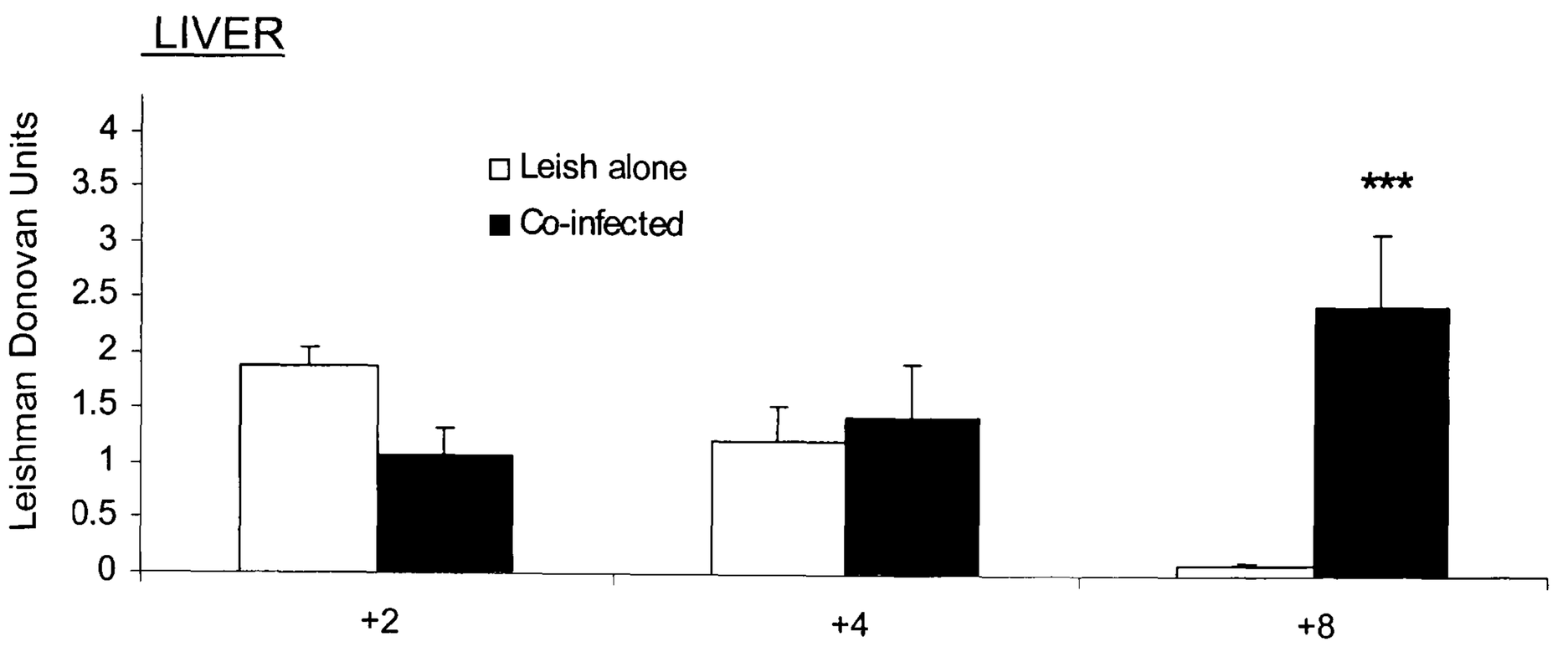


Figure 3.3.3.: Graphs show the mean (±SE) LDU for five mice from the CO-INFECT and LEISH groups. *** Significantly different from LEISH (p=0.0035).** Significantly different from LEISH (p=0.02). Description of the groups see legend to Figure 3.3.1.

In the spleen LDU counts increased between +2 and +4 weeks in both LEISH and CO-INFECT mice but then declined sharply (4.2 fold) between +4 and +8 weeks in the LEISH but increased in the CO-INFECT (3.3 fold) such that there was an 11.3 fold higher LDU count in the CO-INFECT compared with the LEISH alone (p=0.02).

Overall therefore, the LEISH and CO-INFECT mice showed a similar response to the *L. donovani* infection at +2 and +4 wk in both the liver and spleen but between +4 and +8 wk

there was a marked divergence in both organs, the schistosome infected mice failing to control the *L. donovani* infection.

3.3.2. Experimental design - Experiment 2

This is essentially a repeat of Experiment 1 with the same experimental design except that group sizes were increased because a few of the animals in the first experiment had lost condition towards the end of the experiment and consideration had to be given to culling them. Numbers were also increased to try to obtain adequate cell numbers for the liver lymphocyte cytokine determinations. However, one CO-INFECT mouse had to be killed because of illness on day 61, and a SCHISTO mouse on day 65. Several CO-INFECT mice were observed to be sick on day 100 with symptoms similar to middle ear disease *e.g.* tilting of head, ataxic gait and two had to be culled on day 103.

3.3.2.1 Animal and organ weights - Experiment 2

Mouse weights at sacrifice are shown in Figure 3.3.4.

Figure 3.3.4. Mean weights of mice.

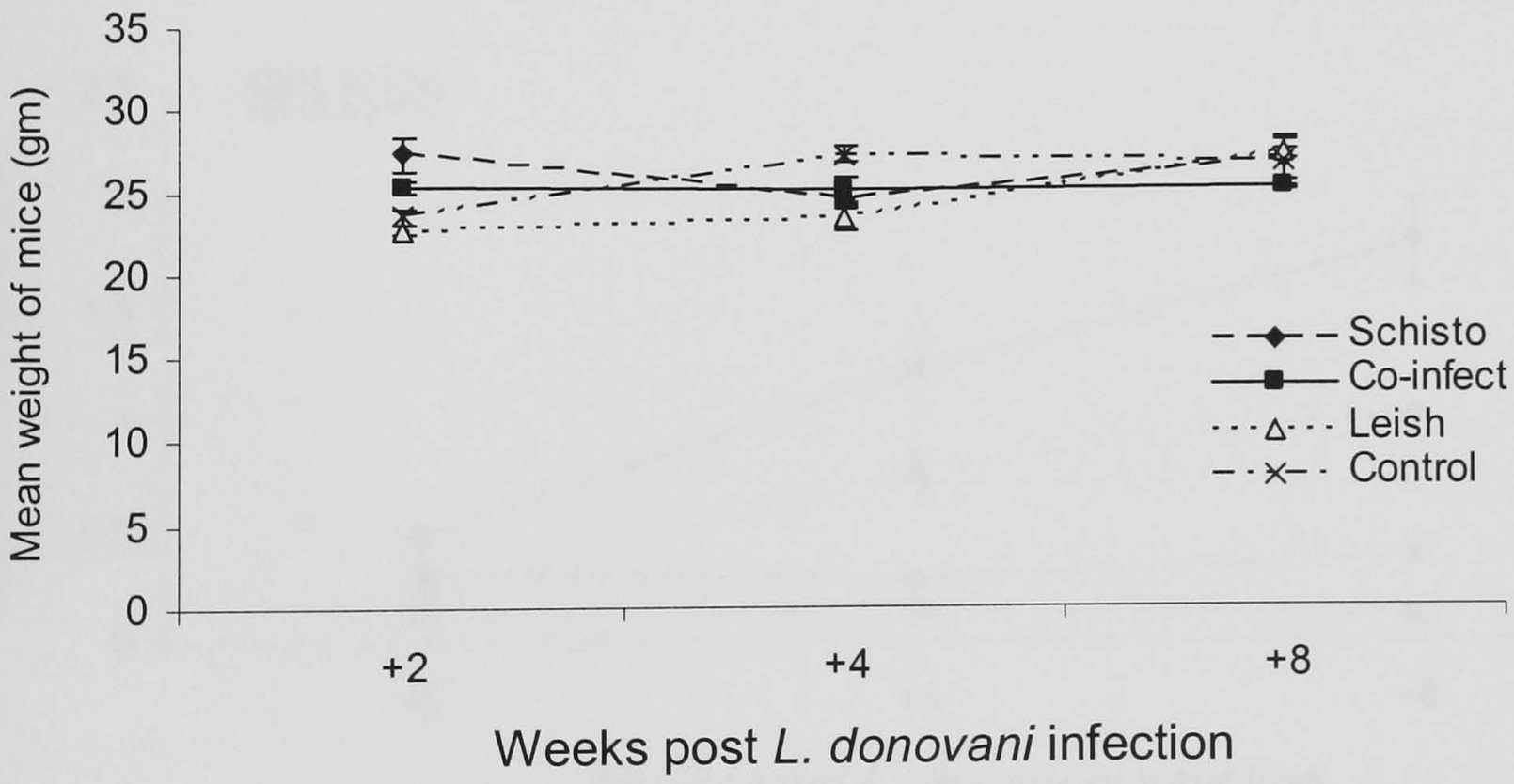


Figure 3.3.4.: Mean weights of mice (±S.E.) at time of sacrifice based on 4 or 5 mice/time point. For description of the groups see legend to Figure 3.3.1.

There were some statistically significant differences between the group weights at particular time points (*e.g.* at +2 weeks the mean SCHISTO group weight was statistically significantly higher than that of the CONTROL [0.0032] and LEISH [0.0021] groups) but such differences were not consistent between different time points and so may have represented random variations. There were no significant differences between the groups at +8 weeks. The weights of the livers and spleens are shown in Figure 3.3.5

Figure 3.3.5: Mean weights of livers and spleens

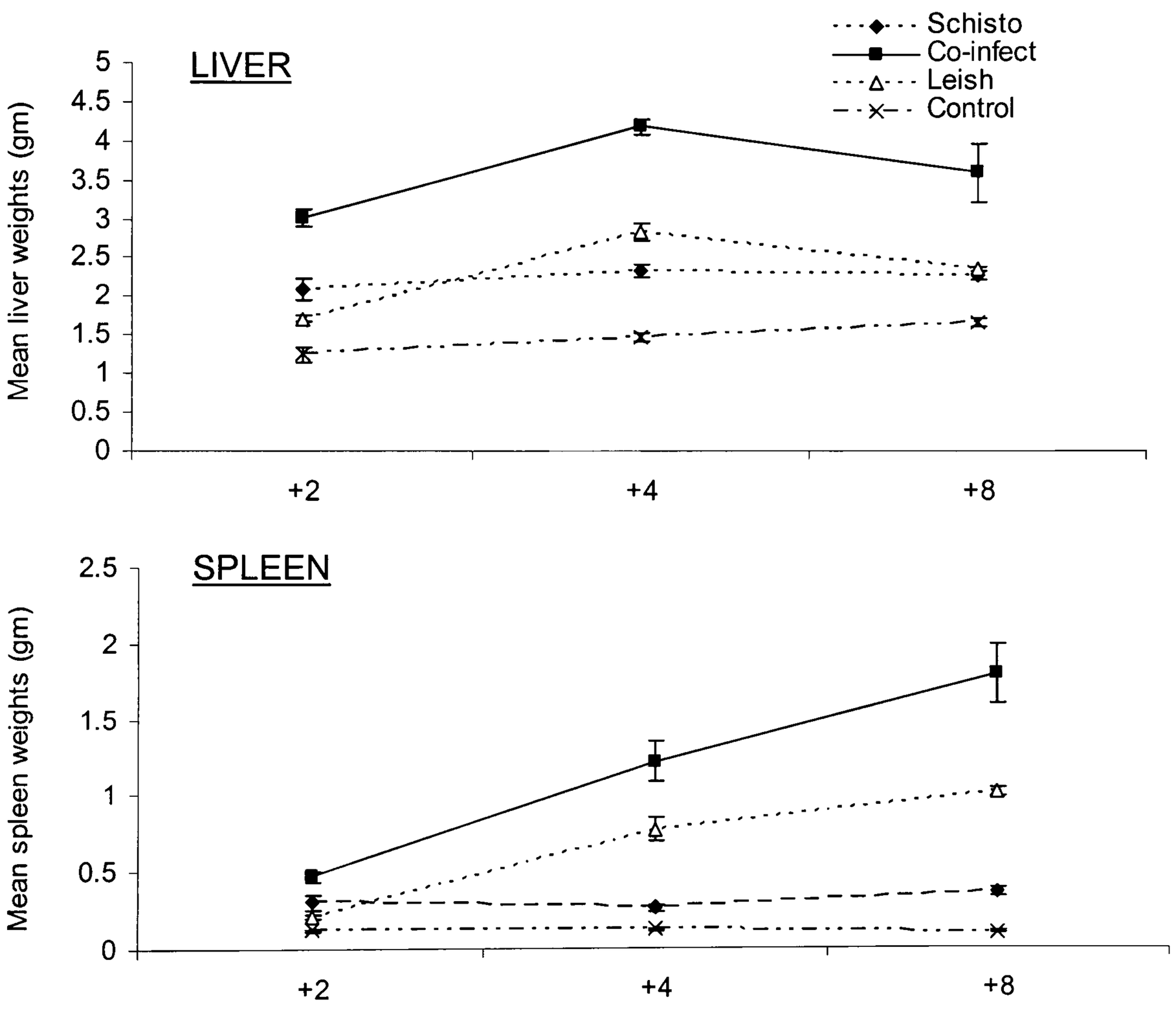


Figure 3.3.5: Mean weights of livers and spleens of mice (±S.E.) at time of sacrifice based on 5 mice/time point. For description of the groups see legend to Figure 3.3.1.

The data is essentially similar to that in the first experiment (Figure 3.3.2.) and so only the key features will be emphasized. The CO-INFECT groups showed the highest weights for both liver and spleen at each time point, significantly higher than the LEISH group at +2, +4 and +8 weeks (respectively $p<0.0001$, <0.0001 and 0.0046 for the liver and <0.0001, 0.023 and 0.0164 for the spleen). The percentage greater mean weights between CO-INFECT and LEISH groups were 76, 48, 52 % at +2, +4 and +8 weeks respectively for the livers and 123, 58 and 76% for the spleens.

3.3.2.2. Parasitological results - Experiment 2

3.3.2.2.1 Effect of *L. donovani* superinfection on the established *S. mansoni* worm burden and egg production – (Experiment 2)

Worm and egg burdens from the SCHISTO and CO-INFECT groups are shown in Table 3.3.2.

Table 3.3.2.: Mean adult worm and geometric mean liver egg burdens (±SD) in Schisto and Co-infect groups at +2, +4 and +8weeks after *L. donovani* super-infection.

Group	Time (wk)	Mean worm count		Geometric mean egg count (±SD)#	Geometric mean eggs/ worm pair* (±SD)
		Total (±SD)	Pairs (±SD)		
SCHISTO	+2	7.5 (±3.1)	2.0 (±1.2)	20706 (±10679)	10353 (±7214)
	+4	7.5 (±4.0)	3.2 (±1.6)	12946 (±4470)	4046 (±2039)
	+8	6.4 (±3.9)	2.2 (±1.3)	12102 (±5603)	5501 (±3977)
CO-INFECT	+2	11.3 (±1.7)	**4.8 (±0.5)	13323 (±4811)	2776 (±902)
	+4	5.4 ± (2.9)	2.0 (±1.2)	10231 (±5828)	5116 (±3484)
	+8	6.7 (±1.8)	2.6 (±0.9)	12815 (±2329)	4929 (±2797)

C57BL/6 mice were infected with 25 *S. mansoni* cercariae and super-infected with *L. donovani* 8 weeks later. # Egg count expressed as total for the whole liver. *Estimated from the number of mature female and male worms recovered in each animal. ** Significantly different worm pair burden from SCHISTO ($p=0.005$). n=5 mice/ group.

There were no significant differences between the mean worm recoveries from the two groups at any of the time points although worm pair numbers were higher in the Co-infect group perfused at +2 wk. The GECs were similar in the CO-INFECT and SCHISTO groups at +4 and +8wk but were lower at +2wk but this difference was not statistically significant. Similarly the GEC/worm pair values were similar for both groups at +4 wk and +8 wk but higher in the SCHISTO group at the +2 wk time point (10353±7214 cf 2776±902) although this difference was not statistically significant (p=0.053).

3.3.2.2.2 Effect of prior *S. mansoni* infection on the superimposed *L. donovani* infection (Experiment 2).

The LDU counts are shown in Figure 3.3.6. The patterns of infection in the LEISH and CO-INFECT groups were similar to Experiment 1 in that the infection resolved in the LEISH group but not in the CO-INFECT group.

At +2 wk after *L. donovani* infection both groups had similar LDU counts from the liver. In the spleen the counts were again much lower in both groups compared with the liver but were already 5 fold higher in the CO-INFECT group than the LEISH group (p=0.011).

Between +2 and +4 wk there was a significant decline in liver LDU in the LEISH group (p=0.0002). LDU in the CO-INFECT mice also showed a significant drop in LDU in the liver between +2 and +4 wks (p=0.02) but this was 2.5 fold higher than in the LEISH group (p=0.005). In the spleen the LDU levels increased but remained low in the LEISH group but in the CO-INFECT group the LDU increased sharply (p=0.0009) to be 13 fold higher than in the LEISH group (p=0.0002).

Between +4 and +8 wk there was a further significant decline in liver LDUs in the LEISH group (3.1 fold, p=0.01 respectively) but the CO-INFECT group showed a 1.5 fold increase in liver LDU which was 11.5 fold higher than in the LEISH group at +8 weeks (p=0.002). In the spleen the mean LDU value again increased, 4 fold, between +4 and +8 weeks in the LEISH group (p=0.002). There was also a 4 fold increase in the spleen LDU for the CO-

INFECT group (p=0.002) but the mean LDU value was 12.5 fold higher for the CO-INFECT group than for the LEISH group (p=0.0006).

Figure 3.3.6.: Comparison of Leishman Donovan Units (LDU) from Liver and Spleen imprints at +2, +4 and +8 weeks post *L. donovani* super-infection.

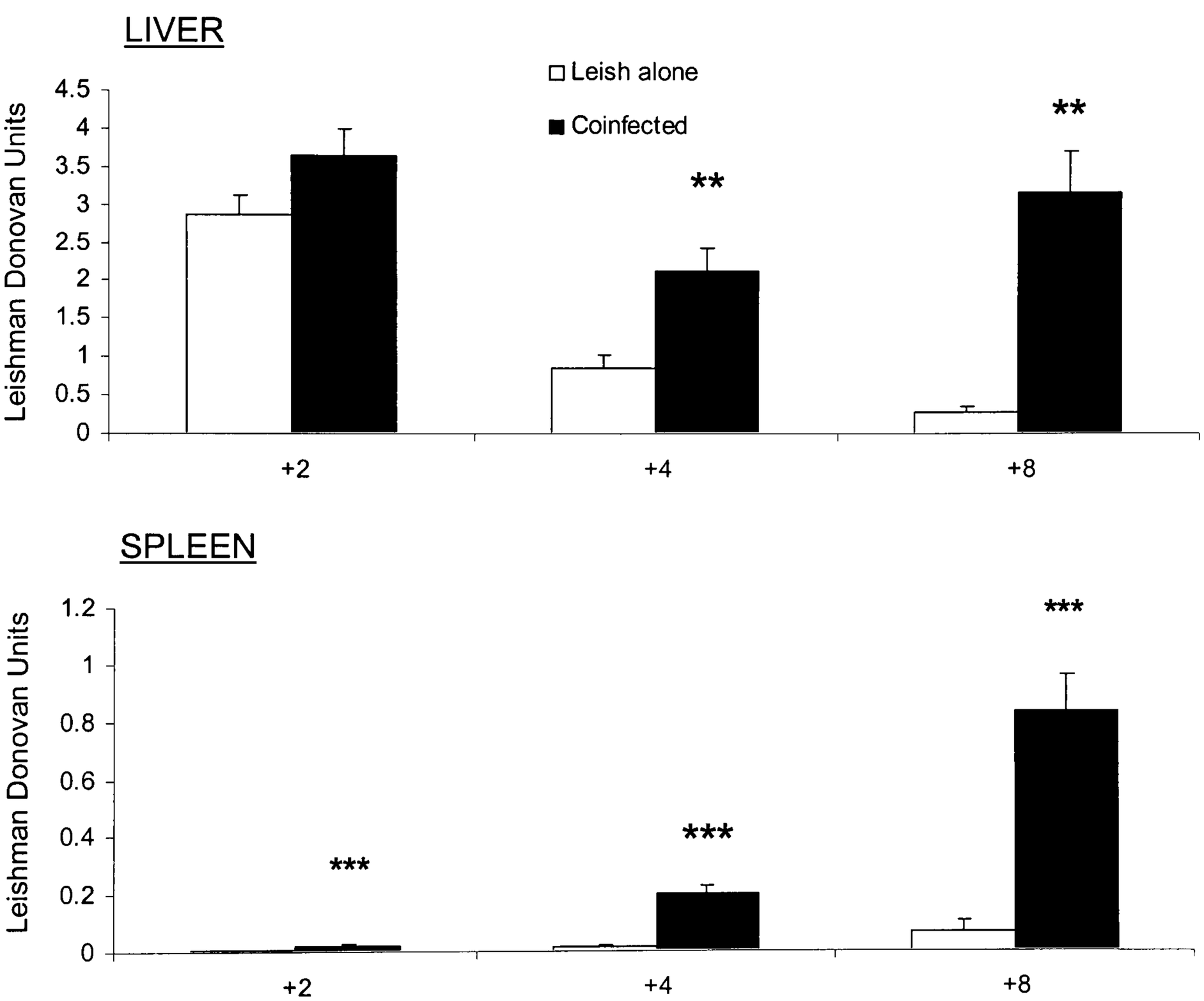


Figure 3.3.6.: Graphs show the mean (±S.E.) LDU for five mice from the CO-INFECT and LEISH groups. ** Significantly different from LEISH group (p=0.005 and 0.002 at +4 and +8 weeks respectively). *** Significantly different from LEISH group (p=0.01, 0.0002, and 0.0006 at +2, +4 and +8 weeks respectively). For description of the groups see legend to Figure 3.3.1.

3.3.1.3 Immunology results - Experiment 1

Cytokine production (IFN-γ and IL-4 as markers of Th1 and Th2 responsiveness respectively) and the immunoregulatory cytokine IL-10, was assessed in supernatants obtained from spleen cells from three individual mice per group and from pooled liver-derived lymphocytes. Cells were cultured *in vitro* at a concentration of $5x10^6$/ml with medium alone, mitogen (ConA), schistosome antigens (soluble worm antigen preparation [SWAP] and soluble egg antigen [SEA]), and leishmanial antigen (formalin-fixed amastigote antigen [FLAA]).

3.3.1.3.1 Detection of IL-4 production from spleen lymphocytes by ELISA

The cytokine data is plotted with the ConA data (Figure 3.3.7.) and also without it (Figure 3.3.8.) so as to emphasize the antigen-specific responses but the description of the data refers to both graphs together.

At +2weeks post LV9 splenocytes from both the SCHISTO and CO-INFECT groups produced raised levels of IL-4 in the presence of ConA compared with CONTROLS in which no IL-4 was detected. Splenocytes from the LEISH group produced low levels of IL-4 to ConA, significantly lower than the SCHISTO group ($p=0.037$) but not significantly different from the CO-INFECT group owing to the high value in one of the mice. With the specific schistosome antigens, SEA and SWAP (see Figure 3.3.7.), IL-4 was raised in both SCHISTO and CO-INFECT groups but undetectable in the CONTROLS. Although the levels of IL-4 were higher for both SEA and SWAP in the CO-INFECT group the differences were not statistically significant. The LEISH group did not show responses to SWAP or SEA and none of the groups showed a response to FLAA.

At +4 weeks post LV9 both the SCHISTO and CO-INFECT groups showed significant IL-4 production to ConA relative to control mice ($p=0.007$, 0.004 respectively).

Figure 3.3.7. Comparison of the levels of IL-4 in supernatants of spleen cell

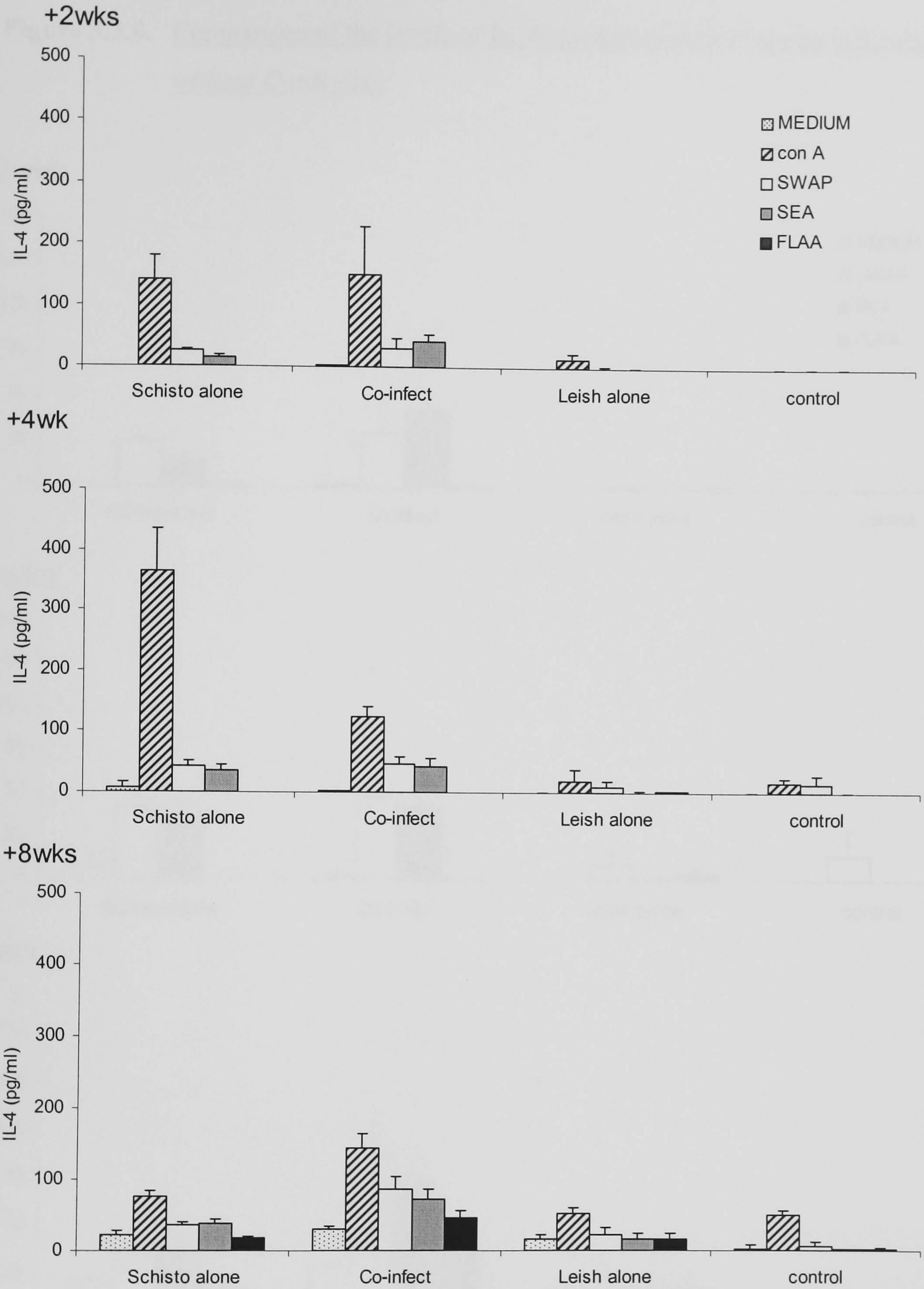
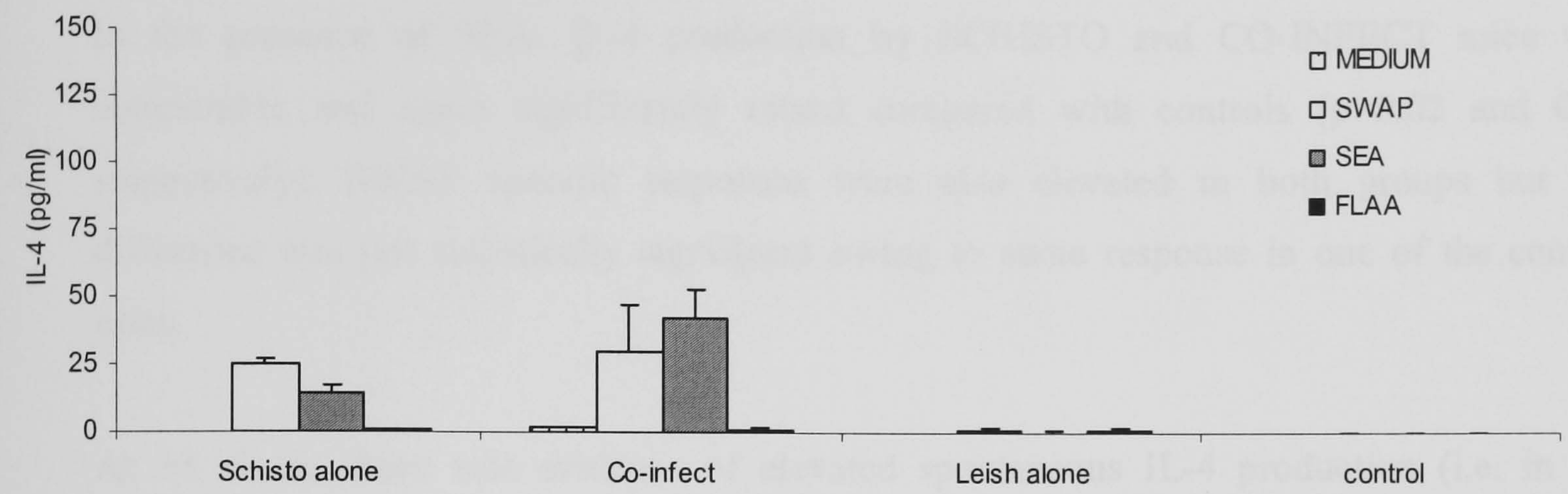
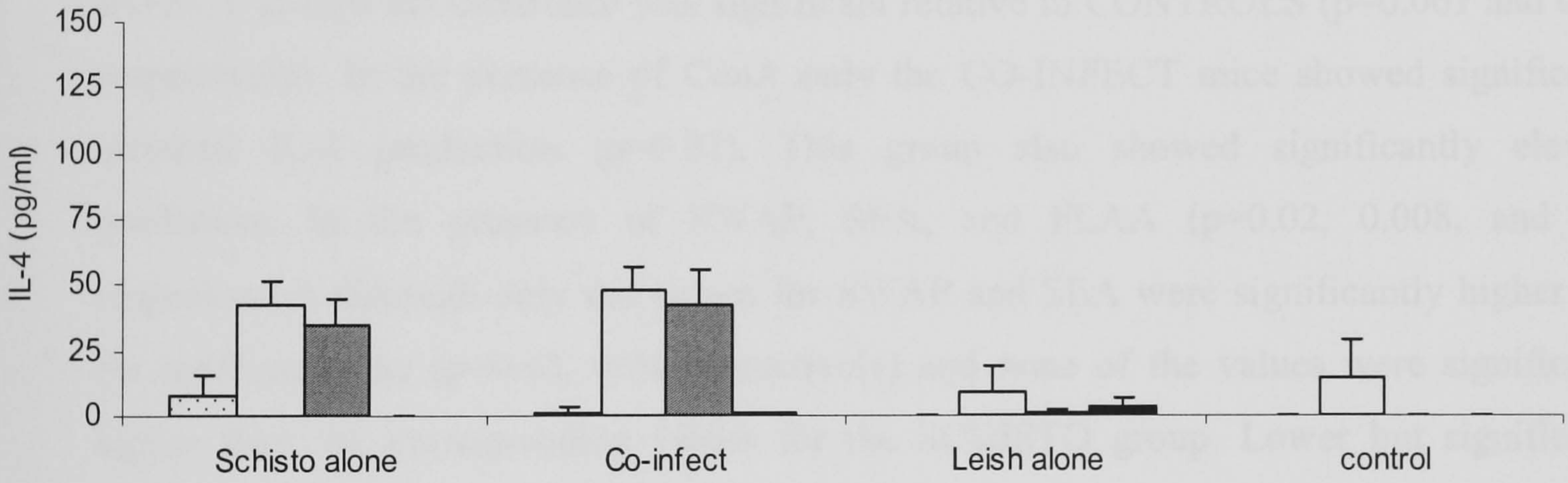


Figure 3.3.7. Graphs showing the means and standard errors from IL-4 detected by ELISA based on three mice from each group sacrificed at +2, +4 or +8 weeks after the *L. donovani* infection. Cultures of $5x10^6$ spleen cells/ml were stimulated with medium alone, ConA at 5μg/ml, schistosomal antigens (SWAP and SEA at 10μg/ml) or fixed leishmanial amastigote antigen (FLAA, at 10^7 amastigotes/ml). For description of the groups see legend to Figure 3.3.1.

Figure 3.3.8. Comparison of the levels of IL-4 in supernatants of spleen cell cultures *without* ConA data.





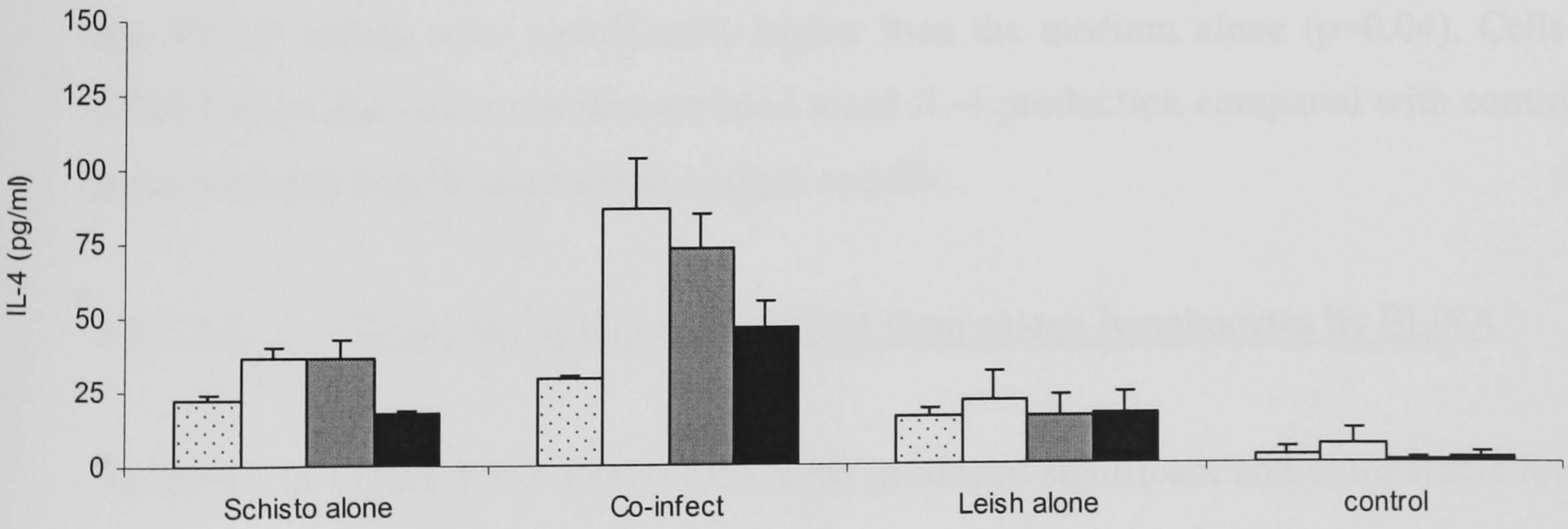


Figure 3.3.8. Data from Figure 3.3.7 redrawn without showing ConA results. See legend to Figure 3.3.1.

The highest ConA-induced IL-4 production was from the SCHISTO group and this was elevated compared with the response in the SCHISTO group at +2 weeks and significantly higher than in both the CO-INFECT and CONTROL groups (p=0.007, 0.028 respectively).

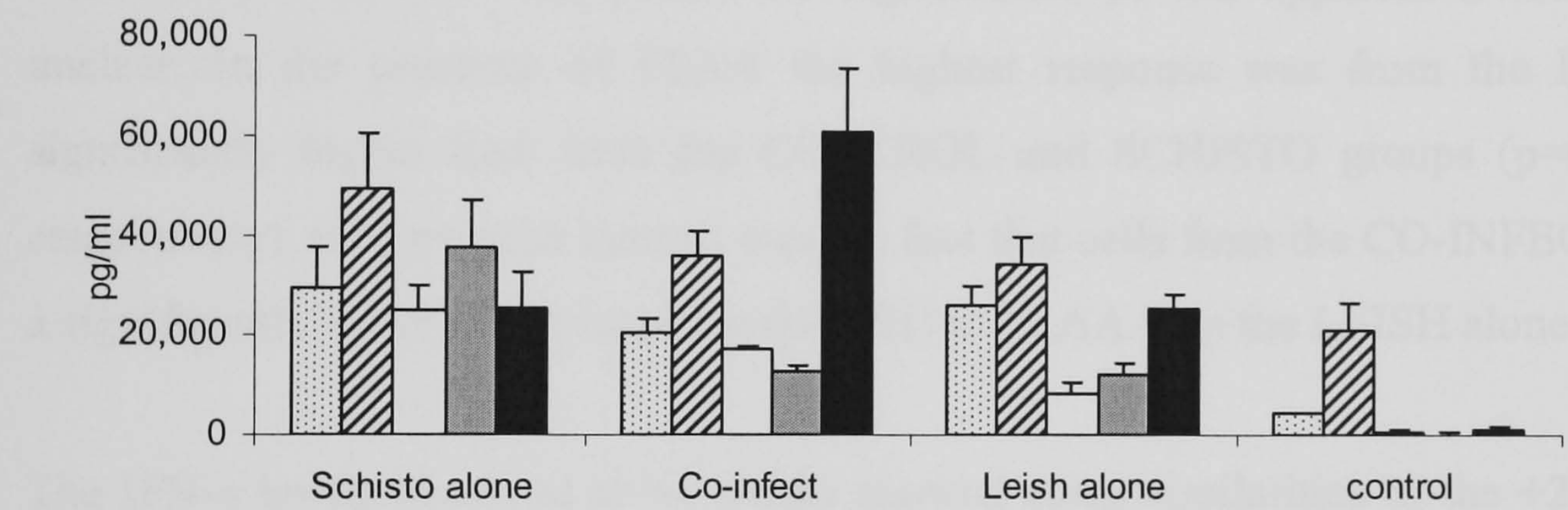
In the presence of SEA, IL-4 production by SCHISTO and CO-INFECT mice was comparable and again significantly raised compared with controls (p=0.02 and 0.03 respectively). SWAP specific responses were also elevated in both groups but the difference was not statistically significant owing to some response in one of the control mice.

At +8 weeks there was evidence of elevated spontaneous IL-4 production (i.e. in the presence of medium alone) in all of the infected groups. For the SCHISTO and CO-INFECT groups the difference was significant relative to CONTROLS (p=0.001 and 0.005 respectively). In the presence of ConA only the CO-INFECT mice showed significantly elevated IL-4 production (p=0.02). This group also showed significantly elevated production in the presence of SWAP, SEA, and FLAA (p=0.02, 0.008, and 0.01 respectively) although only the values for SWAP and SEA were significantly higher than for medium alone (p=0.05, 0.04 respectively) and none of the values were significantly higher than the corresponding values for the SCHISTO group. Lower but significantly raised IL-4 levels compared with CONTROLS were seen with cells from the SCHISTO group with SWAP, SEA, and FLAA (p=0.02, 0.009 and 0.002 respectively), however only the SWAP values were significantly higher than the medium alone (p=0.04). Cells from LEISH mice showed somewhat elevated mean IL-4 production compared with controls but these were not significant and not antigen specific.

3.3.1.3.2. Detection of IFN-γ production from spleen lymphocytes by ELISA

As shown in Figure 3.3.9. CONTROL cells produced significant and comparable levels of IFN-γ at each of the time points in the presence of ConA but in the presence of medium alone or the specific antigens there were minimal responses.

Figure 3.3.9. Comparison of the levels of IFN-γ in supernatants of spleen cell cultures



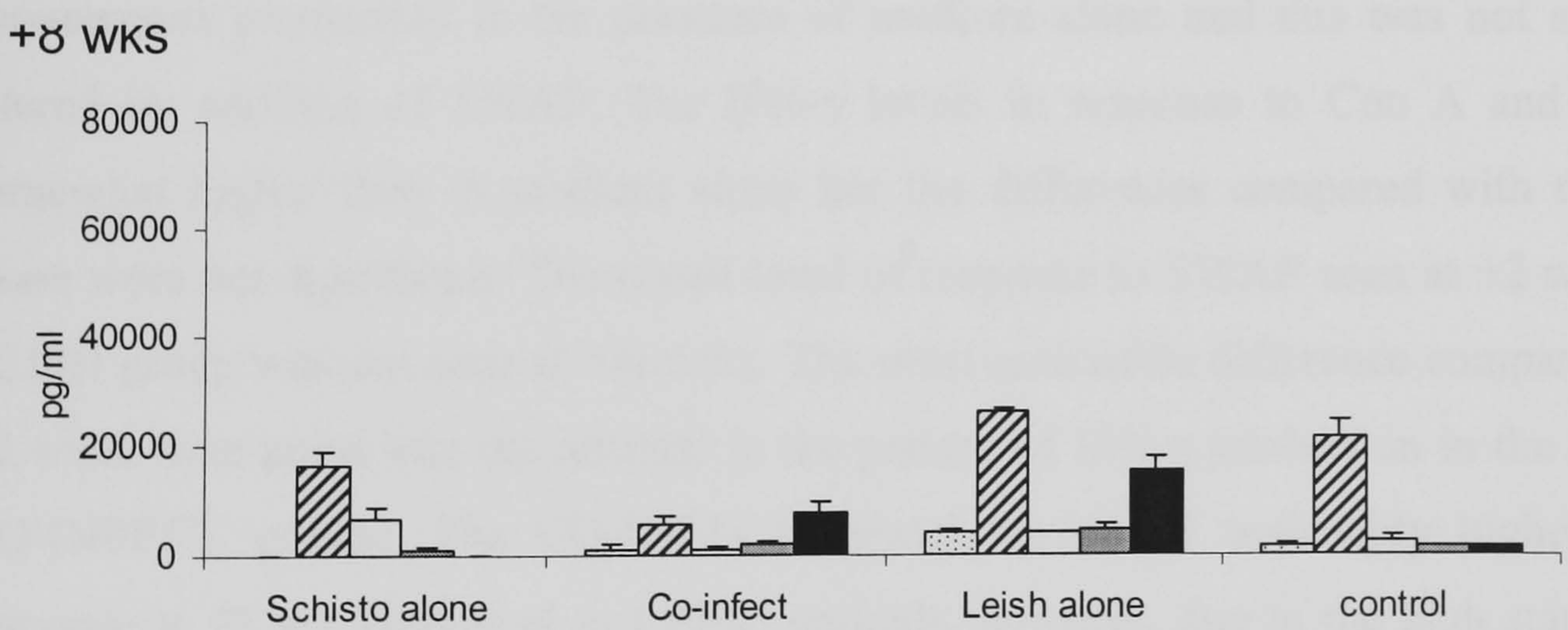


Figure 3.3.9. Graphs showing the means and standard errors for IFN-γ detected by ELISA based on three mice from each group sacrificed at +2, +4 or +8 weeks after the *L. donovani* infection. Cultures of $5x10^6$ spleen cells/ml were stimulated with medium alone, ConA at 5μg/ml, schistosomal antigens (SWAP and SEA at 10μg/ml) or fixed leishmanial amastigote antigen (FLAA, at 10^7 amastigotes/ml). For description of the groups see legend to Figure 3.3.1.

At +2 weeks post LV9 each of the infected groups, SCHISTO, LEISH and CO-INFECT showed raised IFN-γ production compared to the controls in the presence of medium alone and the various parasite antigens. The SCHISTO group showed responses to ConA comparable with the controls but showed raised responses relative to controls with medium alone, SWAP, SEA and FLAA. The schistosome antigens, SEA and SWAP did not stimulate significantly higher responses compared with medium alone indicating a spontaneous production of IFN-γ but no evidence of a specific recall response. The LEISH and CO-INFECT groups showed similar responses to medium, ConA and SEA but there was a surprisingly high response to SWAP in the LEISH group, significantly higher even than was observed in the SCHISTO and CO-INFECT groups ($p=0.008$, 0.0001 respectively) and perhaps suggesting some cross reactivity but since a comparable response was not seen at later time points the significance of this apparent SWAP response is unclear. In the presence of FLAA the highest response was from the LEISH group, significantly higher than from the CONTROL and SCHISTO groups ($p=0.0007$, 0.007 respectively). Of particular interest was the fact that cells from the CO-INFECT mice made a significantly lower IFN-γ response ($p=0.01$) to FLAA than the LEISH alone group.

The IFN-γ levels observed at +4 weeks showed some similarities to the +2 week data in that cells from the SCHISTO and CO-INFECT mice showed raised, apparently spontaneous production in the presence of medium alone and this was not significantly altered by addition of SWAP. The IFN-γ levels in response to Con A and SEA were somewhat higher than in medium alone but the differences compared with the medium alone were not significant. The raised level of response to SWAP seen at +2 weeks in the LEISH group was not seen at +4weeks. The most noticeable difference compared with the +2 week time point was the reversal in the pattern of IFN-γ production in the LEISH and CO-INFECT groups. The CO-INFECT animals produced noticeably higher IFN-γ in response to FLAA compared to LEISH animals, however, due to the high standard error, the CO-INFECT value was not statistically significantly higher with FLAA than the medium alone value ($P=0.064$) and also not significantly higher than the LEISH response to FLAA.

At +8 weeks levels of IFN-γ in response to all antigens were markedly lower than at the earlier time points. There was no longer IFN-γ production in the presence of medium alone by cells from SCHISTO, LEISH or CO-INFECT MICE. Neither the SCHISTO and CO-INFECT group showed noticeable IFN-γ specific recall responses in the presence of schistosome antigens (SWAP, SEA). The LEISH and CONTROL groups showed comparable and significant ConA-induced IFN-γ production in the presence of ConA compared with medium alone ($p<0.0001$ and 0.008 respectively) and the SCHISTO response to ConA was also significant ($p=0.002$). However, the Con A induced response in the CO-INFECT mice was significantly lower than that in the CONTROL, LEISH or SCHISTO groups ($p=0.02$, 0.0005 and 0.048 respectively). Both the CO-INFECT and LEISH groups showed elevated IFN-γ responses to FLAA but this was only significantly higher than the CONTROLS for the LEISH group ($p=0.007$). Notably, IFN-γ production stimulated by FLAA was lower in the CO-INFECT group compared with the LEISH group although this was not statistically significant ($p=0.09$).

3.3.1.3.3. Detection of IL-10 production from splenocytes by ELISA

At +2 weeks, as shown in Figure 3.3.10., Con A stimulated splenocytes from the CO-INFECT and the SCHISTO animals produced the highest levels of IL-10, although the differences compared to CONTROLS and to medium alone were not statistically significant.

Similarly the SCHISTO and CO-INFECT groups produced similar, elevated, levels of IL-10 in response to both schistosome antigens but especially to SEA. The differences relative to CONTROL mice were statistically significant for both SWAP ($p=0.004$, 0.05 respectively) and SEA ($p<0.0001$, 0.01 respectively). Both the LEISH and CO-INFECT groups showed production of IL-10 to FLAA but this was only significant higher than CONTROL values for the LEISH group ($P=0.001$) and this was not significantly higher than the CO-INFECT IL-10 value ($p=0.08$).

At +4 weeks Con A induced IL-10 levels were again high in the SCHISTO and CO-INFECT groups although owing to some variability these values were not significantly different from the CONTROL values ($p= 0.08$ and 0.07 respectively). The SCHISTO and

Figure 3.3.10. Comparison of the levels of IL-10 in supernatants of spleen cell cultures

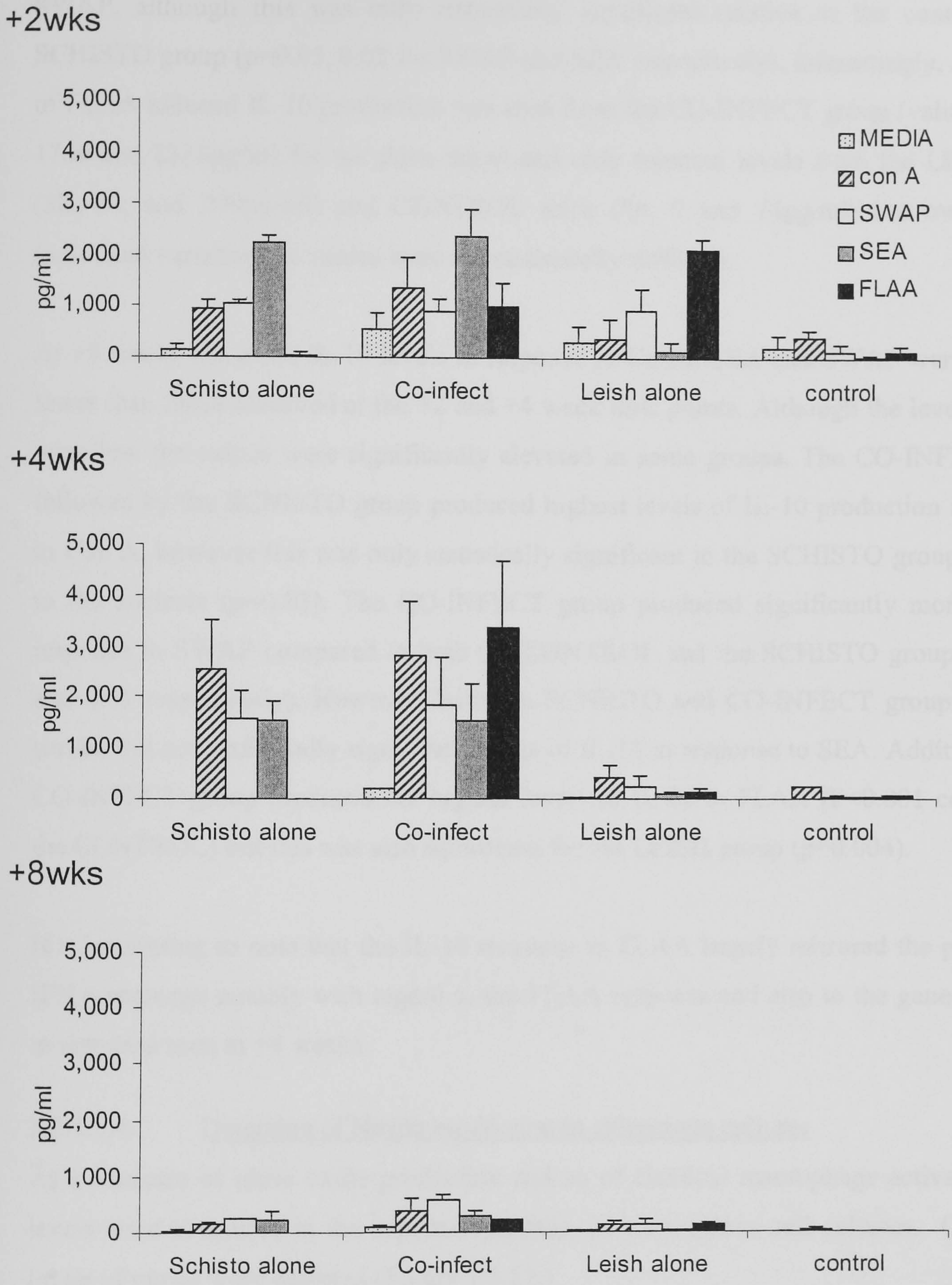


Figure 3.3.10. Graphs showing the means and standard errors for IL-10 detected by ELISA based on three mice from each group sacrificed at +2, +4 or +8 weeks after the *L. donovani* infection. Cultures of $5x10^6$ spleen cells/ml were stimulated with medium alone, ConA at 5μg/ml, schistosomal antigens (SWAP and SEA at 10μg/ml) or fixed leishmanial amastigote antigen (FLAA, at 10^7 amastigotes/ml). For description of the groups see legend to Figure 3.3.1.

CO-INFECT groups also produced similarly raised levels of IL-10 in response to SEA and SWAP, although this was only statistically significant relative to the controls in the SCHISTO group ($p=0.05$, 0.02 for SWAP and SEA respectively). Interestingly, a high level of FLAA induced IL-10 production was seen from the CO-INFECT group (values of 5993, 1760 and 2338pg/ml for the three mice) and only minimal levels from the LEISH group (38, 67, and 299pg/ml) and CONTROL mice (86, 0 and 74pg/ml) but owing to the individual variation the values were not statistically different.

At +8 weeks the mean IL-10 levels in response to ConA, SEA and SWAP were markedly lower than those observed at the +2 and +4 week time points. Although the levels of IL-10 were low the values were significantly elevated in some groups. The CO-INFECT group followed by the SCHISTO group produced highest levels of IL-10 production in response to Con A, however this was only statistically significant in the SCHISTO group compared to the controls ($p=0.03$). The CO-INFECT group produced significantly more IL-10 in response to SWAP compared to both the CONTROL and the SCHISTO groups ($p=0.002$ and 0.01 respectively). However, both the SCHISTO and CO-INFECT groups produced similar but not statistically significant levels of IL-10 in response to SEA. Additionally, the CO-INFECT group produced the highest levels of IL-10 to FLAA ($P<0.001$ compared to the CONTROL) but this was also significant for the LEISH group ($p=0.004$).

It is interesting to note that the IL-10 response to FLAA largely mirrored the pattern of the IFN-γ response notably with regard to the FLAA response and also to the general reduction in response seen at +8 weeks.

3.3.1.3.4. Detection of Nitrite production in splenocyte cultures

As a measure of nitric oxide production and so of classical macrophage activation nitrite levels were measured in the supernatants from *in vitro* spleen cell cultures. Overall low levels of nitrite were detected (Figure 3.3.11.).

At +2weeks, there were no sign of nitrite production in response to ConA, SEA, FLAA antigens in any of the infected or control mice.

Figure 3.3.11.: Levels of Nitrite produced in splenocyte cultures

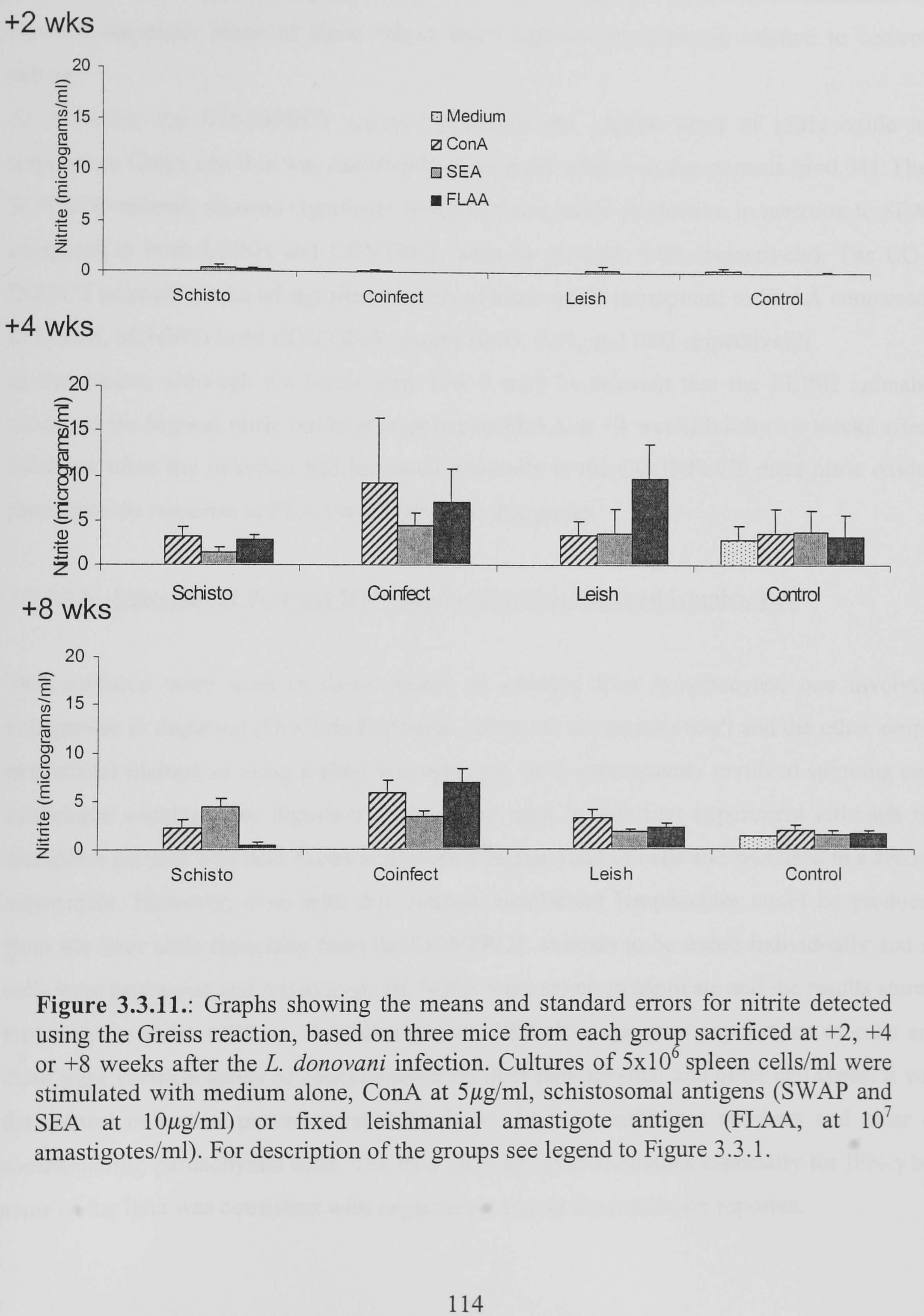


Figure 3.3.11.: Graphs showing the means and standard errors for nitrite detected using the Greiss reaction, based on three mice from each group sacrificed at +2, +4 or +8 weeks after the *L. donovani* infection. Cultures of $5x10^6$ spleen cells/ml were stimulated with medium alone, ConA at 5μg/ml, schistosomal antigens (SWAP and SEA at 10μg/ml) or fixed leishmanial amastigote antigen (FLAA, at 10^7 amastigotes/ml). For description of the groups see legend to Figure 3.3.1.

At +4weeks post *L. donovani* infection the only responses higher than the CONTROL levels were in the CO-INFECT group where the highest mean level of nitrite was in response to ConA followed by FLAA and in the LEISH group which FLAA stimulated an elevated response. None of these values were significantly elevated relative to control values.

At +8weeks, the CO-INFECT animals produced the highest level of nitric oxide in response to ConA and this was statistically significant relative to the controls ($p=0.04$). The SCHISTO animals showed significant levels of nitric oxide production in response to SEA compared to both LEISH and CONTROL animals ($p=0.03$, 0.03 respectively). The CO-INFECT animals produced significant levels of nitric oxide in response to FLAA compared to LEISH, SCHISTO and CONTROL groups (0.03, 0.01, and 0.02 respectively).

In conclusion, although the levels were low it may be relevant that the LEISH animals produced the highest nitric oxide in response to FLAA at +4 weeks but by +8 weeks after infection when the infection had increased markedly in the CO-INFECT mice nitric oxide production in response to FLAA was highest in that group.

3.3.1.3.5. Detection of IL-4 and IFN-γ produced by liver-derived lymphocytes

Two methods were used in these studies to produce liver lymphocytes, one involving collagenase D digestion (Dr Chris Engwerda, 'personal communication') and the other simple mechanical disruption using a glass homogenizer. Both subsequently involved spinning on a histopaque cushion. The digestion method was used in this first experiment although the disruption method was later found to provide a higher yield of cells and was used in a second experiment. However, even with this method insufficient lymphocytes could be produced from the liver cells especially from the CONTROL animals to be tested individually and so cells were processed and tested as pools. Wells were set up in triplicate and the results shown represent the mean of these. Overall it proved difficult to get pure populations of cells and there were variable levels of contamination by liver parenchymal and other cell types. It was the control cells that proved most difficult to obtain in sufficient numbers and clear of contaminating parenchymal cells. The data showed some anomalies especially for IFN-γ but some of the data was consistent with expectation and so the results are reported.

At +2 wk post *L. donovani* super-infection (see Figure 3.3.12a) IL-4 was only detected in the ConA stimulated cultures from the SCHISTO and CO-INFECT groups and in the SEA stimulated culture from the SCHISTO group. The IFN-γ data was difficult to interpret as the medium alone cultures showed quite a high response in the SCHISTO and LEISH groups whereas with FLAA the highest response was with the FLAA and CO-INFECT groups. With SEA there was also a response seen in the CONTROL group. This was comparable in the LEISH group but higher in the CO-INFECT and SCHISTO groups. The IFN-γ production with Con A group showed a pattern more similar to what might be expected as there was a high production in the LEISH group and somewhat lower in the SCHISTO group and no production in the CONTROL cells but the CO-INFECT group also showed a response similar to the SCHISTO group.

At +4 weeks (see Figure 3.3.12b), the IL-4 data was again consistent and as expected showing a similar trend to the +2 week time point. There was evidence of spontaneous IL-4 production in the SCHISTO and CO-INFECT groups in the presence of medium but the significance of this is uncertain because with FLAA there was a comparable level in the CONTROL group. With Con A and SEA the responses were higher and only seen in the SCHISTO and CO-INFECT groups. The IFN-γ data was again puzzling as the CONTROL cells gave high values with both medium alone and the other stimulants. In the Con A and SEA cultures this was particularly high (note the difference in scale on the ConA graph in Figure 3.3.12b).

At +8 weeks (see Figure 3.3.12c), the SCHISTO group again showed comparable IL-4 production in the presence of Con A although no CO-INFECT response was seen in contrast to the +4 time-point. Interestingly the SEA stimulated IL-4 responses in both SCHISTO and CO-INFECT were markedly lower than those recorded at +4 weeks. Similarly, the IFN-γ levels tended to be substantially lower than at the earlier time points, although this time the control levels were lower and closer to what would be expected. With medium alone all of the groups showed low and similar levels.

Figure 3.3.12a: Comparison of IL-4 and IFN-γ produced by cultures of liver-derived lymphocytes.

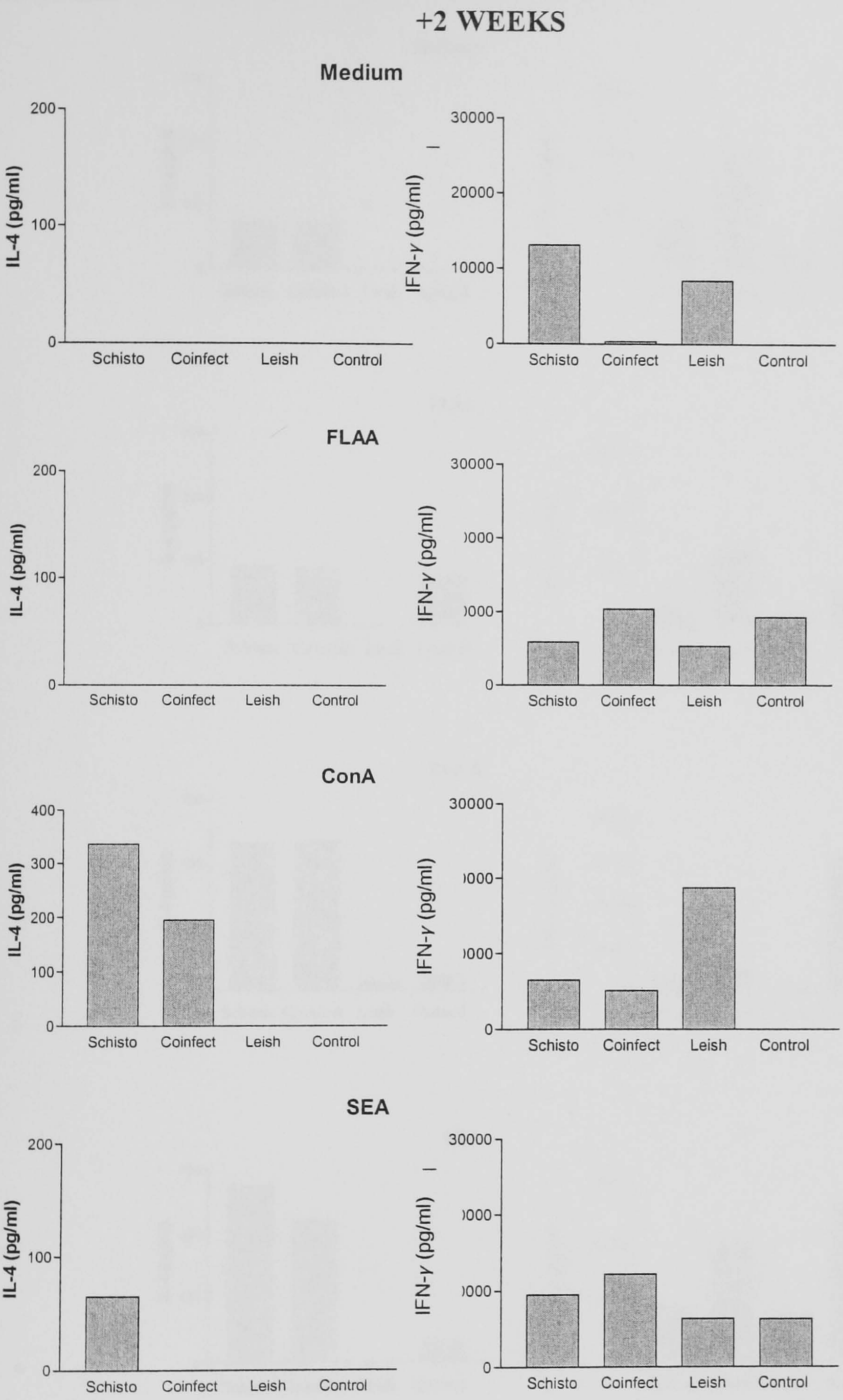


Figure 3.3.12a: Lymphocytes were recovered from livers pooled from 5-7 mice/group at 2 weeks post *L. donovani* infection. Cultures of $5x10^6$cells/ml were stimulated with medium alone, ConA at 5μg/ml, schistosomal antigens (SWAP and SEA at 10μg/ml) or fixed leishmanial amastigote antigen (FLAA, at 10^7amastigotes/ml). Supernatants were recovered at 24hr (for IL-4) or 72hr (for IFN-γ) and assayed by cytokine ELISA. Data represent the mean of three replicate ELISA wells. For description of the groups see legend to Figure 3.3.1.

Figure 3.3.12b: Comparison of IL-4 and IFN-γ produced by cultures of liver-derived lymphocytes.

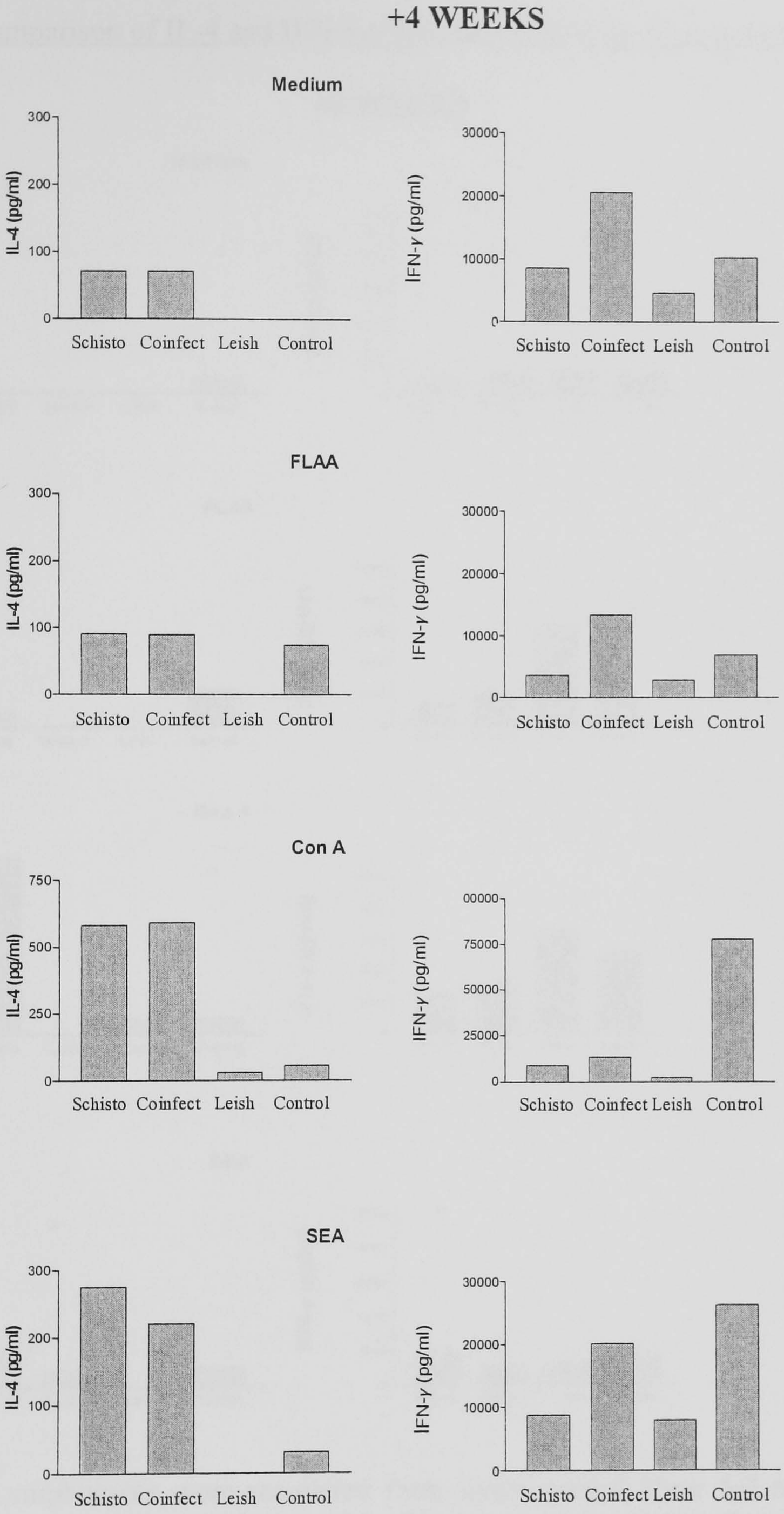


Figure 3.3.12b: Lymphocytes were recovered from livers pooled from 5-7 mice/group at +4 weeks post *L. donovani* infection. Cultures of $5x10^6$ cells/ml were stimulated with medium alone, ConA at 5μg/ml, schistosomal antigens (SWAP and SEA at 10μg/ml) or fixed leishmanial amastigote antigen (FLAA, at 10^7amastigotes/ml). Supernatants were recovered at 24hr (for IL-4) or 72hr (for IFN-γ) and assayed by cytokine ELISA. Data represent the means of three replicate ELISA wells. For description of the groups see legend to Figure 3.3.1.

Figure 3.3.12c: Comparison of IL-4 and IFN-γ produced by cultures of liver-derived lymphocytes.

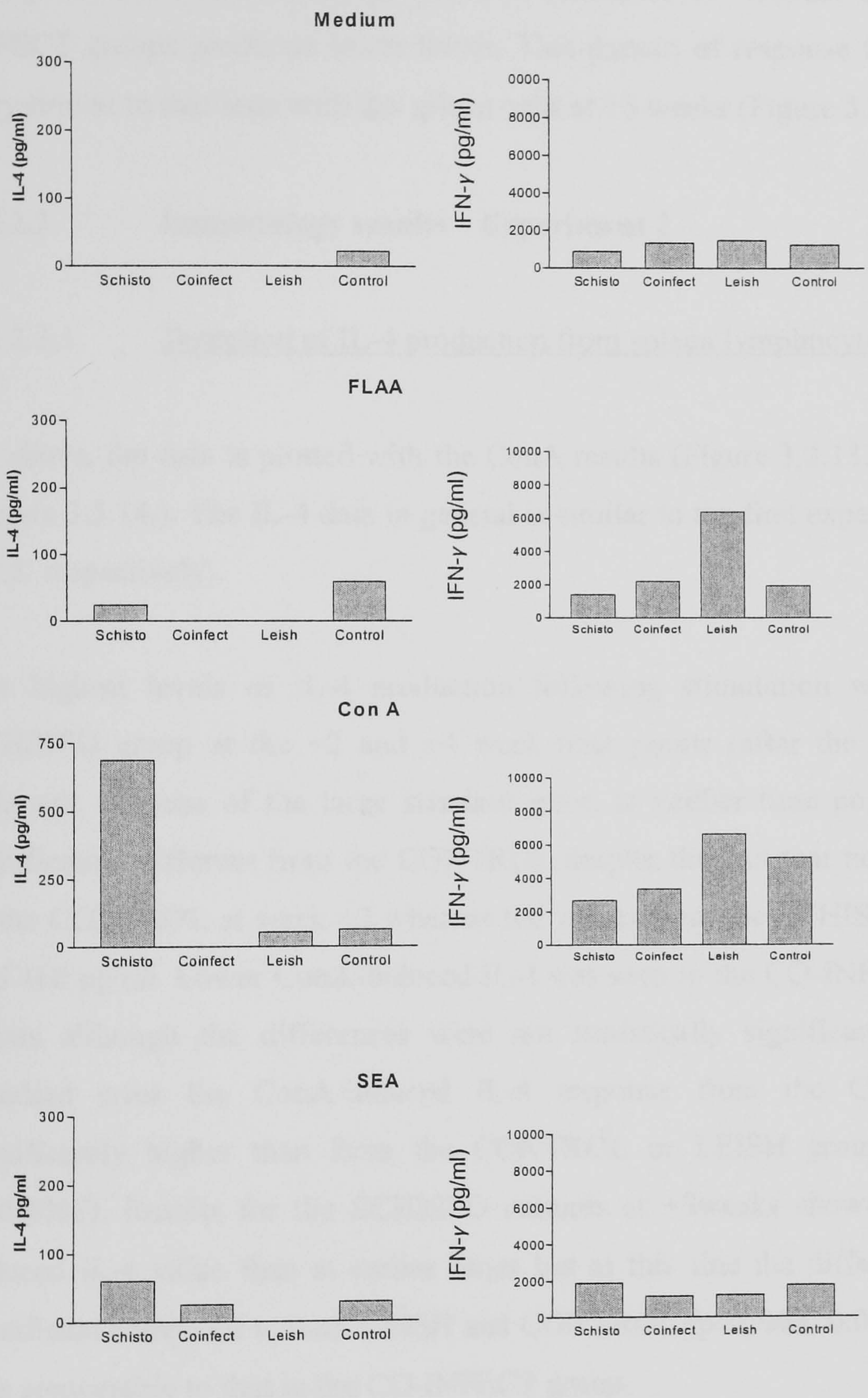


Figure 3.3.12c: Lymphocytes were recovered from livers pooled from 5-7 mice/group at +8 weeks post *L. donovani* infection. Cultures of $5x10^6$cells/ml were stimulated with medium alone, ConA at 5μg/ml, schistosomal antigens (SWAP and SEA at 10μg/ml) or fixed leishmanial amastigote antigen (FLAA, at 10^7amastigotes/ml). Supernatants were recovered at 24hr (for IL-4) or 72hr (for IFN-γ) and assayed by cytokine ELISA. Data represent the means of three replicate ELISA wells. For description of the groups see legend to Figure 3.3.1.

With FLAA the LEISH group showed the highest IFN-γ production and the CO-INFECT group substantially less. With ConA the LEISH cells also produced the highest amount of IFN-γ but the control cells produced a similar level whereas both SCHISTO and CO-INFECT groups produced lower levels. This pattern of response to FLAA and Con A was very similar to that seen with the spleen cells at +8 weeks (Figure 3.3.9.).

3.3.2.3. Immunology results – Experiment 2

3.3.2.3.1 Detection of IL-4 production from spleen lymphocytes by ELISA

As above, the data is plotted with the ConA results (Figure 3.3.13.) and also without them (Figure 3.3.14.). The IL-4 data in general is similar to the first experiment (Figure 3.3.7.and 3.3.8. respectively).

The highest levels of IL-4 production following stimulation with ConA were in the SCHISTO group at the +2 and +4 week time points (after the *L. donovani* infections) although, because of the large standard error, at neither time point was this statistically significantly different from the CONTROL despite the fact that no IL-4 could be detected in the CONTROL at week +2 whereas the values from the SCHISTO mice were 137, 145 and 466 pg/ml. Lower ConA-induced IL-4 was seen in the CO-INFECT group at both time points although the differences were not statistically significant. Because of the low standard error the ConA-induced IL-4 response from the CO-INFECT group was significantly higher than from the CONTROL or LEISH groups at +4wks ($p=0.002$, $p=0.0002$). Results for the SCHISTO cultures at +8weeks showed a lower mean ConA induced IL-4 value than at earlier times but at this tine the differences were statistically significant compared to both LEISH and CONTROL ($p=0.003$, 0.004 respectively) and this was comparable to that in the CO-INFECT group.

Regarding antigen-specific responses (Figure 3.3.13.), significantly raised IL-4 to SWAP was detected at week +2 in the SCHISTO group ($p=0.04$) but not in the CO-INFECT group and there was only a slight and not significant elevation in both groups to SEA. At +4

weeks SEA and SWAP both induced raised levels of IL-4 compared with CONTROLS in both the SCHISTO and CO-INFECT groups but the only significant value was for SEA in the CO-INFECT group ($p=0.04$). There was an anomalous response with one of the mice in the CONTROL group responding to SWAP and FLAA. At +8 weeks raised antigen specific IL-4 production was also seen in the SCHISTO and CO-INFECT groups with both SEA and SWAP but the values were only significantly different ($p=0.04$) from the LEISH and CONTROL groups for the SCHISTO group. There was a slightly elevated but not significant IL-4 response by cells from the LEISH and CO-INFECT groups to FLAA.

Figure 3.3.13. Comparison of the levels of IL-4 in supernatants of spleen cell cultures

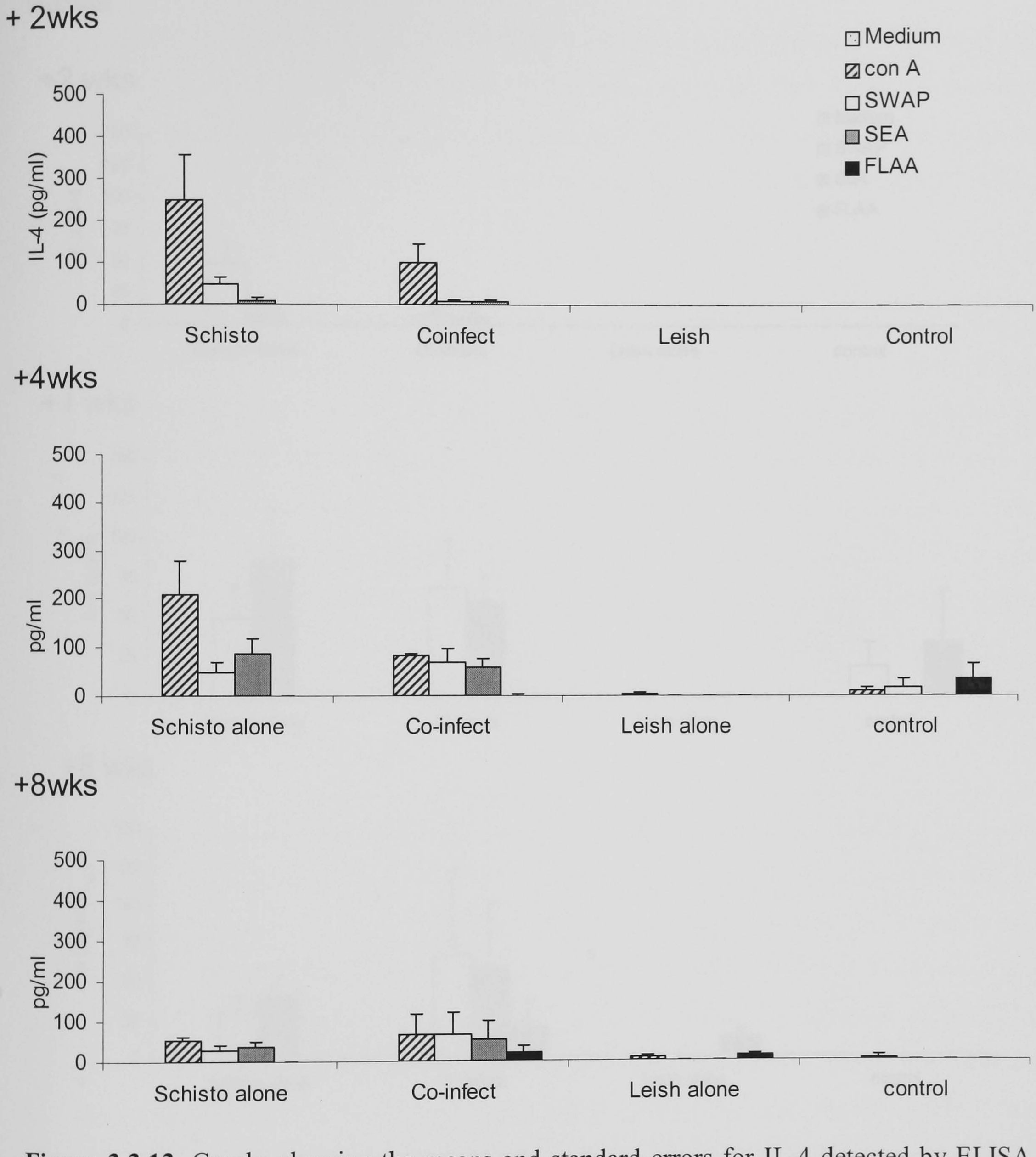


Figure 3.3.13. Graphs showing the means and standard errors for IL-4 detected by ELISA, based on three mice from each group sacrificed at +2, +4 or +8 weeks after the *L. donovani* infection. Cultures of $5x10^6$spleen cells/ml were stimulated with medium alone, mitogen (ConA at 5μg/ml), schistosomal antigens (SWAP and SEA at 10μg/ml) or fixed leishmanial amastigote antigen (FLAA, at 10^7 amastigotes/ml). For description of the groups see legend to Figure 3.3.1.

Figure 3.3.14. Comparison of the levels of IL-4 in supernatants of spleen cell cultures

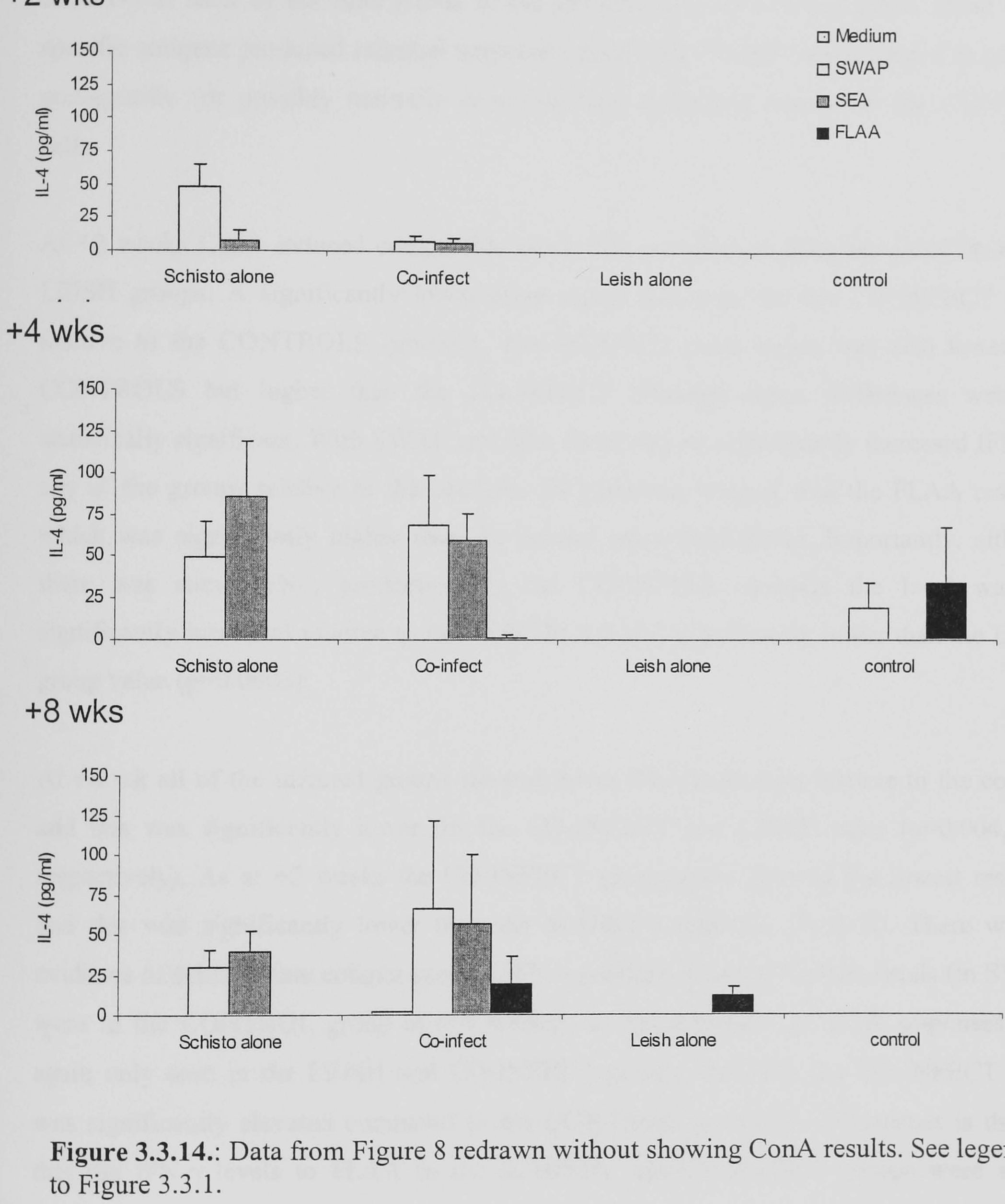


Figure 3.3.14.: Data from Figure 8 redrawn without showing ConA results. See legend to Figure 3.3.1.

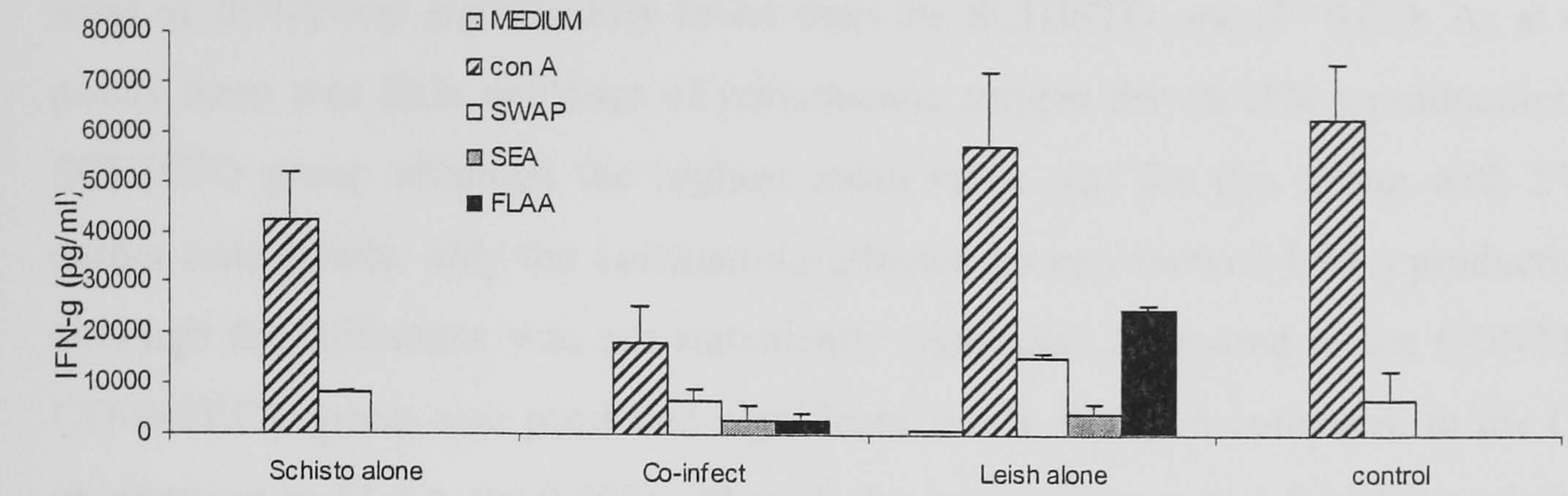
3.3.2.3.2 Detection of IFN-γ production from spleen lymphocytes by ELISA

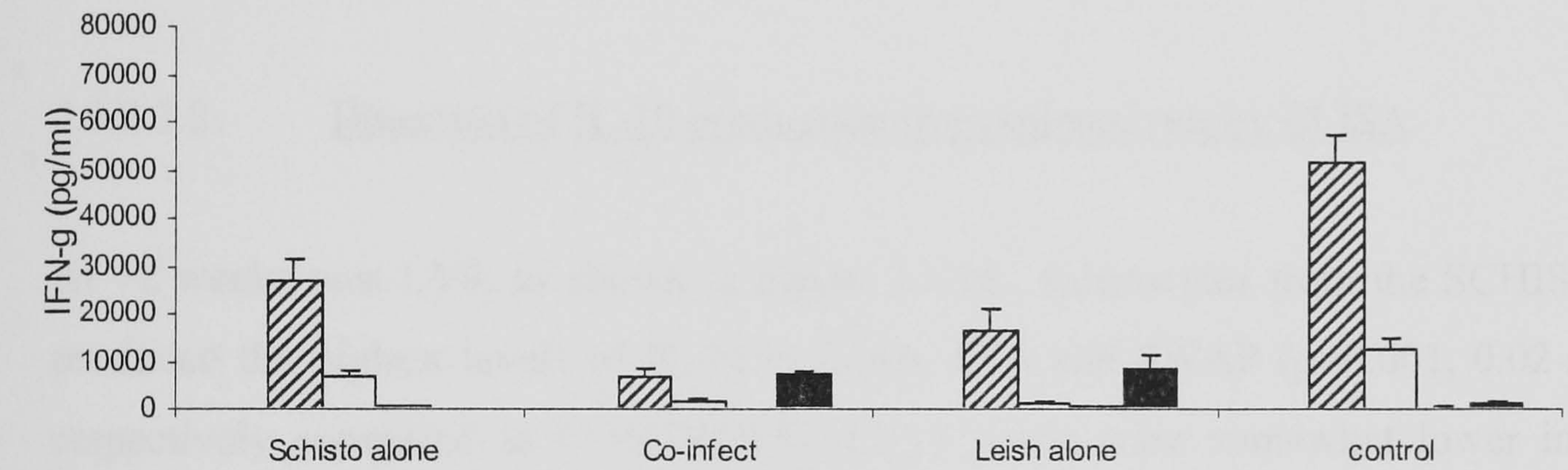
As shown in Figure 3.3.15., CONTROL cells produced significant and comparable levels of IFN-γ at each of the time points in the presence of ConA but medium alone or the specific antigens produced minimal responses apart from SWAP which tended to produce non-specific (or possibly naturally cross-reacting) responses even with the CONTROL cells.

At +2 weeks ConA induced comparable mean IFN-γ responses from the CONTROL and LEISH groups. A significantly lower mean value was seen for the CO-INFECT group relative to the CONTROLS ($p=0.03$). The SCHISTO mean value was also lower than CONTROLS but higher than the CO-INFECT although these differences were not statistically significant. With SWAP and SEA there was no significantly increased IFN-γ in any of the groups relative to the controls. Of particular interest was the FLAA response which was significantly higher than for control mice ($p<0.0001$). Importantly, although there was some IFN-γ production by the CO-INFECT animals the level was not significantly increased relative to the CONTROLS and significantly lower than the LEISH group value ($p=0.0003$).

At +4 wk all of the infected groups showed lower IFN production relative to the controls and this was significantly lower for the CO-INFECT and LEISH mice ($p=0.004$, 0.02 respectively). As at +2 weeks the CO-INFECT group again showed the lowest response and this was significantly lower than the SCHISTO response ($P=0.03$). There was no evidence of schistosome antigen specific IFN-γ production as the highest levels (to SWAP) were in the CONTROL group at (+4 weeks). In the presence of FLAA responses were again only seen in the LEISH and CO-INFECT groups, but only the CO-INFECT value was significantly elevated compared to the CONTROL ($p=0.002$). Of interest is the fact that the IFN-γ levels to FLAA in the SCHISTO and CO-INFECT groups were almost identical unlike the +2 and +8 week times.

Figure 3.3.15. Comparison of the levels of IFN-γ in supernatants of spleen cell cultures





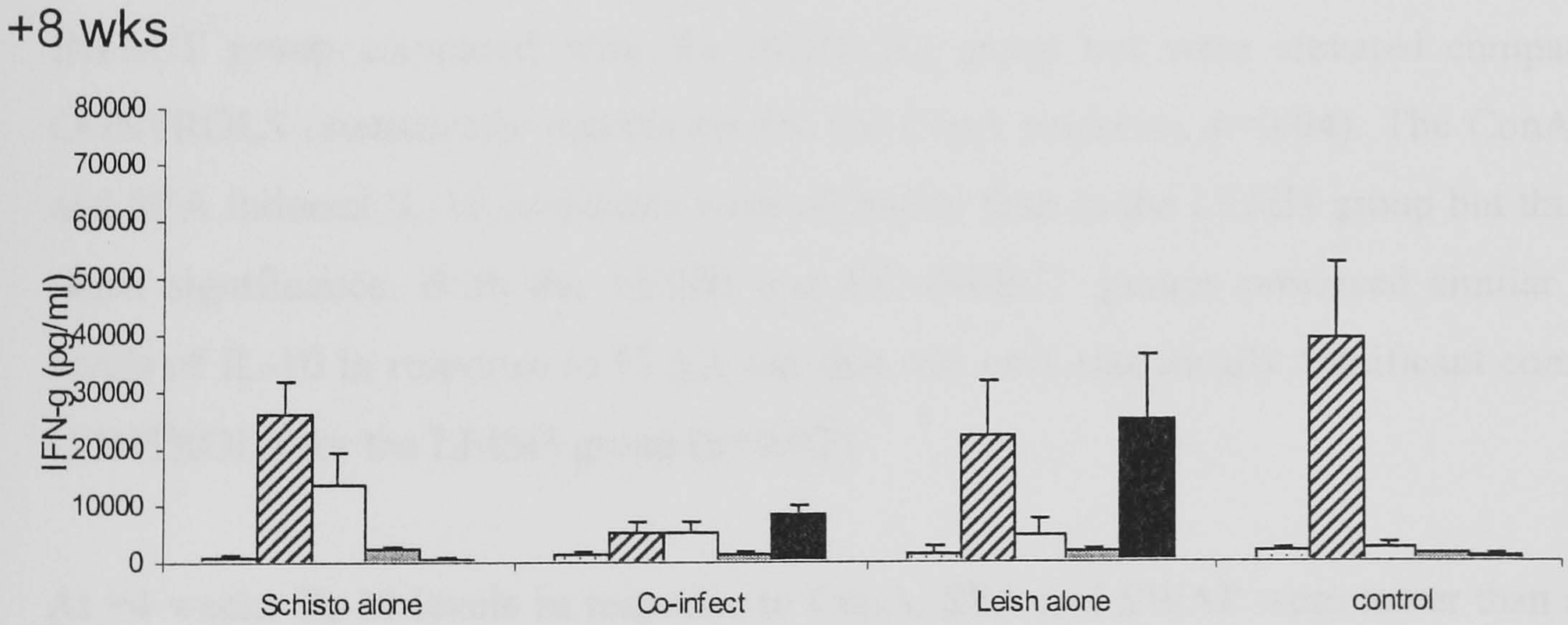


Figure 3.3.15. Graphs showing the means and standard errors for IFN-γ detected by ELISA based on three mice from each group sacrificed at +2, +4 or +8 weeks after the *L. donovani* infection. Cultures of $5x10^6$ spleen cells/ml were stimulated with medium alone, ConA at 5μg/ml, schistosomal antigens (SWAP and SEA at 10μg/ml) or fixed leishmanial amastigote antigen (FLAA, at 10^7 amastigotes/ml). For description of the groups see legend to Figure 3.3.1.

At +8weeks the pattern of ConA-induced IFN-γ production was very similar to the +12 week time points *i.e.* highest from the CONTROLS, lowest in the CO-INFECT mice and at intermediate levels in the other two groups. As at the +4 week time point the CO-INFECT level of IFN-γ was significantly lower than the SCHISTO one (P=0.03). As at earlier time-points there was little evidence of schistosome antigen driven IFN-γ production even in the SCHISTO group although the highest mean value was for this group with SWAP. As at earlier time-points, only the *Leishmania*-infected groups showed IFN-γ production to FLAA although the difference was not statistically significant compared to the CONTROLS. The CO-INFECT group also produced significant levels of IFN-γ compared to the CONTROLS in response to FLAA (p=0.008) although the mean value was 3-fold lower than the LEISH alone value.

3.3.2.3.3 Detection of IL-10 production from splenocytes by ELISA

At +2 weeks post LV9, as shown in Figure 3.3.16., splenocytes from the SCHISTO group produced the highest levels of IL-10 to ConA, SEA and SWAP (p=0.001, 0.02 and 0.001 respectively compared to CONTROLS). IL-10 levels were somewhat lower in the CO-INFECT group compared with the SCHISTO group but were elevated compared with CONTROLS (statistically significant for the ConA response, p=0.04). The ConA, SWAP and SEA induced IL-10 responses were all higher than in the LEISH group but this did not reach significance. Both the LEISH and CO-INFECT groups produced similar elevated levels of IL-10 in response to FLAA but this was only statistically significant compared to CONTROLS for the LEISH group (p=0.02).

At +4 weeks IL-10 levels in response to ConA, SEA and SWAP were lower than at the +2 week time point for the SCHISTO group. The highest Con A-induced IL-10 production was from the SCHISTO group and this was significantly higher than from both the CO-INFECT and LEISH groups (p=0.03, 0.02 respectively). SEA-induced IL-10 responses were again highest in the SCHISTO group, significantly higher than in the CONTROL (p=0.04). The SCHISTO group also produced IL-10 to SWAP.

Figure 3.3.16 Comparison of the levels of IL-10 in supernatants of spleen cell cultures

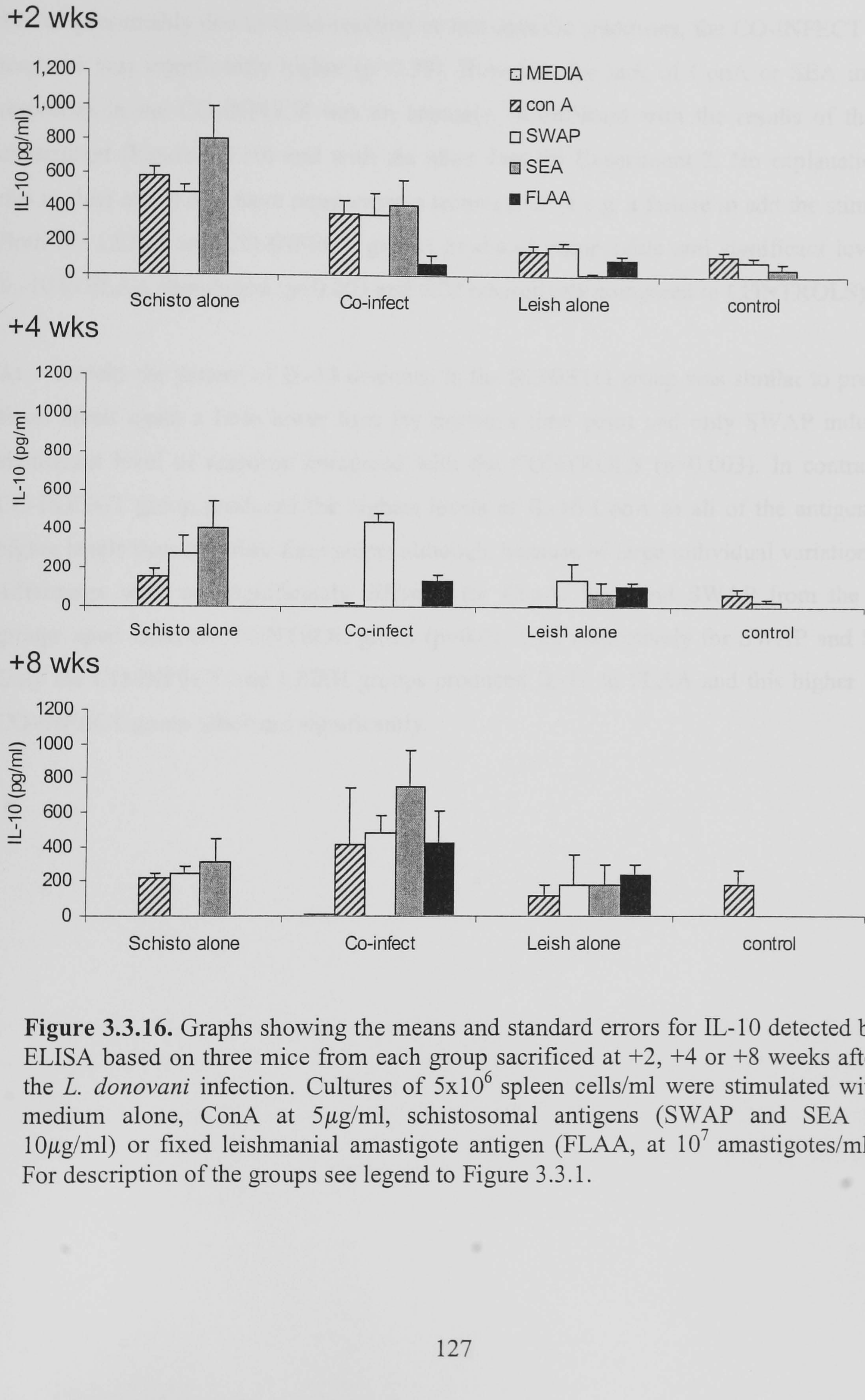


Figure 3.3.16. Graphs showing the means and standard errors for IL-10 detected by ELISA based on three mice from each group sacrificed at +2, +4 or +8 weeks after the *L. donovani* infection. Cultures of $5x10^6$ spleen cells/ml were stimulated with medium alone, ConA at 5μg/ml, schistosomal antigens (SWAP and SEA at 10μg/ml) or fixed leishmanial amastigote antigen (FLAA, at 10^7 amastigotes/ml). For description of the groups see legend to Figure 3.3.1.

The CO-INFECT animals produced significant IL-10 in response to SWAP compared to the CONTROLS ($p=0.001$). Although the LEISH group also produced some IL-10 to SWAP presumably due to cross-reacting or non-specific responses, the CO-INFECT IL-10 response was significantly higher ($p=0.39$). However, the lack of ConA or SEA induced responses in the CO-INFECT was an anomaly, inconsistent with the results of the first experiment (Figure 3.3.10) and with the other data for Experiment 2. No explanation for this is clear and it may have represented a technical error e.g. a failure to add the stimulant. Both the LEISH and CO-INFECT groups produced comparable and significant levels of IL-10 to FLAA stimulation ($p=0.003$ and 0.02 respectively compared to CONTROLS).

At + 8weeks the pattern of IL-10 response in the SCHISTO group was similar to previous times albeit again a little lower than the previous time point and only SWAP induced a significant level of response compared with the CONTROLS ($p=0.003$). In contrast the CO-INFECT group produced the highest levels of IL-10 ConA to all of the antigens and higher levels than at earlier time points although, because of large individual variations, the differences were not significantly different for ConA, SEA and SWAP from the other groups apart from the CONTROL group ($p=0.01$, 0.02 respectively for SWAP and SEA). Only the CO-INFECT and LEISH groups produced IL-10 to FLAA and this higher in the CO-INFECT group albeit not significantly.

3.3.2.3.4. Detection of Nitrite production in splenocyte cultures-Experiment 2

The data (Figure 3.3.17.) were similar to the first experiment (Figure 3.3.11.).

At +2 weeks post-*L. donovani*, there was no significant production of nitric oxide in response to ConA, SEA, FLAA antigens in any of the groups.

At +4 weeks there was no elevated production of nitric oxide in response to mitogen (ConA) in any of the infected groups. Meanwhile, in response to the schistosome antigen (SEA) the CO-INFECT animals produced elevated nitric oxide levels compared to the SCHISTO ($p=0.04$), LEISH ($p=0.02$) and CONTROL animals (not statistically significant). Significantly elevated levels were seen compared with CONTROL mice with FLAA in both the CO-INFECT and LEISH groups ($p=0.02$ and 0.01 respectively) and strikingly, the level of nitrite was significantly higher in the LEISH group compared with the CO-INFECT group ($p=0.02$).

At +8 weeks, elevated responses were seen with ConA in all infected groups but this was only statistically significant relative to CONTROL for the SCHISTO group ($p=0.003$). Interestingly, the SCHISTO mice produced significant levels of nitric oxide production in response to SEA compared to the CO-INFECT, LEISH and CONTROL animals ($p=0.03$, 0.03, and 0.007 respectively) perhaps indicating more of a Th1 role in the chronic granulomatous response. The CO-INFECT animals produced the highest levels of nitric oxide in response to FLAA but this was not statistically significantly higher than the LEISH values and CONTROL.

In conclusion, these results showed a similar pattern to the results obtained from the earlier experiment (Figure 3.3.11.), showing that the LEISH group produced notably higher levels of nitric oxide in response to FLAA compared with the CO-INFECT group at +4weeks but that by +8 weeks this pattern is reversed, the nitrite levels being lower than at +4 weeks in the LEISH and higher in the CO-INFECT (Fig. 3.3.17.).

Figure 3.3.17.: Levels of Nitrite produced in splenocyte cultures

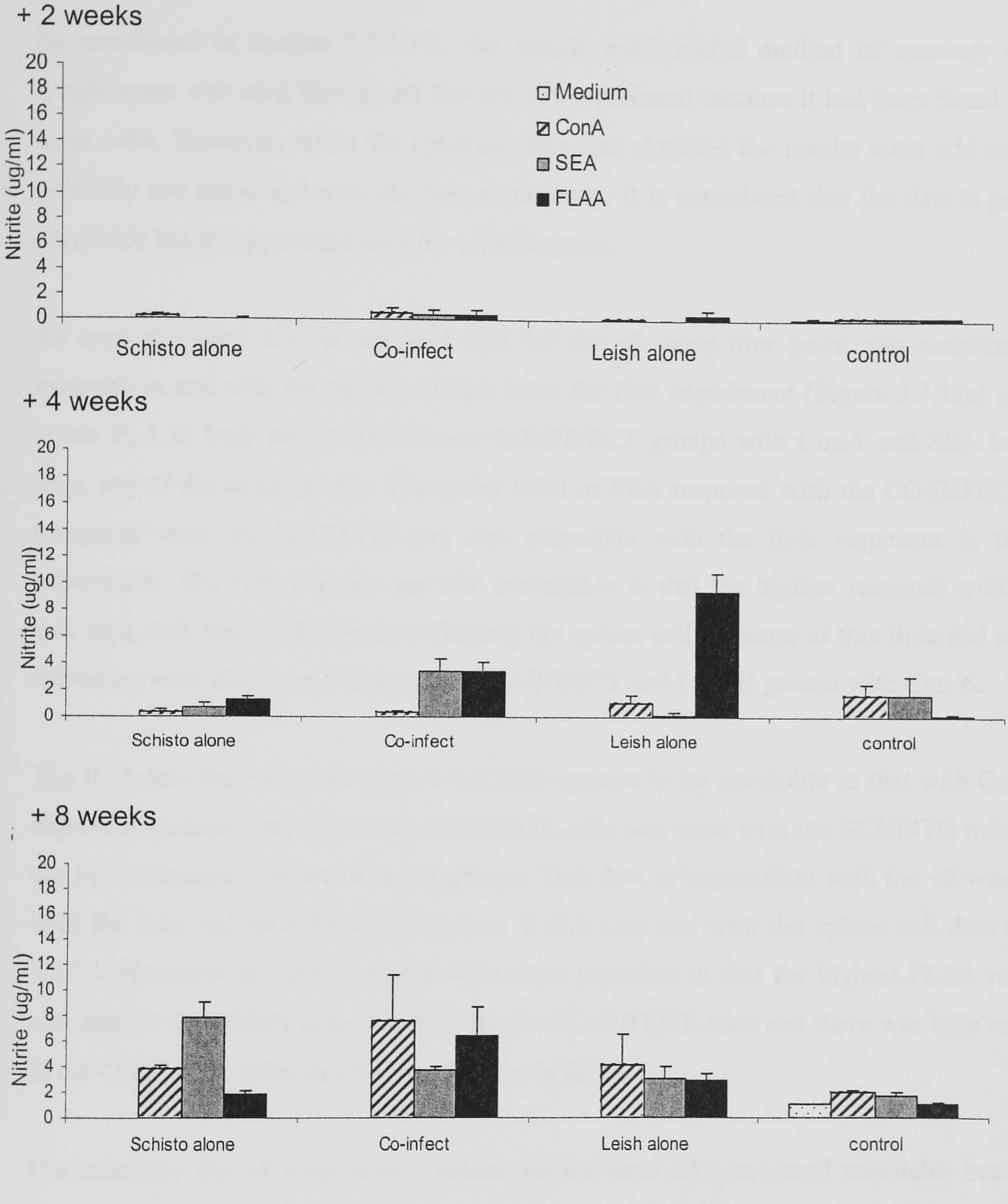


Figure 3.3.17.: Graphs showing the means and standard errors for nitrite detected using the Greiss reaction, based on three mice from each group sacrificed at +2, +4 or +8 weeks after the *L. donovani* infection. Cultures of $5x10^6$ spleen cells/ml were stimulated with medium alone, ConA at 5μg/ml, schistosomal antigens (SWAP and SEA at 10μg/ml) or fixed leishmanial amastigote antigen (FLAA, at 10^7 amastigotes/ml). For description of the groups see legend to Figure 3.3.1.

3.3.2.3.5. Detection of IL-4 and IFN-γ produced by liver-derived lymphocytes by ELISA

As mentioned in section 3.3.1.3.5. the simple mechanichal method of recovery of liver lymphocytes was used throughout this second experiment because it had been found to yield more cells. However, when the cytokine data was obtained the results were odd and were generally not consistent with the first experiment. It is considered that the data is generally unreliable but it is presented here for completeness.

As seen in Figure 3.3.18a the IL-4 data for the +2 week time point was consistent with expectation and with the pattern of data from the first experiment (Figure 3.3.12a) *i.e.* high levels IL-4 in both the SCHISTO and CO-INFECT groups with ConA and SEA but little from any of the other groups. The lower level of SEA response with the CO-INFECT cells compared with the SCHISTO was also consistent with the liver responses in the first experiment. The IFN-γ results are also reasonable in that the highest response with FLAA was seen with the LEISH group reflecting the spleen cell response at this time and elevated responses were also seen with both the CO-INFECT and LEISH groups with Con A.

The IL-4 data for +4 weeks (Figure 3.3.12b) appears to be unreliable in that with ConA the highest responses were seen with CONTROL cells and none with the SCHISTO mice. Also the SEA response was low with all groups. This data is inconsistent with the +2 week data, with the liver cell data from Experiment 1 (3.3.12a) and with the spleen cell data (Figure 3.3.7.). However, the IFN-γ results were more plausible in that the highest FLAA response was seen in the LEISH mice followed by the CO-INFECT mice and there was little response in the CONTROL cells, in medium alone or in SEA.

Unfortunately the +8 week liver cytokine results were all considered unreliable because of very high values for the CONTROL cells and for medium alone (Data not shown).

Figure 3.3.18a: Comparison of IL-4 and IFN-γ produced by cultures of liver-derived lymphocytes.

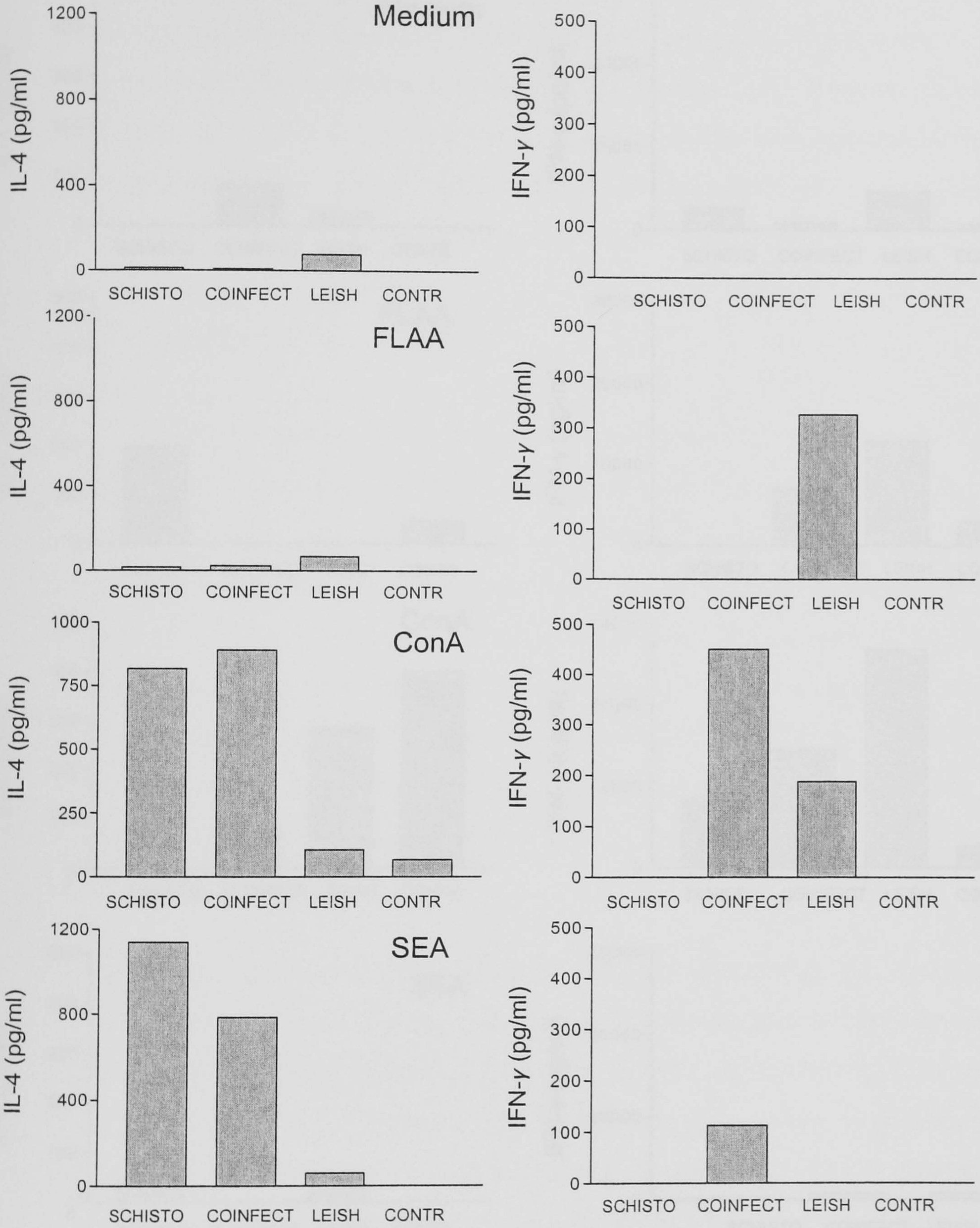


Figure 3.3.18a: Lymphocytes were recovered from livers pooled from 5-7 mice/group at +2 weeks post *L. donovani* infection. Cultures of $5x10^6$cells/ml were stimulated with medium alone, ConA at 5μg/ml, schistosomal antigens (SWAP and SEA at 10μg/ml) or fixed leishmanial amastigote antigen (FLAA, at 10^7amastigotes/ml). Supernatants were recovered at 24hr (for IL-4) or 72hr (for IFN-γ) and assayed by cytokine ELISA. Data represent the means of three replicate ELISA wells. For description of the groups see legend to Figure 3.3.1.

Figure 3.3.18b: Comparison of IL-4 and IFN-γ produced by cultures of liver-derived lymphocytes.

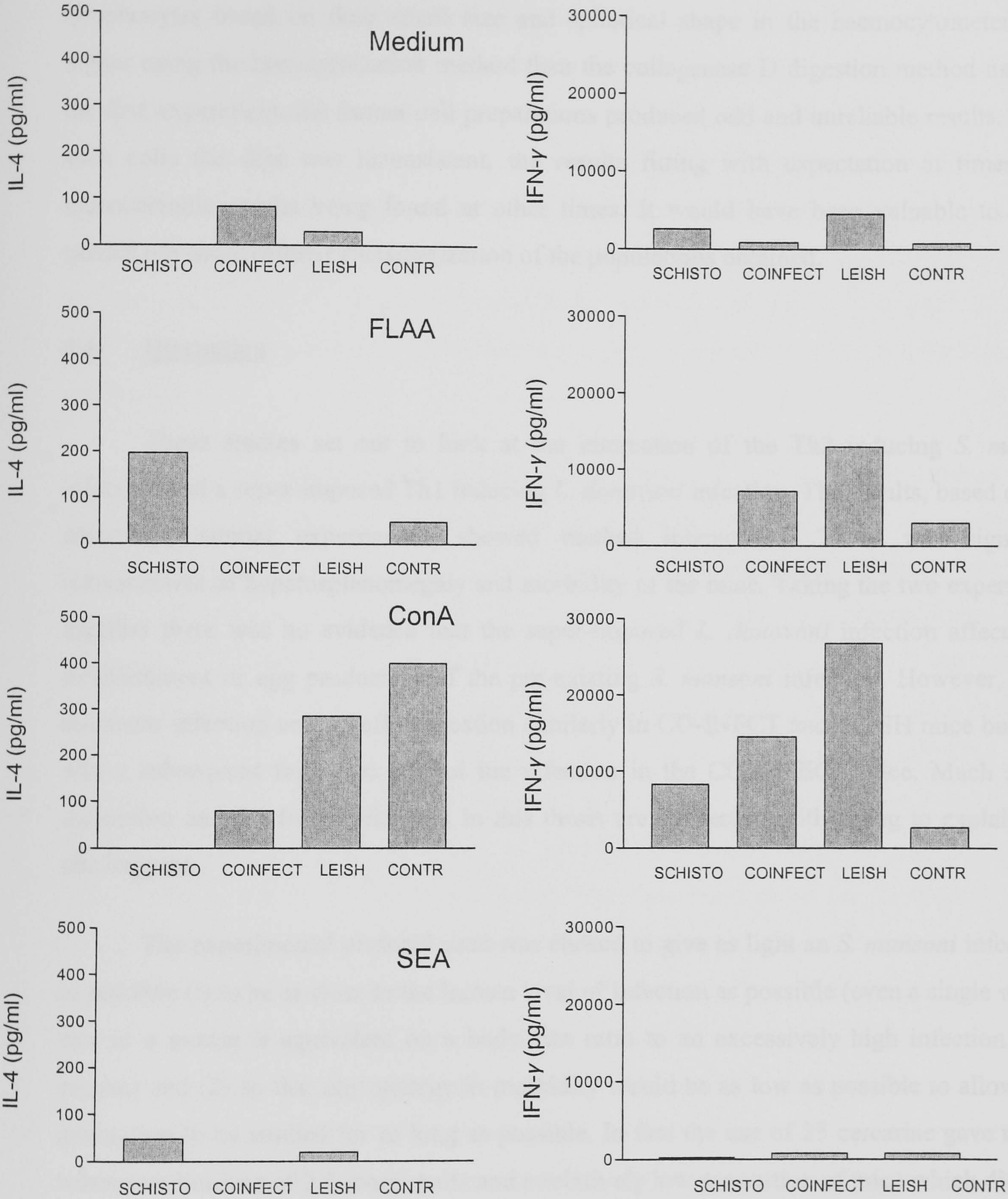


Figure 3.3.18b: Lymphocytes were recovered from livers pooled from 5-7 mice/group at +4 weeks post *L. donovani* infection. Cultures of $5x10^6$cells/ml were stimulated with medium alone, ConA at 5μg/ml, schistosomal antigens (SWAP and SEA at 10μg/ml) or fixed leishmanial amastigote antigen (FLAA, at 10^7amastigotes/ml). Supernatants were recovered at 24hr (for IL-4) or 72hr (for IFN-γ) and assayed by cytokine ELISA. Data represent the means of three replicate ELISA wells. For description of the groups see legend to Figure 3.3.1.

Overall the liver lymphocyte data for this experiment was disappointing and less reliable than the first experiment. It is concluded that although the yield of cells regarded as lymphocytes based on their small size and spherical shape in the haemocytometer was higher using the homogenization method than the collagenase D digestion method used in the first experiment, the former cell preparations produced odd and unreliable results. With such cells the data was inconsistent, the results fitting with expectation at times but inconceivable results being found at other times. It would have been valuable to have carried out some cellular characterization of the populations obtained.

3.4. Discussion

These studies set out to look at the interaction of the Th2 inducing *S. mansoni* infection and a super-imposed Th1 inducing *L. donovani* infection. The results, based on two essentially similar experiments, showed marked interactions. There was significant enhancement of hepatosplenomegaly and morbidity of the mice. Taking the two experiments together there was no evidence that the super-imposed *L. donovani* infection affected the establishment or egg production of the pre-existing *S. mansoni* infection. However, the *L. donovani* infection started off replication similarly in CO-INFCT and LEISH mice but there was a subsequent failure to control the infection in the CO-INFECT mice. Much of this discussion and the further chapters in this thesis are concerned with trying to explain how this happens.

The experimental protocol used was chosen to give as light an *S. mansoni* infection as possible (i) to be as close to the human level of infection as possible (even a single worm pair in a mouse is equivalent on a body size ratio to an excessively high infection in a human) and (2) so that any synergy in morbidity would be as low as possible to allow the interaction to be studied for as long as possible. In fact the use of 25 cercariae gave mean worm pair burdens of 2-3 worm pairs and a relatively low proportion of mice which did not have a bisexual egg producing infection. Nevertheless the CO-INFECT mice did suffer increased morbidity and some died or had to be euthanized. Overall, though, the 25 *S. mansoni* cercariae and $2x10^7$ *L. donovani* amastigotes seemed to represent a balanced level

of infection for the duration of the experiments set up. Regarding the reasons for the increased morbidity/mortality in the CO-INFECT mice the overall weight of the mice in all of the infection and control groups differed little at each time point but both the *L. donovani* and the *S. mansoni* infections caused hepatosplenomegaly and the CO-INFECT mice showed an additive effect having the highest mean spleen and liver weights. Hepatosplenomegaly per se would not necessarily cause morbidity but would increase the stress on the animals, apart from causing portal hypertension and the sequelae of complications ranging from increased incidence of bleeding (GIT hemorrhage) to minor complications such as congestive gastropathy causing anorexia and weight loss and eventually death. Further consideration of the causes of morbidity are dealt with below and in Chapter 5.

S. mansoni worm burdens and importantly worm pair burdens were consistent in the SCHISTO and CO-INFECT groups and at the different time points in both experiments demonstrating that the *L. donovani* infection had no effect on survival of the worms. With regard to possible effects of the LEISH infection on egg production/egg persistence in the liver, the GMEC per worm pair were indeed a little higher in the SCHISTO animals than the CO-INFECT at each of the time points in Experiment 1 but the differences were not significant. In Experiment 2 the GMEC/worm pair was higher in the SCHISTO group compared with the CO-INFECT group at +2 weeks but essentially similar at +4 and +8 weeks. This high GMEC/worm pair at +2 weeks for the SCHISTO group was inconsistent with the data from the first experiment, data from later time points for Experiment 2 and data from other Experiments (data not shown) so this result is considered unreliable. It could have arisen from a failure to recover all of the worms from some of the mice in this group. Overall the data show that the super-imposed LEISH infection has minimal if any effect on the established schistosomes or their egg production and survival. Since the schistosome egg is the principle inducer of the Th2 response in schistosomiasis (Warren, 1978; Pearce *et al.*, 1991; Grzych *et al.*, 1991) the consistency in liver egg burden between CO-INFECT and SCHISTO mice means that direct assessment can be made of any immuno-modulatory effects the *L. donovani* infection might have had on anti-egg immune responses.

The patterns of infection in the LEISH animals are as reported previously in mice *i.e.* control of the parasites in the liver between weeks +4 and +8 eventually resulting in elimination and gradual increase in the spleen LDU leading to persistent infection (Murphy *et al.*, 2001).

It is clear that the major effect observed in the CO-INFECT mice was that they failed to develop this control such that higher *L. donovani* LDU were seen compared with the LEISH alone particularly at +8 weeks. However, it is important to stress that early during the co-infection there were not marked differences. Thus, in the livers of CO-INFECT compared with LEISH animals LDU at +2 weeks post super-infection were 1.7 fold lower in Experiment 1 and comparable in Experiment 2. This means that *L. donovani* shows comparable rates of replication in naïve mice and mice harboring *S. mansoni* infections of 8-10 weeks duration. By +4 weeks LDU were comparable in Experiment 1 and 2.5 fold higher in Experiment 2 indicating that by this time there were signs of a loss of control of the *L. donovani* infection. This was much more marked at +8 weeks when LDU were 27 fold higher in Experiment 1 and 12.5 fold higher in Experiment 2. A similar result was seen in the spleen with LDU at +8 weeks being 11.3 fold higher in the CO-INFECT compared with the LEISH in Experiment 1 and 12.4 fold higher in Experiment 2.

It would obviously have been interesting to know how the *L. donovani* LDUs would have progressed beyond the +8 week time point i.e. would the *L. donovani* infection have been resolved or continued uncontrolled. However, as judged by the morbidity seen in the second experiment in particular it seems unlikely that it will be easy to study this co-infection at later time points using the same combination of mice/schistosome strain and *L. donovani* strain and levels of parasite infection. Twenty five cercariae were used in these experiments and the resulting mean worm burdens were pretty low (mean of 3.1 worm pairs in Experiment 1 and 2.8 in Experiment 2) but by reducing the exposure it would be possible to obtain a lower mean worm burden. Reducing the infection in this way would mean that a higher proportion of mice did not have a mixed egg producing infection and so larger group sizes would be needed. Also the effects on the *L. donovani* LDU might have

been dependent on the level of schistosome infection and so it might have taken longer anyway for the effect on the *L. donovani* to develop.

Various possible explanations have been considered to explain the failure of the CO-INFECT mice to control the infection and much of the rest of this thesis is aimed at investigating the most likely of these possibilities. The hypotheses, which have developed during the work, are laid out next.

(1) *S. mansoni* induced hepatosplenomegaly provides additional tissue sites for *L. donovani* proliferation (*i.e.* there is simply more tissue in general for *L. donovani* to infect);
(2) this added tissue in the liver would comprise active *S. mansoni* egg granulomas rich in recruited macrophages and in the spleen, would comprise blood as a result of hypertension but also increased numbers of immune cells including recruited macrophages from the spleen (King *et al.*, 2001), responding the antigenic challenge of the eggs (*i.e.* there are more suitable host cells [macrophages] to infect); furthermore the local immunological changes in the liver organ particularly in Küpffer cells show a Th2-deviated immune response which may lead to further susceptibility towards *L. donovani* infection (Hayashi *et al.*, 1999). (3) there is a failure to induce a sufficiently strong protective systemic Th1 response to *L. donovani* antigens by the spleen i.e. there was a lower IFN-γ response to FLAA due to:
(i) the Th2-skewed response to *S. mansoni* resulting in direct action of IL-4 on Th1 development (O'Garra, 1998) or (ii) to indirect effects of schistosome or *Leishmania* induced IL-10 (which is produced by Th2 cells but also several other cell types [*e.g.* Treg cells] and not strictly a Th2 cytokine) on antigen presenting cell functions such as downregulation of co-stimulatory molecules and IL-12 production (Bogdan *et al.*, 1991; Moore *et al.*, 2001). (iii) action of other regulatory cytokines *e.g.* TGF-β (Barrel *et al.*, 1995).
(4) lower IFN-γ levels may result in reduced development/reduced rate of maturation of *L. donovani* granulomas (comprising antigen-specific Th1 cells and recruited macrophage and which are most clearly seen in the liver) *i.e.* there would be a reduced frequency of development of mature *L. donovani* granulomas;

(5) *L. donovani* granulomas may physically fail to form correctly within the context of the inflammatory cells of the *S. mansoni* granulomas and so there may be a lack of control in infected macrophages within the egg granuloma; (6) even if morphologically normal mature granulomas form the macrophages have a reduced capacity to become activated and so produce leishmanicidal factors [nitric oxide, ROI and TNF-α]. This could be due to lower IFN-γ [as in (3) above] or: (7) high levels of IL-10 induced by the *S. mansoni* infection serve to down-regulate macrophage activation to develop leishmanicidal activity (Moore *et al.*, 2001; Groux *et al.*, 1999).

(8) inability of the macrophages to become leishmanicidal could be due to the fact that macrophages in schistosome infected mice are alternatively activated [by IL-4 and IL-13 (Linehan *et al.*, 2003)] and so are poor at directing *L. donovani* granuloma formation and/or show poor leishmanicidal activity superoxide and NO production (Gazzinelli *et al.*, 1992);

(9) such effects on impaired macrophage function may be more pronounced within the *S. mansoni* egg granulomas in which the aaMø predominate (Linehan *et al.*, 2003) so that there is preferential *L. donovani* replication within infected macrophages within the egg granulomas;

(10) reduced leishmanicidal activity due to down-regulated IFN-γ production or down-regulated macrophage activation could result in a failure to kill amastigotes resulting in accumulation of amastigotes within *L. donovani* infected macrophages *i.e.* there would be a higher mean numbers of amastigotes in CO-INFECT mice compared with LEISH mice and/or in the infected macrophages within the *S. mansoni* granulomas compared with the parenchyma of CO-INFECT mice.

With regard to (1) and (2), *i.e.* greater organ size and availability of macrophage host cells for infection in the *S. mansoni* infected mice, comparison of the organ weights shows that the percentage differences between organ weights in the LEISH and CO-INFECT groups were small and not sufficient to explain the many fold greater LDUs in the CO-INFECT mice at +8 weeks. The egg granuloma comprises around 30% macrophages (Oswald *et al.*, 1993; Andrade and Cheever, 1995) and this may offer an increased density of potential macrophage host cells although of course the granuloma does displace the normal liver tissue that is rich in Küpffer cells. Although this idea cannot be discounted, there is no evidence

that the spread of the *L. donovani* is limited by available macrophages. The super-infection was given +8 weeks after the *S. mansoni* infection and since egg deposition would have started at 5-6 weeks there was a significant granulomatous response and schistosome induced organ enlargement in the liver at the time. However, the LDU counts were comparable (Experiment 2) or even lower (Experiment 1) in the CO-INFECT mice at +2 weeks post super-infection showing no evidence of enhanced replication in the CO-INFECT mice over the first two weeks of infection. *S. mansoni* egg granulomas were not commonly seen in the spleen in histological sections (data not shown) and so the increased infection in the spleen in the CO-INFECT mice could not be due to increased availability of granuloma macrophages.

The next hypothesis to be considered (3 above) is that there is a failure to induce a sufficiently strong protective systemic Th1 response to *L. donovani* antigens by the spleen due the Th2-skewed response to *S. mansoni* (*i.e.* there was a lower IFN-γ response to FLAA). The effects of IL-4 in inducing Th2 development are dominant over Th1 polarizing cytokines (Hsieh *et al.*, 1993, Seder and Paul, 1994,) *i.e.* if IL-4 reaches a certain threshold during initiation of an immune response Th2 cells will predominate and then by producing increasing levels of IL-4 this will accelerate Th2 development. The above data clearly show that in the SCHISTO alone and the CO-INFECT mice there is elevated IL-4 and IL-10 which in the case of the CO-INFECT were produced in recall responses to both schistosome antigen and FLAA.

It is clear that Th1 responses are required for control of *L. donovani* infections in mice. Processes leading to the induction of the protective Th1 response to *L. donovani* begin soon after infection *i.e.* Th1 chemokine mRNA expression is seen at 2hr post infection and peaks at 5hr (Cottrell *et al.*, 1998; 2000) and splenic DCs show IL-12 production within 5 hours of infection (Gorak *et al.*, 1998). Development of a pro-inflammatory response involving IL-2, IL-12 and IFN-γ develop and are crucial to the control and resolution of the infection (Kaye *et al.*, 1991; Engwerda *et al.*, 1996; 1998). For example, depletion of IL-12 or IFN-γ with monoclonal antibody (mAb) during the initial infection of mice results in markedly increased LDU in the liver at day 14 (Murphy *et al.*, 2001). IFN-$\gamma^{-/-}$ mice show uncontrolled parasite proliferation for 8 weeks post infection although LDU eventually

decline between 8 and 12 weeks which appeared to be controlled by TNF-α as anti-TNF-α treatment reversed the control (Taylor and Murray, 1997).

Regarding the possible modulatory role of IL-4 on progression of *L. donovani* infections there has been considerable interest in the role of endogenous IL-4 induced by the *L. donovani* infection in both mice and humans. In mice IL-4 mRNA expression is seen in the tissues of infected mice soon after infection but this does not seem to progress and is believed to be rapidly overcome by a protective Th1 response (Miralles *et al.*, 1994). Studies using anti-IL-4 antibody treatment (Miralles *et al.*, 1994; Murray, 1994) showed no effect on the kinetics of visceral infection. Furthermore, IL-4 $^{-/-}$ mice did not show enhanced protection (Satoskar and Alexander, 1995) compared with the wild-type mice. So this data suggested that endogenous induction of IL-4 responses play little or no role in controlling experimental *L. donovani* infection.

In fact IL-4 has more recently been shown to have a subtle influence on Th1/Th2 commitment. Studies of *L. major* infection in BALB/c mice which are susceptible to infection and normally make a strongly polarized Th2 response (Reiner and Locksley, 1995; Biedermann *et al.*, 2001) demonstrated that if IL-4 is present (by injection) during the initial activation of DCs (within the first 8hr of infection) the mice are made resistant and DCs in the draining lymph node made large amounts of IL-12 at 16 hrs post infection. At 8 weeks of infection these mice made higher levels of IFN-γ and markedly lower levels of IL-4 mRNA in the lymph node when compared with untreated mice. However, if the injection of IL-4 is extended to 16 and 24 hr, when the DC/CD4 T cell priming is occurring the resistant phenotype is reversed and the cytokine production in the lymph nodes reverts to that of the untreated mice i.e. high IL-4 and low IFN-γ. So the influence of IL-4 on responses to co-administered antigen or infectious organism is crucially dependent on the timing and persistence of the elevated IL-4.

Of direct relevance to hypothesis (3) are studies aimed at experimentally increasing IL-4 levels during *L. donovani* infection. Murray *et al.* (1997) showed that IL-4 transgenic mice which over-expressed IL-4 produced a strong Th2 response as judged by IgE production but showed almost exactly the same pattern of liver LDU as WT mice, effectively resolving

the infection at 8 weeks post infection. However, these mice were on a 129/Sv background which are generally innately resistant to *Leishmania* infection (Bradley *et al.*, 1979) and it was suggested that the failure to show exacerbated disease against *L. donovani* was because the enhanced IL-4 secretion alone was not sufficient to overcome the innate resistance of the 129/Sv strain. No studies seem to have been reported on IL-4 transgenics on a C57BL/6 background as used in the current studies.

However, in BALB/c mice, Murray *et al.* (1997) showed that treatment with exogenous IL-4 complexed with anti-IL-4 on day-1 of *L. donovani* infection led to a transient increase in liver LDU at week 2 post infection and repeated treatment, twice weekly over four weeks, led to a protracted effect (3 fold increase at 4 weeks, and 6 fold increase at 8 weeks). Once the IL-4 treatment stopped the infection was controlled as shown by the parasite loads dropping between 4 and 8 weeks in a similar way to the untreated mice. This data indicated that IL-4 itself could interfere with the development of the protective Th1 response.

To return to the possibility that *S. mansoni* induced Th2 responses may have a modulating effect on co-infecting *L. donovani*, previous studies have shown elevated IL-4 to both ConA and schistosome antigens following *S. mansoni* infection of mice (Grzych *et al.*, 1991, Wynn *et al.*, 1993; 1997). In the present studies, the Th2 bias induced by the *S. mansoni* infection relative to *L. donovani* infection and to naïve cells was clearly seen by both ConA and schistosome antigen specific stimulation of splenocytes which induced elevated levels of IL-4 only in the schistosome infections in both experiments. The liver-derived lymphocytes from the SCHISTO and CO-INFECT mice also showed high ConA and antigen specific IL-4 production. The ConA induced IL-4 responses tended to be lower in the CO-INFECT than the SCHISTO mice at +2 and +4 weeks in both experiments. However, there was essentially no difference between the schistosome specific (SEA and SWAP) IL-4 responses in the SCHISTO and CO-INFECT mice indicating that the proliferation of *L. donovani* in liver and spleen had no significant effect on the nature and level of the schistosome specific cytokine responses.

In the SCHISTO mice the spontaneous, antigen-specific and/or ConA production of IL-4, IFN-γ and IL-10 by splenocytes were generally reduced or markedly reduced at +8 weeks compared with the +4 and/or +2 week time points. SEA induced IL-4 responses in the liver lymphocytes were also lower at +8 weeks (16 weeks post *S. mansoni*) than earlier. Significantly and markedly lowered IL-4, IFN-γ, and IL-10 production and cellular proliferation by MLN or spleen cells to SEA stimulation were also reported in chronic (+15/16 weeks) compared with acute (+8 week) infections (Grzych *et al.*, 1991, Henderson *et al.*, 1992, Chensue *et al.*, 1992, Sadler *et al.*, 2003) and this has been related to the "endogenous desensitization" seen in granulomatous responses in chronically infected mice (Domingo and Warren, 1968). Recently, IL-10 has been implicated in this as in IL-$10^{-/-}$ KO mice both SEA specific IFN-γ and IL-4 production were significantly increased in the acute phase (approx 12 and 6 fold respectively) but, in contrast to normal mice, remained high at the chronic phase. In addition cellular proliferation of spleen and MLN cells were equally high at +15 weeks as at +8 weeks and importantly there was no reduction in granuloma diameter in the chronic infection. These results in IL-10 KO mice suggested that IL-10 functions to depress both Th1 and Th2 cytokines in schistosome infections and that this can be manifest at a time when IL-10 itself is also downregulated (Sadler *et al.*, 2003).

In the CO-INFECT group in the present studies, the drop in IL-4 levels at +8 weeks was not seen in Experiment 1 and in fact the ConA, SEA and SWAP induced IL-4 levels were higher at +8 weeks than at +4 weeks and higher than in the SCHISTO group suggesting that the down regulation in chronic infection associated with long term *S. mansoni* infection may be modulated by the *L. donovani* infection. In Experiment 2 this pattern was less clear but there was still a greater reduction in the IL-4 responses in the SCHISTO compared with the CO-INFECT mice.

Individuals co-infected with schistosomiasis and HIV showed decreased PBMC proliferation and also decreased Th2-like cytokine production, compared with those from HIV-1 negative patients with active schistosomiasis. Those co-infected individuals responded to egg antigen by making dramatically reduced levels of IL-4 and IL-10 but similar (low) amounts of IFN-γ than did HIV-1 negative individuals which indicates that

there was a swing in the overall balance of the response from Th2 and Th1 (Mwinzi *et al.*, 2001). An explanation for the reduced production of Th2 cytokines in PBMC of the schistosome-HIV co-infected patients is that the antigen-activated Th2 cells are more easily infected by HIV-1 (Weissman *et al.*, 1996) and so it is possible that schistosome-antigen-responsive cells were being infected and eliminated by the virus.

In general, minimal or no schistosome antigen specific IFN-γ responses were seen in the splenocyte response from either the SCHISTO or CO-INFECT groups relative to the CONTROL mice and the ConA induced IFN-γ responses were generally also lower than for CONTROL mice. This pattern was also seen with the liver lymphocytes. Interestingly, as with the IL-4 response the CO-INFECT mice produced lower IFN-γ following ConA stimulation than both the SCHISTO and LEISH mice at most of the time points. A possible explanation for this would be that the cells recovered from the CO-INFECT mice would represent a balance between those specific for each infection and so likely to be biased to Th2 (for SCHISTO) and Th1 (for LEISH) but the possibility of inhibitory mechanisms developing in the CO-INFECT animals cannot be excluded. It would be possible to further investigate the frequency and dynamics of antigen specific Th2/Th1 cells in the spleen and liver by antigen specific ELISPOT analysis of IFN-γ or IL-4/5 (Murphy *et al.,* 1997) or by FACS analysis, for example looking at the T1/ST2 marker of Th2 cells and intracellular cytokine staining for IFN-γ for Th1 cells (Kropf *et al.*, 2002).

With regard to the cytokine response to *L. donovani*, neither splenocytes or liver lymphocytes from LEISH mice made FLAA-specific IL-4 responses at any time from +2 to +8 weeks. This is consistent with the demonstration that early IL-4 mRNA expression following *L. donovani* infection is rapidly overcome by a protective Th1 response (Miralles *et al.*, 1994). In contrast FLAA specific IFN-γ production by splenocytes was significantly elevated at +2 weeks in the LEISH mice relative to the CONTROL mice or to cultures with medium alone. By +4 weeks antigen specific IFN-γ levels were lower and in Experiment 1 were lower again at +8 weeks. The spleen data is consistent with Murphy *et al.* (2001) who showed a significant drop in leishmanial antigen specific IFN-γ production between +2 and +4 weeks of infection in C57BL/6 mice.

Compared with the LEISH mice, the FLAA-specific IFN-γ levels were lower in the CO-INFECT mice at +2 and +8 weeks in Experiment 1 although at +4 weeks the CO-INFECT showed higher FLAA specific IFN-γ production compared with the LEISH. In Experiment 2 the same pattern held with the CO-INFECT mice showing lower IFN-γ at +2 and +8 weeks but minimal difference at +4 weeks. It is particularly clear from both experiments that the early +2 week response is reduced in the CO-INFECT mice. But there was evidence of a recovery of the response at +4 weeks followed later by a down-regulation of the IFN-γ response in the CO-INFECT mice at +8 weeks.

This pattern of *Leishmania*-specific IFN-γ production does not correlate directly with the observed ability to control the *L. donovani* infection as judged by the LDU values in the liver or the spleen. Notably, despite the markedly lower IFN-γ production in the CO-INFECT mice at +2 weeks (22,000 cf 62000 in Experiment1, 1000 cf 22000 in Experiment 2) the LDU were comparable or higher in the CO-INFECT compared with the LEISH. There could of course be a delay between the presence of FLAA specific IFN-γ producing T cells in the spleen and the competence to mediate leishmanicidal activity. This could be due to delay in the trafficking of the cells to the appropriate location for example. The data from the liver lymphocytes shows that in Experiment 1 there was no sign of FLAA-specific IFN-γ production by the liver lymphocytes at +2 or +4 weeks whereas in Experiment 2 such IFN-γ production was seen at +2 and + 4 weeks and as in the spleen was markedly higher in the LEISH compared with the CO-INFECT mice. It could be argued that induction of a protective response was more rapid in Experiment 2 than in Experiment 1 and indeed the LDU in the LEISH mice were lower than in the CO-INFECT even at +2 weeks in Experiment 2 whereas the reverse was true in Experiment 1 and there was a marked decline in LDU between +2 and +4 weeks in the LEISH mice in Experiment 2 and a somewhat lower reduction in Experiment 1. With regard to the differences between the two experiments it is worth noting that the mean LDU values at +2 weeks were markedly higher in Experiment 2 than in Experiment 1 which could have influenced the kinetics of development of the immune response. Differences in parasite loads following different inoculation have been reported (Melby *et al.*, 2001) and indeed marked differences in *L.*

donovani establishment can occur between different experimental groups of mice inoculated with the same number of parasites (see Chapter 6).

At +8 weeks, when the LDU in both liver and spleen were markedly higher in the CO-INFECT in both experiments, the levels of FLAA-specific IFN-γ produced by the splenocytes were 2-3 fold higher in the LEISH compared with the CO-INFECT. Also the liver lymphocyte response in Experiment 1 showed three fold higher IFN-γ production in the LEISH. So there is a correlation at this time point between elevated FLAA-specific IFN-γ production and reduced LDUs.

So the data shows that the CO-INFECT mice did make schistosome specific IL-4 and did show reduced levels of *Leishmania*-specific IFN-γ at certain times during the time course of the experiment. Before considering the temporal relationship of these responses the involvement of IL-10 will be considered.

With regard to hypothesis (8), there is evidence from human and murine studies that IL-10 is associated with suppression and/or modulation of responsiveness during *L. donovani* infections and it is clearly established that IL-10 is produced during schistosome infections (Grzych *et al.*, 1991; Pearce *et al.*, 1991; Sadler *et al.*, 2003).

Several studies show that in human visceral leishmaniasis caused by *L. donovani* and *L. chagasi* patients show a marked depression of T cell responses, characterized by the absence of IL-2 and IFN-γ production by lymphocytes on *in vitro* stimulation with *Leishmania* Ag. Furthermore, clinically apparent or progressive disease is associated with a Th2 response (Zwingenberger *et al.*, 1990; Carvalho *et al.*, 1992; Cenini *et al.*, 1993; Carvalho *et al.*, 1994; Sundar *et al.*, 1997). For example, Carvalho *et al.* (1994) studying *L. chagasi* showed that although IFN-γ and IL-4 levels in the supernatants of PBMC lymphocyte cultures were minimal, mRNA for these cytokines and for IL-10 were present and restoration of T cell proliferative responses and IFN-γ production was achieved by addition of a neutralizing mAb against IL-10 but not against IL-4 although mAb against both had a synergistic effect. A more significant role for IL-4 in this modulation in *L. donovani* was suggested by the studies of Sundar *et al.* (1997) who found IFN-γ, IL-4 and

IL-10 activity in 53%, 84%, and 56% of patient samples compared with 90%, 10%, and 20% in healthy co-endemic people. The patients had comparable levels of IFN-γ to the normal but three-and 13-fold increases in IL-10 and IL-4, respectively and the lack of IFN-γ response was associated with the presence of IL-4 alone or in combination with IL-10 but not with IL-10 alone. However, other studies report that IL-4 is also not consistently expressed in human kala-azar (Babaloo *et al.*, 2001; Ghalib *et al.*, 1993) IL-10 production correlates with pathology (Karp *et al.*, 1993).

Other human studies have shown elevation of IFN-γ during active disease (Karp *et al.*, 1993; Kenney *et al.*, 1998) along with elevation of IL-4 and IL-10. The elevated IL-10 was markedly diminished following treatment. This correlation of active disease with elevated IL-10 in the face of elevated IFN-γ supports suggestions that the cellular immune depression that is associated with active disease in humans may be due mainly to the direct inhibitory effects of IL-10 rather than to a lack of IFN-γ (Holaday *et al.*, 1993; de Medeiros *et al.*, 1998).

Studies in mice have concerned both depletion and over-expression of IL-10. Studies on depletion of IL-10 are described in more detail in Chapter 4 but in brief here it is known that treatment with anti-IL-10R antibody (Murray *et al.*, 2002; Murray *et al.*, 2003) or infection of IL-$10^{-/-}$ mice (Murphy *et al.*, 2001) results in a reduction or resolution of the infection indicating that endogenous IL-10 is responsible for down-modulating the protective response in normal mice. Of more direct relevance to the present co-infection study, studies showed that IL-10 transgenic mice which over-express IL-10 had increased LDU compared with controls at 3 weeks (50% increased) and 7 fold increased at 8 weeks (Murray *et al.*, 2002). Also there was an approximately 6 fold reduction in IFN-γ mRNA production in the livers of the transgenic mice and a significant (~5 fold) reduction in the production of parasite-free (resolved) granulomas at 4 and 8 weeks and a 50% and 25% reduction in mature granulomas at 4 and 8 weeks respectively. In addition iNOS expression in liver granulomas at 2 weeks was virtually absent in the transgenics. It was concluded that the effects of the IL-10 over-expression was initially attributable to the general suppression of Th1 responsiveness but eventually granulomas did form and then it is suggested that the

IL-10-directed macrophage inhibitory effects (Bogdan *et al.,* 1991; Moore and O'Garra, 2001) were prominent resulting in heavily parasitized macrophages within structurally normal mature granulomas. So the CO-INFECT mice showed a similar trend to the IL-10 transgenics although the transgenics showed a greater effect in terms of an earlier effect on LDU and a greater fold increase in IFN-γ response although in the present studies *Leishmania*-specific IFN-γ release by splenocytes was measured rather than the non-specific liver mRNA level in the liver. In fact the CO-INFECT animals only showed lowered IFN-γ levels at +2 weeks (2.7 and 9 fold lower in experiments 1 and 2 respectively) and at +8 weeks (2.1 and 2.9 fold lower responses) whereas at +4 weeks there was a 2.5 fold greater response in Experiment 1 and a comparable response in Experiment 2. The difference may reflect the fact that although IL-10 may play a major role in the CO-INFECT mice there are many additional interacting immune responses involved compared with the transgenics. To return to the production of IL-10 during the course of the current co-infections, the SCHISTO and CO-INFECT mouse cell cultures produced significant levels of IL-10 following SEA and/or SWAP stimulation in both experiments at +2 and +4 weeks post super-infection. FLAA specific IL-10 was also produced in the LEISH and CO-INFECT mice and in Experiment 1 at +2 weeks this was somewhat higher in the LEISH than in the CO-INFECT mice. But by +4 weeks in experiment 1 FLAA specific IL-10 was only produced in the CO-INFECT mouse cultures in which it was produced at a high level. Production of IL-10 mRNA and of IL-10 producing cells has been reported during the first two weeks of infection, declining to baseline by +4 weeks (Melby *et al.*, 1998; 2001). At +8 weeks the patterns of production of IL-10 to both schistosome antigens and FLAA were similar in both experiments, highest in the CO-INFECT group with respect to both schistosome antigens and FLAA. Although it is clear from the *in vitro* data that the CO-INFECT mice would generally be producing more IL-10 than the LEISH mice throughout the time course there was, as with IFN-γ, no direct correlation between the *in vitro* IL-10 levels and the LDUs *e.g.* at +2 weeks post infection the LDU were similar for both LEISH and CO-INFECT mice and notably in Experiment 1 significantly higher levels of IL-10 to both the schistosome antigens and FLAA were produced by the CO-INFECT mice *in vitro* but there were comparable LDU levels. It was not until 8 weeks that higher IL-10

production to both schistosome and leishmanial antigens correlated with higher LDU in both experiments.

In summary, it is clear that he CO-INFECT mice did manifest schistosome induced Th2 cytokine responses characterized by IL-4 production in both liver and spleen throughout the time course of these experiments. FLAA specific IFN-γ production by both splenocytes and liver lymphocytes was found in both LEISH and CO-INFECT mice but was markedly and significantly lower in the CO-INFECT than the LEISH mice at +2 weeks post super-infection. It was also lower at +8 weeks. However, despite this down-regulation of the *Leishmania*-specific Th1 response in the CO-INFECT mice there was no corresponding elevated FLAA specific IL-4 production. These results are consistent with the idea that the presence of schistosome specific IL-4 could influence the development of *Leishmania* specific IFN-γ production and that this at least in part explains the loss of control of the *L. donovani* infection. However, there was not a direct temporal correlation between the FLAA specific IFN-γ production and control of the infection e.g. biggest differences between CO-INFECT and LEISH were seen at +2 weeks when the LDUs were either higher in the CO-INFECT or only a little lower. Schistosome specific IL-10 was also produced to the schistosome antigens throughout the time course. In addition, there was FLAA specific IL-10 production which was enhanced in the CO-INFECT mice. IL-10 is involved in IFN-γ production in *L. donovani* infection as is shown by the marked elevation of IFN-γ in infected IL-$10^{-/-}$ mice (Murphy *et al.*, 2001). However, it is worth noting that treatment of the KO mice with mAb to IFN-γ only partially increased the parasite loads but anti-IL-12 treatment had a greater effect in restoring parasite loads. This indicates that only part of the inhibitory effects of IL-10 may be mediated via its effects on reducing IFN-γ in LEISH mice. Alternatively/in addition, the schistosome_induced IL-10 could be acting directly to inhibit macrophage activation (Moore *et al.*, 2001). The above data is not able to distinguish between these two possible alternative effects of IL-10 although the levels of IL-10 to FLAA fitted somewhat better with the LDU values than the IFN-γ levels in that the biggest differences between the CO-INFECT and LEISH animals (more IL-10 in the CO-INFECT) occurred later in the infection when the LDUs also showed the biggest differences (higher in CO-INFECT).

In addition to IL-10, other immuno-regulatory cytokines are known. TGF-β can inhibit generation of IFN-γ by differentiating Th1 cells *in vitro* and has an even greater effect on Th2 development (Heath *et al.*, 2000). Like IL-10, TGF-β is produced at high levels in schistosome infection (Grzych *et al.*, 1991, Pearce *et al.*, 1991). IL-21, another immuno-regulatory cytokine which has been described more recently, is preferentially produced by Th2 cells and acts on Th1 cells to specifically inhibit IFN-γ production by inhibiting IL-12 responsiveness of developing Th1 cells (Wurster *et al.*, 2002). Since neither of these cytokines were measured in these co-infection studies, their possible involvement is only speculation but it would be interesting to know how the patterns of expression of these cytokines related to the course of the *L. donovani* infection. Distinct regulatory CD4+ T cell subsets, known as Tregs or Th3 cells have also been described which can inhibit both inflammatory responses such as those that lead to auto-immunity and also Th2 cell mediated pathologies by processes that involve TGF–β (Powrie and Mason, 1990; Groux *et al.*, 1997; Bridoux *et al.*, 1997; Lafaille *et al.*, 1997). The involvement of these cells in schistosome infections has not yet been established but it is possible that they could also play a role in the immuno-regulation seen.

A couple of other studies are particularly relevant to this co-infection study. Murray *et al.* (1997) set out to look at the effects of biasing the *Leishmania*-specific immune response towards Th2. For this they immunized the mice with four weekly subcutaneous injections with heat-killed *L. major* promastigotes (HKLP) a strategy which had earlier been shown to prevent induction of protective immunity against *L. major* leading to fatal disease (Liew *et al.*, 1985). This resulted in a cross-reacting Th2 response to *L. donovani* as shown by elevated IgE levels before *L. donovani* challenge and even more elevated responses 4 weeks following infection. This protocol resulted in significant increases (2-fold) in LDU by 2 weeks post infection and this higher level was maintained in the vaccinated mice although it had declined to a very low level by 8 weeks. Treatment of these vaccinated mice with either anti-IL-4 or anti-IL-10 resulted in normal levels of control of the infection showing that in this model neither cytokine acting alone could undermine the immune control and suggesting that both IL-4 and IL-10 mediated processed were operating. La

showing that in this model neither cytokine acting alone could undermine the immune control and suggesting that both IL-4 and IL-10 mediated processed were operating. La Flamme *et al.* (2002) recently studied the effect of concurrent *S. mansoni* infection on the course of *L. major* which also requires a strong IFN-γ mediated response to control the infection (Reiner *et al.*, 1995). In this study the *L. major* infection was given after 2 weeks of the *S. mansoni* infection so that the peak Th1 response to the *L. major* (at +4 weeks) corresponded with the peak Th2 response to *S. mansoni* (at 6-7 weeks post infection). They found a complex dynamic relationship in which parasite burdens were comparable at +4 weeks but at +8 weeks remained constant in the CO-INFECT but dropped by 50% in the LEISH. Thereafter parasite burden declined in both to resolve by +16 weeks. At +4 weeks IL-4 response to *L. major* antigen was seen in popliteal lymph node cells in the CO-INFECT but not the LEISH mice and there was lower antigen specific IFN-γ, TNF-α and NO production. At +8-+16 weeks the *Leishmania*-specific IFN-γ showed little change in the LEISH but increased progressively in the CO-INFECT to overshoot the LEISH levels. So the lower IFN-γ at +4 weeks did not correspond to higher parasite loads in the CO-INFECT at that time but led to a subsequent delay in parasite clearance which was seen at +8 weeks at which time the IFN-γ levels were in fact higher than in the LEISH animals. This lag between the apparent inhibition of *Leishmania*-specific IFN-γ production and lack of control of the *Leishmania*-infection is similar to the results obtained in the present study. It was further shown that, in the presence of IFN-γ, peritoneal macrophages from schistosome infected mice were less able to kill *L. major in vitro* than normal macrophages and this response was greatest in the presence of the non-adherent PEC population of cells which include T, B and granulocytes. Preliminary data was referred to indicating that IL-10 from such cells was responsible for the impaired responses. It was suggested that the Th2 response to *S. mansoni* modulates the development of the protective Th1 response to *L. major* with the result that there is a delay in lesion development and to control of the parasite load. As this increases however, so the Th1 response eventually develops and overshoots in the face of increasing parasite load and eventually controls the infection. As in the present studies with *L. donovani*, the extent to which the delay in control is due to impaired IFN-γ production or to enhanced IL-10 production is unclear.

In conclusion, the work in this chapter has shown that a superimposed *L. donovani* infection has no significant effect on a pre-existing *S. mansoni* worm burden or egg production but the there is a progressive failure to control the *L. donovani* infection. Various hypothesis have been put forward to explain this. Of these it is possible that IL-4 and/or IL-10 production which is induced by the schistosome infection in the CO-INFECT mice could be responsive for down-regulation of Leishmanial specific IFN-γ which is required for protection although there is not a direct temporal association. Between the levels of these cytokines and loss of control of the Ld infection. The immuno-regulatory/immuno-suppressive properties of both IL-4 and IL-10 on Th1 responses are clearly established (Powrie *et al.,* 1993). The present studies were not able to distinguish which was the most important in the co-infection model. Studies in the next chapter set out to try to investigate more directly the role of IL-10. Studies in Chapter 5 relate to other

Chapter 4

Investigation of the role of IL-10 on the progression of *L. donovani* in co-infected mice using B-cell deficient (μMT) mice or C57BL/6 mice treated with anti-IL-10R mAb.

4.1. Introduction

The most significant observation from the studies in Chapter 3 was the failure of *S. mansoni*-infected C57BL/6 mice to control progression of a superimposed *L. donovani* infection with the result that at +8 weeks post infection there were high levels of LDUs in the livers and spleens of the co-infected mice (CO-INFECT) and minimal levels in mice only infected with the *L. donovani* infection (LEISH). The cytokine analysis showed that the *S. mansoni* infection had, as expected (Grzych *et al.*, 1991; Pearce *et al.*, 1991; Kaplan *et al.*, 1998) induced a Th2-biased response as judged by IL-4 production by spleen cells to Con A, SEA and SWAP but little IFN-γ to the same stimuli. The Th2-biased response was also generally demonstrated in the response of liver-derived lymphocytes (Hayashi *et al.*, 1999; King *et al.*, 2001). Also as expected (Solbach and Laskay, 2000) the LEISH infection induced strong IFN-γ production but no IL-4 to leishmanial antigen (FLAA) i.e. a Th1-biased response. The CO-INFECT animals showed similar Th2 responses to the SCHISTO mice but the anti-FLAA IFN-γ response was reduced especially at +2 weeks and then again at +8 weeks compared to the LEISH mice. The early difference in IFN-γ levels suggests that the Th2 environment due to the schistosome infection may have interfered with the development of the IFN-γ response to *L. donovani* through the inhibitory action of IL-4 on the expansion of Th1 cells (Lehn *et al.*, 1989; Miralles *et al.*, 1994; Carvalho *et al.*, 1994). However, at +4 weeks the IFN-γ response in the CO-INFECT mice was comparable or greater than in the LEISH alone group suggesting that the development of the Th1 response was not prevented but apparently delayed. Nevertheless at +8 weeks post *L. donovani* super-infection the FLAA-specific IFN-γ response was again lower in the CO-INFECT mice.

The other cytokine response demonstrated during the *S. mansoni* infection which has also been previously demonstrated in *S. mansoni* infected mice (Flores Villanueva *et al.*, 1994a; Wynn *et al.*, 1998; Sadler *et al.*, 2003) and humans (King *et al.*, 1996) was the induction of IL-10 responses as seen following ConA and schistosome antigen stimulation of spleen cells *in vitro*. In addition there was evidence that in the CO-INFECT mice there was an enhanced IL-10 response to *L. donovani* antigen, FLAA. IL-10 has been shown to be produced during *L. donovani* infection in mice and to be a critical cytokine in:- inhibiting Th1 cell responses predominantly through effects on APC function (Murray *et al.*, 2002; Moore *et al.*, 2001; Groux *et al.*, 1999); inhibiting the intracellular killing of *L. donovani* (Bhattacharya *et al.*, 2001a; Murray *et al.*, 2002); and controlling susceptibility to *L. donovani* (Heinzel *et al.*, 1991, Melby *et al.*, 1998) as further shown by rapid control of infection in IL-10 KO mice (Murphy *et al.*, 2001; Murray *et al.*, 2002; Kane and Mosser, 2001), loss of control of infection in IL-10 transgenic mice which over-express IL-10 (Murray *et al.*, 2002; Groux *et al.*, 1999) or following neutralization of IL-10 (Ishida *et al.*, 1992; Ghalib *et al.*, 1993; Romani *et al.*, 1994; Chatelain *et al.*, 1992; 1998; Bhattacharyya *et al.*, 2001; Castro *et al.*, 2000; Murray, 2000; 2001; Murray *et al.*, 2002; 2003).

Moreover, IL-10 is responsible for progression of other intracellular and extracellular organisms by effectively disabling the afferent arm of the Th1 cell-associated responses that are required for induction and expression of cell-mediated immunity leading to disease persistence and reactivation e.g. *Mycobacterium tuberculosis,* HIV, *Toxoplasma gondii*, *malaria, Leishmania* species, and *Trypanosoma cruzi* (Gerosa *et al.,* 1999; Ostrowski *et al.,* 2001; Plebanski *et al.,* 1999; Neyer *et al.,* 1997; Ghalib *et al.,* 1993; Gasim *et al.,* 1998).

So experiments were planned to try to further implicate IL-10 in mediating the enhanced effects on *L. donovani* infections. Consideration was given to using IL-10 gene targeted mice (IL-10 KO), but various experiments had shown that such mice are essentially resistant to *L. donovani* infection (Murphy *et al.*, 2001; Murray *et al.*, 2002; Kane and Mosser, 2001) which itself shows the importance of this cytokine in controlling Th1 responses. Therefore other approaches were considered to investigate the role of IL-10.

Although originally considered a Th2 cytokine, IL-10 is now thought of as a generally regulatory cytokine and is known to have a variety of cell sources e.g. activated Th2 cells, Treg cells, B cells, basophils, monocytes, macrophages, thymocytes, keratinocytes and to be produced during both Th2 and Th1 responses (Belkaid *et al.*, 2001; 2002; Shevach *et al.*, 2001; De Waal *et al.*, 1991; Mizuno *et al.*, 1994; Thompson-Snipes *et al.*, 1991). Work by Harn and colleagues has implicated B cells as a likely major source of IL-10 during *S. mansoni* infection (Harn *et al.*, 1989). Thus during *S. mansoni* infection, Velupillai and Harn (1994) found that spleen cells from infected BALB/c mice 8 weeks post infection proliferated in response to the schistosome egg oligosaccharide, Lacto-*N*-fucopentaose III which contains the Lewis^x trisaccharide which is a highly antigenic component of the egg antigen. The responding population was found to be B220$^+$, CD4$^-$ CD8$^-$ i.e. B-cells and purified B cells from the infected mice produced high levels of IL-10 in response to the oligosaccharide. The B cells also produced PGE2 which also has a negative effect on development of Th1 cells (Betz and Fox, 1991). Palanivel *et al.* (1996) have also shown ligand-specific activation of IL-10-producing B cells in leishmaniasis and lymphatic filariasis models in which Th2 activation occurs. In these experiments peritoneal exudate B cells were shown to secrete large amounts of IL-10 *in vitro* in response to stimulation with soluble leishmanial extract in *L. major* infections and soluble microfilarial extract. The conclusion from these studies was that there was a correlation and perhaps causal relationship between expansion of ligand-specific IL-10-producing B and B-1 cells with dominance of Th2-type T cells.

In view of this demonstration of a key role for B cells in production of IL-10 in *S. mansoni* infected mice an experiment was planned to carry out the co-infection in B-cell deficient mice i.e. μMT strain mice (Smelt *et al.*, 1997; Jankovic *et al.*, 1998), in comparison with Wild Type (WT), C57BL/6 mice as above. If the greater *L. donovani* infection observed in the *S. mansoni* infected mice was due to the effects of B cell-dependent IL-10 production then it would be expected that the percentage increase in LDU would be less in the μMT mice.

The other approach taken was to administer anti-IL-10R mAb to CO-INFECT mice starting at week 6 after super-infection to see if it was possible, by inhibiting the action of IL-10, to reverse the loss of control of the *L. donovani* infection which was seen most dramatically at 8 weeks after superinfection. At the time of setting up this experiment it was known that IL-10KO mice showed greatly enhanced resistance to infection as discussed above (Murphy *et al.*, 2001; Murray *et al.*, 2002) but the later papers on IL-10 receptor administration to *L. donovani* infected mice (see below) were not published. However, it had been shown that established *L. major* infections could be controlled by anti-IL-10R treatment. In these experiments there was little or no increase in IFN-γ expression or secretion or additional inflammation suggesting that it was macrophage responsiveness to the existing Th1 cell response which was enhanced by removal of the modulating effects of IL-10 by the anti-IL-10R treatment (Belkaid *et al.*, 2001). It was anticipated that similar IL-10R treatment in *L. donovani* infection was likely to lead to enhanced control and, subsequent to carrying out this experiment, it was indeed shown by Murray *et al.* (2002) that administration of anti-IL-10R mAb to BALB/c *L. donovani* infected mice on day +1 post infection and again at weekly intervals led to a suppression of the infection which was virtually resolved by week 3 when the liver LDU in control mice was still near the peak level. In a later study anti-IL-10R mAb was administered to an established *L. donovani* infection at day 14 post infection and this also had a significant effect in reducing liver parasite burden on day 21 in both BALB/c (reduced 68%) and C57BL/6 mice (reduced 57%) (Murray *et al.*, 2003a). The treatment resulted in a 2.5 fold increase in serum IL-12 but a dramatic 55-fold increase in IFN-γ i.e it had a marked effect on the afferent arm of the immune response.

In spite of the anticipated and subsequently demonstrated enhanced control of *L. donovani* infection in LEISH alone mice the use of anti-IL-10R treatment at the time when the loss of control in the CO-INFECT mice was increasing was envisaged as testing the anti-IL-10R treatment as a therapy for the uncontrolled infection in CO-INFECT mice which it was postulated was due to *S. mansoni* induced IL-10.

4.2. Results

4.2.1. Co-infection of *μMT and C57BL/6 mice.*

4.2.1.1. Experimental design

The experimental design was similar to the first two experiments with SCHISTO, CO-INFECT, LEISH and CONTROL groups. Appropriate groups of mice of each strain (10 mice/group) were infected percutaneously with 25 cercariae of *S. mansoni* and/or with $2x10^7$ amastigotes of LV9 strain by tail-vein injection. The *L. donovani* infection/co-infection was given 8 weeks post *S. mansoni* infection. It had been intended that groups of mice would be sacrificed at +8wks p.i. with *L. donovani* superinfection but after 5 weeks of the *L. donovani* infection a viral infection was discovered in a different section of the animal unit and this meant that the unit had to be closed and the animals under experimentation had to be culled. So this experiment had to be terminated at just 5 weeks after co-infection.

4.2.1.2. Co-infection of μMT and C57BL/6 mice - effect of prior *S .mansoni* infection on *L. donovani* LDU.

At +5wks p.i. with *L. donovani,* the LDU obtained from both liver and spleen imprints of infected C57BL/6 mice showed similar results to the 8 week time points in the previous experiments (Figure 4.1). Thus the CO-INFECT animals showed increased parasitic burden in both liver (3.25 fold higher than the LEISH group, p= 0.0052) and spleen (2.8 fold higher, but not significant, p=0.091).

The *L. donovani*-infected B-cell deficient mice showed much lower LDUs in both liver and spleen compared to infected C57BL/6 mice. In the liver the CO-INFECT μMT mice showed 6.8 fold lower LDU than the CO-INFECT C57BL/6 (p=0.003) and in the corresponding LEISH groups LDU were 3.4 fold lower in the μMT (0.003). The same pattern was observed in the spleens of μMT mice, with 4.4 fold lower LDU in the CO-INFECT group (but not significant, p=0.055) and 5 fold lower in the LEISH group (p= 0.038).

Figure 4.1. Comparison of Leishman Donovan Units (LDU) from liver and spleen imprints following infection or co-infection of B-cell deficient (μMT) and C57BL/6 mice

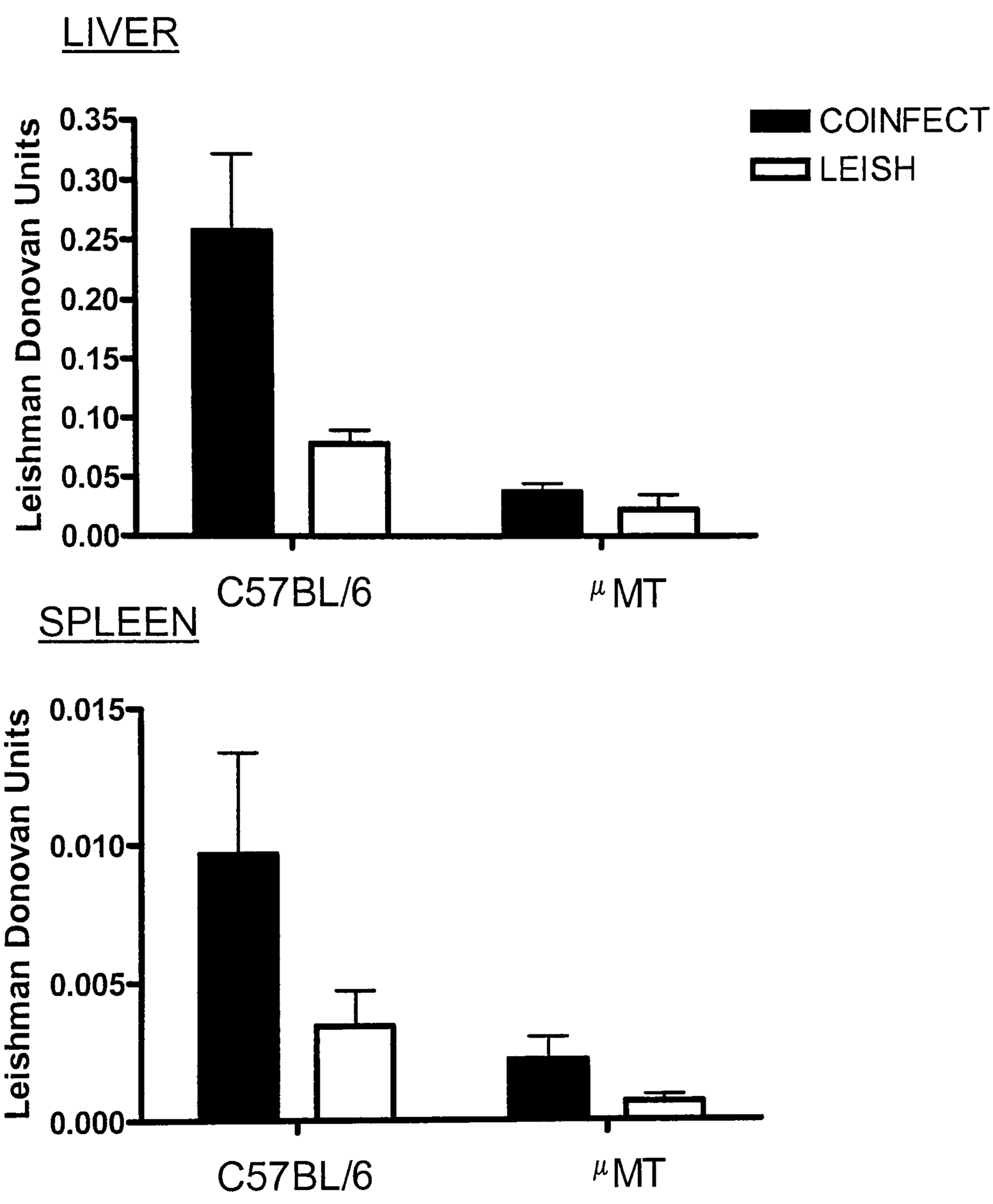


Figure 4.1: Graphs showing the mean (±S.E.) LDU from the livers and spleens of mice from the CO-INFECT and LEISH groups of infected C57BL/6 and μMT mice (7-10 mice/group). For description of the groups see legend to Figure 3.3.1.

The markedly lower LDUs in μMT mice was an expected complication (Smeldt *et al.*, 2000) which prevents statistical comparisons between the two strains but with regard to the comparison of the *L. donovani* infection in naïve and *S. mansoni* infected mice of both strains, there was a greater fold increase in the LDUs in the livers of the CO-INFECT

C57BL/6 mice (2.25 fold, P= 0.0052) than the μMT mice (0.63 fold, NS, p=0.36). In the spleens however, in which there were very low counts, the fold increases were more similar, 2.8 for the C57BL/6 mice and 3.25 for the μMT mice but neither were significant (0.054 and 0.055 respectively).

In conclusion, it was observed that the percentage increase in liver LDU in the CO-INFECT compared with the LEISH mice was less in the μMT compared with the C57BL/6 mice supporting the notion that *S. mansoni* induced B cell derived IL-10 may play a role in inhibiting anti-*L. donovani* immunity.

4.2.1.3. Co-infection of μMT and C57BL/6 mice – Cytokine responses

Spleen cell cultures were set up as described in Chapter 3 section 3.3.7. except that the concentration of antigen was doubled in this experiment following experience of other experiments in the laboratory (Quentin Bickle, 'personal communication'). Liver cell preparations were made using the homogenisation method.

IL-10 production: (i) from splenocytes

In the C57BL/6 (wild type) mice:

As shown in Figure 4.2., ConA levels in the infected groups were comparable to or lower than in the CONTROLS. There was an elevated and comparable level of IL-10 to SWAP in the CONTROL, LEISH and CO-INFECT groups but the most notable response was seen in the SCHISTO mice which showed the highest IL-10 production in response to SWAP significantly higher than in the LEISH and CONTROL groups (p=0.02, 0.03 respectively). Moreover, the schistosome infected animals also showed markedly elevated IL-10 in response to SEA, significantly higher than in CO-INFECT, LEISH and CONTROL groups (p=0.006, 0.0002, and <0.0001 respectively). However, there was little sign of enhanced IL-10 responsiveness in the CO-INFECT group. The mean level of IL-10 production to SEA in this group was higher than the CONTROL and LEISH groups but this was not

statistically significant. There was a low level of IL-10 production in response to FLAA in the LEISH group but little in the CO-INFECT group.

Figure 4.2. Comparison of the levels of IL-10 in supernatants of spleen cell cultures from C57Bl/6 mice and B-cell deficient

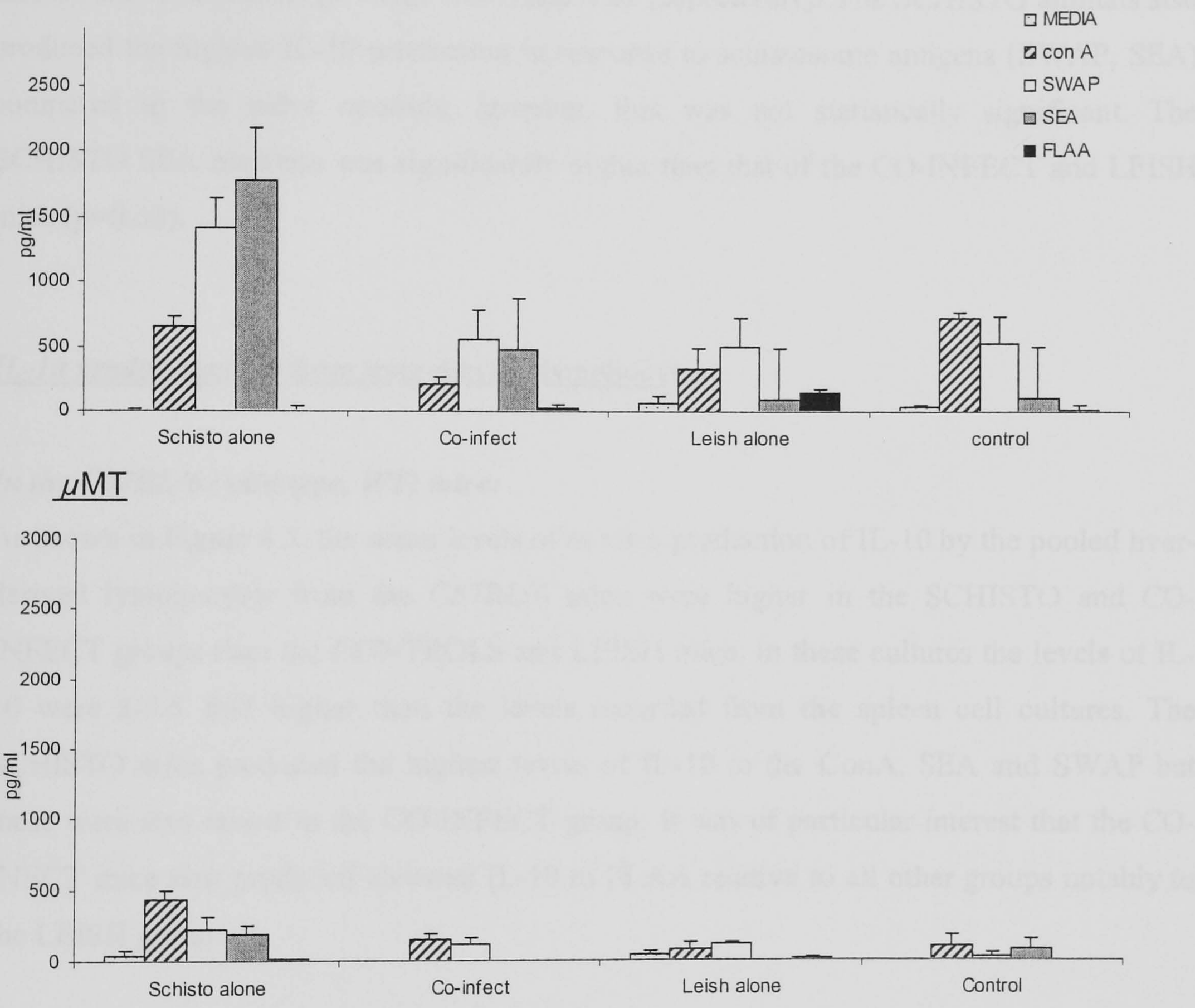


Figure 4.2 : Graphs showing the means and standard errors for IL-10 detected by ELISA based on three mice from each group sacrificed at +5 weeks after the *L. donovani* infection. Cultures of $5x10^6$ spleen cells/ml were stimulated with medium alone, mitogen (ConA at 5μg/ml), schistosomal antigens (SWAP and SEA at 20μg/ml) or fixed leishmanial amastigote antigen (FLAA, at 10^7 amastigotes/ml). For description of the groups see legend to Figure 3.3.1.

In the B-cell deficient mice:

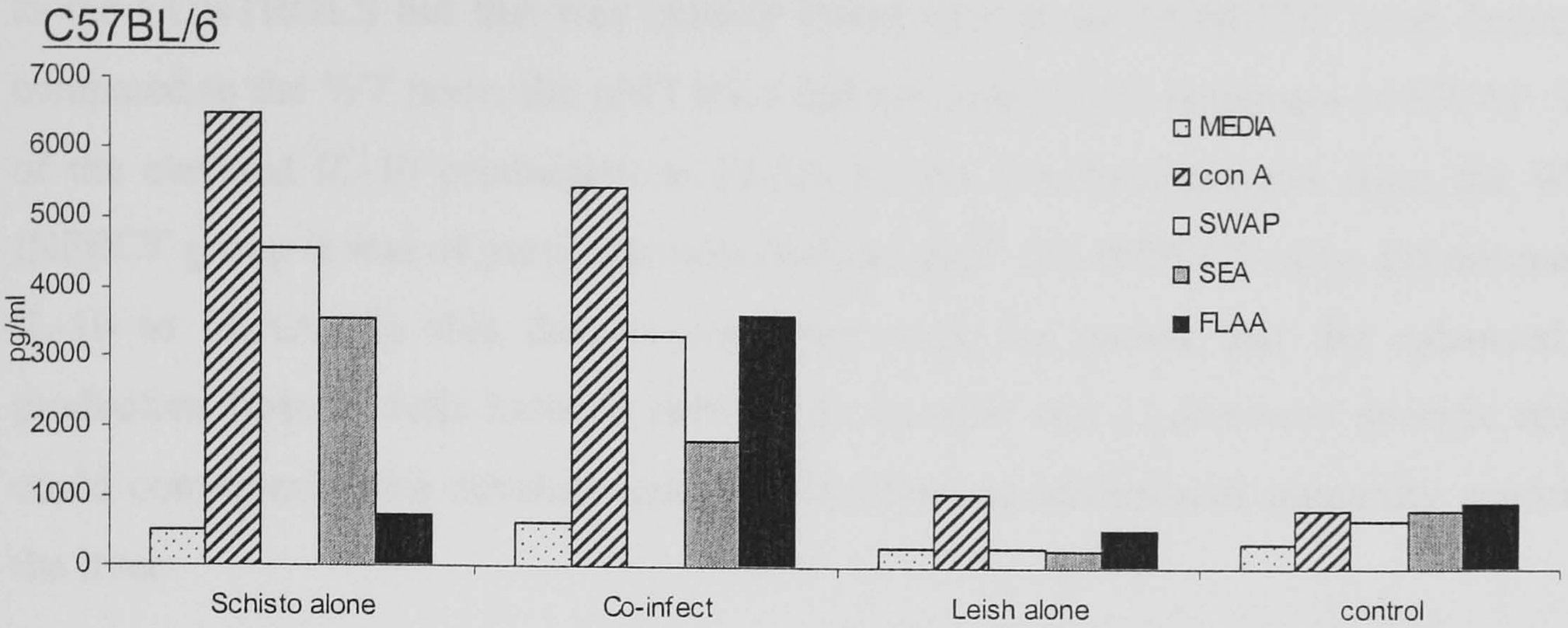
In the B-cell deficient mice IL-10 production was notably lower (5-7 fold) in all groups supporting the notion that B cells may be a general source of IL-10 responses. With ConA, the SCHISTO mice showed the highest production of IL-10 compared to the CO-INFECT, LEISH and CONTROL ($p=0.02$, 0.009, and 0.03 respectively). The SCHISTO animals also produced the highest IL-10 production in response to schistosome antigens (SWAP, SEA) compared to the naïve controls, however, this was not statistically significant. The SCHISTO SEA response was significantly higher than that of the CO-INFECT and LEISH mice ($p=0.03$).

IL-10 production: (ii) from liver-derived lymphocytes

In the C57BL/6 (wild type, WT) mice:

As shown in Figure 4.3, the mean levels of *in vitro* production of IL-10 by the pooled liver-derived lymphocytes from the C57BL/6 mice were higher in the SCHISTO and CO-INFECT groups than the CONTROLS and LEISH mice. In these cultures the levels of IL-10 were 3-3.5 fold higher than the levels recorded from the spleen cell cultures. The SCHISTO mice produced the highest levels of IL-10 to the ConA, SEA and SWAP but these were also raised in the CO-INFECT group. It was of particular interest that the CO-INECT mice also produced elevated IL-10 to FLAA relative to all other groups notably to the LEISH group.

Figure 4.3. Comparison of the levels of IL-10 in supernatants of pooled liver-derived lymphocytes from C57Bl/6 mice and B-cell deficient mice.



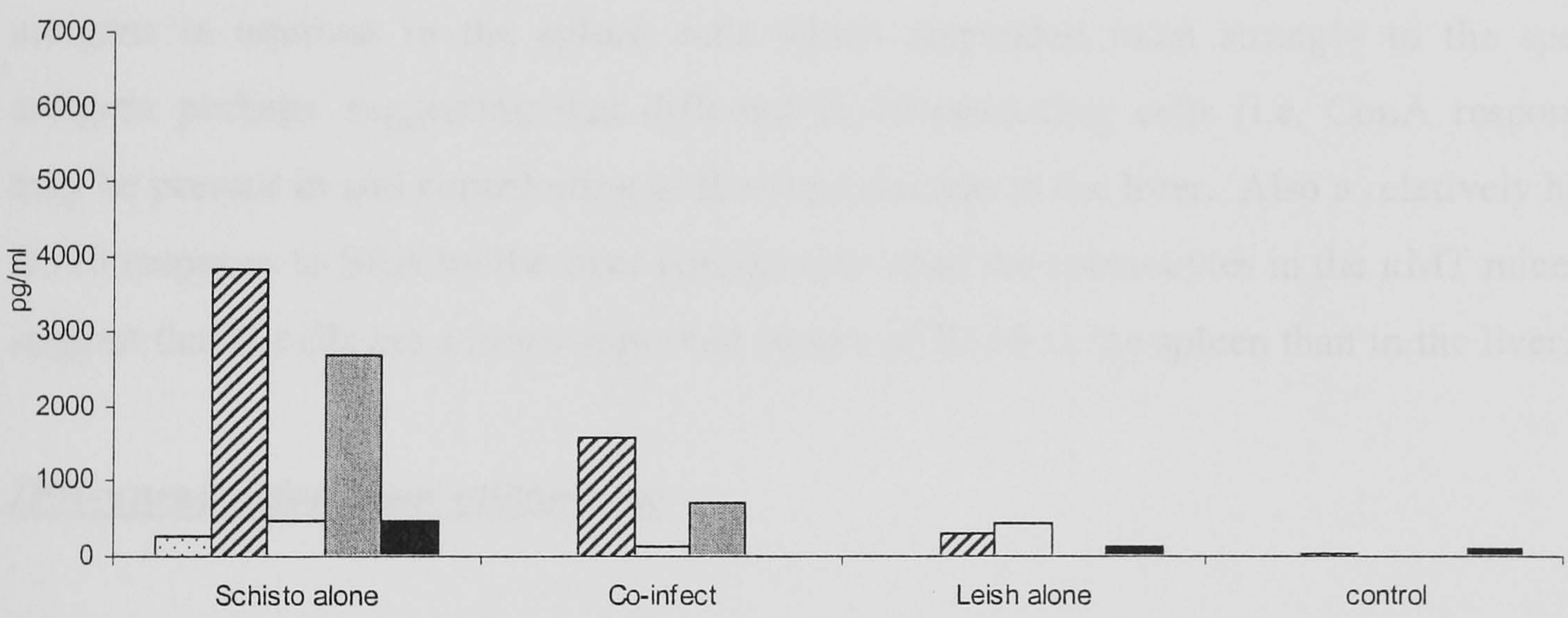


Figure 4.3 : Graphs showing the IL-10 detected by ELISA in 72hr cultures of liver-derived lymphocytes mice from mice sacrificed at +5 weeks after the *L. donovani* infection. Cultures of $5x10^6$ liver-derived lymphocytes/ml pooled from 5-7 mice were stimulated with medium alone, mitogen (ConA at 5μg/ml), schistosomal antigens (SWAP and SEA at 20μg/ml) or fixed leishmanial amastigote antigen (FLAA, at 10^7 amastigotes/ml). For description of the groups see legend to Figure 3.3.1.

In the B-cell deficient mice:

The pattern of response by the liver-derived lymphocytes from the B-cell deficient mice was similar to the wild-type (WT) mice in that elevated responses were only seen in the SCHISTO and CO-INFECT groups. As in the spleen the levels of IL-10 were generally lower than in the WT mice but less so than with the spleen cell cultures. The SCHISTO

animals produced the highest levels of IL-10 in response to ConA, SWAP, and SEA. The CO-INFECT mice showed some production of IL-10 in response to ConA and SEA relative to the CONTROLS but this was notably lower than in the SCHISTO mice. Interestingly compared to the WT mice, the μMT mice did not make IL-10 responses to SWAP. In view of the elevated IL-10 production to FLAA by the liver lymphocytes from the WT CO-INFECT group it was of particular note that the μMT CO-INFECT mice did not make any IL-10 to FLAA. So this data is consistent with the notion that the enhanced IL-10 production from B cells induced both by *S. mansoni* and *L. donovani* specific responses could compromise the development of protective anti-leishmanial immunity especially in the liver.

The highest responses by the liver lymphocytes were to ConA rather than the specific antigens in contrast to the spleen cells which responded most strongly to the specific antigens perhaps suggesting that different IL-10-producing cells (i.e. ConA responsive) may be present in and contributing to IL-10 production in the liver. Also a relatively higher IL-10 response to SEA by the liver lymphocytes than the splenocytes in the μMT mice may suggest that B cells are a more important source of IL-10 in the spleen than in the liver.

IFN-γ production from splenocytes

In the C57BL/6 (wild type) mice:

In response to ConA all groups showed elevated IFN-γ levels (Figure 4.4) which were highest in the CONTROL cells as was previously observed (Figures 3.3.9 and 3.3.15, Chapter 3). The IFN-γ production by the SCHISTO mice was significantly lower than the CONTROLS ($p=0.009$). The SCHISTO group produced the highest IFN-γ in response to SWAP but this was not significantly higher than that in the CONTROLS although it was significantly higher than the LEISH group ($p<0.0001$). Minimal responses were seen with

Figure 4.4. Comparison of the levels of IFN-γ in supernatants of spleen cell cultures from C57BL/6 and μMT mice

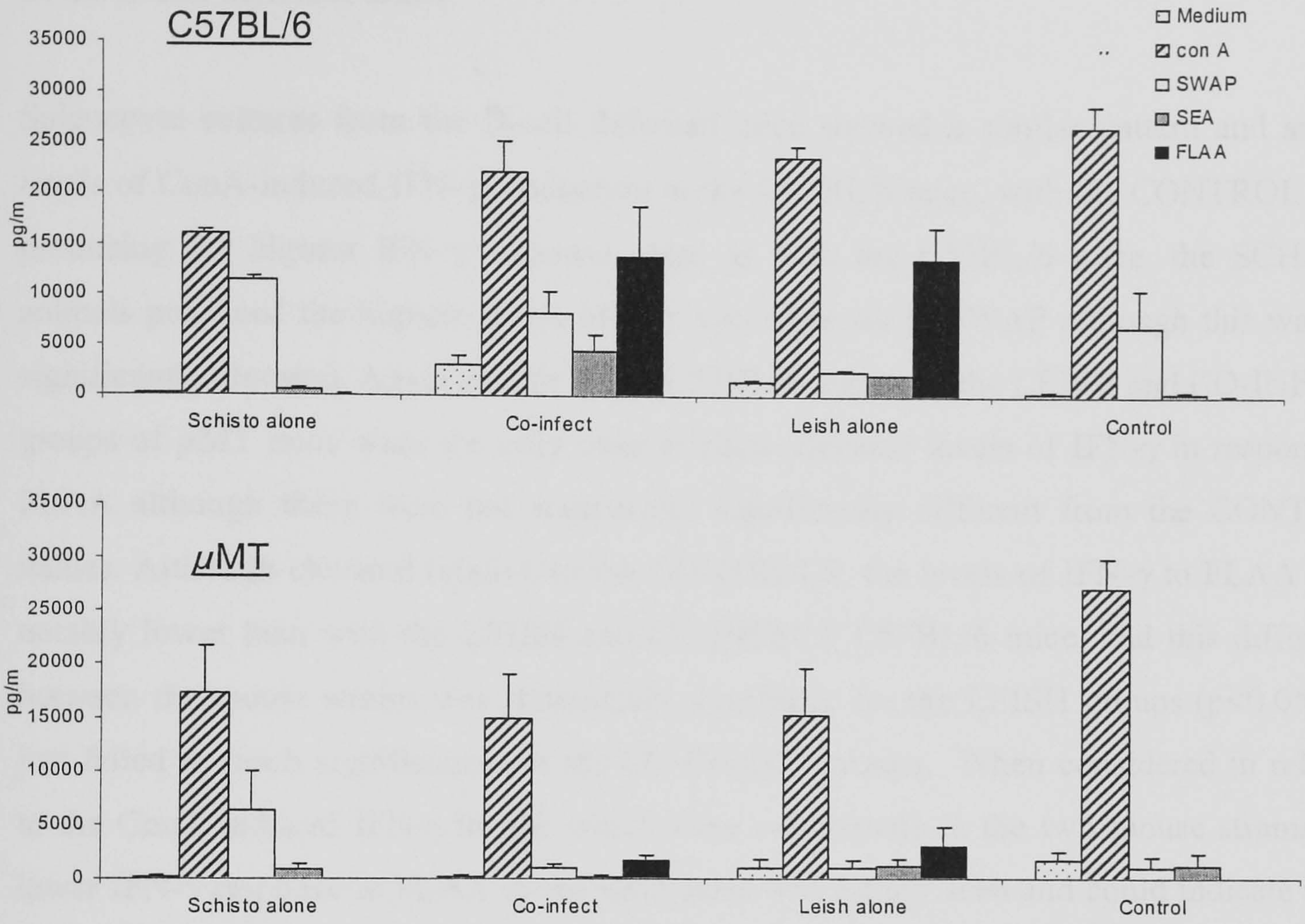


Figure 4.4 : Graphs showing the means and standard errors for IFN-γ detected by ELISA based on three mice from each group sacrificed at +5 weeks after the *L. donovani* infection. Cultures of $5x10^6$ spleen cells/ml were stimulated with medium alone, mitogen (ConA at 5μg/ml), schistosomal antigens (SWAP and SEA at 20μg/ml) or fixed leishmanial amastigote antigen (FLAA, at 10^7 amastigotes/ml). For description of the groups see legend to Figure 3.3.1.

SEA and only in the CO-INFECT and LEISH groups although this was significant for the LEISH group relative to the CONTROLS ($p=0.002$) possibly due to cross reactivity at this higher level of SEA re-stimulation (20μg/ml).

Most importantly, only the CO-INFECT and LEISH groups produced elevated levels of IFN-γ in response to FLAA compared to both the SCHISTO and CONTROL mice ($p=0.05$, 0.05 respectively relative to the former and $p=0.02$, 0.02 respectively to the latter). Notably

there was no difference in the levels of IFN-γ between the CO-INFECT and LEISH groups in spite of the marked differences in *L. donovani* parasite burdens.

In the B-cell deficient mice:

Splenocyte cultures from the B-cell deficient mice showed a similar pattern and similar levels of ConA-induced IFN-γ production to the C57BL/6 mice, with the CONTROL mice producing the highest IFN-γ response. Also as with the C57BL/6 mice, the SCHISTO animals produced the highest levels of IFN-γ in response to SWAP although this was not significantly elevated. Again similar to the C57BL/6 response, the LEISH and CO-INFECT groups of μMT mice were the only ones to show elevated levels of IFN-γ in response to FLAA although these were not statistically significantly different from the CONTROL values. Although elevated relative to the CONTROLS, the levels of IFN-γ to FLAA were notably lower than with the LEISH and CO-INFECT C57BL/6 mice, and this difference between the mouse strains was statistically significant for the LEISH groups ($p<0.05$) but just failed to reach significance for the CO-INFECT groups. When considered in relation to the ConA-induced IFN-γ levels, which were comparable in the two mouse strains, the lower IFN-γ response to FLAA in the μMT mice was clearly seen and could indicate some specific involvement of B cells in the generation of the FLAA IFN-γ response in addition to the IL-10 response.

<u>IL-4 production from splenocytes</u>

In the C57BL/6 (wild type, WT) mice:

The data including the ConA results are shown in Figure 4.5 and redrawn without the ConA data in Figure 4.6.

There was a similar pattern of low IL-4 responsiveness as seen in earlier experiments (see Figures 3.3.7, 3.3.8, 3.3.13, 3.3.14 in Chapter 3). With ConA stimulation the SCHISTO animals produced the highest IL-4 response but only the CO-INFECT animals showed

significantly different levels compared to the LEISH group (p=0.03). Once again, the SCHISTO animals produced the highest levels of IL-4 in response to (SWAP, SEA) although this was not significantly elevated compared to other groups. There was no IL-4 production in response to FLAA in any of the groups.

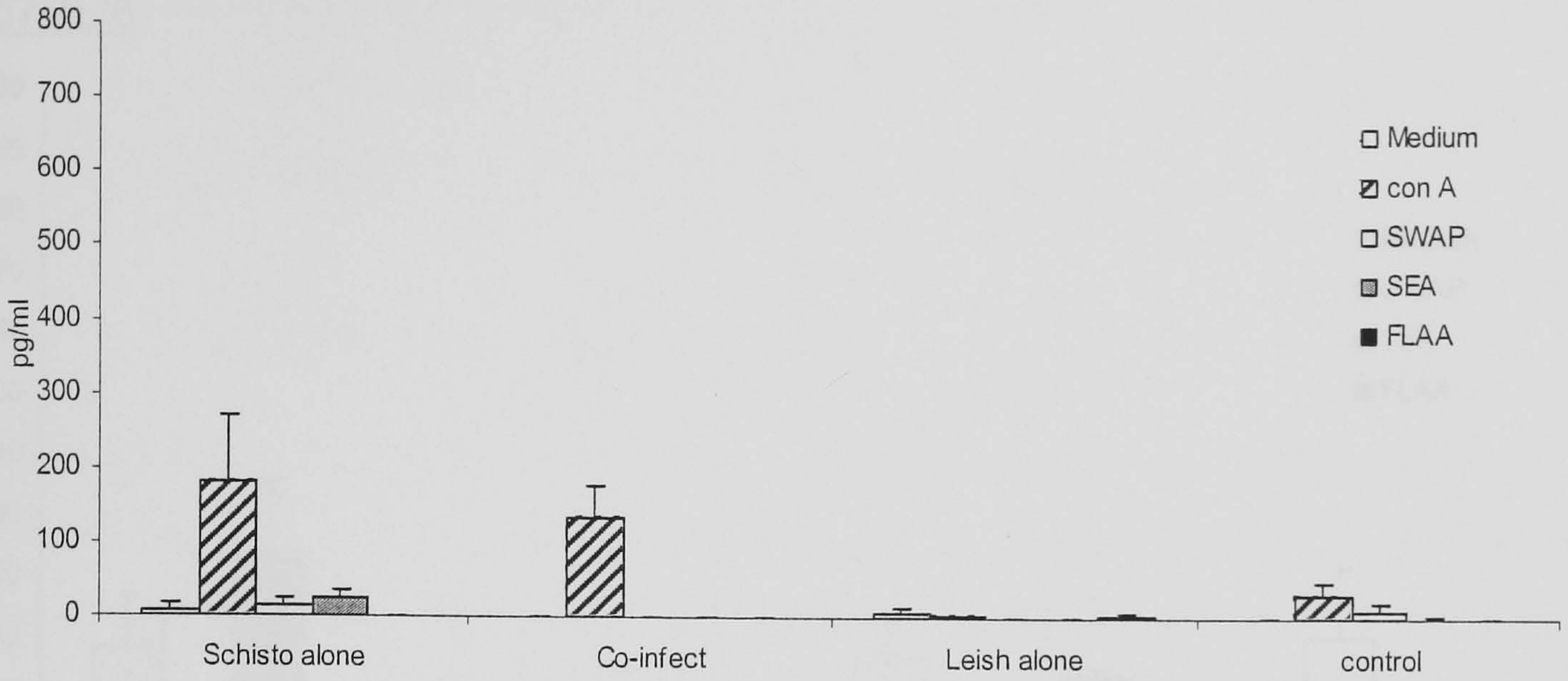
In the B-cell deficient mice:

A similar pattern of IL-4 production to that of the C57BL/6 mice was obtained in the different groups of μMT mice. Thus, the SCHISTO and CO-INFECT animals produced the highest IL-4 in response to ConA. The responses in the LEISH and CONTROL groups were significantly lower (p=0.001, 0.0006 respectively to the former, p=0.05, 0.03 respectively to the latter). Once again, the SCHISTO animals produced the highest production of IL-4 in response to SEA and this was higher than in the CO-INFECT mice although it was not statistically significantly higher than in any of the other groups. There were minimal responses to FLAA in any of the groups.

Intriguingly the SCHISTO and CO-INFECT groups of μMT mice produced noticeably higher IL-4 levels in response to ConA compared to the same groups in the C57BL/6 mice and the difference was statistically significant for the SCHISTO groups (p=0.01). However, this difference was not seen in relation to the antigen specific responses.

Figure 4.5. Comparison of the levels of IL-4 in supernatants of spleen cell cultures from C57BL/6 and μMT mice

C57BL/6



μMT

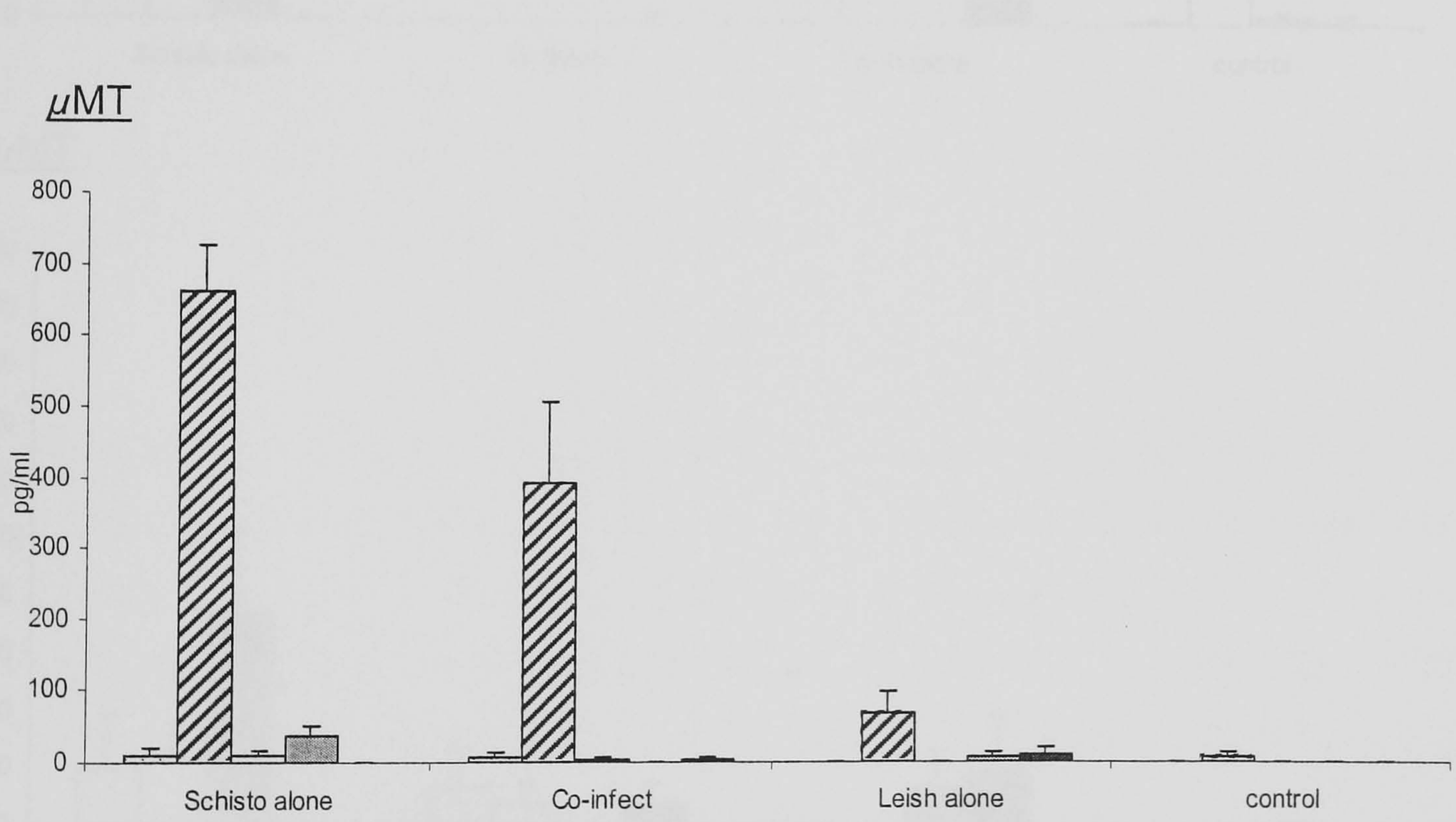
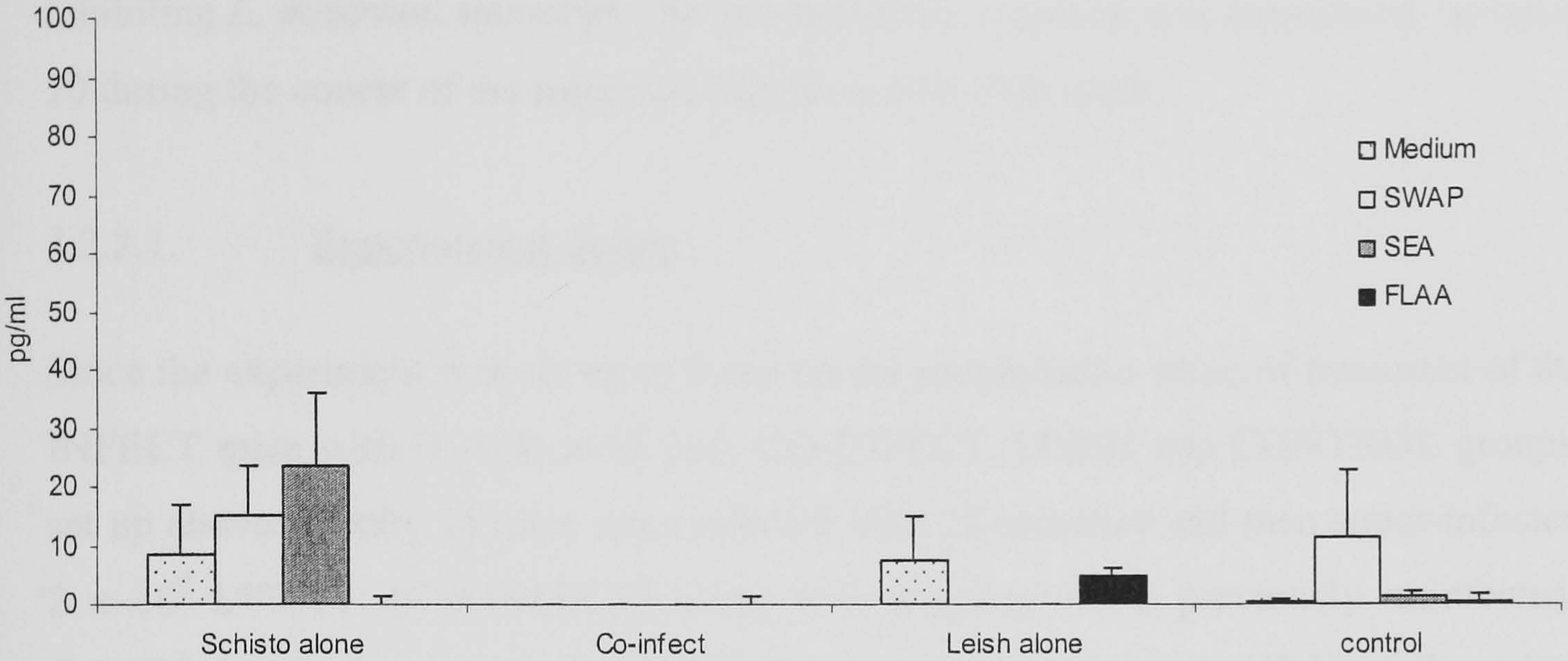


Figure 4.5 : Graphs showing the means and standard errors for IL-4 detected by ELISA based on three mice from each group sacrificed at +5 weeks after the *L. donovani* infection. Cultures of $5x10^6$ spleen cells/ml were stimulated with medium alone, mitogen (ConA at 5μg/ml), schistosomal antigens (SWAP and SEA at 20μg/ml) or fixed leishmanial amastigote antigen (FLAA, at 10^7 amastigotes/ml). For description of the groups see legend to Figure 3.3.1.

Figure 4.6. Comparison of the levels of IL-4 in supernatants of spleen cell cultures from C57BL/6 and μMT mice – as in Figure 4.5 but without ConA data

C57BL/6



μMT

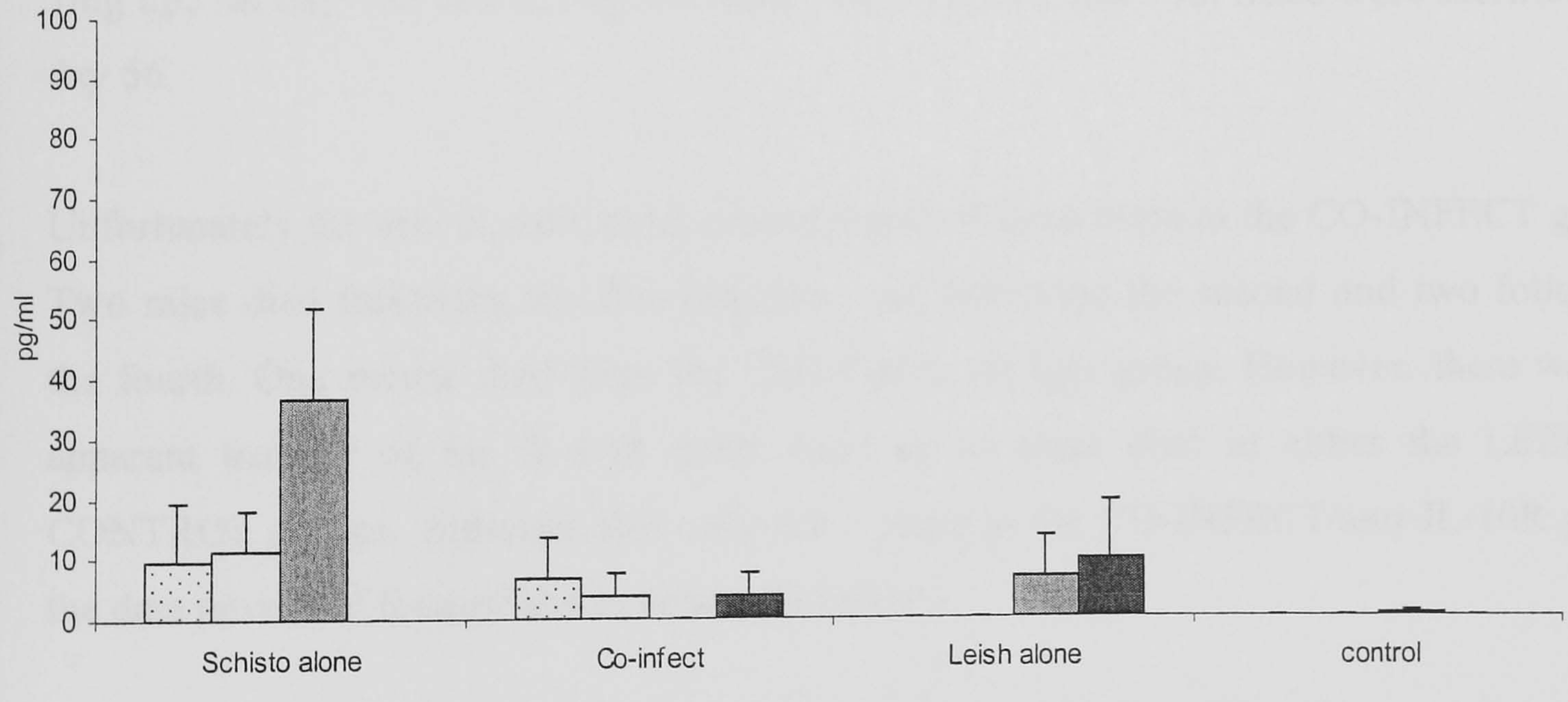


Figure 4.6: For legend see Figure 4.5.

4.2.2 Effect of IL-10 neutralization using IL-10R mAb on *S. mansoni/L. donovani* co-infection.

Because of the intrinsic resistance of the μMT mice to *L. donovani*, the above experiment was not conclusive in establishing if *S. mansoni* induced IL-10 was responsible for inhibiting *L. donovani* immunity. So an alternative approach was considered, to inhibit IL-10 during the course of the infection with an anti-IL-10R mAb.

4.2.2.1. Experimental design

Since the experiment was set up to focus on the prophylactic value of treatment of the CO-INFECT mice with IL-10R mAb only CO-INFECT, LEISH and CONTROL groups were set up above. Briefly 16 mice were infected with 25 cercariae and then super-infected with 2 x 10^7 LV9 i.v. (CO-INFECT) along with a group of 16 previously uninfected mice (LEISH). A further group of CONTROL mice was also set up. Half of the mice were injected i.p. with the anti-IL-10 mAb and the other half with the control rat Ig at the rate of 1mg i.p./ on day +42 and 0.5mg on days +46, +49,+ 52 and +55. Mice were sacrificed on day 56.

Unfortunately the anti-IL-10R mAb caused death of some mice in the CO-INFECT group. Two mice died following the first injection, one following the second and two following the fourth. One mouse died from the CONTROL rat IgG group. However, there was no apparent toxicity of the IL-10R mAb itself as no mice died in either the LEISH or CONTROL groups. Although this only left 3 mice in the CO-INFECT/anti-IL-10R group the data proved of interest and is presented below.

4.2.2.2. LDU values following i*n vivo* treatment with IL-10R mAb

One mouse in the CO-INFECT group treated with control rat IgG showed extremely high LDU values (12.65 for the liver and 1.585 for the spleen) relative to the others in the group. This was so much higher than the rest of the group that the values for this mouse were

removed from the data plotted in Figure 4.7 But it is included in the data plotted in Figure 4.8.

Figure 4.7. Comparison of Leishman Donovan Units (LDU) from liver and spleen imprints following infection or co-infection of C57BL/6 mice treated with anti-IL-10R mAb.

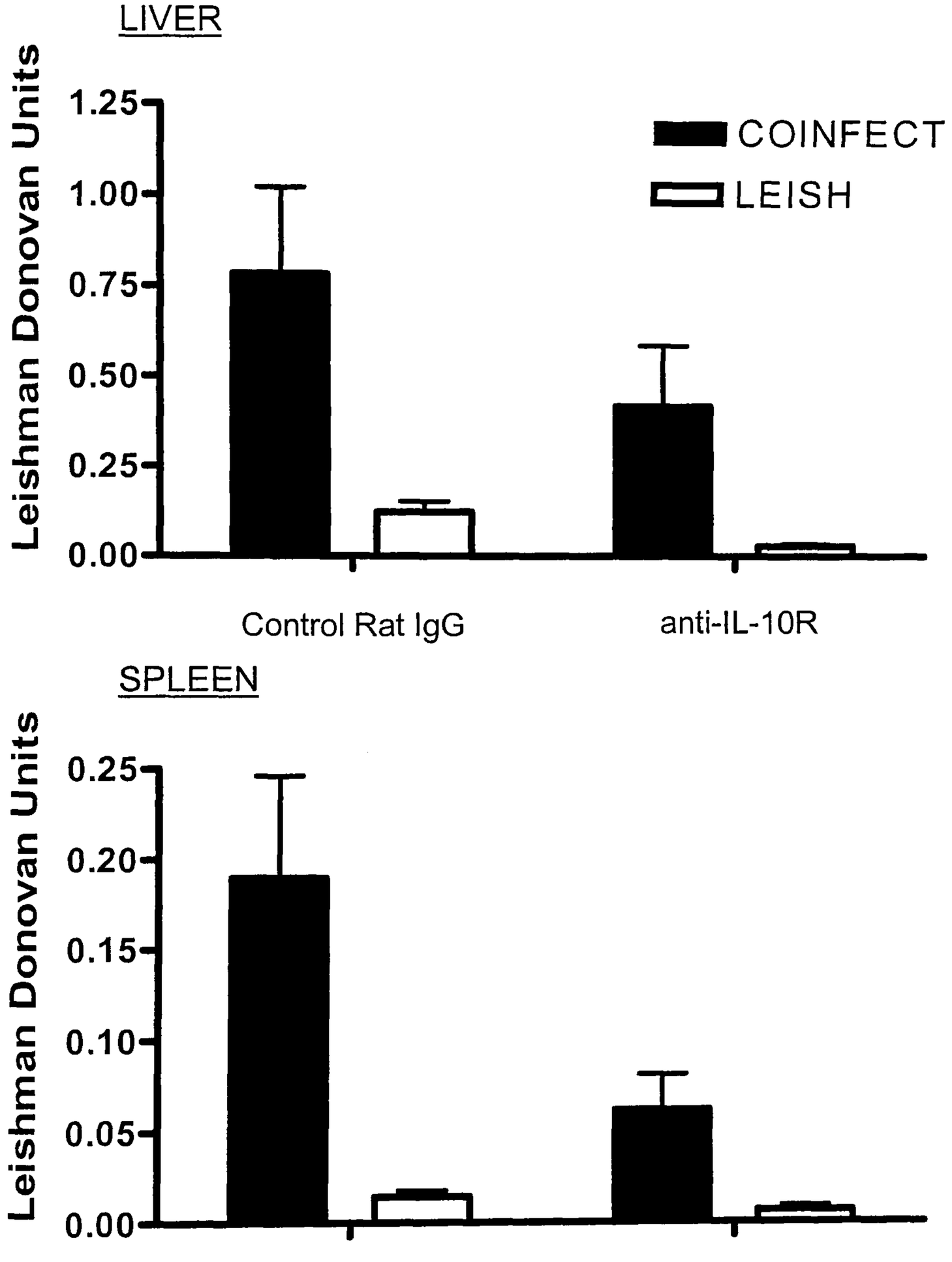


Figure 4.7: Graphs showing the mean (±SE) LDU for the CO-INFECT and LEISH groups treated i.p. with rat monoclonal anti-mouse IL-10R antibody (1mg on day +42, 0.5mg on days +46, 49, 52 and 55). They were sacrificed on day +56. These graphs exclude one mouse from the CO-INFECT group which had very high LDU counts. Data is based on 6 mice for the CO-INFECT plus rat IgG, 3 mice for the CO-INFECT with anti-IL-10R and 5 for each of the LEISH groups. For descriptions of the groups see legend to Figure 3.3.1.

Figure 4.8 Comparison of Leishman Donovan Units (LDU) from liver and spleen imprints following infection or co-infection of C57BL/6 mice treated with anti-IL-10R mAb- Data as in Figure 4.7 but including one outlier mouse result from the CO-INFECT which was not included in figure 4.7.

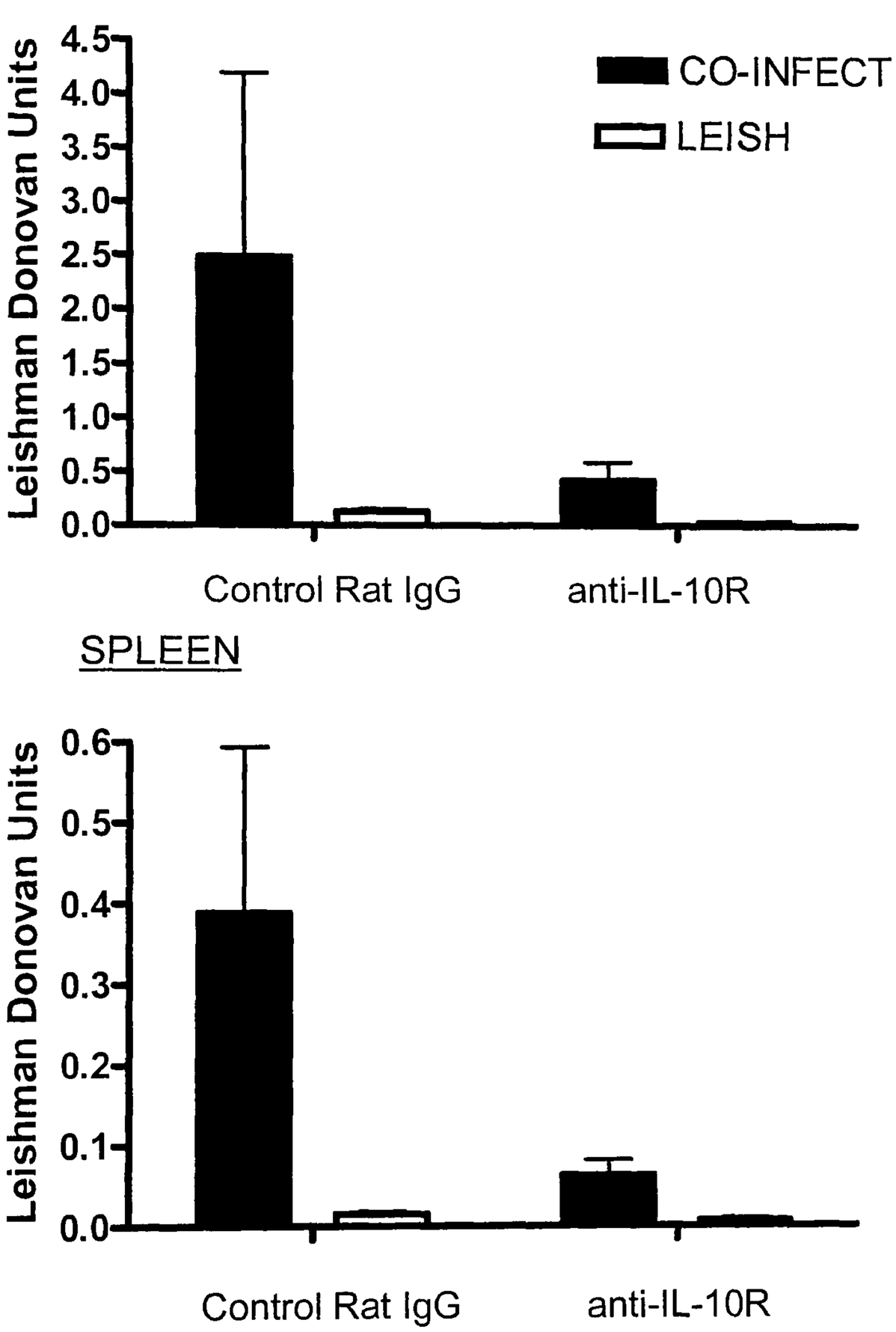


Figure 4.8: Graphs showing the mean (±SE) LDU for the CO-INFECT and LEISH groups treated i.p. with rat monoclonal anti-mouse IL-10R antibody (1mg on day +42, 0.5mg on days +46, 49, 52 and 55). They were sacrificed on day +56. These graphs include one mouse from the CO-INFECT group which had very high LDU counts. Data is based on 7 mice for the CO-INFECT plus rat IgG, 3 mice for the CO-INFECT with anti-IL-10R and 5 for each of the LEISH groups. For descriptions of the groups see legend to Figure 3.3.1.

As seen in Figure 4.7. and 4.8., as in the previous co-infection experiments, the CO-INFECT mice showed a loss of control of the *L. donovani* infection compared with the LEISH mice. Thus in the groups receiving the control rat IgG the CO-INFECT mice had a 6.3 fold increase in liver LDU compared with the LEISH ($p=0.0338$) and 20 fold increase if the mouse with the high value is included although because of the high values this difference was not statistically significant. For the spleen LDUs the reductions were 13 fold ($p=0.02$) or 27 fold, with the high value included.

Moreover, the *Leishmania* infected mice treated with Rat IgG showed significantly lower liver and spleen LDUs compared to CO-INFECT animals ($p=0.03$, 0.02 respectively).

Generally, the CO-INFECT mice treated with IL-10R mAb showed a reduction of LDUs compared to those treated with rat IgG. With the high LDU mouse excluded the CO-INFECT mice treated with IL-10R mAb showed a 1.9 fold reduction in liver LDUs and with the mouse included a 6 fold reduction. The LDUs in the spleen were 3.1 fold less with the mouse excluded and 6.3 fold lower with this mouse included. But given the low numbers surviving and the large variations none of these differences were statistically significant.

So in spite of the mouse deaths in the CO-INFECT group there was evidence that the anti-IL-10 treatment was able to reduce the high *L. donovani* LDU in the CO-INECT mice but it was not possible to conclude that this was due to inhibition of *S. mansoni* mediated IL-10 responses as the LEISH infected mice treated with IL-10R mAb also showed a reduction in the spleen LDUs (2.2 fold lower) and a marked reduction in liver LDUs (4.5 fold lower) compared to the LEISH group treated with Rat IgG, and this difference was statistically significant for the liver LDUs ($p=0.012$).

4.2.2.3 Detection of cytokine production

Note: Both IL-10 and IFN-γ production by cells from the CONTROL/anti-IL-10R treated mice showed very high levels of IL-10 and IFN-γ with all treatments including medium alone. All of the other groups showed variable responses with the different treatments and it

is concluded that the data for this group was unreliable and this was not included in the analysis (shown as N.S. [not shown] in Figures 4.9 and 4.10).

*Levels of **IL-10** from spleen cells* in vivo *treated with anti-IL-10R or rat-IgG*

The pattern of IL-10 production (see Figure 4.9.) shared some similarities was the data for the 8 week time points in the two experiments in Chapter 3 (Figure 3.3.10 and 3.3.16). As with both of these earlier sets of data there was IL-10 production to FLAA in both the LEISH and CO-INFECT groups and as in the earlier data this was higher in the CO-INFECT although the difference was not significant. There was however, no specific IL-10 production to the SEA above that seen in the cultures in medium alone at this time. This is consistent with the down-regulated IL-10 production seen Figure 3.3.10 but Figure 3.3.16 showed higher levels especially in the CO-INFECT. The reason for this difference is not apparent.

Figure 4.9. Comparison of the levels of IL-10 in supernatants of pooled splenocytes from mice treated with anti-IL-10R antibody

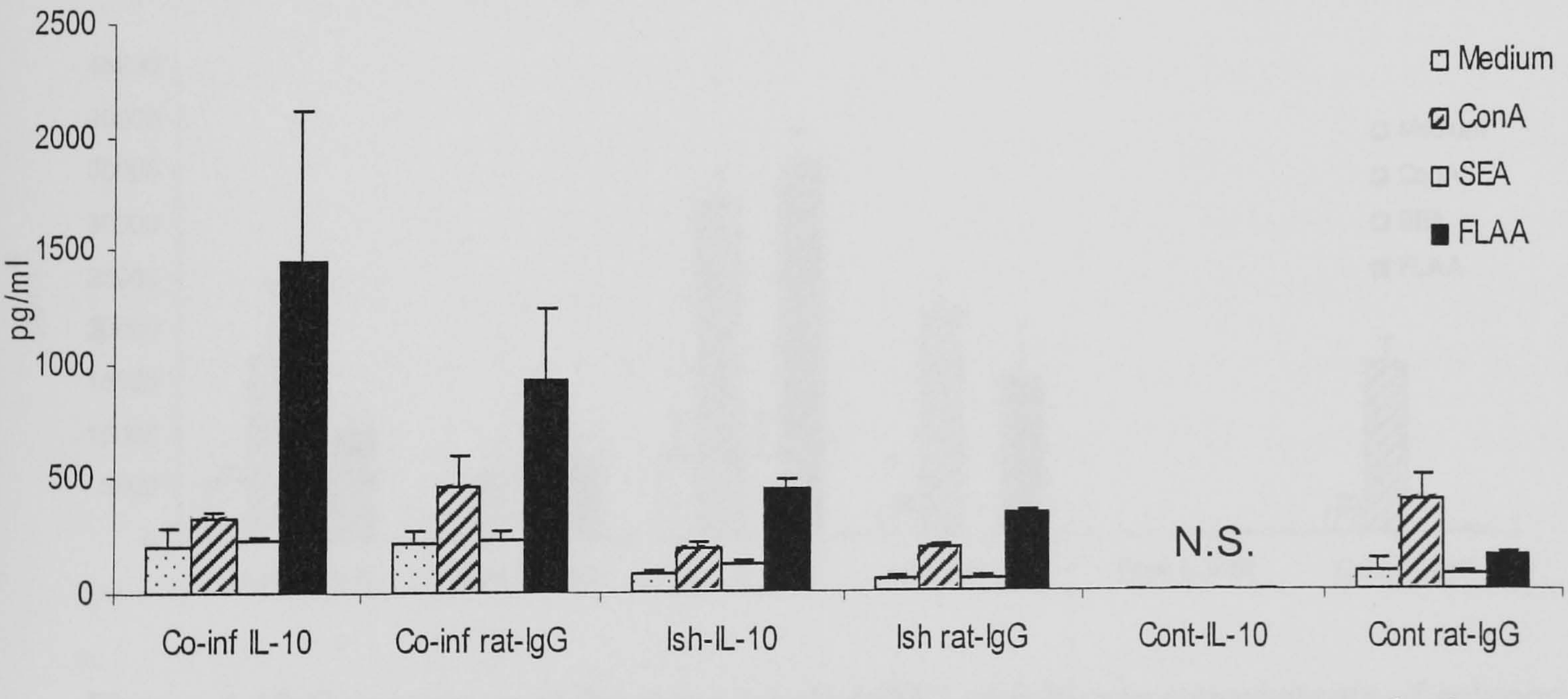


Figure 4.9 Comparison of the mean levels (±SE) of IL-10 in supernatants of splenocytes from CO-INFECT, LEISH and CONTROL groups treated i.p. with rat monoclonal anti-mouse IL-10R antibody (1mg on day +42, 0.5mg on days +46, 49, 52 and 55). They were sacrificed on day +56. For descriptions of the groups see legend to Figure 3.3.1.

Regarding the effects of the anti-IL-10R treatment this had no significant effect on the levels of IL-10 production in either the CO-INFECT or LEISH mice.

*Levels of **IFN-γ** from spleen cells* in vivo *treated with anti-IL-10R or rat-IgG*

Figure 4.10 shows IFN-γ production from spleen cells from mice treated *in vivo* with anti-IL-10R or rat-IgG. The pattern of IFN-γ responses notably to ConA and FLAA in the CO-INFECT and LEISH groups treated with the control IgG were consistent with the +8 week time points in the earlier experiments (Figures 3.3.9 and 3.3.15, Chapter 3) i.e. the level of IFN-γ production was higher in the LEISH than in the CO-INFECT mice and this was significant for the ConA data ($p=0.02$). There were no significant responses to SEA above the medium alone values.

Figure 4.10. Comparison of the levels of IFN-γ in supernatants of pooled splenocytes from mice treated with anti-IL-10R antibody

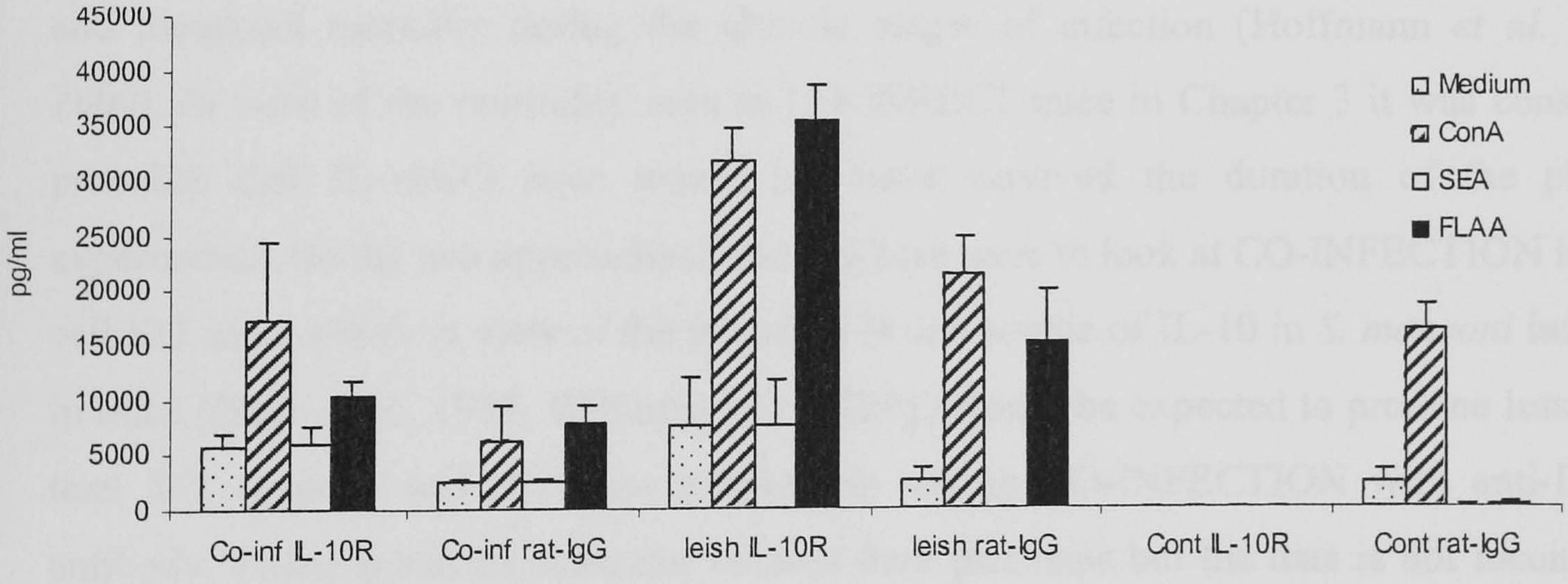


Figure 4.10 Comparison of the mean levels (±SE) of IFN-γ in supernatants of splenocytes from CO-INFECT, LEISH and CONTROL groups treated i.p. with rat monoclonal anti-mouse IL-10R antibody (1mg on day +42, 0.5mg on days +46, 49, 52 and 55). They were sacrificed on day +56. For descriptions of the groups see legend to Figure 3.3.1.

The *in vivo* treatment with anti-IL-10R antibody enhanced the level of IFN-γ production by splenocytes from the LEISH mice in response to ConA and FLAA as compared with the

response by cells from the LEISH/control rat IgG antibody group. The difference was statistically significant for the FLAA response (p=0.02). There was little evidence of enhanced IFN-γ production in the CO-INFECT following the anti-IL-10 treatment as compared with the control IgG treatment. The ConA response was increased somewhat but not significantly and the FLAA response not noticeably different.

4.3 Discussion

These studies were concerned with trying to obtain supportive evidence for the immuno-regulatory role of *S. mansoni* induced IL-10 on the failure to control *L. donovani* infection in CO-INFECT mice which was postulated in Chapter 3. Consideration of using IL-10 gene targeted mice (IL-10 KO) was ruled out as various experiments had shown that such mice are essentially resistant to *L. donovani* infection (Murphy *et al.,* 2001; Murray *et al.*, 2002; Kane and Mosser, 2001). Furthermore, IL-10KO mice show greater susceptibility to *S. mansoni* infection as judged by increased morbidity with the development of large schistosomal granulomas (Hoffmann *et al.,* 2000; Metwali *et al.,* 1996; Wynn *et al.*, 1997), and increased mortality during the chronic stages of infection (Hoffmann *et al.*, 1999; 2000). In view of the morbidity seen in CO-INFECT mice in Chapter 3 it was considered probable that IL-10KO mice would not have survived the duration of the planned experiments. So the two approaches followed here were to look at CO-INFECTION in (i) B cell KO mice which in view of the proposed B cell source of IL-10 in *S. mansoni* infection in mice (Harn *et al.,* 1989; Williams *et al.,* 2001) would be expected to produce less IL-10 than WT controls and (ii) mice treated late during CO-INFECTION with anti-IL-10R antibody. These protocols were not without their problems but the data is not inconsistent with the proposed immuno-regulatory role for *S. mansoni*-induced IL-10.

In the studies using μMT mice, the parasitological data from the WT (C57BL/6) control mice was consistent with the results from Chapter 3 in that even though the experiment had to be terminated at +5 weeks post *L. donovani* infection there was a comparable lack of control of the *L. donovani* infection in the CO-INFECT mice as at the +8 week time point

in Chapter 3, with parasite loads in the liver and spleen the CO-INFECT mice 3-fold higher than in the LEISH alone group.

However, the μMT mice showed generally lower susceptibility to the *L. donovani* infection, the LEISH alone mice showing 3-fold lower LDUs compared with the WT mice. Such lowered susceptibility had previously been shown by Smelt *et al.* (1997) who reported significantly lower LDU in μMT mice as early as 15 days post infection. Unexpectedly they found enhanced neutrophil infiltration into the *L. donovani* granulomas in the liver early after infection which they suggested may be due to a lack of B cell derived IL-10 which is known to regulate neutrophil production/activation. It was suggested that ROI produced by TNF-α activated neutrophils may be the basis of the enhanced control in μMT mice as ROI have been shown to play an important role especially early after infection (Murray and Nathan, 1999). Unfortunately this differential susceptibility in the LEISH WT and μMT animals in the present experiments meant that it was not possible to directly compare the LDU values in the μMT and WT CO-INFECT mice. However, in the livers, the relative increase in LDUs in μMT mice in the CO-INFECT mice over the LEISH mice was lower in the μMT than the C57BL/6 mice (increased only 0.63 fold in the CO-INFECT compared with the LEISH mice [N.S.] in μMT and increased 2.25 fold in the CO-INFECT compared with the LEISH in the C57BL/6 mice [P= 0.0052]). In other words in the absence of B cells there was relatively little effect of the *S. mansoni* infection on the control of the *L. donovani* infection. So could this be due to a lack B cell produced IL-10 in the *S. mansoni* infected mice?

As in the experiments in Chapter 3, in *in vitro* splenocyte cell culture, the SCHISTO WT type (C57BL/6) mice showed markedly elevated IL-10 in response to SEA, significantly higher than in CO-INFECT, LEISH and CONTROL groups ($p=0.006$, 0.0002, and <0.0001 respectively). However, in the CO-INFECT mice there was little evidence of raised IL-10 responses relative to the LEISH mice or the CONTROLS except for the SEA response but this was not statistically significant. This was somewhat different from the experiments in Chapter 3 both of which showed elevated IL-10 responses in the CO-INFECT mice to both schistosome and leishmanial antigens. The reasons for this difference are not clear.

However, the liver lymphocytes in CO-INFECT WT mice did show elevated IL-10 production to both schistosome and leishmanial antigens compared with the LEISH and CONTROL mice. Such higher levels of antigen specific cytokine production by granuloma macrophages compared with splenocytes has been reported previously for both IL-10 (King *et al.,* 2001) and IL-4 (Hayashi *et al.,* 1999). So, the liver IL-10 data at least demonstrates elevated levels of IL-10 to both schistosome and leishmanial antigen in the CO-INFECT WT mice.

In the μMT mice *in vitro* cell culture from both the livers and spleens of the SCHISTO μMT mice showed markedly lowered IL-10 compared to the WT mice. Given that the levels of schistosome infections in the μMT and C57BL/6 mice were comparable, this difference in IL-10 response supports the notion (Harn *et al.,* 1989; O'Garra *et al.,* 1992) that B cells are an important source of IL-10 in *S. mansoni* infection and that this can be induced in response to the schistosome products. The IL-10 levels were also lower in the spleen and notably the liver responses of the μMT compared with the WT CO-INFECT mice. So the IL-10 cytokine data is consistent with the idea that B cells lead to enhanced IL-10 production in *S. mansoni* infected mice and that during *L. donovani* co-infection this could serve to compromise the development/action of anti-leishmanial immunity.

Whereas the higher IL-10 levels in the liver of CO-INFECT compared to LEISH WT mice correlated with the higher LDU in the CO-INFECT mice, the levels of splenocyte IFN-γ were comparable between the groups suggesting that IFN-γ does not mediate this difference in parasite control. However, in both of the two earlier experiments (in Chapter 3) there was evidence of lowered FLAA specific IFN-γ production in the CO-INFECT mice compared with thee LEISH at +2 and + 8 weeks but either similar or higher production in the CO-INFECT at + 4 weeks. So it is possible that the higher LDU in this experiment (terminated at +5 weeks post super-infection) reflects earlier deficiency in IFN-γ production in the CO-INFECT mice.

Given the immuno-regulatory role of IL-10 it might have been expected that the proposed absence of B cell-derived IL-10 would have led to an increase in IFN-γ levels in the μMT compared with the WT mice. However, the μMT mice *in vitro* cell cultures from both the livers and spleens of the SCHISTO and CO-INFECT μMT mice showed lowered IFN-γ responses to schistosome antigen than the WT mice, giving no indication that the lack of B cells and the IL-10 they may produce had skewed the immune response away from a Th2 response to a Th1 response. In fact very low IFN-γ levels to FLAA were seen in the μMT mice. IFN-γ levels were not reported by Smelt *et al.* (2000) but the lack of IFN-γ may be explained simply by the low parasite burdens which develop in these animals as a result of the proposed neutrophil mediated attrition resulting in low antigen stimulation.

Alternative to the effects on IFN-γ production, the elevated IL-10 production in the liver of the CO-INFECT mice could mediate immuno-modulatory effects via modulation of macrophage activation (Moore *et al.*, 1999) making them refractory to activation by IFN-γ for intracellular killing (Moore *et al.*, 1993; Kane and Mosser, 2001; Gazzinellini *et al.*, 1992).

Unexpectedly the SCHISTO and CO-INFECT groups of μMT mice produced noticeably higher IL-4 levels in response to ConA compared to the same groups in the C57BL/6 mice and the difference was statistically significant for the SCHISTO groups ($p=0.01$). The reasons for this are unknown. In the papers showing that *S. mansoni* infection induced B cell outgrowth and production of IL-10 from B cells stimulated with egg carbohydrates it was suggested that this route of IL-10 production might promote the Th2 responses seen during acute schistosomiasis in mice (Fitch *et al.*, 1993). So it would have been expected to have seen lower Th2 responses in mice lacking B cells and so lacking the ability to produce IL-10 via this route. However, this difference was not seen in relation to the antigen specific responses and why it was restricted to the Con A response is not clear.

To conclude this experiment it could be argued that the relatively lower fold increase in LDU in the liver of the μMT mice (CO-INFECT vs. LEISH) compared with the WT mice (CO-INFECT vs. LEISH) could be due to lack of B-cell derived IL-10 inhibiting macrophage-mediated killing of *L. donovani* in the μMT mice. However, it should be emphasized that the µMT mice are much less susceptible to infection with *L. donovani* than the C57BL/6 mice and that this is believed to be due to the rapid cytotoxic effects of recruited neutrophils (Smelt *et al.*, 1997) and it is unclear how the schistosome infection would affect this potent neutrophil mediated killing.

The complications which arose from the relative resistance of B cell deficient mice meant that this was not a profitable approach to addressing the role of IL-10 and so this experiment was not repeated. Instead an attempt was made to see if treatment of the established co-infection with anti-IL-10R mAb would serve to prevent or reverse the trend of increasing *L. donovani* LDU in the CO-INFECT mice.

Unfortunately, the anti-IL-10R mAb caused death of some mice in the CO-INFECT group but there was no apparent toxicity of the IL-10R mAb itself, as no mice died in either the LEISH or CONTROL groups. A SCHISTO alone group was not included in this study as the intention was only to look at the effects on the loss of *L. donovani* control. In retrospect it would have been a useful control.

Regarding the reasons for the mortality seen, Sadler *et al.,* (2003) reported a higher mortality in *S. mansoni* infected IL-$10^{-/-}$ mice over a 15 week period, but showed that the knockout (KO) mice had larger, less well defined schistosome granulomas but no appreciable difference in cellular composition. Extensive areas of hepatocellular necrosis were seen around the granulomas, significantly greater at both 8 and 15 weeks post *S. mansoni* infection. They ruled out differences in antibody which were similar in the two strains levels but reported markedly higher SEA-induced IFN-γ levels in MLN and spleen cell cultures and suggested that excessive production of inflammatory mediators such as nitric oxide caused the liver damage. There is evidence that the proposed role for "alternatively activated" granuloma macrophages in inhibiting

cellular proliferation and the pro-inflammatory component of the anti-egg response involves IL-10 (Flores Villanueva *et al.,* 1994a) and mice which are unable to generate AAMϕ suffer inflammatory disease and death (Herbert *et al.,* 2004). This is consistent with the pathology and high mortality seen in Th1 polarized (IL-4/IL-10 KO) mice (Hoffmann *et al.*, 2000). In the present study, therefore, it is likely that the administration of anti-IL-10 antibody during the chronic phase would have similarly led to unregulated pro-inflammatory responses and to tissue damaging NO production perhaps notably around the schistosome granulomas.

Apart from the effects that blockage of IL-10 function may have on the schistosome granulomatous response the CO-INFECT mice in this study would also have had high *L. donovani* counts at the time of neutralization. So the sudden neutralization of IL-10 function is likely to have resulted in rapid and marked increases in production of NO in the liver due to pro-inflammatory anti-*L. donovani* responses. This is in agreement with Murray *et al.,* (2003a) who showed that anti- IL-10R mAb treatment of early 14 day *L. donovani* infections resulted in a dramatic 55-fold increase in IFN-γ leading to rapid control of the *L. donovani* infection via the activation of NO and reactive oxygen species. Although this early 14 day infection was controlled without mouse morbidity, in the present studies the inflammatory response in the liver is likely to have been much greater due to the development of the Th1 response and the high parasite loads. It is concluded that blockage of IL-10 function is likely to have led to sudden increases in the pro-inflammatory responses to both *L. donovani* and *S. mansoni* granulomas in the liver leading to inflammatory damage and death.

Despite the death of several of the animals three were left to allow an assessment to be made of the effect of blocking IL-10 function on reversing the schistosome-induced inhibition of *L. donovani* immunity. As in the previous co-infection experiments, the CO-INFECT mice treated with the control antibody showed a loss of control of the *L. donovani* infection compared with the LEISH mice. In the current experiment, the LEISH mice treated with control rat IgG showed significantly lower liver (6.3 fold) and spleen (13 fold) LDUs compared to CO-INFECT animals treated with same antibody ($p=0.03$, 0.02 respectively).

The CO-INFECT mice treated with IL-10R mAb showed lower liver LDUs (1.9 fold reduction) and spleen LDUs (3.1 fold reduction) compared to the CO-INFECT mice treated with control rat IgG. One of the CO-INFECT mice developed exaggerated and progressive parasitic burden in both liver (16 fold higher values than mean liver LDU in other mice of the same group) and spleen (8.4 fold higher values) and if this mouse was included the difference would have been greater. Thus the anti-IL-10R treatment did serve to reduce the uncontrolled *L. donovani* proliferation in CO-INFECT mice suggesting that IL-10 was exerting a negative influence on killing of *L donovani* and that this could be relatively rapidly reversed. As a therapy therefore the anti-IL-10R treatment was effective. However, although they had lower LDU than in the CO-INFECT mice the LEISH alone mice also showed lower liver LDUs (4.4 fold reduction) and spleen LDUs (2.3 fold reduction) when treated with the anti-IL-10R mAb compared to the LEISH mice treated with control rat IgG. Similarly reduced LDU following anti-IL-10R mAb treatment in *L. donovani* infected BALB/c mice were recently reported by Murray *et al.*, (2003a).

How the inhibition of IL-10 function led to apparently greater control in both the LEISH and CO-INFECT groups in the current experiment is uncertain. IFN-γ production to FLAA was increased in LEISH mice treated with anti-IL-10R mAb compared to the control rat IgG ($p=0.02$). So the data showing reduced *L. donovani* parasite loads in the anti-IL-10R treated LEISH mice fits with the idea that the treatment enhances IFN-γ production. This also came in agreement with other studies showing that anti-IL-10R mAb treatment enhances Th1 responses (Castro *et al.*, 2000). However, the level of increased IFN-γ production was only 2.3 fold compared with the control IgG treated mice. By contrast, Murray *et al.* (2003) reported a 55 fold increase in IFN-γ in mice following treatment with the same mAb but there were differences compared with this experiment. Murray *et al.* (2003) measured serum levels, they used BALB/c mice and the treatment started at day 14 post infection rather than the 42 days post infection in the experiment described here.

It was particularly interesting that although the anti-IL-10R treatment led to lower LDU in the livers and spleens of the CO-INFECT mice this treatment did not induce elevated

Leishmania specific IFN-γ production. This suggests that in the CO-INFECT mice the lowered FLAA specific IFN-γ response which was seen at +8 weeks in the various co-infection experiments in this thesis is not principally due to IL-10 mediated suppression. So in both the LEISH and CO-INFCT mice other IL-10 mediated effects may be more important in the lack of *L. donovani* control than effects on IFN-γ levels i.e. (i) induced blockade of IL-10R signalling (Miralles *et al.,* 1994), via restoring the PKC activity in the parasitized macrophages (Bhattacharyya *et al.,* 2001b); (ii) blocked inhibition of nitric oxide, ROI and murine TNF-α release by the infected macrophages (Bhattacharyya *et al.* 2000; Murray *et al.*, 2003), affecting granuloma assembly (Murray *et al.,* 2002). (iii) APC/Th1 cell interactions via costimulatory molecules (iv) Th1 cell proliferation (Ghalib *et al.,* 1993).

In chronic cutaneous *L. major* infection, anti-IL-10R injections eliminated persisting parasites apparently by increasing macrophage responsiveness to existing level of Th1 cell reactivity. Neither increased levels IFN-γ expression and/or local cellular responses were up-regulated in treated C57BL/6 mice (Belkaid *et al.,* 2001).

In conclusion, in spite of the increased mouse deaths in the CO-INFECT group there was evidence that the anti-IL-10 treatment was able to reduce the high *L. donovani* LDU in the CO-INECT mice but it was not possible to conclude with certainty that this was due to inhibition of *S. mansoni* mediated IL-10 responses as the LEISH infected mice treated with anti-IL-10 mAb also showed a reduction. The fact that the anti-IL-10R treatment did not result in increased IFN-γ production suggests that the prime effect of the IL-10 is to inhibit macrophage function reducing granuloma formation and leishmanicidal killing within the granulomas.

Chapter 5

Biochemical and histopathological responses in the liver to *S. mansoni/L. donovani* Co-infection

5.1. Introduction

The work in Chapters 3 and 4 had shown: (i) that *L. donovani* parasites were able to proliferate more effectively in mice with a pre-existing *S. mansoni* infection than in naïve mice (ii) that the co-infected mice showed elevated IL-4 and IL-10 responses to schistosome antigens and IL-10 to leishmanial infections (iii) that the co-infected mice showed lower leishmanial specific IFN-γ responses. In section 3.4. of Chapter 3 various hypotheses were outlined to explain the lack of control in the CO-INFECT mice. Prominent amongst these was the idea that the *S. mansoni* induced cytokines notably IL-4 and IL-10 could suppress the afferent and efferent arm of the protective Th1 response against *L. donovani i.e.* the generation around Ld infected KCs in the liver of IFN-γ mediated granulomas comprising activated macrophages secreting TNF-α and the leishmanidical ROI and NO. The data from Chapter 4 although not conclusive were consistent with the idea that IL-10 could be involved in the failure to control *L. donovani* in the co-infected mice.

Among the hypotheses were the following: (Hypothesis 2) that *S. mansoni* hepatic egg granulomas are rich in recruited macrophages and so there may be a higher density of foci of *L. donovani* infection within the egg granulomas compared with the parenchyma; (Hypothesis 4) that lower IFN-γ levels may result in reduced development/reduced rate of maturation of *L. donovani* granulomas (comprising antigen-specific Th1 cells and recruited macrophage and which are most clearly seen in the liver) *i.e.* there would be a reduced frequency of development of mature *L. donovani* granulomas; (Hypothesis 5) that *L. donovani* granulomas may physically fail to form correctly within the context of the inflammatory cells of the *S. mansoni* granulomas and so there may be a lack of control in infected macrophages within the egg granuloma; (Hypothesis 6) that even if morphologically normal mature granulomas form, the constituent macrophages have a

reduced capacity to become activated and so to produce leishmanicidal factors which may result in higher mean numbers of amastigotes in *L. donovani* foci in CO-INFECT mice compared with LEISH mice; (Hypothesis 7) Alternatively activated macrophages which predominate in the schistosome egg granulomas may be poor at directing *L. donovani* granuloma formation and/or show poor leishmanicidal activity. In this case amastigotes may accumulate within *L. donovani* infected macrophages in the egg granuloma resulting in higher mean numbers of amastigotes in the infected macrophages within the *S. mansoni* granulomas compared with the parenchyma of CO-INFECT mice and/or LEISH mice.

The studies in this chapter were concerned principally with the functional consequences of the proposed immune modulation by the *S. mansoni* infection on the resolution of the *L. donovani* infection. For this purpose attention was focused on the generation of *L. donovani* granulomas or other foci of Ld infection in the liver because in singly (LEISH) infected mice the evolution of mature granulomas and the resultant leishmanicidal activities within these are believed to be necessary for control of the infection. So attention was focused on histological or immuno-histological analysis of the *L. donovani* and the *S. mansoni* granulomas in the CO-INFECT and LEISH mice. In addition, the data in Chapter 3 also showed that although the superimposed Ld infection did not induce a significant modulation of the *S. mansoni* immune response from Th2 to Th1, the CO-INFECT mice did produce IFN-γ to leishmanial antigens and it was considered possible that this could have influenced the Th2 response to the *S. mansoni*. So histological observations on the *S. mansoni* egg granulomas were also made.

Immune granulomas, both the *S. mansoni* and the *L. donovani* induced ones are localised inflammatory reactions containing T cells and represent a form of delayed-hypersensitivity. They share a number of common features including prolonged antigenic stimulation that is not easily cleared by phagocytic cells. In the case of the schistosome eggs this is due to the prolonged release of soluble egg antigens through microspores in the egg shell and the large size of the schistosome granuloma formed as host protective immune reaction. In the case of *Leishmania* the parasite chooses to live inside phagocytic cells and is able to persist there. Granulomas are often associated with the fusion of macrophages to form flattened

epithelioid and multinucleated giant cells. The recruited T cells contribute to the attraction of myeloid inflammatory effector cells which can vary between granulomas of different origin (Chensue and Boros, 1979; Kunkel *et al.*, 1998).

During acute infection of mice, the *S. mansoni* egg granuloma is a Th2 cytokine dominated response, with the granuloma cells producing IgG1, IgE, and large quantities of Th2 cytokines IL-4, IL-5 (Metwali *et al.*, 2002) and IL-13 (Fallon *et al.*, 2000). IL-13 is responsible in mice for fibrosis and consequently for schistosome pathology (Fallon *et al.*, 2000). However, egg antigen specific Th1 cytokines are also produced in *S. mansoni* infected mice and studies in double knock out mice (IL-4/IL-10$^{-/-}$, Th1 biased; IL-12/IL-10$^{-/-}$, IFN-γ/IL-10$^{-/-}$, Th2 biased) suggest that the granulomatous response is finely balanced in normal mice. However, if this is skewed in the Th1 direction it results in severe inflammatory disease and animal death, whereas if the granulomatous response is skewed towards a Th2 gives rise to increased fibrosis and increased mortality (Hoffmann *et al.*, 2000). This is because mediators associated with Th1 responses, *e.g.* IFN-γ, IL-12, TNF-α and NO can prevent IL-13 mediated fibrosis (Hesse *et al.*, 2002). Both Th2 and Th1 cytokines are produced in humans to egg antigens and are also likely to counter regulate each other to minimize damage to the host. For example, there is evidence that fibrosis in human schistosomiasis may be linked to polymorphisms in the IFN-γ receptor 1 gene associated with lack of effectiveness of IFN-γ in suppressing fibrosis (Dessein *et al.*, 1999). However, the role of IL-13 has yet to be firmly established in human disease (Pearce and MacDonald, 2002). There is evidence of an association of hepatosplenic disease with elevated Th1 responses and plasma levels of soluble TNF-α receptor I and II (Mwatha *et al.*, 1998), although the link between hepatosplenic disease and fibrosis in humans is still uncertain (Dessein *et al.*, 1999). Nevertheless it is clear from mice that increased IFN-γ can downregulate the Th2 mediated granulomatous response. The administration of IL-12 together with egg antigen, promotes a more Th1 response and results in reduced Th2 responses, liver fibrosis and granuloma size (Cheever and Yap, 1997; Wynn *et al.*, 1994). In these present studies the induction of elevated IFN-γ response to the superimposed *L. donovani* infection in the liver could modulate the *S. mansoni* Th2 response leading to reduced Th2 responses and granuloma size.

The liver apart from providing an altered cytokine environment for *L. donovani* development, in a *S. mansoni* infected mouse also presents a markedly altered cellular environment *i.e.* the schistosome egg granulomas contain predominantly eosinophils, lymphocytes, fibroblasts and macrophages (Weinstock and Boros, 1983). The granuloma macrophages derived from blood monocytes are recruited to the granuloma from the periphery (King *et al.,* 2001); Initiation and regulation of disease in schistosomiasis: Mahmoud AAF, editor. Schistosomiasis. London: Imperial College Press, 213-264.) and may offer additional sites for *L. donovani* infection. In addition it is clear that the schistosome granuloma macrophages are of the "alternatively activated macrophage (aaMø)'' phenotype which are characteristically immuno-suppressive and anti-inflammatory. AAMøs were first defined as such by Stein *et al.* (1992) who showed that IL-4 treatment of murine macrophages deactivated the macrophages and upregulated mannose receptor (MR) expression. Recently by staining for MR, Linehan *et al.* (2003) showed that GM were MR positive whereas GM in IL-4R$\alpha^{-/-}$ mice were not. The term aaMø was originally applied to describe macrophages activated by IL-4 or IL-13 (Stein *et al.*, 1992) and to distinguish such cells from those deactivated by IL-10 but the concept of alternatively activated macrophages is now used more broadly and considered in terms of their function (Noel *et al.,* 2004).

"Classical activation" of macrophages is mediated by Th1 cytokines which promotes the secretion of proinflammatory cytokines, the up-regulation of NO and O_2^- production and enhances the capacity of macrophages to activate T cells *i.e.* the processes typically involved in the generation of resistance to *L. donovani* infection. In contrast the Th2 cytokines, IL-4 and IL-13, have been shown to promote aaMø from bone marrow derived macrophages *in vitro* (Goerdt *et al.,* 1999) and these cells secrete anti-inflammatory cytokines, they do not produce significant levels of NO and superoxide and have non-specific anti-proliferative properties (Gordon, 2003). A key feature of aaMø's is that IL-4 and IL-13 induce the expression of arginase (Hesse *et al.,* 2001), which converts L-arginine to L-ornithine which can lead to collagen production and fibrosis. Arginase-1 is heavily expressed in *S. mansoni* egg granulomas but is not induced in Th2 deficient mice (Hesse *et al.,* 2001). Importantly L-arginine is also the substrate for iNOS which is activated by IFN-γ ligation and which

leads to production of NO and citrulline. An intermediate in this pathway is L-hydroxyarginine which inhibits arginase activity and so yet another way in which the Th2 granulomatous response leading to fibrosis, is modulated by the concurrent Th1 response (Hesse *et al.,* 2001). However, the activation of arginase-1 by Th2 cytokines reduces the availability of L-arginine for NO production and this seems to be critical in preventing Th1 mediated nitrosative damage such as occurs in *S. mansoni* infected Th2 deficient mice, *e.g.* IL-4$^{-/-}$ mice (Brunet *et al.,* 1997). The critical role of Th2 activated aaMø's in *S. mansoni* infection was elegantly shown recently in mice with a selective deletion of IL-4Rα in macrophages and so which cannot develop aaMø show rapid wasting and death with elevated iNOS production in both the liver and gut. It is concluded that the alternatively activated granuloma macrophages are unable to produce tissue damaging NO or superoxide, counter-balancing the destructive potential of *S. mansoni* egg-induced Th1 inflammation (Herbert *et al.,* 2004).

In fact Stadecker *et al.* (1990) were the first to show the presence of immuno-modulatory macrophages in schistosomiasis. They found that isolated granuloma macrophages (GM) strongly inhibited the ability of splenic APC to stimulate SEA-specific CD4+ve Th1 cell clones *in vitro* and exposure to GM plus SEA renders the Th1 cells anergic to restimulation. This effect was dependent on IL-10, and not TGF-β both of which inhibitory factors can be produced by macrophages (de Waal Malefyt *et al.,* 1991; Assoian *et al.,* 1987). The GM showed downregulated expression of costimulatory molecules, B7-1 and B7-2 which could be restored by inhibition of the endogenous IL-10 production (Flores Villanueva *et al.,* 1994b). Furthermore transfer of purified GMs *in vivo* down-regulated egg granuloma formation and was associated with a marked decrease in IL-12 production (Flores Villanueva *et al.,* 1994a). This earlier work supports the conclusions that the GM have profoundly immune-suppressive and anti-inflammatory properties which serve to prevent damaging inflammatory responses in schistosomiasis but which are likely to have profound effects on the leishmanicidal response to *L. donovani* infection of GM or of adjacent macrophages.

The development, structure and function of *Leishmania donovani* granulomas have been thoroughly reviewed by Murray, (2001). The parasite targets tissue macrophages and resistance depends on T cells, Th1 type cytokines and activated mononuclear phagocytes recruited to the developing granulomas. In the liver the resident macrophages (Küpffer cells [KCs]) are initially infected and then a central core of fused parasitized KCs becomes surrounded by cytokine secreting specific T cells (IFN-γ producing) and infiltrating blood monocytes. Within the mature granuloma, macrophages are activated by IFN-γ and TNF-α leading to the production of leishmanicidal components (NO and ROI) killing the intracellular amastigotes (Murray and Cartelli, 1983, Murray, 1982; Melby *et al.,* 1998; Taylor and Murray, 1997; Engwerda *et al.,* 1996). Although this is the classical response leading to parasite attrition in animals which have developed specific T cell responses, the presence of structurally intact mature granulomas does not necessarily lead to parasite killing. For example, ineffective granulomas form in TNF-α KO mice, (Murray and Nathan, 1999). Since IL-10 has been shown to inhibit macrophage secretion of mediators such as IL-1β, IL-6 and TNF-α (Fiorentino *et al.,* 1991a; Chensue *et al.,* 1989; Bogdan *et al.*, 1991; Ralph *et al.,* 1992), it is possible that the elevated levels of IL-10 in CO-INFECT mice could inhibit leishmanicidal activity within formed *L. donovani* granulomas without necessarily affecting the development of mature granulomas as judged by histological appearance. Anti-leishmanial effects can also occur in the absence of tissue reactions (invisible granulomas) and enlarged granulomas (hypertrophied granulomas) can lead to enhanced killing. Furthermore, typical *L. donovani* granulomas do not form in the spleen.

In order to gain some insight into which of these potential mechanisms may be most important in causing the higher parasite loads in CO-INFECT mice, the work in this Chapter was principally aimed at examining the histopathological response in the liver with a particular focus on the nature of the *L. donovani* granulomas *i.e.* their development/maturation, tissue distribution and level of amastigote loads. Some data is also presented on histological analysis of the possible effects of the *L. donovani* infection on the formation of *S. mansoni* egg granulomas and before considering the histopathology, data is presented on another aspect of the pathological response; the evidence for liver damage based on biochemical analysis of liver enzyme release into the serum of infected mice.

5.2. Results

5.2.1. Liver enzyme concentrations

5.2.1.1. Blood concentration of liver enzymes (ALT/AST)

Experiment 1

In the co-infection experiments in Chapters 3 and 4 some of the CO-INFECT mice showed greater morbidity than the SCHISTO mice whereas none of the LEISH mice showed marked signs of illness. Because *S. mansoni* and *L. donovani* both infect the liver it was anticipated that there would be enhanced damage to the liver in the CO-INFECT mice and so serum was taken in order to test for liver enzymes in the experiments in Chapter 3 and 4. Data is presented for the first two experiments in Chapter 3. As seen in Figure 5.1. each of the infected groups showed higher enzyme levels at most of the time points compared with the control levels.

At +2wk (post LV9) the highest level of liver damage (ALT/AST) was among the CO-INFECT followed by the LEISH and then by the SCHISTO animals but the results were only statistically significant different from the CONTROL for the SCHISTO mice ($p=0.002$ and 0.03 respectively for ALT and AST) but these were not significantly different from the other groups of infected mice. The enzyme levels for the SCHISTO group remained at a relatively constant level at the subsequent +4 and +8 week time points.

At +4 weeks the SCHISTO values were significantly higher than in the CONTROLS ($p=0.02$, 0.005 respectively for ALT and AST). Between +2 and +4 weeks both the ALT and AST generally increased in both the LEISH and CO-INFECT groups to reach peak values and for both infected groups these were approximately 8 and 4 fold higher than CONTROLS the differences being statistically significant ($p=0.001$, 0.02 respectively for LEISH and $p=0.0005$, and 0.02 respectively for the CO-INFECT). For the LEISH group the increase between +4 and +8 weeks was significant for both ALT and AST (0.01 and 0.01 respectively).

Figure 5.1. Comparisons of the degree of liver damage in different infected groups as judged by serum liver enzymes, ALT/AST – Experiment 1

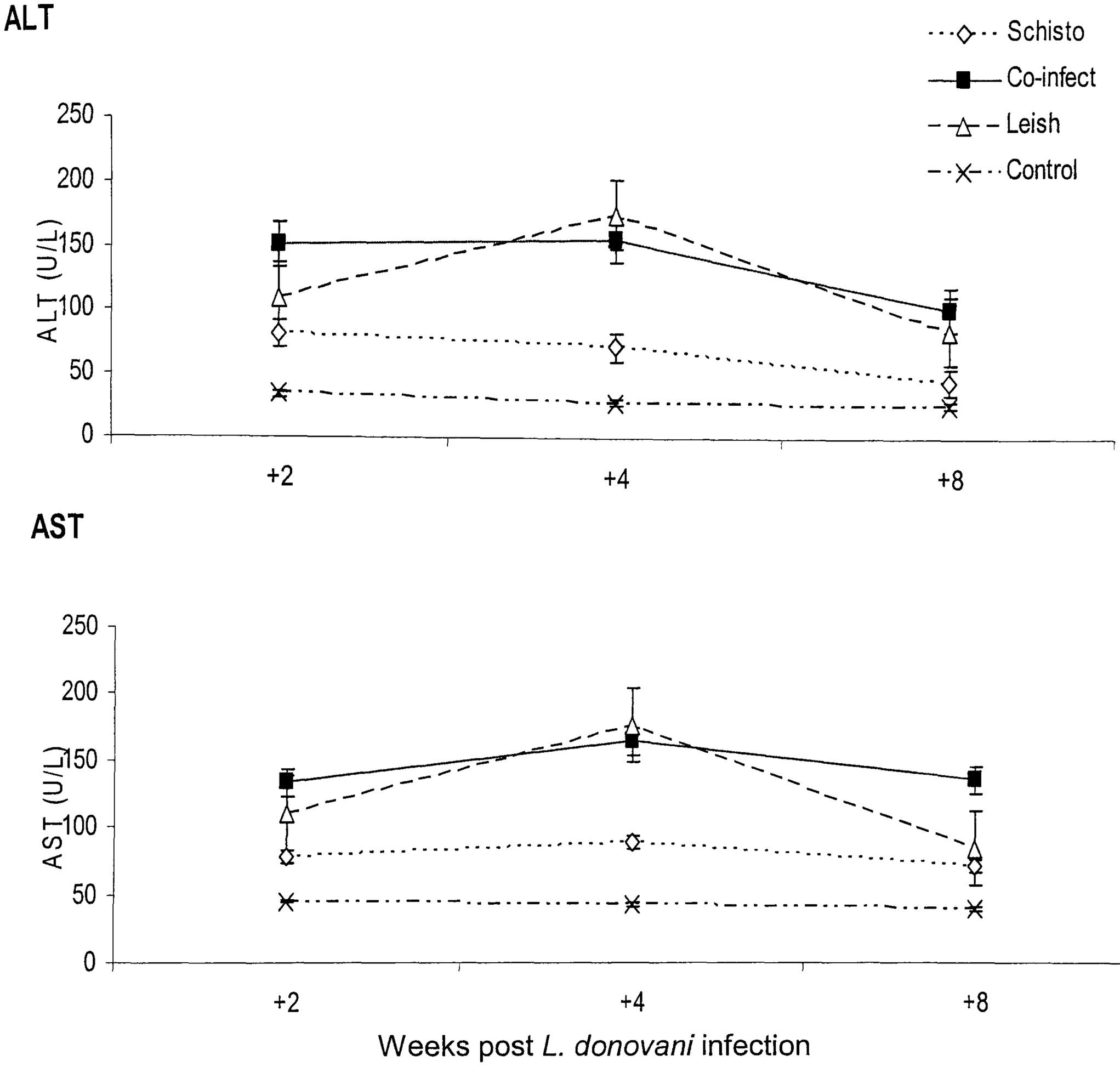


Figure 5.1. Graphs showing showing the Mean ± SE levels of Liver transaminases [Aspartate aminotransaminase (AST), Alanine Aminotransaminase (ALT)] in Units/L in the blood of infected animals at +2, +4 and +8wks p.i. with LV9 for Experiment 1. For description of groups see legend to Figure 3.3.1. The data is from 5 mice/group

At +8weeks the CO-INFECT animals had the highest ALT/AST values followed by the LEISH group but these were not significantly different and not significantly higher than the

CONTROL values. Meanwhile, the SCHISTO group because of the low variation, still showed significantly raised ALT values compared with CONTROLS ($p=0.03$).

5.2.1.2. Blood concentration of liver enzymes (ALT/AST) : Experiment 2

As seen in Figure 5.2 the results are remarkably similar to those in Figure 5.1

Figure 5.2. Comparisons of the degree of liver damage in different infected groups as judged by serum liver enzymes, ALT/AST – Experiment 2

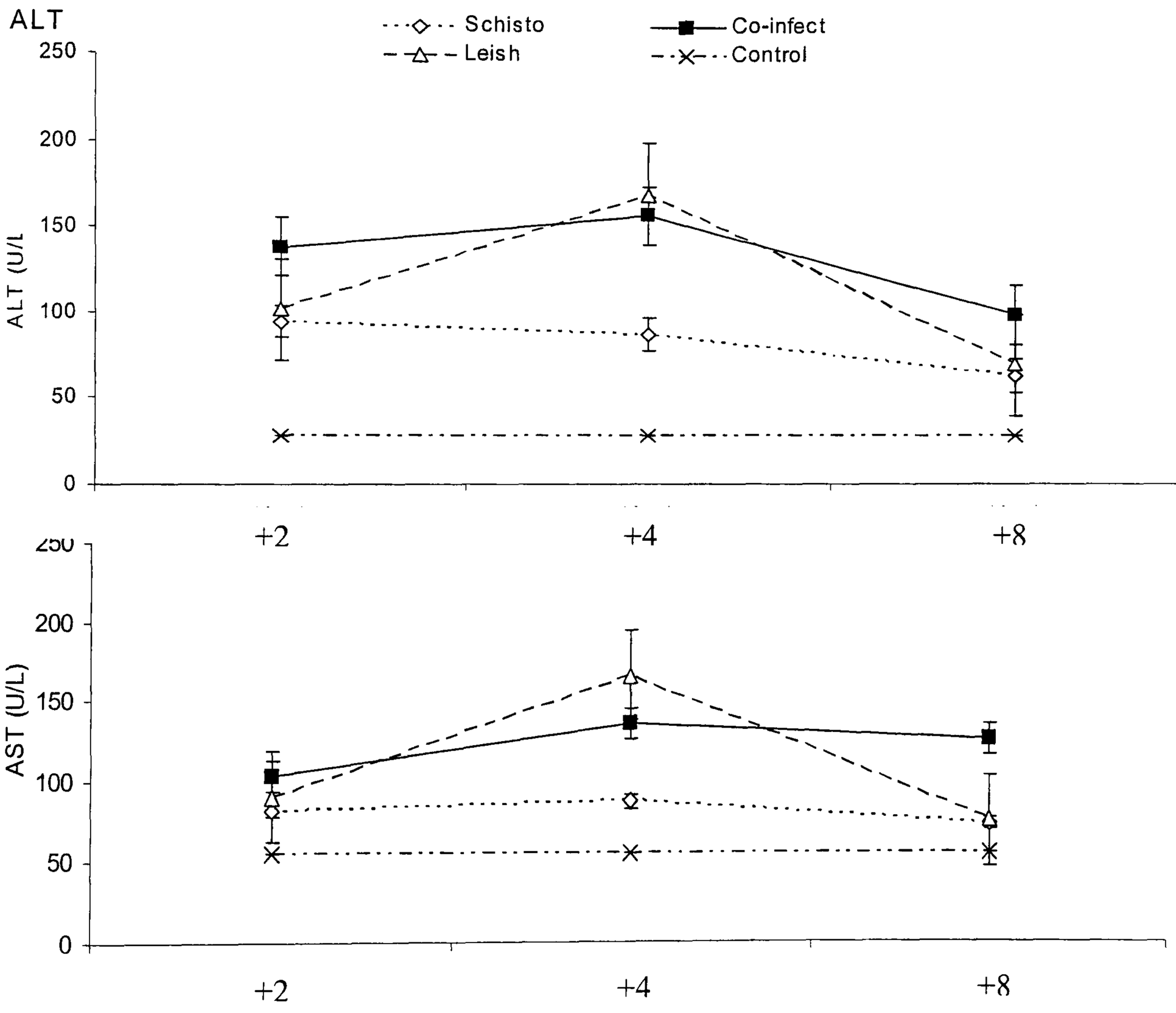


Figure 5.2. Graphs showing showing the Mean ± SE levels of Liver transaminases (ALT/AST) in Units/L in the blood of infected animals at +2, +4 and +8wks p.i. with LV9 for Experiment 2. For description of groups see legend to Figure 3.3.1. The data is from 5 mice/group

At each of the time points the ranking of levels of serum enzymes was similar to the previous experiment.

At +2wk the highest level of liver damage (ALT/AST) was again among the CO-INFECT animals compared to the controls ($p=0.0005$, 0.001 respectively) followed by the LEISH animals ($p<0.0001$, $p=0.003$ respectively) and the SCHISTO group ($p=0.002$ and 0.02 respectively). As before the enzyme levels for both the SCHISTO group and CONTROLS remained at a relatively constant level at the subsequent +4 and +8 week time points but the CO-INFECT and LEISH groups increased noticeably between +2 and +4 weeks.

At +4weeks the LEISH group showed the highest liver damage ($p=0.0003$, 0.002 respectively relative to CONTROLS) but the CO-INFECT values were very similar and also significantly higher than the CONTROLS ($p=0.004$, and 0.0003).

Moreover, the SCHISTO group developed significant liver damage for ALT and AST relative to the controls ($p=0.001$, 0.03 respectively) but this was significantly lower compared to levels in the CO-INFECT animals ($p=0.04$, 0.009 respectively), or LEISH animals ($p=0.004$, 0.01 respectively).

At +8wk generally the levels of ALT/AST remained similar in the SCHISTO animals gradually declined. The LEISH values showed a drop in level of both enzymes compared with the +4 week time point but these were still significantly raised compared with controls ($p=0.02$, 0.04 respectively). The CO-INFECT values were the highest ($p=0.02$, 0.03 respectively compared to the CONTROL) remaining at the same level as at +4 weeks for AST and showing some decline in ALT levels but less than for the LEISH ALT levels.

5.2.2. Effect of *Leishmania* superinfection on the schistosome granuloma

To assess whether the superimposed and ultimately uncontrolled *L donovani* infection affected the immuno-pathological response in the liver to schistosome eggs histopathological analysis was carried out into the size and cellular composition of individual granulomas in SCHISTO compared with CO-INFECT mice. Animals with

comparable numbers of recovered adult worms and geometeric mean egg counts were selected and analysis carried out on tissue from 3 mice of each. Observations were made at +4 and +8 week time points, +4 weeks being the time of the peak Th1 response in *L. donovani* infections (Murray, 2001a) and +8 weeks being the time of peak *L. donovani* parasite loads. To ensure that the different granuloma sections were comparable only reactions with a central egg present were assessed. In addition because the granulomas in a particular liver could be of a range of ages depending on when the inducing egg was produced and so either newly formed, maximal in size or resolving, only granulomas with an egg containing a miracidium were assessed as shown in Figure 5.3.

Figure 5.3. Section of liver tissue showing the cellular composition of a typical *S. mansoni* egg granuloma.

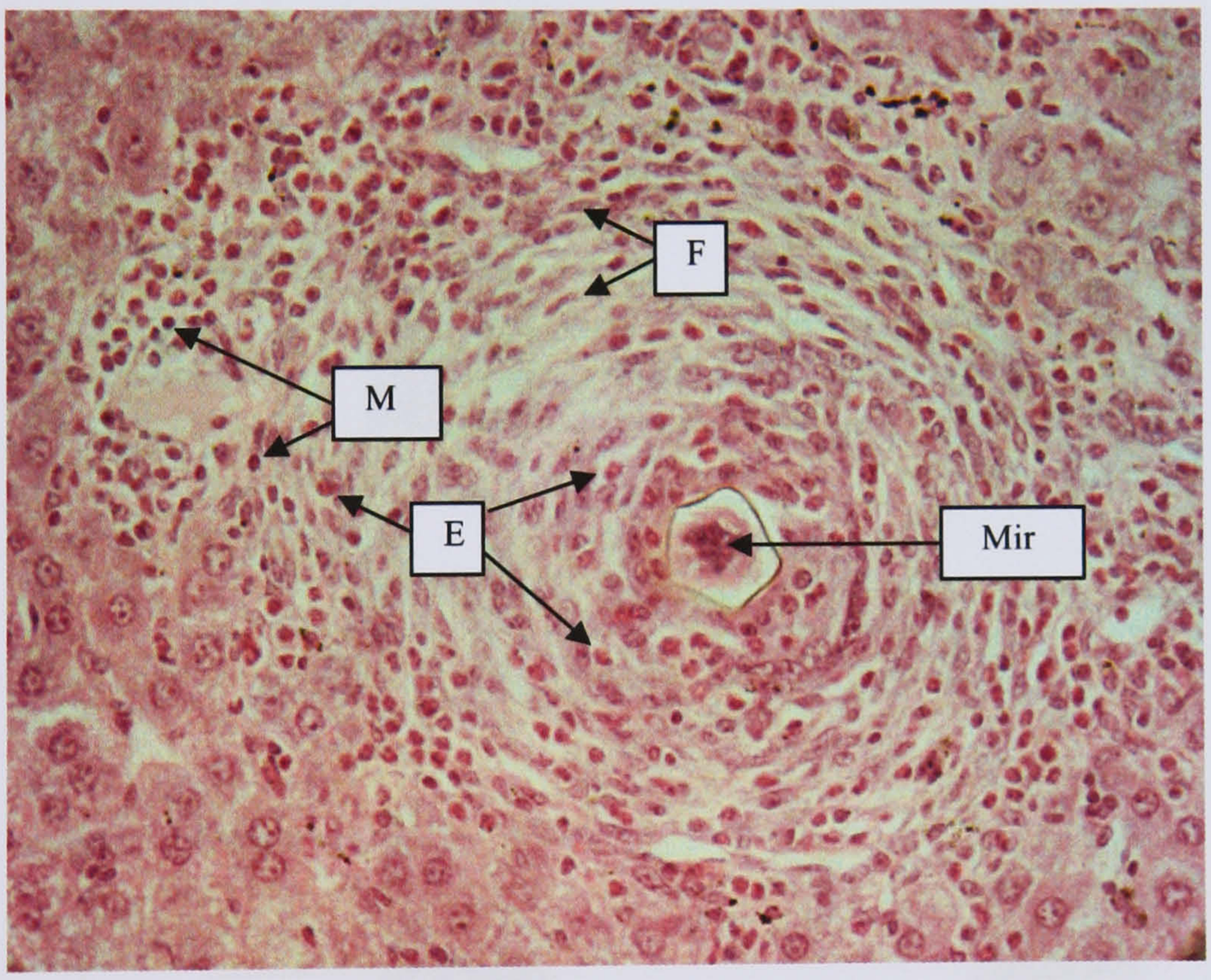


Figure 5.3. Section of liver tissue from a SCHISTO mouse at +8 weeks post *L. donovani* superinfection (+16 weeks post schistosome infection) stained with haematoxylin and eosin (H&E) showing an *S. mansoni* granuloma containing a central egg containing a miracidium (**Mir**) and surrounding granulomatous tissue containing fibroblasts (**F**), eosinophils (**E**) and mononuclear cells (**M**). Magnification x 400

The longest granuloma diameter was recorded together with the diameter at 90° to this. From the mean of these two values the liver volume was calculated and the comparison of the SCHISTO and CO-INFECT data is shown in Figure 5.4. It is clear from both experiments that at +4 weeks the mean egg granuloma volumes in the CO-INFECT mice were higher than in the SCHISTO alone mice, however the difference was not statistically significant. At +8 weeks there was an increase in granuloma size in the SCHISTO mice but this was not statistically significant. As a consequence there was no difference between egg granuloma sizes at +8 weeks in the SCHISTO and CO-INFECT mice.

Figure 5.4. *S. mansoni* egg granuloma size in SCHISTO and CO-INFECT mice

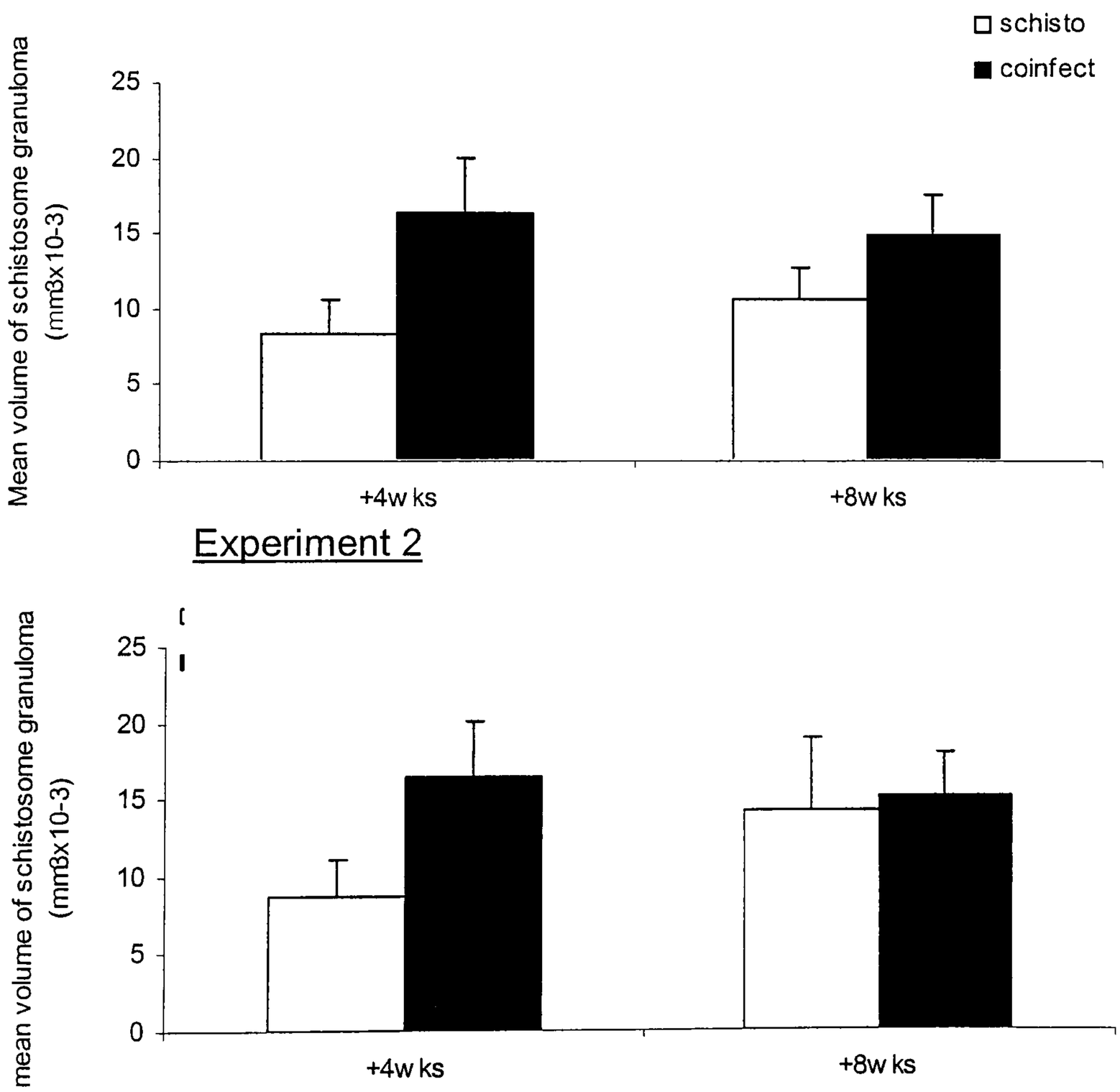


Figure 5.4. Mean and SE of the volume of granulomas from livers of SCHISTO and CO-INFECT animals at +4 and +8 weeks post *L. donovani* superinfection in experiments 1 and 2 (see Chapter 3). Histological sections were stained with (H&E) and transverse and longitudinal diameters were measured from 21 mature granulomas/mouse from 3 mice using standardized micrometry. The volumes of granulomas were calculated from the mean radius (i.e.$4/3\pi R^3$). The mean ±SE are based on 63 observations.

To determine if the effect of the *L. donovani* infection on the schistosome egg granulomas could be related to changes in the cellular composition of the egg granulomas, semi-quantitative estimates were made of the major identifiable cell types represented in the granuloma *i.e.* mononuclear cells, eosinophils, and fibroblasts. As demonstrated in (Figure 5.5. A and B) it is clear that the percentage of these three cell types was very constant with no obvious or significantly significant differences between +4 and +8weeks in either groups nor between the SCHISTO and CO-INFECT groups.

Figure 5.5.A Comparison of cellular composition of *S. mansoni* egg granuloma in SCHISTO and CO-INFECT mice at +4, and +8weeks – *Experiment 1.*

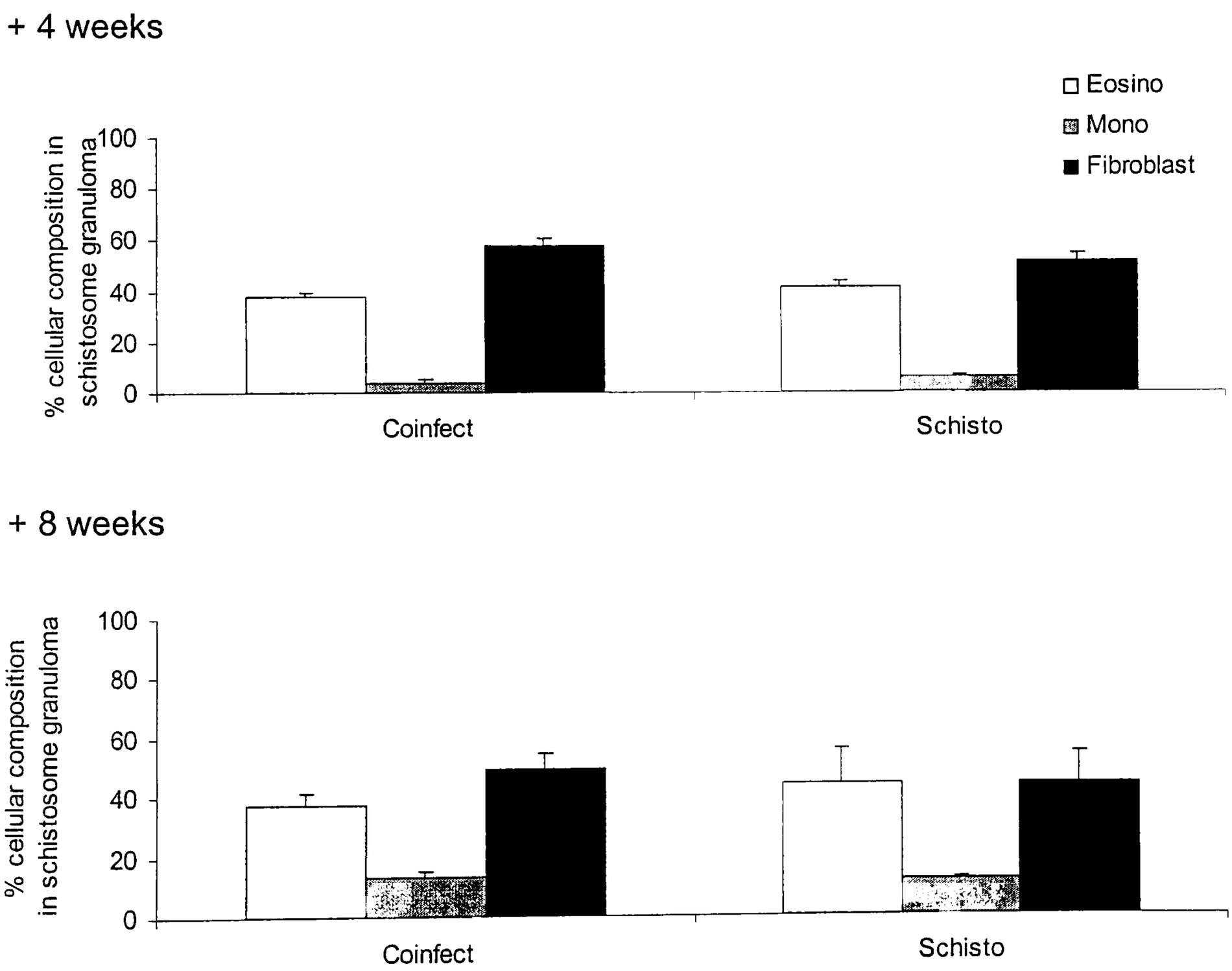


Figure 5.5.A. Experiment 1. Graphs showing the mean (±SE) percentage frequency of eosinophils, mononuclear cells and fibroblasts in the *S. mansoni* egg granulomas analysed in Figure 5.4. (see legend) *i.e.* from SCHISTO and CO-INFECT mice at +4 and +8weeks post superinfection.

Figure 5.5.B. Comparison of cellular composition of *S. mansoni* egg granuloma in SCHISTO and CO-INFECT mice at +4, and +8weeks – *Experiment 2.*

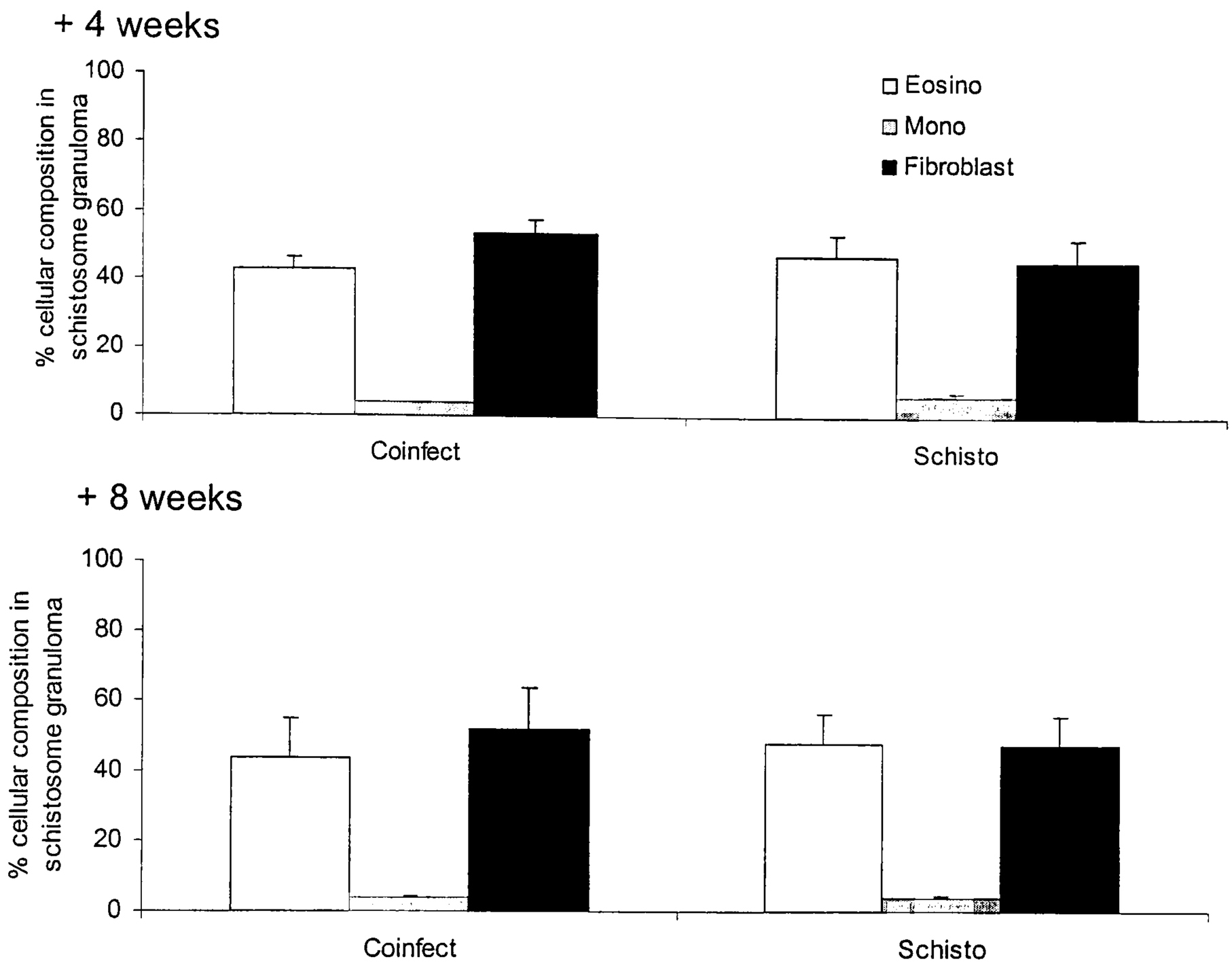


Figure 5.5.B. Experiment 2. Graphs showing the mean (±SE) percentage frequency of eosinophils, mononuclear cells and fibroblasts in the *S. mansoni* egg granulomas analysed in Figure 5.4 (see legend) *i.e.* from SCHISTO and CO-INFECT mice at +4 and +8weeks post superinfection.

5.2.3. Effect of *S. mansoni* infection on *L. donovani* granulomas- IFAT/propidium iodide staining.

Immune killing of *L. donovani* amastigotes in mice is due to the development of small inflammatory granulomas formed of cellular reactions around amastigote infected Küpffer cells of comprising CD4+ve T cells lymphocytes and macrophages (Wilson *et al.*, 1996; Bradley and Kirkley, 1977). As the amastigotes multiply within the Küpffer cell (KC) so the granuloma develops through identifiable stages which have been characterized as: Infected Küpffer cell: one or more than one adjacent infected cell with no associated inflammatory cells; Fused Küpffer cell: Fusion of two or more infected KCs without adjacent inflammatory cells; Immature granuloma: Infected KCs incompletely surrounded

with more than 3 and less than 10 cells adjacent cells; Mature granuloma: Infected KCs completely surrounded with more than 10 cells; Sterile granuloma: Collection of inflammatory cells with no amastigotes, in which the amastigotes may have been destroyed by immune components. A couple of typical *L donovani* mature granulomas are shown in Figure 5. 6.

Figure 5.6 Typical mature *L. donovani* granulomas in LEISH mice

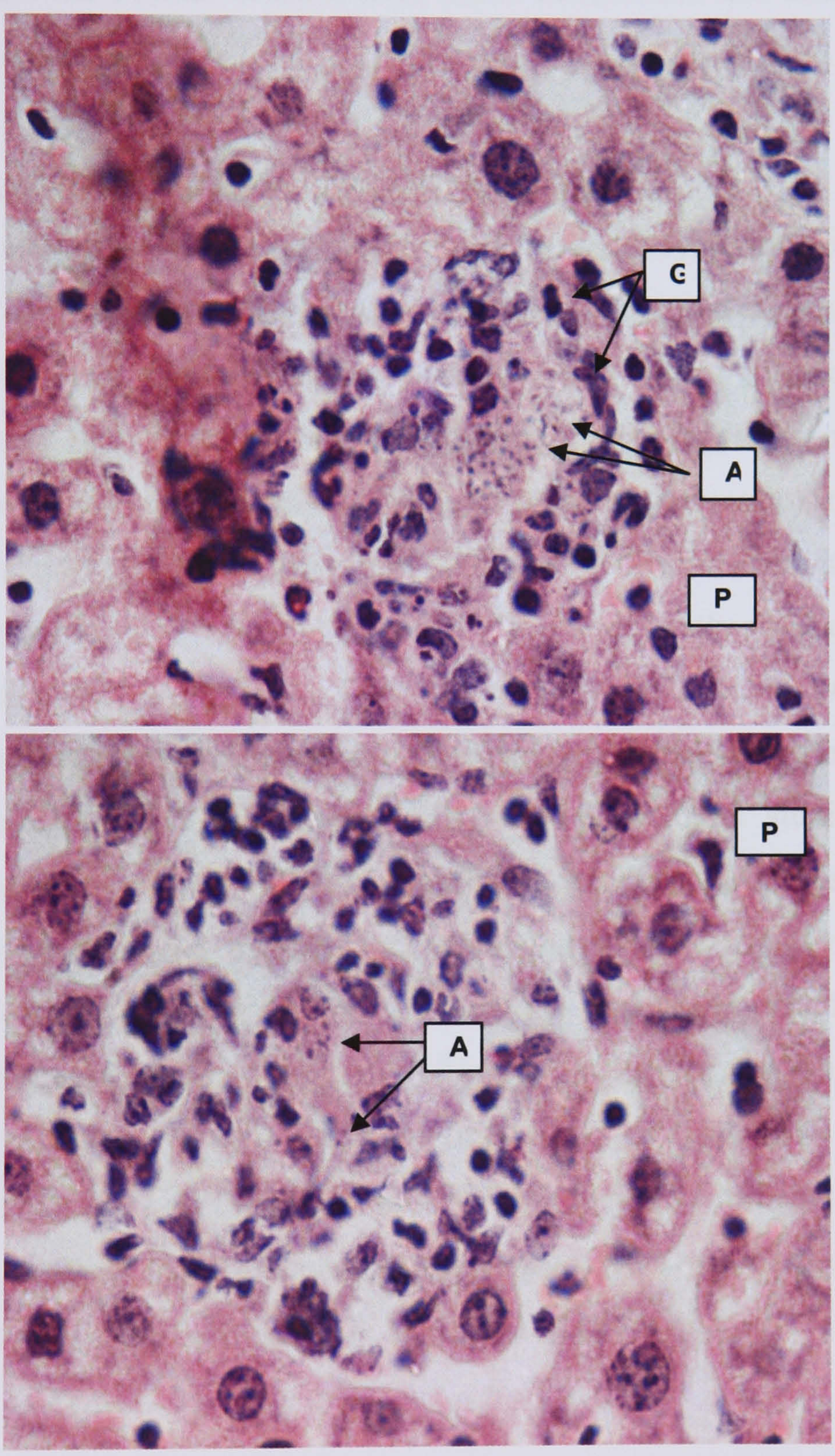


Figure 5.6 Photomicrographs of liver sections from a LEISH mouse at +8 weeks post-infection stained with H&E showing a mature granuloma containing many amastigotes (**A**) (TOP) and one in which most of the amastigotes have been killed (BOTTOM). Sections were cut at 5 μM. **G** = *L. donovani* granuloma cells. **P** = liver parenchymal cells

The production of TNF-α (Engwerda *et al.*, 1998) and NO (Roach *et al.*, 1993) within the granulomas has been shown to increase as the granuloma matures and is believed to be responsible for parasite destruction. The failure of *S. mansoni* infected mice to control the *L. donovani* infection at 8 weeks post infection suggests that there may be a failure in development of the *L. donovani* granulomas. In order to investigate this, studies were planned to compare the cellular composition, tissue distribution and amastigote content of granulomas in liver tissue.

5.2.3.1. Complications caused by haematin pigment.

Histopathology using paraffin sections stained with haemotoxylin and eosin was tried first. In the first series of sections prepared from Experiment 1 (Chapter 3) it was considered that this histological approach would have limitations, in particular for looking at numbers of amastigotes, because it was clear from the CO-INFECT histology that many of the KCs in the *S. mansoni* infected mice contained *S. mansoni* pigment (haematin) including the KC in *L. donovani* granulomas (see Figure 5.7. Top). This meant that it was difficult to distinguish the amastigotes from the pigment. It was subsequently realized that the staining of these initial sections was not optimal for identification of *L. donovani* amastigotes and that this could be improved by increasing the blueing step for the haemotoxylin staining (see for example Figure 5.7. bottom) and this was used in later studies (section 5.2.4). Another complication for the CO-INFECT mice was that in the *S. mansoni* infected mice, in addition to the circumoval granulomas, inflammatory foci also formed around KCs containing schistosome pigment in the parenchyma and these reactions resembled *L. donovani* granuloma (see Figure 5.8. Top). So in CO-INFECT mice if such reactions were seen to contain amastigotes they could be defined as *L. donovani* granulomas but if amastigotes could not be seen it was not certain if the reactions were just pigment reactions, sterile (resolved) *L. donovani* granulomas (as in Figure 5.8. bottom) or mature granulomas in which it was not possible to distinguish amastigotes from pigment.

Figure 5.7. Mature *L. donovani* granulomas in a CO-INFECT mouse stained with different protocols of haematoxyolin and eosin.

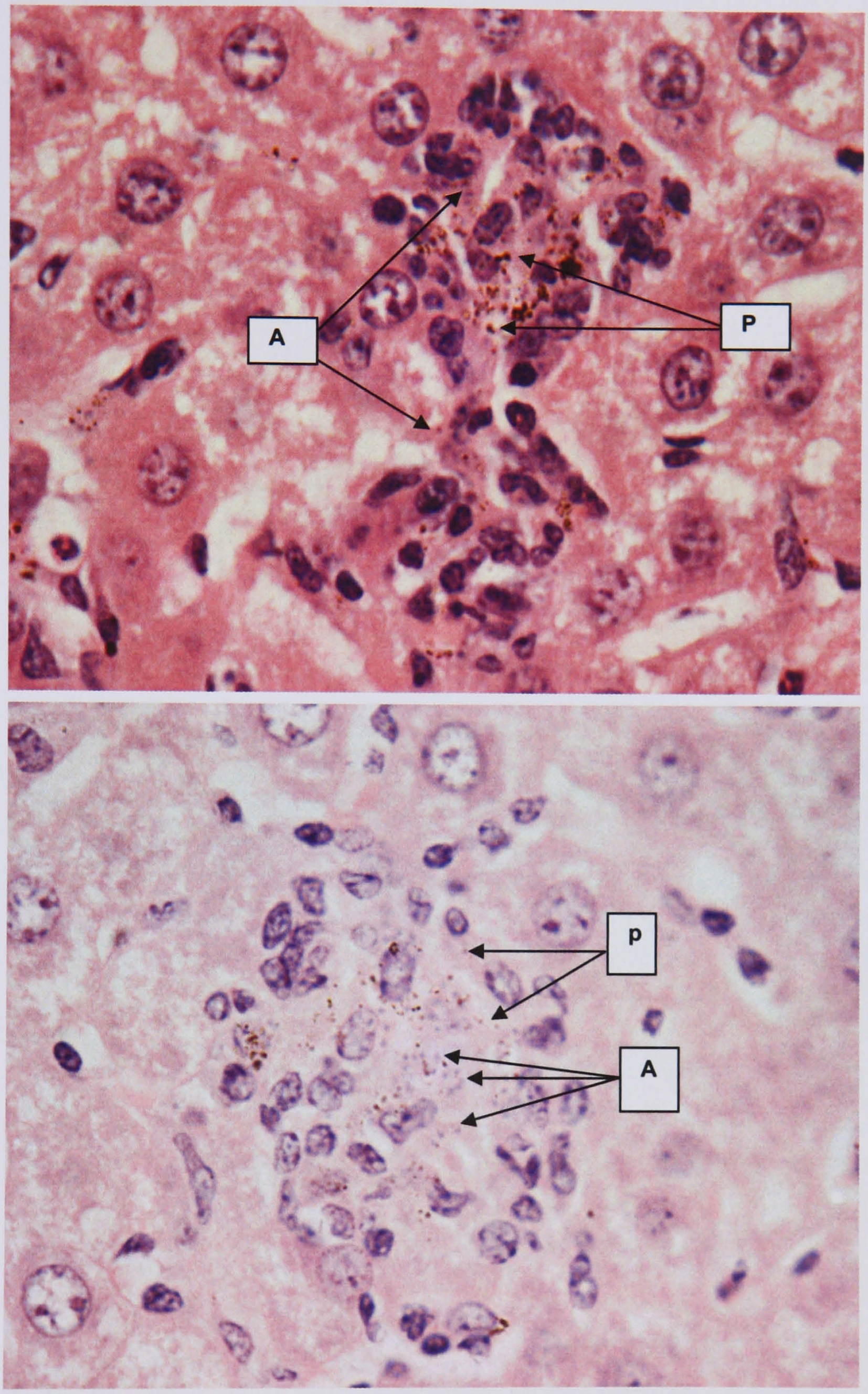


Figure 5.7. Photomicrographs of sections of liver tissue from a CO-INFECT mouse at +8 weeks post infection showing (Top) the initial H&E staining in which distinguishing amastigotes and schistosome pigment was difficult and (Bottom) modification of the staining (increasing blueing step with haemotoxylin) to improve detection of *L. donovani* amastigotes (**A**).

Figure 5.8. Comparison of an inflammatory focus containing haematin pigment in a SCHISTO mouse and a sterile granuloma in a LEISH mouse.

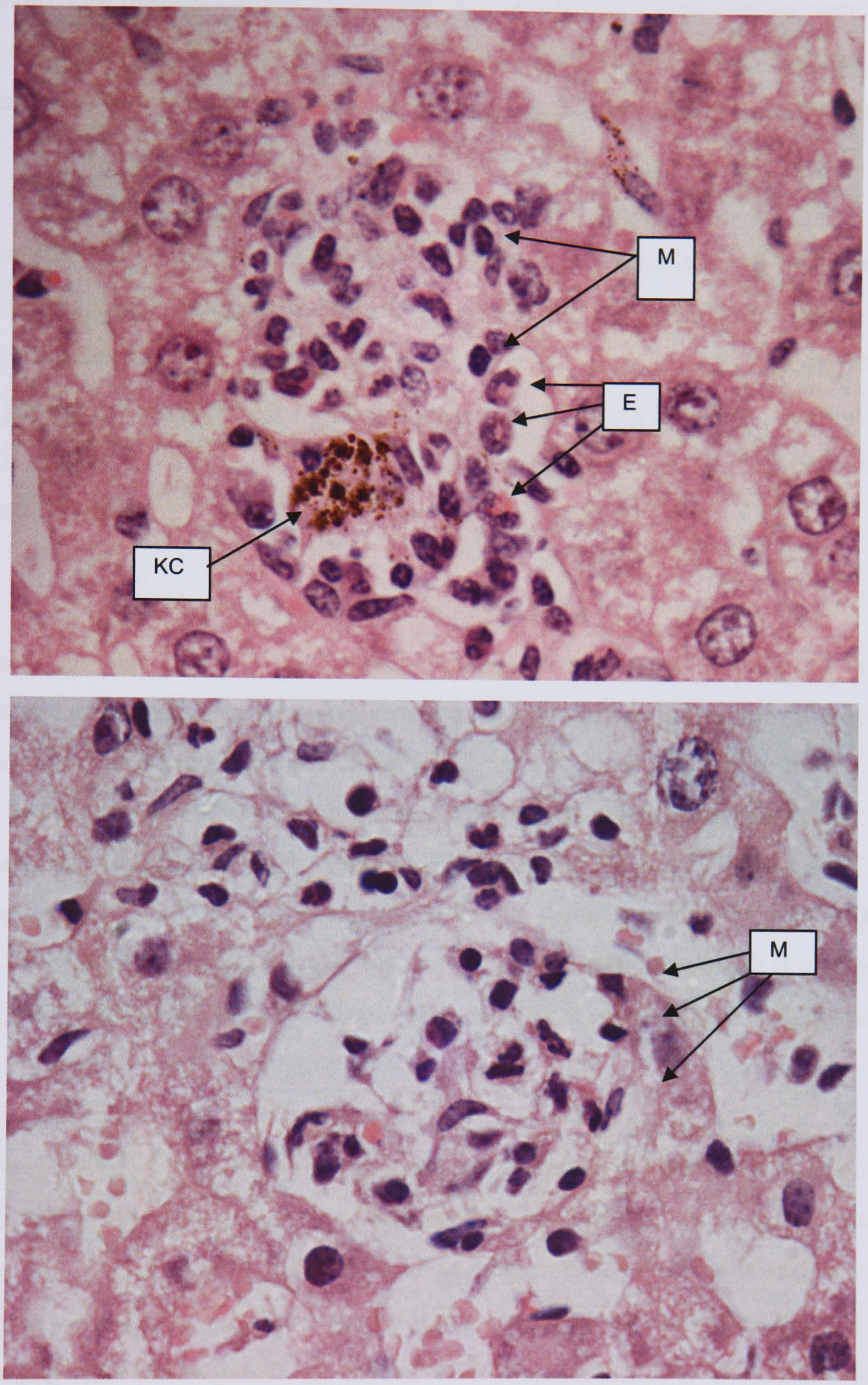


Figure 5.8.. Photomicrograph of sections of liver tissue from (TOP) a SCHISTO mouse at +8 weeks post infection showing an inflammatory focus around KCs containing *S. mansoni* pigment and resembling a *L. donovani* granuloma. (BOTTOM) a sterile (resolved) granuloma from a LEISH mouse at +8 weeks. **KC**= haematin filled Kupffer cells; **E** = eosinophils; **M**= mononuclear cells.

To try to overcome these problems caused by the haematin pigment studies were carried out to try to immuno-label the amastigotes inside granulomas using hamster anti-*L. donovani* (anti-Ld) immune serum followed by a range of labeled anti-hamster antibodies.

5.2.3.2. Immunohistochemistry with chromogenic substrates

Initially attempts were made to use immuno-histochemical methods using enzyme linked antibodies and a range of different colour chromogenic substrates [*e.g.* Vector V.I.P. peroxidase substrate kit (gives pink color), peroxidase substrate kit AEC SK-4200 (gives red color), peroxidase substrate kit DAB SK (gives brown color). However, even with blue substrates it was not possible to get a clear distinction between labeled parasites and pigment (data not shown).

5.2.3.3. Immunofluorescent staining of amastigotes

A further attempt was made using indirect immuno-fluorescence. In order to visualize amastigotes and surrounding inflammatory cells at the same time propodium iodide which stains nuclei of inflammatory foci red under UV light was tried (see Figure 5.9 A). Using this system it proved possible to identify the amastigotes as sharp florescent green objects and the nuclei of surrounding inflammatory cells were stained red (Figure 5.9 [C]). The staining was specific for *L. donovani* and there was no staining in the livers of SCHISTO alone mice (Figure 5.9 [D]). By focusing up and down with the microscope it was possible to distinguish individual amastigotes pretty well and this seemed to give a fairly accurate estimate of amastigote numbers (Figure 5.9 [C]).

So this method (referred to as IFAT/PI) was used to assess the development of *L. donovani* granulomas. Figure 5.10 shows how the propodium iodide staining was used to characterize granulomas. The white rectangle delineates a mature granuloma (>10 cells and completely surrounding the amastigotes) and the yellow rectangle an immature one (<10 cells and/or not surrounding the amastigotes) in the parenchyma adjacent to a schistosome egg granuloma. Foci of amastigote infection also occurred within the egg granulomas but owing to the presence of propodium iodide staining egg granuloma cell nuclei it was not possible to determine accurately whether discrete *L. donovani* granulomas formed around these foci

(see blue rectangle). Consequently in this analysis scoring of the maturation status of the *L. donovani* granulomas was only made for reactions in the parenchyma of both the CO-INFECT and LEISH animals. However, amastigote counts were made from all reactions including those within the egg granulomas.

Figure 5.9. IFAT staining of *L. donovani* amastigotes and propidium iodide staining of cell nuclei in *L. donovani* granulomas.

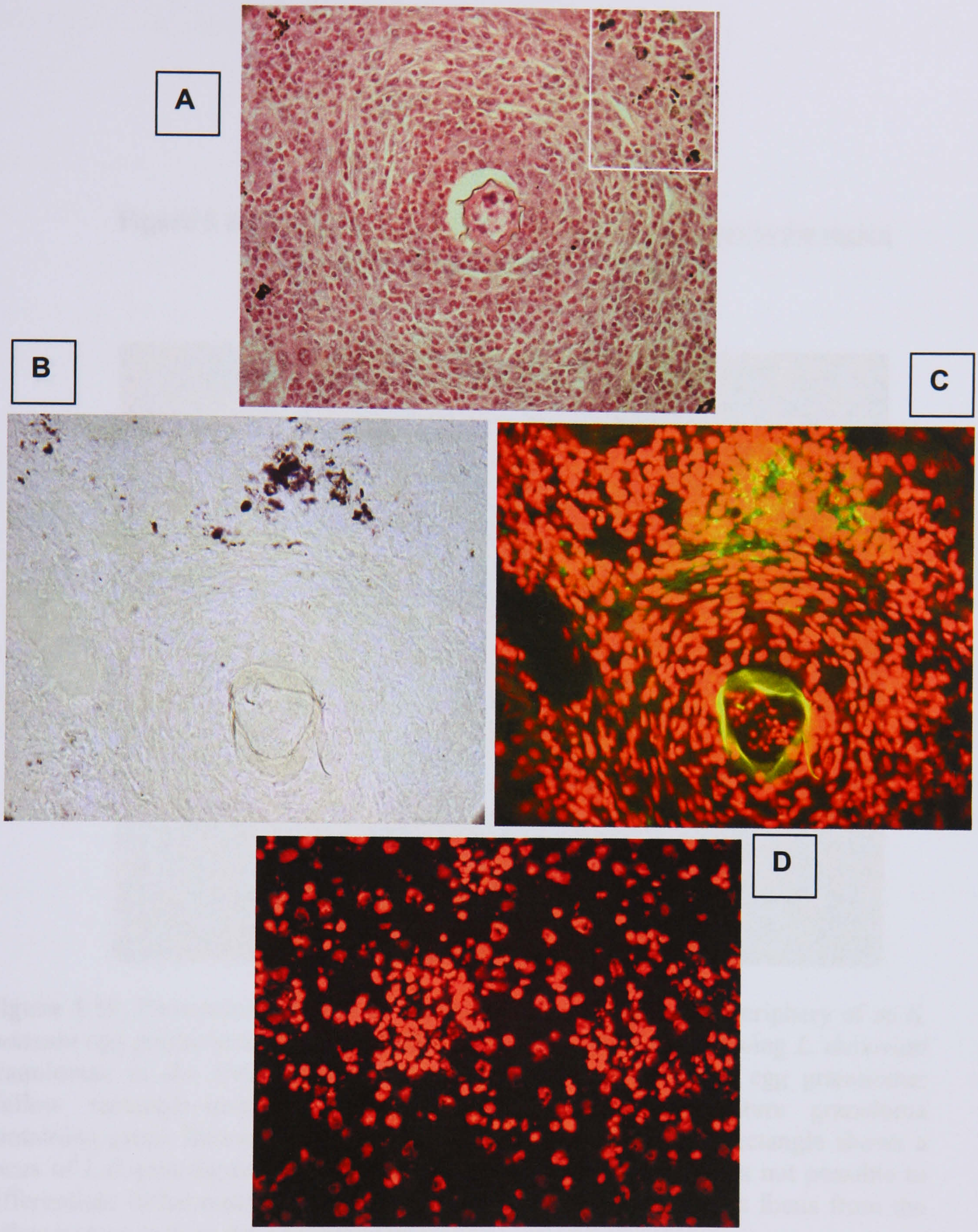


Figure 5.9. Photomicrographs from the liver of a CO-INFECT mouse at + 8 weeks. [A] – paraffin embedded liver section using light microscope at power x40 showing a schistosome granuloma with multiple eccentric macrophages containing haematin pigment (in white rectangle); [B]-cryosection of tissue from the same mouse showing a similar granuloma stained with hamster Anti-LV9 serum (1:100 dilution) followed by FITC labeled Goat anti-hamster antibody (1:250 dilution) and counter stained with propodium iodide– Viewed under tungsten light; [C]– as in [B] but viewed under U.V. light (green staining amastigotes and red staining host cell nuclei can be seen); [D]– cryosection of a granuloma from the liver of a SCHISTO mouse stained as in [B] (there is no green fluorescence).

Figure 5.10. IFAT staining of *L. donovani* granulomas in the region of *S. mansoni* granulomas.

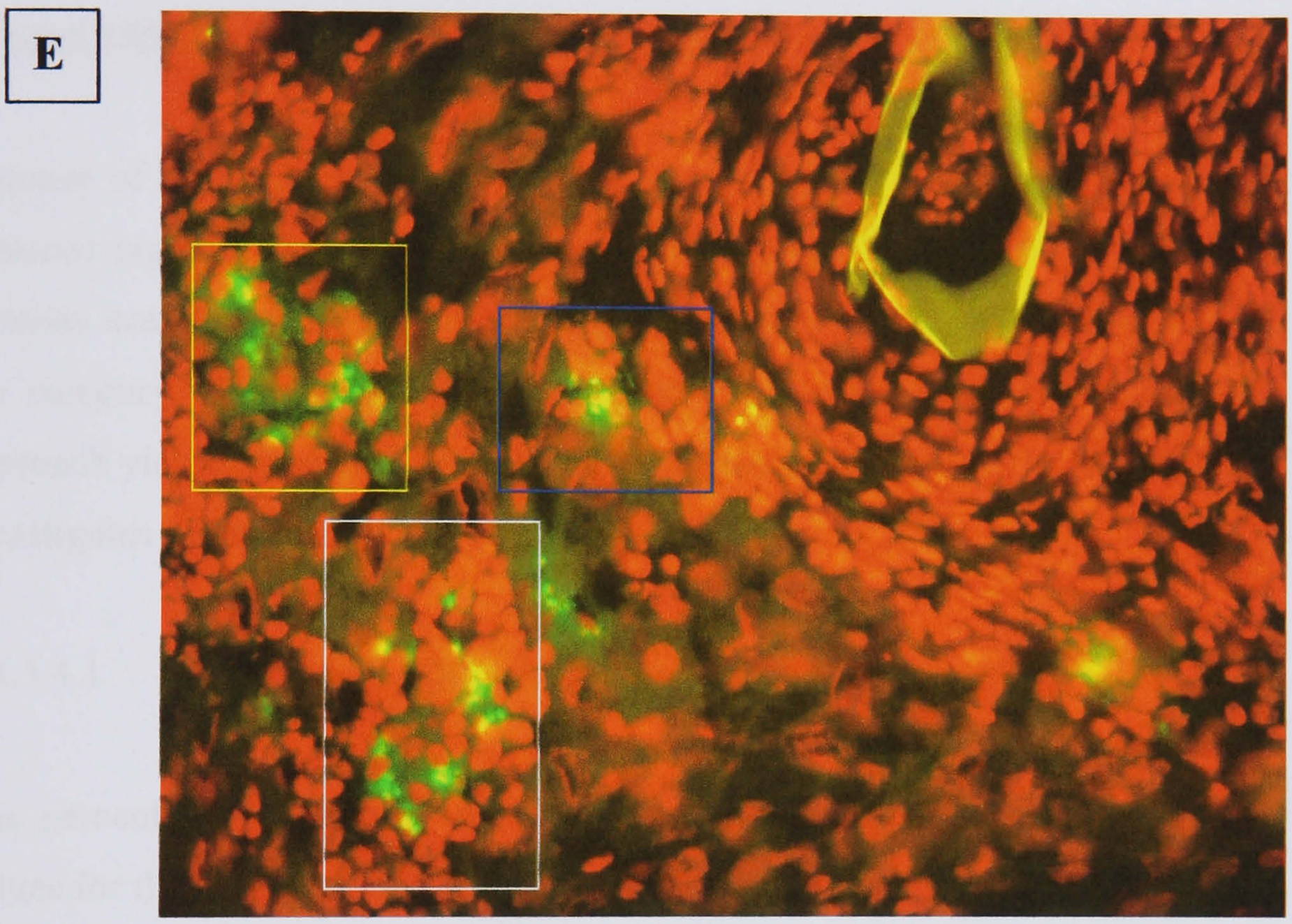


Figure 5.10. Photomicrograph of *L. donovani* granulomas on the periphery of an *S. mansoni* egg granuloma for a CO-INFECT mouse at +4 weeks showing *L. donovani* granulomas in the liver parenchyma adjacent to a schistosome egg granuloma: Yellow rectangle–immature granuloma; white rectangle– mature granuloma containing green fluorescing *L. donovani* amastigotes. The blue rectangle shows a focus of L.d amastigotes at the edge of the egg granuloma and it is not possible to differentiate inflammatory cells which may be associated with this focus from the inflammatory cells in the egg granuloma

5.2.3.4 *L. donovani* granulomatous response and associated amastigote number.- Experiment I

Attention was focused on the +4 and +8 weeks time points which correspond to the times when the CO-INFECT mice showed a loss of control of the *L. donovani* infection. Using the above method, stained sections from 4-5 mice at each time period were assessed using the criteria for categorizing *L. donovani* granulomas described above (section 5.2.3.) *i.e.* infected küpffer cell(s), Immature granuloma, Mature granuloma.

Because of the problem of distinguishing Sterile granulomas of *L. donovani* from the *S. mansoni* pigment reactions this category of granuloma was left out. For each mouse the sections scanned and 30 strictly adjacent microscope fields were carefully examined and the category of granuloma recorded along with the number of amastigotes seen. This approach yielded results on the percentage of each granuloma type and the mean number of amastigotes per reaction.

5.5.3.4.1 *L. donovani* Granulomatous responses -Experiment I

The percentage of reactions of each type were calculated for each mouse and the mean values for the mice are shown in Figure 5.11.

At +4 weeks there was no significant difference between the percentage of each of the different types of reactions between the CO-INFECT and LEISH animals although the CO-INFECT mice did show a higher percentage of Infected KCs than the LEISH animals.

Between +4 and +8weeks the LESIH animals showed a marked increase in the proportion of Mature granulomas (from 40 to 73%, p=0.004). However, the CO-INFECT mice did not show a significant increase (40-48%) of Mature granulomas, the LEISH group showed a significantly higher percentage than the CO-INFECT at +8weeks (73 cf 48%, p=0.025). In parallel with the higher percentage of Mature granulomas there was conversely more

Immature granulomas and significantly more IKCs in the CO-INFECT than the LEISH mice (32 cf 15%, p=0.015).

Figure 5.11. The percentage of different types of *L. donovani* inflammatory foci characterized using IFAT/PI at +4 and +8weeks after super-infection. –Experiment I.

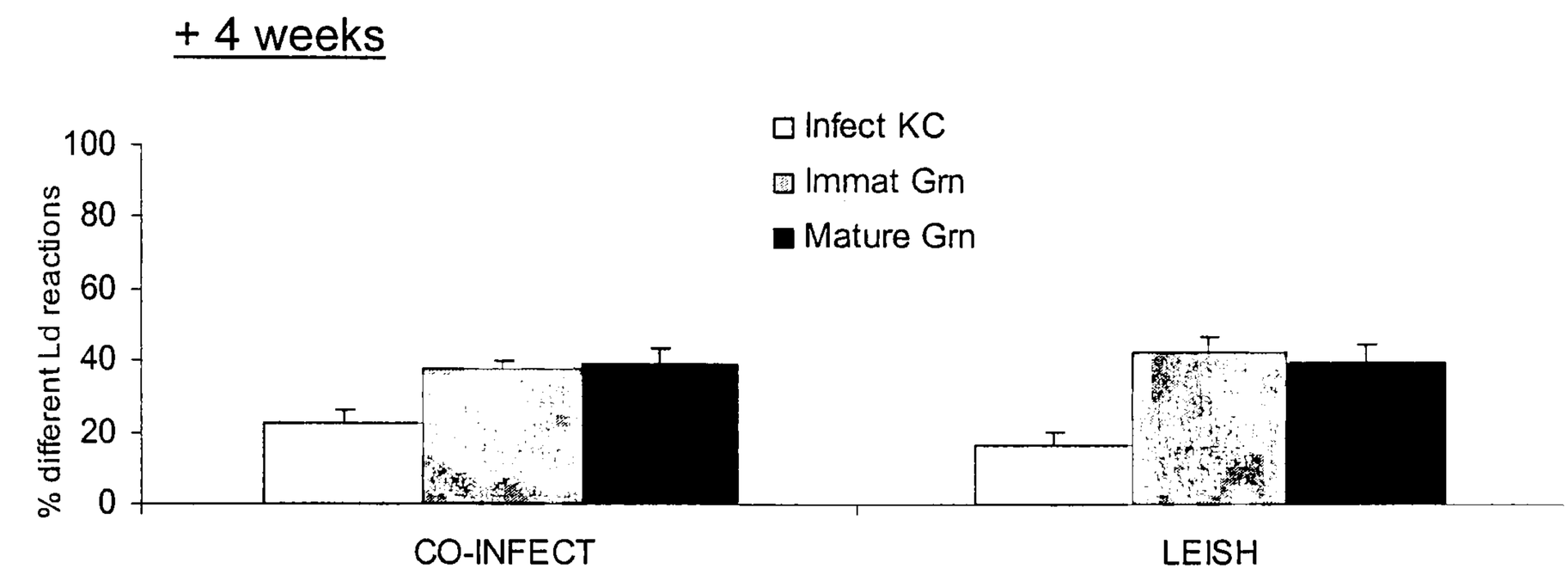


Figure 5.11. Graphs showing the mean (±SE) percentage of Infected Küpffer cells, Immature granulomas, or Mature granulomas in the livers of mice from the CO-INFECT and LEISH groups at +4 and +8 weeks post-infection. Note: Sterile granulomas are not included in this analysis because in the CO-INFECT sections it was not possible to distinguish these from the reactions around *S. mansoni* pigment. Reactions in 30 adjacent microscope fields were counted and the data represents the mean values from 5 mice in each group.

5.2.3.4.2. *L. donovani* amastigote numbers – Experiment I

The mean numbers of amastigotes recorded in each of the different categories of *L. donovani* reactions is shown in Figure 5.12.

Figure 5.12. Mean number of amastigotes in the different types of *L. donovani* granulomas at +4 and +8 weeks – Experiment I

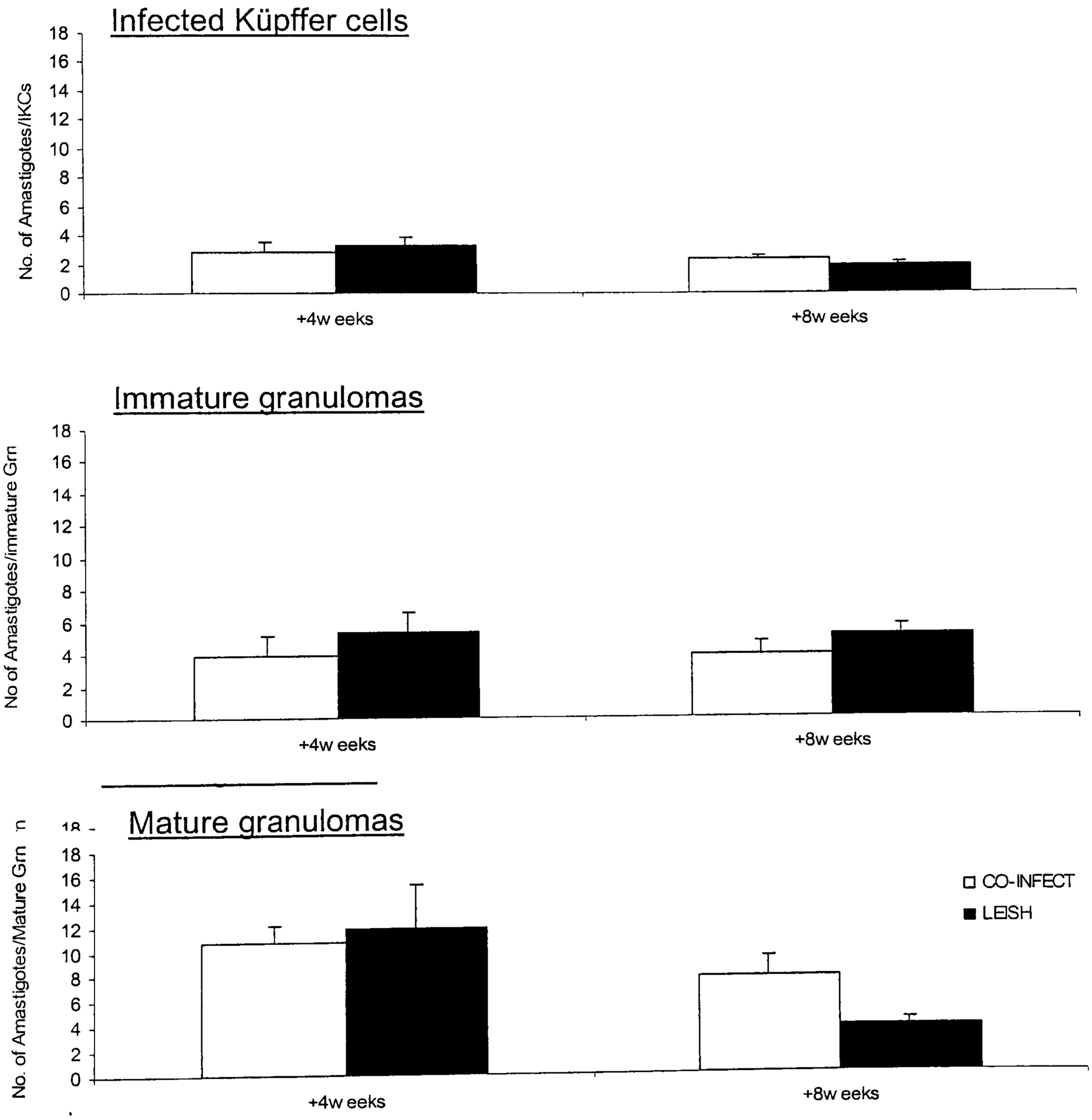


Figure 5.12. Graphs showing the mean (± S.E.) number of amastigotes/Mature granuloma, Immature granuloma or Infected Küpffer cell in 5 mice from the CO-INFECT and LEISH groups at +4 and +8 weeks. Reactions in 30 adjacent microscope fields were counted and the data represents the mean values from 5 mice in each group.

There were low but comparable numbers of amastigotes in the IKCs of both LEISH and CO-INFECT mice at both +4 and +8 weeks.

Similarly, both the LEISH and the CO-INFECT animals showed comparable numbers of amastigotes per Immature granuloma at both +4 and +8weeks.

With the Mature granulomas, the mean number of amastigotes were comparable in both groups at +4 weeks. Between +4 and +8 weeks, there was a marked reduction in the mean number of amastigotes per Mature granuloma in the LEISH group (p=0.05). The reduction in the CO-INFECT group was less. This meant that at +8 weeks the amastigote counts in the CO-INFECT group was significantly higher than in the LEISH group (p=0.048).

5.2.3.5. Use of anti-Ld IFAT and propidium iodide staining to characterize the *L. donovani* granulomatous response and associated amastigote number. – Experiment II

5.2.3.5.1 *L. donovani* Granulomatous responses –Experiment II

The results of the repeat experiment are shown in Figure 5.13. Similar results were obtained to the earlier experiment. There was little difference in the frequency of the different types of *L. donovani* granulomas at +4 weeks. As before there was a significant increase (from 50.7 to 77.6%, p=0.001) in the percentage of Mature granulomas in the LEISH animals between weeks +4 and +8. In contrast there was no significant change in the frequency of Mature granulomas in the CO-INFECT group (54.3 cf 51.5% at +4 and +8 weeks). This meant that the percentage of Mature granulomas in the LEISH mice was significantly higher than in the CO-INFECT mice at +8 weeks (p=0.001).

Figure 5.13. The percentage of different types of *L. donovani* inflammatory foci characterized using IFAT/PI at +4 and +8weeks after super-infection. –Experiment II.

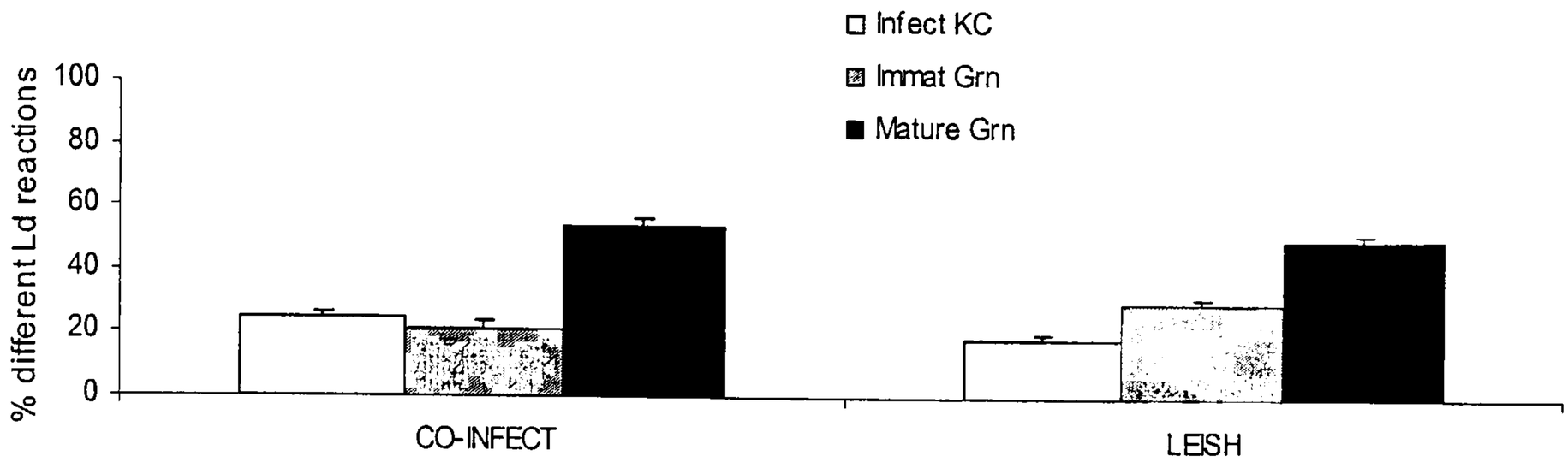
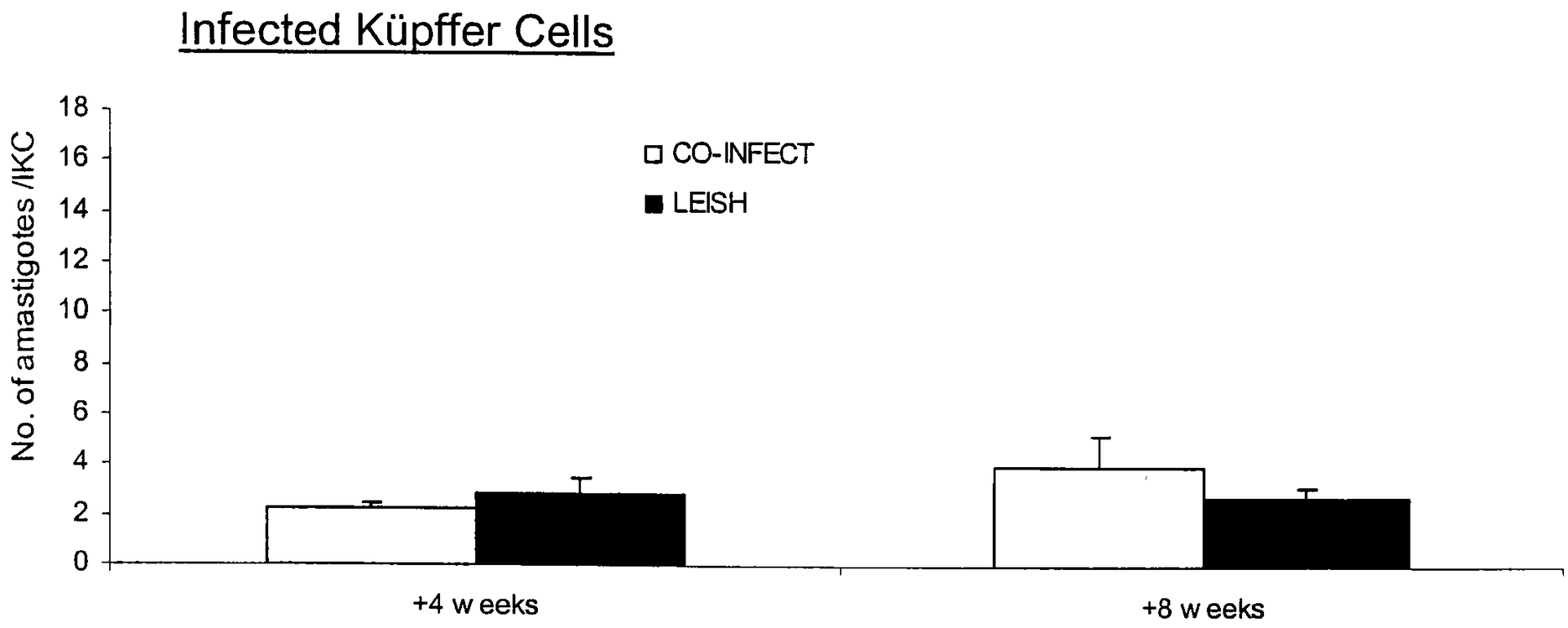
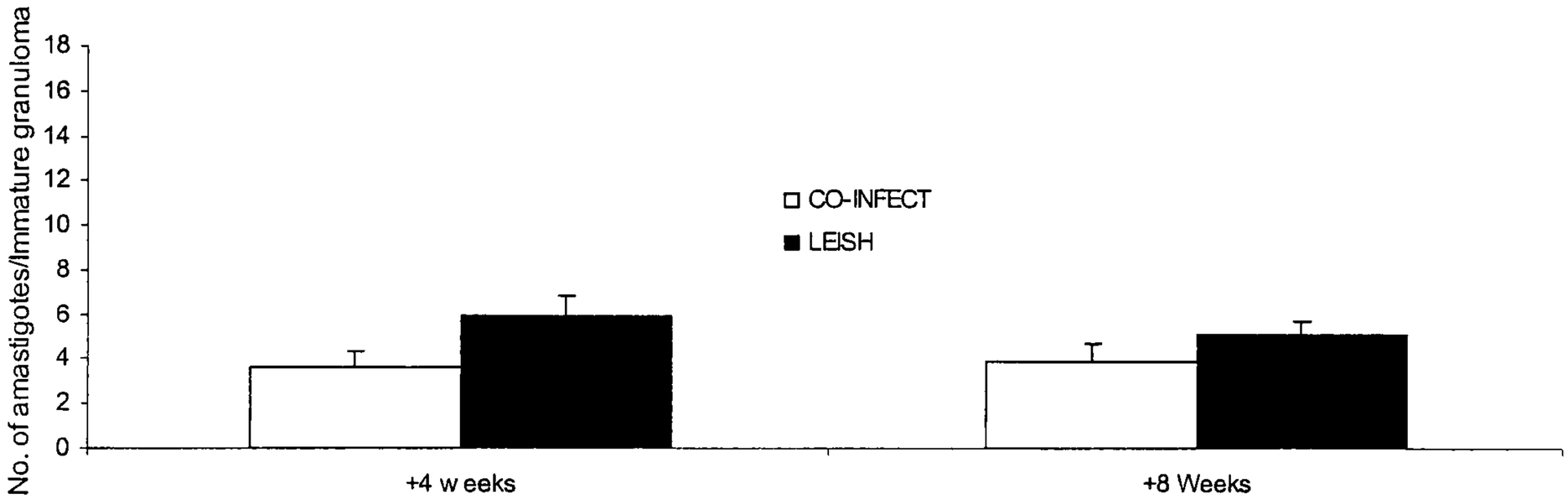


Figure 5.13. Graphs showing the mean (±SE) percentage of Infected Küpffer cells, Immature granulomas, or Mature granulomas in the livers of mice from the CO-INFECT and LEISH groups at +4 and +8 weeks post-infection. Note: Sterile granulomas are not included in this analysis because in the CO-INFECT sections it was not possible to distinguish these from the reactions around *S. mansoni* pigment. Reactions in 30 adjacent microscope fields were counted and the data represents the mean values from 5 mice in each group.

The data was similar to the previous experiment in several aspects (Figure 5.14). Again there was no evidence of significantly greater numbers of amastigotes in the IKCs or immature granulomas of CO-INFECT compared with LEISH mice at either +4 or +8 weeks. The results are different from Experiment I because the amastigote counts were not reduced at +8 weeks compared to +4 weeks but were comparable in the LEISH animals

Figure 5.14. Mean number of amastigotes in the different types of *L. donovani* granulomas at +4 and +8 weeks – Experiment II





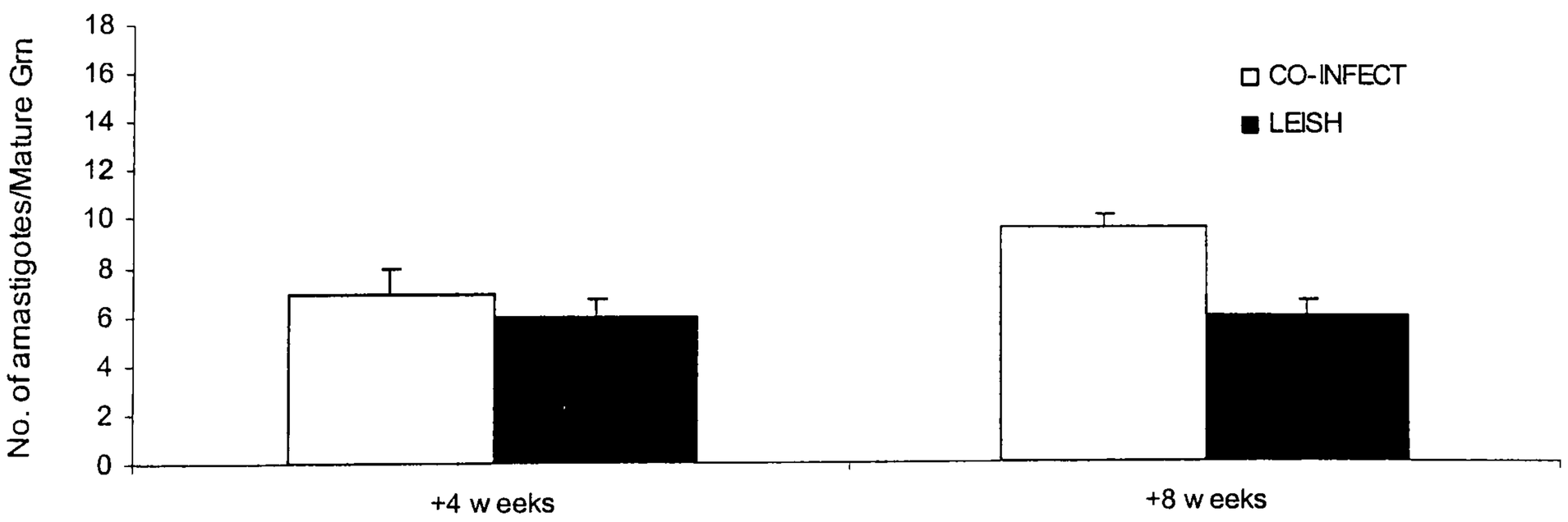


Figure 5.14.: Graphs showing the mean (± S.E.) no.of amastigotes/Mature granulomas, Immature granulomas or Infected Küpffer cell(s) in 5 mice from the CO-INFECT and LEISH groups at +4 and +8 weeks. Reactions in 30 adjacent microscope fields were counted and the data represents the mean values from 5 mice in each group.

and significantly increased in the CO-INFECT group (p=0.04). However, like Experiment I the amastigote count at +8 weeks was significantly higher in the CO-INFECT group than the LEISH group (p=0.002).

This IFAT/PI method proved to be valuable in allowing investigation of the numbers of amastigotes and the frequency of the different types of granulomas. It was introduced as the original haematoxylin and eosin staining of sections from experiments I and II had been too red and did not allow the discrimination of *L. donovani* amastigotes from *S. mansoni* pigment both of which were found inside kupffer cells of CO-INFECT mice. However, during the sectioning of a different experiment the sections produced by the LSHTM histology unit were much bluer and the morphology of the granulomas was much clearer as shown in Figure 5.7. It was realized that the H&E method was superior to the IFAT/PI method for morphological characterization of the *L. donovani* granulomas and also for the accurate quantitation of the amastigote numbers and so was used to confirm and extend the observations made using IFAT/PI. The analysis was extended to include the +2 week time points.

5.2.4. Effect of *S. mansoni* infection on the *L. donovani* granulomas – Haemotoxylin and eosin staining.

So essentially this was a repeat of the above method but using H and E staining which allowed greater detail of the granulomatous response to be analyzed. As for the IFAT/PI staining, 30 adjacent fields were counted and, unlike in the IFAT/PI analysis, the distribution of the *L. donovani* granulomas in the liver parenchyma and within different regions of the *S. mansoni* granulomas themselves was recorded. It was hoped that the data would allow (i) a detailed characterization of the morphology of the foci in CO-INFECT compared to LEISH mice and quantification of (ii) the differences in density of the *L. donovani* foci between LEISH and CO-INFECT mice and (iii) the differences in density between the parenchyma and *S. mansoni* egg granulomas of the CO-INFCT mice, (iv) the progression of these to maturity (perhaps a measure of the degree of Ld specific immune

responsiveness, (v) the amastigote numbers per focus (perhaps a measure of the degree of immunological control).

5.2.4.1. (i) Qualitative observations on the morphology of *L. donovani* foci

Histological sections were cut from mice at the different time points for all co-infection experiments in Chapters 3 and 6. For the qualitative analysis described below attention was focused on the +8 week post *L. donovani* infection time point when the major differences between the LEISH and CO-INFECT LDUs were seen.

As shown in Figure 5.15 reactions were seen in both the liver parenchyma and within the *S. mansoni* egg granulomas. *L. donovani* granulomas in the parenchyma can be clearly distinguished from the surrounding parenchyma. In contrast, for the majority of reactions, L. donovani infected cells present within the egg granulomas (arrowed below) were not surrounded with discrete inflammatory foci which could be distinguished from inflammatory cells of the egg granuloma itself.

Figure 5.15. Low power images of liver tissue from CO-INFECT mice at +8 weeks post infection.

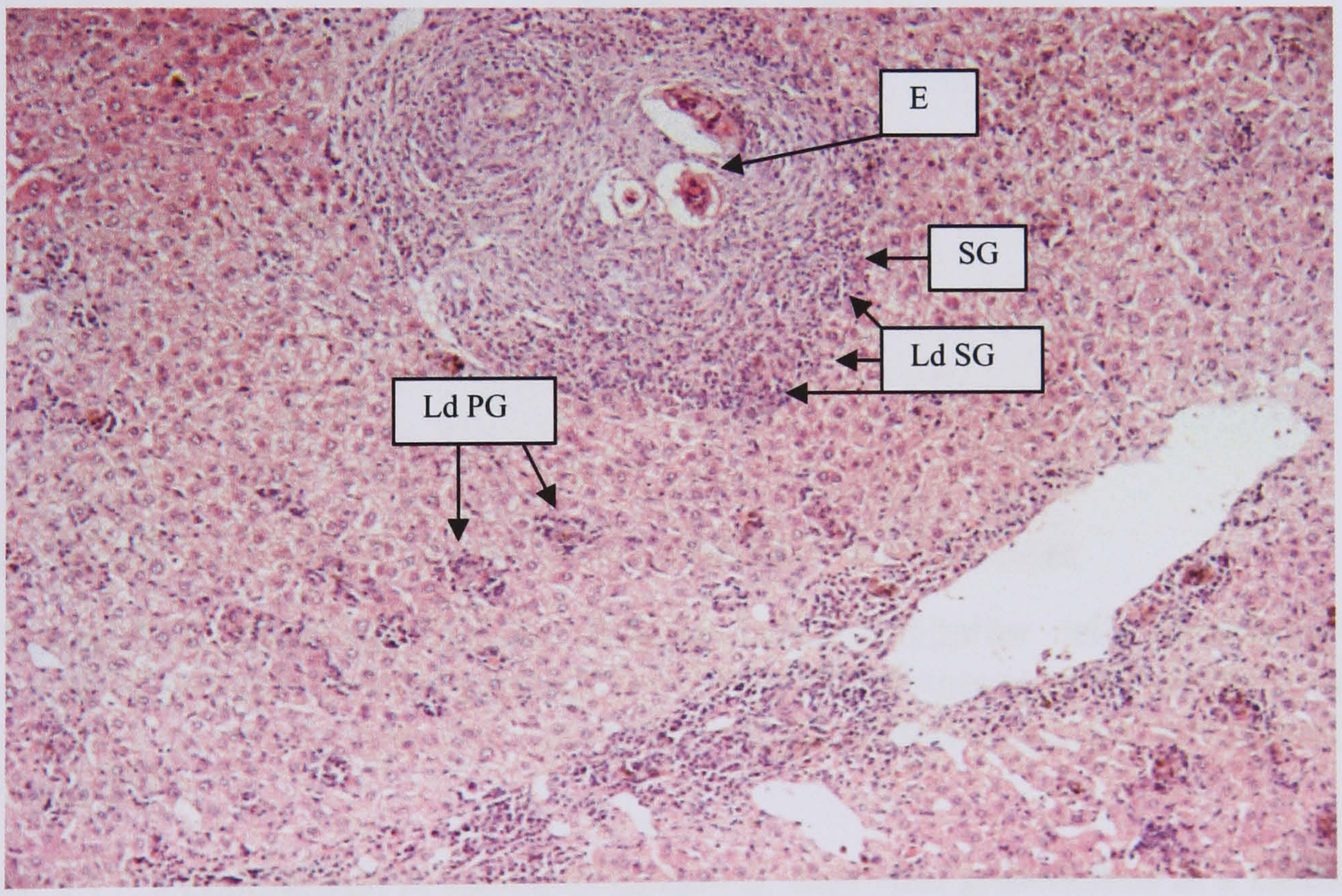


Figure 5.15. Photomicrograph of low power image showing *L. donovani* granulomas in the liver parenchyma and a *S. mansoni* egg granuloma containing numerous *L. donovani* infected cells. This was from a CO-INFECT mouse +8 weeks after co-infection with *L. donovani*. **LdPG** – *L. donovani* parenchymal granuloma, **LdSG** – foci of *L donovani* amastigotes within the *S. mansoni* egg granuloma. **SG** – *S. mansoni* egg granuloma. **E** – *S. mansoni* egg.

The nature of the *L donovani* reactions within the egg granulomas are considered below but first the reactions in the parenchyma are described.

5.2.4.1.1. *L. donovani* granulomas in the parenchyma.

The various categories of *L. donovani* granulomas defined in section 5.2.3 of this Chapter were seen in both LEISH and CO-INFECT sections. Obvious differences were the presence of schistosome pigment and of a proportion of eosinophils in the CO-INFECT *L. donovani* granulomas. In Figure 5.16 schistosome pigment is shown in an infected single Küpffer cell (IKC) containing many amastigotes but lacking any surrounding cellular infiltration.

Figure 5.16: Infected Küpffer cell in a CO-INFECT mouse showing amastigotes together with schistosome pigment.

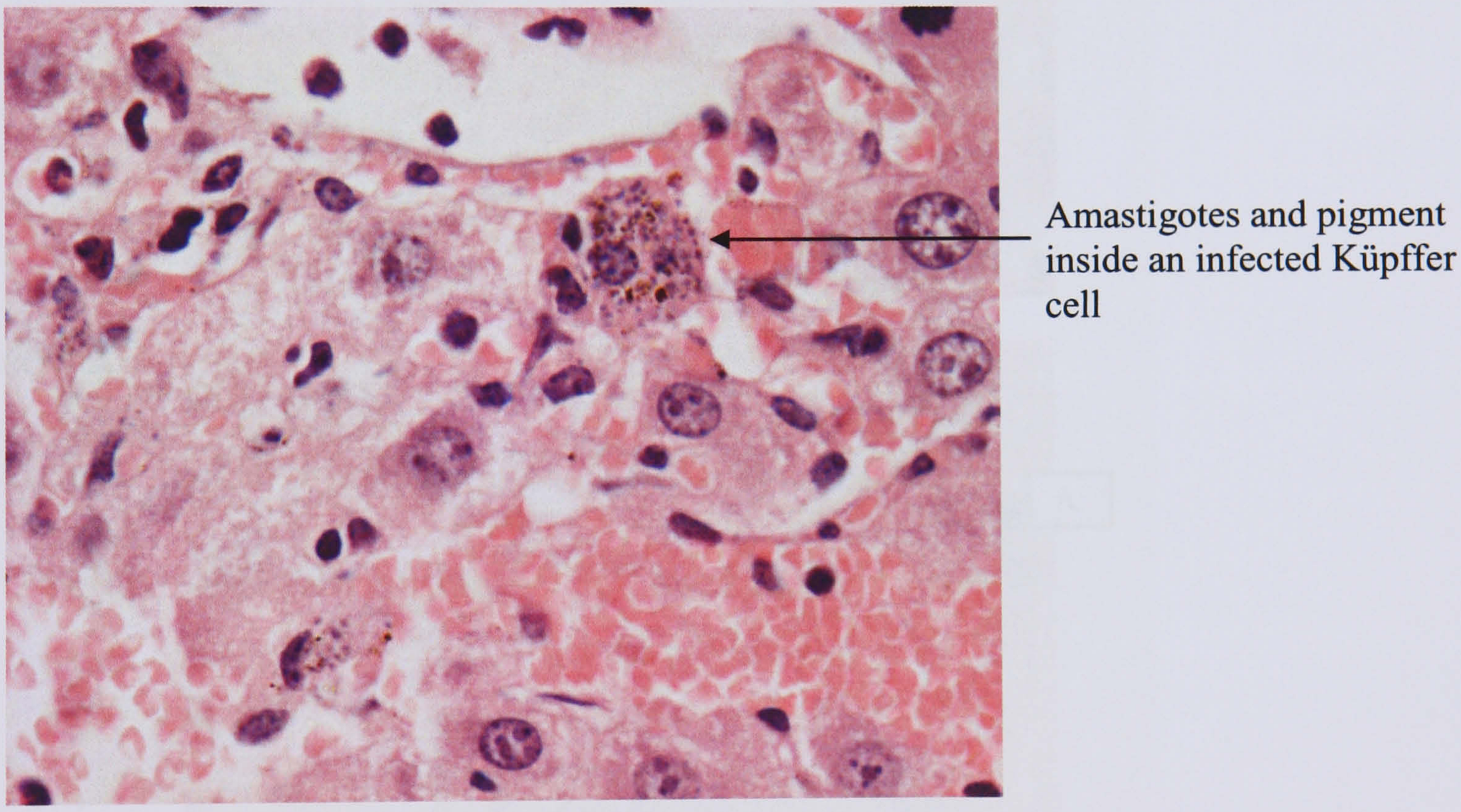


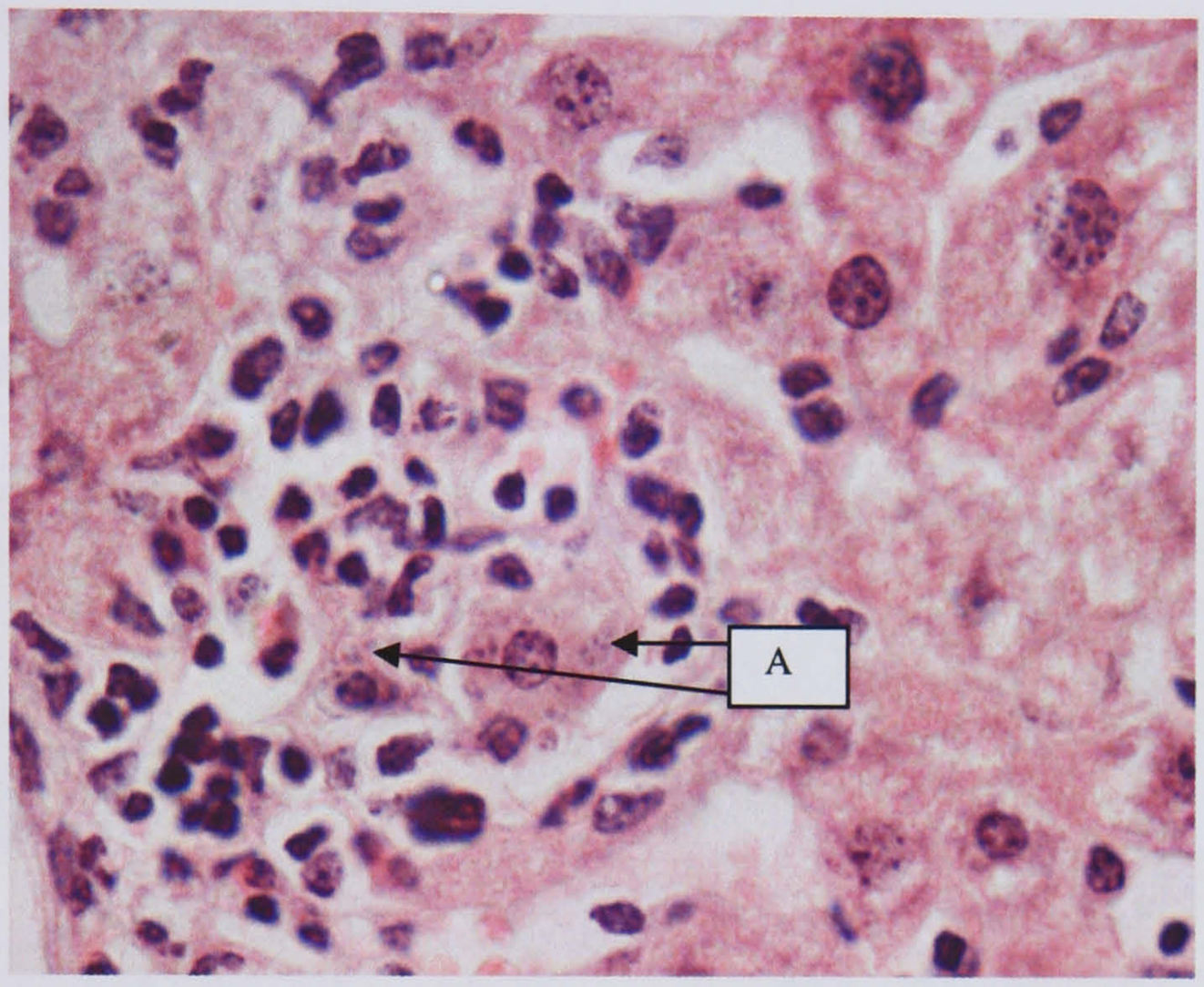
Figure 5.16. Photomicrographs showing infected Küpffer cells containing *L. donovani* and schistosome pigment in a CO-INFECT mouse at +8 weeks post *L. donovani* superinfection. For description of the different groups see legend to Figure 5.2.3.

In Figure 5.17. a comparison is shown of mature *L. donovani* granulomas in a LEISH and a CO-INFECT mouse. Superficially the granulomas appear similar; consisting of a mononuclear cell infiltration. Amastigotes contained in separate cells within the granuloma are indicated along with schistosome pigment and eosinophils in the CO-INFECT mouse.

In the CO-INFECT section groups of more isolated amastigotes outside of the mature granuloma are also arrowed. Such isolated groups outside the main focus were designated as separate foci.

Figure 5.17. Mature granulomas in the parenchyma of LEISH and CO-INFECT mice.

LEISH



CO-INFECT

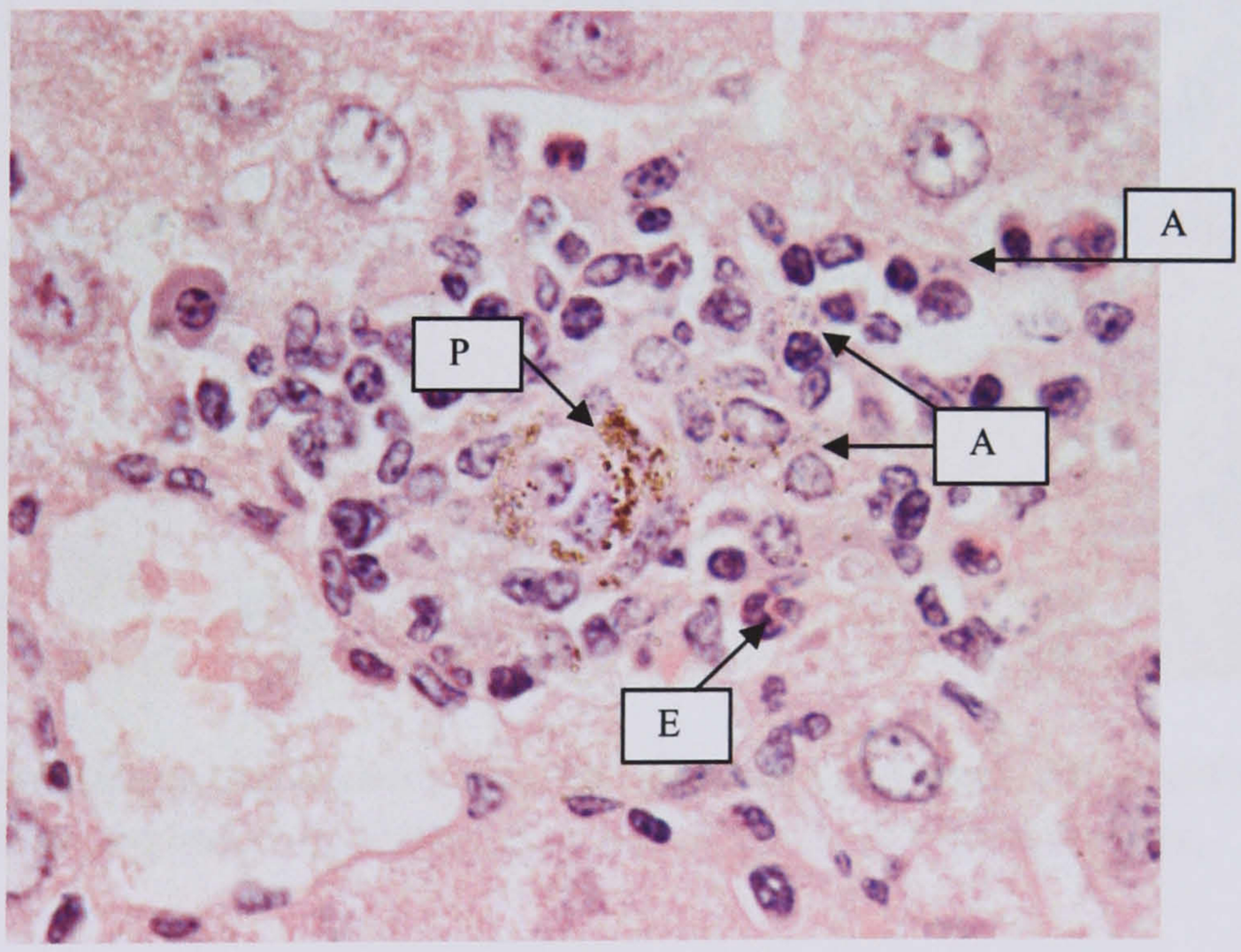


Figure 5.17. Photomicrographs showing mature granulomas from a LEISH and a CO-INFECT mouse at +8 weeks post *L. donovani* superinfection. For description of the different groups see legend to Figure 5.2.3. **P** = schistosome pigment. **E** = eosinophils. **A** = amastigotes, **A*** = groups of amastigotes outside the main focus.

Some *L. donovani* granulomas in CO-INFECT mice showed a large percentage of eosinophils and heavy schistosome pigment as shown in Figure 5.18. The eosinophils in such reactions could be a reaction to the schistosome pigment present as they were seen in the pigment reactions around macrophages containing schistosome pigment in the SCHISTO mice infected with *S. mansoni* alone (Figure 5.8). Such reactions posed some difficulty for interpretation as they appeared to be completely surrounded by cells but the cells concerned were mainly eosinophils rather than mononuclear cells.

Figure 5.18: Mature *L. donovani* granulomas in a CO-INFECT mouse showing a large percentage of eosinophils and schistosome pigment.

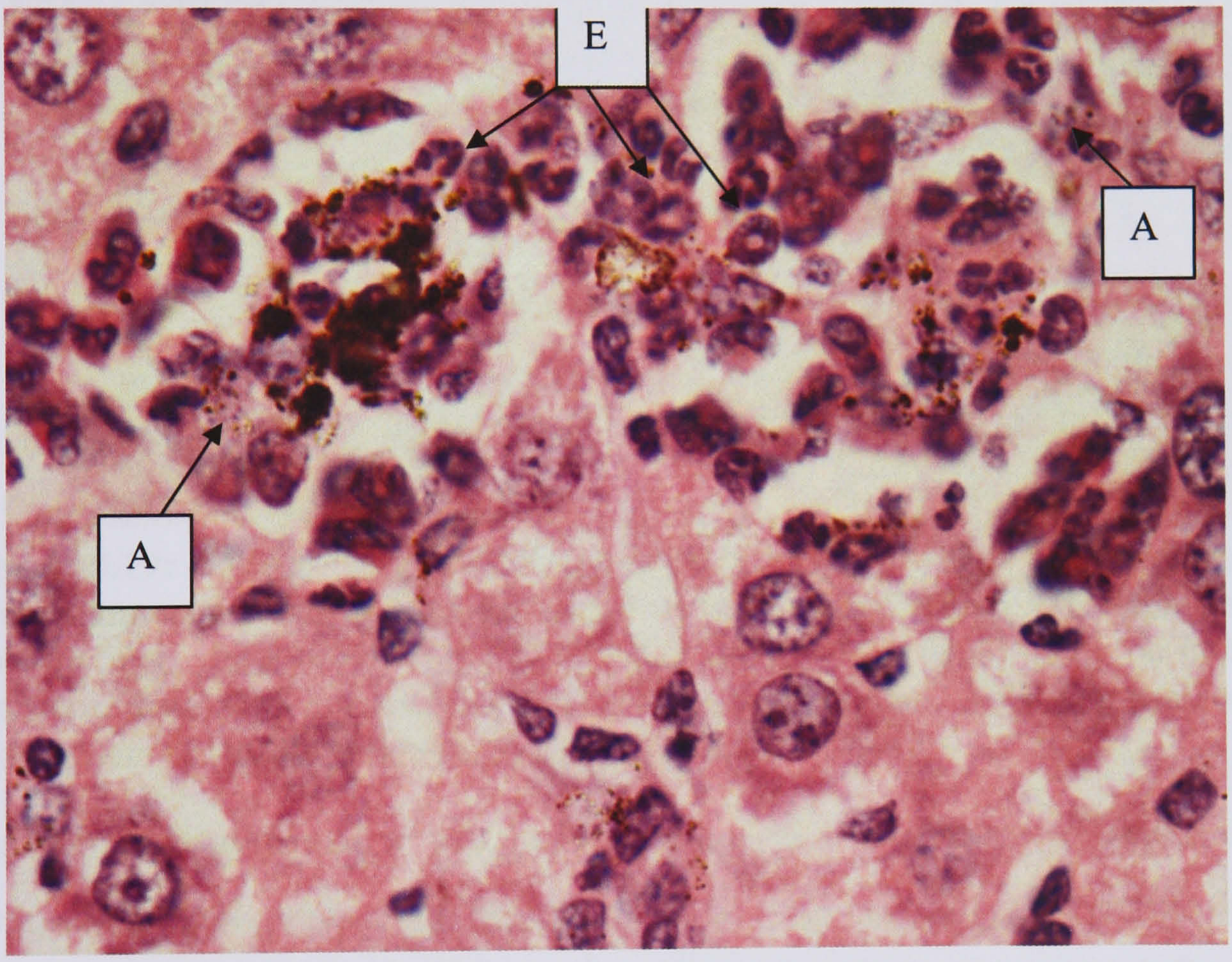


Figure 5.18: Photomicrographs showing mature granulomas from a CO-INFECT mouse at +8 weeks post *L. donovani* superinfection. **E** = eosinophils. **A** = amastigotes.

5.2.4.1.2. *L. donovani* reactions inside the *S. mansoni* granulomas

Foci of *L. donovani* infected cells were commonly seen inside the *S. mansoni* granulomas and could be found throughout the egg granulomas. Figure 5.19. shows a relatively low power image comparing the appearance of leishmanial granulomas in the parenchyma.

Figure 5.19: Comparison of *L. donovani* reactions in the parenchyma and within the schistosome egg granulomas of CO-INECT mice at +8 weeks post *L. dononvani* superinfection

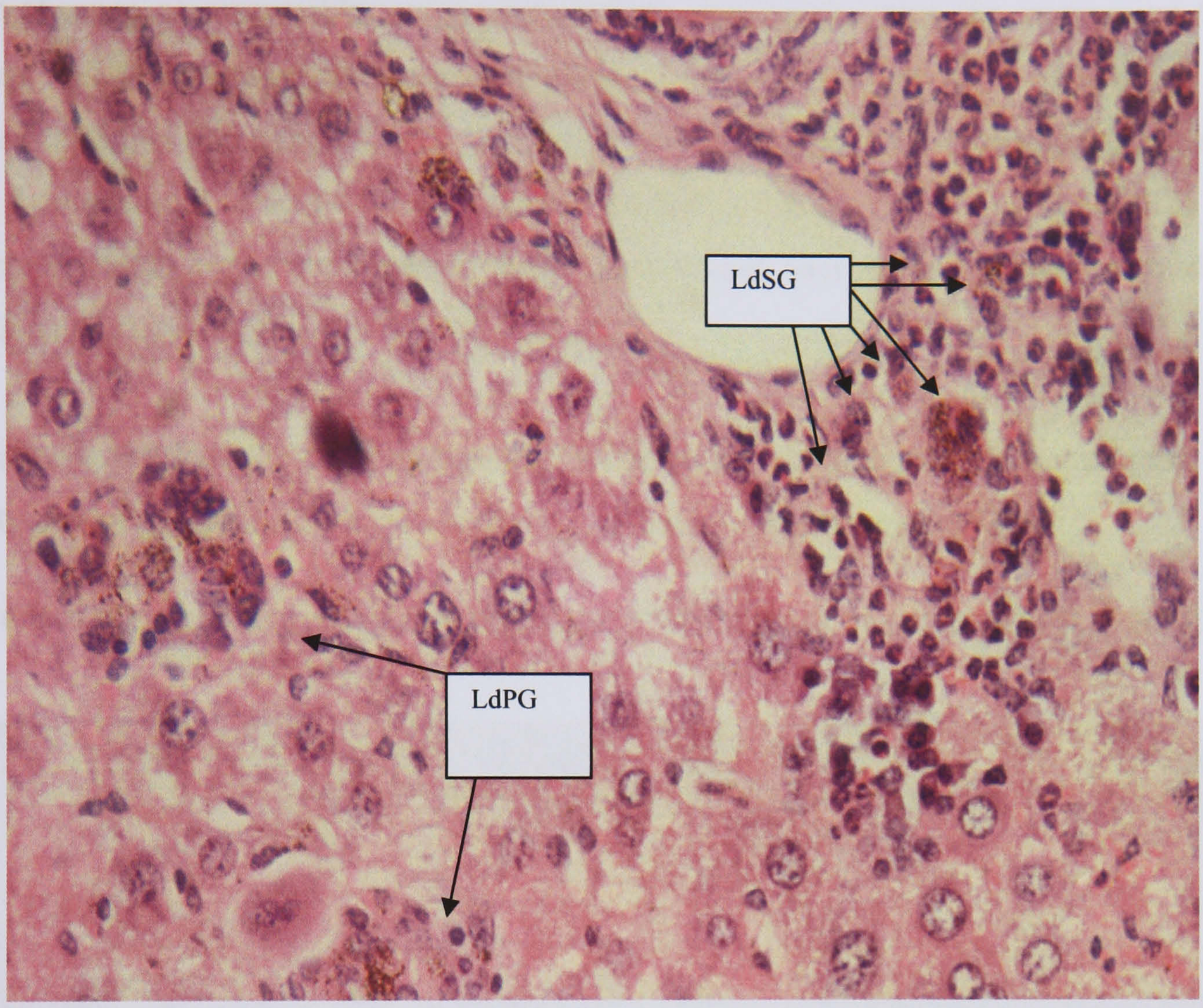


Figure 5.19: Photomicrograph showing mature *L. donovani* granulomas in the liver parenchyma compared with *L. donovani*-infected cells within the cells comprising a *S. mansoni* egg granuloma of a CO-INFECT mouse +8 weeks after co-infection with *L. donovani*. **LdPG** – *L. donovani* parenchymal granuloma, **LdSG** – foci of *L. donovani* amastigotes within the *S. mansoni* egg granuloma.

The cellular organization making up mature granulomas in the parenchyma can be clearly seen. In contrast, within the egg granulomas focal cellular organisation around the *L. donovani*-infected cells could not be distinguished from the other inflammatory cells comprising the egg granuloma which in this instance were around 50% eosinophils. As a consequence it was particularly difficult to characterize a region comprising several distinct amastigote-containing cells as representing one focus or several. This was a particular problem regarding counting the number of amastigotes per focus (see below). Although somewhat subjective it did appear that within the egg granulomas the areas of *L. donovani* infection commonly comprised foci from just one or two to numerous amastigotes. This

can be seen in the lower power image in Figure 5.19 in which 6 apparently discrete foci of *L. donovani* infection are indicated (**LdSG** arrowed) containing from 3 to >15 amastigotes. The absence of discernible surrounding cellular infiltration gave the impression that the focus of *L. donovani* infection was spreading.

Other common features of foci of *L. donovani* infection within schistosome egg granulomas are demonstrated in several other sections shown in Figures 5.15, 5.19, 5.20, 5.21 [top & bottom], 5.22, 5.23 (top) which show *L donovani* foci in association with different regions/cell types within the egg granulomas.

L. donovani infected cells were commonly seen in the periphery of the granulomas. Figure 5.20. shows the boundary between the egg granuloma and the parenchyma.

Figure 5.20 <u>*L. donovani* infected cells are common in the periphery of the *S. mansoni* egg granulomas.</u>

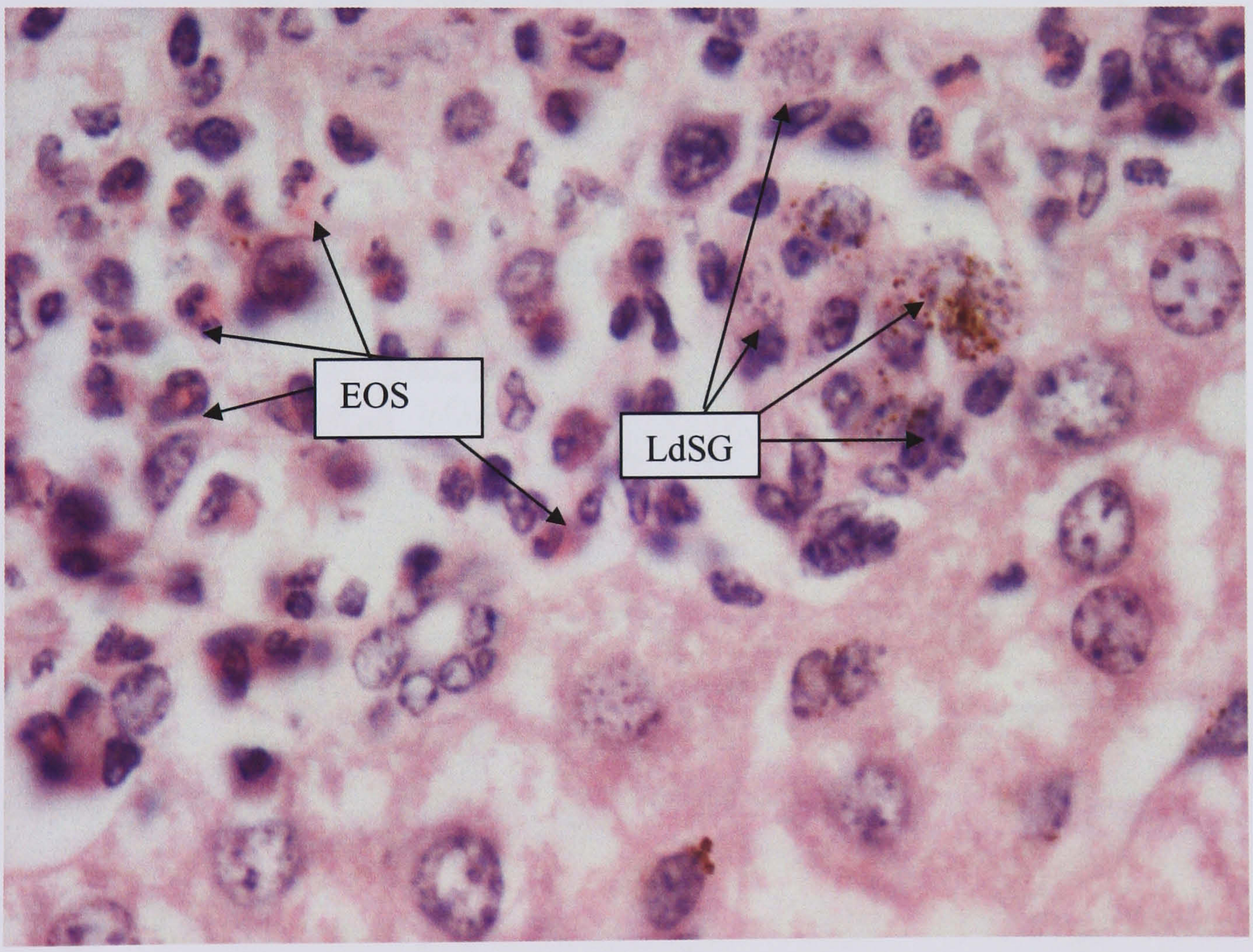


Figure 5.20: Photomicrograph showing several adjacent *L. donovani* infected cells in the periphery of an *S. mansoni* egg granuloma from a CO-INFECT mouse at +8 weeks after superinfection. **EOS** = eosinophils. **LdSG** = *L. donovani* foci within the *S. mansoni* egg granuloma.

Numerous eosinophils can again be seen. Although there seems to be some indication of organization of cells around the area of infected cells in this section this cannot be clearly distinguished as a *L. donovani* granuloma. Foci of *L. donovani* infection were also found deeper within the egg granuloma. Figure 5.21 an shows infected macrophage at the fibroblast/eosinophil boundary.

Figure 5.21. Focus of infection in a fibroblast/eosinophils region of a schistosome egg granuloma.

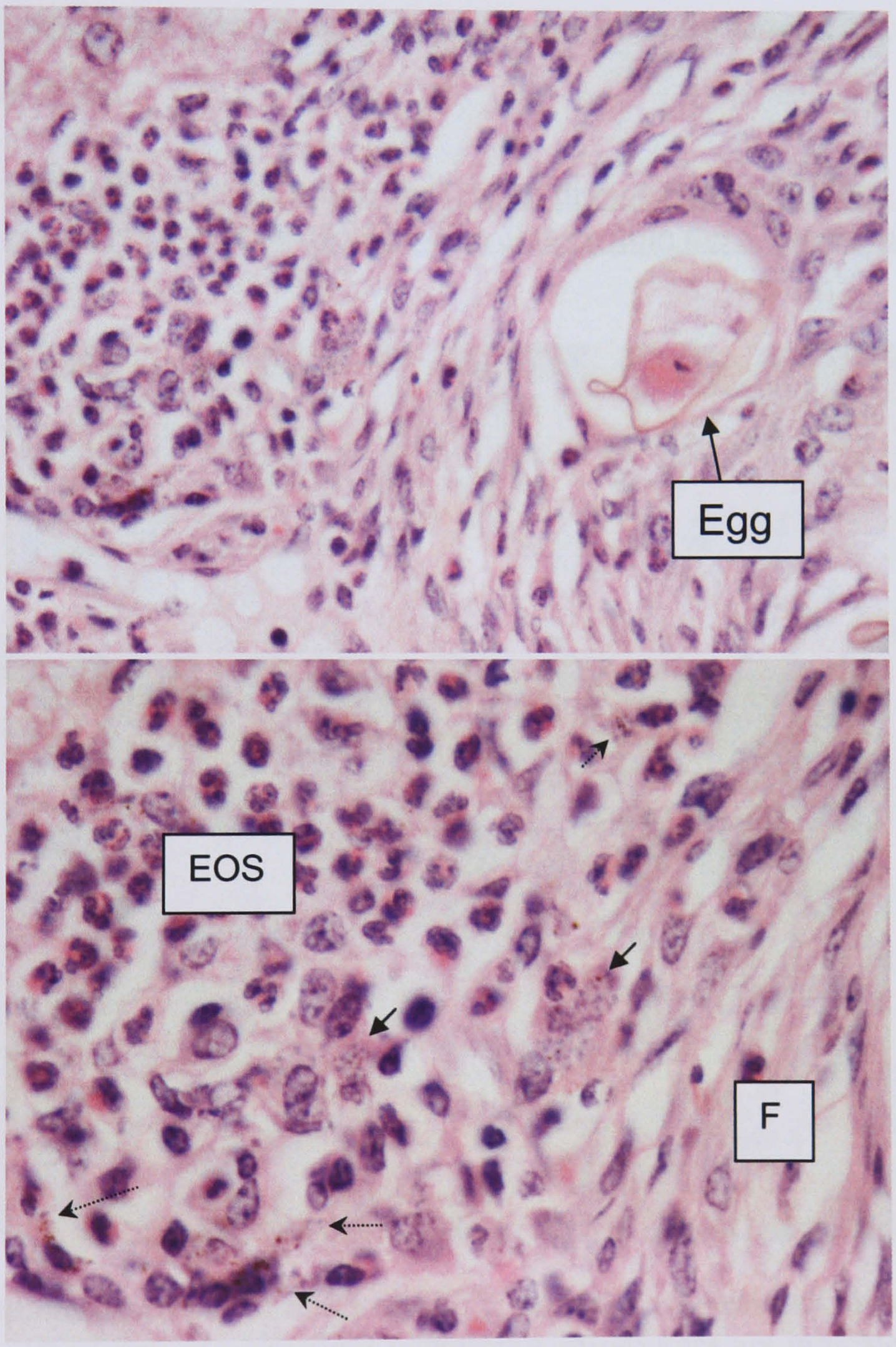


Figure 5.21. Photomicrographs at low (x40) and higher (x100) magnification from a CO-INFECT mouse at +8 weeks after superinfection showing an area of *L. donovani* infection within a schistosome egg granuloma. **Egg**= *S. mansoni* egg, **EOS** =eosinophil rich area, **F** = area of fibroblasts and collagen deposition. Isolated groups of single or a few amastigotes which may be free or in cells are indicated by arrows with dotted lines. Solid arrows show more heavily infected cells.

Again it is clear (i) that there is no discrete cellular organization around the infected cells and (ii) that the region contains several infected cells some with many and other with just a few amastigotes.

Figure 5.22 shows an area rich in lymphocytes and macrophages including some which are heavily infected with amastigotes and containing lots of schistosome pigment. Again it is not possible to distinguish any discrete organization of cells around the numerous *L. donovani*-infected cells.

Figure 5.22 : Multiple foci of infection and lack of organisation of *L. donovani* granulomas in mononuclear-rich areas within the *S. mansoni* egg granuloma.

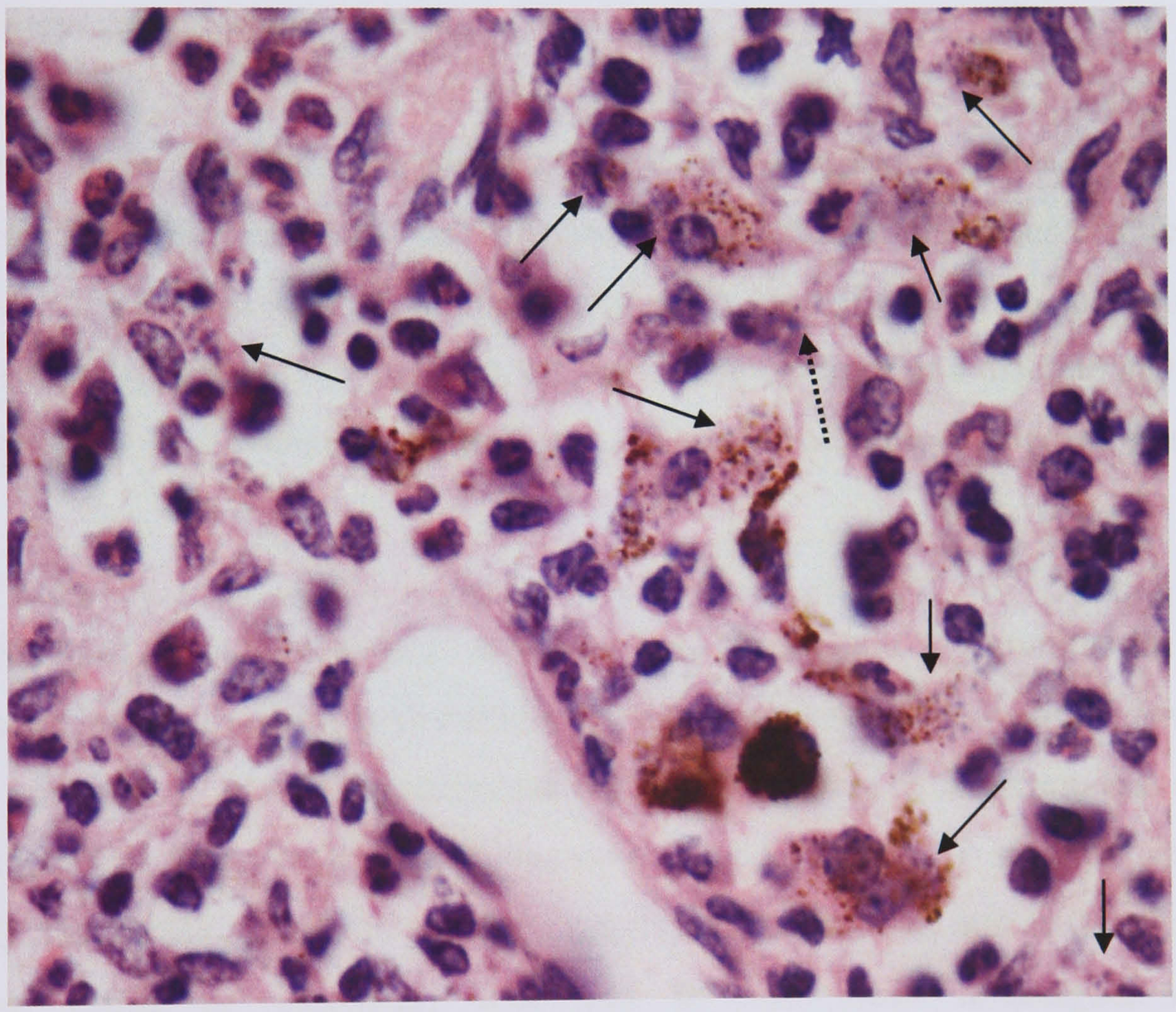


Figure 5.22 : Photomicrograph from a CO-INFECT mouse at +8 weeks of infection from within an *S. mansoni* egg granuloma. Macrophages containing amastigotes are indicated by arrows with solid lines. An isolated small group of amastigotes apparently free and not contained within a cell is indicated by the arrow with dotted lines.

For the purpose of trying to quantify the number of amastigotes within infected macrophages in order to compare amastigote attrition between the parenchymal and egg granuloma regions of CO-INFECT mice or between LEISH and CO-INFECT mice, the inability to discern discrete *L. donovani* granulomas within the egg granulomas posed a problem. This is exemplified in the comparison shown in Figure 5.23 [top and bottom] of a region of infection within the egg granuloma and in the parenchyma of the same mouse. In the top section from within the egg granuloma no discrete *L. donovani* granuloma(s) can be seen and there are several groups of amastigotes ranging from 3 to 10 per group. The difficulty is in deciding whether all of the amastigotes in this section should be taken as one *L. donovani* focus or each focus counted separately in this section the small groups of amastigotes outside the main area were regarded as separate foci of infection and so their amastigote content is recorded separately.

This point is emphasized because as is shown later the mean amastigote count for the focus shown in Figure 5.23 (top) was lower than if it had been counted as one focus of infection. In contrast in the *L. donovani* focus in the parenchyma (Figure 5.23 bottom) the amastigotes are also spread out but clearly occur within the confines of a discrete mature *L. donovani* granuloma. In this instance all of the amastigotes would be counted as from one focus.

These examples of the *L. donovani* foci reactions within *S. mansoni* egg granulomas show (i) that it is not possible to distinguish cellular recruitment specifically to the *L donovani* infected cells within the *S. mansoni* granulomas and so not possible to be certain that any *L. donovani* granulomas form within the *S. mansoni* granuloma and if so their state of maturation (ii) that the lack of the delineating *L. donovani* granulomas makes it difficult to determine the limits of individual foci and so the mean amastigote count.

Figure 5.23 Comparison of amastigote distribution in foci of *L. donovani* infection within an egg granuloma (top) and in the parenchyma (bottom)

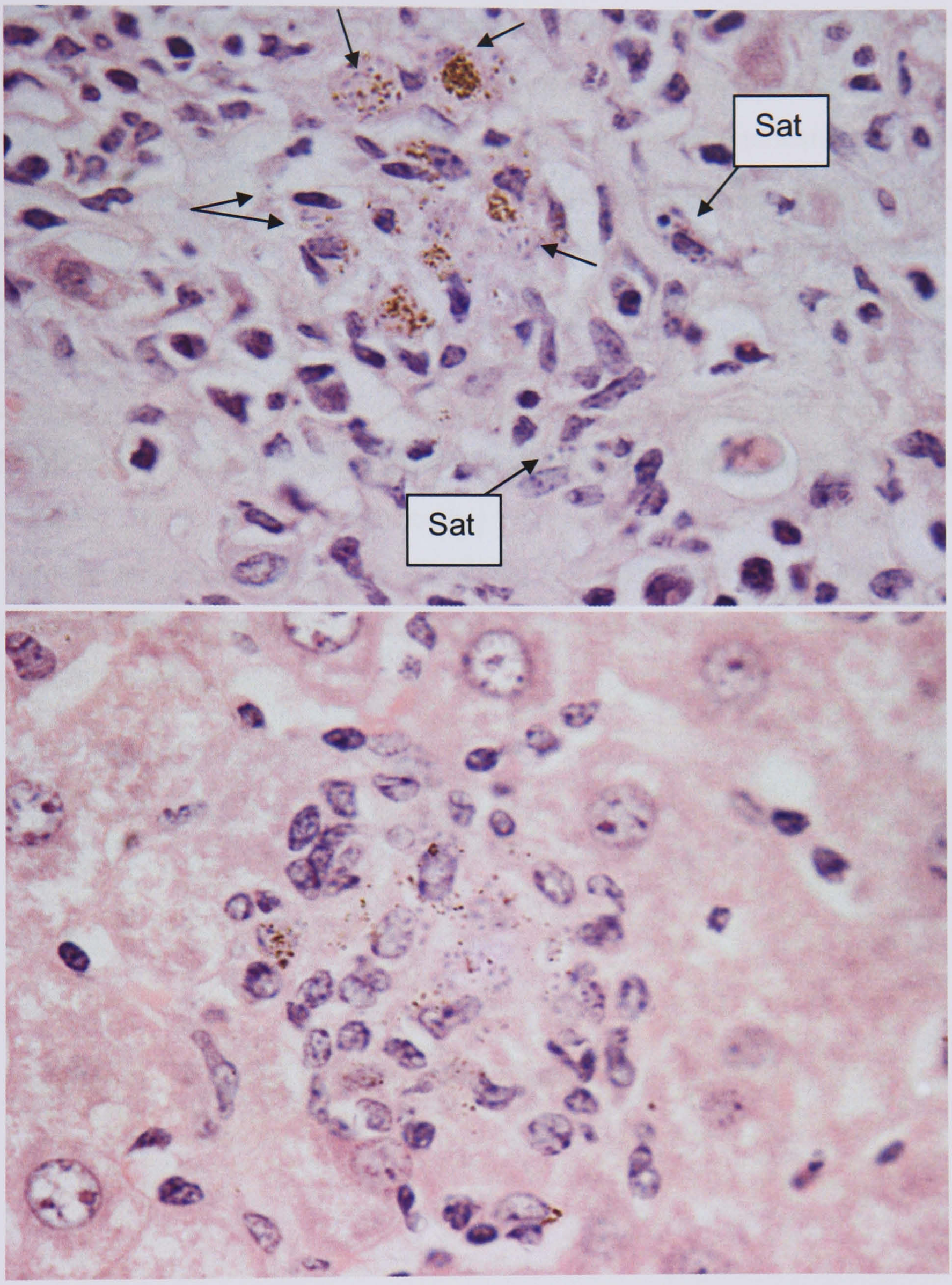


Figure 5.23. Photomicrographs from a CO-INFECT mouse +8 weeks after *L. donovani* superinfection showing a region of *L. donovani* infection inside an *S. mansoni* egg granuloma (Top section) and a discrete mature granuloma in the parenchyma (bottom section). The arrows indicate separate infected macrophages that are regarded as part of the same inflammatory focus. Apparent spread of the infection to satellite areas (SAT) outside this focus are also arrowed. Granulomas apparently leads to dissemination of amastigotes is indicated by arrows.

5.2.4.1.3. *L. donovani* infection inside the *S. mansoni* egg shells.

L. donovani infected macrophages were seen throughout *S. mansoni* egg granulomas. In a proportion of the empty egg shells, at the centre of older granulomas, large numbers of amastigotes could be seen infecting large giant cells (multinucleate macrophages). Examples of this are shown in Figure 5.24. The detailed morphology of these reactions is difficult to relate to the *in vivo* organisation. There appears to be a space around the egg shell which is surrounded by fibrin-like material. Whether this is truly an area lacking cells *in vivo* or the apparent space is an artifact of preservation and sectioning is unclear. In Figure 5.24. [top] it appears that the macrophages are outside the egg shell whereas in Figure 5.24. [bottom] it appears that a giant cell is inside the shell as well as cells outside which are making contact with the surrounding fibrin-like tissue. Even allowing for some artifact of fixation and sectioning it does not appear that there was intimate contact between the infected cells and inflammatory cells. Again numerous eosinophils can be seen in the surrounding cellular response.

Figure 5.24. *L. donovani* infection of macrophages associated with the *S. mansoni* egg shell at the centre of egg granulomas.

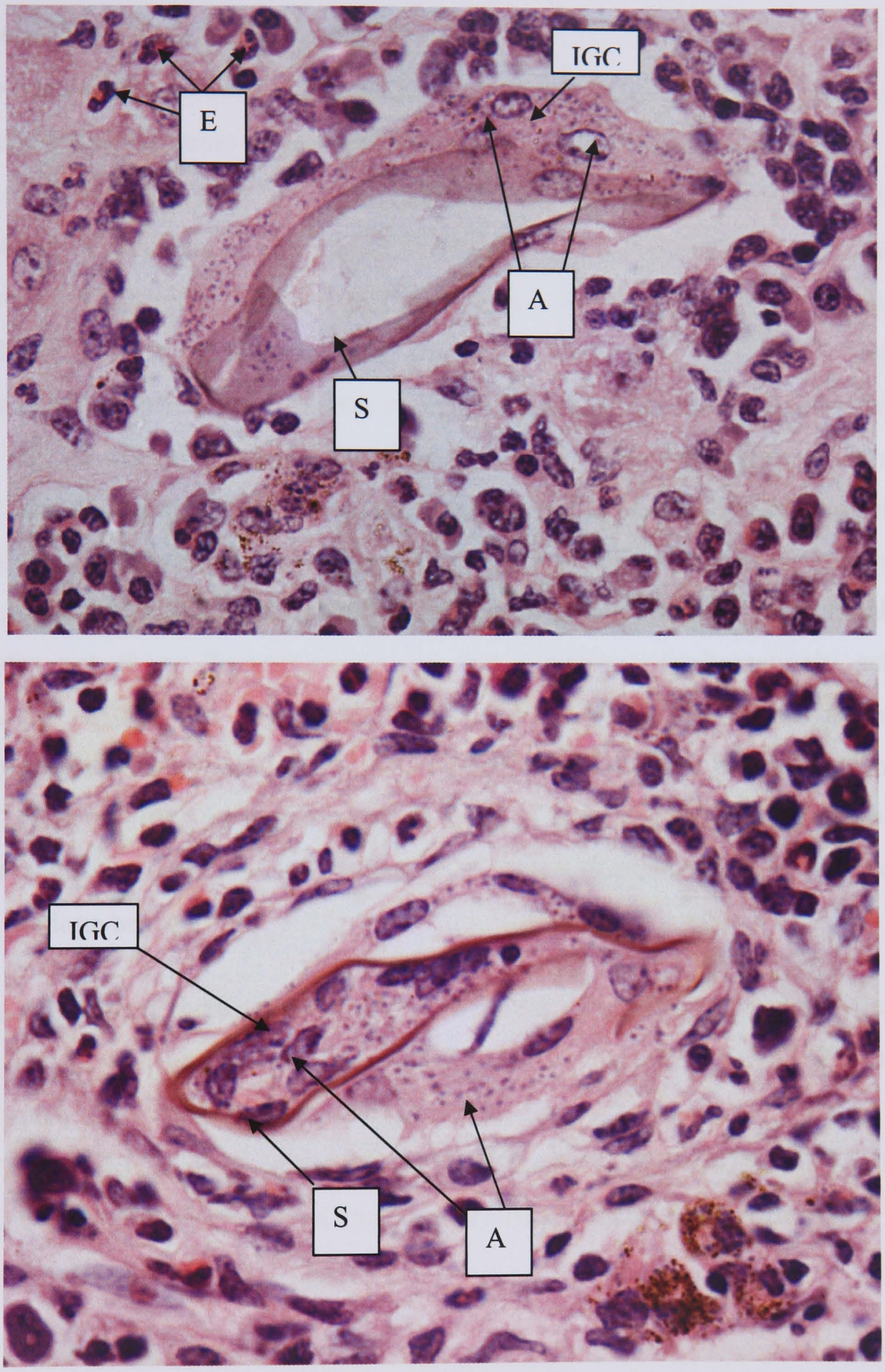


Figure 5.24. Photomicrographs from CO-INFECT mice +8 weeks after *L. donovani* superinfection showing sections of the egg shells at the centre of *S. mansoni* egg granulomas with associated *L. donovani* infected macrophages. **A** = amastigotes, **S**= *S. mansoni* egg shell, **IGC** = infected giant cell, **E** = eosinophils.

5.2.5.2. Quantitative observations.

The LDU data from Chapter 3 had shown substantially higher numbers of total amastigotes in the livers and spleens of CO-INFECT mice compared with LEISH mice at +8 weeks. In the final section of this Chapter analysis of the histological material was carried out to address the following hypotheses which might explain this finding: (i) that the *S. mansoni* egg granuloma macrophages provide an additional and possibly higher density of alternative sites for *L. donovani* infection than the liver parenchyma macrophages [hypothesis 2 in Chapter 3 discussion](ii) that reduced leishmanicidal activity results from reduced progression of *L. donovani* granulomas to the mature state in CO-INFECT mice [hypothesis 4 in Chapter 3 discussion] (iii) that *L. donovani* granulomas may physically fail to form correctly within the context of the inflammatory cells of the *S. mansoni* granulomas [hypothesis 5]. This was reported in section 5.2.4.1.2. and the extensive characterization of foci of *L. donovani* infection during this although no quatification is recorded (iv) that reduced leishmanicidal activity around foci of *L. donovani* infection in CO-INFECT mice results in increased mean numbers of amastigotes per focus [hypothesis 6] (v) that infection of alternatively activated macrophages within the schistosome egg granulomas would result in reduced amastigote killing compared with infected Kupffer cells in the parenchyma of CO-INFECT mice resulting in higher mean amastigote counts in the foci of *L. donovani* infection in the egg granulomas [hypothesis 7]. These alternatives (i) –(v) are referred to in the sections below.

Histological sections were cut and stained with haematoxylin and eosin. Microscopic analysis was carried out on 30 adjacent fields in the parenchyma of LEISH and CO-INFECT mice and similar number of fields inside the schistosome granuloma of the CO-INFECT mice. This analysis was carried out for 3 mice per group. For the CO-INFECT group mice were chosen which had comparable schistosome parasitic burdens in terms of adult worm pairs and geometric egg counts.

5.2.5.2.1. Comparison of the density of sites of *L. donovani* amastigote infection in the parenchyma of LEISH animals, in the parenchyma of CO-INFECT mice and inside the *S. mansoni* egg granulomas of CO-INFECT mice [(i)].

This analysis yielded data on the density of reactions which is shown in Figure 5.25 for both Experiments I and II. The overall trend is clear and similar in both experiments i.e.

there is a progressive decline in the density of foci detected in the LEISH mice from +2, +4, +8 weeks (p=0.05, 0.003 respectively expt 1) (p=0.02, 0.003 respectively expt 2) whilst in the CO-INFECT mice the density in the CO-INFECT mice increases.

Figure 5.25. Comparison of the density of foci of *L. donovani* infection in the parenchyma or egg granulomas of LEISH and CO-INFECT mice.

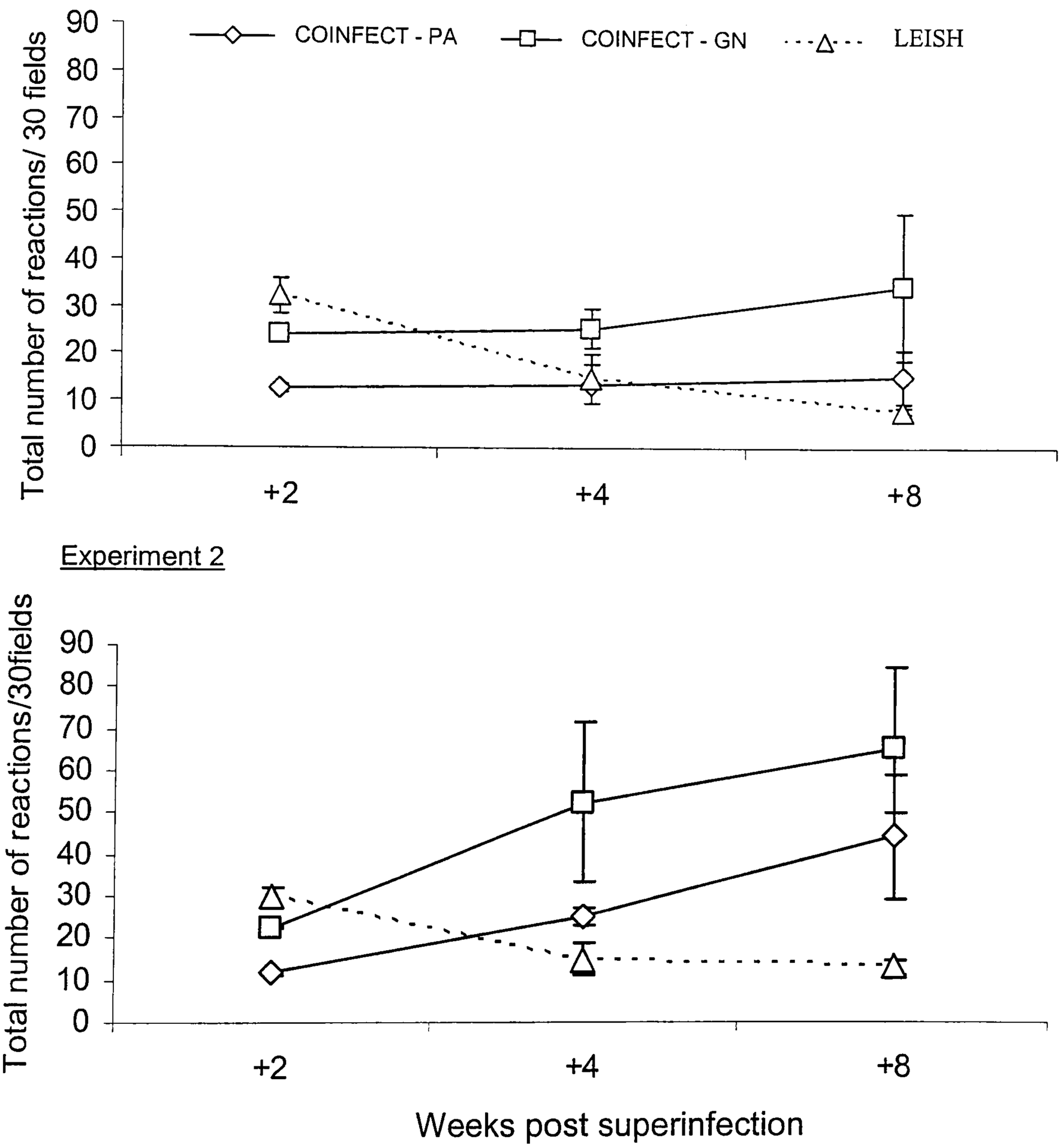


Figure 5.25.: Comparison of mean density of total inflammatory reactions in LEISH animals compared with those in the parenchyma or within the *S. mansoni* egg granulomas of CO-INFECT mice (CO-INFECT–PA and CO-INFECT-GN, respectively). The values represent the mean (± SE) from 5 mice for each of which the number of *L. donovani* foci of infection were counted in 30 adjacent fields from microscopic examination of histological sections at +2, +4 and +8 weeks post *L. donovani* superinfection.

However at +2weeks in experiment I, the LEISH infected animals actually showed a significantly higher mean density of infected foci in the parenchyma compared with the CO-INFECT (p=0.007). Similarly in experiment II, the LEISH infected animals actually showed a significantly higher mean density of infected foci in both the CO-INFECT parenchyma and inside the schistosome granuloma (p=0.002, 0.04 respectively).

The other prominent observation was that CO-INFECT animals showed a higher density of reactions inside the schistosome granuloma compared with within the parenchyma at all time points in both repeat experiments although this was only statistically significant at +2wks (p=0.002, <0.0001 respectively). Overall there were around 1.5 fold more foci in the same area of granulomatous tissue compared with the parenchyma at all times. So this does show some support for the idea that there is preferential development of *L. donovani* within the egg granulomas (hypothesis 2).

5.2.5.2.2. Analysis of the maturation state of *L. donovani* granulomas [(ii)]

The mean frequency of the following types of *L. donovani* foci of infection were determined in 30 fields from CO-INFECT and LEISH mice: infected Küpffer cells (in the parenchyma) and macrophages (Infect Kpf), fused Küpffer cell (Fused Kpf), immature granulomas (Immature Grn) and mature granulomas (Mature Grn). Because it was not possible to distinguish *L. donovani* granulomas associated with foci of infection within the schistosome egg granulomas this analysis was only done on reactions in the parenchyma. The data from Experiments 1 and 2 are shown in Figures 5.26. and 5.27. respectively.

It is clear from both experiments that the LEISH infected animals showed a progressive increase in the frequency of mature granulomas as the infection was controlled. But this progression was much less pronounced in the CO-INFECT mice.

At +2 weeks, there was little difference between the groups apart from a higher frequency of FKCs in the LEISH (p=0.03, 0.04) with a corresponding higher frequency of immature granulomas in the CO-INFECT (NS in Experiment 1, P=0.03 in Experiment 2).

Figure 5.26 Frequency of different types of *L. donovani* granulomas in the parenchyma of CO-INFECT and LEISH mice – Experiment 1.

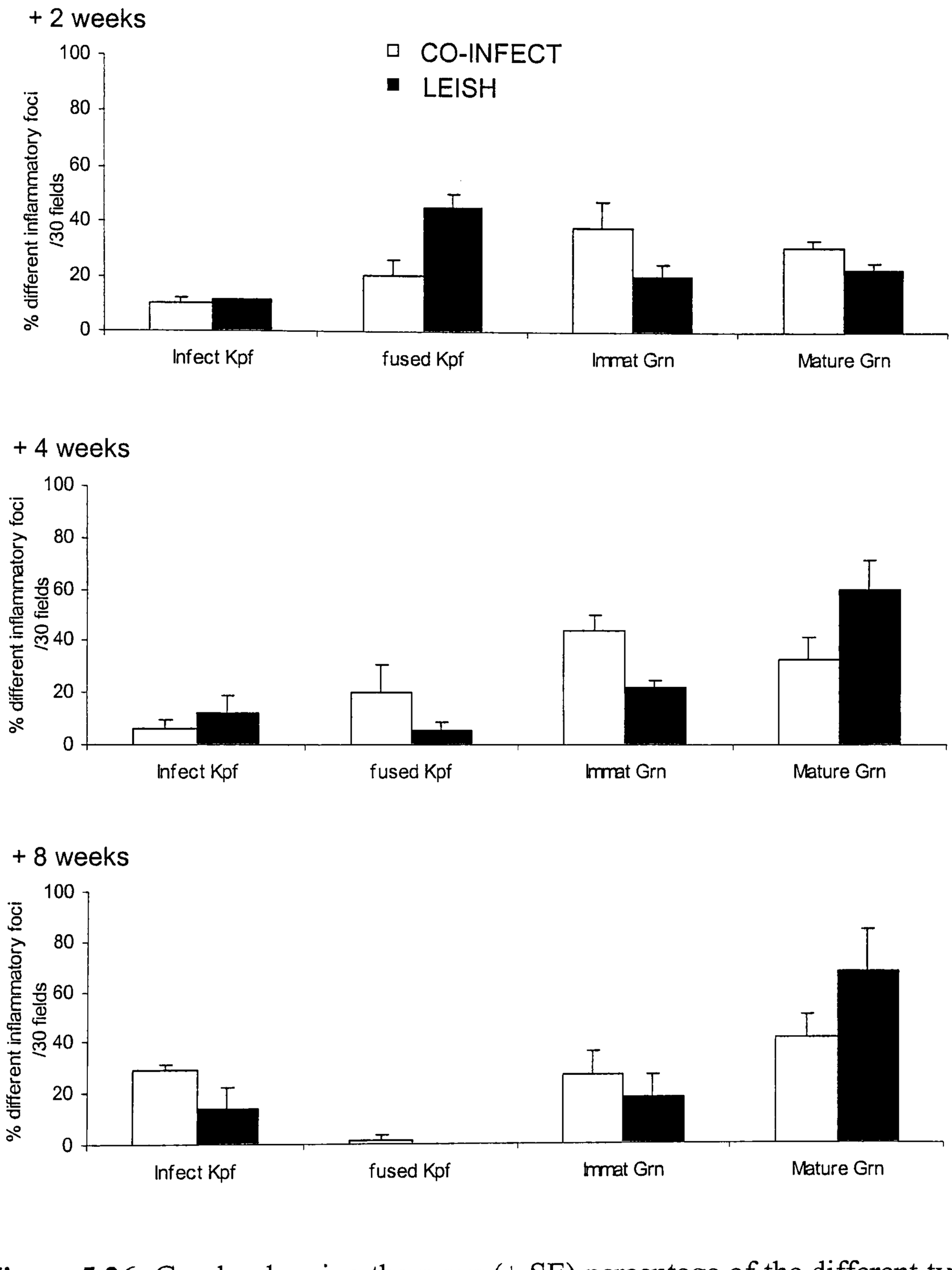
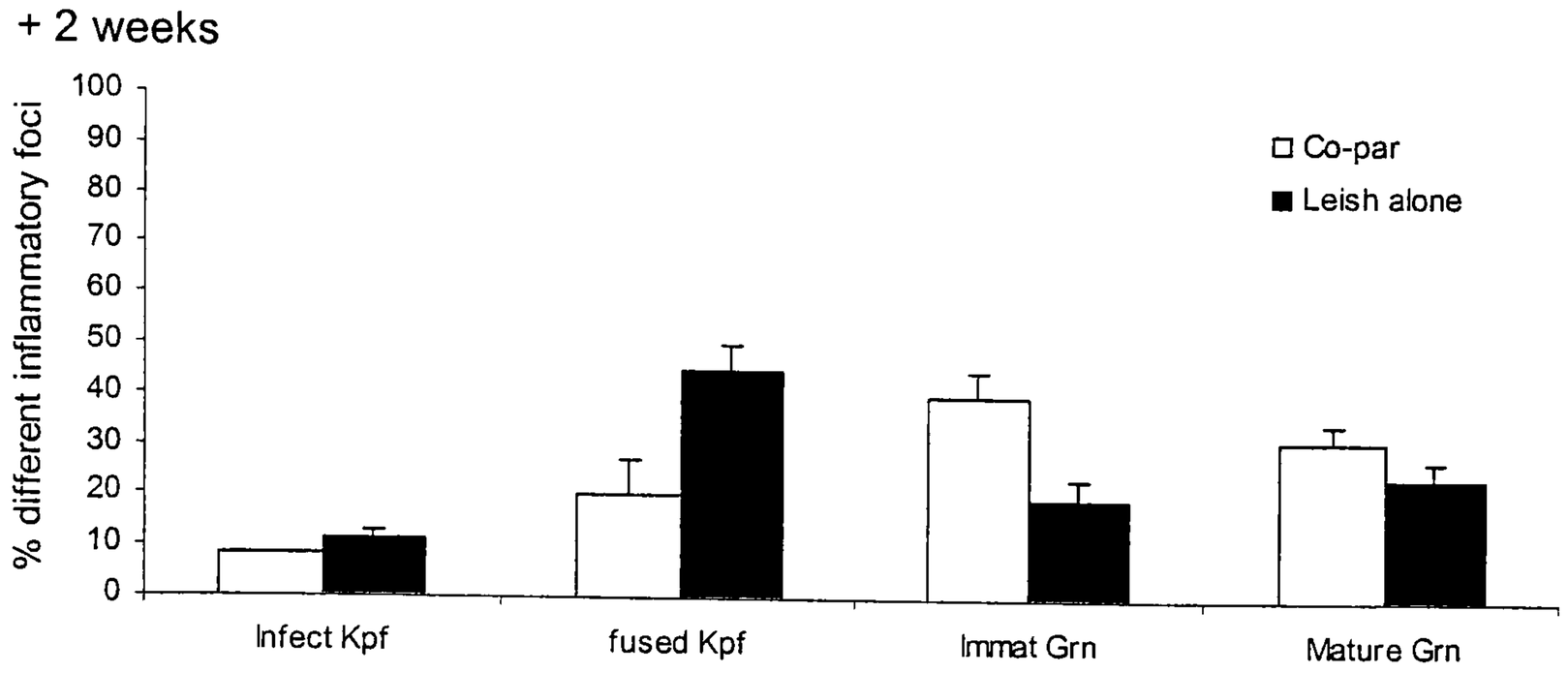
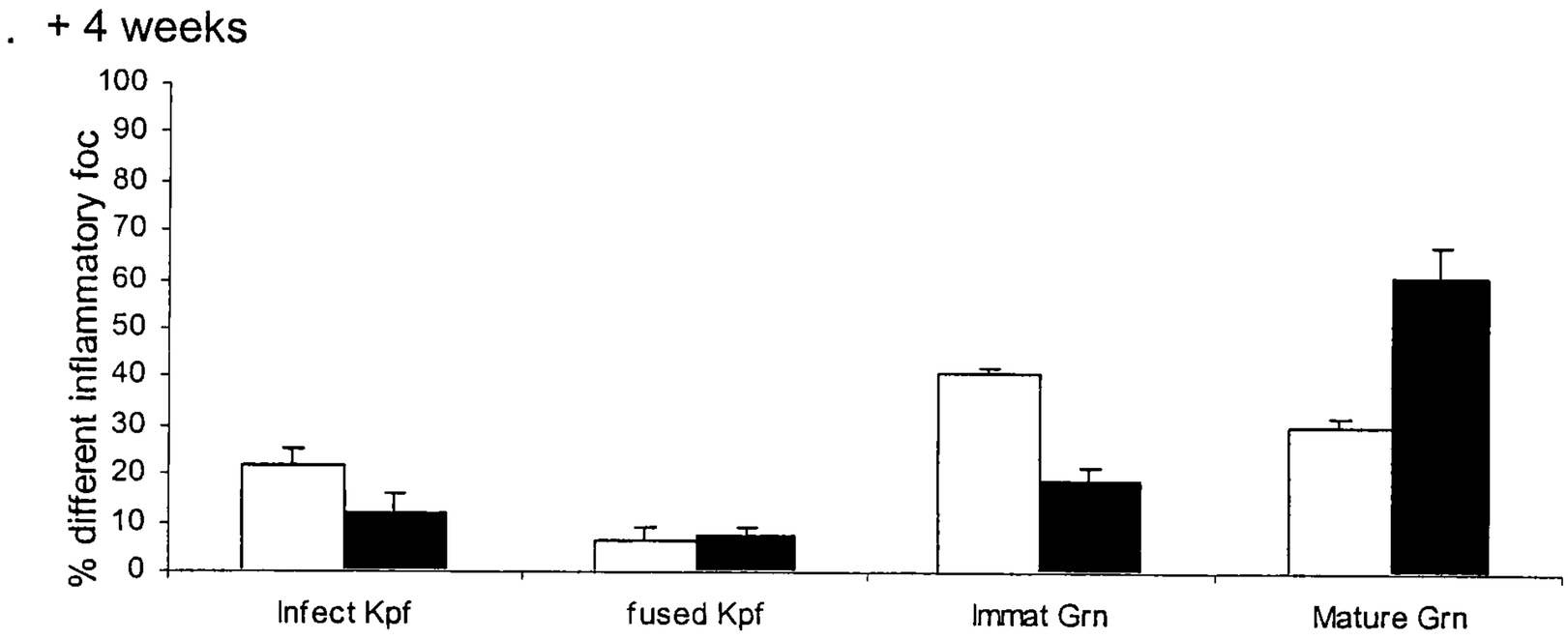


Figure 5.26. Graphs showing the mean (± SE) percentage of the different types of foci of *L. donovani* infection containing amastigotes: Infect Kpf – infected Küpffer cell, fused Küpffer cell, Immat Grn – immature granuloma, Mature Grn – mature granuloma ($p=0.03$). For definition of these reactions see section 5.2.3. Data is based on the foci counted in 30 fields from each mouse and 3mice/group.

Figure 5.27 Frequency of different types of *L. donovani* granulomas in the liver parenchyma of CO-INFECT and LEISH mice – Experiment 2





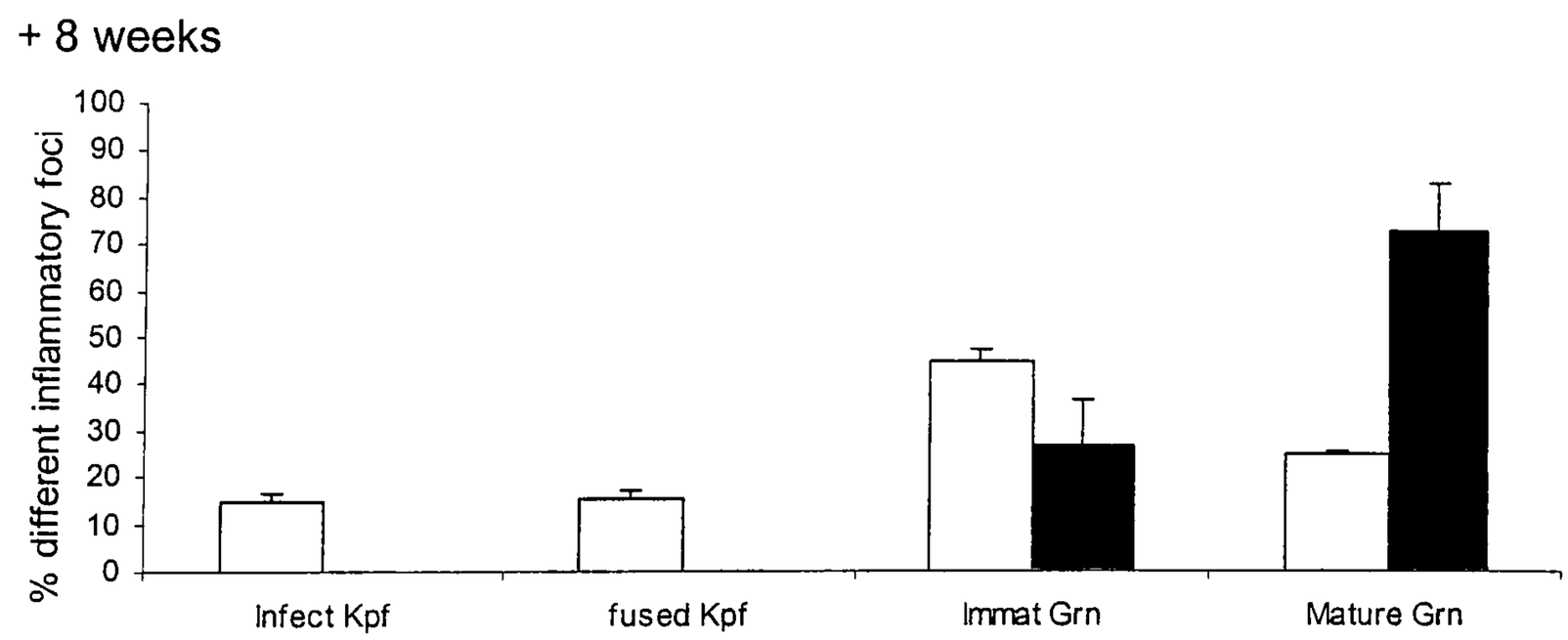


Figure 5.27. Graphs showing the mean (± SE) percentage of the different types of foci of *L. donovani* infection containing amastigotes: Infect Kpf – infected Küpffer cell, fused Küpffer cell, Immat Grn – immature granuloma, Mature Grn – mature granuloma (p=0.03). For definition of these reactions see section 5.2.3. Data is based on the foci counted in 30 fields from each mouse and 3mice/group.

At +4weeks, the LEISH animals showed an increased frequency of mature granulomas compared to CO-INFECT animals (NS in Experiment 1, P=0.01 in Experiment 2). Reciprocally, the CO-INFECT animals showed higher frequencies of immature granulomas compared to the LEISH animals (p=0.03 in Experiment 1, p=0.002 in Experiment 2).

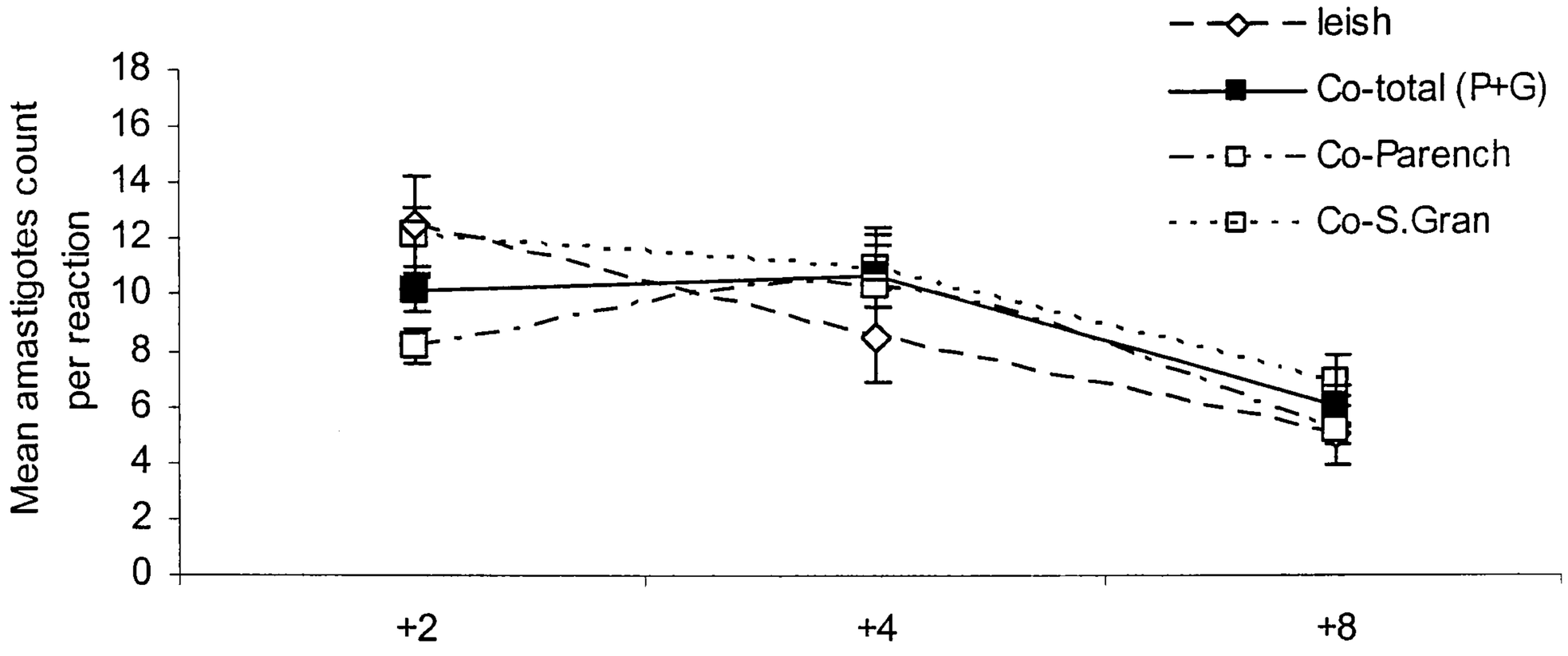
At +8weeks, the LEISH animals showed a higher frequency of mature granulomas compared to the CO-INFECT (NS in Experiment 1, p= 0.009 in Experiment 2) and reciprocally less formation of newly IKCs, FKCs and immature granulomas.

5.2.5.2.3. Comparison of the mean numbers of *L. donovani* amastigotes per focus of infection in the LEISH and CO-INFECT mice [(iv) and (v)].

This data is shown in Figure 5.28. The mean number of amastigotes at +2 weeks post infection were rather similar in both groups (between 8-13 amastigotes) and also between the parenchyma and granulomatous tissue of the CO-INFECT mice but in both experiments the LEISH animals had the highest numbers. In Experiment 1 this was significantly higher than in the parenchyma of CO-INFECT (p=0.04) and in Experiment 2 was significant compared to both the CO-INFECT parenchyma and schistosome granuloma foci (p=0.01, 0.03 respectively). Subsequently, amastigote counts progressively decreased in the LEISH animals between +2 and +8wks (p=0.007 in experiment 1 and p=0.002 in experiment 2). The amastigote count in the CO-INFECT animals in the parenchyma remained fairly constant between +2 and +4 weeks but also showed a decline between +4 and +8 weeks in both experiments (p=0.05 experiment 1, p=0.02 in experiment 2), however, there were NS in amastigote count in CO-INFECT animals inside the schistosome granuloma between both time points +4 and +8 weeks).

Overall, it is clear from this analysis of mean amastigote levels that there was little indication that *L. donovani* granulomas in CO-INFECT mice showed enhanced accumulation of amastigotes compared with LEISH alone animals even for foci of infection within the egg granulomas.

Figure 5.28. Comparison of the mean number of *L. donovani* amastigotes in all foci of infection in the parenchyma and/ within the *S. mansoni* granulomas of LEISH and CO-INFECT mice.



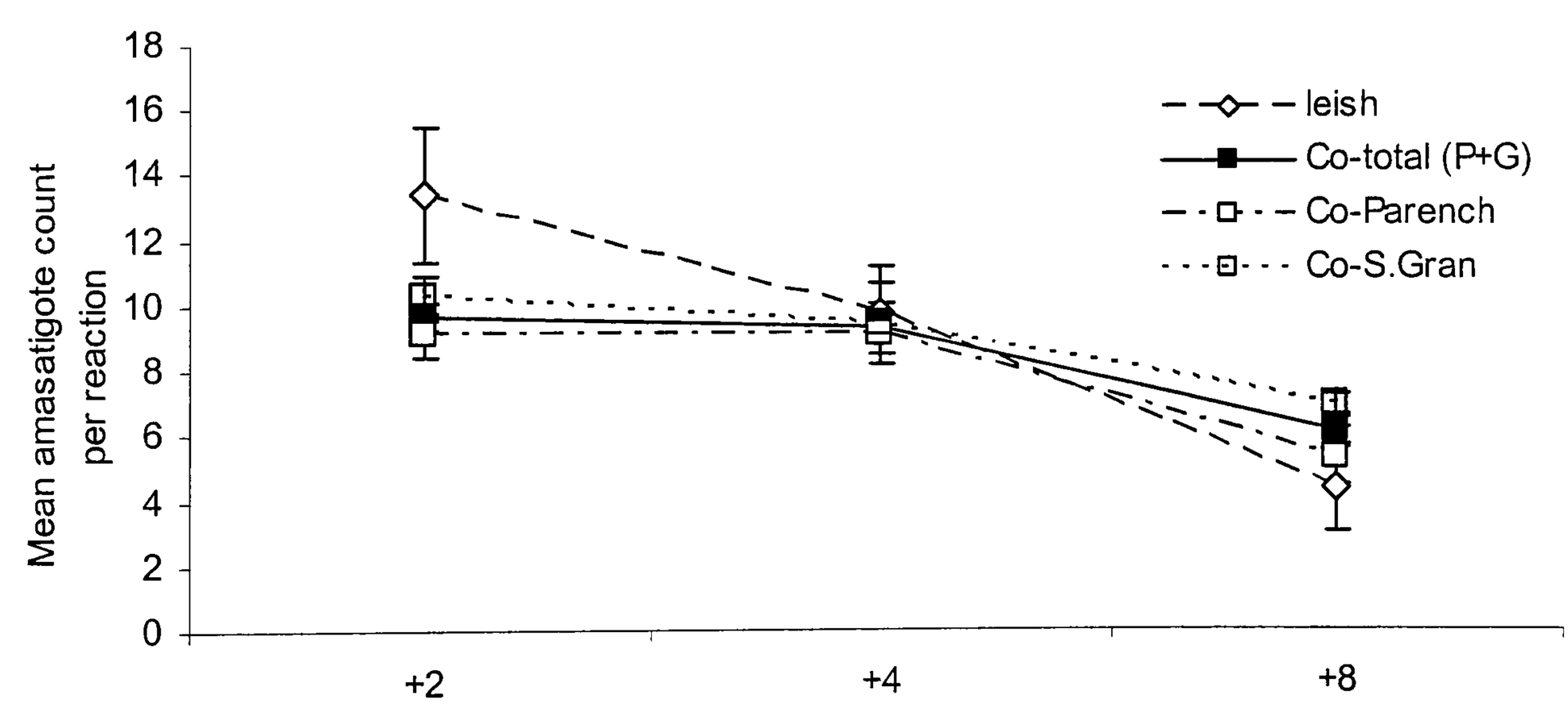


Figure 5.28. Mean ± SE of the number of amastigotes in all foci of infection: leish – data from foci in the LEISH parenchyma, Co-total (P+G) – data from foci in the parenchyma and the granulomas of CO-INFECT mice. Co-Parench – data just from the parenchyma of CO-INFECT mice, Co-S. Gran – data from foci within the schistosome egg granulomas of CO-INFECT mice. Data is based on the foci counted in 30 fields from each mouse and 3 mice/group.

It was considered possible that the mean amastigote levels from all reactions might hide selective differences between the leishmanicidal activities of different types of *L. donovani* granulomas in CO-INFECT compared with LEISH animals. So the data was analyzed to look at amastigote count by type of focus. Because of the failure to distinguish Ld granulomas within the Schistosome egg granulomas this analysis is only done on the parenchymal data. The data is shown in Figure 5.29.

Figure 5.29. Comparison of the mean numbers of amastigotes in the different types of foci of *L. donovani* infection in CO-INFECT and LEISH mice.

Figure 5.29. Comparison of the mean (± SE) amastigote counts in the different *L. donovani* foci of infection: IKC – Infected Küpffer cell, FKC – Fused Küpffer cell, Imm grn – Immature granuloma, Mat grn – mature granuloma. LSH – LEISH mice, Co-I – CO-INFECT mice. Data is based on the analysis of reactions in 30 fields from 3 mice as above.

The data on the mean number of amastigotes in IKC or FKCs is not particularly consistent between the two experiments. On balance there was no consistent difference in the mean numbers of amastigotes in the IKCs or FKCs of the LEISH or CO-INFECT mice at any of the time points. It was of particular interest to know if the *L. donovani* granulomas which formed in the CO-INFECT mice were characterized by a failure to control the infection and accumulation of amastigotes. The mean number of amastigotes in the immature and mature *L. donovani* granulomas were higher at +2 and +4 weeks in both experiments in the CO-INFECT than LEISH, in the immature granuloma at +2wks and +4wks ($p=0.02$, and NS in experiment 1) and ($p=0.001$ and 0.02 respectively in experiment 2). Similarly, the mean count of amastigote count in the mature granulomas were higher in both experiments at +2 and +4 weeks in the CO-INFECT than LEISH ($p=0.006$, 0.003 respectively in experiment 2), however it was NS at both timepoints in experiment I. In the first experiment a higher mean amastigote count in the immature and mature granulomas of CO-INFECT compared with LEISH mice was also seen at the +8 week time point but the opposite trend was seen in the second experiment ($p=0.002$ in immature granuloma, and NS in the mature granuloma). So overall there was evidence of higher mean amastigote numbers in the immature and mature granulomas at +2 and +4 weeks in both experiments and in the first experiment also at +8 weeks. However, the differences were generally not statistically significant.

The amastigote counts in Figure 5.28 did show that there was a higher mean amastigote count in the foci of infection within the schistosome egg granulomas particularly at week +8. The histopathology showed that the foci of infection were difficult to define and it appeared that a proportion of the reactions appeared to be diffuse (see section 5.2.4.1.2. and figure 5.19, 5.20, 5.22). A number of these "satellite" reactions would be recorded as containing relatively few amastigotes (figure 5.23). So it was reasoned that there may be *L. donovani* infected macrophages, particularly alternatively activated macrophages in the *S. mansoni* granulomas which may have been unable to control the *L. donovani* proliferation resulting in accumulation of high number of amastigotes but the inclusion of the low amastigote counts from the newly infected macrophages would bring the mean value down. To check to see if there was evidence of a proportion of highly infected macrophages in the CO-INFECT mice the frequency distribution of the amastigote counts was plotted.

5.2.5.2.4. Frequency distribution of the amastigote counts in CO-INFECT and LEISH mice.

The data is shown in Figures 5.30 and 5.31. It can be seen that there is a wide standard error for the values but two relevant points can be made. Firstly in both experiments the majority of reactions contained 5-10 amastigotes at all of the time points in both CO-INFECT and LEISH mice. Secondly there was no evidence for a population of more highly infected cells in the CO-INFECT compared with the LEISH mice and there was also no evidence for a higher frequency of heavily infected reactions in the CO-INFECT mice than the LEISH mice. Furthermore, there was no indication for an increased frequency of such heavily infected cells in the *S. mansoni* egg granulomas than in the parenchyma of CO-INFECT mice.

Figure 5.30. Frequency (percentage) of the different numbers of amastigotes in *L. donovani* foci in CO-INFECT and LEISH mice –Experiment 1.

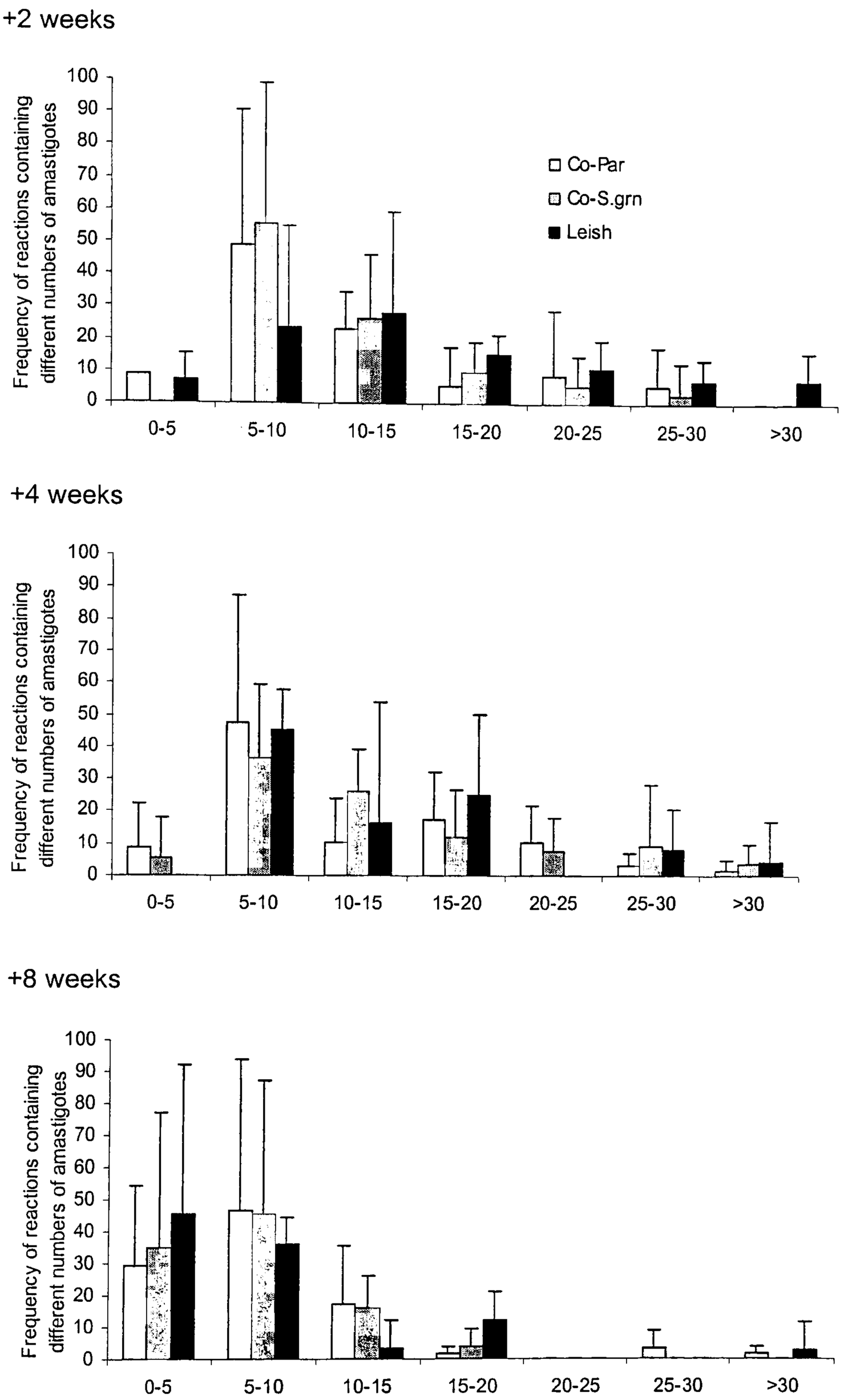


Figure 5.30. Percentage of the total *L. donovani* foci with particular numbers of contained amastigotes. Co-par – reactions in the parenchyma of CO-INFECT mice, Co-S.grn – reactions within the *S. mansoni* granulomas of CO-INFECT mice, Leish – reactions in the parenchyma of LEISH mice. Data is based on 30 fields and represents the mean ± SE from 3 mice.

Figure 5.31. Frequency (percentage) of the different numbers of amastigotes in *L. donovani* foci in CO-INFECT and LEISH mice –Experiment 2.

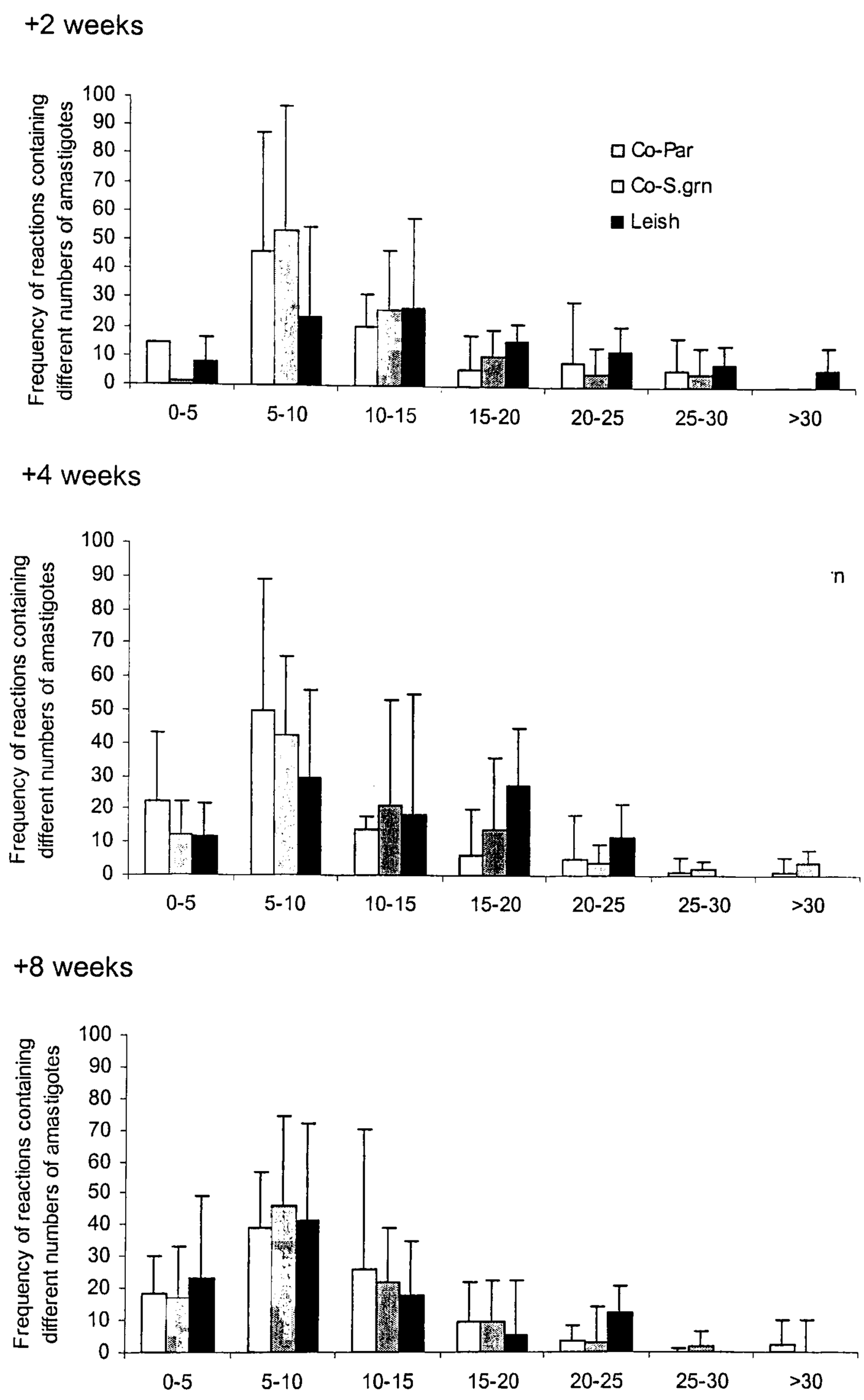


Figure 5.31. Percentage of the total *L. donovani* foci with particular numbers of contained amastigotes. Co-par – reactions in the parenchyma of CO-INFECT mice, Co-S.grn – reactions within the *S. mansoni* granulomas of CO-INFECT mice, Leish – reactions in the parenchyma of LEISH mice. Data is based 30 fields and represents the mean ± SE from 3mice.

5.3. Discussion

This chapter was concerned with three aspects of the co-infection: the possible synergistic effect of both infections on damage to the liver function; effects on the size and cellular composition of the *S. mansoni* egg granulomas; and effects on the distribution, maturation and amastigote content of *L. donovani* granulomas.

Aspartate aminotransaminase (AST), and Alanine Aminotransaminase (ALT) showed similar patterns to each other and in both experiments. Both the *S. mansoni* and *L. donovani* infections alone induced some elevation of liver enzyme levels, rather more with *S. mansoni* which was also characterized by a rise in enzyme levels between +2 and +4 weeks (+10 and +12 weeks post schisto) and a decline between +4 and +8 weeks. This decline would be consistent with the reported decline in egg granuloma sensitivity as the infection becomes chronic (Colley, 1975; Hernandez *et al.,* 1997) but in this study as described below such a decline in granuloma volume was not seen between +4 and +8 weeks. The CO-INFECT mice showed a similar level of response to the SCHISTO mice rather than an additive one except for the final time point when the levels were higher in the CO-INFECT than the SCHISTO which corresponds to the time when the *L. donovani* LDU levels were rising. It is possible that this evidence of increased liver damage at +8 weeks contributed to the observed morbidity and mortality in some of the CO-INFECT mice, compared to the SCHISTO alone mice which showed a stable and consistent rise of liver enzymes (ALT/AST) (Pyrrho *et al.,* 2004; Chiaramonte *et al.,* 2001).

The analysis of the size and cellular composition of the *S. mansoni* egg granulomas was undertaken because it is clear that this response is tightly controlled by interacting Th1 and Th2 cytokines and the action of Th2 cytokine-induced alternatively activated granuloma macrophages (Pearce and MacDonald, 2002). Marked skewing of the response towards a Th1 response as occurs in IL-4/IL-10$^{-/-}$ mice results in high mortality and elevated inflammatory mediators (Hoffmann *et al.,* 2000) but less extreme diversion, by immunization with rIL-12 plus schistosome eggs results in lowered Th2 cytokine responses, smaller granulomas and reduced fibrosis (Wynn *et al.,* 1995). So it was conceivable that a superimposed *L. donovani* infection by inducing Th1 responses in the liver would serve to lower the egg granuloma volume. However, the data showed

a larger volume in the CO-INFECT than the SCHISTO alone at both +4, +8 weeks in both experiments, however there were no significant differences in both timepoints in the two repeat experiments. Clearly in both experiments there was a dramatic reduction volume of the granuloma at +8wks. This schistosome granuloma modulation is in agreement with other studies in human (Falcao *et al.,* 1998) and murine studies showing peak granuloma size which occurs at the acute phase approximately 8-10 wk post infection there is a decrease in mean granuloma size (Warren *et al.*,1967; Colley *et al.*, 1975).

There was no clear indication from the splenocytes, anti-egg cytokine responses how this down-modulation could have occurred as schistosome antigen-specific IL-4, IL-10 and IFN-γ levels were essentially similar at +4 weeks between the CO-INFECT and SCHISTO mice. There was no effect on the schistosome granuloma volume in other co-infection studies as reported in *S. mansoni*-infected mice exposed to recombinant *Vaccinia* virus (Actor *et al.,* 1993; 1994). However there was a marked reduction in schistosome granuloma volume as reported by Marshall *et al.* (1999) who showed that infection with pre-existing *Toxoplasmosis gondii*, and superinfection with *Schistosome mansoni* skewed the Th1-like/Th2-like balance towards a Th2-like immune response. This was accompanied by a marked decline in IFN-γ, NO production and a slight increase in IL-4 and IL-5 to virus (Actor *et al.,* 1993; 1994) and Toxoplasmosis antigens (Marshall *et al.,* 1999). The other point to note was that in the SCHISTO alone mice there was an apparent increase in egg granulomas size between +4 and +8 weeks (+12 and +16 weeks post *S. mansoni* infection) in contrast to the reduction in granuloma volume ("endogenous desensitization") which has been reported to occur between the acute and chronic state of the infection and which is attributed to a variety of different immuno-regulatory responses (reviewed in King, C.L. (2001) Initiation and regulation of disease in schistosomiasis. In Mahmoud, A.A.F. (Ed). Schistosomiasis. Imperial College Press, London p213-264). However, this modulation can already occur by +12 weeks (Diab *et al.,* 1989) and in a recent study in C57BL/6 mice used here there was only around 23% reduction between the vigorous acute, 8 week granuloma and those at 15 weeks (Sadler *et al.,* 2003). There was essentially no difference in the cellular composition of the egg granulomas between the different groups or the different time points. It would be interesting to extend this analysis to the +2 week time point and to

confirm the result, but attention was not focused on this here because the apparent effect on granuloma volume in the CO-INFECT mice was transient and there was a greater interest in the potential significance of the increased *L. donovani* response.

As discussed in the results section the initial haematoxylin and eosin staining of liver histology did not give good differentiation of *L. donovani* amastigotes from schistosome pigment. Following attempts at immuno-staining of cryosections for amastigotes using *L. donovani* infected hamster serum a method was developed using IFAT. This method yielded some useful data but the eventual refinement of the haematoxylin staining to clearly show the amastigotes was the best method for morphological and quantitative analysis.

To investigate the possibility that the macrophages of the *S. mansoni* egg granulomas offer a favorable site for *L. donovani* infection, data on the density of foci containing *L. donovani* amastigotes in the parenchyma and egg granulomatous tissue was collected from the haematoxylin and eosin stained tissue as the demarcation of the schistosome granuloma from the parenchymal tissue was more clearly defined than by the IFAT method. The term "focus of *L. donovani* infection" is used to refer to the infection sites within the *S. mansoni* granulomas because characteristic *L. donovani* granulomas did not form in this location (see later). In the LEISH mice there was a progressive decline in the density of foci from +2 to +4 to +8 weeks. However this decline was much less sharp in percentage terms than the LDU values. In contrast there was a progressive increase in the density of foci in the CO-INFECT mice. At + 8 weeks the percentage differences in the density of foci of CO-INFECT compared with LEISH mice were 1.9 and 3.4 fold in the parenchyma and 4.25 and 5.05 fold higher in the granulomatous tissue for Experiments I and II respectively. These compare with the corresponding fold increases in LDU values of 27 fold (Experiment I) and 12.5 fold (Experiment II). The LDU values reflect both the density of foci and the amastigote content and also allows for the organ weights and at +8 weeks these were approximately 1.75 fold higher for the livers of CO-INFECT compared with LEISH mice. If the liver weight component is removed from the LDU values the amastigote "density" as judged by the impression smears would become 15.4 and 7.1 higher in CO-INFECT than LEISH. This is still higher than the density of *L. donovani* reactions recorded in the histological analysis of

the sections and this could reflect a higher mean number of amastigotes per reaction in the foci which are present in the CO-INFECT tissue.

In relation to hypothesis 2, that *S. mansoni* hepatic egg granulomas are rich in recruited macrophages and so there may be a higher density of foci of *L. donovani* infection within the egg granulomas compared with the parenchyma it was interesting to note that there was indeed a somewhat higher density of reactions containing *L. donovani* amastigotes in the schistosome granulomatous tissue than in the parenchymal tissue (1.5 fold higher density). This appears to give support to the idea that there is preferential development of *L. donovani* within the egg granulomas in the CO-INFECT mice (hypothesis 2). However, this apparent bias to infection of the egg granuloma macrophages compared with the parenchymal Küpffer cells was seen at a similar level at +2, +4 and +8 weeks. Since the density of foci in the parenchyma of LEISH animals was actually higher at +2 weeks than in either the parenchyma or egg granulomas of the CO-INFECT mice, this indicates that although there may be some preference for infection of the granuloma macrophages even early in infection this does not lead to an overall higher density of infection in the CO-INFECT mice. This suggests that there is not a limitation in the availability of suitable cells for invasion in the normal liver that can be overcome by the presence of increased egg granulomas. Furthermore, as is seen by the comparable or higher LDU values in the LEISH compared with the CO-INFECT mice at +2 weeks, this early infection of egg granuloma macrophages does not result in more rapid proliferation of *L. donovani*.

This is interesting because the infected cells in the schistosome granulomas may well be AAMø (Stein *et al.,* 1992; Goerdt and Orfanos, 1999) which have immuno-suppressive properties; with decreased IL-12R; NO; IL-1β, IL-6 and TNF-α, paralleled with suppression in the proliferative response (Loke *et al.*, 2000). These AAMø show increased Th2 cytokines and chemokines and produce high levels of anti-inflammatory IL-10 and TGF-β and reduced co-stimulatory molecules CD80, CD86, and MHC class II expression in comparison to expression induced by bacterial stimulants (MacDonald *et al.,* 2001). So it does not seem that during the first two weeks of Ld infection of the liver (*S. mansoni* granulomas would have been there from the start) L.d can proliferate any more effectively in the AAMøs than in the classically activated macrophages of

normal LEISH mice. It may well be that the levels of anti-Ld IFN-γ were insufficient to activate the infected KCs for leishmanicidal activity during this first two weeks.

Analysis of the frequency of the different stages of *L. donovani* granuloma maturation showed that the schistosome infection did not interfere with the development/maturation of *L. donovani* granulomas in the parenchyma during the first two weeks of infection at which time the percentage of mature granulomas was comparable between CO-INFECT and LEISH. Subsequently, in the LEISH animals the control of the infection between +2 and +8 weeks was accompanied by an increase in the frequency of mature granulomas and a decrease in the frequency of IKC and FKC (Murray, 2001a; Taylor and Murray, 1997; Murray *et al.,* 2000; Satoskar *et al.,* 2000; Murray, 2001b). In contrast in the CO-INFECT mice it is clear from both experiments and from both the IFAT and the histology that the distribution of the different types of *L. donovani* granulomas did not change much with time so that there were ultimately significantly lower percentages of mature granulomas in the CO-INFECT mice than the LEISH mice at +8 weeks. This seems to support hypothesis 4, that lower IFN-γ levels may result in an impaired/reduced rate of maturation of *L. donovani* granulomas but another explanation is that any mechanism leading to higher amastigote replication in the CO-INFECT mice would mean that there were more amastigotes surviving to infect new KCs and so there would be a higher proportion of IKC and FKCs. Certainly the LDU counts (Chapter 3) showed that there was a sharp decline in amastigote counts in the LEISH animals between weeks +4 and +8 (3.1 – 13.4 fold drop) but an increase in the CO-INFECT mice. As the number of amastigotes available to establish new foci of infection dropped in the LEISH animals so the proportion of mature (older) reactions would increase and the converse would be true in the CO-INFECT mice. In conclusion these studies based purely on the morphology of the *L. donovani* granulomas are not able to demonstrate if there is impairment in the rate of development of individual *L. donovani* granulomas which would account for impaired leishmanicidal activity.

Regarding Hypothesis 5, that *L. donovani* granulomas may physically fail to form correctly within the context of the inflammatory cells of the *S. mansoni* granulomas and so there may be a lack of control in infected macrophages within the egg granuloma, it was clearly not possible to distinguish any cellular accumulation associated with the *L. donovani*-infected macrophages from the cells comprising the *S. mansoni* egg

granulomatous tissue. Such foci of infection were most abundant in the periphery of the granulomas but *L. donovani* reactions and amastigotes were also found throughout the *S. mansoni* granuloma and surrounded by the various cell types found in the different regions of the egg granuloma *e.g.* eosinophils, fibroblasts, mononuclear cells. Although it was clear that the lymphocyte/macrophage rich granulomas typical of *L. donovani* infection in the parenchyma of LEISH animals did not form within the egg granulomas it was not possible to be certain if any of the surrounding cells were leishmanial antigen specific or recruited by leishmanial specific responses and thus possibly able to produce pro-inflammatory cytokines, IFN-γ and TNF-α. The *L. donovani* infected giant cells inside the empty egg shells at the centre of *S. mansoni* appeared to be isolated from close cellular attention. It was suggested that egg granulomas, by providing a microenvironment Vaccinia, preferentially infected the granuloma macrophages, in combination with the cytokine imbalance present during schistosome infection, can promote the expansion of *vaccinia virus* and possibly other viral agents (Actor *et al.* 1993; 1994).

It would have been useful to have been able to immunostain the *L. donovani* granulomas for TNF-α and other immuno-modulatory cytokines *e.g.* TGF-β to look at the activation state of the constituent macrophages but use of the same reagents as used by Gorak *et al.* (1998) was unsuccessful owing to the masking caused by the presence of the haematin pigment in many of the CO-INFECT *L. donovani* granulomas and even use of different coloured substrates as described in section 5.2.3.2. was unsuccessful.

In LEISH infected mice there would be amastigote replication within macrophages in parallel with the recruitment of anti-leishmanicidal cells as the immune response developed. In LEISH mice the action of antigen specific Th1 cells would eventually lead to macrophage activation and so to intracellular killing of the amastigotes. If this intracellular killing was compromised in CO-INFECT mice it might be predicted that there would be uncontrolled replication in the infected cells leading to high peak numbers with eventual cell destruction and amastigote release (Hypothesis 7). Analysis of the mean number of amastigotes per focus of infection showed an interesting pattern declining from a mean of 12-14 at +8 weeks to 4-6 at +8 weeks in the LEISH animals. Given that there was progression to a higher percentage of mature granulomas relative to IKC or FKCs +8 weeks, this lowered mean value presumably reflects increasing

leishmanicidal activity of the mature granulomas resulting in intracellular killing of amastigotes. However, the CO-INFECT animals also showed a decline in mean amastigote counts between +4 and +8 weeks despite the increasing numbers of LDU. This does not necessarily mean that the peak amastigote counts were comparable in LEISH and CO-INFECT mice because, as discussed above, the uncontrolled proliferation would lead to establishment of new infections of KCs or macrophages; a proportion of which would contain low numbers of parasites which would reduce the mean count.

Restricting analysis of the amastigote numbers to the immature or mature *L. donovani* granulomas, which reflects the ability of the *Leishmania* specific immune response to develop leishmanicidal activity showed a more interesting trend. Overall there was evidence of higher mean amastigote numbers at +2 and +4 weeks in both experiments and in the first experiment also at +8 weeks. In conclusion this analysis of parenchymal foci gives support the idea that although morphologically comparable *L. donovani* granulomas can form in the CO-INFECT mice these granulomas have a reduced ability to kill the contained amastigotes. IL-10, causes deactivating macrophages by suppressing the afferent and/or efferent arms of the Th1 cell-associated mechanism (Moore *et al.,* 2001; Groux *et al.,* 1999). IL-10 also suppresses antigen-presenting cells (Mitra *et al.*, 1995), T cell co-stimulatory mechanisms, T cell activation, specific T cell proliferation and inhibits type 1 cytokine synthesis and production, in particular IFN-γ, TNF-α, IL-1β, IL-6, IL-12 by Th1 clones (Mosmann and Moore, 1991; Fiorentino *et al.,* 1991).

Hypothesis 7 was infected alternatively activated macrophages within the schistosome egg granulomas may show particularly poor leishmanicidal activity and that this might lead to amastigote accumulation resulting in higher mean numbers of amastigotes in the infected macrophages within the *S. mansoni* granulomas compared with the parenchyma of CO-INFECT mice and/or LEISH mice. There was slightly higher mean amastigote counts in the foci of infection within the schistosome egg granulomas particularly at week +8 although this was not significantly different from that in the LEISH mice. It was reasoned that heavily infected cells might occur but that if there was rapid proliferation there would also be many newly infected cells with few amastigotes.

However, analysis of the frequency distribution of amastigote counts gave no evidence for the presence of a population of more highly infected cells in the CO-INFECT compared with the LEISH mice.

A possible explanation for the failure to see increased amastigote numbers in the CO-INFECT mice is that amastigotes may fail to be killed within macrophages in the CO-INFECT mice but, owing to a limit in the numbers which can be contained within the macrophages, they may simply break out to invade additional cells rather than be killed with the result that differences in mean amastigote counts would not be seen. Some of the reactions especially within the egg granulomas gave the appearance of a "spreading focus of infection" with a range of levels of infection in macrophages in adjacent areas. One place where high numbers of *L. donovani* amastigotes were seen was inside the giant cells which had invaded the egg shells at the centre of the *S. mansoni* granulomas. It could be argued that the existence of such heavily infected cells argues against the idea that macrophages will only support a relatively finite number of amastigotes but clearly these infected cells represent a special case. They were not in close contact with any other cells and there was no evidence of any form of a *L. donovani* granulomatous response. These *L. donovani* infected egg shells were not seen very frequently but they graphically suggest that the infected macrophages at the centre of the Th2 dominated *S. mansoni* egg granuloma are not likely to have effective leishmanicidal activities.

It is concluded that (i) although the schistosome egg granuloma provides a source for infection for *L. donovani* the mere presence of this additional source of macrophages does not account for the proliferation of Ld in CO-INFECT (hypothesis 2) (ii) in the hepatic parenchyma there was an increased frequency of mature *L. donovani* granulomas in the LEISH as the infection resolved but not in the CO-INFECT but it is likely that this reflects the lack of parasites for establishment of the immature granuloma stages in the LEISH rather than a failure in progression to the mature stage in the CO-INFECT (hypothesis 4) (iii) there were higher mean amastigote numbers in the immature and mature granulomas in the CO-INFECT mice suggesting a failure of leishmanicidal activity within the granulomas (hypothesis 6) (iv) typical hepatic *L. donovani* granulomas do not form around foci of infection within the schistosome egg granulomas and reduced killing of amastigotes due to the lack of granuloma formation (hypothesis 5) or to the presence of immuno-suppressive alternatively activated

macrophages (hypothesis 7) leads to the slightly increased *L. donovani* amastigote numbers (v). However, there was no evidence of heavy accumulation of amastigote numbers within foci of infection in either the parenchyma or granulomatous tissue of CO-INFECT mice and it is concluded that a variety of mechanisms could account for a reduced killing of amastigotes within infected macrophages in CO-INFECT mice in particular within the egg granuloma but that this does not result in accumulation of amastigotes within the cell compared with LEISH mice but rather results in cell breakdown and subsequent infection of new cells rather than amastigote killing.

The various ways in which the strongly polarised Th2 response could inhibit anti–*L. donovani* immunity have been described earlier (5.1). Apart from influencing the development of Th1 cells themselves, IL-4, IL-10 and the other Th2 cytokine IL-13 are also able to inhibit the microbicidal activities of macrophages exposed to Th1 cytokines, by inducing arginase-1 production which competes with inducible NO synthase for L-arginine, the common substrate for both enzymes (Modolell *et al.,* 1995). IL-10 strongly synergizes with IL-4 and IL-13 by strongly upregulating IL-4Rα. (Lang *et al.,* 2002). IL-10 also inhibits macrophage migration inhibition factor (MIF) which like IFN can also induce NO production from human monocytes (Wu *et al.*, 1993). IL-10 has recently been shown to inhibit the expression of a large proportion of the genes activated by the Th1 stimulus LPS including numerous pro-inflammatory genes *e.g.* IL-12, IL-6 and TNF-α (Lang *et al.,* 2002).

As also shown in the present studies spleen cells from infected mice are able to produce IL-10 in response to SEA stimulation and this was shown to inhibit APC function resulting in the suppression of SEA mediated proliferation of SEA specific Th1 cell clones (Flores Villanueva *et al.,* 1993). This activity started at 5 weeks post *S. mansoni* and peaked at +8 weeks thereafter declining a little to +14 weeks but still being significant at +23 weeks. La Flamme *et al.* (2002) have shown that peritoneal macrophages from 6-8 week *S. mansoni* infected mice activated with IFN-γ were markedly less able to kill *L. major in vitro* as judged by increased rates of infected macrophages and this corresponded to impaired production of NO. The fact that this effect was seen with peritoneal macrophages shows the systemic nature of the inhibition of leishmanicidal activity. Such inactivation could result from the effects of IL-10 or TGF-β both of which are produced in high amounts in schistosome infection (Grzych *et*

al., 1991; Pearce *et al.*, 1991) and which have been shown to inhibit IFN-γ mediated killing of *L. major* by macrophages (Barral-Netto *et al.*, 1992; Vouldoukis *et al.*, 1997). IL-10 has also been shown to inhibit such killing of *L. infantum* (Vouldoukis *et al.*, 1997). *L. donovani* amastigotes infecting human monocytes *in vitro* can be killed by treatment with MIF but this is also prevented by prior exposure of human monocytes to IL-10 (Wu *et al.*, 1993). As an extension of this work it would be valuable to assess the *in vitro* leishmanicidal activity of activated peritoneal macrophages from *S. mansoni* infected mice for *L. donovani* as carried out for *L. major* (La Flamme *et al.*, 2002) and to use macrophages from the different time points in the infection studied here (+2 - +8 weeks). It would also be interesting to compare this activity with that of egg granuloma macrophages. Finally it would also be valuable to compare the "alternatively activated" status of *L. donovani* infected macrophages within the liver parenchyma, egg granulomas and the spleen of CO-INFECT mice as judged by double staining for macrophage mannose receptor using the specific monoclonal antibody (Linehan *et al.*, 2003) and for amastigotes using the immune hamster serum as used above.

Chapter 6

Interaction between an established *L. donovani* infection and a superimposed *S. mansoni* infection

6.1. Introduction

The experiments in this chapter were designed to give insight into the effects of prior exposure to *L. donovani* on a subsequent *S. mansoni* infection. In contrast to the studies in the previous chapters in which a *S. mansoni* infection was established first and a Th2>Th1 environment created, the design in this Chapter meant that the Th1>Th2 response induced by prior *L. donovani* was established first.

A number of possible effects were considered possible as outlined below:

As described in earlier sections and demonstrated in the mouse experiments in Chapter 3 *L. donovani* results in a Th1>Th2 response (Engwerda *et al.,* 1996, Miralles *et al.,* 1994) in which the key endogenous cytokines leading to control of the disease are IL-12, IL-2, IFN-γ, TNF-α, and GM-CSF (Murray *et al.,* 1993; 1995; 2000; Tumang *et al.,* 1994; Murray. 1997; Engwerda *et al.,* 1998; Satoskar *et al.,* 2000; Murray and Delph-Etienne, 2000). In C57BL/6 mice this Th1 response increases to a peak at around 4 weeks of infection when parasite numbers plateau and start to decline (Murray, 2001a). Superinfection of *L. donovani* infected mice with *S. mansoni,* once this Th1 response had developed, would result in the schistosome larvae migrating through the lungs when this Th1 response was ongoing. The lung stage of schistosome migration is particularly susceptible to immune mediated trapping including by Th1 (IFN-γ) mediated inflammation in immune mice exposed to irradiated cercariae (Coulson, 1997; Wilson and coulson, 1999). A significant proportion of lung stage schistosomula appeared to be trapped in the lungs of normal animals (Mangold and Dean. 1992) and it was considered possible that enhanced levels of systemic Th1 response might be present as a result of the *L. donovani* infection and lead to

bystander enhancement of the innate trapping response leading to reduced survival of larvae due to the promotion of innate trapping of schistosomula.

The acute response to the schistosome egg results in rapid induction of a Th-biased response in which Th2 cytokines, IL-4, IL-5 and IL-13 predominate and the resulting egg granuloma comprises a large proportion of eosinophils and fibroblasts, components of the immune response associated with IL-5 and IL-13 respectively (Hesse *et al.,* 2001; Chiaramonte *et al.,* 1999; Kaplan *et al.,* 1998). Despite the Th2 dominated response, immuno-modulating Th1 responses are present as seen by the increase in granuloma size following IFN-γ neutralisation (Luckacs and Boros, 1993). If the Th1 response to the egg antigens is promoted by prior immunization with eggs plus rIL-12, the granuloma size and fibrosis is reduced resulting in enhanced survival (Wynn *et al.,* 1994). However, if the response to the egg is strongly skewed to Th1, as in infected IL-4 KO (Brunet *et al.,* 1997) or IL-4/IL-10 double KO (Hoffmann *et al.,* 2000) there is high production of pro-inflammatory cytokines and higher mortality. So in the present studies it was considered possible that the nature or size of the schistosome granuloma and morbidity might be altered by the Th1 biased response induced by a pre-existing *Leishmania donovani* infection. Depending on the level of putative skewing towards a Th1 response to the egg antigens this could either reduce the egg granuloma size and reduce disease or lead to damaging pro-inflammatory responses.

However, although the acute phase of *L. donovani* infection in mice results in strong Th1 responses which control the infection it is known that *L. donovani* causes T cell immuno-suppression (Alvar, 1994; Zwingenberger *et al.* 1990; Bogdan *et al.,* 1996) and that the morbidity and mortality associated with infection is often due to secondary infections (Kadivar *et al.,* 2000; Desjeux, 1992). Immuno-deficiency may occur during the chronic stage of the disease in both humans and mice (Ho *et al.,* 1983; Gomes *et al.,* 1998; Dasgupta *et al.,* 1999) and T cell anergy to multiple antigens and PHA has been described in visceral leishmaniasis patients (Aikat *et al.,* 1979; Ho *et al.,* 1983).

Leishmania parasites have been shown to induce a variety of immune evasion mechanisms which results in their persistence (Bogdan and Rollinghoff, 1998; Zambrano-Villa *et al.,* 2002). Although *L. donovani* infection in the liver induces mRNA expression of Th1 (IFN-γ, IL-2, IL-12, TNF-α), immuno-regulatory cytokines associated with Th2 responses (IL-4, and IL-10) are also expressed in the liver over the first 28 days of infection (Engwerda *et al.,* 1996, Miralles *et al.*, 1994), more so in certain strains of mice than others. Th2 associated cytokines (IL-4, PGE2, TGF-β and IL-10) appear important in regard to pathogenicity since they can suppress specific T cell proliferation and inhibit type 1 cytokine synthesis and production and/or host defense effects of activating cytokines, including IFN-γ and IL-2 (Howard, 1986; Mosmann and Coffman, 1989; Silva *et al.,* 1992). Endogenous IL-10 might inhibit cellular immune responses not just to *Leishmania* antigens but also to bystander antigens resulting in T cell anergy to multiple antigens and PHA as observed in some VL patients (Aikat *et al.,* 1979; Ho *et al.,* 1983). With respect to the current study on superimposed *S. mansoni*, IL-10 has been implicated in inhibiting the size of acute stage granulomas and in bringing about the down-regulation of granuloma size in chronic infection (Sadler *et al.,* 2003). Leishmanial infection also suppresses: antigen presenting activity of macrophages by reduced expression of MHC-II molecules through inhibition of PKC activity (Bhattacharyya *et al.,* 2001a; 2001b); co-stimulatory molecule expression such as B7.1, B7.2, and ICAM-1 (Saha *et al.,* 1995); production of oxygen intermediates (ROI), especially H_2O_2 (Murray, 1982; Murray *et al.*, 1983) and reactive nitrogen intermediates (RNI) (Roach *et al.,* 1993) leading to reduced macrophage leishmanicidal activity. Recently Engwerda *et al.* (1998) have shown splenic marginal zone macrophages disappear at 28 days post infection of mice with *Leishmania donovani*. This disruption of lymphoid architecture persists and may cause insufficient antigen presentation to T cells and also effect lymphocyte trafficking during chronic leishmaniasis (Engwerda and Kaye, 2000; Smelt *et al.,* 1997). Dendritic cells also failed to migrate from the marginal zone (MZ) to the peri-arteriolar regions (PALs) of the spleen during chronic *L. donovani* infection and showed reduced CCR7 expression, and poor responsiveness to the CCR7 ligands CCL21 and CCL19 (Ato *et al.,* 2002). The stromal cells were also fewer resulting in the associated loss of CCL21 and CCL19 expression and of extracellular matrix components (the reticular network) which are produced by the stromal cells in the T cell

area (Ato *et al.,* 2002). This stromal cell loss during chronic infection would effect the chemokine-mediated interactions between DCs and T cells. So chronic infection of the spleen resulting in disruption of the microarchitecture of both the B cell follicles and the MZ is considered likely to result in reduced presentation of antigens from other infections causing increased susceptibility to concomitant infections (Engwerda and Kaye, 2000; Engwerda *et al.,* 2002; Smelt *et al.,* 1997). Other immunosuppressive effects reported include disruption to lymphocyte migration (Garside *et al.*, 1998; Sallusto *et al.,* 1999), and loss of $CD45RO^+$ memory T cells, a process which is reversed by chemotherapy (Cillari *et al.*, 1995). There are also mechanisms resulting in suppression of IFN-γ production and it's principal inducer, IL-12 (Kaye *et al.,* 1991; Gorak *et al.,* 1998; Reiner *et al.,* 1994; Engwerda *et al.*, 1998; Taylor and Murray, 1997; Murray *et al.,* 2000) and of TNF-α production (Theodos *et al.,* 1991). Various of these effects are believed to contribute to the immuno-compromised status of patients with VL and influence the response to concomitant infections. Despite the common acceptance that immuno-suppression is a key feature of *L. donovani* infection in humans and also in mice, there have been no experimental investigations to demonstrate this. The superinfection experiments planned in this chapter were designed so that the schistosome eggs would be produced at a time when the disruption to the splenic architecture and function was known to have occurred i.e. 8 weeks post *L. donovani* infection.

Another alternative outcome considered possible was that the strong Th2 response associated with the eggs might still develop and serve to reduce the protective Th1 response that normally develops at 2 weeks and peaks at 4 weeks post *L. donovani* infection, and would be expected to have reduced the *L. donovani* parasite load dramatically by the time the eggs were produced. As judged by PCR analysis, IL-4 and IL-10 mRNAs are also expressed by week 2 after *L. donovani* infection and it has been suggested that this Th2 cell response might contribute to the logarithmic increase in parasite replication during the first weeks after challenge (Murray *et al.,* 1987) before the Th1 response dominates. So if the strong Th2 cytokine response induced by the incoming *S. mansoni* eggs could inhibit the Th1 response controlling the *L. donovani* infection directly or by restimulating the *L. donovani* specific Th2 component of the immune response there could be a recrudescence

of LDU. Furthermore, the *S. mansoni* egg granulomas containing numerous anti-proliferative, alternatively activated marcrophages (Flores Villanueva *et al.*, 1994a) could provide additional and favourable sites for *L. donovani* development as discussed in Chapter 5.

So the aims of this work were:-

1 To study the effect of the predominant Th1 cytokine response associated with a pre-exsisting *L. donovani* infection on the establishment of a superimposed *S. mansoni* infection and on the production of schistosome eggs.

2 To determine if a Th2 response induced by the superimposed *S. mansoni* infection could inhibit or reverse the development of Th1 mediated control of the *L. donovani* infection leading to enhanced LDU.

3 To determine if the immune response to the schistosome infection was altered by the pre-existing *L. donovani* infection resulting in altered egg granuloma formation.

4 To determine if the immune response to the *L. donovani* infection is altered by the superimposed *S. mansoni* infection

6.2. Results

For the experiments described in this chapter it was planned that the *L. donovani* infection would be given and then 2 weeks later the mice superinfected with *S. mansoni*. This meant that the Th1 response to the *L. donovani* infection would (i) be establishing at the time when the larval schistosomes were migrating through the lungs (1 week post *S. mansoni* and 3 weeks post *L. donovani* infection) during which stage they are susceptible to Th1 mediated attrition in immune animals (Coulson, 1997) and (ii) would be fully developed at a time when the *S. mansoni* eggs were first produced (5-6 weeks post *S. mansoni* infection, 8 weeks post *L. donovani* infection) and when they would normally induce a marked Th2 response. This timing also meant that the egg production in the liver would start when the

L. donovani infection in the liver was reducing but not resolved and so could be affected by a developing Th2 response to the eggs.

Several problems were encountered in trying to carry out this experiment and four separate attempts were made. In the first experiment, using 25 cercariae per mouse there was poor *S. mansoni* infectivity so that at perfusion, insufficient numbers of mice had bisexual infections. Therefore in subsequent experiments the number of cercariae given was increased to 50 and then to 100. A second experiment had to be terminated because of a viral infection in the Biological Services Unit which necessitated all mice being culled. Two further experiments were completed and the data for both is presented below but, in the first of these, the *L. donovani* infectivity was low. Eventually two experiments were completed.

Parasitological results – Experiment 1.

6.2.1. Experimental design – Experiment I

Thirty two female C57BL/6 mice were divided into 4 groups. Initially 16 mice were each infected with *Leishmania donovani* strain at $2x10^7$ amastigotes of LV9/ml i.v. via the tail vein. Two weeks after the *L. donovani* infection 8 mice were super-infected with 50 *Schistosoma mansoni* cercariae via the percutaneous route (CO-INFECT). The remaining 8 mice were given the schistosome infection only (SCHISTO). A further group of 8 mice were left with the *Leishmania* infection alone (LEISH). Finally a group of 8 mice were untreated (CONTROLS). All mice were sacrificed at +8 weeks post *L. donovani* infection.

6.2.2. Parasitological results (Experiment I)

6.2.2.1. <u>*S. mansoni* worm and egg burdens.</u>

Worm and egg burdens from the SCHISTO and CO-INFECT groups are shown in Table 6.1.

Table 6.1. Mean adult worm and geometric mean liver egg burdens (±SD) in SCHISTO and CO-INFECT groups at +8 week after *S. mansoni* super-infection.

Group	Time (wk)	Mean worm count		#Geometric mean egg count (±SD)	Geometric mean eggs/ worm pair (±SD)
		Total (±SD)	Pairs* (±SD)		
SCHISTO	+8	25 (±2.01)	9.3 (±1.38)	22141 (±6922)	8516 (±3914)
CO-INFECT	+8	22.9 (±1.7)	9.6 (±0.4)	16273 (±3658)	4282 (±1197)

Table 6.1. C57BL/6 mice were infected i.v. with *L. donovani* and 2 weeks later superinfected with 50 *S. mansoni* cercariae. The mice were perfused at +8 weeks and livers digested for egg counting. # Egg count expressed as total for the whole liver. *Estimated from the number of mature female and male worms recovered in each animal. Data is based on the mean number of animals which developed bisexual, egg producing schistosome infections (6 CO-INFECT, 7 SCHISTO mice).

All mice which had been exposed to schistosome cercariae were found to have adult worms. There were no significant differences between the mean total worm or worm pair recoveries from the two groups. Despite this the (geometric mean egg count) GECs were lower in the CO-INFECT group compared with the SCHISTO group. Consequently, the GEC/worm pair values were lower for the CO-INFECT group compared with the SCHISTO giving some suggestion of fecundity suppression/more rapid egg destruction following the *Leishmania* infection. However, these results were not statistically significantly different.

6.2.2.2. *L. donovani* parasite load.

The Leishman Donovan units (LDU) are shown in Figure 6.1.

It was clear from looking at the impression smears in this experiment that amastigotes were very scanty and this is reflected in the LDU counts. In spite of this there were no significant differences between either the liver or spleen LDU counts in the CO-INFECT and LEISH mice.

Figure 6.1. Comparison of Leishman Donovan Units (LDU) from Liver and Spleen imprints at +8 weeks post *S. mansoni* superinfection.

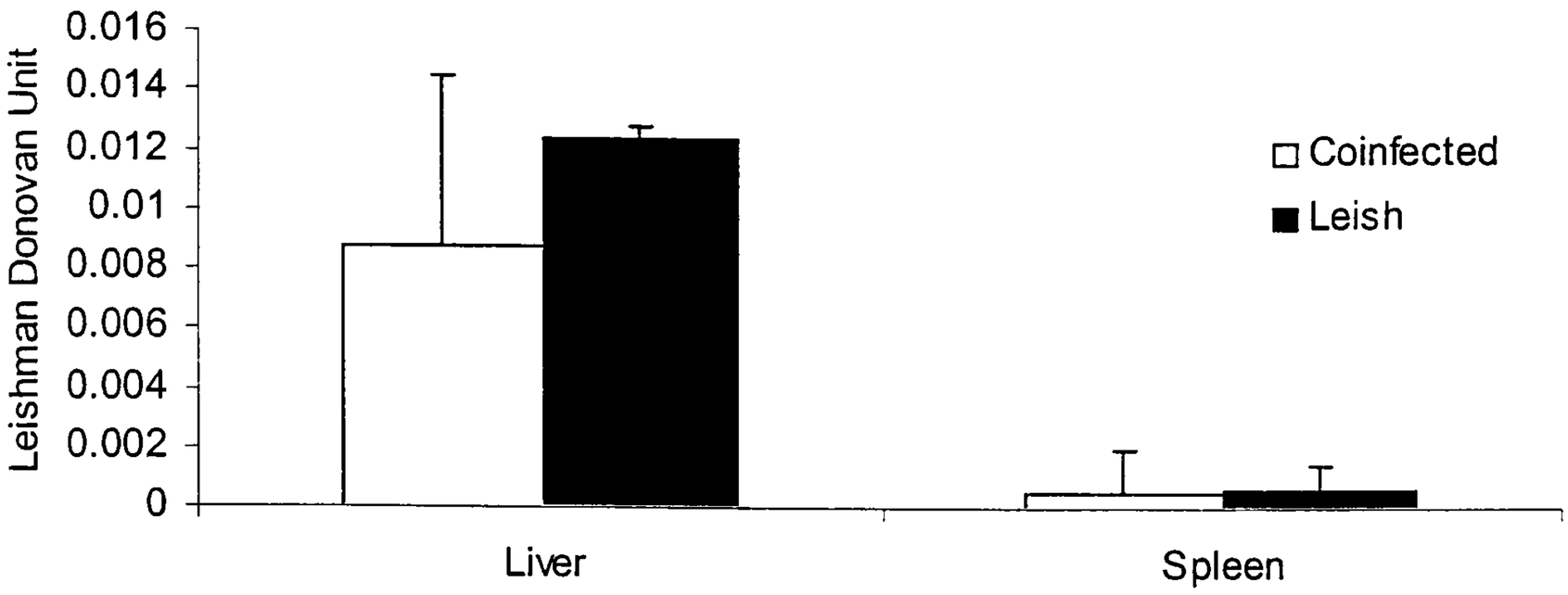


Figure 6.1. Graphs show the mean (±SE) LDU for 7/8 mice from the CO-INFECT and LEISH groups. LEISH and CO-INFECT mice were infected i.v. with *L. donovani* and the CO-INFECT mice superinfected with 50 *S. mansoni* cercariae 2 weeks later. Mice were sacrificed 8 weeks later (+8 weeks).

6.2.3 Experimental design – Experiment II

This is essentially a repeat experiment with the same experimental design as in Experiment I (6.2.1) except that the *S. mansoni* infection was doubled to 100 cercariae in order to ensure a potent schistosome infection. There were 8 mice per group for CO-INFECT SCHISTO and CONTROLS and 5 mice for LEISH.

6.2.4. Parasitological results (Experiment II)

6.2.4.1. *S. mansoni* worm and egg burdens.

Worm and egg burdens from the SCHISTO and CO-INFECT groups are shown in Table 6.2.

In this repeat experiment, the overall *S. mansoni* worm burden was higher than in the first experiment as expected from the higher number of cercariae in the infection. Unlike the first experiment the CO-INFECT mice showed a lower infection rate compared with the SCHISTO animals. This was seen with both the total worm burdens (24.5% lower in CO-INFECT cf SCHISTO, p= 0.03) and in worm pairs (29.3% lower, p=0.05). The total liver egg burden was also reduced by a similar percentage in the CO-INFECT (26%) but this

was not statistically significant. The tissue eggs/worm pair were almost exactly comparable giving no indication of fecundity suppression.

Table 6.2. Mean adult worm and geometric mean liver egg burdens (±SD) in SCHISTO and CO-INFECT groups at +8 week after *S. mansoni* superinfection.

Group	Time (wk)	Mean worm count		#Geometric mean egg count (±SD)	Geometric mean eggs/ Worm pair (±SD)
		Total (±SD)	Pairs (±SD)		
SCHISTO	+8	41.3 (±2.4)	18.4*(±1.4)	53823 (±8915)	2925.2 (±6367.9)
CO-INFECT	+8	30.8 (±3.8)	13 (±2.3)	38789 (±10156)	2983.8 (±4415.7)

Table 6.2. C57BL/6 mice were infected i.v. with *L. donovani* and 2 weeks later, superinfected with 100 *S. mansoni* cercariae. The mice were perfused at +8 weeks and livers digested for egg counting. # Egg count expressed as total for the whole liver. *Estimated from the number of mature female and male worms recovered in each animal. Data is based on the mean number of animals which developed bisexual, egg producing schistosome infections (5 mice/group).

6.2.4.2. *L. donovani* parasite load

The mean Leishman Donovan units (LDU) are shown in Figure 6.2. In this experiment the spleen and liver LDU values were more similar to the expected values for a 10 week *L. donovani* infection (*e.g.* similar to the values in Experiments 1 and 2, Chapter 3) than in Experiment 1 above. Unexpectedly and interestingly the CO-INFECT mice produced markedly lower levels of parasitic burden (LDU) in both liver and spleen imprints compared to the LEISH animals (95.5% lower for the liver values, p=0.04, and 93.8% lower for the spleen values 0.05 respectively).

Figure 6.2. Comparison of Leishman Donovan Units (LDU) from Liver and Spleen imprints at +8 weeks post *S. mansoni* superinfection.

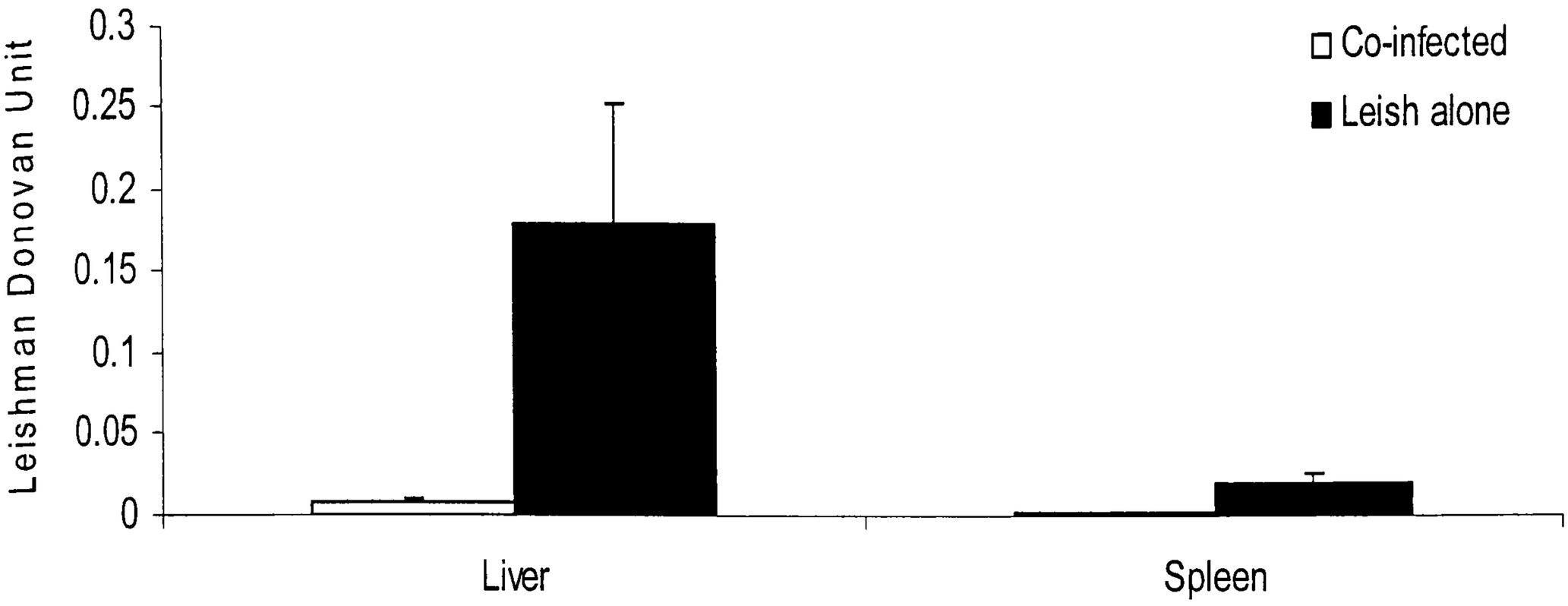


Figure 6.2. Graphs show the mean (±SE) LDU for 5/8 CO-INFECT mice and 5/5 mice in LEISH groups. LEISH and CO-INFECT mice were infected i.v. with *L. donovani* and the CO-INFECT mice superinfected with 100 *S. mansoni* cercariae 2 weeks later. Mice were sacrificed 8 weeks later (+8 weeks).

6.2.5. Immunology results - Experiment I

6.2.5.1. Detection of cytokine production from spleen lymphocytes by ELISA

Cytokine production (IFN-γ and IL-4 as markers of Th1 and Th2 responsiveness respectively), was assessed in supernatants obtained from spleen cells from three individual mice per group. Cells were cultured *in vitro* at a concentration of $5x10^6$/ml with medium alone, mitogen (ConA), schistosome soluble egg antigen [SEA], and leishmanial antigen (formalin fixed amastigote antigen [FLAA]).

6.2.5.1.1. *Levels of IL-4*

As shown in Figure 6.3, at 10 weeks (+8 wks post *S. mansoni*) the splenocyte *in vitro* cell cultures from both SCHISTO and CO-INFECT groups produced raised levels of IL-4 in the presence of mitogen compared with the CONTROLS (p=0.05 and 0.0005 respectively) and the LEISH animals (p=0.04 and 0.0004 respectively). Moreover, both CO-INFECT and SCHISTO animals produced raised levels of IL-4 in response to SEA although this difference was only statistically significant for the CO-INFECT animals relative to the

LEISH animals and the CONTROLS (p=0.0003 and 0.0007 respectively). There were no significant differences between the IL-4 levels in the CO-INFECT and SCHISTO groups stimulated with either ConA or SEA. There was no IL-4 detectable in the presence of FLAA from any of the other groups.

Figure 6.3. Comparison of the levels of IL-4 in supernatants of spleen cell cultures

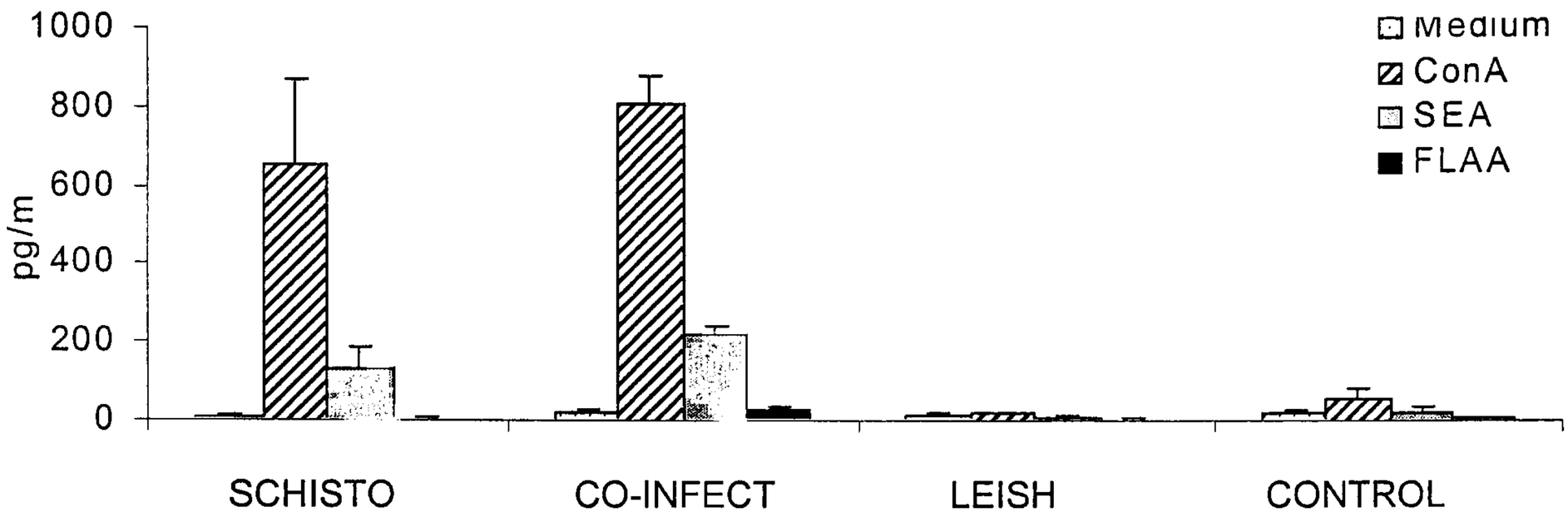


Figure 6.3. Graphs showing the means and standard errors for IL-4 production by spleen cells from three mice in each group sacrificed at +8 weeks after the *S. mansoni* superinfection. Cultures of $5x10^6$cells/ml were stimulated with medium alone, ConA at 5μg/ml, SEA at 20μg/ml or fixed leishmanial amastigote antigen (FLAA) at 10^7/ml. LEISH and CO-INFECT mice were infected i.v. with *L. donovani* and the CO-INFECT mice superinfected with 100 *S. mansoni* cercariae 2 weeks later along with a previously uninfected group SCHISTO, CONTROL mice were uninfected naïve mice.

6.2.5.1.2. *Levels of IFN-γ*

As shown in Figure 6.4, the LEISH and CONTROL animals produced higher levels of IFN-γ in response to Con A compared with the SCHISTO and CO-INFECT mice but this was only significantly higher for the CONTROL animals (p=0.05, and 0.01 relative to CO-INFECT and SCHISTO respectively).

In the presence of FLAA high IFN-γ responses were seen with both the LEISH and CO-INFECT groups and these were significantly higher than the levels in the CONTROL (p=0.0002 and 0.008 respectively), and SCHISTO animals (p=0.0002 and 0.008 respectively). IFN-γ production stimulated by FLAA was lower in the CO-INFECT group

compared with the LEISH group but this was not significantly significant. Minimal levels of IFN-γ to SEA were seen in the SCHISTO and CO-INFECT animals.

Figure 6.4. Comparison of the levels of IFN-γ in supernatants of spleen cell cultures

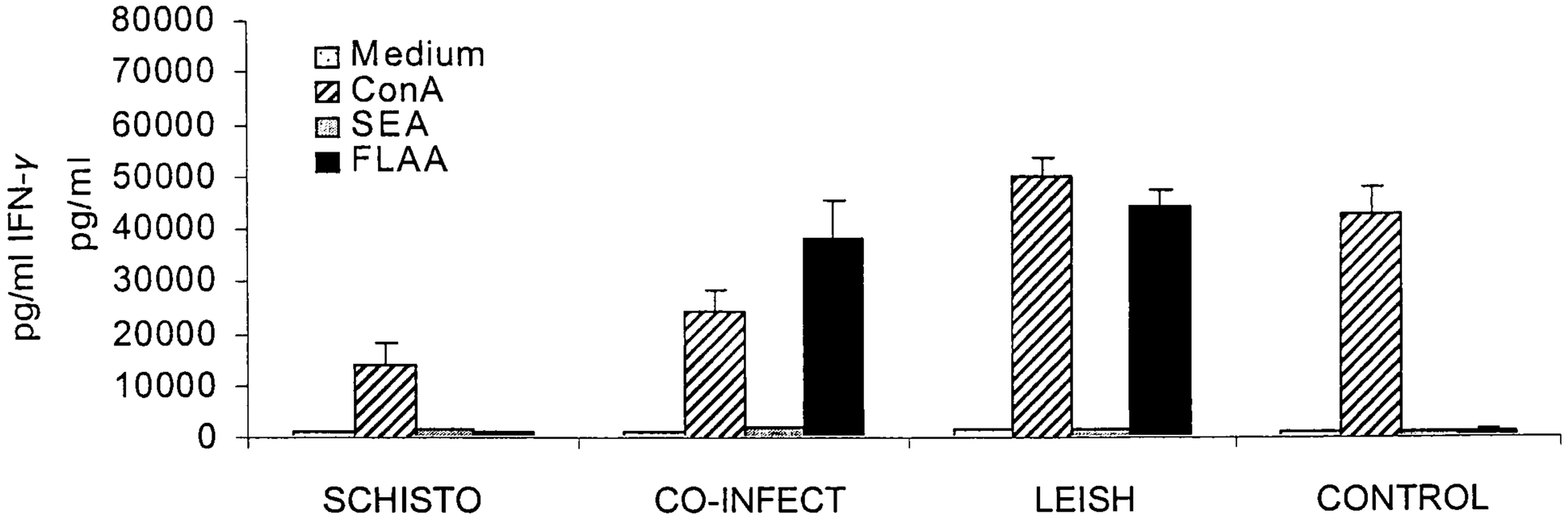


Figure 6.4. Graphs showing the means and standard errors for IFN-γ production by spleen cells from three mice in each group sacrificed at +8 weeks after the *S. mansoni* superinfection. Cultures of $5x10^6$cells/ml were stimulated with medium alone, ConA at 5μg/ml, SEA at 20μg/ml or fixed leishmanial amastigote antigen (FLAA), at 10^7/ml. For definition of groups see legend to Figure 6.3.

6.2.5.1.3. *Levels of Nitric Oxide (Nitrite).*

The level of nitrite in the spleen cultures should be a reflection of the balance of the various influences acting to activate or modulate activation of the macrophages in the cultures. The results are shown in Figure 6.5.

Although the levels of nitrite were modest, cells from the CO-INFECT animals produced the highest level of nitric oxide in response to mitogen (ConA) ($p=0.03$ relative to CONTROLS). The SCHISTO animals showed significantly elevated nitrite levels compared to both the LEISH and CONTROL animals ($p=0.009$ and 0.01 respectively). In response to FLAA, the CO-INFECT animals showed the highest levels of nitrite but the significance of this is uncertain as the SCHISTO animals also showed higher levels of nitrite than the LEISH animals.

Figure 6.5. Comparison of the levels of nitrite in supernatants of spleen cell cultures

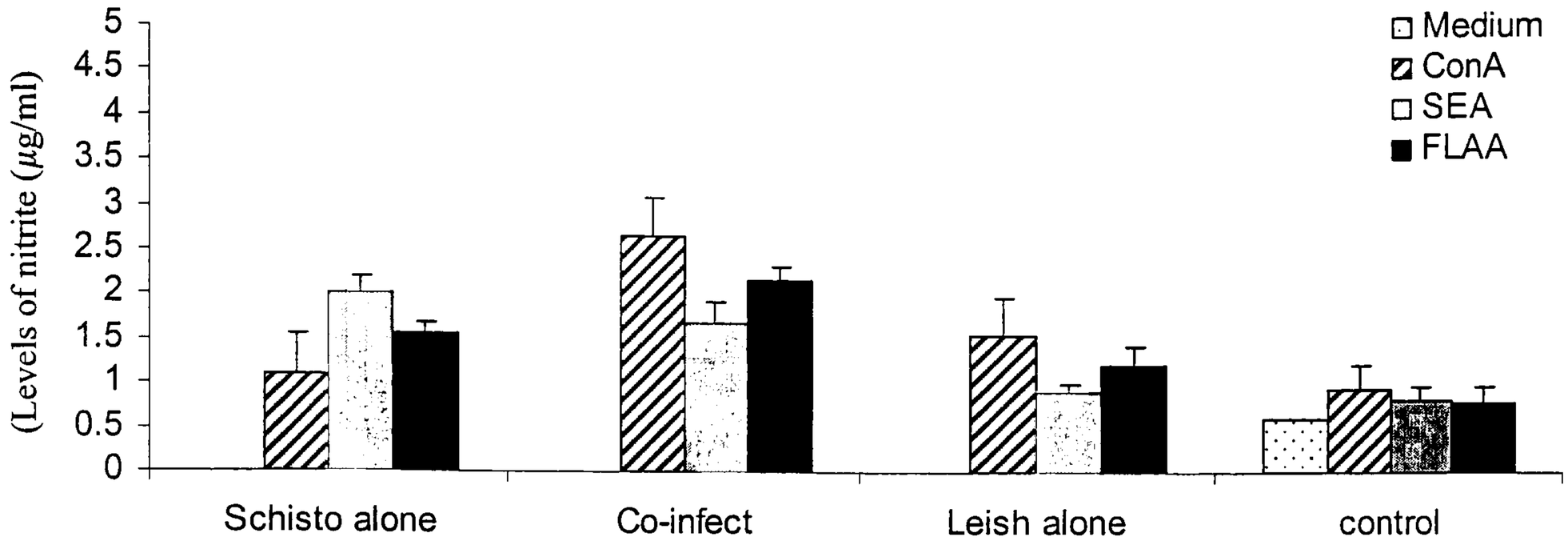


Figure 6.5. Graphs showing the means and standard errors for nitrite production by spleen cells from three mice in each group sacrificed at +8 weeks after the *S. mansoni* superinfection. Cultures of $5x10^6$cells/ml were stimulated with medium alone, ConA at 5μg/ml, SEA at 20μg/ml or fixed leishmanial amastigote antigen (FLAA) at 10^7/ml. For definition of groups see legend to Figure 6.3.

6.2.6. Immunology results - Experiment II

6.2.6.1. Detection of cytokine production from spleen lymphocytes by ELISA

6.2.6.1.1. *Levels of IL-4*

As can be seen in Figure 6.6 the pattern of IL-4 production was essentially similar to that in Experiment I although the levels of IL-4 were lower than in the above experiment (Figure 6.3.) which is surprising given the higher level of worm and egg burdens in this second experiment.

As shown in Figure 6.6, the splenocyte cell cultures from both SCHISTO and CO-INFECT groups produced raised levels of IL-4 compared with the CONTROLS in the presence of medium alone (P=0.04 and 0.01 respectively), ConA (p=0.009 and 0.007 respectively), and SEA (p=0.004 and <0.0001 respectively). Moreover, both the SCHISTO and CO-INFECT groups produced raised levels of IL-4 compared with the LEISH animals in the presence of medium alone (P=0.01 and 0.005 respectively), mitogen conA (p=0.02 and 0.02

respectively), and SEA (p=0.002 and <0.0001 respectively). There was no IL-4 detectable in the presence of FLAA for any of the other groups.

Figure 6.6. Comparison of the levels of IL-4 in supernatants of spleen cell cultures

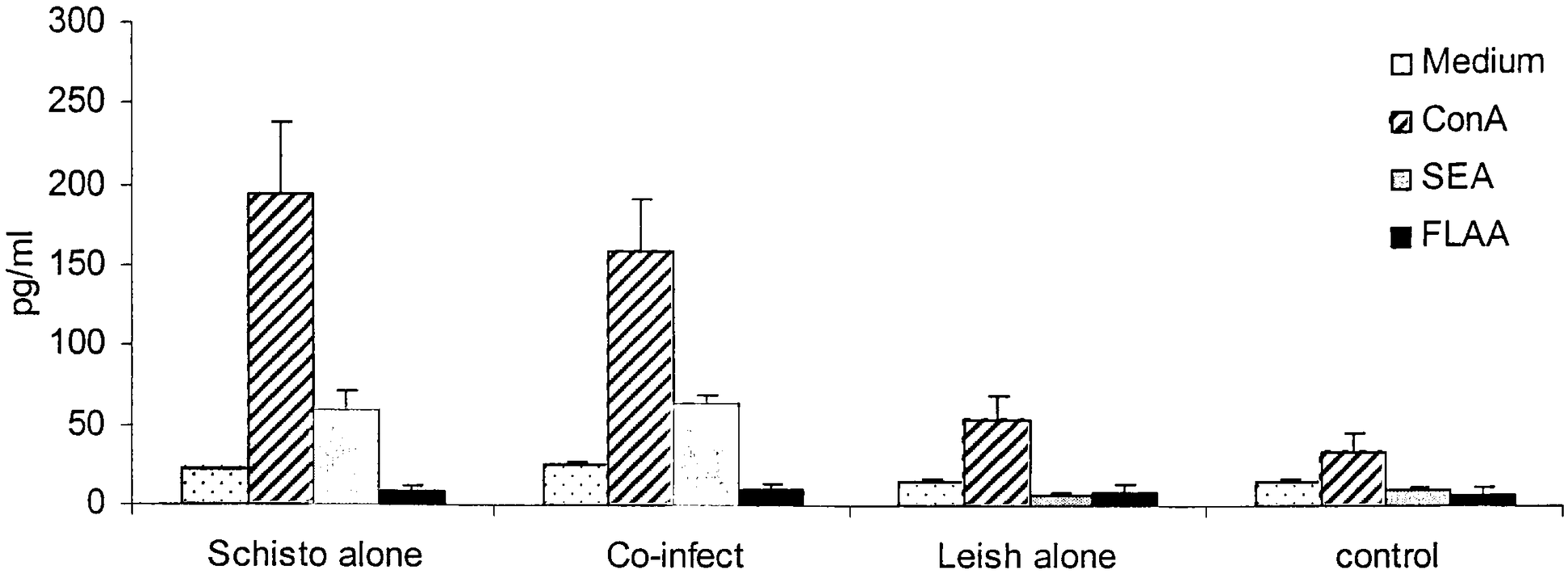


Figure 6.6. Graphs showing the means and standard errors for IL-4 production by spleen cells from three mice in each group sacrificed at +8 weeks after the *S. mansoni* super-infection. Cultures of $5x10^6$cells/ml were stimulated with medium alone, ConA at 5μg/ml, SEA at 20μg/ml or fixed leishmanial amastigote antigen (FLAA) at 10^7/ml. For definition of groups see legend to Figure 6.3.

6.2.6.1.2. *Levels of IL-5*

In view of the low levels of IL-4 recorded, an IL-5 cytokine ELISA was also used on the supernatant in this experiment.

As shown in Figure 6.7, none of the splenocyte *in vitro* cell cultures showed significant IL-5 production in medium alone. However, in response to ConA, both the SCHISTO and CO-INFECT groups produced high levels of IL-5, significantly higher than in the CONTROLS and LEISH animals ($P<0.0001$ for all). Similarly SEA induced markedly elevated IL-5 production by the SCHISTO and CO-INFECT animals which was significantly elevated compared with the LEISH and CONTROL groups ($p=0.007$ and 0.002 respectively for the SCHISTO groups and $p=0.05$ and 0.003 for the CO-INFECT). None of the groups made significant levels of IL-5 to FLAA.

Figure 6.7. Comparison of the levels of IL-5 in supernatants of spleen cell cultures.

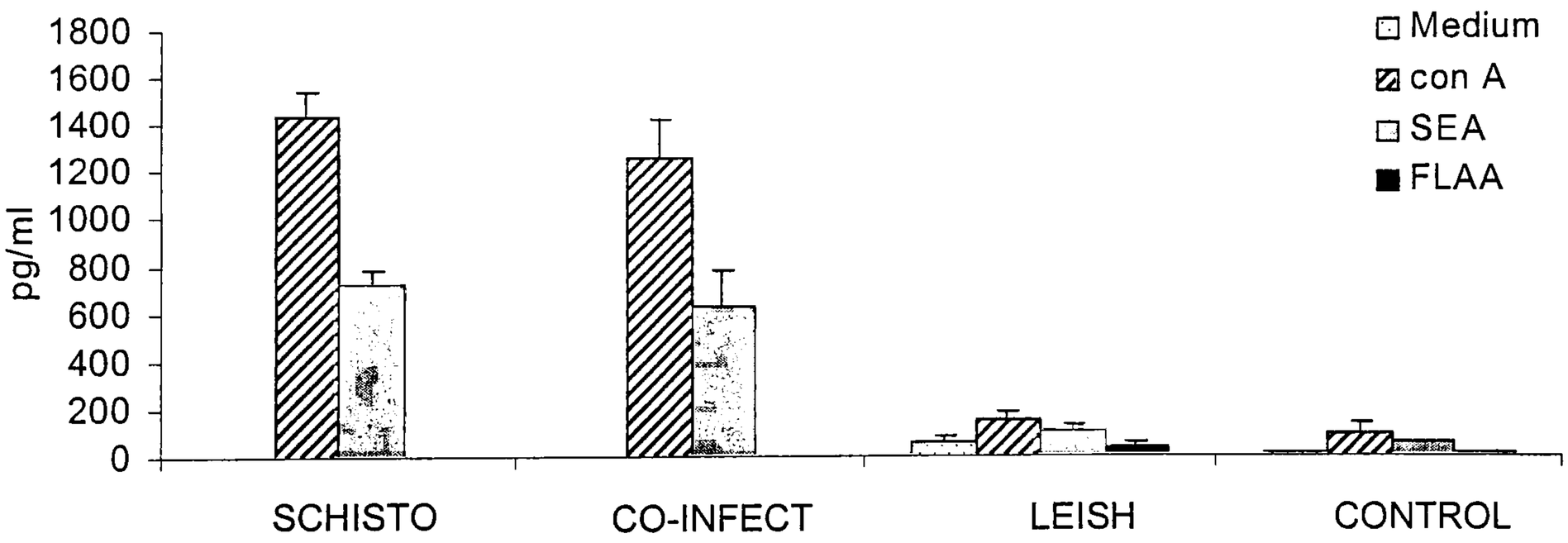


Figure 6.7. Graphs showing the means and standard errors for IL-5 production by spleen cells from three mice in each group sacrificed at +8 weeks after the *S. mansoni* superinfection. Cultures of $5x10^6$cells/ml were stimulated with medium alone, ConA at 5μg/ml, SEA at 20μg/ml or fixed leishmanial amastigote antigen (FLAA) at 10^7/ml. For definition of groups see legend to Figure 6.3.

6.2.6.1.3. *Levels of IFN-γ*

As shown Figure 6.8., all groups produced comparable levels of IFN-γ in the presence of medium alone.

In the presence of ConA, as in the first experiment (Figure 6.4.) the LEISH animals produced the highest levels of IFN-γ but this was not statistically significantly different from the other groups. However, the CONTROL cells produced somewhat lower IFN-γ than in the first experiment whereas the SCHISTO and CO-INFECT were similarly low.

In response to SEA, minimal levels of IFN-γ were produced in any of the groups. In the presence of FLAA the highest response was from the LEISH group (p=0.05) relative to CONTROLS. The CO-INFECT animals showed noticeably lower levels of IFN-γ compared with LEISH animals although this was also significant compared to CONTROLS (p=0.03). The difference in IFN-γ production between the CO-INFECT and LEISH groups was not significantly significant but did parallel the difference in LDU values (i.e. both IFN-γ and LDU low in the CO-INFECT).

Figure 6.8. Comparison of the levels of IFN-γ in supernatants of spleen cell cultures

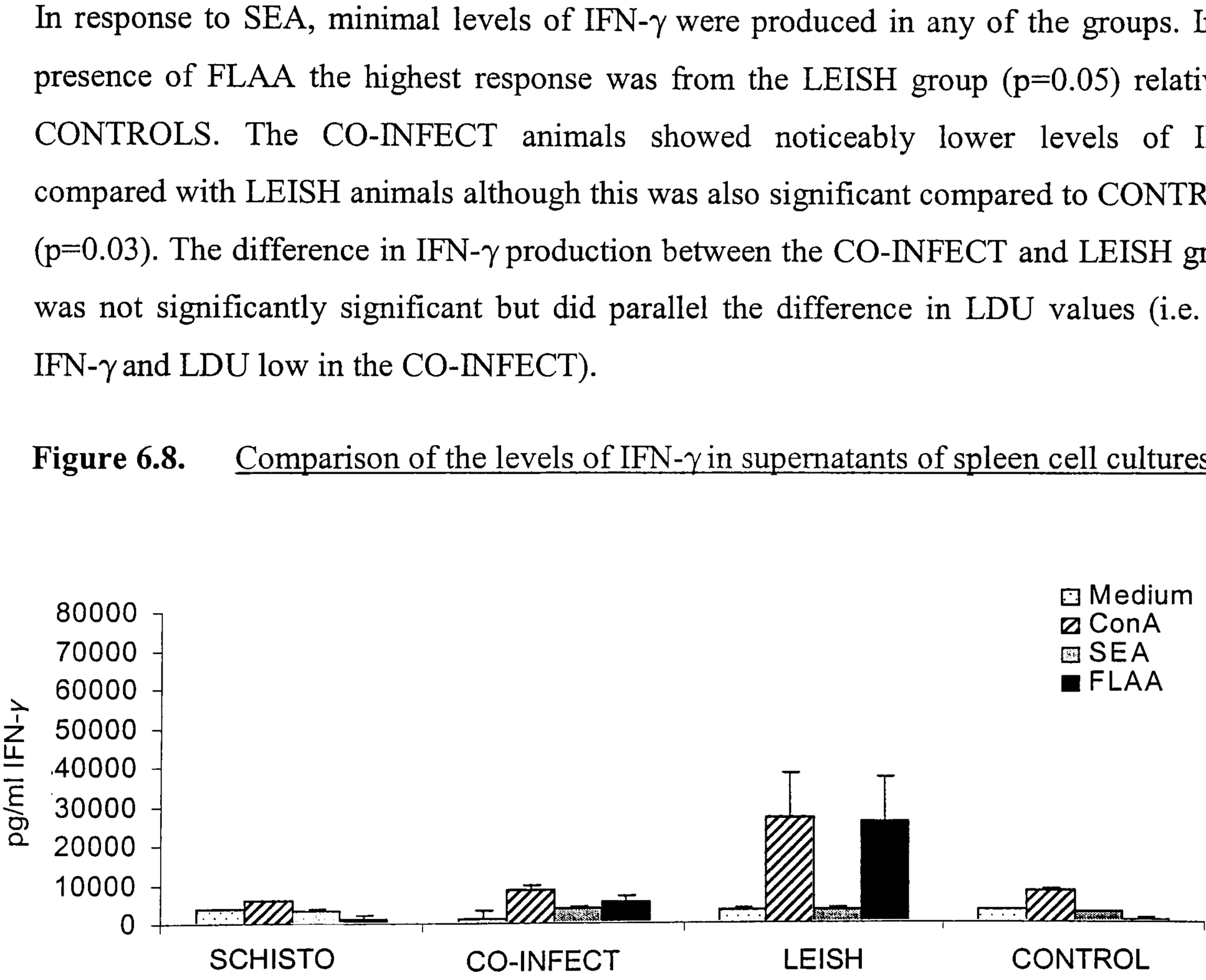


Figure 6.8. Graphs showing the means and standard errors for IFN-γ production by spleen cells from three mice in each group sacrificed at +8 weeks after the *S. mansoni* superinfection. Cultures of $5x10^6$cells/ml were stimulated with medium alone, ConA at 5μg/ml, SEA at 20μg/ml or fixed leishmanial amastigote antigen (FLAA) at 10^7/ml. For definition of groups see legend to Figure 6.3.

6.2.6.1.4. Levels of Nitric Oxide (Nitrite).

The levels of nitrite detected were low and very similar to the first experiment. The CO-INFECT animals produced the highest level of NO production in response to ConA, significantly higher than in the CONTROLS and LEISH group (p=0.01 and 0.05 respectively). However, in response to SEA, the SCHISTO animals produced the highest nitric oxide production (p=0.0005, 0.0009 and <0.0001 respectively compared to CO-INFECT, LEISH and CONTROLS). In response to FLAA, the CO-INFECT animals produced the highest levels of nitric oxide which was significantly higher than the LEISH animals (p=0.02) but as in the earlier experiment SCHISTO alone mice also produced a higher response than CONTROLS (p=0.01).

Figure 6.9. Comparison of the levels of nitric oxide in supernatants of spleen cell cultures

Figure 6.9. Graphs showing the means and standard errors for nitrite production by spleen cells from three mice in each group sacrificed at +8 weeks after the *S. mansoni* superinfection. Cultures of $5x10^6$cells/ml were stimulated with medium alone, ConA at 5μg/ml, SEA at 20μg/ml or fixed leishmanial amastigote antigen (FLAA) at 10^7/ml. For definition of groups see legend to Figure 6.3.

6.2.7. Pathological responses

6.2.7.1. Effect on the schistosome granuloma size and cellular composition

- Experiment I

To assess whether leishmaniasis altered the course of disease in superimposed *S. mansoni* infections histopathology was carried out. For this, tissue from animals with comparable numbers of recovered adult worms and geometric egg counts in the liver were chosen for sectioning. Histological examination showed there to be no obvious differences in the schistosome-induced granulomatous and peri-granulomatous responses.

Nevertheless granuloma size and cellular composition were measured. As previously, the granulomas measured were those containing an identifiable miracidium and the widest diameter of each was recorded together with the diameter at right angles to this. The mean was then used to calculate the granuloma volume. For each sample 30 granulomas were measured. As shown in Figure 6.10. there was no significant difference between the mean granuloma volumes of CO-INFECT and SCHISTO animals.

Analysis of the percentage cellular composition of the egg granulomas showed an essentially similar cellular composition in the CO-INFECT and SCHISTO animals although the SCHISTO animals showed a slightly higher frequency of eosinophils compared to CO-INFECT animals ($p=0.05$).

Figure 6.10. Volume and cellular composition of *S. mansoni* granulomas

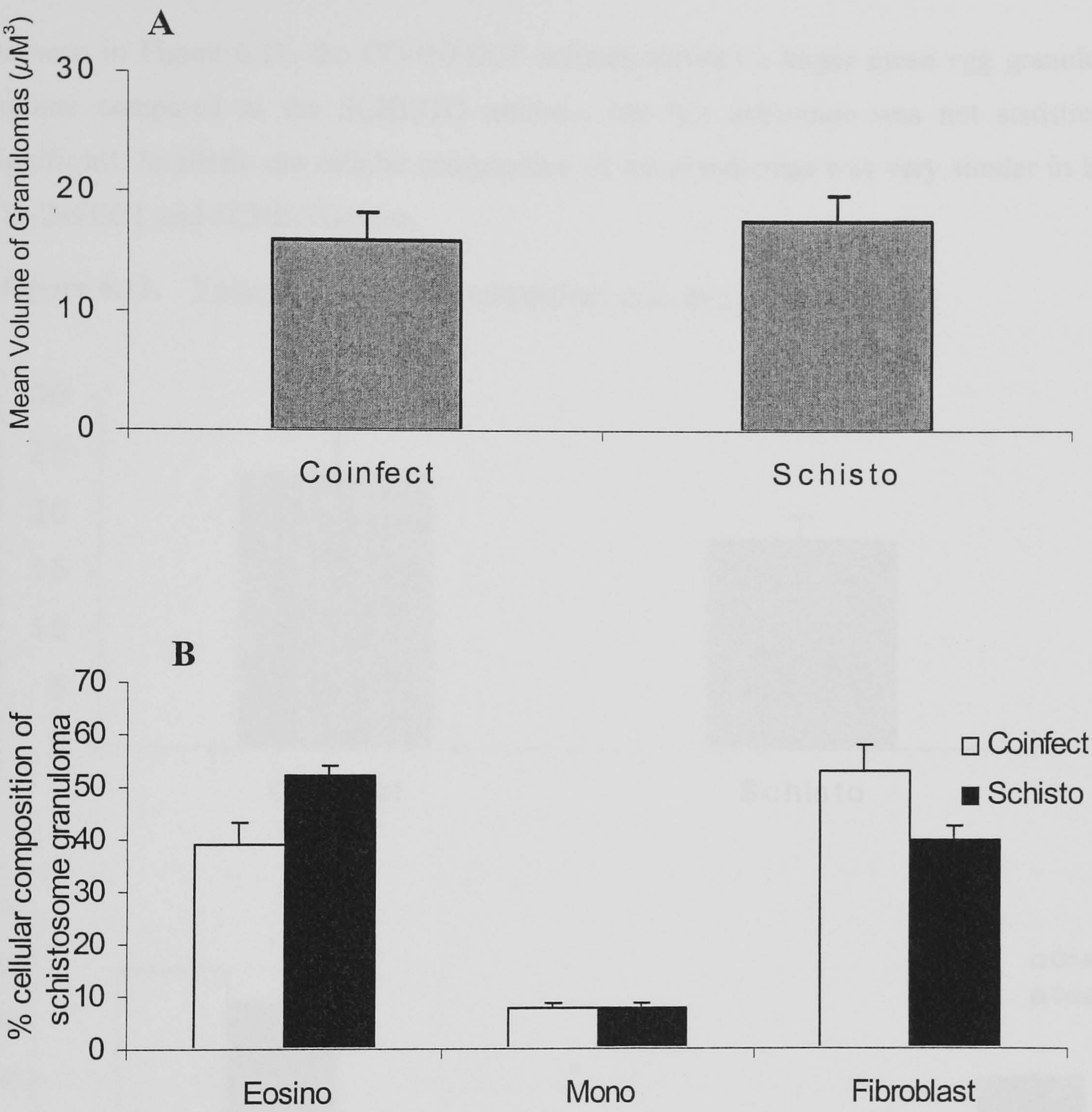


Figure 6.10. A. Graphs showing the mean (±SE) volume of *S. mansoni* egg granulomas from livers of SCHISTO and CO-INFECT animals at +8 weeks post *S. mansoni* superinfection. Histological sections were stained with H&E and transverse & longitudinal diameters were measured for 30 granulomas/mouse (with visible viable miracidium inside the schistosome egg). **B.** Graphs showing the mean (±SE) cellular composition of the above granulomas based on the data from three mice per group.

6.2.7.2. Effect on the schistosome granuloma size and cellular composition -*Experiment II*

As seen in Figure 6.11. the CO-INFECT animals showed a larger mean egg granuloma volume compared to the SCHISTO animals, but this difference was not statistically significant. Similarly the cellular composition of the granulomas was very similar in both CO-INFECT and SCHISTO mice.

Figure 6.11. Volume and cellular composition of *S. mansoni* granulomas

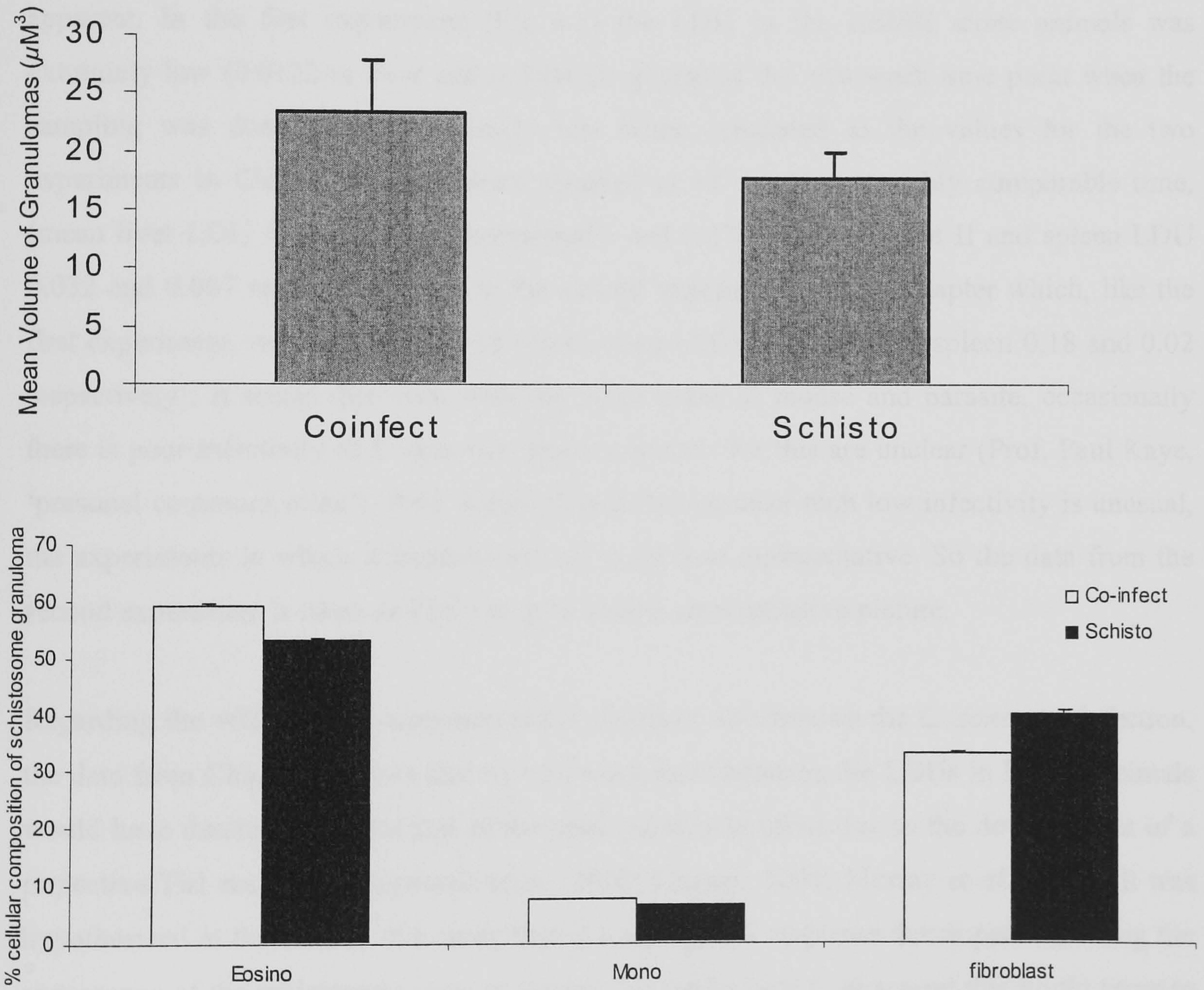


Figure 6.11. A. Graphs showing the mean (±SE) volume of *S. mansoni* egg granulomas from livers of SCHISTO and CO-INFECT animals at +8 weeks post *S. mansoni* superinfection. Histological sections were stained with H&E and transverse & longitudinal diameters were measured from 30 mature granulomas/mouse with visible viable miracidium inside the schistosome egg. **B.** Graphs showing the mean (±SE) cellular composition of the above granulomas based on the data from three mice per group.

6.3 Discussion

Owing to variation in infectivity of either the *Leishmania* or the schistosome infections several attempts had to be made before appropriate levels of parasite infection and numbers of surviving animals were achieved. Nevertheless the final experiment worked well in this regard and gave clear results several of which were supported by data from an earlier experiment in which there was a low *Leishmania* infection.

The reasons for the variation in *L. donovani* infection in the two experiments reported are not apparent. In the first experiment (Fig 6.1) the LDU in the LEISH alone animals was extremely low (0.0122 in liver and 0.0004 in spleen) at the +10 week time point when the sampling was done. This was clearly low when compared to the values for the two experiments in Chapter 3 which were sampled at +8 weeks, a roughly comparable time, (mean liver LDU were 0.09 for Experiment I and 0.276 in Experiment II and spleen LDU 0.032 and 0.067 respectively) and to the second experiment in this Chapter which, like the first experiment, was sampled at +10 weeks (mean LDU for liver and spleen 0.18 and 0.02 respectively). It seems that even with the same strain of mouse and parasite, occasionally there is poor infectivity of *L. donovani* but the reasons for this are unclear (Prof. Paul Kaye, 'personal communication'). Prof. Kaye advises that because such low infectivity is unusual, the experiments in which it happens are not treated as representative. So the data from the second experiment is taken as likely to give a more representative picture.

Regarding the effect of the superimposed *S. mansoni* infection on the *L. donovani* infection, the data from Chapter 3 shows that by +10 week post infection the LDUs in LEISH animals would have declined to a fraction of the peak parasite burdens due to the development of a protective Th1 response (Engwerda *et al.,* 2002; Murray, 2001; Murray *et al.,* 1997). It was hypothesized at the start of this study that if a strong Th2 response developed following the appearance of the schistosome eggs at around 7-8 weeks post *L. donovani* this might serve to reduce the anti-*L. donovani* Th1 response possibly resulting in a recrudescence of the infection. However, although it was clear that a strong Th2 response did indeed develop to schistosome antigens and to ConA, this did not result in a recrudescence but, on the contrary,

the *L. donovani* infection was markedly and significantly lower in the CO-INFECT group in both the liver ($p=0.04$) and spleen ($p=0.05$) in the second experiment. This was particularly interesting given the opposite effect seen in Chapter 3 when the *L. donovani* was given second, during the Th2 response established by the prior *S. mansoni* infection. A major difference, therefore between the protocols was that in the *S. mansoni/L. donovani* combination the immune response to the *L. donovani* had to develop in the context of the Th2 biased response whereas in the *L. donovani/S. mansoni* protocol the anti-leishmanial response developed in a naïve immune context. The crucial question is how the superimposed *S. mansoni* infection might result in lower LDU than the LEISH alone?

A possible explanation is that the prepatent period of *S. mansoni* infections in mice are characterized by elevation of Th1 cytokines. Thus splenocytes from C57BL/6 mice showed significantly elevated IL-2 and IFN-γ at 1-6 weeks post infection in the presence of both ConA and schistosome antigens (larval, adult and egg antigens) (Grzych *et al.,* 1991; Pearce *et al.,* 1991). It was only following the production of eggs at +6 weeks that the Th1 response declined and the Th2 cytokines came to dominate. Such effects were seen with only 10-50 cercariae (Grzych *et al.,* 1991; Pearce *et al.,* 1991) so the 50-100 cercariae used in the current infections should similarly have promoted splenic Th1 responses during weeks +3 - +8 of the *L. donovani* infection *i.e.* during the whole period of development of the protective Th1 response. More recently, Mountford *et al.* (2001) have shown that both unirradiated and irradiated infections induce IL-12 production in the skin soon after infection and it is suggested that this is the cause of the pro-Th1 response which develops. So it is possible that the *S. mansoni* induced production of IFN-γ and IL-2 could enhance the early development of the protective Th1 response to *L. donovani*. However, in the second experiment, in which the LDU differences were most pronounced, the levels of IFN-γ both to ConA and FLAA were lower in the CO-INFECT mice than in the LEISH mice which appears not to support the notion that the prepatent Th1 response induced by the schistosome infection in the CO-INFECT mice boosts the anti-leishmanial response. A possible explanation for this is that any boost to the anti-leishmanial immunity would have occurred early after the *S. mansoni* superinfection leading to a rapid and early reduction in the *L. donovani* parasite loads. In this case there would have been a reduction in the level and duration of *L. donovani* antigen

stimulation leading to the lower FLAA specific IFN-γ production observed in the CO-INFECT mice compared to the LEISH. To investigate this further it would be particularly interesting to carry out cytokine analysis earlier during the co-infection *i.e.* at +2 and +4 weeks post *S. mansoni* superinfection.

Possible boosting of a protective anti-leishmanial immune response by a concurrent infection may explain the demonstration by Rousseau *et al.* (1997) that pre-infection with *T. spiralis* 7 days before visceral leishmaniasis due to *L. infantum*, resulted in the hepatic and splenic amastigote burden in the co-infected mice being significantly lower than in mice infected with *L. infantum* alone both in the acute (+18d p.i) and chronic (+70d p.i.) phases. Despite an increase in IFN-γ, IL-4 and IL-5 mRNA levels locally (in the liver and spleen) and systemically (in the blood) in the co-infected mice, their spleen cells produced significantly less IFN-γ than spleen cells from the mice with *L. infantum* alone.

Regarding the effect of the pre-existing *L. donovani* infection on the superimposed *S. mansoni* infection the two experiments showed some differences. In the first experiment worm and worm pair numbers were very similar but in the second experiment both were significantly lower in the CO-INFECT mice compared with the SCHISTO mice. This difference was also reflected in the total schistosome egg count. Obviously this observation has to be confirmed but it is conceivable that the Th1 response induced by the *L. donovani* infection could enhance the IFN-γ response which larval schistosomes induce soon after infection (Grzych *et al.*, 1991; Pearce *et al.*, 1991, Mountford *et al.*, 2001). Enhancement of this response or its earlier induction could lead to accelerated and enhanced IFN-γ mediated inflammatory responses to the migrating larvae as have been shown to mediate killing of challenge larvae in the lungs of mice sensitized with irradiated cercariae (Coulson, 1997). However, there was no evidence of enhanced IFN-γ responses to ConA or SEA in the CO-INFECT mice compared with SCHISTO mice at the +8 week sampling time although the Th2 bias following the start of egg production would have down-regulated the pre-existing Th1 responses characteristic of the prepatent period (Grzych *et al.*, 1991; Pearce *et al.*, 1991). So as with the responses to FLAA it would be interesting to investigate the IFN-γ

responses at +2 and +4 weeks after superinfection and to include larval antigen against which there is the strongest Th1 response (Pearce *et al.*, 1991).

Once the worms were established there was no evidence of an effect on the production of eggs as there were no significant differences in the mean numbers of eggs/worm pair in the SCHISTO and CO-INFECT groups. TNF-α is stimulated during *L. donovani* infections being a key cytokine in mediating leishmanicidal activity in *L. donovani* granulomas (Tumang *et al.*, 1994; Engwerda *et al.*, 2002; D'Oliveira *et al.*, 2002) and an early study implicated TNF-α in enhancing worm fecundity (Amiri *et al.*, 1992) although there has since been some problems with confirming this data (Cheever *et al.*, 1999; Davies *et al.*, 2004).

Given the finely balanced immune responses controlling schistosome granuloma formation (Pearce and MacDonald, 2002; Herbert *et al.*, 2004) it was possible that the Th1 environment of the *L. donovani*-infected liver and spleen would have modulated the developing anti-egg response resulting in altered immune responses and so immuno-pathology. For instance, a strong pro-inflammatory response to the egg as occurs in IL-4/IL-10 double KO mice results in rapid death and up-regulated Th1 cytokines (Hoffmann *et al.*, 2000) although a less strong Th1 inducement by immunizing with eggs and rIL-12, results in less pathology, the granulomas being smaller and lacking eosinophils and fibrosis and the animals showing enhanced survival (Wynn *et al.*, 1994). However, in the present study there was no evidence of alteration of the cytokine response to egg antigens. Thus, *L. donovani* co-infection had no significant effect on the ConA- or schistosome egg antigen (SEA)-specific Th2 cytokine responses (IL-4 and IL-5) in either experiment. Similarly there was no evidence of stimulation of an SEA-specific IFN-γ response in the CO-INFECT animals.

However, although the schistosome egg specific immune response was apparently not altered by the co-infection, there was IFN-γ production to the *L. donovani* infection and, in the liver, this could have direct bystander effects on macrophage function, for example, counteracting the development of AAMØs (by the Th2 cytokines) which are crucial for suppressing damaging pro-inflammatory responses (Herbert *et al.*, 2004). Nevertheless, in the current

study, there was no evidence of either enhanced inflammatory responses to the eggs or inhibition of Th2 mediated fibrosis as a consequence of the *L. donovani* infection as judged by granuloma volume, cellular composition or morbidity.

Little has been done previously on the effects of prior protozoal infections on the development of schistosome immuno-pathology. Given the susceptibility of both the *L. donovani* and *S. mansoni* granulomas to immuno-modulation, it had been considered that either or both the *L. donovani* and *S. mansoni* parasite counts or disease states would have been affected during this co-infection. In fact there was essentially no effect of an established *L. donovani* infection on the establishment of the worms, their egg production or the immunological and pathological response to the eggs *i.e.* a strong Th2 response developed in spite of the altered spleen architecture which has been shown to develop relatively early during infection in mice and which is considered a possible mechanism for the immuno-suppression seen in human visceral leishmaniasis (Engwerda *et al.,* 2002). However, in spite of the loss of marginal zone macrophages no functional immune deficiency as a consequence has been shown (or tested) previously in mice and the relevance of the altered splenic architecture to the severe immuno-suppression seen in patients with active kala-azar is uncertain but it is possible that the *S. mansoni* infection in such patients may have a more severe outcome than is suggested by these mouse studies. The proposed reactivation of *L. donovani* infection following subsequent *S. mansoni* infection also did not occur but rather the *L. donovani* seemed to decline more quickly in the CO-INFECT mice. Again the significance of this for co-infected humans remains to be elucidated. Finally the observations in this chapter were based on limited number of experiments and the experiments need to be repeated to confirm some of the tentative conclusions.

Chapter 7

CONCLUSIONS

Polyparasitism is a common phenomenon in many parts of the world and due to the wide geographical distribution of schistosome infection, schistosomiasis is frequently found together with other infectious organisms, viruses, bacteria, protozoa and other worms. The aims of this study were to model the effects of prior *S. mansoni* infection on *L. donovani* infection and vice versa. This was particularly interesting because the schistosome infection is characterised by strong Th2 responses and the *L. donovani* infection is controlled by Th1-dependent responses.

The results in Chapter 3 showed that a superimposing *L. donovani* infection did not significantly affect a pre-existing *S. mansoni* infection as judged by worm burden or egg production. However, there was some evidence of an increased granuloma volume at +4 weeks following the *L. donovani* super-infection although this difference was not seen at +8 weeks. The basis of this increase in size was not apparent from the cellular composition of the granulomas (percentage of eosinophils, fibroblasts and mononuclear cells) or the schistosome antigen specific cytokine responses (IL-4, IL-10 and IFN-γ being comparable in the CO-INFECT and SCHISTO mice). In further studies on this aspect it would be valuable to determine the granuloma volumes at the +2 week time point. A possible explanation for an increased granuloma volume is that, as shown in Chapter 5, *L. donovani* infection was common in macrophages in the periphery of the egg granulomas in CO-INFECT mice *i.e.* there was a higher density of amastigotes than either further inside the egg granuloma or in the parenchyma. Although foci of infection in this granuloma periphery did not form typical *L. donovani* granulomas a degree of leishmania-specific cellular infiltration is likely and this would have led to an increase in cellular infiltration into this area and an apparent increase in egg granuloma diameter.

In contrast to the lack of effect on the schistosome worm burden it was clear from the experiments in Chapter 3 and 4 that the pre-existing schistosome infection resulted in a lack of control of the *L. donovani* infection resulting in significant and marked increases in LDU

in both the liver and spleen. This effect was not seen at +2 weeks post super-infection but was seen in two out of three experiments at +4 - +5 weeks and in three out of three experiments at +8 weeks, when the difference was most marked. No experiments were taken beyond this time as, even with the low *S. mansoni* infections used, the CO-INFECT animals showed significant morbidity at the later time points. So it is not known if the *L. donovani* infections would have subsequently resolved or continued to rise. In future work it would be interesting in future work to study infections in strains of mice which develop weaker Th1 responses to *L. donovani* and show a slower progression and resolution of *L. donovani* infection *e.g.* BALB/c mice (Murphy *et al.*, 2001).

Trying to determine the reasons for this loss of control became the major focus of much of the subsequent work. Various hypotheses were put forward to explain this:

(1) *S. mansoni* induced hepatosplenomegaly provides additional tissue sites for *L. donovani* proliferation (*i.e.* there is simply more tissue in general for *L. donovani* to infect);
(2) The added tissue would consist of *S. mansoni* egg granulomas rich in recruited macrophages in the liver and, in the spleen would comprise blood as a result of hypertension but also increased numbers of immune cells including recruited macrophages responding to the antigenic challenge of the eggs. In both cases there would be more suitable host cells [macrophages] to infect. A measurable consequence of this might be a higher density of foci of *L. donovani* infection within the *S. mansoni* egg granulomas in the liver compared with foci in the parenchyma;
(3) There is a failure to induce a sufficiently strong protective systemic Th1 response to *L. donovani* antigens by the spleen *i.e.* there was a lower IFN-γ response to FLAA due to:

(i) the Th2-skewed response to *S. mansoni* resulting in direct action of IL-4 on Th1 development (O'Garra, 1998) or
(ii) indirect effects of schistosome or *Leishmania* induced IL-10 (which is produced by Th2 cells but also several other cell types and not strictly a Th2 cytokine) on antigen presenting cell functions such as down-regulation of costimulatory molecules and IL-12 production (Bogdan *et al.*, 1991; Moore *et al.*, 2001) or (iii) action of other regulatory cytokines *e.g.* TGF-β.

(4) lower IFN-γ levels may result in reduced development/reduced rate of maturation of *L. donovani* granulomas (comprising antigen-specific Th1 cells and recruited macrophage and which are most clearly seen in the liver) *i.e.* there would be a reduced frequency of development of mature *L. donovani* granulomas;

(5) *L. donovani* granulomas may physically fail to form correctly within the context of the inflammatory cells of the *S. mansoni* granulomas and so there may be a lack of control in infected macrophages within the egg granuloma;

(6) even if morphologically normal mature granulomas form, the constituent macrophages have a reduced capacity to become activated and so to produce leishmanicidal factors [nitric oxide, NO and TNF-α]. This could be due to lower IFN-γ (as in (3) above) or to the direct suppressive action of IL-4, IL-13 and IL-10 on macrophage activation (Vouldoukis *et al.*, 1997). If this is true there may be an accumulation of amastigotes within *L. donovani* infected macrophages *i.e.* there would be higher mean numbers of amastigotes in macrophages of CO-INFECT mice compared with LEISH mice.

(7) As an extension to hypothesis (6) Alternatively Activated Macrophages which predominate in the schistosome egg granulomas (Linehan *et al.,* 2003; Hesse *et al.,* 2001; Noel *et al.,* 2004) may be infected and may be poor at directing *L. donovani* granuloma formation and/or show poor leishmanicidal activity. In this case amastigotes may accumulate within *L. donovani* infected macrophages in the egg granuloma *i.e.* there would be higher mean numbers of amastigotes in the infected macrophages within the *S. mansoni* granulomas compared with the parenchyma of CO-INFECT mice and/or LEISH mice.

With regard to (1) and (2), *i.e.* that greater organ size and availability of macrophage host cells for infection in the *S. mansoni* infected mice simply provide more sites for *L. donovani* infection, comparison of the organ weights (in Chapter 3) shows that the percentage differences between organ weights in the LEISH and CO-INFECT groups were small and not sufficient to explain the many fold greater LDUs in the CO-INFECT mice at +8 weeks. The egg granuloma comprises around 30% macrophages (Oswald *et al.,* 1993; Andrade and Cheever, 1995) and this may offer an increased density of potential macrophage host cells although of course the granuloma does displace the normal liver tissue that is rich in Küpffer cells. However, it is unlikely that the increase in LDU in CO-

INFECT mice arises simply from the greater availability of macrophages in the egg infected liver because at the +2 week time point, when the LDUs were similar in the CO-INFECT and LEISH animals, egg loads in the CO-INFECT liver were comparable to the later +4 and +8 week time points. So there would have been comparable numbers of egg granuloma macrophages available for infection at each of these times but this did not result in increased LDUs in the CO-INFECT mice relative to the LEISH mice at +2 weeks. Furthermore, in the histological studies in Chapter 5, although there was an apparent bias to *L. donovani* infection of the egg granuloma macrophages compared with the parenchymal Küpffer cells (60% foci were seen associated with the egg granulomas) this was also comparable at +2, +4 and +8 weeks. The histological studies also showed that the density of foci in the parenchyma of LEISH animals was actually higher at +2 weeks than in either the parenchyma or egg granulomas of the CO-INFECT mice. This indicates that although there may be some preference for infection of the granuloma macrophages even early in infection this does not lead to an overall higher density of infection in the CO-INFECT mice. This supports the notion that there is not a limitation in the availability of suitable cells for invasion in the normal liver that can be overcome by the presence of increased egg granulomas.

With regard to the idea (hypothesis 3) that an egg-induced Th2 response inhibits the development of the anti-Leishmanial immune response it was clear from the studies in Chapter 3 that the *S. mansoni* infection did induce significant IL-4 and IL-10 but not IFN-γ responses to schistosome egg and worm antigens as previously reported (Grzych *et al.*, 1991) *i.e.* a Th2 biased response. The LEISH infection induced *Leishmania* antigen (FLAA) specific IFN-γ production but not IL-4. The level of FLAA-specific IFN-γ which is crucial to resolution of *L. donovani* infection (Murray *et al.*, 1983; 1987; Murray and nathan, 1999; Murray *et al.*, 2003*)* was shown to be markedly lower at +2 weeks post infection in the CO-INFECT compared with the LEISH mice indicating a delay in development of the response. However, at +4 weeks the IFN-γ levels were comparable or even higher in the CO-INFECT animals but by +8 weeks they were again lower. In addition there was evidence of a higher FLAA specific IL-10 response at later time points. A delay in development of *Leishmania*-specific IFN-γ responses was also seen in *S.*

mansoni infected mice super-infected with *L. major* (La Flamme *et al.,* 2002) at +4 weeks post infection and this was followed by an increase in parasite burdens in the co-infected mice at +8 and +12 weeks relative to the mice infected with *L. major* alone, despite an overshoot at these times in the IFN-γ response in the co-infected animals. It appeared that the delay in inducing the *L. major* specific IFN-γ response allowed the infection to proliferate to high levels which eventually led to induction of high IFN-γ levels which were then able to control the infection by +16 weeks. So as in the present study there was not a direct temporal relationship between the higher IFN-γ levels and lower LDU values. The immuno-regulatory/immuno-suppressive properties of both IL-4 and IL-10 on Th1 responses are clearly established and both could have down-regulated the levels of specific IFN-γ seen in the CO-INFECT mice and also other aspects of leishmanicidal macrophage activation (IL-4 and IL-10 synergise to reduce CMI (Powrie *et al.,* 1993). The above studies of cytokine levels do not distinguish which of these cytokines or regulatory processes were the most important in this CO-INFECTION model.

Studies were carried out (Chapter 4) to try to investigate more directly the role of IL-10. Two approaches were tried. Firstly similar co-infection experiments were carried out in μMT mice which lack B cells. This was because it has been reported that B cells are a major source of IL-10 in *S. mansoni* infected mice (Harn *et al.,* 1989). The results show that the egg glycoproteins were found to stimulate production of B-1 cells ($CD5^+$ $B220^+$) which appeared in the peritoneal cavity of mice at the time of egg production (Harn *et al.*, 1989), and are capable of producing large amounts of IL-10 (O'Garra *et al.,* 1992). Indeed there was lower IL-10 production in the μMT mice and also a relatively lower fold increase in LDU in the liver (but not the spleen) of the μMT mice (CO-INFECT vs. LEISH) compared with the WT mice (CO-INFECT vs. LEISH). Although this is consistent with a relative lack of B-cell derived IL-10 inhibition of macrophage-mediated killing of *L. donovani* in the CO-INFECT μMT mice, this strain of mouse was also much less susceptible to infection with *L. donovani* alone than the C57BL/6 mice. This had been reported previously and is believed to be due to the rapid cytotoxic effects of recruited neutrophils (Smelt *et al.*, 1997). The lowered burdens in the LEISH alone μMT mice meant that it was not possible to directly compare the *L. donovani* infections in the CO-INFECT μMT and WT mice.

Given the operation of an additional leishmanicidal mechanism in these mice it is difficult to interpret the significance of these results and it is concluded that use of the μMT mice gave little insight into the role of IL-10 in the CO-INFECT model. In the second approach co-infections were set up as above and the mice treated with anti-IL-10 receptor antibody at on day +42 and 0.5mg on days +46, +49,+ 52 and +55. Unfortunately the anti-IL-10R treatment resulted in the death of some of the CO-INFECT mice. Despite this there was evidence that the anti-IL-10 treatment was able to reduce the high *L. donovani* LDU in the CO-INFECT mice but it was not possible to conclude with certainty that this was due to inhibition of *S. mansoni* mediated IL-10 responses as the LEISH infected mice treated with anti-IL-10 mAb also showed a reduction in LDU as previously reported in IL-10 KO mice (Castro *et al.*, 2000) and in IL-10R neutralization (Murray *et al.*, 2002). The anti-IL-10R treatment did not result in increased *Leishmania* specific IFN-γ production and so this shows that the *S. mansoni*-induced IL-10 can act to inhibit *L. donovani* immunity in the absence of IFN-γ effects. This suggests that the prime effect of the IL-10 in the CO-INFECT animals is to inhibit macrophage function resulting in reduced granuloma formation and leishmanicidal killing within the granulomas (hypothesis 6).

In order to visualise events in the liver of CO-INFECT and LEISH mice histological and immuno-histological studies were carried out (Chapter 5). It was demonstrated that there was an increased frequency of mature *L. donovani* granulomas relative to immature granulomas or newly infected Küpffer cells in the LEISH mice as the infection resolved (*i.e.* between weeks +2 and +8) but, in contrast, this ratio was comparable at all time points in the CO-INFECT mice. Although this could be taken as evidence that there was a relative failure to develop mature granulomas in the CO-INFECT mice it was reasoned that the lack of newly infected KCs and immature granulomas in the LEISH animals arose because the maturation of an effective immune response meant that there were few amastigotes surviving to establish new infections. In contrast, a failure to control the infection in the CO-INFECT mice (for whatever reason) would result in continued sources of new and immature foci of infection. The demonstration that morphologically normal mature granulomas could form in the CO-INFECT mice even at +8 weeks means that the loss of control in the CO-INFECT mice is due to a failure in progression of *L. donovani*

granulomas to the mature stage (hypothesis 4). However, the histological studies did show that there were higher mean amastigote numbers in the immature and mature granulomas in the CO-INFECT mice suggesting a failure of leishmanicidal activity within the granulomas (hypothesis 6).

The histological studies in Chapter 5 also showed that foci of *L. donovani* infection were seen in various locations within the egg granulomas but most commonly in a ring around the outside of the granulomas. Typical hepatic *L. donovani* granulomas did not form around these foci of infection and it was often difficult to define the limits of a focus which appeared to spread showing satellites of infection around a centre containing just a few amastigotes. The difficulty in deciding whether the satellite foci should be considered separate foci meant that estimation of the mean amastigote count/focus was not clear-cut. Even so, it was concluded that there was a slightly higher mean amastigote count per focus within the granuloma compared with either the parenchyma of either CO-INFECT or LEISH mice (hypotheses 5 and 7). Nevertheless, there was no evidence of heavy accumulation of amastigote numbers within foci of infection in either the parenchyma or granulomatous tissue of CO-INFECT mice. So in the livers of the CO-INFECT mice at +8 weeks there are many more foci of infection and a much higher LDU count but not a marked increase in the mean numbers of amastigotes/*L. donovani* granuloma or even evidence of heavy amastigote accumulation in a proportion of foci. It is concluded that there must be a lack of killing within the *L. donovani* granulomas but that this does not result in accumulation of amastigotes within the infected cells compared with LEISH mice. Rather the infected cells may only be able to accommodate a certain number of amastigotes before these escape or the infected cell breaks down releasing the amastigotes to infect new cells.

It is suggested that morphologically normal mature *L. donovani* granulomas can form in CO-INFECT mice but that the leishmanicidal activity of these is low owing to reduced *Leishmania*-specific IFN-γ and increased IL-10 as a result of the strongly polarised Th2 response induced by the *S. mansoni* infection. IFN-γ is crucial for leishmanicidal activity as it is essential for macrophage activation in *L. donovani* infection (Taylor and Murray,

1997). IL-10 could exert its effects in a variety of ways. It has recently been shown to inhibit the expression of a large proportion of the genes activated by the Th1 stimulus, LPS, including numerous pro-inflammatory genes *e.g.* IL-12, IL-6 and TNF-α. (Lang *et al.,* 2002) and is known to affect antigen presenting cell functions such as down-regulation of costimulatory molecules and IL-12 production (Bogdan *et al.,* 1991; Moore *et al.,* 2001). IL-10 has been shown to inhibit IFN-γ mediated killing of *L. major* and *L. infantum* by macrophages (Barral-Netto *et al.,* 1992; Vouldoukis *et al.,* 1997). *L. donovani* amastigotes infecting human monocytes *in vitro* can be killed by treatment of the cells with macrophage migration inhibition factor (MIF) which, like IFN-γ, can also induce NO production but this is also prevented by prior exposure of the monocytes to IL-10 (Wu *et al.,* 1993). La Flamme *et al.* (2002) have shown that peritoneal macrophages from 6-8 week *S. mansoni* infected mice activated with IFN-γ were markedly less able to kill *L. major in vitro* as judged by increased rates of infected macrophages and this corresponded to impaired production of NO. The fact that this effect was seen with peritoneal macrophages shows the systemic nature of the inhibition of leishmanicidal activity and it was suggested that IL-10 was a likely factor in such inhibition.

Ultimately the mature *L. donovani* granuloma is able to mediate killing of the surrounded amastigotes by the production of nitric oxide and Murphy *et al.* (2001) demonstrated that in IL-10 KO BALB/c mice *L. donovani* granulomas developed with the same kinetics and degree of maturity as in WT BALB/c. However, iNOS staining was barely detectable in granulomas of WT mice at this time whereas it was readily detected in the granulomas of KO mice. This shows that in WT mice, IL-10 can inhibit the leishmanicidal activity in *L. donovani* infection even in morphologically normal mature granulomas. Similarly, neutralisation of TNF-α does not affect the granuloma inflammation (Tumang *et al.*, 1994) but does prevent control and resolution of infection. So, in the present co-infection studies, although the schistosome infection seemed to have little effect on the development of morphologically normal *L. donovani* granulomas, the action of IL-10 may have had marked effects on leishmanicidal activity of the granulomas. Despite the difficulties encountered in the present studies with immuno-histochemistry owing to the presence of schistosome pigment in many of the *Leishmania* granulomas in CO-INFECT mice it is likely that

further attempts to improve the system would provide additional definitive information on the leishmanicidal potential of the *L. donovani* granulomas in the CO-INFECT and LEISH mice. Notably, if the technical problems could be overcome, staining for iNOS and TNF-α would be of great value as this would show definitively if the leishmanicidal activity of the *L. donovani* granulomas in the parenchyma or foci of infection in the periphery of the egg granulomas of CO-INFECT mice was reduced compared to the LEISH mice.

Such proposed inefficient leishmanicidal activity may be even more pronounced for the *L. donovani* infected macrophages within the egg granulomas. These are likely to be alternatively activated macrophages which are induced by the concerted action of IL-4 and IL-13 and which can be further promoted by the ability of IL-10 to strongly upregulate IL4Rα (Lang *et al.,* 2002). The concentrations of these cytokines would be greatest around the egg granulomas owing to the constant stimulation of surrounding T cells by the egg antigen released by the egg. These Th-2 cytokines induce arginase-I production which competes with inducible NO synthase for L-arginine, the common substrate for both enzymes (Modolell *et al.,* 1995) and so they show strongly inhibited microbicidal activities. An alternative consequence of arginase I induction in aaMø has also recently been demonstrated. Iniesta *et al.* (2001; 2002), demonstrated that arginase I induction in macrophages by Th2-type cytokines results in enhanced proliferation of intracellular *Leishmania major* amastigotes and specific inhibition of arginase I by using N(omega)-hydroxy-nor-L-arginine (nor-LOHA) reverted growth of *L. major* and *L. infantum* in infected macrophages. Arginase I synthesizes *L-ornithine* which can be used by the parasite to generate polyamines which are required for growth and differentiation of *Leishmania* (Mukhopadhyay and Madhubala, 1995; Fairlamb and Cerami, 1992). Addition of L-ornithine and putrescine, to *in vitro* infected cultures of macrophages could enhance growth of intracellular *Leishmania* amastigotes. It is concluded that arginase induction in the context of a Th2 predominant response might promote amastigote proliferation through increasing the availability of polyamines (Iniesta *et al.*, 2002). It was particularly interesting to note heavy *L. donovani* infection in the giant cells invading the empty schistosome egg shell although these were relatively rarely seen and so not likely to contribute markedly to the enhanced proliferation in CO-INFECT mice.

Future studies on the CO-INFECT livers could investigate if the "spreading" foci of *L. donovani* infection in the periphery of the egg granulomas are associated with AAMø by carrying out co-localization experiments using the anti-mannose receptor (MR) antibody (MR is a surface marker of AAMø) (Linehan *et al.,* 2003) and anti-*L. donovani* infection sera to localise amastigotes.

The final section of these studies was concerned with the effects of an established *L. donovani* infection on a superimposed *S. mansoni* infection. In view of the susceptibility of both the *L. donovani* and *S. mansoni* granulomas to immuno-modulation it had been thought that either or both the *L. donovani* and *S. mansoni* parasite counts or disease states could have been modulated. In fact there was no effect on the establishment of the schistosome worms, their egg production or the immunological and pathological response to the eggs *i.e.* a strong Th2 response developed. In these experiments the *L. donovani* infection was around 8 weeks of duration when the strong Th2 response was induced by the superimposed *S. mansoni* infection and so the *L. donovani* infection was well controlled by this time and it had been thought that the Th2 skewing might result in a recrudescence. In contrast the *L. donovani* seemed to decline even more quickly in the CO-INFECT mice than in the LEISH alone and it is suggested that the early phase of the schistosome infection which is characterised by Th1 responses (Grzych *et al.,* 1991) may have promoted the anti-leishmanial response. In these experiments only the +8 week post *S. mansoni* time point was investigated and so it was not known what effect the *S. mansoni* infection had on *L. donovani* immune responses and proliferation earlier during infection *e.g.* at +2 weeks post *S. mansoni.* So in future experiments it would be important to look at earlier time points.

In view of the experimental demonstration of exacerbated *L. donovani* infection during *S. mansoni* co-infection in mice it would be of interest to carry out epidemiological investigations into the existence, prevalence and significance of *L. donovani* and schistosome co-infections in humans. In the South-Eastern regions of Sudan, (Prof. el-Hassan 'personal communication' Department of Epidemiology and Clinical Sciences, Institute of Endemic Diseases, University of Khartoum, Khartoum, Sudan) the likelihood of visceral leishmaniasis and schistosomiasis co-infections in humans is thought to be likely

particularly due to ongoing displacement and movement of people between endemic areas for these infections (el-Hassan and Zijrich, 2001). The likelihood of co-infections occurring is heightened by the chronic nature of both *L. donovani* and schistosome infections.

Furthermore, nematode infections, both with filarial worms and with gut nematodes are also chronic infections associated with high IL-10 which is proposed to have important immuno-regulatory processes in humans (Faulkner *et al.*, 2002; Turner *et al.*, 2003; King *et al.*, 1993) and also in mice (Schopf *et al.*, 2002; Lawrence and Devaney, 2001) and also with the induction of AAMøs (Allen and Loke, 2001). So it is possible that such infections may also exacerbate *L. donovani* infection. Given the high global prevalence of gut nematode and filarial infections many co-infections with *L. donovani* and helminths are likely to occur. So epidemiological investigations into *L. donovani* disease states in relation to these co-infecting worms would also be worthy of investigation.

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