IMMUNOENDOCRINOLOGICAL CONTROL OF MURINE LEISHMANIASIS

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To my parents

their constant encouragement and love
have made this work possible

ABSTRACT

Female DBA/2 mice have been shown to be relatively resistant to infection *L. mexicana* as compared with male mice (Alexander,1988). The resistance of female mice was associated with the preferential expression of IFN-γ transcripts in their draining lymph nodes. Susceptible male mice on the other hand had transcripts for TNF-α, but only rarely produced transcripts for IFN-γ. At this time both the sexes had IL-4 and IL-12 mRNA transcripts. Administration of neutralising IFN-γ monoclonal antibodies to *L. mexicana* infected females enhanced lesion growth, whereas treatment of infected males with poly I:C, to induce IFN-γ production from NK cells, conferred partial protection. Further analysis revealed that females produced more parasite-specific IgG2a than males. Males displayed significant titres of IgG1 antibodies as early as week 6 post-infection. This antibody subclass could not be detected in females until week 10 and at level significantly less than males. Significantly, antigen stimulated lymph node cells from *L. mexicana* infected female mice produced IFN-γ, whereas this cytokine could not be detected in equivalent cultures from infected male mice. By 12 weeks post-infection B cell expansion was greater in male than female mice. Treatment of ovarectomised female mice with di-hydroxy testosterone (DHT) resulted in exacerbation of *L. mexicana* infection.

Sex differences in susceptibility to visceral leishmaniasis were also observed in non-cure BALB/c and cure C57BL/10 ScSn mice following *L. donovani* infection. In both strains male mice were relatively susceptible to *L. donovani* infection as compared with females.

As no differences could be observed in the production of IL-4 transcripts between resistant female and susceptible male DBA/2 mice following *L. mexicana* infection I used IL-4 gene deficient mice to evaluate the *in vivo* role of this cytokine during *L. mexicana* and *L. donovani* infection. Following infection with *L. mexicana*, IL-4+/+ mice developed large, non healing, cutaneous lesions, whereas no lesions whatsoever developed in IL-4-/- mice. The antigen stimulated lymph node cells from both IL-

4+/+ and IL-4-/- mice produced IFN-γ and those from IL-4+/+ mice also produced IL-5. Furthermore, at this time point, antigen stimulated splenocytes from IL-4-/- mice produced significantly higher amounts of IFN-γ as compared with those from IL-4+/+. This was also associated with the development of a significant delayed-type-hypersensitivity (DTH) response in IL-4-/- mice. As the infection progressed IL-4+/+ mice, unlike IL-4-/- mice developed significant titres of parasite-specific IgG1, indicating a Th2 influenced response in IL-4+/+ mice. Flowcytometric analysis revealed a significant increase in B cell population in the draining lymph nodes from IL-4+/+ mice. At the site of infection, IL-4-/- mice displayed substantial amounts of inducible nitric oxide synthase (iNOS) unlike immunocompetant control. Although, IL-4-/- mice failed to develop lesions following *L. mexicana* infection, viable parasites could be cultured from cutaneous inoculation sites *in vitro*. Following *L. donovani* infection IL-4-/- mice developed higher hepatic parasite burdens than their wild-type control counterparts. However, the differences were only significant at 15 days post-infection.

Lastly, I compared the growth of *L. mexicana* and *L.donovani* in IFN-γR, TNFR1, IL-6 and MHC class II gene deficient mice with appropriate immunocompetant controls IFN-γR -/- mice developed non healing, cutaneous lesions which were significantly larger than in control mice. Following *L. donovani* infection IFN-γR-/- mice liver parasite burdens were less than the control mice. However, the differences were significant only at day 15 post-infection. No significant differences were observed in the cutaneous growth of *L.mexicana* between TNFR1-/- and TNFR1+/+ mice. At 4 months post-infection the lesions healed in TNFR1-/- mice but still persisted in TNFR1+/+ mice. There were no differences in the growth of *L.donovani*. No significant differences could be noted in disease progression of cutaneous and visceral leishmaniasis between IL-6-/- and IL-6+/+ mice. While control MHC class II+/+ mice developed large, non healing cutaneous lesions at week 4 post-infection following inoculation with *L. mexicana*, MHC class II -/- mice either developed no lesions or lesion development was greatly delayed compared with controls.

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ABBREVIATIONS

Ag	Antigen
ALP	alkaline phosphatase
B cell	bursa matured lymphocyte
CL	cutaneous leishmaniasis
Con A	concanavalin A
CD	clusters of differentiation
cDNA	complementary DNA
DCL	diffuse cutaneous leishmaniasis
DNA	deoxyribonucleic acid
DHT	di-hydroxy testosterone
DTH	delayed type hypersensitivity
ELISA	enzyme linked immunoabsorbant assay
FCS	foetal calf serum
GKO	gene knock out
GP	glycoprotein
H & E	haematoxylin and eosin
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
Kd	kilodaltons
LCL	localised cutaneous leishmaniasis
L-NMAA	L-N^G-monomethylarginine
LPG	lipophosphoglycan
LPS	lipopolysaccharide
M	molar
mAb	monoclonal antibody
MCL	mucocutaneous leishmaniasis
MHC	major histocompatibility complex
MIF	migration inhibition factor
min	minutes
ml	millilitre
mM	millimolar
mm	millimetre
mRNA	messenger- ribonucleic acid
NK	natural killer
NHS	normal human serum
NRS	normal rabbit serum

NO	nitric oxide
nM	nanomolar
nm	nanometer
OCT	optimum cutting temperature
PBS	phosphate buffered saline
PCR	polymerase chain reaction
R	receptor
SCID	severe combined immunodeficient
SE	standard error
T cell	thymus matured lymphocyte
Th	T helper
TNF	tumour necrosis factor
Tris	tris(hydroxymethyl) aminomethane
Tween 20	polyoxyethylenesorbitan monolaurate
U	units
µg	microgram
µl	microlitre
VL	visceral leishmaniasis
v/v	volume for volume
w/v	weight for volume

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Chapter 1

General Introduction

1.1 Leishmania and the *Leishmaniases*

The leishmaniases comprise a group of diseases caused by various species of the intracellular protozoan parasite *Leishmania* . Taxonomically ,*Leishmania* is classified as follows :
Kingdom:PROTISTA, Sub–kingdom:PROTOZOA,
Phylum:SARCOMASTIGOPHORA,Sub–Phylum:MASTIGOPHORA,
Class:ZOOMASTIGOPHOREA,Order:KINETOPLASTIDA,
Sub–Order:TRYPANOSOMATINA,Family:TRYPANOSOMATIDAE,
Genus: *Leishmania* (Levine *et al.*, 1980, Lainson and Shaw, 1987).
Leishmaniasis is endemic in the tropics of Latin America, Africa, the Indian sub-continent regions, south-west Asia, the Mediterranean and south-east Asia (Ashford *et al.*, 1992, Desjeux, 1992). It is one of the six ear-marked diseases of the UNDP/World Bank/ WHO Special Programme for Research and Training.

Leishmania amastigotes were first reported in skin biopsies of patients suffering from Delhi Boil (Cunnigham,1885) and the viscera of patients who had died from kala azar (Leishman, 1903; Donovan, 1903) in India. The global importance of leishmania infection as a major public health problem is clearly evident from the WHO technical report on *Leishmania* in 1990 which indicated an annual incidence of 600,000 new cases and a world-wide prevalence of 12 million with a further 3,500 million people at risk of contracting the disease (WHO,1990).

There are at least 20 known species of *Leishmania* infecting mammals, many of which also infect man although *L. donovani* may be the only species comprising an anthorpozoonosis (Desjeux, 1992; Grimaldi and Tesh, 1993). In humans, leishmaniasis is caused by different *Leishmania* species which can be classified into distinct groups based on the clinical pattern of the disease displayed. Clinical manifestations of the disease may be confined to the skin,

may involve the nasal or oropharyngeal mucosa, or may be widely disseminated throughout the organs comprising the reticuloendothelial system.

Cutaneous leishmaniasis (CL) is the most common disease form seen in the "Old World" which is mainly caused by *L. major* or *L. tropica* and occasionally by *L. infantum* and *L. donovani*. Diffuse cutaneous leishmaniasis is mostly associated with *L. aethiopica*. It is normally manifest as a localised self-healing skin lesion which may occasionally evolve to multiple lesions resulting in disfiguring scars. Mucocutaneous leishmaniasis (MCL) or espundia, is prevalent in the "New World" and is primarily caused by parasites of the *L. braziliensis* complex, although occasionally associated with *L. mexicana* infection (Walton *et al.*, 1977). Clinically, it is characterised by involvement of the nasal and oropharyngeal mucosa with extensive tissue destruction. Chiclero's ulcer is a classical clinical characteristic feature of the *Leishmania mexicana* complex which causes CL in the "New World". This infection may involve the cartilage of the pinna of the ear and may lead to mutilation of the ear (Walton,1987). Diffuse cutaneous leishmaniasis (DCL) caused by *L. aethiopica* or *L. amazonensis* is associated with extensive nodular skin lesions, except in the palms of the hands and the soles of the feet. Visceral leishmaniasis (VL) is the most severe clinical form of this disease spectrum as measured by mortality resulting from wide dissemination of parasites throughout the reticuloendothelial system. It is clinically characterised by hepatosplenomegaly, fever, abdominal pain, diarrhoea, coughing, bleeding episodes, general malaise and weight loss. It is also associated with an earthy–grey pigmentation of the skin and hence also known widely as kala–azar i.e "black sickness" (Chance,1981). Post–kala–azar dermal leishmaniasis (PKDL) is a late complication of visceral leishmaniasis which sometimes occurs following chemotherapy and it is characterised by the development of multiple nodules (Rees and Kager, 1987) and responds very slowly to chemotherapeutic treatment (WHO, 1990).

Leishmaniasis is known to exist in all the continents except Australia and Antarctica and is believed to be endemic in at least 97 countries (Ashford *et al.*, 1992). However, more than 90% of all cases of visceral leishmaniasis have been reported from Bangladesh, India, Brazil and Sudan, whereas cutaneous leishmaniasis is mostly prevalent in Afghanistan, Brazil, Saudi Arabia, Iran and Syria (Desjeux, 1992).

The diagnosis of leishmaniasis is usually based on educated deduction as a result of the clinical aspects presented by patients in particular *Leishmania* endemic regions (Manson-Bahr,1987). However, appropriate parasitological and serological investigations as well as highly sensitive molecular biological techniques such as the polymerase chain reaction (PCR) are required for accurate diagnosis (Weiss,1995).

Chemotherapy by intravenous inoculation of pentavalent antimonial drugs such as sodium stibogluconate and glucantine provide the standardised treatment for leishmaniasis (Moss and Wilson, 1992; Herwaldt *et al.*, 1992). However the efficacy of drugs such as Amphoterecin B, Paramomycin and Rifampicin have also been evaluated with variable success (Davidson and Croft,1993). Topical treatment of cutaneous lesions has been attempted using both chemical and physical means. Drugs such as berberine acid sulphate, mepacrine, emetine hydrochloride, the pentavalent antimonials and paramomycin sulphate have also been used to treat the lesions of *Leishmania major* (El-On *et al.*, 1992), *Leishmania mexicana* (El–On *et al.*, 1993) and nodules in PKDL (Stosiek *et al.* 1992). In addition, physical methods such as curettage, irradiation (Dostrovsky and Sagher, 1942; Giannini and De Fabo, 1989), local heat (Navin *et al.*, 1990; Berman and Neva, 1981; Junaid *et al.*, 1986) and cryotherapy (Bassiouny *et al.*, 1982) have been used for local treatment of cutaneous leishmaniasis in its early stage.

In recently reported clinical and experimental studies, cytokines such as IL-12, IL-2, IFN–γ and GM-CSF either alone, or in combination with chemotherapy have been used successfully to treat the different forms of leishmaniasis (Tables1,2,3).

1.2 Life Cycle of *Leishmania*

Leishmaniasis is transmitted by female sand flies belonging to *Lutozomyia spp* in the New World and *Phlebotomus spp* in the Old World (Desjeux, 1992; Grimaldi and Tesh, 1993). Many species of sand flies bite man but the vast majority of leishmanial infections occur as zoonoses of domestic or wild animals. Only *L. donovani* and *L. tropica* produce anthroponotic infections where man is believed to be the only host (Desjeux, 1992; Grimaldi and Tesh, 1993).

Reservoir hosts, which include man or domestic wild animals, may be infected with species of *Leishmania* belonging either to the sub genus *Leishmania* or the sub genus *Viannia* (Desjeux, 1992; Grimaldi and Tesh, 1993). In the life cycle of the species of the sub genus *Leishmania,*the parasite is limited to the midgut and foregut of the insect host, but if the life cycle of the parasite includes a prolonged phase of development as paramastigotes attached to the wall of the sandfly hindgut it is a member of the subgenus *Viannia* (Saf'janvo, 1982).

1.2.1 Life cycle in the sandfly:

Over the last few years it has become increasingly evident that the development of infective forms of leishmanial parasites in the sandfly gut is a complex process and it is these infective forms which initiate natural infection in humans (Killick–Kendrick,1990; Schlein, 1993).

Following a blood meal, amastigotes are believed to be released from infected macrophages by rupture, either due to mechanical trauma during the feeding process or because they are broken down by the cibarial teeth (Lewis, 1975). Only a few amastigotes survive (Franke *et al.*, 1987) and these divide prior to their transformation to non-infective log phase promastigotes (Warburg *et al.*, 1986; Sacks and Perkins, 1984). The parasites resist the activity of the proteolytic enzymes in the sandfly gut and divide within the sac like peritrophic membrane secreted by gut epithelial cells around the blood meal (Schlein, 1993). Excreted factor (EF) released by parasites and protease gp63, which is also expressed by the parasites in the sandfly gut are believed to protect promastigotes against the action of proteolytic enzymes (Schlein and Romano, 1986; Davies *et al.*, 1990). The chitinolytic enzymes formed by dividing parasites digest the peritrophic membrane (Schlein *et al.*, 1991) to release the promastigotes which enter anteriorly into the thoracic midgut and cardiac valve. Parasites migrate firstly into the pharynx from the abdominal gut as attached paramastigotes and secondly as stationary phase infective metacyclic promastigotes which are found in the proboscis of the sandfly (Reviewed by Killick–Kendrick, 1990). The migration of promastigotes along the gut is believed to be due to a chemical gradient which may result from the release of stored sugars in very high concentrations just posterior to the pharynx (Bray, 1983).

Metacyclic promastigotes differ from log phase promastigotes in their morphology, lectin binding (Sacks *et al.*, 1985), LPG structure (Turco and Descoteaux, 1992), gp63 surface expression (Ramamoorthy *et al.*, 1992), resistance to killing by normal human serum (Howard *et al.*, 1987) and enzyme content (Mallinson and Coombs, 1989a,b). Parasites appear in the midgut of the sandfly on, or before, day 3 post-infection and their number increases until day 7 (Sacks and Perkins, 1984). Occasionally, migration of parasites from the midgut to the pharynx can take up to 14 days

(Killick–Kendrick and Roux, 1981).

1.2.2 Life cycle in vertebrates:

The infective metacyclic promastigotes are transmitted to vertebrates by infected female sand flies during a blood meal (Killick-Kendrick and Molyneux, 1981). Transmission to the vertebrate host may occur as a consequence of damage to the cardiac valve in infected sand flies, which results in suction of the pump in both directions and ejection of parasites into host tissue (Schlein, 1993). On rare occasions leishmaniasis has been reported to be transmitted sexually (Symmers 1960) and also through blood transfusions (Bruce–Chwatt 1972). In dogs, the oral route is thought to be important in the transmission of leishmaniasis (Killick–Kendrick, 1979).

In vertebrates the parasites primarily infect the cells of the mononuclear phagocyte system (Alexander and Russell,1992). Internalisation of promastigotes by the cells of the mononuclear phagocyte system is essential for the successful propagation of the life cycle. Not all promastigotes inoculated by sand flies in the extracellular environment at the site of the bite are capable of initiating infection (Adler and Ber, 1941), and a recently identified potent vasodialator peptide, maxadilan, released by the *Lutzomyia* sandfly has been shown to enhance the infectivity of the promastigotes (Lerner and Shoemaker,1992).

Once phagocytosed by the host macrophage, the parasites become lodged in phagosomes to which lysosomes fuse (Alexander and Vickerman,1975). The interaction between the parasites and the macrophage membrane system influences their survival in the phagolysosome (Russell,1995). Transformation of promastigotes to amastigotes is accompanied by significant biochemical and morphological changes. In a resistant macrophage, the

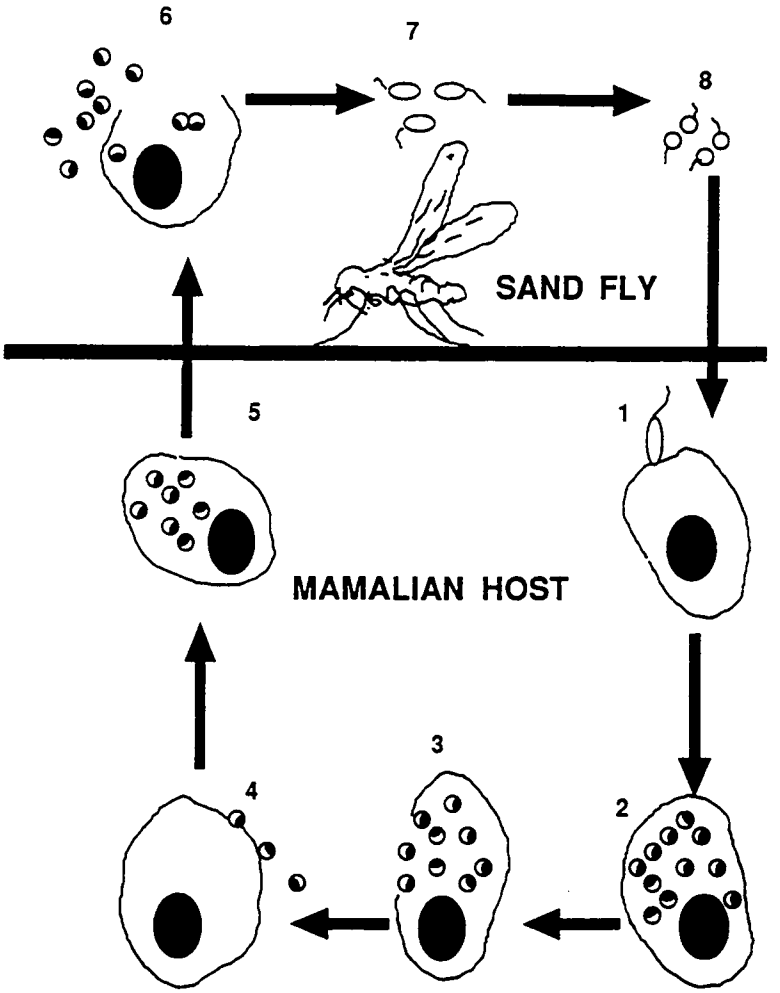

Fig.1: *Leishmania* parasites enter the mammalian host through the skin following the bite of an infected sandfly (1) which are phagocytosed by macrophages (2). Inside the macrophage the promastigotes transform into the ovoid amastigotes (2). The amastigotes multiply inside the macrophages and rupture the cell to release free parasites (3). These free amastigotes infect neighbouring macrophages (4). The infected macrophages are ingested by the sandfly during blood meal (5). Cell lysis within the sandfly gut releases amastigotes which transform into motile promastigotes (6). Following extensive multiplication within abdominal midgut they migrate anteriorly to the proboscis as infective metacyclic promastigotes ready to initiate further infection(7, 8).

parasites are killed in the phagolysosome by macrophage leishmanicidal mechanisms, whereas within a susceptible macrophage amastigotes continue to divide until large numbers of parasites occupy the parasitophorous vacuole. The infected cell eventually bursts releasing large numbers of amastigotes which in turn infect other cells of the mononuclear phagocyte system.

1.3 Genetic Control of Leishmaniasis

Both MHC and non-MHC immune response genes are involved in genetic control of murine cutaneous and visceral leishmaniasis (Reviewed by Blackwell, 1988). Five genes influence, in different ways, parasite infection in the mouse (Table 1), and the chromosomes to which they have been mapped have homology in the human genome (Reviewed by Alexander and Russell, 1992; Blackwell *et al.*, 1994).

				L. mexicana	
Gene	Chromosome	*L.donovani*	*L.major*	Primary cutaneous	Metastatic /visceral
Lsh (Nramp)	1	++	-	-	++
H-2	17	++	+	-	++
H-11	nk	++	++	+	++
Scl-1	8(11)	-	++	+	-
Scl-2	4	-	-	++	-

++, major influence; +, minor influence; -, no influence; nk, not known.

Table 1. Mouse genes controlling *Leishmania* infection with proven or probable macrophage involvement (after Alexander and Russell, 1992, Blackwell *et al.*, 1994).

1.4 Immunology of Leishmaniasis

Both innate and acquired immune responses interact to determine the outcome of *Leishmania* infections (Fig. 2).

1.4.1 Innate Immunity in Leishmaniasis:

Innate immune responses, primarily mediated by macrophages and NK cells play an important role in early defence against leishmanial infection. Cytokines such as IL-12 and IFN-γ produced sequentially during interactions between macrophages and NK cells respectively, are involved in the subsequent development and modulation of protective T - cell responses.

Macrophages:

Although macrophages and other cells of the mononuclear phagocytic system are primary target cells for *Leishmania*, they are also the principle effector cells involved in intracellular parasite killing and elimination. Macrophage-activating cytokines such as IFN-γ and TNF-α have been shown to play a key role in inducing macrophage anti-leishmanial activity (Belosevic *et al.*1988; Sadick *et al.*,1986; Titus *et al.*, 1984b; Squires *et al.*,1989; Weiser *et al.*,1987).

Parasite surface molecules such as LPG and gp63 have also been shown to play an important role in macrophage-parasite interaction (Sacks *et al.*, 1990; Russell and Wilhelm, 1986; Wilson and Hardin, 1988). In the absence of serum, gp63 binds to the CR3 integrin and LPG binds to the integrins p150/95, CR3 and LFA-1, which are present on the macrophage membrane. In the presence of non-immune serum the metacyclic promastigotes activate complement and become opsonised by C3b which is the ligand to the CR1 integrin (Reviewed by Russell and Talamas- Rhoana, 1989). Although the *Leishmania* promastigotes are capable of infecting all phagocytes, they can only survive in macrophages and immature monocytes (Alexander and

Russell, 1992, Beil *et al.*, 1992).

Following entry into the host macrophage, the parasites reside in an intracytoplasmic acidic parasitophorous vacuole (Chang, 1980; Antoine *et al.*, 1990), which is now accepted to be late endosomal to lysosomal in nature (Kornfeld and Mellman,1988). Thereafter, the outcome of infection is determined by the balance between the capacity of activated host macrophage to kill parasites by different intracellular leishmanicidal mechanisms and the ability of the parasite to inhibit macrophage function and resist killing.

Until recently it was thought that oxygen (O_2) dependent microbiocidal mechanisms in activated macrophages were responsible for the killing of leishmanial parasites (Murray,1981; Murray,1982; Haidaris and Bonventre,1982; Pearson *et al.*,1982; Buchmuller–Roiller and Mauel,1986; Hughs,1988). However the lack of correlation between macrophage leishmanicidal activity and production of toxic metabolites of oxygen (Liew *et al.*, 1990b; Mauel *et al.*, 1991) questioned the role of O_2 dependent pathways in the killing of *Leishmania* . Later Nitric oxide (NO), generated through an L- arginine dependent pathway was shown to be the principle effector mechanism involved in the killing of *Leishmania* in activated macrophages (Green *et al.* ,1990a; Liew *et al.*, 1990b).

Nitric oxide (NO) is generated during the chemical reaction between the amino acid L-arginine and molecular oxygen. This reaction is catalysed by the enzyme nitric oxide synthase (NOS) (Hibbs *et al.*,1988; Marletta *et al.*, 1988). NO is an unstable molecule with 3-15 seconds half -life and is rapidly converted to the stable end products nitrite (NO2-) and nitrate (NO3-). The enzyme nitric oxide synthase (NOS) mainly exists in two forms (Moncada *et al.*,1991; Werner-Felmayer *et al.*, 1991; Green *et al.*, 1990a; Liew *et al.*, 1990c,d), either as a constitutive enzyme present in endothelial

cells, platelets and the brain (Snyder, 1992), or as an inducible enzyme expressed in macrophages (Stuehr,*et al.*,1989), neutrophils (McCall *et al.* ,1991), mast cells (Salvemini *et al.*, 1990), hepatocytes (Curran *et al.*, 1989), fibroblasts, articular chondrocytes, cardiac myocytes and keratinocytes (Stueher and Griffith, 1992 ; Nathan,1992; Marletta, 1993).

Many *in vitro* and *in vivo* experimental studies indicate that NO is the major macrophage effector mechanism involved in the killing of leishmanial parasites (Reviewed by Liew and O'Donell, 1993; Liew and Cox, 1991). Thus, following *in vitro* stimulation, macrophages from genetically resistant mouse strains expressed significantly high amounts of iNOS and generated more NO as compared with their susceptible counterparts (Liew *et al.*,1991b). Furthermore, NO was shown to have a direct cytotoxic effect on *L. major* (Liew *et al.*, 1990b) and *L. enrietti* (Mauel *et al.*,1991) promastigotes. Treatment of resistant as well as susceptible mouse strains with the arginine analogue L-NMMA, known to inhibit NO production, caused rapid disease progression following infection with *L. major* (Liew *et al.*, 1990b; Evans *et al.*, 1993). Furthermore, in one *in vivo* study, resistant mice showed significantly higher expression of iNOS in lesions and draining lymph nodes than their susceptible counterparts (Stenger *et al.*,1994). Additionally, infection of iNOS gene-knockout mice with *L. major* resulted in progressive disease, thus once again indicating the *in vivo* protective role of NO in leishmaniasis (Wei *et al.*, 1995).

As the macrophage is the major effector cell involved in the killing of leishmanial parasites, it is perhaps not surprising that the ability of leishmanial parasites to cause clinical disease depends on their successful survival inside the host macrophage. Amastigotes are rich in different proteases which are capable of degrading parasite as well as host derived polypeptides (Pupkis *et al.*, 1986; North *et al.*, 1990), inhibiting NK cell

derived IFN-γ production (Ryan *et al.*,1995) and also, by cleaving immunoregulatory molecules such as CD4 from host immune cells (Hey *et al.*, 1994). Additionally, *Leishmania* have also been shown to alter macrophage function by downregulating MHC class I and class II expression (Reiner,1987), signal co-stimulatory molecule (Kaye *et al.*,1994) surface expression, inhibiting cytokine mediated macrophage activation (Mauel, 1984) and altering macrophage signal transduction (Moore *et al.*,1993). Recent experimental studies have shown that infection of macrophages with leishmanial parasites significantly reduces their ability to present 'irrelevant' antigens (Fruth *et al.*,1993; Prina *et al.*, 1993).

Langerhans cells:

Langerhans cells (LC) belong to the dendritic cell lineage and are derived from bone marrow precursors. Their characteristic feature is the presence of Birbeck granules in their cytoplasm (Schuler,1990). They express high levels of MHC class II molecules and are potent stimulators of antigen specific T cell responses (Stingel *et al.*, 1978).

In murine models of cutaneous leishmaniasis caused by *L. major*, parasitised epidermal Langerhans cells have been shown to transport parasites from infected skin to draining lymph nodes for antigen presentation to T cells (Blank *et al.*,1993; Moll *et al.*, 1993; Moll, 1993; Axelrod *et al.*, 1994).

NK cells:

Natural killer (NK) cells are large granular lymphocytes, derived from the bone marrow which were originally identified by their cytotoxic activity against tumour cells (Reviewed by Trinchieri, 1989). Since then, various experimental studies have established a role for NK cells in defence against bacteria, fungi, viruses and parasites including *Leishmania* (Reviewed by Bancroft, 1993).

Their importance in leishmanial infection was first identified in NK cell deficient C57BL/6bg/bg mutant mice, which failed to effectively clear parasites following infection with *L. donovani,* though not with *L. major* (Kirkpatrick and Farrell, 1982, 1984; Kirpatrick *et al.* ,1985). Additionally, *in vivo* depletion of NK cells prior to infection with *L. major* exacerbated the disease in a genetically resistant mouse strain while *in vivo* NK cell activation using poly I:C treatment resulted in milder disease in a susceptible mouse strain (Laskay *et al.*, 1993). However, in nude BALB/c mice infected with *L. donovani,* NK cells failed to induce anti-leishmanial activity (Murray *et al.*, 1995a). A clinical study carried out in patients suffering from American cutaneous leishmaniasis showed substantial numbers of NK cells in skin lesions (Ridel *et al.*, 1988). Recent experimental studies indicate that IL-12 activates NK cells and increases IFN-γ production from them during the early phase of *L. major* infection (Afonso *et al.*, 1994; Scharton-Kersten *et al.*, 1995). IFN-γ produced by activated NK cells, as well as activating macrophages, is critical for the differentiation of CD4+ T cells to the protective Th1 subset (Scharton and Scott 1993).

1.4.2 Acquired Immunity in Leishmaniasis:

Acquired immunological responses in leishmaniasis are mainly mediated by T- cells, although B cells and antibodies may influence disease progression. Generally, the development of protective immunity is associated with the presence of a strong delayed type hypersensitivity (DTH) response and low antibody titres, whereas disease progression is associated with high levels of immunoglobulin (Ig) in absence of DTH (Turk and Bryceson, 1971).

B lymphocytes and antibodies:

Although, anti-leishmanial antibodies are capable of enhancing parasite phagocytosis in the presence of complement (Herman 1980) and killing parasites *in vitro* (Pearson and Steigbigel,1980; Mosser and Edelson,1984)

, a similar role *in vivo* is questionable. Transfer of hyperimmune serum or antibody from a donor previously infected with *L. major* failed to confer protection in susceptible BALB/c mice (Howard *et al.*, 1984). In addition, a mouse strain with a genetically impaired humoral immune response (Biozzi AB/L) was extremely resistant to *L. major* infection and developed small healing lesions (Hale and Howard,1981). Interestingly, B cell depletion in susceptible BALB/c mice using anti-IgM antibodies made them resistant to *L. major* whereas similar treatment of resistant C3H/HeN mice made them susceptible (Sacks *et al.*,1984; Scott *et al.*,1986). These differences are believed to be related to the antigen presenting capacity of B cells, which may be required for the generation of protective as well as counterprotective T cells in resistant and susceptible mice respectively (Liew and O'Donnell,1993). Nevertheless, some studies have reported *in vivo* protection induced by monoclonal antibodies in *L. mexicana* infection (Anderson *et al.*,1983; Champsi and McMahon-Pratt,1988).

Gamma-delta T cells:

These are T cells with their T cell receptor comprising of gamma and delta instead of alpha and beta chains (Raulet,1989). Most γδ T cells do not express CD4 or CD8 and are mainly present in the gut mucosa, pulmonary mucosa, reproductive organs and epidermis (Fukushima *et al.*,1991, Raulet,1989). Although little is known about the immunological function of the γδ T cell, it has been shown to display nonspecific cytolytic activity against tumour cells and also has strong cytotoxic capacity (Brenner *et al.*,1987; Matis *et al.*,1987). In addition to their anti-tumor activity, γδ cells have been implicated as playing a role in bacterial (Hiromatsu *et al.*,1992; Janis *et al.*,1989), viral (De Paoli *et al.*,1990) as well as parasitic infections (Minoprio *et al.*,1989, Raziuddin *et al.*,1992; Modlin *et al.*,1989; Uyemura *et al.*,1992).

Clonally selected γδ T cells have been detected in substantial proportion in skin lesions from patients suffering from american cutaneous leishmaniasis caused by *L. braziliensis* (Modlin *et al.*,1989; Uyemura *et al.*,1992). In addition, patients suffering from cutaneous, mucosal as well as visceral leishmaniasis were shown to have increased numbers of γδ T cells in their blood indicating a substantial involvement of γδ T cells in various clinical forms of leishmaniasis (Russo *et al.*,1991). In a further clinical study , children suffering from visceral leishmaniasis showed a marked increase in γδ T cells in their peripheral blood. These were found to secrete B cell growth factors which may contribute to the polyclonal B cell expansion observed during visceral leishmaniasis (Raziuddin *et al.*,1992). Recently, expansion of γδ T cells in BALB/c mice following *L. major* infection was shown to be dependent on CD4+ T cells belonging to Th2 subset (Rosat *et al.*, 1995).

Although earlier experimental studies in the murine model failed to demonstrate any role for γδ T cells in pathogenesis of cutaneous leishmaniasis caused by *L. major* (Lohoff *et al.*, 1991; Titus *et al.*, 1991), a recent study showed that activated γδ T cells expressing a Vδ4 gene segment were involved in host defence against *L. major* in resistant as well as susceptible mouse strains (Rosat *et al.*,1993; Rosat *et al.*,1994).

CD4+ T cells:

It is widely accepted that CD4+ T cells play the major role in determining the ultimate disease outcome in leishmaniasis. Initial studies demonstrated that T cell deficient mice were extremely susceptible to leishmaniasis (Preston *et al.*, 1972; Skov and Twohy, 1974; Handman *et al.*, 1979; Mitchell *et al.*, 1980) but became resistant to infection following adoptive syngenic transfer of immune CD4+ T cells (Preston and Dumonde *et al.*, 1976; Rezai *et al.*, 1980; Mitchell *et al.*, 1980, 1981; Moll *et al.*, 1988; Liew *et al.*, 1982, 1984).

Later studies showed that CD4+ T cells could be subdivided into two functional subsets, Th1 or Th2, producing different cytokines, following their activation (Mosmann *et al.*, 1986; Cher and Mosmann, 1987; Cherwinski *et al.*, 1987). Th1 cells produce IFN-γ and IL-2, enhance B cell IgG2a production and mediate DTH, whereas Th2 cells produce IL-4, IL-5, IL-6 and IL-10 and promote IgE and IgG1 production but do not mediate DTH (Mosmann *et al.*,1986; Cherwinski *et al.*,1987; Fiorentino *et al.*,1989). Both Th1 and Th2 cells produce GM-CSF, IL-3, TNF and macrophage inflammatory proteins (MIP) in varying amounts (Zurawski *et al.*, 1986; Mosmann *et al.*, 1986; Brown *et al.*, 1989; Cherwinski *et al.*, 1987).

Many experimental studies carried out in murine models of cutaneous leishmaniasis caused by *L. major* clearly indicate that resistance to infection is predominantly mediated by the Th1 subset whereas the Th2 subset is responsible for disease progression (Scott, 1989, 1990; Locksley *et al.*, 1989, 1991; Liew, 1989, 1992). Thus, following infection with *L.major*, resistant C57BL/6 mice showed the presence of IFN-γ transcripts in their draining lymph nodes and spleens in contrast to susceptible BALB/c mice which expressed IL-4 transcripts (Heinzel *et al.*,1989). In addition, following *in vitro* stimulation, T cells from a *L. major* infected susceptible mouse strain predominantly produced, IL-4 and IL-5, whereas those from a resistant strain produced IFN-γ (Scott *et al.*,1989; Boom *et al.*,1990; Coffman *et al.*, 1991). *In vivo* neutralisation of IL-4 using anti-IL4 monoclonal antibodies rendered BALB/c mice resistant to infection with *L. major* (Sadick *et al.*,1990; Chatelain *et al.*,1992), while similar treatment of C3H/He and BALB/c mice with neutralising anti-IFN-γ monoclonal antibodies made them susceptible to *L. major* and *L.donovani* infections respectively (Belosevic *et al.*,1989; Squires *et al.*, 1989). Although, SCID mice reconstituted with Th1 clones were resistant to infection with *L.major* and those reconstituted with Th2 clones were susceptible (Holaday *et al.*,1991), BALB/c T cell reconstituted

C.B-17 SCID as well as nude mice developed protective Th1 responses which healed following infection with *L.major* (Varkila *et al.*,1993; Mitchell *et al.*,1981). Immunisation of BALB/c mice with lethally irradiated, heat killed or sonicated promastigotes by the intravenous route, but not subcutaneously, led to development of long lasting protective immunity (Howard *et al.*,1982) which could be transferred to syngenic recipients by IFN-γ producing CD4 + T cells (Liew *et al.*,1984,1987). Conversely, in some experimental studies, *in vivo* transfer of a IFN-γ forming cell line resulted in disease exacerbation following infection with *L. major* (Titus *et al.*, 1984a,1991).

The clear Th1/Th2 pattern of disease development demonstrated for *L. major* has not been observed in non-curing visceral leishmaniasis (Kaye *et al.*, 1991; Murray *et al.*, 1992; Miralles *et al.*, 1994), and cutaneous *L. amazonensis* infection (Afonso and Scott, 1993). Additionally, in one experimental study, CD4+ T cells responsible for disease progression could not be classified into Th1 or Th2 subsets based on cytokine production following *L. major* infection (Moll and Rollinghof, 1990). Similar results have been observed in recent clinical studies, where both parasite reactive Th1 and Th2 clones have been isolated from patients recovered from visceral leishmaniasis (Kemp *et al.*,1993b).

CD8+ T cells:

Although, different functional subsets of CD4+ T cells play an important role in determining disease outcome in leishmaniasis, some experimental studies do suggest a protective role for cytotoxic CD8+ T cells in leishmaniasis.

Following infection with *L.major*, both resistant CBA, as well as susceptible BALB/c mice showed a marked increase in parasite-specific CD8+ T cells in their draining lymph nodes (Milon *et al.*,1986; Titus *et al.*,

1987). Furthermore, depletion of CD8+ T cells using anti-CD8 monoclonal antibodies exacerbated *L. major* infection in resistant as well as susceptible mice (Titus *et al.*, 1987; Farrel *et al.*, 1989). Conversely depletion of CD4+ T cells from BALB/c mice using anti-CD4 monoclonal antibodies made them resistant to subsequent infection (Titus *et al.*,1985; Hill *et al.*,1989; Muller *et al.*,1991). In a murine model of visceral leishmaniasis, an influx of CD8+ T cells was associated with a decrease in parasite growth in the liver (McElrath *et al.*,1988). In addition, both CD4+ and CD8+ Tcells from euthymic BALB/c mice were necessary for the transfer of resistance against *L. donovani* to their athymic litter mates (Stern *et al.*,1988).

Activated CD8+ T lymphocytes have been shown to produce IFN-γ (Kaufmann,1988; Fong and Mosmann,1990) which can enhance the leishmanicidal activity of macrophages (Green *et al.*, 1991; Lorsbach and Russell, 1992). Incubation of *Leishmania* infected macrophages with a H-2^d-restricted, cytotoxic CD8+ T cell line specific for the circumsporozoite protein of *Plasmodium berghei* resulted in killing of parasites which was IFN-γ dependent and was believed to be due to activation of bystander macrophages (Smith *et al.*,1991). In fact, CD8+ T lymphocytes were shown to produce significant amounts of IFN-γ in *L. m. amazonensis* infected C57BL/6 mice (Chan,1993) .

However, in other experimental studies, although *L. mexicana* promastigotes could induce *in vivo* cytotoxic T cells (CTL) responses against parasite antigen, these CTLs were unable to recognise infected macrophages (Lopez *et al.*,1993) and targeted activation of CD8+ T cells failed to protect susceptible BALB/c mice against *L.major* infection (Wang *et al.*, 1993a). In addition CD 8+ T cell deficient β2-microglobulin knockout mice failed to demonstrate a functional role for CD8+ T cells during *L. major* or *L. mexicana* infection (Wang *et al.*, 1993a; Overath and Harbecke, 1993).

1.4.3 Cytokines in Leishmaniasis:

Cytokines derived from different cells of the immune system play an important role in modulation of host defence mechanisms during leishmaniasis. The control of *L. major* growth in genetically resistant mice has been clearly associated with the expansion of the Th1 lymphocyte subset of the CD4+ T cell population and the production of the cytokines IFN-γ,IL-2,TNF-α, MIF and IL-12 (Tables 2 -5) (Liew and O'Donnell, 1993; Heinzel ,1994). Conversely, non-healing responses in susceptible mice have been related to the expansion of the Th2 subset and production of cytokines such as IL-3, IL-4, IL-10 and TGF-β (Table 6) (reviewed by Liew and O'Donnell,1993).

Although, IFN-γ and TNF-α have been clearly shown to exert their protective effect against *Leishmania* infection by inducing macrophage leishmanicidal activity (Table 2 and 7) , IFN-γ has also been shown to be directly cytostatic for the parasites (Bhattacharya *et al.*, 1993). Despite a wealth of knowledge about our significant understanding of the basic immunological mechanisms involved in the control of *Leishmania* infection, the role of the various cytokines throughout the course of a infection appears to be complex and a number of apparently contradictory reports have been published. Thus, for example, IL-2 in combination with IFN-γ not only enhanced leishmanicidal activity of macrophages *in vitro* (Belosevic *et al.*, 1988), but also reduced liver parasite burdens in experimental visceral leishmaniasis following *in vivo* treatment (Murray *et al.*, 1993). However, in *L. major* (Heinzel *et al.*, 1993b) as well as *L.mexicana* infected susceptible mice, IL-2 enhanced parasite growth and lesion development (Lezama-Davila *et al.*, 1992; Mazingue *et al.*, 1989). Similarly, while the Th2 derived counterprotective cytokines IL-4 (Liew *et al.*, 1989b, Scott *et al.*, 1989) and IL-10 (Heinzel *et al.*, 1991) have clearly been shown in some studies to inhibit macrophage leishmanicidal mechanisms (Table 2 and 7) and enhance

disease progression in susceptible mice infected with *L.major* (Table 4), in other studies IL-4 has also been shown to activate macrophages (Crawford *et al.*, 1987) and enhance their IFN-γ induced leishmanicidal activity (Belosevic *et al.*, 1988; Bogdan *et al.*, 1991). Furthermore, intralesional administration of IL-4 into *L.major* as well as *L.mexicana* infected susceptible mice during the initial stage of infection resulted in disease exacerbation , whereas, its late administration led to protection (Lezama-Davila *et al.*, 1992). A similar protective effect was observed in *L. major* infected BALB/c mice following intralesional injection of IL-4 in a slow release gel preparation (Carter *et al.*,1989). This led to complete resolution of lesions within 10 weeks and the development of protective immunity which could be transferred to naive recipients by splenic T cells. IL-3 which inhibits IFN-γ mediated leishmanicidal activity of macrophages is nevertheless produced by both protective Th1 and counterprotective Th2 cells (Liew *et al.*,1989b) and has been implicated in promoting disease progression in *L. major* infection (Lelchuk *et al.*, 1988; Feng *et al.*, 1988; Louis *et al.*, 1987). Additionally, IL-3 has been thought to be responsible for recruitment of immature *Leishmania* -permissive rather than mature *Leishmania* -resistant macrophages into the site of infection which further exacerbates disease (Mirkovich *et al.*, 1986). Similarly, different experimental studies have reported variable roles for GM-CSF in leishmaniasis (Table 2 and 4). Although, some *in vitro* and *in vivo* studies indicated that GM-CSF was beneficial in *L. major* (Handman and Burgess, 1979; Solbach *et al.*, 1987a), *L. donovani* (Weiser *et al.*, 1987) and *L. amazonensis* (Ho *et al.*, 1990) infections, others reported a disease exacerbating role for this cytokine (Greil *et al.*, 1988). In fact, it has been suggested that *in vitro* GM-CSF acts as a growth factor for *L. mexicana* promastigotes (Charlab *et al.*,1990).

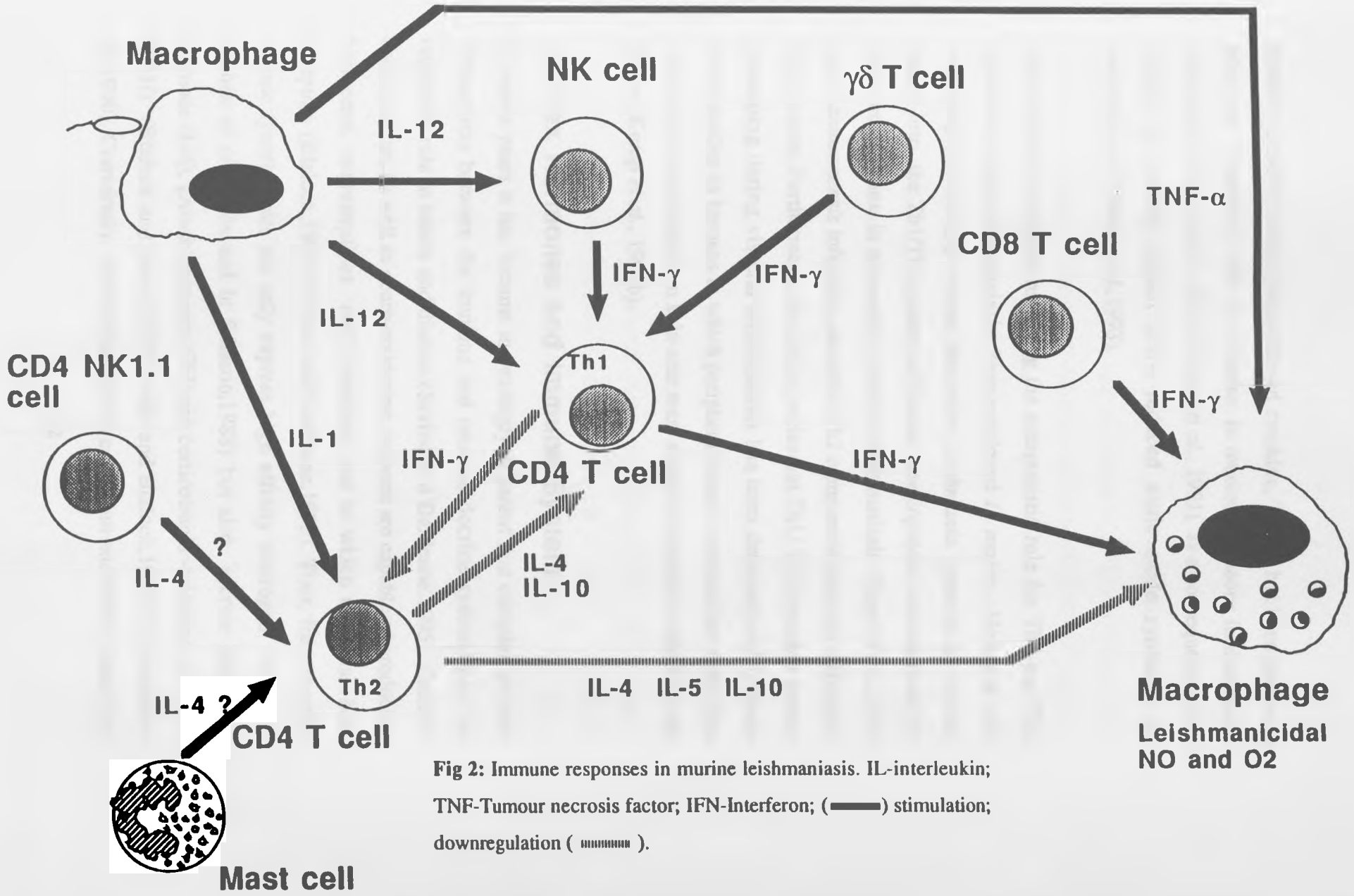

Fig 2: Immune responses in murine leishmaniasis. IL-interleukin; TNF-Tumour necrosis factor; IFN-Interferon; (▬▬) stimulation; downregulation (▪▪▪▪▪▪▪▪).

Recently, anothe macrophage derived cytokine, TGF-β has been shown to play an important role in disease in murine cutaneous leishmaniasis progression (Nacy *et al.*, 1991; Nelson *et al.*, 1991) as a consequence of its ability to directly inhibit IFN-γ induced nitric oxide synthesis by macrophages (Cunha *et al.*,1993).

The majority of studies regarding the antagonistic role for Th1 and Th2 cytokines during leishmaniasis have employed *L. major* . However it is becoming increasingly evident that other *Leishmania* species do not fall clearly into the Th1/Th2 pattern of disease development demonstrated for this species. Thus, in non-curing visceral leishmaniasis (Kaye *et al.*,1991) and *L. amazonensis* infections, an active Th2 component has been difficult to demonstrate. Furthermore, the lack of a clearcut Th1/ Th2 cytokine pattern developing during visceral leishmaniasis has been demonstrated in some recent studies in humans in which peripheral blood mononuclear cells from individuals recovered from kala-azar were shown to produce both IL-4 and IFN-γ (Kemp *et al.*, 1993b).

1.5 Sex Hormones and Immune System

In recent years it has become increasingly apparent that complex *in vivo* interactions between the immune and neuro-endocrine systems play an important role in health and disease (Savino and Dardenne,1995). Cells of both immune, as well as neuro-endocrine systems are capable of producing hormones, neuropeptides and cytokines, and to which they also bear receptors (Blalock,1994; Savino and Dardenne,1995). Thus, for example, splenic lymphocytes not only express high affinity oestrogen receptors (Cohen *et al.*,1983a and b; Stimson,1988) but also secrete lutenizing hormone (LH), growth hormone (GH) and corticotropin -releasing hormone (CRH) (Blalock and Smith,1980; Smith and Blalock,1981; Stephanou *et al.*,1990). Conversely, neurons as well as cells from endocrine glands not

only produce IL-1 but also express the IL-1 receptor (IL-1R) (Cunningham and DeSouza, 1993).

Sex hormones have been shown to have profound influence on the modulation of immune responses and the outcomes of various diseases in males and females (reviewed by Schurrs and Verheul,1990; reviewed by Alexander and Stimson,1988). Generally, females have better antibody mediated (Rhodes *et al.*,1969; Patty *et al.*,1976; Spencer *et al.*,1977; Michaels and Rogers,1971; London and Drew,1977) but poorer cell mediated immune responses than males (Inman,1978). Hormonal manipulation studies in experimental animals (Stimson and Alexander, 1988; Viselli *et al.*, 1995; Benten *et al.*, 1991; Benten *et al.*, 1992) and clinical observations during human pregnancy (Reviewed by Brabin and Brabin,1992) have clearly shown how sex hormones can affect *in vivo* immune responses. Thus, following ovarectomy, resistant female DBA/2 mice became susceptible to *L. mexicana* infection, whereas oestrogen treatment of male DBA/2 mice enhanced their resistance against *L. mexicana* (Khamis,1990). Furthermore, at physiological concentrations, oestrogen enhanced *in vitro* leishmanicidal activity of male and female DBA/2 peritoneal macrophages against *L. mexicana* (Khamis,1990). Such protective effects of oestrogen have also been observed in murine bacterial infections (Wheater and Hurst,1961; Nicol *et al.*,1964). However, in some experimental studies, oestrogens were found to suppress immunological responses against some antigens (Slijivic and Warr,1973), viruses (Friedman *et al.*,1972) and parasites (Alexander and Stimson,1988). The role of androgens in modulation of *in vivo* immunological responses is equally diverse and sometimes different reports are contradictory. Thus, one group observed increased (Healy and Leeman,1967) whereas another group observed decreased (Fujii *et al.*,1975) anti-sheep red blood cell (SRBC) antibody responses in male mice following castration. Although testosterone has been shown to suppress protective

immunity against protozoan parasites such as *Leishmania* (Mock and Nacy, 1988; Alexander and Stimson,1988) and *Plasmodium* (Benten *et al.*, 1991; Wunderlich *et al.*,1988; Kamis and Ibrahim,1989) , it is also believed to be responsible for resistance against *Toxoplasma* in guinea pigs and mice (Kittas *et al.*,1980; Kittas *et al.*,1979).

In humans, pregnancy-related hormones have been shown to depress cell-mediated immunity (Finn *et al.*, 1972; Thong *et al.*, 1973) and influence B cell lymphopoiesis (Medina and Kincade, 1994).

Although effects exerted by sex steroids on the immune system may be mediated by more than one mechanism , at least one seems to be the ability of hormones to regulate gene transcription (Parker, 1983). Hence, they are able to influence *in vivo* immunological responses indirectly, either by modifying the local environment in lymphoid tissue or by increasing the release of immunoregulatory factors (Stimson,1987). Recently, oestrogens have been shown to modulate the expression of TNF-induced endothelial cell adhesion molecules E-selectin, intercellular adhesion molecule type 1 (ICAM-1) and vascular cell adhesion molecule type 1 (VCAM-1)(Cid *et al.*,1994).

Macrophages and sex hormones:

Oestrogen binding sites have been identified in normal human mononuclear cells (Danel *et al.*, 1983), rodent peritoneal macrophages (Gulshan *et al.*, 1990) and also in the human monocyte leukemia line, J111 (Gulshan *et al.*, 1990). In humans, elevated levels of oestrogens during pregnancy and the menstrual cycle were thought to be responsible for increased number of peripheral blood monocytes (reviewed by Alexander and Stimson ,1988). In addition, oestriol has also been shown to enhance the phagocytic capacity of rat peritoneal macrophages (Stimson ,1983; Stimson,1987), increase the bactericidal capacity of liver macrophages (Morahan *et al.*, 1984) and

modulate the production of reactive oxygen intermediates and nitrite release (Chao *et al.*, 1994; AbuAmer and BarShavit, 1994). Furthermore, treatment of experimental animals with natural or synthetic oestrogens enhanced the phagocytic activity of cells of the reticulo endothelial system (RES) (Loose and DiLuzio,1976; Nicol *et al.*,1964; Trejo *et al.*,1972) and also elevated the numbers of peripheral blood monocytes, peritoneal macrophages (Boorman *et al.*, 1980) and Kupffer cells (DenDulk *et al.*,1979). However, it was also observed that oestrogen induced RES stimulation was mainly due to enhancement of RES cell activity and the consequent generation of efficient antigen presenting cells (APC) (Fruhman,1973) rather than due to increase in RES cell number (Loose and DiLuzio,1976; Warr and Sligivic,1973; Morahan *et al.*,1984). Furthermore, *in vivo* studies have shown oestrogen to be the most potent inducer of macrophage phagocytic activity (Nicol *et al.*,1965). Besides all the above activities, sex hormones have also been shown to play a role in the modulation of cytokine production from macrophages (Kuang *et al.*,1988; Flynn,1986; Girasole *et al.*,1992; Ralston *et al.*,1990).

NK cells and sex hormones:

In humans, oestrogens have been shown to suppress NK activity during pregnancy (Gabrilovac *et al.*,1988) and the periovulatory period (Sulke *et al.*, 1985). Conversely, the oestrogen antagonist tamoxifen was shown to stimulate NK cell activity (Mandeville *et al.*,1984). However, a recent, contradictory study reported *in vitro* enhancement of the NK activity of YT-N17 (a human NK-like cell line) by 17β oestradiol, which could be blocked by tamoxifen (Sorachi *et al.*, 1993).

T cells and sex hormones:

It is, perhaps, not surprising that sex steroids can exert direct regulatory effect on T cell function as receptors for these hormones have been shown to

be present on T cells (Cohen *et al.*,1983a; Meikle *et al.*,1992; Stimson, 1988). In humans, the oestrogen receptor has been shown to be expressed by resting peripheral CD8+ T cells but not resting CD4+ T cells (Cohen *et al.*,1983b). These CD8+ T cells have been shown to exhibit classical suppressor/cytotoxic activity (Stimson ,1988). Although initial studies failed to demonstrate androgen receptors in circulating lymphocytes (Cohen *et al.*,1983a; Stimson,1988; Kovacs and Oleson, 1987), androgen binding sites have been recently identified in human peripheral blood mononuclear cells which comprise 70-80% T lymphocytes (Kuhnle *et al.*, 1994).

Castrated male rats showed significant increase in their thymic CD4 T helper and CD8 suppressor/cytotoxic T cell populations (Grossman ,1989). Similarly castrated male mice not only showed a decrease in their splenic CD8+ T cell activity (Weinstein and Berkovich,1981), but also in peripheral T cell number (Viselli *et al.*,1995). In humans, untreated hypogonadal male patients had significantly higher numbers of T-helper cells as compared with androgen treated patients (Kiess *et al.*,1991). Further, clinical and experimental studies showed that androgen treatment increased the CD8+T cell population (Novotny *et al.*,1983; Ahmed *et al.*,1985b), whereas oestrogens not only reduced CD8+ cells but also suppressed their functional activity (Paavonen *et al.*,1981; Cosma, 1973). Increased levels of oestrogens during pregnancy have been shown to deplete thymic CD4+CD8+ T cells (reviewed by Clarke and Kendall, 1994). However, the immunomodulatory effect of sex hormones on T cells is dose dependent. Thus, although, supraphysiological levels of sex hormones inhibited phytohaemagglutinin (PHA) stimulated *in vitro* lymphocyte proliferation (Weetman*et al.*,1981; Neifield *et al.*,1979) , neither oestrogen nor progesterone , in concentrations up to 10^{-6} M altered Con A induced proliferative responses of PBMC from healthy post menopausal women (Yron *et al.*,1991). Additionally, sex hormones have also been shown to have a profound influence on the

production of immunoregulatory cytokines from T cells (Fox *et al.*, 1991; Grasso and Muscettola, 1990; Araneo *et al.*,1991; Wang *et al.*,1993b; Weinstein *et al.*,1984).

B cells and sex hormones:

Females are known to have higher levels of the major immunoglobulins (IgM,IgG,IgA) as compared with males (Rhodes *et al.*, 1969, Eidinger and Garrett,1972; Butterworth *et al.*, 1972; Ainbender *et al.*, 1968; Astorquiza *et al.*, 1987). Hence, it is not surprising, that, although females can mount greater antibody responses against various infectious agents (Rhodes *et al.*, 1969; Michaels and Rogers,1971; Patty *et al.*,1976; Spencer *et al.*,1977; London and Drew,1977), they are also prone to auto-immune diseases (Inman,1978; Theofilopoulos and Dixon,1983; Lahita,1985; Baum and Rothschild,1981).

In humans, oestrogen as well as oestrogen antagonists, tamoxifen and FC-1157a, enhanced *in vitro* Ig synthesis from pokeweed mitogen (PWM) - stimulated B lymphocytes (Sthoeger *et al.*,1988; Paavonen and Anderson, 1985; Paavonen *et al.*, 1981). On the other hand, treatment of PWM-stimulated human B cells with testosterone either suppressed Ig synthesis (Sthoeger *et al.*,1988) or had no effect at all (Paavonen *et al.*,1981). In SLE prone NZB/NZW mice, testosterone therapy suppressed autoimmunity (Roubinian *et al.*, 1977) whereas oestrogen treatment resulted in disease exacerbation (Roubinian *et al.*,1978). A recent experimental study demonstrated *in vitro* that although splenocytes from castrated male mice, produced comparable amounts of the major immunoglobulin subclasses (IgM,IgG,IgA) to normal male mice, castrated males exhibited high titres of at least two different autoreactive antibodies in their splenocyte culture supernatants (Viselli *et al.*,1995).

Cytokines and sex hormones:

Sex hormones can directly regulate the production of different cytokines from macrophages and T cells and this can produce widespread effects on various aspects of the immune response. A number of studies in the past have documented the regulatory influence of sex steroids on cytokine production (Tables 11 and 12) (Flynn,1984; Hu *et al.*,1988; Tabibzadeh *et al.*,1989; Daynes *et al.*,1990; Fox *et al.*,1991). Thus, for example, macrophages from mature female rats have been shown to secrete higher amounts of IL-1 than those from males (Hu *et al.*,1988). However, ovarectomy resulted in a decrease in IL-1 production which could be restored by oestrogen (Kuang *et al.*,1988; Hu *et al.*,1988). In humans, *in vitro* production of IL-1 by peripheral blood monocytes was enhanced by physiological but was inhibited by supraphysiological levels of oestrogen and progesterone (Polan *et al.*,1988). Additionally, 17β-estradiol has recently been shown to suppress IL-1-and/or TNF-induced IL-6 production from normal human bone marrow-derived cells (Girasole *et al.*,1992) as well as inhibiting the release of TNF from PBMCs in postmenopausal women (Ralston *et al.*,1990).

Lymphocytes from female mice were shown to produce higher amounts of the Th1 derived cytokines IFN-γ and IL-2 than those from males following appropriate stimulation (Huygen and Palfilet,1984; McFarland and Bigley,1989; Weinstein *et al.*,1984; Araneo *et al.*,1991). Conversely, splenocytes from castrated male mice produced significantly higher levels of IL-2 and IFN-γ than controls (Viselli *et al.*,1995). Following treatment with testosterone, splenocytes from female mice produced lower levels of IL-2 comparable to those observed in normal males (Weinstein *et al.*, 1984). In a clinical study, both oestradiol and progesterone increased *in vitro* production of IFN-γ by PBMC from non pregnant women (Grasso and Muscettola,1990). These were believed, at least in part, to be due to the effects of sex steroids on IFN-γ gene expression. Later, it was shown that

the sex steroid 17β oestradiol markedly increased the activity of the IFN-γ promoter in lymphoid cells (Fox *et al.*,1991). Several other experimental studies have demonstrated that sex hormones can regulate the expression of IL-2, IL-3, IL-5 (Wang *et al.*,1993b) as well as IL-6 genes (Girasole *et al.*,1992; Potratz *et al.*, 1994). Progesterone and testosterone have been shown to induce the expression of IL-2, IL-3 and IL-5 mRNA in the murine T lymphoma, EL-4 cell line. On the other hand, 17 β-oestradiol only weakly induced expression of IL-5 mRNA expression in EL-4 (Wang *et al.*,1993b) but inhibited IL-6 gene expression in humans (Pottratz *et al.*,1994). Furthermore,*in vitro* incubation of T lymphocytes with dihydroxytestosterone (DHT) substantially reduced IL-4 and IFN-γ production, but had no effect on IL-2 secretion (Araneo *et al.*, 1991).

1.6 Sex Hormones and Parasitic Diseases

Sex hormones clearly play an influential role in control of parasitic diseases by their ability to modulate different components of the immune response. Nevertheless, it is broadly accepted that, though by no means without exception, males are more susceptible to parasitic infection than females (Alexander and Stimson, 1988). Although, in human diseases, epidemiological and social factors may occasionally contribute to gender-associated differences, they have also been observed in different laboratory disease models (Alexander and Stimson, 1988; Brabin and Brabin, 1992). Elevated levels of oestrogen, progesterone and corticosterone are implicated in suppression of existing immunity and increased susceptibility to parasitic infections during pregnancy (Van Zon *et al.*,1982; Mibrahtu *et al.*, 1988; Brabin *et al.*,1990; Brabin and Brabin ,1992).

In humans, males have a higher incidence of helminthic infections such as schistosomiasis (Goble and Konopka,1973), lymphatic filariasis (Brabin,

1990; Weller *et al.*,1983) and onchocerciasis (Kirkwood *et al.*,1983; Brandling-Bennet *et al.*,1981) than females. Similarly, male C57BL/6 mice exhibited significantly higher *Strongyloides ratti* parasite burdens as compared to their female counterparts which decreased following orchidectomy (Kiyota *et al.*,1984). Testosterone is implicated in susceptibility of jirds (Ash,1971) and mice (Nakanishsi *et al.*,1989) to *Brugia pahangi* infection.

The role of sex hormones in modulating in protozoan infections is more complex. Although, females exhibit better resistance against *T. cruzi* (Hauschka,1947; Laranja *et al.*,1956; Kierzenbaum *et al.*,1974), *T.brucei* (Greenblatt and Rosentreich,1984), *G. lamblia* (Faubert *et al.*,1985), *L. mexicana* (Alexander,1988) and *Plasmodium chabaudi* (Wunderlich *et al.*,1988) infections, males are more resistant to *Trichomonas vaginalis* (Coombs *et al.*,1985), *Toxoplasma gondii* (Roberts *et al.*,1995; Kittas and Henry,1979; Kittas and Henry,1980) and *L. major* (De Tolla *et al.*,1981; Giannini ,1986; Alexander,1988) infections.

Sex differences have been observed in human as well as in experimental leishmaniasis . In humans , males have a higher incidence of infection with *L. donovani* than females (Goble and Konopka,1973; Wijers *et al.*,1969) while non healing cutaneous ulcers caused by *L. tropica* are more common in females (Greenblatt,1980). Although, social or/and epidemiological factors may be responsible for these sex differences in human leishmaniasis, gender related differences are also reported in experimental animal models of leishmaniasis. In murine cutaneous leishmaniasis, female DBA/2 mice were relatively resistant to *L.mexicana* but susceptible to *L.major* (Alexander,1988). On the other hand, male DBA/2 were found to be resistant to *L. major* but developed large progressive lesions following infection with *L.mexicana* (Alexander,1988). Similarly, differences were

observed in C57BL/10 mice following *L. major* infection where males were more resistant to cutaneous leishmaniasis than their female counterparts (Giannini,1986). Resistance of DBA/2 mice against *L. mexicana* is oestrogen dependent and can be transferred to male DBA/2 mice by adoptive syngenic transfer of immune splenocytes from female mice (Khamis,1990). Furthermore, following i.v inoculation with *L. major*, male BALB/cAnpt,DBA/2N as well as DBA/2J mice developed higher liver parasite burdens than their female counterparts (Mock and Nacy,1988).

1.7 Aims of the Present Study

Despite of our detailed understanding of the immunological responses involved in *L. major* infection, immunoregulatory control of *L.mexicana* and *L.donovani* and the immunological mechanisms underlying sex differences in murine leishmaniasis still remain to be studied.

In the past gender-related differences in susceptibility to leishmaniasis have been observed in humans (Goble and Konopka,1973; Wijers *et al.*, 1969). Although, social , epidemiological and nutritional factors may account for some of these differences (Bundy, 1988), similar differences are observed in mice following infection with *L. major* (Mock and Nacy,1988) and *L. mexicana* (Alexander, 1988). Hormonal manipulation studies imply that these gender-related differences are hormone dependent and are possibly mediated by hormone-influenced immunological mechanisms.

The first part of the present study was undertaken to elucidate the immunological mechanisms underlying the sex differences observed in male and female DBA/2 mice during *L. mexicana* infection and investigate whether similar differences exist in murine VL following *L. donovani* infection. Understanding of these immunological differences between male

and female following *Leishmania* infection could have a significant impact on treatment and prevention of leishmaniasis.

It is well known from human and murine studies that cellular immunity is of paramount importance in determining the outcome of leishmaniasis. Disease progression following infection with *L. major*, as demonstrated in the mouse, is associated with specific immunosuppression and mediated by Th2 cells in contrast with disease control, which is associated with immunocompetence and mediated by Th1 cells (Liew, 1992). However, such a clear Th1/Th2 dichotomy can not be demonstrated following infection with *L. donovani* (Kaye *et al.*, 1991) and parasites belonging to *L. mexicana* complex (Afonso and Scott, 1993; Satoskar and Alexander, 1995).

Thus, in the second part of this study we used gene-knockout mice generated by homologous recombination to evaluate the *in vivo* role of cytokines and investigate the exact immunological mechanisms involved in control of murine *L. donovani* and *L. mexicana* infection. This was done in detail in IL-4 deficient mice in the first instance but preliminary studies in other gene-knockout mice have also been studied.

Table 2: *In vitro* cytokine modification of murine *Leishmania* infection

Cytokine	Species	Cell Type	Mouse Strain	Protection	Exacerbation	Reference
IL-2	*L.amazonensis*	-	-	no	yes	Mazingue *et al.* (1989)
IL-2	*L.donovani*	-	-	no	yes	Mazingue *et al.* (1989)
IL-2	*L.major*	PEM/BMDM	CBA/T6T6	no	no	Titus *et al.* (1984b)
IL-2+IFN-γ	*L.major*	RPM/BMDM	C3H/HeN	yes	no	Belosevic *et al.* (1988)
IL-3	*L.major*	macrophages	not specified	no	yes	Louis *et al.*(1987)
IL-3	*L.major*	PEM	CBA/T6T6	no	yes	Liew *et al.* (1989b)
IL-4	*L.major*	PEM	CBA/T6T6	no	yes	Liew *et al.* (1991a)
IL-4	*L.major*	RPM/PEM	BALB/c	no	no	Bogdan *et al.* (1991a)
IL-4	*L.major*	PEM	BALB/c	yes	no	Stenger *et al* (1991)
IL-4+IFN-γ	*L.major*	PEM	BALB/c	yes	no	Stenger *et al.* (1991)
IL-4+IFN-γ	*L.major*	RPM/PEM	BALB/c	yes	no	Bogdan *et al.* (1991a)
IL-4+IFN-γ	*L.major*	RPM	BALB/c	yes	no	Scott *et al.*(1989)
IL-4+IFN-γ	*L.major*	RPM/BMDM	C3H/HeN	yes	no	Belosevic *et al.* (1988)
IL-4+TNF	*L.major*	PEM	BALB/c	no	no	Bogdan *et al.* (1990b)
IL-7	*L.major*	PEC/BMM	BALB/c	yes	no	Gessner *et al.* (1993b)
IL-7	L.major	PEC/BMM	C57BL/6	yes	no	Gessner *et al.*

						(1993b)
IL-10	*L.major*	RPM/BMDM	-	no	yes	Vieth *et al.* (1994)
IFN-γ	*L.enrietti*	PEM	C57BL/6	yes	no	Titus *et al.* (1984b)
IFN-γ	*L.donovani*	P388D1 (cell line)	BALB/c	no	no	Reiner *et al.* (1988)
IFN-γ	*L.donovani*	PEM	BALB/c	no	no	Reiner *et al.* (1987)
IFN-γ	*L.donovani*	RPM	BALB/c	yes	no	Mosser *et al.* (1992)
IFN-γ	*L.donovani*	PEM	BALB/c	yes	no	Murray *et al.* (1985)
IFN-γ+LPS	*L.donovani*	RPM/BMDM	B10(LshR)	yes	no	Roach *et al.* (1991)
IFN-γ+LPS	*L.donovani*	RPM/BMDM	B10(LshS)	yes	no	Roach *et al.* (1991)
IFN-γ	*L.major*	RPM/PEM	BALB/c	yes	no	Bogdan *et al.* (1991a)
IFN-γ	*L.major*	PEM	BALB/c	yes	no	Stenger *et al.* (1991)
IFN-γ	*L.major*	PEM/BMDM	CBA/T6T6	yes	no	Titus *et al.* (1984b)
IFN-γ	*L.major*	RPM	BALB/c	yes	no	Mosser and Handmann(1992)
IFN-γ	*L.major*	RPM	C57BL/6	yes	no	Wyler *et al.* (1987)
IFN-γ	*L.major*	RPM	BALB/c	yes	no	Scott *et al.*(1989)
IFN-γ	*L.major*	RPM/BMDM	C3H/HeN	no	no	Belosevic *et al.* (1988)
IFN-γ	*L.major*	PEM	C3H/HeN	yes	no	Davis *et al.*(1988)
IFN-γ+LPS	*L.major*	PEM	CBA/T6T6	yes	no	Liew *et al.* (1991b)

IFN-γ+LPS	*L.major*	RPM	C3H/HeN	yes	no	Green *et al.* (1990b)
IFN-γ+TNF	*L.major*	PEM	BALB/c	yes	no	Stenger *et al.* (1991)
IFN-γ+TNF	*L.major*	PEM	BALB/c	yes	no	Bogdan *et al.* (1990b)
IFN-γ+TNF + LPS	*L.major*	PEM	CBA/T6T6	yes	no	Liew *et al.* (1990d)
IFN-γ + GM-CSF	*L.major*	RPM/BMDM	C3H/HeN	yes	no	Belosevic *et al.* (1988)
IFN-γ	*L.donovani*	RPM	BALB/c	yes	no	Murray *et al.* (1995a)
IFN-γ	*L.donovani*	RPM	BALB/c (nu/nu)	yes	no	Murray *et al.* (1995a)
TNF	*L.major*	-	-	no	no	Moll *et al.*(1990)
TNF	*L.major*	PEM	C3H/HeN	yes	no	Theodos *et al.* (1991)
TNF+LPS	*L.major*	PEM	CBA/T6T6	yes	no	Liew *et al.* (1990c)
TNF+LPS	*L.major*	PEM	CBA/T6T6	yes	no	Liew *et al.* (1990a)
M-CSF	*L.major*	PEM/BMDM	CBA/T6T6	no	no	Titus *et al.* (1984b)
GM-CSF	*L.amazonensis*	-	-	no	yes	Charlab *et al.* (1990)
GM-CSF	*L.tropica*	RPM	C3H/HeN	no	no	Ralph *et al.* (1983)
GM-CSF	*L.tropica*	RPM	C3H/HeN,BALB/c, CBA/H	yes	no	Handman & Burgess (1979)
GM-CSF	*L.major*	SM	BALB/c	no	yes	Greil *et al.*(1988)
GM-CSF	*L.major*	PEM/BMDM	CBA/T6T6	no	no	Titus *et al.* (1984b)

Table 3: *In vitro* cytokine activity associated with murine *Leishmania* infection

Cytokine	Species	Cell Type	Mouse Strain	Healing	Stimulating Antigen	Cytokine Activity	Reference
IL-1	*L.donovani*	RPM	BALB/c	no	LPS	decreased	Reiner (1987)
IL-1	*L.donovani*	PEM	BALB/c	no	fixed *Staph. aureus*	decreased	Reiner (1987)
IL-1	*L.major*	P388D1(cell line)	BALB/c	-	-	increased	Cillari *et al.* (1989)
IL-1	*L.major*	RPM	BALB/c	no	-	increased	Cillari *et al.* (1989)
IL-1	*L.major*	PEM	BALB/c	no	*L. major* amastigotes	increased	Cillari *et al* (1989)
IL-1	*L.major*	P388D1(cell line)	BALB/c	-	*L.major* amastigotes	increased	Cillari *et al.* (1989)
IL-1	*L.major*	SM	BALB/c	no	*L.major* FrTP	increased	Wagner *et al.* (1991)
IL-1	*L.major*	SM	C57BL/6	yes	*L.major* FrTP	decreased	Wagner *et al.* (1991)
IL-1	*L.major*	SM	BALB/c	no	*L.donovani* FrTP	decreased	Wagner *et al.* (1991)
IL-1	*L.major*	RPM	BALB/c	no	*L.major* promastigotes	increased	Chakkalath &Titus (1994)
IL-1	*L.major*	RPM	C3H/HeN	yes	*L.major* promastigotes	increased	Chakkalath &Titus (1994)
IL-1α	*L.major*	RPM	C3H/HeN	yes	*L.major*	increased	Delfino *et al.* (1995)
IL-1α	*L.infantum*	RPM	C3H/HeN	yes	*L.major*	increased	Delfino *et al.* (1995)
IL-2	*L.donovani*	spleen	C57BL/6	no	Con A	decreased	Olivier *et al.* (1989a)
IL-2	*L.donovani*	spleen	BALB/c	no	PHA	decreased	Reiner & Finke

							(1983)
IL-2	*L.donovani*	spleen	BALB/c	yes	*L.donovani* lysate Ag	increased	Murray *et al.* (1987)
IL-2	*L.donovani*	spleen	BALB/c(nu/nu)	no	*L.donovani* lysate Ag	decreased	Murray *et al.* (1987)
IL-2	*L.major*	LNC	BALB/c	no	*L.major* FrTP	decreased	Solbach *et al.* (1987b)
IL-2	*L.major*	LNC	C57BL/6	yes	*L.major* FrTP	increased	Solbach *et al.* (1987b)
IL-2	*L.amazonensis*	LNC	C57BL/6	yes	*L.amazonensis* promastigotes	decreased	Chan (1993)
IL-2	*L.amazonensis*	LNC	BALB/c	no	*L.amazonensis* promastigotes	increased	Chan (1993)
IL-3	*L.major*	spleen	BALB/c	no	*L.major* SAg	increased	Lelchuk *et al.* (1988)
IL-3	*L.major*	spleen	CBA	yes	*L.major* SAg	decreased	Lelchuk *et al.* (1988)
IL-3	*L.major*	spleen	irradiated BALB/c	yes	*L.major* SAg	decreased	Lelchuk *et al.* (1988)
IL-3	*L.major*	spleen	Biozzi Low	yes	*L.major* SAg	decreased	Lelchuk *et al.* (1988)
IL-3	*L.major*	spleen	Biozzi High	no	*L.major* SAg	increased	Lelchuk *et al.* (1988)
IL-3	*L.major*	spleen	αCD4mAB treatedBALB/c	yes	*L.major* SAg	decreased	Liew *et al.* (1989a)
IL-3	*L.major*	spleen	αCD4mAB treatedCBA/T6T6	yes	*L.major* SAg	increased	Liew *et al.* (1989a)
IL-3	*L.major*	spleen	BALB/c	no	*L.major* FFP	increased	Liew *et al.* (1989b)
IL-4	*L.major*	spleen	BALB/c	no	*L.major* FFP	increased	Liew *et al.* (1989b)
IL-4	*L.major*	LNC	BALB/c	no	-	increased	Scott (1991b).
IL-4	*L.major*	LNC	C3H/HeN	yes	-	increased	Scott (1991b).

IL-4	*L.major*	Spleen	129/Sv IL-4 transgenic	no	*L.major* Ag	increased	Leal *et al.* (1993)
IL-4	*L.amazonensis*	LNC	C57BL/10	no	*L.amazonensis* FTP	increased	Afonso&Scott (1993)
IL-4	*L.amazonensis*	Spleen	C57BL/10	no	*L.amazonensis* FTP	decreased	Afonso&Scott (1993)
IL-4	*L.major*	LNC	C57BL/10	yes	*L.major* FTP	decreased	Afonso&Scott (1993)
IL-4	*L.major*	Spleen	C57BL/10	yes	*L.major* FTP	decreased	Afonso&Scott (1993)
IL-4	*L.major*	LNC	C57BL/6	no	-	increased	Wang *et al.* (1994)
IL-4	*L.major*	LNC	IFN-γ-/- αCD4mAB treatedBALB/c	yes	*L.major* Ag	decreased	Mocci&Coffman (1995)
IL-4	*L.major*	LNC	C57BL/6	yes	*L.major* Ag	decreased	Mocci&Coffman (1995)
IL-4	*L.major*	LNC	129/J IL-4T	yes	*L.major* Ag	decreased	Mocci&Coffman (1995)
IL-4	*L.major*	LNC	129/J	yes	*L.major* FTP	decreased	Mocci&Coffman (1995)
IL-4	*L.major*	LNC	C57BL/6	yes	-	decreased	Wang *et al.* (1994)
IL-4	*L.amazonensis*	LNC	C57BL/6	yes	*L.amazonensis* promastigotes	decreased	Chan (1993)
IL-4	*L.amazonensis*	LNC	BALB/c	yes	*L.amazonensis* promastigotes	increased	Chan (1993)
IL-4	*L.major*	LNC	αIL-1R treated C57BL/6	yes	*L.major* promastigotes	decreased	Theodos *et al.* (1994)
IL-4	*L.major*	LNC	αIL-1R treated BALB/c	no	*L.major* promastigotes	decreased	Theodos *et al.* (1994)
IL-4	*L.major*	LNC	C57BL/6	yes	*L.major* promastigotes	decreased	Theodos *et al.* (1994)
IL-4	*L.major*	LNC	BALB/c	yes	*L.major*	increased	Theodos *et al.*

					promastigotes		(1994)
IL-4	*L.major*	LNC	αIL-12 treated C57BL/6	no	*L.major* Antigen	increased	Heinzel *et al.* (1995)
IL-6	*L.major*	RPM	BALB/c	no	*L.major* promastigotes	decreased	Chakkalath &Titus (1994)
IL-6	*L.major*	RPM	C3H/HeN	yes	*L.major* promastigotes	decreased	Chakkalath &Titus (1994)
IL-9	*L.major*	LNC/Spleen	C57BL/6	yes	*L.major* FTP	increased	Gessner *et al.* (1993a)
IL-9	*L.major*	LNC/Spleen	BALB/c	no	*L.major* FTP	increased	Gessner *et al.* (1993a)
IL-10	*L.major*	LNC	C57BL/6	yes	*L.major* FTP	decreased	Mocci&Coffman (1995)
IL-10	*L.major*	LNC	129/J	yes	*L.major* FTP	decreased	Mocci&Coffman (1995)
IL-10	*L.major*	LNC	129/J IL-4T	yes	*L.major* FTP	decreased	Mocci&Coffman (1995)
IL-10	*L.major*	RPM	C3H/HeN	yes	*L.major* promastigotes	decreased	Chakkalath &Titus (1994)
IL-10	*L.major*	RPM	BALB/c	no	*L.major* promastigotes	decreased	Chakkalath &Titus (1994)
IL-12 p40	*L.major*	LNC	C3H/HeN	yes	-	increased	Scharton-Kersten *et al.* (1995)
IL-12 p40	*L.major*	LNC	BALB/c	no	-	increased	Scharton-Kersten *et al.*(1995)
IFN-γ	*L.major*	LNC	C57BL/6	yes	*L.major* promastigotes	increased	Theodos *et al.* (1994)
IFN-γ	*L.major*	LNC	BALB/c	yes	*L.major* promastigotes	decreased	Theodos *et al.* (1994)
IFN-γ	*L.major*	LNC	αIL-1R treated C57BL/6	yes	*L.major* promastigotes	increased	Theodos *et al.* (1994)
IFN-γ	-	Spleen	C.B-17scid	no	*L.donovani* amastigotes	decreased	Kaye&Bancroft (1992)

IFN-γ	*L.major*	LNC	BALB/c	no	-	decreased	Scott(1991b)
IFN-γ	*L.major*	LNC	C3H/HeN	yes	-	increased	Scott(1991b)
IFN-γ	*L.major*	PBMC	Vervet monkey	yes	*L.major* FFP	increased	Olobo *et al.* (1992)
IFN-γ	*L.major*	LNC	C57BL/6	yes	*L.major* FTP	increased	Sadick *et al.* (1986)
IFN-γ	*L.major*	LNC	BALB/c	no	*L.major* FTP	decreased	Sadick *et al.* (1986)
IFN-γ	*L.major*	LNC	irradiated BALB/c	yes	*L.major* FTP	increased	Sadick *et al.* (1986)
IFN-γ	*L.amazonensis*	LNC	C57BL/6	yes	*L.amazonensis* promastigotes	increased	Chan (1993)
IFN-γ	*L.amazonensis*	LNC	BALB/c	no	*L.amazonensis* promastigotes	decreased	Chan (1993)
IFN-γ	*L.major*	LNC	PolyI:C treatedBALB/c	yes	-	increased	Laskay *et al.* (1993)
IFN-γ	*L.major*	LNC	αNK1.1mAB treatedC57BL/6	no	-	decreased	Laskay *et al.* (1993)
IFN-γ	*L.major*	LNC	C57BL/6	yes	*L.major* FTP	increased	Mocci&Coffman (1995)
IFN-γ	*L.major*	LNC	129/J	yes	*L.major* FTP	increased	Mocci &Coffman (1995)
IFN-γ	*L.major*	LNC	129/J IL-4T	yes	*L.major* FTP	increased	Mocci&Coffman (1995)
IFN-γ	*L.major*	LNC	129/Sv IL-4 transgenic	no	*L.major* FTP	decreased	Leal *et al.* (1993)
IFN-γ	*L.major*	LNC	IFN-γ+/- C57BL/6	yes	-	increased	Wang *et al.* (1994)
IFN-γ	*L.amazonensis*	LNC	C57BL/10	no	*L.amazonensis* FTP	decreased	Afonso & Scott (1993)
IFN-γ	*L.major*	LNC	C57BL/10	yes	*L.major* FTP	increased	Afonso & Scott (1993)
IFN-γ	*L.amazonensis*	Spleen	C57BL/10	no	*L.amazonensis* FTP	decreased	Afonso & Scott

							(1993)
IFN-γ	*L.major*	Spleen	C57BL/10	yes	*L.major* FTP	increased	Afonso & Scott (1993)
IFN-γ	*L.major*	LNC/spleen	IFN-γR -/- C57BL/6	no	*L.major* Ag	increased	Swihart *et al.* (1995)
IFN-γ	*L.major*	LNC	αIL-12 treated C57BL/6	no	*L.major* Antigen	decreased	Heinzel *et al.* (1995)
IFN-γ	*L.chagasi*	Spleen	C3H.HeJ	yes	*Lcr1* antigen	increased	Wilson *et al.* (1995)
IFN-γ	*L.chagasi*	Spleen	BALB/c	no	*Lcr1* antigen	increased	Wilson *et al.* (1995)
TNF	*L.major*	Spleen	C.B-17scid	no	*L.donovani* amastigotes	decreased	Kaye&Bancroft (1992)
TNF	-	PEM	C3H/HeN	yes	*L.major* amastigotes	increased	Theodos *et al.* (1991)
TNF	-	RPM	C3H/HeN	yes	*L.major* amastigotes	increased	Green *et al.* (1990b)
TNF	*L.donovani*	BMDM	BALB/c	no	LPS	decreased	Descoteaux & Matlashewski (1989)
TNF	*L.major*	LNC	C3H/HeN	yes	*L.major* promastigotes	increased	Titus *et al.*(1989)
TNF	*L.major*	LNC	BALB/c	no	*L.major* promastigotes	decreased	Titus *et al.*(1989)
TNF	*L.major*	Spleen	BALB/c	no	*L.major* promastigotes	increased	Moll *et al.* (1990)
TNF	*L.major*	Spleen	C57BL/6	yes	*L.major* promastigotes	increased	Moll *et al.*(1990)
TGF-β	*L.amazonensis*	RPM	BALB/c	no	*L.amazonensis* promastigotes	increased	Barral-Netto *et al.* (1992)

IFN - interferon; TGF - transforming growth factor; TNF - tumour necrosis factor; IL - interleukin; LPS - lipopolysaccharide; con A -

concanavalin A; PHA - phytohaemagglutinin; T - transgenic; FFP - formalin fixed promastigotes; FrTP - frozen and thawed promastigotes; SAg - soluble antigen; Ag - antigen; aCD4mAb - anti-CD4 monoclonal antibody; PEM - peritoneal exudate macrophages; RPM - resting peritoneal macrophages; BMDM - bone marrow derived macrophages; LNC - lymph node cells; PBMC - peripheral blood mononuclear cells; SM - splenic macrophages

Table 4: *In vivo* modification of murine *Leishmania* infection by cytokine therapy.

Cytokine	Species	Route of Adminstration	Mouse Strain	Protection	Exacerbation	Reference
IL-1	*L. donovani*	not specified	B10.D2/n	yes	no	Curry & Kaye (1992)
IL-2	*L. amazonensis*	s.c. local	BALB/c	no	yes	Mazingue *et al.* (1989)
IL-2	*L. amazonensis*	osmotic pump i.p.	BALB/c	no	no	Mazingue *et al.* (1989)
IL-2	*L. donovani*	i.v. & i.p.	BALB/c	no	no	Murray *et al.* (1987)
IL-2	*L.major*	s.c. local	BALB/c	no	no	Lezama-Davila*et al.*(1992)
IL-2	*L.mexicana*	s.c. early	BALB/c	no	yes	Lezama-Davila*et al.*(1992)
IL-2	*L.mexicana*	s.c. late	BALB/c	no	no	Lezama-Davila*et al.*(1992)
IL-2+ Pentostam	*L. donovani*	i.p.	BALB/c (nu/nu)	yes	no	Murray *et al.* (1989)
IL-2	*L.donovani*	osmotic pump s.c.	BALB/c	yes	no	Murray *et al.* (1993)
IL-2+IFN-γ	*L.donovani*	osmotic pump s.c.	BALB/c	yes	no	Murray *et al.* (1993)
IL-3	*L. major*	osmotic pump i.p.	BALB/c	no	yes	Feng *et al.*(1988)
IL-3	*L. major*	osmotic pump i.p.	CBA/J	no	yes	Feng *et al.*(1988)

IL-3	*L. major*	not specified	BALB/c	no	yes	Louis *et al.* (1987)
IL-4	*L. major*	s.c. local	C3H/HeN	no	yes	Chatelain*et al.* (1992)
IL-4	*L. major*	s.c. local	BALB/c	yes	no	Carter *et al.* (1989)
IL-4	*L.major*	s.c. early	BALB/c	no	yes	Lezama-Davila *et al.*(1992)
IL-4	*L.major*	s.c. late	BALB/c	yes	no	Lezama-Davila *et al.*(1992)
IL-4	*L.mexicana*	s.c. early	BALB/c	no	yes	Lezama-Davil *et al.*(1992)
IL-4	*L.mexicana*	s.c.late	BALB/c	no	no	Lezama-Davila *et al.*(1992)
IL-4	*L.major*	-	IL-4 Transgenic	no	yes	Leal *et al.* (1993)
IL-12	*L.major*	i.p	BALB/c	yes	no	Heinzel *et al.* (1993a)
IL-12	*L.major*	i.p	BALB/c	yes	no	Sypek *et al.* (1993)
IL-12	*L.major*	s.c	BALB/c	yes	no	Afonso *et al.* (1994)
IL-12+ Leish SAg	*L.major*	s.c	BALB/c	yes	no	Afonso *et al.* (1994)
IL-12	*L.donovani*	osmotic pump s.c.	BALB/c	yes	no	Murray *et al.* (1995a)
IL-12+ Pentostam	*L.major*	intralessional	BALB/c	yes	no	Nabors *et al.* (1995)
IFN-γ	*L.major*	-	IFN-γ deficient	no	yes	Wang *et al.* (1994)
IFN-γ	*L.major*	-	IFN-γR deficient	no	yes	Swihart*et al.* (1995)
IFN-γ	*L.donovani*	i.v. & i.p.	BALB/c	yes	no	Murray *et al.* (1987)

IFN-γ	*L.donovani*	i.v., i.p. & i.m.	BALB/c	yes	no	Murray *et al.* (1985)
IFN-γ	*L.donovani*	i.v.	BALB/c	yes	no	Kiderlen & LohmannMatth
IFN-γ	*L.donovani*	i.p.& osmotic pump i.p.	BALB/c	yes	no	Murray (1990)
IFN-γ +Pentostam	*L.donovani*	i.p.	BALB/c (nu/nu)	yes	no	Murray *et al.* (1989)
IFN-γ +Pentostam	*L.donovani*	i.p.	BALB/c	yes	no	Murray *et al.* (1988)
IFN-γ	*L.donovani*	osmotic pump s.c.	BALB/c	yes	no	Murray *et al.* (1993)
IFN-γ	*L.major*	i.p.	BALB/c	no	no	Sadick *et al.* (1990)
IFN-γ	*L.major*	s.c. &i.v.	BALB/c	yes	no	Kiderlen & LohmannMatthes (1989)
IFN-γ+TNF	*L.major*	s.c.	CBA/T6T6	yes	no	Liew *et al.* (1990a)
IFN-γ	*L.donovani*	i.p. & osmotic pump	BALB/c (nu/nu)	no	no	Murray *et al.* (1995a)
IFN-γ	*L.donovani*	i.p. & osmotic pump	BALB/c	yes	no	Murray *et al.* (1995a)
IFN-γ	*L.donovani*	osmotic pump s.c.	BALB/c	yes	no	Murray *et al.* (1995b)
TNF	*L.major*	i.v.	C3H/HeN	yes	no	Titus *et al.* (1989)
TNF	*L.major*	i.v.	BALB/c	yes	no	Titus *et al.* (1989)
TNF	*L.major*	s.c.	CBA/T6T6	yes	no	Liew *et al.* (1990a)
TNF + *L.major* SAg	*L.major*	s.c.	CBA/T6T6	yes	no	Liew *et al*. (1991c)
GM-CSF	*L.major*	i.p.(vaccinia insert)	BALB/K	no	no	Cocoran *et al.* (1988)

GM-CSF	*L.major*	-	GM-CSF Transgenic	no	no	Cocoran *et al.* (1988)
GM-CSF	*L.major*	i.p.	BALB/c	no	yes	Greil *et al.*(1988)
GM-CSF	*L.major*	i.p.	BALB/c	no	yes	Solbach *et al.* (1987a)
GM-CSF	*L.donovani*	osmotic pump s.c.	BALB/c	yes	no	Murray *et al.* (1995b)

GM-CSF - granulocyte macrophage colony stimulating factor; IFN - interferon; TNF - tumour necrosis factor; IL - interleukin; R - receptor; S.C. - subcutaneous; i.p. - intraperitoneal; i.m. - intramuscular; i.v. - intravenous; Pentostam - sodium stibogluconate; SAg - soluble antigen

Table 5: *In vivo* modification of *Leishmania* infection by anti-cytokine and anti-cytokine receptor neutralizing antibodies

Antibody	**Species**	**Route of Adminstration**	**Mouse Strain**	**Protective**	**Exacerbative**	**Reference**
αIL-1RmAb	*L.major*	i.p.	BALB/c	yes	no	Theodos *et al.* (1994)
αIL-1RmAb	*L.major*	i.p.	C57BL/6	yes	no	Theodos *et al.* (1994)
αIL-2mAb	*L.donovani*	i.p.	BALB/c (nu/+)	no	yes	Murray *et al.* (1991)
αIL-2mAb	*L.major*	i.p.	BALB/c	yes	no	Heinzel *et al.* (1993b)
αIL-2mAb	*L.donovani*	i.p.	BALB/c	no	yes	Murray *et al.* (1993)
αIL-4mAb	*L.major*	i.p.	BALB/c	yes	no	Chatelain *et al.* (1992)
αIL-4mAb	*L.major*	i.p.	BALB/c	yes	no	Sadick *et al.* (1990)
αIL-4mAb	*L.major*	i.p.	BALB/c(nu/nu)	no	no	Sadick *et al.*

						(1990)
αIL-4mAb	*L.amazonensis*	i.p.	BALB/c	yes	no	Afonso & Scott (1993)
αIL-4mAb	*L.amazonensis*	i.p.	C57BL/10	no	no	Afonso & Scott (1993)
αIL-12mAb	*L.major*	i.p.	C3HeB/FeJ	no	no	Scharton-Kersten *et al.* (1995)
αIL-12mAb	*L.major*	i.p.	C57BL/6	no	no	Sypek *et al.* (1993)
αIFN-γmAb	*L.donovani*	i.p.	BALB/c	no	yes	Squires *et al.* (1989)
αIFN-γmAb	*L.donovani*	i.p.	BALB/c(nu/+)	no	yes	Murray *et al.* (1991)
αIFN-γmAb	*L.major*	i.p.	C3H/HeN	no	yes	Belosevic *et al.* (1990)
αIFN-γmAb	*L.major*	i.p.	BALB/c	no	yes	Belosevic *et al.* (1990)
αIFN-γmAb	*L.major*	i.p.	C3H/HeN	no	yes	Scott (1991a)
αIFN-γmAb	*L.donovani*	i.p.	BALB/c	no	yes	Murray *et al.* (1993)
αIFN-γmAb	*L.mexicana*	i.p.	DBA/2	no	yes	Satoskar & Alexander (1995)
αIFN-γmAb +Pentostam	*L.donovani*	i.p.	BALB/c	yes	no	Nabors and Farrell (1995)
αTNFpAb	*L.major*	i.v.	C3H/HeN	no	yes	Titus *et al.* (1989)
αTNFpAb	*L.major*	i.v.	BALB/c	no	yes	Titus *et al.* (1989)
αTNFpAb	*L.major*	i.v.	CBA/T6T6	no	yes	Liew *et al.* (1990a)
αTNFpAb	*L.major*	i.v.	irradiated BALB/c	no	yes	Liew *et al.* (1990a)
αTGF-βmAb	*L.amazonensis*	s.c.	BALB/c	yes	no	Barral-Netto *et al.*

α GM-CSFAb	*L. donovani*	i.p.	BALB/c	no	yes	(1992) Murray *et al.* (1995b)

IFN - interferon; TNF - tumour necrosis factor; TGF - transforming growth factor; IL- interleukin; a - anti; mAb - monoclonal antibody; pAb - polyclonal antibody; i.p. - intraperitoneal; i.v. - intravenous

Table 6: *In vivo* cytokine activity associated with murine *Leishmania* infection

Cytokine	Species	Cell type	Mouse Strain	Healing	Cytokine activity	Reference
IL-2	*L.donovani*	Liver	BALB/c	no	increased	Mirallese*t al.* (1994)
IL-4	*L.donovani*	Liver	BALB/c	no	increased	Mirallese*t al.* (1994)
IL-4	*L.major*	spleen & LNC	C57BL/6	yes	decreased	Heinzel *et al.* (1989)
IL-4	*L.major*	spleen & LNC	BALB/c	no	increased	Heinzel *et al.* (1989)
IL-10	*L.donovani*	Liver	BALB/c	no	increased	Mirallese*t al.* (1994)
IFN-γ	*L.major*	spleen & LNC	C57BL/6	yes	increased	Heinzel *et al.* (1989)
IFN-γ	*L.major*	spleen & LNC	BALB/c	no	decreased	Heinzel *et al.* (1989)
IFN-γ	*L.mexicana*	LNC	Male DBA/2	no	decreased	Satoskar & Alexander (1995)
IFN-γ	*L.mexicana*	LNC	Female DBA/2	yes	increased	Satoskar & Alexander (1995)
IFN-γ	*L.donovani*	Liver	BALB/c	no	increased	Mirallese*t al.* (1994)

TNF	*L.major*	sera	BALB/c	no	increased	Moll *et al.* (1990)
TNF	*L.major*	sera	C57BL/6	yes	decreased	Moll *et al.* (1990)
TNF	*L.mexicana*	LNC	Male DBA/2	no	increased	Satoskar & Alexander (1995)
TNF	*L.mexicana*	LNC	Female DBA/2	yes	decreased	Satoskar & Alexander (1995)
TGF-β	*L.major*	skin lesion	BALB/c	no	increased	Stenger *et al.* (1994)
TGF-β	*L.major*	skin lesion	C57BL/6	yes	decreased	Stenger *et al.* (1994)

IFN - interferon; TNF - tumour necrosis factor; IL - interleukin; TGF - transforming growth factor; LNC - lymph node cells.

Table 7: *In vitro* modulation of human *Leishmania* infection.

Cytokine	Species	Cell Type	Protection	Exacerbation	Reference
IL-1β	*L.amazonensis*	PBMC	yes	no	Hatzigeorgiou *et al.* (1993)
IL-3	*L.amazonensis*	PBMC	yes	no	Ho *et al.*(1991)
IL-3	*L.amazonensis*	PBMC	yes	no	Ho *et al.* (1992a)
IL-4	*L.amazonensis*	PBMC	no	yes	Ho *et al.* (1992b)
IL-6	*L.donovani*	PBMC	no	yes	Hatzigeorgiou *et al.* (1993)
IL-10	*L.donovani*	PBMC	no	yes	Wu *et al.* (1993)
IFN-γ	*L.amazonensis*	PBMC	yes	no	Ho *et al.* (1992b)
IFN-γ	*L.aethiopica*	THP1(cell line)	yes	no	Mohamed *et al.* (1992)
IFN-γ	*L.donovani*	PBMC	yes	no	Hoover *et al.*(1985)
IFN-γ	*L.donovani*	PBMC	no	no	Engelhorn *et al.*(1990)
IFN-γ	*L.donovani*	PBMC	yes	no	Weiser *et al.* (1987)
IFN-γ+Pentostam	*L.donovani*	PBMC	yes	no	Muray *et al.* (1988)
IFN-γ	*L.major*	PBMC	no	no	Hoover *et al.* (1985)
IFN-γ	*L.mexicana*	PBMC	yes	no	Murray *et al.*(1984)
TNF	*L.amazonensis*	PBMC	yes	no	Ho *et al.*(1992b)
GM-CSF	*L.amazonensis*	PBMC	yes	no	Ho *et al.* (1990)
GM-CSF	*L.donovani*	PBMC	yes	no	Weiser *et al.* (1987)
GM-CSF	*L.donovani*	PBMC	yes	no	Hatzigeorgiou *et al.* (1993)
MIF	*L.donovani*	PBMC	yes	no	Weiser *et al.* (1991)

GM-CSF - granulocyte macrophage colony stimulating factor; IFN - interferon; - tumour necrosis factor; MIF - migration inhibitory factor; PBMC - peripheral blood mononuclear cells (monocytes); Pentostam - sodium stibogluconate

Table 8: *In vitro* cytokine activity associated with human *Leishmania* infection

Cytokine	Species	Disease	Cell Type	Cell Stimulation	Cytokine Activity	Reference
IL-1	-	-	PBMC	*L.donovani* amastigotes	decreased	Reiner *et al.* (1990)
IL-2R	*L.braziliensis*	MCL	PBMC	*L.mexicana* whole Ag	increased	Castes *et al.* (1988)
IL-2R	*L.mexicana*	LCL	PBMC	*L.mexicana* whole Ag	increased	Castes *et al.* (1988)
IL-2	*L.chagasi*	VL	PBMC	*L.chagasi* FrTP	decreased	Carvalho *et al.* (1985a)
IL-2	*L.donovani*	VL	PBMC	PHA	decreased	Cillari *et al.* (1988)
IL-2	*L.donovani infantum*	VL	PBMC	*L.donovani infantum* FrTP	decreased	Vitale *et al.* (1992)
IL-2	*L. major*	VL	PBMC	*L.major* SAg	increased	Passwell *et al.* (1987)
IL-2	*L.major*	CL	PBMC	*L.major* promastigotes	increased	Frankenburg *et al.*(1993)
IL-2	*L.tropica*	CL	PBMC	PHA ;αCD3mAb	decreased	Raziuddin *et al.* (1994)
IL-2	*L.donovani*	VL	PBMC	PHA ;αCD3mAb	decreased	Raziuddin *et al.* (1994)
IL-4	*L.donovani*	VL	PBMC	PMA+αCD3mAb	increased	Zwingenberger *et al.* (1990)
IL-4	*L.major*	MildCL	PBMC	*L.major* crude Ag; LDA; gp63	decreased	Gaafar *et al.* (1995)
IL-4	*L.major*	SevereCL	PBMC	*L.major* crude Ag; LDA; gp63	increased	Gaafar *et al.* (1995)
IL-4	-	-	PBMC	*L.major* crude SAg	increased	Kurtzhals *et al.*(1995)

IL-4	-	-	PBMC	*L.donovani* crude SAg	increased	Kurtzhals *et al.*(1995)
IL-4	*L.major*	CL	PBMC	*L.major* crude SAg; gp63	decreased	Kemp *et al.* (1994)
IL-4	-	Leishmanin positive	PBMC	*L.major* crude SAg; gp63	decreased	Kemp *et al.* (1994)
IL-4	*L.tropica*	CL	PBMC	PHA ;αCD3mAb	increased	Raziuddin *et al.* (1994)
IL-4	*L.donovani*	VL	PBMC	PHA ;αCD3mAb	increased	Raziuddin *et al.* (1994)
IL-4	*L.donovani*	VL	PBMC	*L.major* & *L.donovani* gp63	increased	Kemp *et al.* (1995)
IL-4	*L.donovani*	VL	PBMC	LPGAP	increased	Kemp *et al.* (1993a)
IL-6	*L.donovani*	VL	PBMC	LPS ; αCD3mAb	increased	Raziuddin *et al.* (1994)
IL-8	*L.donovani*	VL	PBMC	LPS ;αCD3mAb	increased	Raziuddin *et al.* (1994)
IL-10	*L.chagasi*	VL	PBMC	*L.chagasi* Ag	increased	Holaday *et al.* (1993)
IL-10	*L.braziliensis*	MCL/CL	PBMC	rLeIf Antigen	decreased	Skeiky *et al.* (1995)
IL-12	*L.braziliensis*	MCL/CL	PBMC	rLeIf Antigen	increased	Skeiky *et al.* (1995)
IFN-γ	*L.braziliensis*	MCL/CL	PBMC	rLeIf Antigen	decreased	Skeiky *et al.* (1995)
IFN-γ	*L.braziliensis*	MCL	PBMC	*L.mexicana* whole Ag	increased	Castes *et al.* (1988)
IFN-γ	*L.braziliensis*	MCL	PBMC	*L.amazonensis* SAg	increased	Carvalho *et al.* (1985b)
IFN-γ	*L.chagasi*	VL	PBMC	*L.chagasi* FrTP	decreased	Carvalho *et al.* (1985a)
IFN-γ	*L.donovani*	VL	PBMC	*L.donovani* SAg	decreased	Sacks *et al.*

						(1987)
IFN-γ	*L.donovani*	VL	PBMC	PMA+aCD3mAb	decreased	Zwingerberger *et al.* (1990)
IFN-γ	*L.major*	LCL	PBMC	*L.major* SAg	increased	Passwell *et al.* (1987)
IFN-γ	*L.mexicana*	LCL	PBMC	*L.mexicana* FrTP	decreased	Murray *et al.* (1984)
IFN-γ	*L.mexicana*	LCL	PBMC	*L.mexicana* whole Ag	increased	Castes *et al.* (1988)
IFN-γ	*L.mexicana*	DCL	PBMC	*L.mexicana* whole Ag	decreased	Castes *et al.* (1988)
IFN-γ	*L.pifanoi*	DCL	PBMC	*L.pifanoi* crude Ag	decreased	Rada *et al.*(1987)
IFN-γ	*L.pifanoi*	MCL	PBMC	*L.pifanoi* crude Ag	increased	Rada *et al.*(1987)
IFN-γ	*L.pifanoi*	LCL	PBMC	*L.pifanoi* crude Ag	decreased	Rada *et al.*(1987)
IFN-γ	*L.major*	MildCL	PBMC	*L.major* crude Ag	increased	Gaafar *et al.* (1995)
IFN-γ	*L.major*	SevereCL	PBMC	*L.major* crude Ag	decreased	Gaafar *et al.* (1995)
IFN-γ	*L.major*	CL	PBMC	*L.major* promastigotes	increased	Frankenburg *et al.*(1993)
IFN-γ	-	-	PBMC	*L.major* crude SAg	increased	Kurtzhals *et al.*(1995)
IFN-γ	-	-	PBMC	*L.donovani* crude SAg	increased	Kurtzhals et al.(1995)
IFN-γ	*L.major*	CL	PBMC	*L.major* crude SAg; gp63	increased	Kemp *et al.* (1994)
IFN-γ	-	Leishmanin positive	PBMC	*L.major* crude SAg; gp63	increased	Kemp *et al.* (1994)
IFN-γ	*L.major*	CL	PBMC	*L.major* & *L.donovani* gp63	increased	Kemp *et al.* (1995)
IFN-γ	*L.donovani*	VL	PBMC	LPGAP	increased	Kemp *et al.* (1993a)
TNF	*L.major*	CL	PBMC	*L.major*	increased	Frankenburg

				promastigotes		*et al.*(1993)
TNF	-	-	PBMC	*L.donovani*	decreased	Reiner *et al.* (1987)
TNF-α	*L.braziliensis*	MCL/CL	PBMC	rLelf Antigen	decreased	Skeiky *et al.* (1995)
TNF-α	*L.tropica*	CL	PBMC	LPS ;αCD3mAb	increased	Raziuddin *et al.* (1994)
TNF-α	*L.donovani*	VL	PBMC	LPS ;αCD3mAb	increased	Raziuddin *et al.* (1994)
GM-CSF	*L.tropica*	CL	PBMC	LPS ;αCD3mAb	decreased	Raziuddin *et al.* (1994)
GM-CSF	*L.donovani*	VL	PBMC	LPS ;αCD3mAb	decreased	Raziuddin *et al.* (1994)

IFN - interferon; TNF - tumour necrosis factor; GM-CSF - granulocyte monocyte colony stimulating factor; IL- interleukin; PBMC - peripheral blood mononuclear cells; R - receptor; LCL - localized cutaneous leishmaniasis; MCL - mucocutaneous leishmaniasis; DCL - diffuse cutaneous leishmaniasis; Ag - antigen; SAg - soluble antigen; LDA - *Leishmania donovani* amstigotes; LPGA - *Leishmania* protein co-isolated with lipophosphoglycan; PHA - phytohaemagglutinin; PMA - phobol myristate acetate; LPS - lipopolysaccharide; gp - glycoprotein; FrTP - frozen and thawed promastigotes; αCD3mAB - anti- CD3 monoclonal antibody.

Table 9: *In vivo* modification of human *Leishmania* infection by cytokine therapy

Cytokine	Species	Route of Adminstration	Disease	Protective	Exacerbative	Reference
IL-2	*L.aethiopica*	i.d.	DCL	yes	no	Akuffo *et al.* (1990)
IFN-γ	*L.guyanensis*	i.d.	MCL	yes	no	Harms *et al.* (1989)

IFN-γ	*L.major*	i.d.	LCL	yes	no	Harms *et al.* (1989)
IFN-γ+ Glucantime	*L.amazonensis*	i.m.	DCL	yes	no	Badaro & Johnson (1989)
IFN-γ+ Glucantime	*L.chagasi*	i.m.	VL	yes	no	Badaro *et al.* (1990)
IFN-γ+ Glucantime	*L.chagasi*	i.m.	VL	yes	no	Badaro & Johnson (1993)
IFN-γ+ Glucantime	*L.tropica*	s.c.	LCL	yes	no	Kurkcuoglu & Tandogdu (1990)
IFN-γ	*L.tropica*	i.d.	LCL	yes	no	Harms *et al.* (1991)
IFN-γ+ Antimony	*L.mexicana/ L.braziliensis*	s.c.	LCL	yes	no	Arana *et al.* (1992)
IFN-γ+ Antimony	*L.braziliensis* complex	s.c.	MCL	yes	no	Bottasso *et al.* (1992)
IFN-γ+ Antimony	*L.mexicana*	i.m.	DCL	yes	no	Melby *et al.* (1992)
IFN-γ+ Antimony	*L.donovani*	i.m.	VL	yes	no	Squires *et al.* (1993)
IFN-γ+ Antimony	*L.donovani*	s.c.	VL	yes	no	Sundar *et al.* (1993a,b)
IFN-γ+ Antimony	*L. chagasi*	s.c.	VL	yes	no	Harms *et al.* (1993)
GM-CSF+ Antimony	*L.chagasi*	s.c.	VL	yes	no	Badaro *et al.* (1993)
GM-CSF	*L.chagasi*	s.c./i.v.	VL	yes	no	Badaro *et al.* (1994)

GM-CSF - granulocyte macrophage colony stimulating factor; IFN - inteferon; IL - interleukin; Glucantime - N-methyl glucamine; i.d. - intradermal; s. c. - subcutaneous; i. m. - intramuscular; DCL - diffuse cutaneous leishmaniasis; MCL - mucocutaneous leishmaniasis; LCL - localized cutaneous leishmaniasis; VL - visceral leishmaniasis; Pentostam - sodium stibogluconate

Table 10: *In vivo* cytokine activity associated with human *Leishmania* infection

Cytokine	Species	Disease	Cell Type	Cytokine activity	Reference
IL-2R	*L. donovani*	VL	sera	increased	Barral-Netto *et al.* (1991)
sIL-2R	-	DCL	sera	increased	Calcagno *et al.* (1995)
sIL-2R	-	LCL	sera	increased	Calcagno *et al.* (1995)
IL-4	*L. donovani*	VL	sera	increased	Zwingenberger *et al.* (1990)
IL-5	-	DCL	sera	increased	Calcagno *et al.* (1995)
IL-5	-	MCL	sera	increased	Calcagno *et al.* (1995)
IL-5	-	LCL	sera	decreased	Calcagno *et al.* (1995)
IL-5	-	DCL	sera	increased	Calcagno *et al.* (1995)
IL-6	*L. donovani*	VL	sera	increased	Cenini *et al.* (1993)
IL-10	-	VL	BMA	increased	Karpet *al.* (1993)
IFN-γ	*L. donovani*	VL	sera	decreased	Zwingenberger *et al.* (1990)
IFN-γ	-	VL	BMA	increased	Calcagno *et al.* (1995)
IFN-γ	*L. donovani*	VL	sera	increased	Cenini *et al.* (1993)
TNF	*L. donovani*	VL	sera	increased	Cenini *et al.* (1993)
TNF	*L. aethiopica*	VL	sera	increased	Pisa *et al.* (1990)
TNF	*L. aethiopica*	DCL	sera	increased	Pisa *et al.* (1990)
TNF	*L. aethiopica*	LCL	sera	decreased	Pisa *et al.* (1990)
TNF	-	DCL	sera	increased	Calcagno *et al.* (1995)
TNF	-	MCL	sera	increased	Calcagno *et al.* (1995)

IFN - interferon; TNF - tumour necrosis factor; IL - interleukin; R - receptor; BMA - bone marrrow aspirate; VL - visceral leishmaniasis; DCL - diffuse cutaneous leishmaniasis; LCL - localized cutaneous leishmaniasis

(Tables modified after Alexander and Russell,1992 and Williams,1993)

Table 11: Modulation of human cytokine activitiy by sex hormones

Cytokine	Cell type	Hormone
IL-1	Macrophage	17β-estradiol
IL-1	Monocyte	Progesteron 10^{-9} mol/l
IL-1	Monocyte	Progesteron $> 10^{-7}$ mol/l
IL-1	Monocyte	17β-estradiol 10^{-10}-10^{-9} mol/l
IL-1	Monocyte	17β-estradiol $>10^{-8}$ mol/l
IL-2	EL-4 T cell lymphoma cell line	Progesterone
IL-2	EL-4 T cell lymphoma cell line	Testosterone
IL-3	EL-4 T cell lymphoma cell line	Progesterone
IL-3	EL-4 T cell lymphoma cell line	Testosterone
IL-4	CD4+ T cell line	Progesterone
IL-5	EL-4 T cell lymphoma cell line	Progesterone
IL-5	EL-4 T cell lymphoma cell line	Testosterone
IL-5	EL-4 T cell lymphoma cell line	17β-estradiol
IL-6	ER transfected HeLa cell line	17β-estradiol
IL-6	BMDC	17β-estradiol

Cytokine activity	Reference
increased	Hu *et al.*(1988)
increased	Polan *et al.* (1988)
decreased	Polan *et al.* (1988)
increased	Polan *et al.* (1988)
decreased	Polan *et al.* (1988)
increased	Wang *et al.* (1993b)
increased	Wang *et al.* (1993b)
increased	Wang *et al.* (1993b)
increased	Wang *et al.* (1993b)
increased	Piccinni *et al.* (1995)
increased	Wang *et al.* (1993b)
increased	Wang *et al.* (1993b)
increased	Wang *et al.* (1993b)
decreased	Pottratz *et al.* (1994)
decreased	Girasole *et al.* (1992)

IFN-γ	PBMC from non-pregnant women	17β-estradiol	increased	Grasso & Muscettola (1990)
IFN-γ	PBMC from non-pregnant women	Progesteron	increased	Grasso & Muscettola (1990)
IFN-γ	SEA stimulated PBMC	Progesteron	decreased	Grasso & Muscettola (1990)
IFN-γ	SEA stimulated PBMC from non-pregnant women	Progesteron	increased	Grasso & Muscettola (1990)
TNF	PBMC from postmenopausal women	17β-estradiol 10^{-12}-10^{-6} mol/l	decreased	Ralston *et al.* (1990)
TNF	PBMC	DHT	no effect	Ralston *et al.* (1990)

IFN - interferon; TNF - tumour necrosis factor; IL - interleukin; ER - estrogen receptor; BMDC -bone marrow derived cells; DHT - dihydroxy testosterone; PBMC -peripheral blood mononuclear cells; SEA - staphylococcal enterotoxin A

Table 12: Modulation of murine cytokine activity by sex hormones

Cytokine	**Cell type**	**Hormone**	**Cytokine activity**	**Reference**
IL-1	PEM	17β-estradiol	increased	Khamis (1991)
IL-2	Helper T cell	Dehydroepiandrosterione	increased	Daynes *et al.* (1990)
IL-2	Splenocytes	Oestrogen	decreased	Ansar Ahmed *et al.*(1985b)
IL-2	Splenocytes	Testosterone	increased	Ansar Ahmed *et al.*(1985b)

IL-2	NIMP-TH1 T cell hybrid	Progesterone	increased	Wang *et al.* (1993b)
IL-2	NIMP-TH1 T cell hybrid	Testosterone	increased	Wang *et al.* (1993b)
IL-2	activated T-cells	Dihydrotestosterone	no effect	Araneo *et al.* (1991)
IL-3	NIMP-TH1 T cell hybrid	Progesterone	increased	Wang *et al.* (1993b)
IL-2	NIMP-TH1 T cell hybrid	Testosterone	increased	Wang *et al.* (1993b)
IL-4	activated T-cells	Dihydrotestosterone	decreased	Araneo *et al.* (1991)
IL-5	activated T-cells	Dihydrotestosterone	decreased	Araneo *et al.* (1991)
IL-5	NIMP-TH1 T cell hybrid	Progesterone	increased	Wang *et al.* (1993b)
IL-5	NIMP-TH1 T cell hybrid	Testosterone	increased	Wang *et al.* (1993b)
IL-5	NIMP-TH1 T cell hybrid	17β-estradiol	increased	Wang *et al.* (1993b)
IL-6	BM derived stromal cell lines	17β-estradiol	decreased	Girasole *et al.* (1992)
IL-6	Nontransformed osteoblast cell lines	17β-estradiol	decreased	Girasole *et al.* (1992)
IFN-γ	activated T-cells	Dihydrotestosterone	decreased	Araneo *et al.* (1991)
IFN-γ	splenocytes	17β-estradiol	increased	Fox *et al.* (1991)

IFN - interferon; TNF - tumour necrosis factor; IL - interleukin; PEM - peritoneal exudate macrophages

Chapter 2

General Materials and Methods

Animals

The inbred mouse strains used during these studies were BALB/c, C57BL/10, C57BL/6, DBA/2, 129/SvEv and 129/Svj. Interleukin-4 gene deficient (GKO) (129/SvxC57BL/6) F2 mice, MHC class II GKO (C57BL/6) mice, IL-6 GKO (129/Sv) mice, TNFR1 GKO (129/Svj) mice and IFN-γR GKO (129/Sv) mice were generated by Dr. H. Bluethmann at F. Hoffmann-La Roche AG, Basel, Switzerland. All the mice used throughout the experiments were bred and maintained at the University of Strathclyde.

Parasites

Leishmania mexicana (MNYC/BZ/62/M379) was maintained by serial passage of 10^6 amastigotes inoculated subcutaneously into the shaven rumps of BALB/c mice. Amastigotes for use in experimental studies were isolated as described by Alexander and Vickerman (1975) by grinding excised cutaneous from an infected mouse through a metal sieve into RPMI 1640 (Gibco, Paisley, Scotland). The parasites were washed two times at 350 g in RPMI 1640, passed through 26G needle and were enumerated using an improved Naubauer hematocytometer.

Leishmania donovani (L82) was maintained by serial passage of amastigotes into hamsters. Parasites used for infecting mice were isolated from the spleens of infected hamsters as described by (Kaye,1987). Briefly, the spleens from the infected hamsters were excised, mashed through a metal sieve into RPMI 1640 and were centrifuged at 350 g for 5 minutes. The pellet obtained was resuspended in 0.05% (w/v) saponin (Sigma) in RPMI1640 for 2 minutes at 37°C to lyse the cells prior to centrifugation at 350 g for 5 minutes. The amastigotes obtained were washed two times in RPMI 1640 and were enumerated using an improved Neubauer hematocytomer.

Following infection with *L. donovani*, the mice were sacrificed at different time points and the parasite burdens in their viscera were assessed as described by Stauber

(1958).

Stationary phase promastigotes (SPP) were produced by *in vitro* culture of *L. mexicana* and *L. donovani* amastigotes in Schneider's Insect Medium (Sigma Chemical Co. Ltd., Poole, Dorset, UK) supplemented with 20% (v/v) fetal calf serum (Sera-Lab Ltd., Sussex, UK) ,streptomycin (50 mg/ml, Gibco), penicillin (50 U/ml , Gibco, Paisley , Scotland).

Extraction of total RNA from draining lymph nodes

Draining inguinal lymph nodes were excised at different time points following infection. Total RNA was extracted using the modified acid-guanidium thiocyanate-phenol-chloroform method (Hunter *et al.*, 1992) and was precipitated at -70°C using ethanol.

Briefly, inguinal lymph nodes removed from the mice were homogenised in 1ml of denaturing solution (4M guanidinium thiocyanate (BDH), 25mN sodium citrate pH 7.0 (BDH), 0.5% (w/v) m-laurel sacrosine (Sigma) and 0.1 M 2-mercaptoethanol (Sigma))using glass-Teflon hand homogenisers (BDH Ltd., Glasgow,Scotland) and were transferred to 15ml Falcon centrifuge tubes.These homogenates were further mixed with 100µl of 2M sodium acetate pH 4, 1ml of Phenol and 200µl of chloroform-isoamyl alcohol (49:1) and vortexed for 10 seconds. Following the incubation on ice for 10 minutes the samples were centrifuged at 10000 g for 20 minutes at 4°C in a Sorvall RC-5B refrigerated super speed centrifuge (DU Pont Company, Connecticut, U.S.A.). The aqueous phase containing the RNA was transferred to a 1.5 ml microcentrifuge tube. The RNA was precipitated with 1ml of ice-cold isopropanol (BDH) at -70°C for 60 minutes. Following precipitation the samples were further centrifuged at 10000 g for 20 minutes at 4°C and the pellets obtained were redissolved in 300µl of denaturing solution and precipitated again at -70°C for 60 minutes using 600µl of ice-cold ethanol. The samples were again centrifuged at 10000 g for 20 minutes at 4°C and the pellets obtained were

resuspended in 300ml of sterile deionised water. These were vigorously mixed with equal volumes of phenol:chloroform (1:1) and centrifuged as described above. The upper phase obtained following centrifugation was transferred to another microcentrifuge tube and RNA was precipitated by the addition of 1/10 volume of 7 M ammonium acetate (Sigma) and 2 1/2 volumes of ethanol as described above. The RNA pellets were obtained by centrifugation as described above. The pellets were resuspended and were washed twice in 70% ethanol at room temperature each time followed by centrifugation at 13000 g before lypholisation and dissolution in an appropriate volume of sterile water. RNA was quantified by measuring absorbance at 280nm and the integrity of RNA was also confirmed by visualising RNA bands following ethidium bromide stained 1.5% agarose gel electrophoresis and ultraviolet illumination.

Reverse transcription and polymerase chain reaction (RT-PCR)

Oligonucleotide primers required for reverse transcription as well as cDNA amplification of β-actin, IFN-γ, tumour necrosis factor-α(TNF-α), interleukin-4 (IL-4), IL-10 and IL-12 were purchased either from Clontech (Cambridge, GB) or Cruachem Ltd (Glasgow, GB). Appropriate cytokine cDNA or transfected plasmids (obtained from Dr.Craig Roberts,Univ. of Strathclyde and Prof. F. Y. Liew, Univ. of Glasgow)were used as a positive controls.

For each sample, reverse transcription was carried out at 43°C for 45 minutes using approximately 5μg of RNA in a total volume of 20μl of 1x Taq polymerase buffer (Promega, Madison, W1) containing 200U of Moloney murine leukemia virus reverse transcriptase (BRL, Uxbridge, UK), 20 U ribonuclease Inhibitor (Promega,U.S.A.), 2.5nM of each dNTP (Pharmacia, Uppsala, Sweden) and 0.5μg of appropriate 3' primer to be used in PCR. For positive controls approximately 2 μg of cDNA was used for amplification .

Following reverse transcription 80 μl of 1 x Taq polymerase buffer containing 0.5μg

of 5' primer and 1x Taq polymerase was added to the reaction mixture and the PCR was carried out using 30 cycles of amplification at 94°C for 1 min, 60°Cfor 2 min and 72°C for 3 min using a Hyabid thermocycler. The PCR products were analysed using ethidium bromide-stained agarose gel electrophoresis and ultraviolet illumination. Computer scanning was used for further image enhancement if required.

Nucleotide sequences for oligonucleotides 5' and 3' primers, respectively were as follows:
B-actin, GTGGGCCGCTCTAGGCACCAA AND CTCTTTGATGTCACGATTTC; IFN-γ,TGAACGCTACACTGCATCTTG and CGACTCCTTTTTCCGCTTCCT-GAG; IL-4, ATGGGTCTCAACCCCCAGCTAGT and GCTCTTAGGCTT-TCCAGGA AGTC; IL-10, GTGAAGACTTTCTTTCAAACAAAG and CTGCTCC-ACTGCCTTGCTTATT; TNF-α, ATGAGCACAGAAAGCATGATCCGC and CCAAAGTAGACCTGCCCGGACTC; IL-12 , GACCCTGCCCATTGAACTGGC and CAACGTTGCATCCTAGGATCG.

Maintenance of R4-6A2 hybridoma and purification of IFN-γ neutralising antibodies
The R4-6A2 rat-mouse hybridoma secreting monoclonal antibody capable of neutralising murine IFN-γ provided by Dr. E.A. Havell (Trudeau Institute, New York) was maintained and grown in RPMI 1640 tissue culture medium supplemented with 10% FCS. Hybridoma supernatants were concentrated using an Amicon ultrafiltration system.The antibodies in concentrated supernatants were further purified using Protein G column chromatography as follows. Briefly, the Protein G-sepharose column was washed with binding buffer (0.02M sodium phosphate, pH7.0) for 30 minutes. Following calibration of the uv monitor, the sample was loaded and the column was washed further with binding buffer to remove unbound protein. Antibody bound to the column was eluted using eluting buffer (0.1M glycine-HCL pH 2.7) and fractions containing antibody were collected into neutralising buffer (1M Tris.HCL, pH 9.0). The antibody concentration was determined by measuring the optical density at 280nm.

Preparation of soluble leishmanial antigen

Soluble leishmanial antigen was prepared from stationary phase promastigotes of *L.mexicana.* Promastigotes were washed twice in ice cold PBS by centrifugation at 10,000 g and were resuspended in hypotonic buffer consisting of 10mN Tris-HCL, 2mM EDTA, pH 7.8 with 50mM N-p-tosyl-L-lysine chlormethylketone (TLCK) and 15mM leupeptin (Sigma, Poole, UK).Following 15 minutes incubation on ice the promastigotes were disrupted in a Braun homogeniser and centrifuged at 10,000 g for 60 minutes at 4°C. The supernatant containing soluble leishmanial antigen was dialysed against PBS (pH 7.4) overnight at 4°C and the protein concentration was determined using a Bradford Assay (Bradford, 1976). The antigen was stored at -20°C until used.

Isotype Enzyme Linked Immunosorbent Assay (ELISA)

Following infection with *Leishmania* plasma samples were obtained at different time points by collecting blood into a heparinised capillary tube via the tail vein and separating the plasma portion following centrifugation at 200 g .These samples were used to determine the titres of IgG1 and IgG2a *Leishmania* specific antibodies. Briefly, each well of a 96 well microtitre plate was coated with 1µg of soluble leishmanial antigen in PBS (pH 9.0). Following a 1hr incubation at 37°C with serially diluted test plasma (1:100 starting dilution), plates were washed 3 times and either rat anti-mouse IgG1 horseradish peroxidase conjugate (Southern Biotechnology Associates,Alabama, USA - 1:8000 dilution) or rat anti-mouse IgG2a horseradish peroxidase conjugate (Jackson Laboratories,1:2000 dilution) was applied. After a further 1hr incubation at 37°C and 3 washes, tetramethyl benzidine in a sodium acetate buffer (pH 5.5) containing H_20_2 was added. The reaction was stopped after 20 min by adding 10% H_2SO_4 and the absorbance measured at 450nm on a Titertek Multiscan plate reader.

T cell proliferation assay

Following infection with *L. mexicana* the spleens and draining lymph nodes were

removed aseptically at different time points and cell suspensions prepared by gentle teasing in RPMI 1640 supplemented with 10% foetal calf serum (heat inactivated), 2mM L-glutamine, 100 units/ml penicillin, 100μg/ml streptomycin and 0.05 mM β-mercaptoethanol (Gibco, Paisley, UK). The cells were centrifuged at 200g for 5 minutes and the erythrocytes lysed by resuspending spleen cells in 3ml of Boyle's solution (Boyle,1968) (0.17MTris, 0.16 M ammonium chloride: BDH Ltd., Dorset, UK) and incubating at 37°C for 3 minutes. Boyle's lysis was not carried out for lymph node cells. Following two washes, the viable cells were counted by trypan blue exclusion using an improved Nuebaer haemocytometer and cell suspensions were adjusted to 5 x 10^6 cells/ml for spleen cells and 3 x 10^6 cells/ml for lymph node cells in complete RPMI medium. Aliquots (100μl) containing 5 x 10^5 spleen cells were added in triplicate to the wells of 96-well flat bottomed tissue culture plates (Costar, Cambridge, MA, USA) containing 100 μl per well of soluble *L. mexicana* antigen at concentrations of 50, 10, 5 and 0 μg/ml or concanavalin A (Con A; 5mg/ml). For lymph node cells Aliquots (100 μl) containing 3 x 10^5 lymph node cells from individual mice were added in triplicate to the wells containing 100μl per well antigen at concentrations of 25 and 0 μg/ml or concanavalin A (Con A; 2.5μg/ml). Following incubation at 37°C in 5% CO_2 for 60 hours, 100μl of the culture supernatant was removed from each well (stored at -70°C for cytokine assays) and replaced with 100μl of complete medium containing the appropriate amount of antigen or Con A. At the same time, the cells were pulsed with 0.25 μCi tritiated thymidine (specific activity 35 Ci/mmol; ICN/Flow, High Wycombe, UK) and following a further 12 hrs incubation at 37°C were harvested onto filter paper (ICN/Flow) using a cell harvester (Skatronas, Lier, Norway). Thymidine uptake was measured by liquid scintillation on a β-counter (Pharmacia LKB Biotech, Milton Keynes, UK) using 1ml of Optiscint (Pharmacia Biosystems) added to the filter discs in vials (Hughes and Hughes, Somerset, UK) and counted for 5 min each.

IL-2,IL-5,IFN-γ and IL-10 measurement by capture ELISA

The culture supernatants and standards, consisting of murine recombinant IL-2 (0-50

U/ml), IL-5 (0-10000 pg/ml), IL-10 (0-10000 pg/ml) or IFN-γ (0-7000 pg/ml) (Cambridge Bioscience, Cambridge, UK) were added in duplicate to the wells of a microtitre plate which had been pre-coated 12 hours previously with the appropriate capture antibody in PBS pH9.0 at 4°C. Following incubation at 37°C for 120 minutes, the wells were washed three times with PBS/Tween (pH 7.4. 0.05% Tween 20) and appropriate secondary biotinylated antibodies were added. After a further incubation at 37°C for 60 min, the plates were washed as above and 100µl of detection complex (streptavidin-linked alkaline phosphatase, 1:2000 dilution) was added to each well. The plates were further incubated at 37°C for 45 min. in the dark and the wells were washed 3 times with PBS/Tween before the addition of 100µl of *p*-nitrophenylphosphatase prepared in glycine buffer (pH 10.1). After a further 30 min incubation at 37°C the absorbances were measured at 405nm on a Titertek Multiskan plate reader. Cytokine concentrations were determined from the standard curve (regression coefficient r =0.995).

Following antibodies were used for detecting the cytokines (all purchased from Cambridge Bioscience, Cambridge, UK).

Cytokine		Clone	Concentration
IL-2	Capture	S4B6	2µg/ml
	Detection	JES6-5H4	1µg/ml
IL-5	Capture	TRFK-5	2µg/ml
	Detection	TRFK-4	1µg/ml
IL-10	Capture	JES5-2A5	2µg/ml
	Detection	SXC-1	1µg/ml
IFN-γ	Capture	R4-6A2	2µg/ml
	Detection	XMG 1.2	1µg/ml

Flowcytometric analysis

Mice were killed at different time points following infection with *L. mexicana*. Their

draining lymph nodes were removed and single cell suspensions were prepared by gentle teasing in complete RPMI 1640 medium. Following two washes the cells were resuspended in 1ml of 1% BSA-PBS/azide (pH7.2) and enumerated by trypan blue exclusion as described above. About 10^5 to 10^6 cells were dispensed per tube in 1ml of 1%BSA-PBS/azide and incubated separately with either PE conjugated rat-anti-mouse CD4 (1:5000 dilution) or PE conjugated rat-anti-mouse CD8.1 (1:5000 dilution) or FITC conjugated rat anti-mouse B220 (1:5000 dilution) for 45 minutes at room temperature. The cells were then washed three times with 1%BSA-PBS/Azide at 200g for 2 min and resuspended as a single cell suspension in 200µl of 1%BSA/Azide and later fixed by adding 1ml of 1% formaldehyde in PBS. Flowcytometric analysis was done using an EPICS Flow Cytometer (Coulter Corporation, Florida, USA).

Preparation of tissue sections and Histopathological examination

Following infection with *L. mexicana* mice were sacrificed at different time points and their skin lesions, draining lymph nodes and spleens were excised ,snap frozen in liquid nitrogen and stored at -70°C to await further processing. The tissues were embedded in optimal cutting temperature (OCT) compound (Diatec, Hallstadt / Bamberg, Germany) and cryostat sections (4-8µm) were cut at a temperature of -22°C and thawed onto polylysine coated slides. The sections were air-dried for 5 minutes, and fixed in cold (-20°C) acetone for 10 minutes.

Following fixation, the sections were rinsed in Tris-PBS to wash off OCT compound. Subsequently, sections were stained by the routine haematoxyline-eosin (HE) staining technique for histopathological analysis.

Chapter 3

Sex determined susceptibility and differential immunological responses in DBA/2 mice infected with *Leishmania mexicana*.

3.1 Summary

Female DBA/2 mice have been shown to be relatively resistant to infection with *L.mexicana* when compared with male mice. They either do not develop lesions at the site of parasite inoculation or develop smaller lesions than male mice all of which develop non healing lesions. In order to determine the immunological basis behind this difference, the immunological responses of male and female DBA/2 mice infected with *L. mexicana* were analysed *in vivo* and *in vitro*. The expression of mRNA transcripts for IFN-γ, TNF-α, IL-4, IL-10 and IL-12 were analysed in draining inguinal lymph nodes at different time points following infection using the reverse transcription- polymerase chain reaction (RT-PCR). Resistant female mice showed preferential expression of IFN-γ transcripts in their draining lymph nodes as compared with susceptible male mice which preferentially expressed TNF-α. The greatest lesion sizes were recorded from those mice expressing TNF-α but not IFN-γ. No differences in expression of IL-4 or IL-12 mRNA were noted with transcripts for both cytokines present in both sexes. Treatment of female mice following infection with IFN-γ neutralising antibody resulted in lesion growth equivalent to male mice whereas treatment of infected male mice with poly I:C, to induce IFN-γ production from NK cells, resulted in transient inhibition of lesion growth compared with control mice. Although both males and females produced parasite specific IgG2a during the course of disease, only males displayed significant titers of IgG1 antibodies indicating Th2 influenced responses which could not be detected in females until week 10. Following *in vitro* stimulation, lymphocytes from the draining lymph nodes of female mice had significantly higher antigen specific proliferative responses than male mice and produced significant quantities of IFN-γ. IFN-γ could not be detected in culture supernatants from male mice, indicating a Th1 influenced response in resistant females and its absence in susceptible males. At this time point lymph node cells from infected male mice produced higher quantities of IL-2 and IL-5 than those from female mice following *in vitro* antigenic stimulation . However these differences were not significant statistically. When the proliferative response of splenocytes from

infected mice were examined those from males exhibited significantly higher antigen specific *in vitro* proliferative responses than than those from females. Although none of the culture supernatants contained IFN-γ, IL-10 could be detected. Following *in vitro* infection with *L. mexicana* , resident peritoneal macrophages from male as well as female DBA/2 mice produced equal quantities of IL-6. By 5 weeks post- infection onwards male mice displayed significant increases in CD4 T cells, CD8 T cells and B cells in their draining lymph nodes. However, no increase in these cell populations could be observed in female mice until week 12 following infection. Further studies showed that treatment of ovarectomised female DBA/2 mice with di-hydroxy testosterone (DHT) exacerbated *L.mexicana* infection. Interferon-gamma production and the predominance of a Th1-like response would, therefore, appear to be responsible for limiting the growth of *L.mexicana* in female DBA/2 mice while TNF-α production in the absence of IFN-γ and the development of a Th2 response are associated with disease progression in male mice.

3.2 Introduction

Gender-related differences in the susceptibility to *L. mexicana* have been clearly demonstrated in DBA/2 mice where the females are generally resistant to cutaneous lesion growth following infection with amastigotes and males develop rapidly growing non-healing lesions (Alexander, 1988). Resistance comes under single gene control and has been designated *Scl-2* which is provisionally mapped to chromosome 4 (Blackwell and Alexander, 1986; Roberts *et al.*, 1990). Recently, tyrosine kinases genes *Jak1* and *Jak2* have been suggested as the possible candidates for the *Scl-2* gene (Blackwell *et al.*, 1994). Interestingly a similar phenomenon has been observed among patients presenting with lesions due to *L.mexicana* infection where females developed the strongest skin test reactivity (DTH) and consequently a rapid healing response (Lynch *et al.*, 1982). Total and specific IgE levels amongst the patients were also sex dependent and inversely correlated with DTH. This is broadly in agreement with murine studies of cutaneous leishmaniasis due to *L.major* where DTH-eliciting IFN-γ producing Th1 cells are associated with resistance and IgE promoting, IL-4 producing Th2 cells are associated with susceptibility (Liew and O'Donnel, 1993).

Although preliminary studies indicate that oestrogen and testosterone can influence *L.mexicana* lesion growth in DBA/2 mice (Alexander and Stimson, 1988), the immunological mechanisms underlying differences in disease progression between the sexes awaits elucidation. Hence this study was undertaken to examine the different immunological pathways generated in male and female DBA/2 mice and to determine how sex hormones influenced these pathways. The results obtained may ultimately help to determine the mode of action of the *Scl-2* gene.

3.3 Material and Methods:

Animals and Infection

In all experiments, eight to twelve week old DBA/2 mice of both sexes were inoculated with $5x10^6$ amastigotes subcutaneously into the shaven rumps. All the animals were individually ear marked. Disease progression was monitored in individual mice by measuring lesion diameter weekly up to 12 weeks.

General Experimental procedures

Isotype specific ELISAs, T cell proliferation assays, cytokine assays, flowcytometric and histopathological analysis were carried out as described in detail in Chapter 2.

Extraction of total RNA and RT-PCR:

Draining inguinal lymph nodes were excised at 2, 5, 8 and 12 weeks post infection. Total RNA was extracted and RT-PCR was performed as described in Chapter 2.

Treatment of female DBA/2 mice with IFN-γ neutralising antibodies:

R4-6A2 antibodies were purified from the hybridoma supernatants as described in Chapter 2. Female DBA/2 mice received 0.4 mg of purified R4-6A2 monoclonal antibody (mAb) in 1ml phosphate-buffered saline(PBS) intraperitoneally 12 hr before infection with 5 x 10^6 *L. mexicana* amastigotes and weekly thereafter up to 8 weeks. Control mice received equivalent amounts of purified rat IgG (Sigma Chemicals, Poole,UK). The disease progression was monitored by weekly measurements of lesion diameters

Treatment of male DBA/2 mice with poly I:C

Male DBA/2 mice received intraperitoneal (i.p) injections of 100μg of poly I:C 18 hrs and 50μg 2 hrs before infection with *L. mexicana* followed by daily i.p injections of 50μg of poly I:C up to 4 weeks as described before by Laskay *et al.* (1993). Control mice received daily i.p injections of sterile PBS . Disease progression was monitored as described above.

Preparation of tissue sections and Histopathological examination

Following infection with *L. mexicana* male and female mice were sacrificed at week 8 and their skin lesions, draining lymph nodes and spleens were excised for histopathological analysis as described previously in Chapter 2.

Isolation and stimulation of resting peritoneal macrophages

Age matched male and female DBA/2 mice were killed by cervical dislocation. Peritoneal lavage was carried out using 3ml of complete tissue culture medium (RPMI-1640) to obtain resting peritoneal macrophages.Cells obtained from 5 mice of either sex were pooled together, washed twice in complete RPMI-1640 and were enumerated by trypan blue exclusion. The cell suspensions were adjusted to 10^6 cells/ml in complete RPMI medium. Aliquots (500µl) containing 5 x 10^5 cells were added in triplicate to the wells of 24 well flat bottomed tissue culture plates (Costar, Cambridge, MA,USA). The cells were incubated at 37°C for 2 hours for macrophage adherence. Following incubation ,the wells were washed three times with prewarmed complete RPMI medium to remove non-adherent cells. Stationary phase *L. mexicana* promastigotes were added in triplicate to wells containing adherent macrophages at a ratio 3:1 parasites:macrophage. Following incubation at 37°C in 5% CO2 for 18 hours, 150µl of the culture supernatants were removed from each well and were analysed for cytokine production .

IL-6 assay

IL-6 production by stimulated (LPS and *L. mexicana* promastigotes) and non stimulated peritoneal macrophages from male and female DBA/2 mice was measured by capture ELISA as described Chapter 2. The neat supernatants and standards consisting of murine recombinant IL-6 (0-20000 pg/ml) (Cambridge Bioscience, Cambridge, UK)were analysed in duplicate using capture antibodies [Rat anti-mouse IL-6, clone: MP5-20F3 (Cambridge Bioscience, Cambridge, UK)] and secondary biotinylated anti-IL-6 [Rat anti-mouse IL-6, clone: MP5-32C11 (Cambridge Bioscience, Cambridge, UK)] antibodies.

Treatment of female DBA/2 mice with dihydroxy-testosterone (DHT)

Following general anaesthesia, oophorectomy was performed as described by Kittas and Henry (1979,1980). Two weeks later, silastic capsules containing 7 mg of dihydroxy testosterone (DHT) were implanted subcutaneously into the dorsal region just below the neck through small subcutaneous incision. Control sham oophorectomised female mice were similarly implanted with empty silastic capsules. The incisions were closed using Michel Clips (7.5 x 1.75 mm) and two weeks later the mice were infected with 5 x 10^6 *L. mexicana* amastigotes subcutaneously into the shaven rump. All animals were individually ear marked and disease progression was monitored in individual mice by measuring lesion diameter every two weeks upto week 10.

Statistical analysis

The significance of differences between the groups was determined by the Student's unpaired *t* test.

3.4 Results

Growth of L. mexicana in male and female DBA/2 mice

Male DBA/2 mice were extremely susceptible to *L.mexicana* when compared with female mice and developed rapidly large non-healing lesions (Fig.1). Female mice produced no lesions, small lesions which healed or slow growing non-healing lesions (Fig.1).

Kinetics of cytokine mRNA expression in draining lymph nodes

The production of IFN-γ and TNF-α mRNA transcripts was slow in both sexes, and no transcripts for IFN-γ or TNF-α could be detected in either sex before week 5 (Fig.2). By week 8 clear differences were observed between susceptible male and resistant female mice. In one representative experiment,all female mice (5/5) expressed mRNA transcripts for IFN-γ in their draining lymph nodes and one also expressed TNF-α (Fig.3). However, although transcripts for TNFα were detected in 4 out of 5 males, only 2 expressed transcripts for IFN-γ. At this time point, mice expressing TNF-α but no IFN-γ had the largest lesions (Table 1). No differences in IL-4, IL-10 or IL-12 expression were noted in either sex. IL-10 mRNA transcripts were detectable in the draining lymph nodes of both sexes at all time points as well as in uninfected controls (data not shown). Transcripts for IL-4 and IL-12 mRNA were expressed in all mice of both sexes by week 8 (Fig.4). By week 12 all males had large non-ulcerating nodules and of 4 mice examined none had transcripts for IFN-γ and 2 had transcripts for TNF-α mRNA. At this time many females did not have lesions and of 3 examined 2 had transcripts for IFN-γ in the draining lymph nodes but TNF-α mRNA could not be detected (Fig.5). A repeat experiment revealed a similar dichotomy between male and female mice in the preferential expression of TNF-α and IFN-γ mRNA.

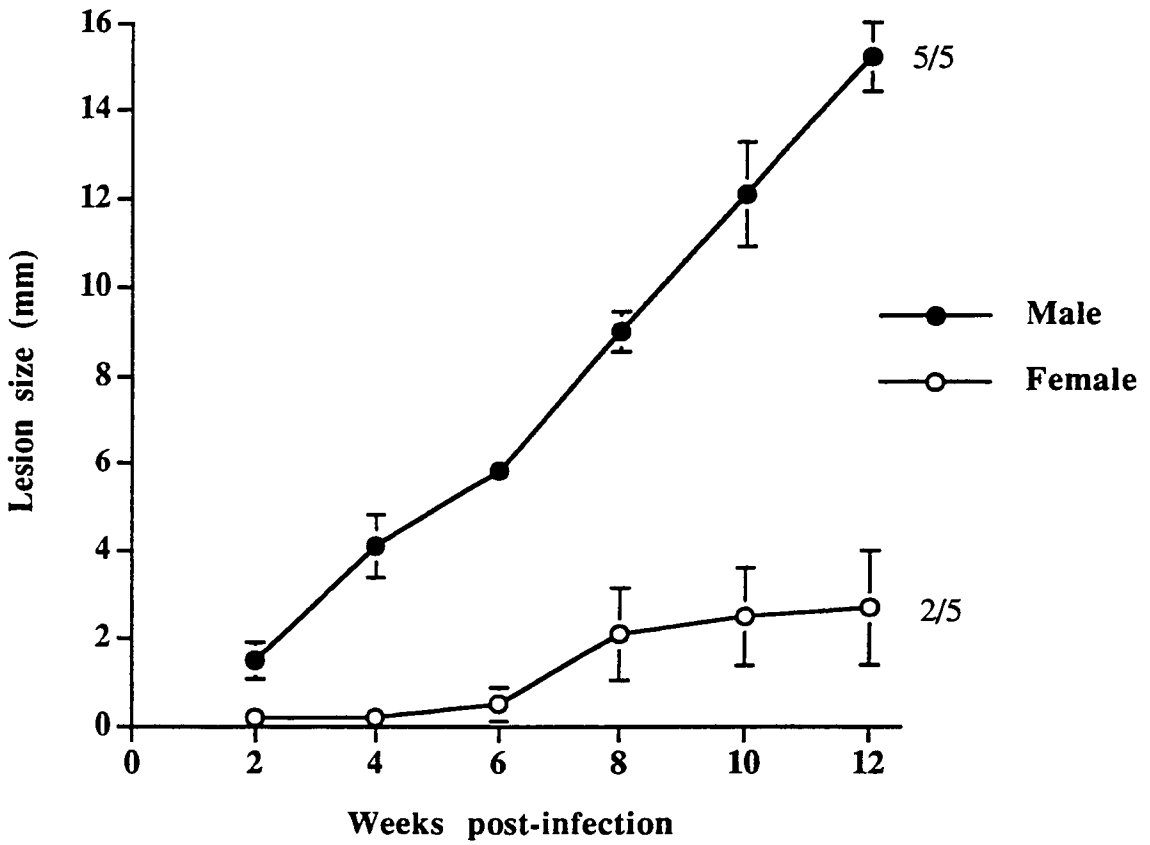

Fig.1 : Mean ± SE lesion growth in the shaven rumps of male (●) and female (○) DBA/2 mice infected with *L. mexicana*. The numbers represent animals with lesions at week 12.

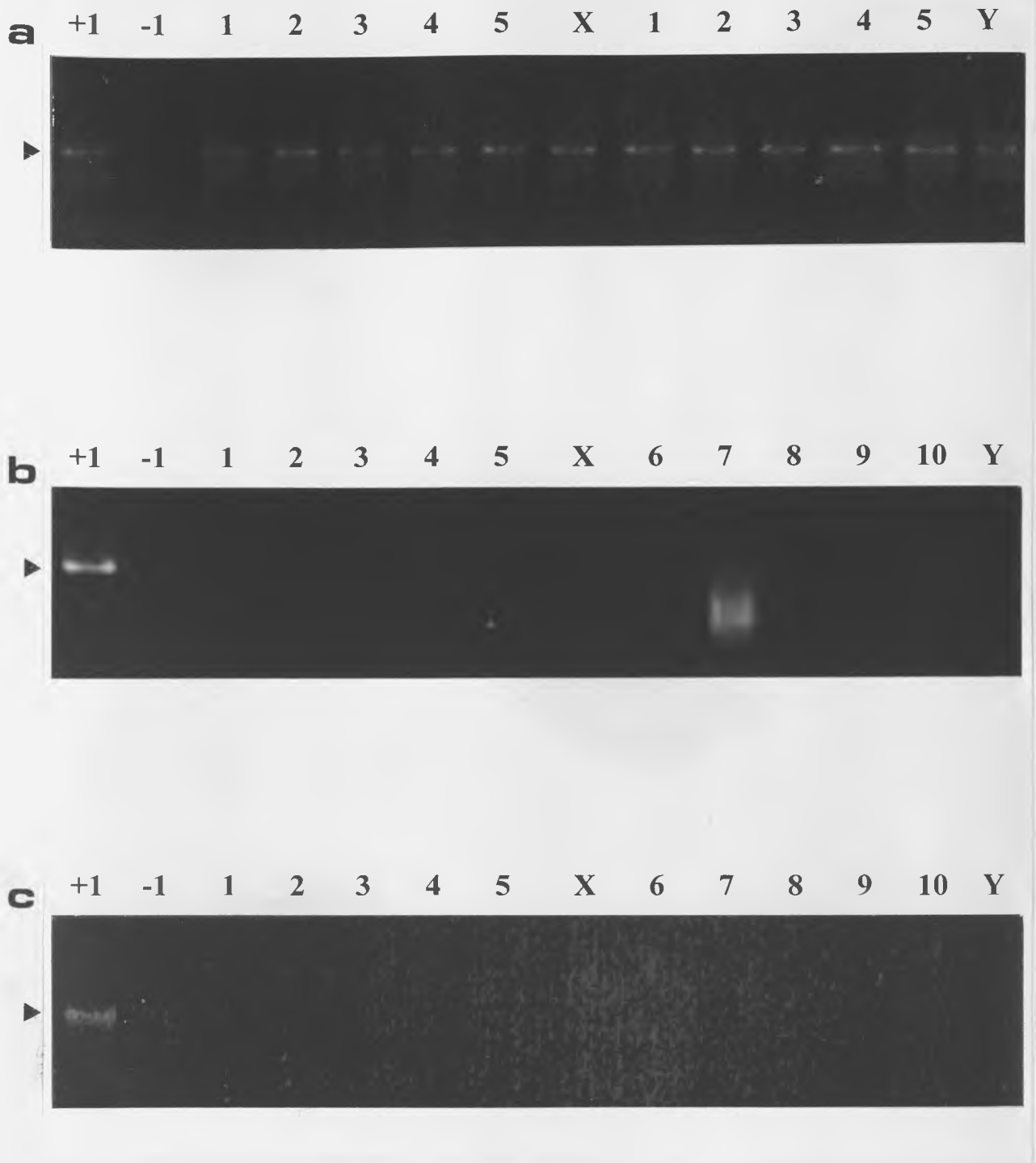

Fig.2 : Computer-scanned prints of ethidium bromide-stained agarose gels showing PCR products for β-actin (a); IFN-γ (b) and TNF-α (c) 5 weeks post-infection. A positive control (+1) and a negative control (-1) are also included. Lanes 1-5 are female mice; lanes 6-10 male mice. Lanes X and Y are the actin controls for uninfected female and male mice respectively.

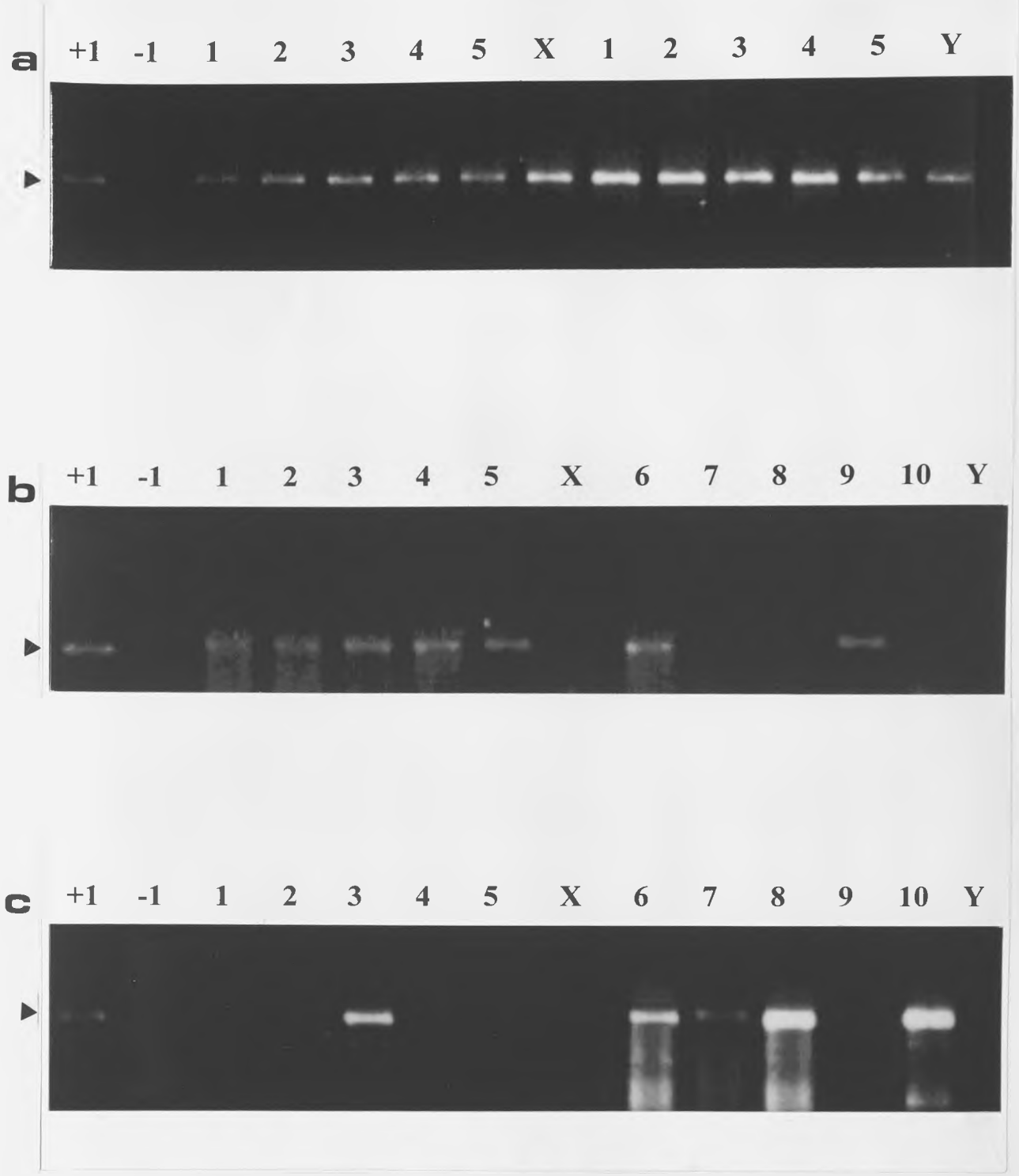

Fig.3 : Computer-scanned prints of ethidium bromide-stained agarose gels showing PCR products for β-actin (a); IFN-γ (b) and TNF–α (c) 8 weeks post-infection. A positive control (+1) and a negative control (-1) are also included. Lanes 1-5 are female mice; lanes 6-10 male mice. Lanes X and Y are the actin controls for uninfected female and male mice respectively.

Lesion size (mm)	Sex	Transcript present	
9	Male	TNF-α	
8	Male	TNF-α	
8	Male	TNF-α	
6	Male		IFN-γ
4	Male	TNF-α	IFN-γ
4	Female	TNF-α	IFN-γ
4	Female		IFN-γ
3	Female		IFN-γ
3	Female		IFN-γ
0	Female		IFN-γ

Table 1: Lesion size (mm) and the presence of mRNA transcripts for TNF-α and IFN-γ in male and female DBA/2 mice at week 8 post-infection.

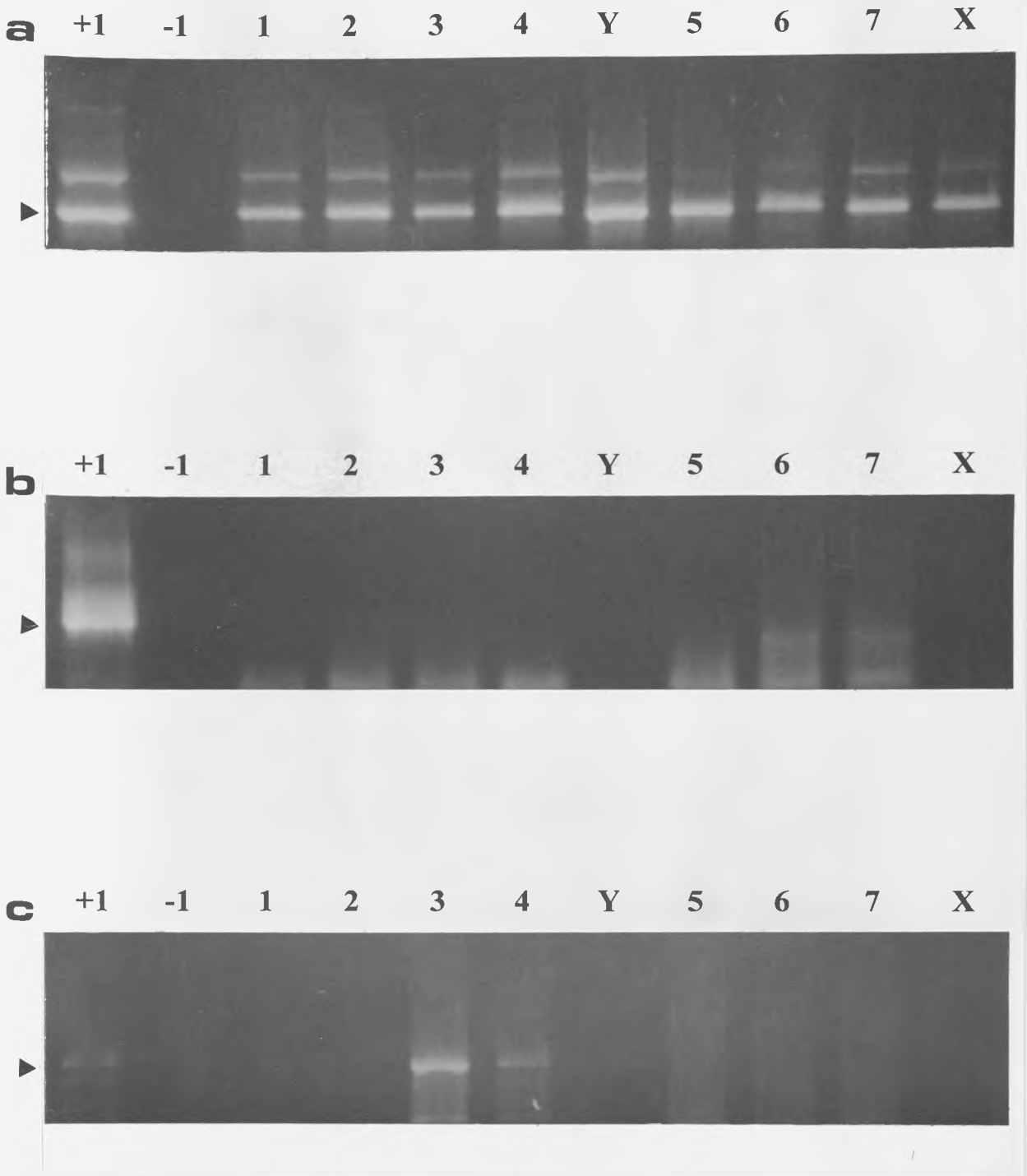

Fig.4 : Computer-scanned prints of ethidium bromide-stained agarose gels showing PCR products for β-actin (a); IFN-γ (b) and TNF-α (c) 12 weeks post-infection. A positive control (+1) and a negative control (-1) are also included. Lanes 1-4 are male mice; lanes 5-7 female male mice. Lanes X and Y are the actin controls for uninfected female and male mice respectively.

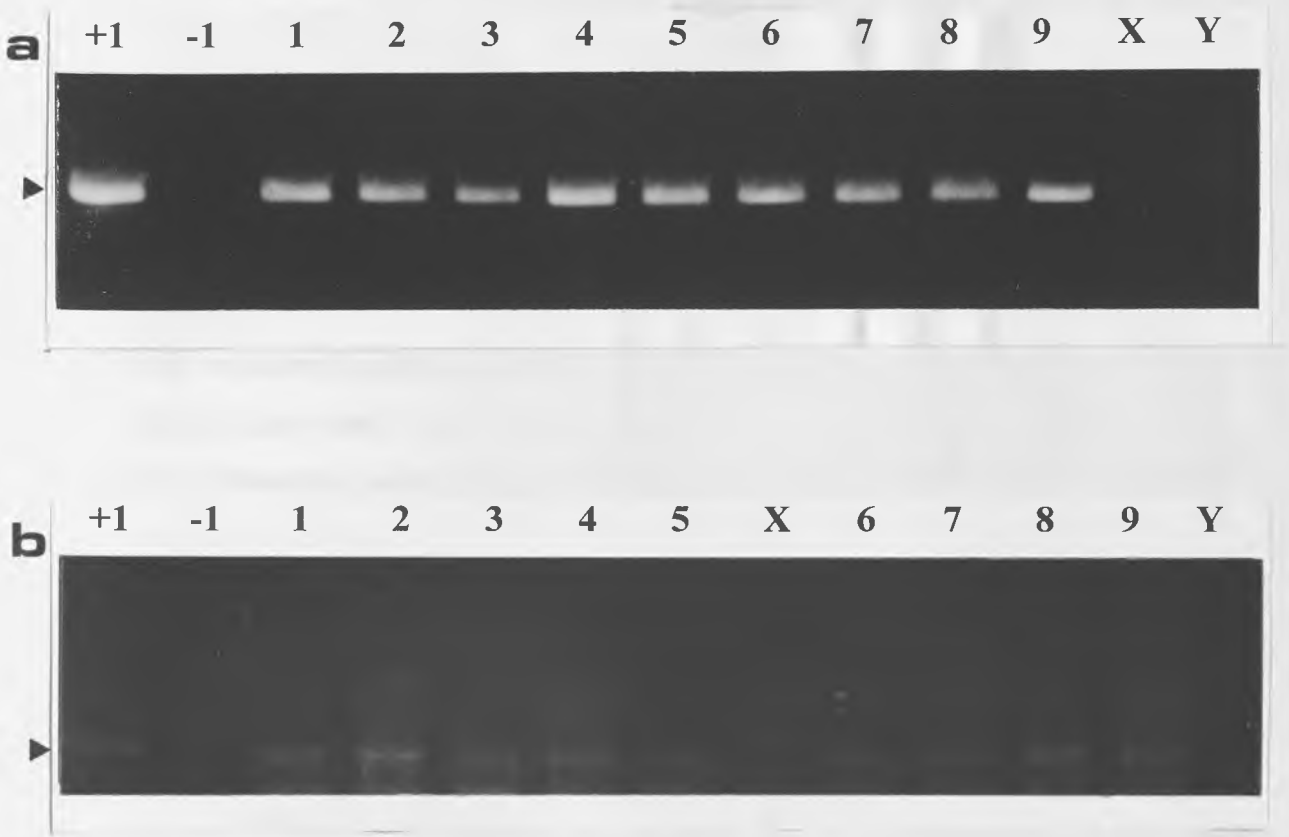

Fig.5 : Computer-scanned prints of ethidium bromide-stained agarose gels showing PCR products for (a) IL-4 and (b) IL-12 8 weeks post-infection. A positive control (+1) and a negative control (-1) are also included. Lanes 1-5 are male mice; lanes 6-9 female male mice. Lanes X and Y are the actin controls for uninfected female and male mice respectively.

Kinetics of L. mexicana specific antibody responses in male and female DBA/2 mice

Specific antibodies against *L. mexicana* could not be be detected in either sex until 6 weeks following infection. At this time point males but not females produced significant titres of *L. mexicana* specific IgG1 which increased significantly ($p < 0.025$) thereafter as infection progressed (Fig.6b). *L. mexicana* specific IgG2a antibodies were detectable in both sexes by week 8 post-infection and thereafter. However, *Leishmania* specific IgG1 was detected only in plasma from female mice at week 10 following infection but not before this time (Fig.6c).

In vitro spleen and draining lymph node T cell proliferative responses

At week 10 post-infection splenocytes as well as lymph node cells from *L. mexicana* infected resistant female mice and susceptible male mice exhibited significant *L. mexicana* antigen specific and ConA induced proliferative responses ($p < 0.05$) (Fig. 7 and 8). However, antigen specific and ConA induced splenocyte proliferative responses were significantly higher ($p < 0.05$) in males than females (Fig.7). On the other hand,resistant female mice displayed significantly greater Con A and antigen induced lymph node T cell proliferative responses than susceptible male mice (Fig.8).

Cytokine assays

Cytokine production by splenocytes

The splenocyte culture supernatants were analysed for the presence of the Th1 associated cytokine, IFN-γ and the Th2 associated cytokine, IL-10 by capture ELISA (Fig.9). Supernatants from ConA stimulated splenocytes from resistant female mice produced significantly higher quantities of IFN-γ than those from male mice (Fig.9). On the other hand, *L. mexicana* antigen stimulated as well as unstimulated splenocytes from both sexes produced comparable levels of IL-10 and either little or no IFN-γ (Fig.9).

Cytokine production by draining lymph node cells

The supernatants from the above lymph node T cell proliferation assay were also

analysed for the Th1 associated cytokines, IL-2 and IFN-γ, and for Th2 associated cytokines, IL-5 and IL-10 as described before. Following ConA as well as antigenic stimulation, lymph node cells from infected female mice produced significantly higher quantities of IFN-γ than those from male mice which failed to produce IFN-γ following antigenic stimulation (Fig.10). Unstimulated lymph node cell culture supernatants from female mice also displayed significant levels of IFN-γ whereas those from male mice had no detectable IFN-γ (Fig.10).

Surprisingly, lymph node cell from resistant female mice also produced significantly higher amounts of IL-10 ($p < 0.05$) than males following stimulation with *L. mexicana* antigen as well as ConA (Fig.10). No differences in IL-10 production could be detected from unstimulated lymph node cells cultures from either sexes (Fig.10). No significant differences in the production of IL-2 and IL-5 could be detected following antigenic stimulation of male or female draining lymph node cells (Fig .11).

Effect of in vivo neutralisation of IFN-γ on disease progression in L. mexicana infected female DBA/2 mice

In order to establish the functional role of IFN-γ in mediating protection against *L. mexicana*, resistant DBA/2 female mice were treated with IFN-γ neutralising antibodies. Female DBA/2 mice receiving intraperitoneal injections of IFN-γ neutralising antibodies at weekly intervals during infection showed significant disease exacerbation and developed significantly larger ($p < 0.025$) lesions than similarly infected female control mice (Fig.12). The lesions that developed in IFN-γ neutralising antibody treated female mice were comparable in size to those found in their untreated male counterparts (Fig.12).

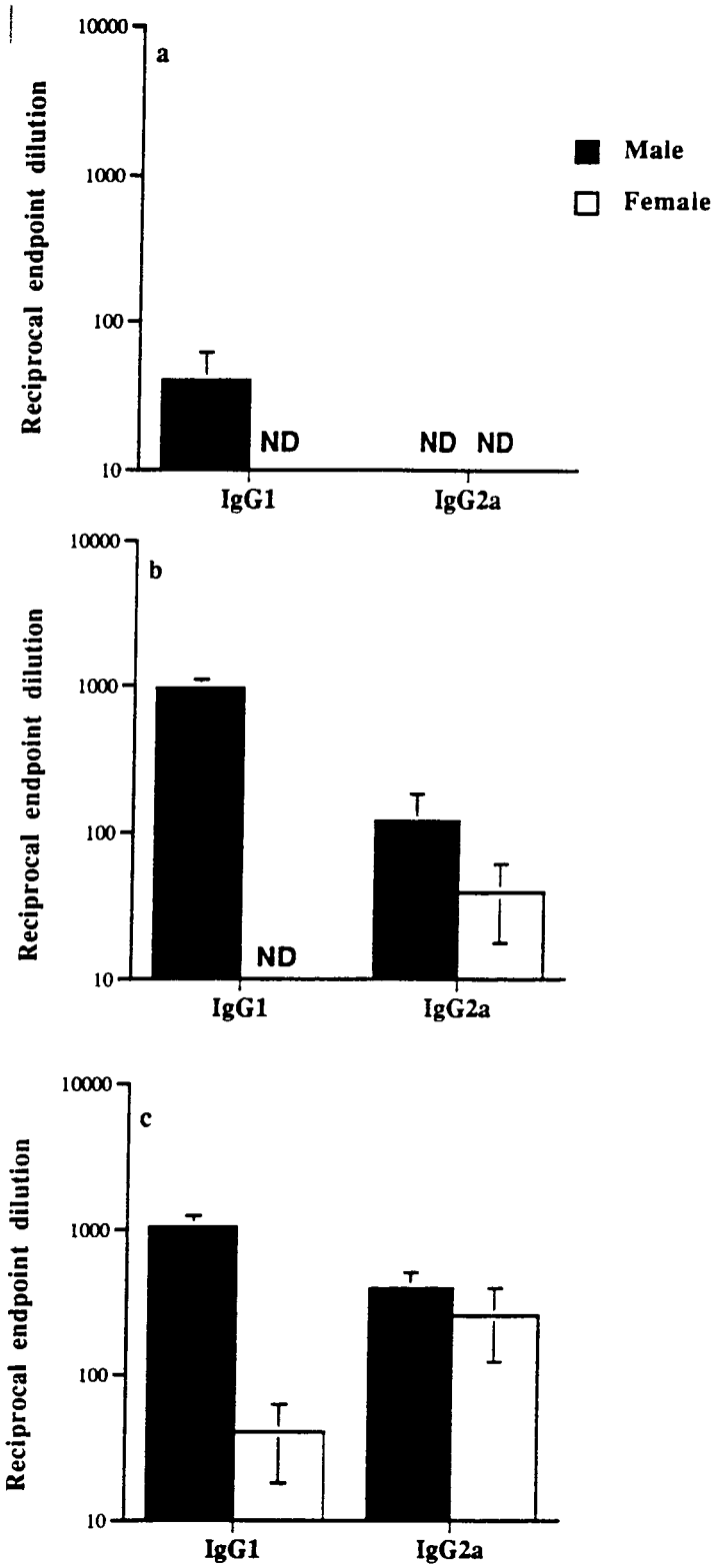

Fig.6: *L. mexicana* specific IgG1 and IgG2a production in male (closed column) and female (open column) mice at week 6 (Fig. 6a), week 8 (Fig.6b) and week 10 (Fig.6 c) post-infection. Data presented as Mean Reciprocal end point dilution ± SE . (ND= no antibody detected at 1: 100 dilution)

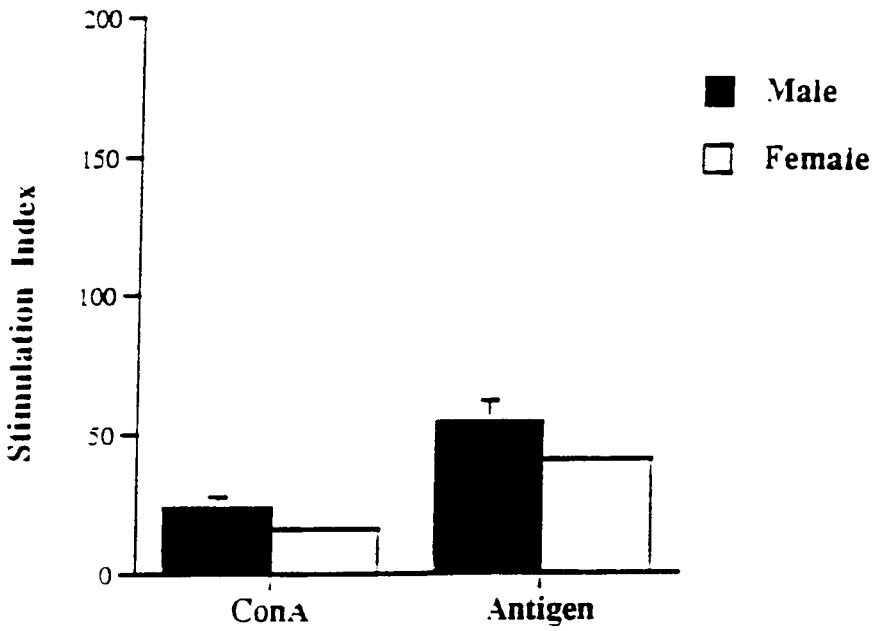

Fig.7: Con A (5µg/ml) and *L. mexicana* soluble antigen (50µg/ml) induced splenocyte proliferation responses in *L. mexicana* infected male (closed column) and female (open column) DBA/2 mice at week 10 post-infection expressed as Mean Stimulation Index ± SE.

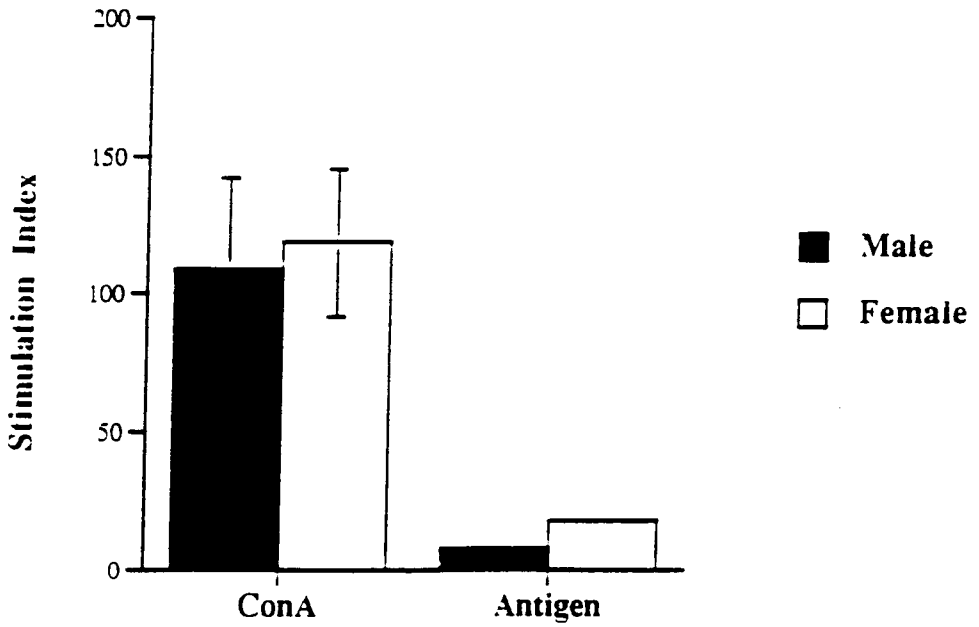

Fig.8 : Con A (2.5μg/ml) and *L. mexicana* soluble antigen (25μg/ml) induced lymph node cell proliferation responses in *L. mexicana* infected male (closed column) and female (open column) DBA/2 mice at week 10 post-infection expressed as Mean Stimulation Index ± SE.

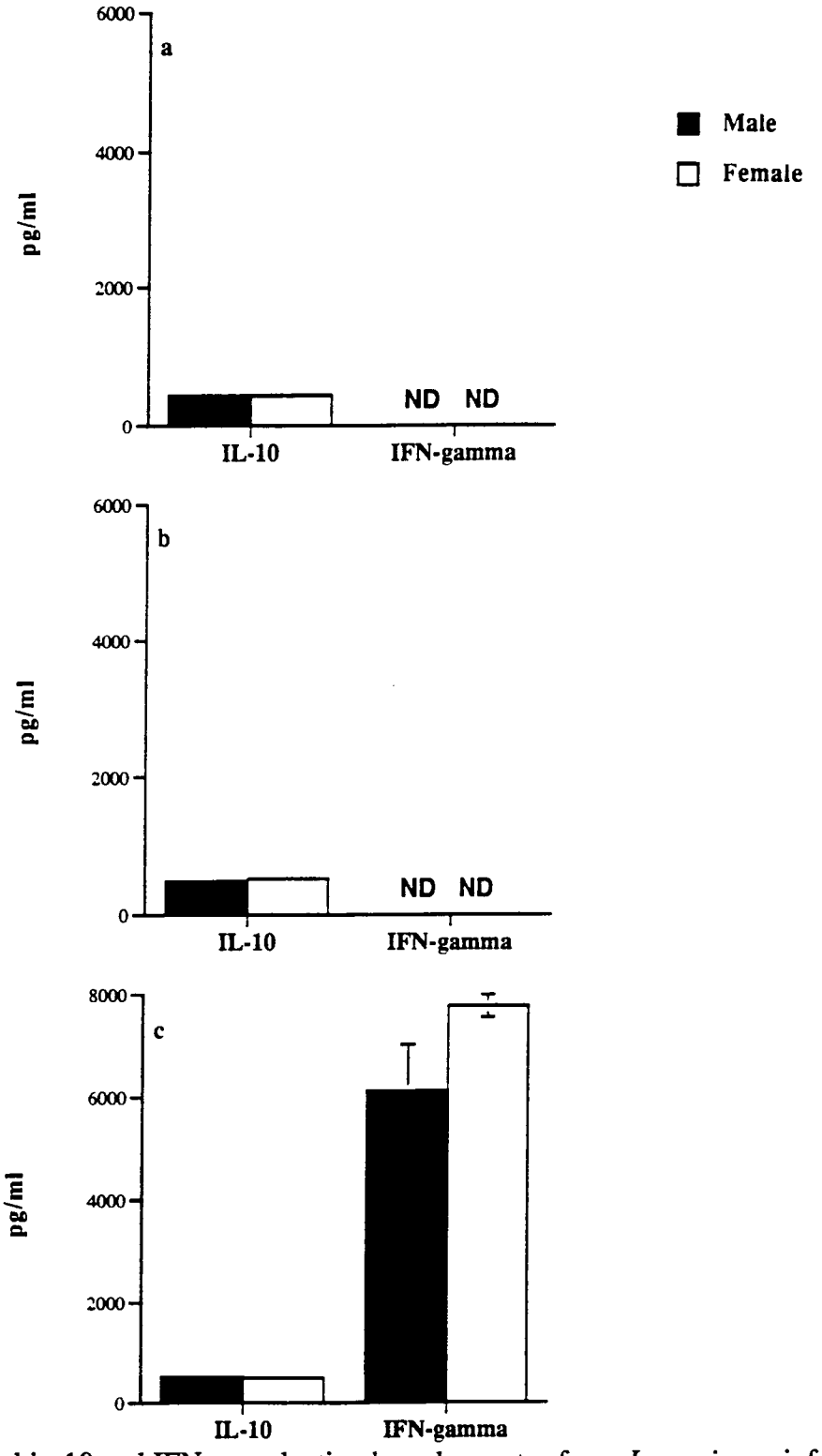

Fig.9: Interleukin-10 and IFN-γ production by splenocytes from *L. mexicana* infected male (closed column) and female (open column) mice at week 10 post -infection in the absence of antigen (Fig.9a), in the presence of 50μg/ml *L. mexicana* soluble antigen (Fig.9b) and in the presence 5μg/ml ConA (Fig.9c). (ND= not detected).

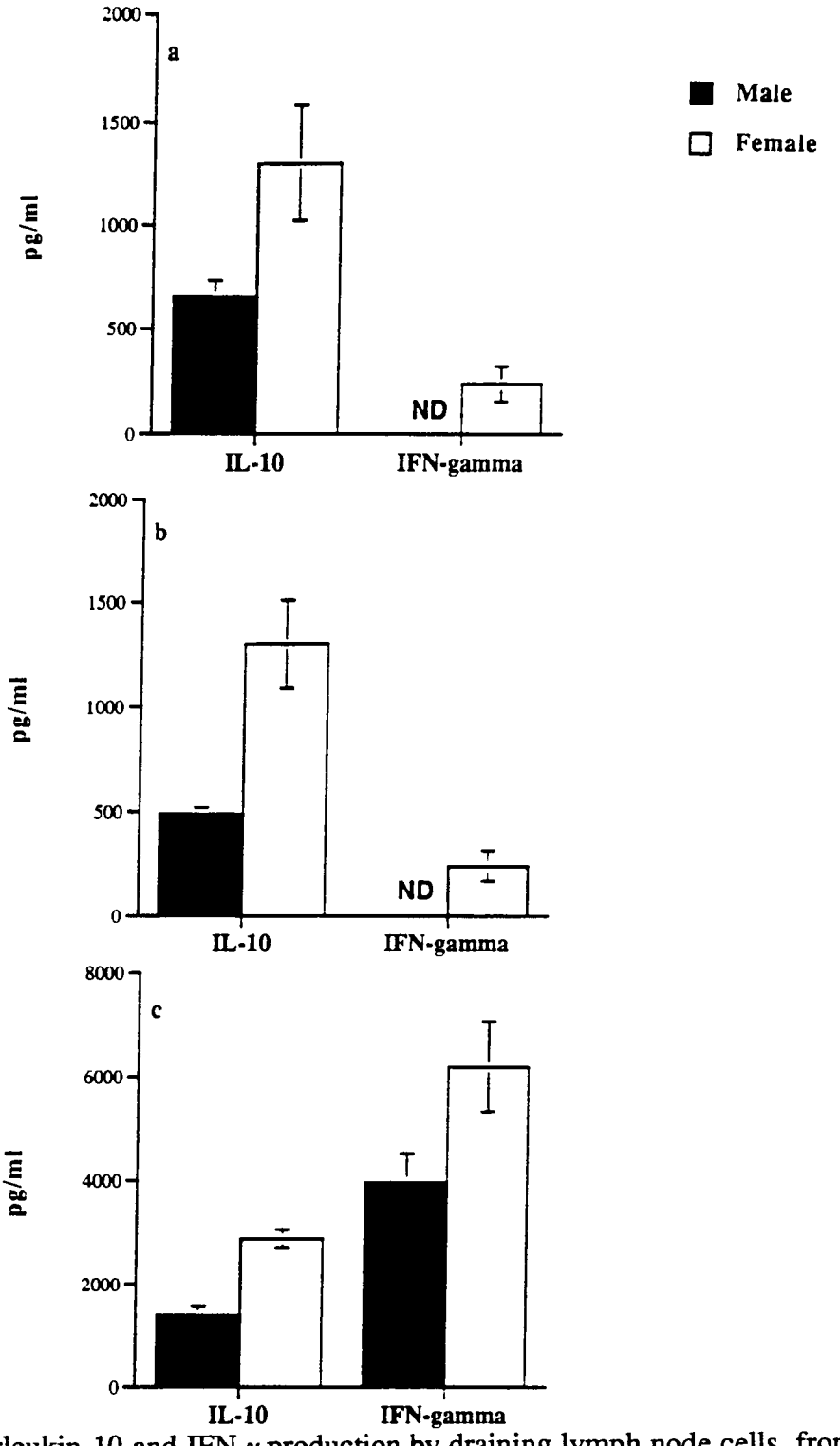

Fig.10: Interleukin-10 and IFN-γ production by draining lymph node cells from *L. mexicana* infected male (closed column) and female (open column) mice at week 10 post-infection in the absence of antigen (Fig. a), in the presence of 25μg/ml *L. mexicana* soluble antigen (Fig. b) and in the presence 2.5μg/ml ConA (Fig. c). (ND = not detected).

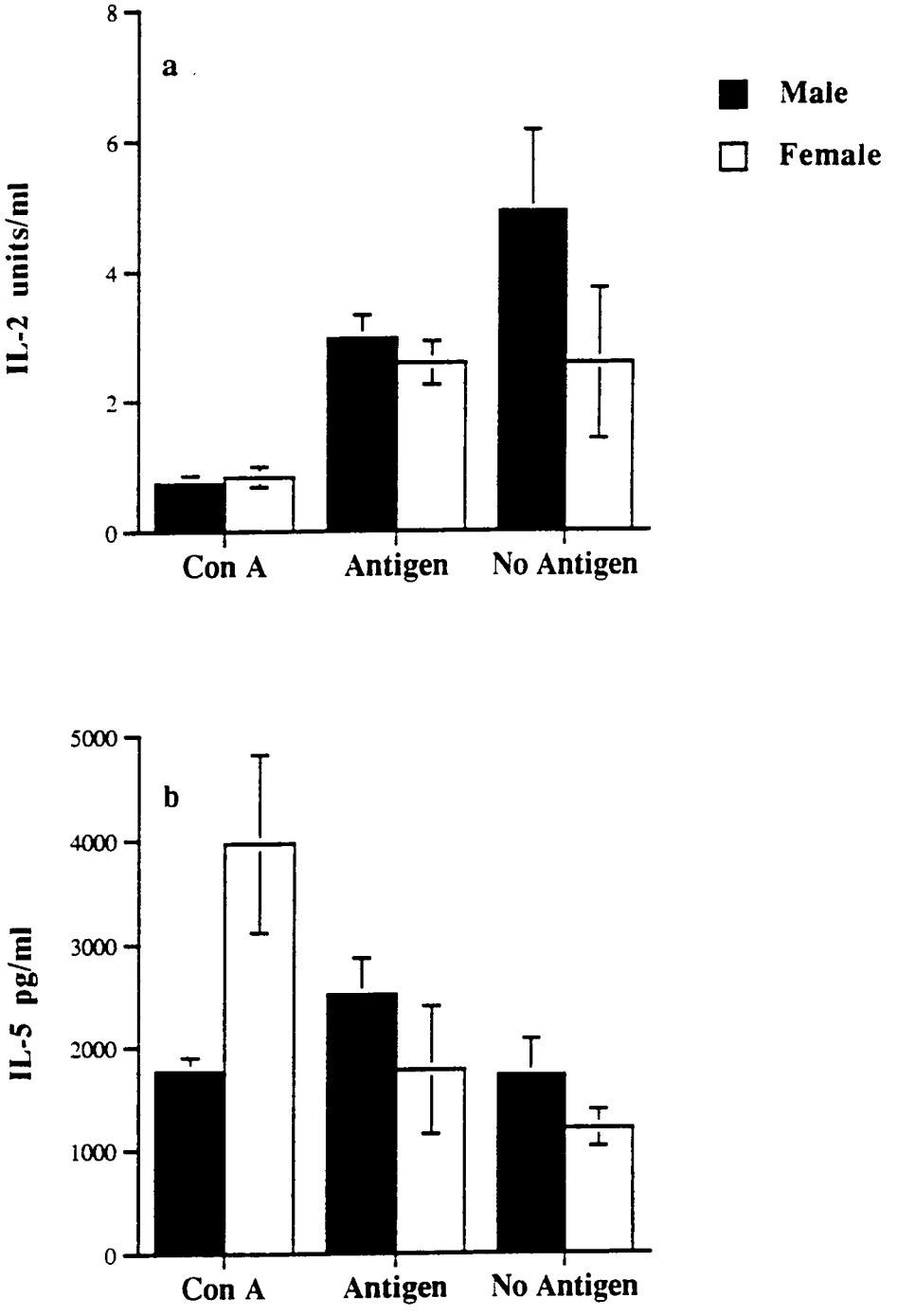

Fig.11: Interleukin-2 (Fig. 11a) and IL-5 (Fig. 11b) production by draining lymph node cells from *L. mexicana* infected male (closed column) and female (open column) mice at week 10 post-infection in the absence of antigen, in the presence of 25µg/ml *L. mexicana* soluble antigen and 2.5µg/ml ConA .

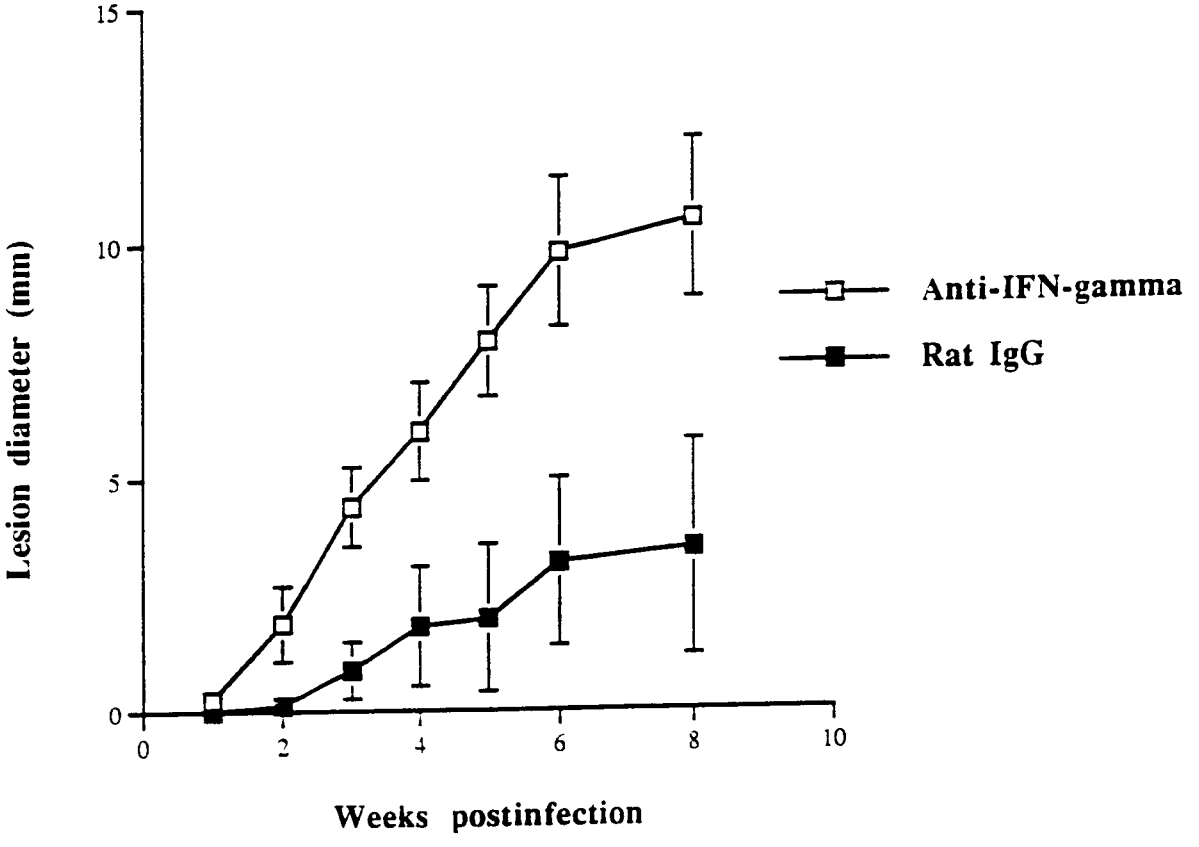

Fig.12: Effect of anti-IFN-γ treatment on course of *L. mexicana* infection in female DBA/2 mice. Data expressed as Mean lesion size (mm) ± SE.

Effect of poly I:C treatment on L. mexicana infection in male DBA/2 mice

Poly I:C has been shown to stimulate *in vivo* and *in vitro* NK cell activity and upregulate their IFN-γ production (Djeu *et al.*,1979). As an alternate strategy to confirm the functional role of IFN-γ in mediating resistance against *L. mexicana* infection in DBA/2 mice, the effect of poly I:C induced *in vivo* IFN-γ production from NK cells was studied in susceptible male DBA/2 mice. Following infection with *L. mexicana*, male mice receiving poly I:C injections showed enhanced resistance against *L. mexicana* and developed significantly smaller lesion sizes as compared with similarly infected control males as long as poly I:C treatment continued (Fig.12). However, after poly I:C treatment was stopped at week 4 post-infection, treated male mice showed rapid disease progression and developed lesions comparable in size to those of control males.

Analysis of draining lymph node cell phenotypes in male and female DBA/2 mice infected with L. mexicana

To assess the general immunological responses in male and female DBA/2 mice during *L. mexicana* infection, cell populations in the spleen and draining lymph nodes were analysed for CD4+ T cells, CD8+ T cells and B cells at different time points following infection by flow cytometry.

At week 5 following infection with *L. mexicana* and thereafter, male DBA/2 mice displayed significantly higher numbers of CD4+, CD8+ as well as B cells in their draining lymph nodes (Table 2). On the other hand, no significant increase in CD4+, CD8+ and B220+ cell populations could be demonstrated in draining lymph nodes from infected female mice before week 12 post infection (Table 2). At this time point draining lymph nodes from both male as well as female mice had comparable numbers of CD4+, CD8+ and B cells (Table 2) .

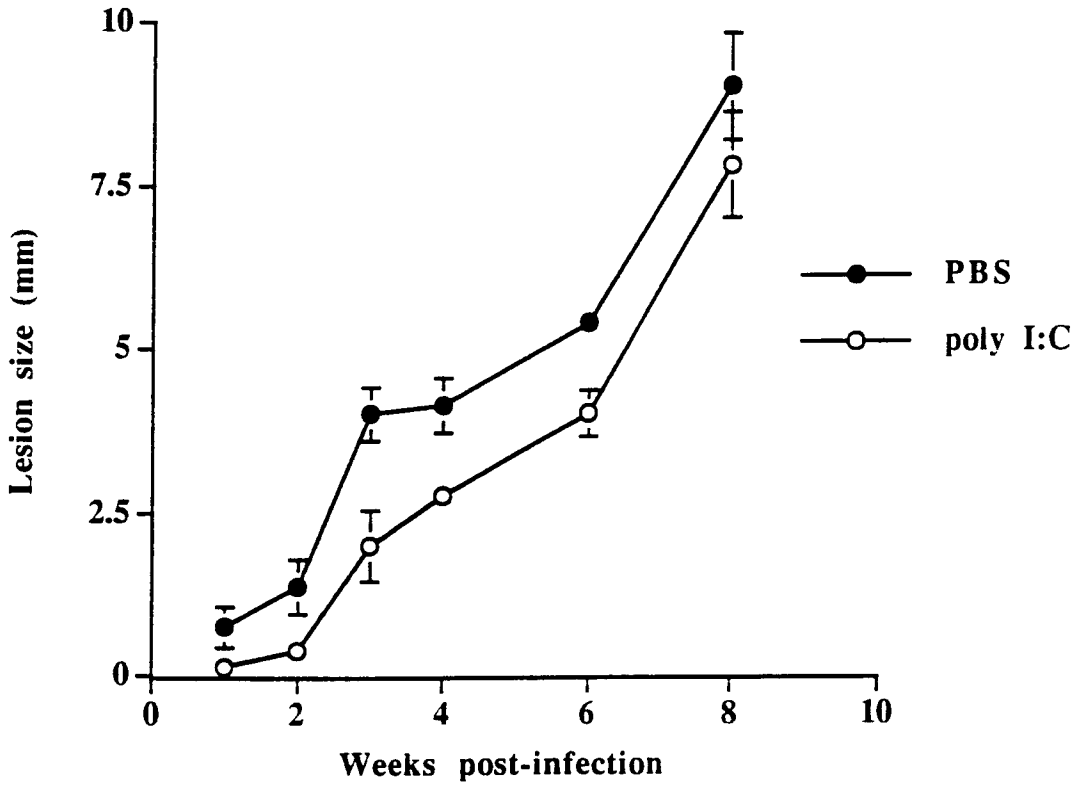

Fig.13: Effect of poly I:C treatment on the course of *L. mexicana* infection in male DBA/2 mice. Data expressed as Mean lesion size (mm) ± SE.

Table 2: Lymphocyte populations in the draining lymph nodes of *L. mexicana* infected male and female DBA/2 mice as determined by flowcytometry at day 0, week 5, 8, and 12 post-infection.

Mice	Weeks postinfection	CD4+T cells	CD8+T cells	B cells
Male	0	0.87±0.08	0.12±0.01	0.29±0.02
	5	1.32±0.28*	0.43±0.07***	0.53±0.07****
	8	1.72±0.43**	0.58±0.14***	1.08±0.28***
	12	2.00±0.47***	0.81±0.16****	2.66±0.27****
Female	0	0.78±0.09	0.25±0.04	0.48±0.05
	5	0.73±0.14	0.23±0.07	0.85±0.12
	8	1.00±0.26	0.37±0.12	0.65±0.18
	12	1.33±0.27***	0.66±0.07***	1.66±0.27***

* $p < 0.05$

**$p<0.025$

***$p<0.005$

****$p<0.0005$

Data expressed as Mean of total number of cells in both inguinal lymph nodes /10^6 ± standard error of the mean for animals per group.

Effect of L. mexicana infection on in vitro production of IL-6 by resting peritoneal macrophages from male and female DBA/2 mice

Resting peritoneal macrophages from male and female DBA/2 mice were infected with *L .mexicana* promastigotes at a ratio of 3 promastigotes /macrophage. Culture supernatants were harvested 18 hrs following infection and were analysed for the presence of IL-6 by capture ELISA. No differences were observed in IL-6 production between male and female mice as *L. mexicana* infected as well as uninfected resting peritoneal macrophages from both sexes produced significant amounts of IL-6 (Fig. 14).

Histopathological analysis of spleen , draining lymph nodes and skin lesions

Skin/Inoculation site

At both time points, skin lesions from male DBA/2 mice showed extensive subcutaneous tissue damage by marked inflammatory infiltrate which predominantly consist of parasitised macrophages, neutrophils, eosinophils and lymphocytes (Fig. 15a). On the other hand, female DBA/2 mice showed markedly less inflammatory infiltrate with few parasite laden macrophages at the site of inoculation (Fig. 16a).

Lymph node

The draining lymph nodes from both *L. mexicana* infected male and female DBA/2 mice showed complete effacement of normal lymph node architecture which was accompanied by distention and engorgement of lymphatic sinusoids with histiocytes (Fig. 15b and 16b).

Spleen

Spleens from both sexes showed follicular and reticuloendothelial hyperplasia at both the time points examined (Fig. 15c and 16c).

Effect of dihydroxy testosterone (DHT) on L. mexicana infection in DBA/2 female mice

This study was carried out to investigate the possible disease exacerbative role of androgens in disease progression in male DBA/2 mice during *L. mexicana* infection.

As compared with normal female mice and oophorectomised female mice bearing empty implants, oophorectomised female DBA/2 mice with DHT implants developed rapidly growing lesions (Fig.17) .

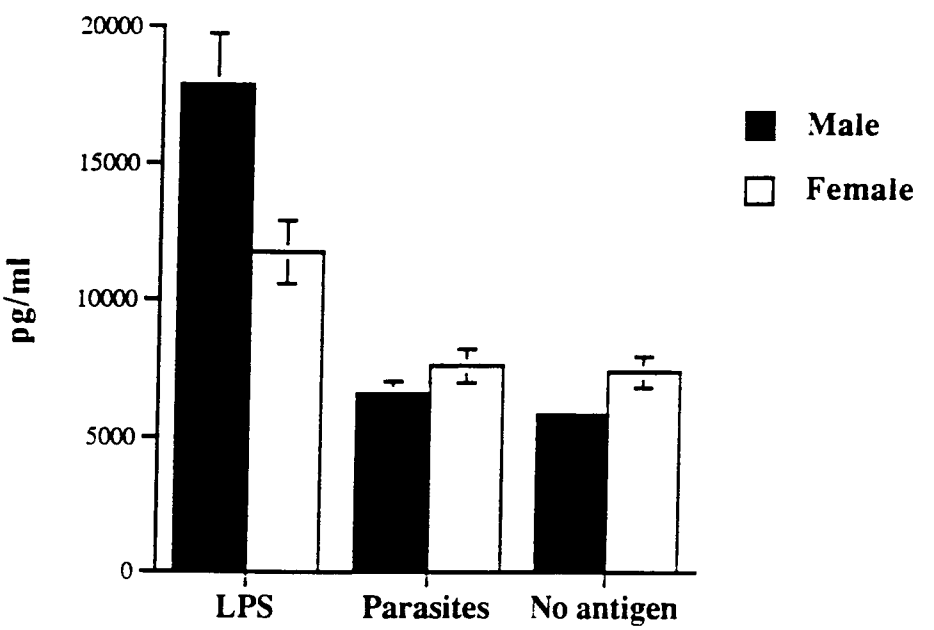

Fig.14: *In vitro* IL-6 production by resting peritoneal macrophages from male and female DBA/2 mice after 18 hours in the presence of *L. mexicana* promastigotes , LPS (5 μg/ml) and in the absence of antigen.

Fig.15: Histopathological examination of inoculation sites, the draining lymph nodes and spleens from *L. mexicana* infected male DBA/2 mice at week 8 post-infection.(a) Section from the skin lesion stained with hematoxylin-eosin showing extensive inflammatory infiltrate comprising of parasitised macrophages (arrows), neutrophils and lymphocytes. (b) Section from a draining lymph node showing the collapse of normal lymph node architecture with marked distention and engorgement of lymphatic sinusoids with histiocytes (arrows). (c) Section from the spleen showing reticuloendothelial hyperplasia characterised by engorgement of splenic sinusoids with histiocytes. CA is the central arteriole.

Fig.16: Histopathological examination of inoculation sites, the draining lymph nodes and spleens from *L. mexicana* infected female DBA/2 mice at week 8 post-infection.(a) Section from the the skin lesion stained with hematoxylin-eosin showing inflammatory infiltrate predominantly comprising of macrophages, lymphocytes and a few neutrophils. (b) Section from a draining lymph node showing the collapse of normal lymph node architecture with distention and engorgement of lymphatic sinusoids with histiocytes. (c) Section from the spleen showing reticuloendothelial hyperplasia characterised by engorgement of splenic sinusoids with histiocytes. CA is the central arteriole.

The draining lymph nodes from age matched uninfected male and female DBA/2 control mice showed normal architecture with prominant primary follicles in the cortex and normal sinuses lined by mononuclear cells.

The spleens from age matched uninfected male and female DBA/2 control mice showed normal splenic architecture with white pulp comprising of central arteriole and peiarterial sheaths of lymphoid cells and red pulp predominantly comprising of vascular sinusoids.

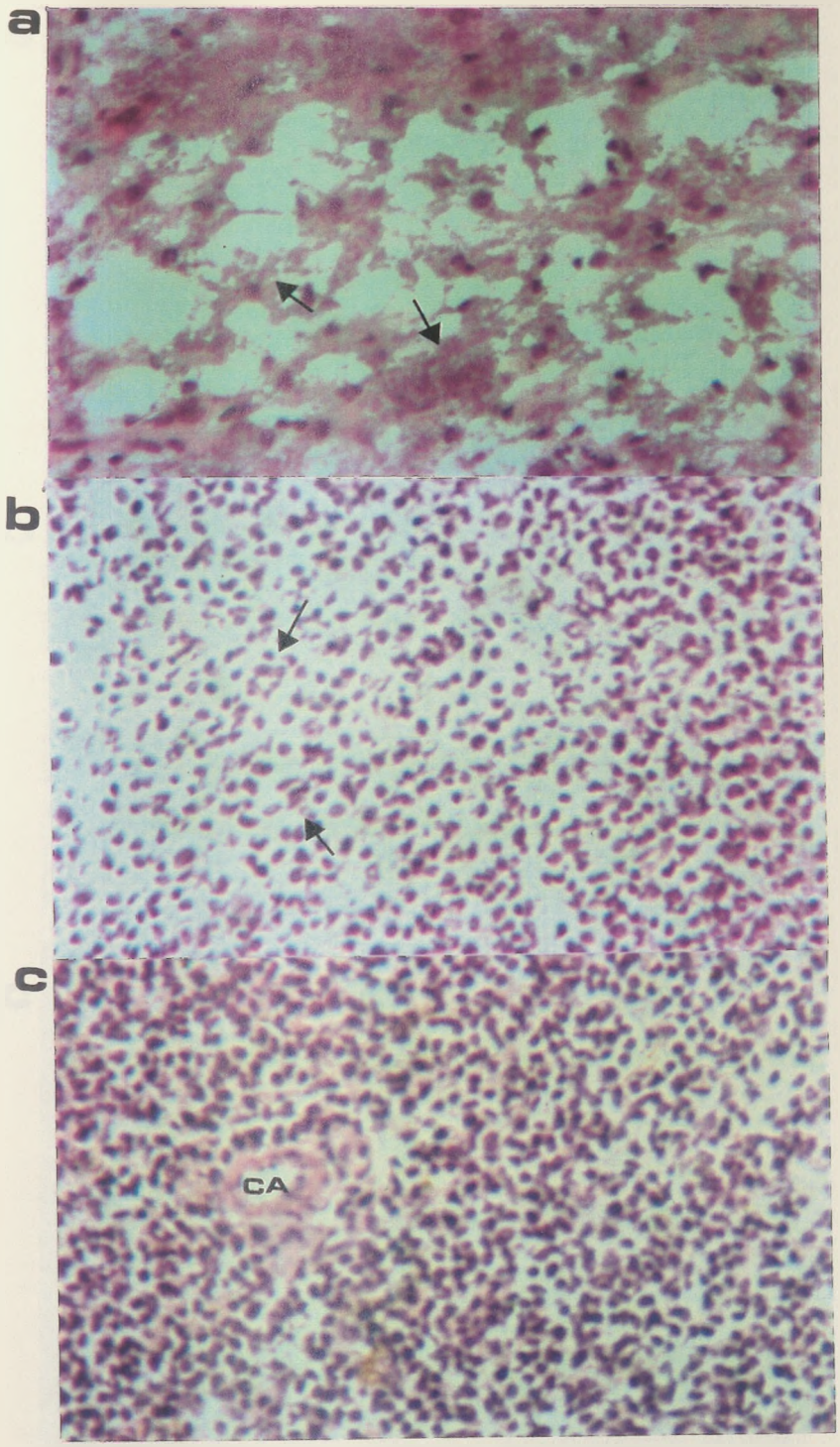

Fig.14 : Hematoxylin & Eosin stained skin lesion (a); draining lymph node (b) and spleen (c) from *L. mexicana* infected male DBA/2 mice at week 8 post-infection. Original magnification x 400.

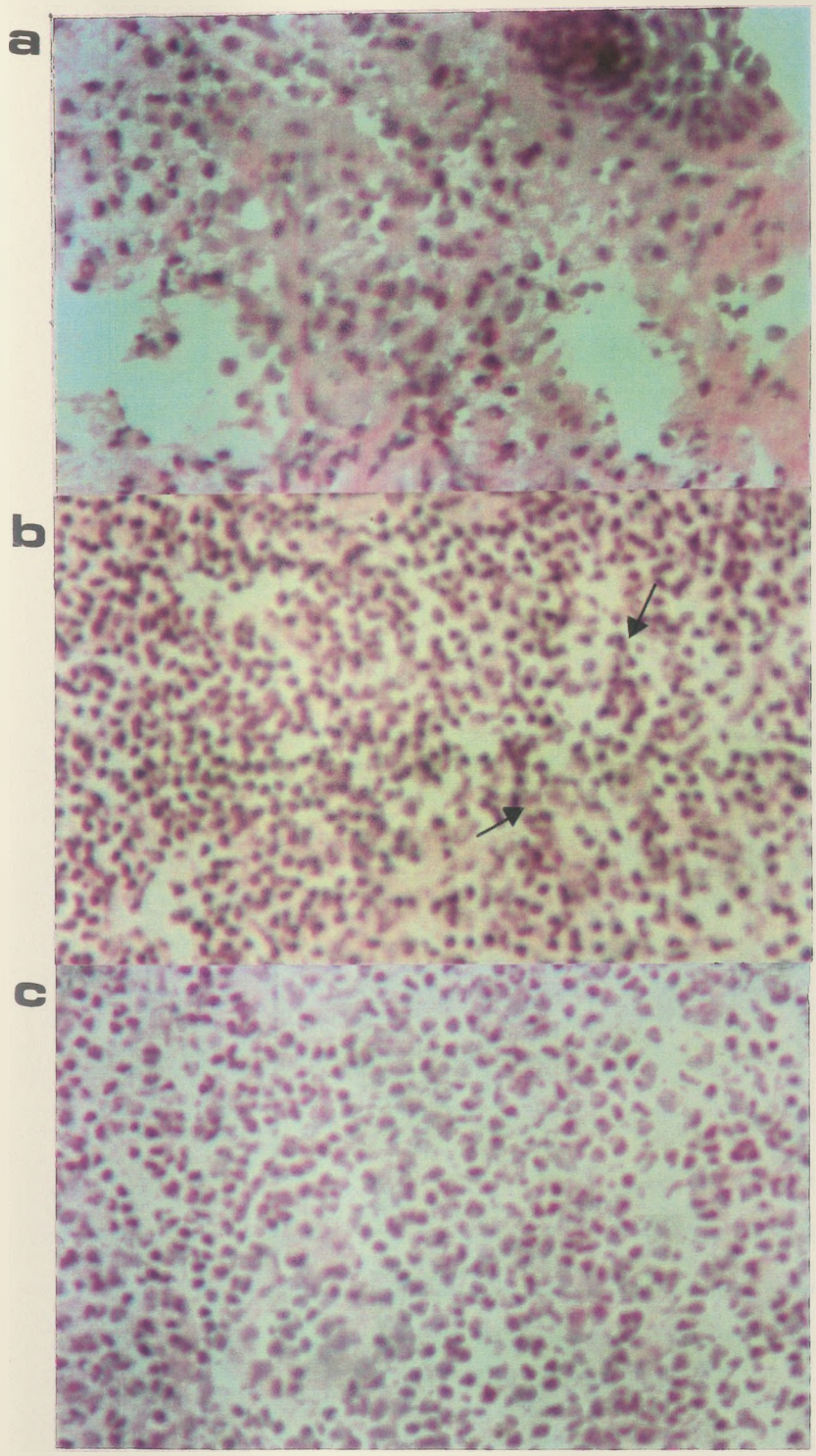

Fig.15 : Hematoxylin & Eosin stained lesion (a); draining lymph node (b) and spleen (c) from *L. mexicana* infected female DBA/2 mice at week 8 post-infection. Original magnification x 400.

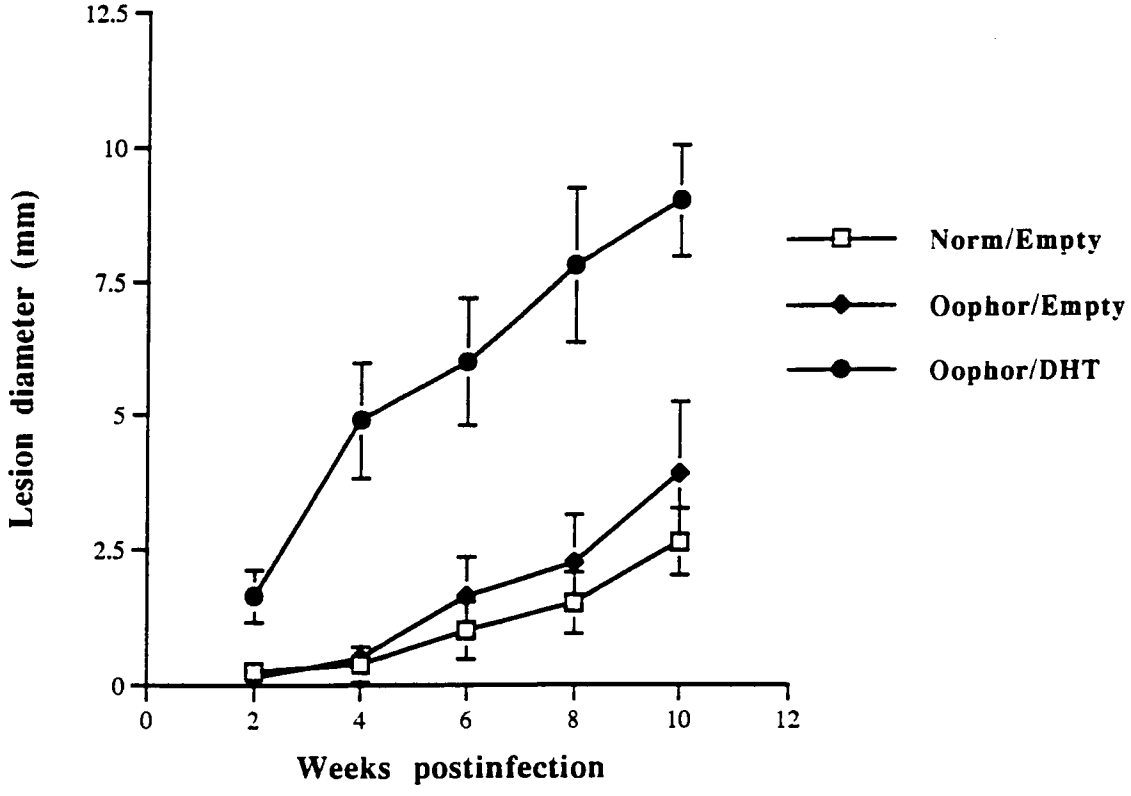

Fig. 17: Effect of DHT on disease progression in *L. mexicana* infected female DBA/2 mice. Data expressed as Mean lesion diameter ± SE.

3.5 Discussion

Previous experimental studies have reported sexual dimorphism in susceptibility to leishmaniasis in mice. As compared with female mice, males were found to be more susceptible to cutaneous *L. mexicana* infection (Alexander, 1988) as well as visceral *L. major* infection (Mock and Nacy, 1988). In this study we confirmed previously reported sex mediated differences observed in DBA/2 mice following *L. mexicana* infection and investigated the immunological basis behind these.

The evolution of the immune response during *L.mexicana* infection is slow compared with *L.major*. While the cytokine profiles, reflecting the progress and outcome of disease in the draining lymph nodes are well established by 4 weeks during infection in resistant and susceptible mice infected with *L.major* (Heinzel *et al.*, 1989), cytokine mRNA production could not be identified from the draining lymph nodes of *L.mexicana* infected DBA/2 mice 5 weeks after infection. Furthermore, *Leishmania*-specific antibodies were barely detectable by week 6 following infection. It is well established that *L.mexicana* is under different genetic and immunological controls to *L.major* (Roberts *et al.*, 1990; Alexander and Kaye, 1985). In addition during *L. mexicana* infection, the lesions tend to be slower growing and non-ulcerating with minimal lymphocyte infiltration (Alexander and Phillips, 1978). This may be a direct result of this parasite's ability to inhibit the release of antigen from macrophages (Russell *et al.*,1992) and/or due to inefficient antigen presentation by infected macrophages (Wolfram *et al.*,1995). Nevertheless, by 8 weeks post infection transcripts for IFN-γ were found in all resistant female DBA/2 mice but rarely in susceptible male mice where those for TNF-α were invariably present. At this time point, following *in vitro* antigenic stimulation, lymphocytes from the draining lymph nodes of infected male and female mice displayed significant differences in the magnitude of the proliferative response as well as cytokine production.Unlike male mice lymphocytes from resistant female mice not only displayed significant antigen specific proliferative responses, but also produced significant amounts of IFN-γ compared with males who failed to produce any detectable IFN-γ.

We further confirmed the *in vivo* functional role of IFN-γ in resistant female DBA/2 mice by treating them with IFN-γ neutralising antibodies following *L. mexicana* infection. Following such a treatment, female mice developed rapidly growing lesions comparable to those found in untreated males. It is, perhaps, not surprising that IFN-γ was detectable in the resistant females as this cytokine has been shown to be of paramount importance not only in helping to promote a TH1 response but in inducing macrophage leishmanicidal activity via the generation of nitrogen intermediates (Liew and O,Donell, 1993; Reed and Scott, 1993). Furthermore, oestrogen has been shown to enhance expression of IFN-γ mRNA in murine spleen cells and may influence production of this cytokine (Fox *et al.*, 1991). Gonadectomy, however, did not have an effect on lesion growth which was increased using testosterone. Surprisingly, lymphocytes from the draining lymph nodes of infected female mice also produced significantly higher levels of IL-10 than male mice following antigenic stimulation. IL-10 is secreted by Th2 cells and has been implicated in disease progression in cutaneous and visceral leishmaniasis in susceptible mouse strains (Heinzel *et al.*, 1991) as well as humans (Karp *et al.*, 1993; Holaday *et al.*, 1993). However, although IL-10 is a predominantly Th2 cytokine (Fiorentino *et al.*, 1989), it can be also produced by other cell types, including monocytes (deWaal Malefyt *et al.*, 1991), mast cell lines (Moore *et al.*, 1990), macrophages and B cells (Howard and O'Garra, 1992).

No differences were observed in IL-2 production , as lymphocytes from both male as well as female mice produced significant amounts of IL-2 following *in vitro* antigenic stimulation. These results were similar to those published previously where both resistant and susceptible mice were shown to express comparable levels of IL-2 mRNA in their draining lymph nodes and spleens following infection (Locksley *et al.*, 1987). However, much of this IL-2 mRNA was predominantly derived from B cells, although, in resistant mice it was also expressed in CD4+ T cells (Heinzel *et al.*, 1991). Although, *in vitro*, IL-2 has been shown to enhance IFN-γ induced leishmanicidal activity of macrophage (Belosevic *et al.*, 1988), *in vivo* studies

indicate that IL-2 may be responsible for disease progression in susceptible mice during *L.major* (Heinzel *et al.*, 1993b) and *L. mexicana* infections (Mazingue *et al.*, 1989; Lezama-Davila *et al.*, 1992). The disease exacerbative role of IL-2 in murine leishmaniasis may result from the ability of this cytokine to induce *in vivo* and *in vitro* IL-4 production and facilitate differentiation of CD4+ T cells towards Th2 expansion (Ben-Sason *et al.*, 1990; Le Gros *et al.*, 1990). A further study indicated that IL-2 enhances parasite growth directly (Mazingue *et al.*, 1989). Similarly, significant differences were also observed in the magnitude of antigen-specific splenocyte proliferative responses between infected male and female mice at this time point. Unlike, the draining lymph node lymphocytes, splenocytes from susceptible males exhibited significantly higher antigen specific responses than those from females. Surprisingly, neither IFN-γ nor IL-10 could be detected in culture supernatants from antigen-stimulated splenocytes from either sex.

While several studies strongly suggest that IFN-γ and TNF-α can synergise to increase macrophage leishmanicidal capabilities (reviewed by Liew and O'Donell, 1993; Bogdan *et al.*, 1990a; Liew *et al.*, 1990a,b,c,d) further studies cast doubt on a protective role for TNF-α independently (Bogdan *et al.*,1990a). The results from this study would certainly imply that while IFN-γ production alone by female DBA/2 mice is enough to control the cutaneous growth of *L.mexicana* lesions, TNF-α production by male mice is insufficient to control disease. In fact a disease exacerbative role for TNF-α would be implied from the studies reported here as *L. mexicana* cutaneous lesions in TNFR1 deficient mice heal while control mice have non-healing lesions (Chapter 6).

As IL-6 has recently been shown to down modulate the *in vitro* cytokine enhanced antileishmanial activity of human macrophages (Hatzigeorgiou *et al.*, 1993), we studied *in vitro* IL-6 production from male and female DBA/2 derived resting peritoneal macrophages following infection with *L. mexicana* promastigotes. We observed that infected as well as uninfected peritoneal macrophages from both sexes

indicate that IL-2 may be responsible for disease progression in susceptible mice during *L.major* (Heinzel *et al.*, 1993b) and *L. mexicana* infections (Mazingue *et al.*, 1989; Lezama-Davila *et al.*, 1992). The disease exacerbative role of IL-2 in murine leishmaniasis may result from the ability of this cytokine to induce *in vivo* and *in vitro* IL-4 production and facilitate differentiation of CD4+ T cells towards Th2 expansion (Ben-Sason *et al.*, 1990; Le Gros *et al.*, 1990). A further study indicated that IL-2 enhances parasite growth directly (Mazingue *et al.*, 1989). Similarly, significant differences were also observed in the magnitude of antigen-specific splenocyte proliferative responses between infected male and female mice at this time point. Unlike, the draining lymph node lymphocytes, splenocytes from susceptible males exhibited significantly higher antigen specific responses than those from females. Surprisingly, neither IFN-γ nor IL-10 could be detected in culture supernatants from antigen-stimulated splenocytes from either sex.

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As IL-6 has recently been shown to down modulate the *in vitro* cytokine enhanced antileishmanial activity of human macrophages (Hatzigeorgiou *et al.*, 1993), we studied *in vitro* IL-6 production from male and female DBA/2 derived resting peritoneal macrophages following infection with *L. mexicana* promastigotes. We observed that infected as well as uninfected peritoneal macrophages from both sexes

produced comparable quantities of IL-6. Similar results have been obtained previously in male and female DBA/2 mice using an indirect biological assay for measuring IL-6 activity (Khamis, 1990).

Surprisingly, no differences were noted between the resistant and susceptible sexes in the generation of IL-12 and IL-4 mRNA. These cytokines have been shown to promote Th1 expansion and disease protection and Th2 expansion and disease susceptibility respectively in mice infected with *L.major* (Scharton-Kersten *et al.*, 1995; Heinzel *et al.*, 1993). However, other experimental studies have failed to demonstrate the clear Th1/Th2 dichotomy in *L. donovani* (Kaye *et al.*, 1991) and *L.amazonensis* infections (Afonso and Scott, 1993). This is further investigated in Chapter 5.

We further analysed *in vivo* immunological responses in male and female DBA/2 mice by measuring serum titres of the Th2 associated IgG1 and Th1 associated IgG2a *Leishmania* specific antibodies (Snapper and Paul, 1987) and by using flow cytometric analysis to monitor changing lymphocyte populations in the draining lymph nodes and spleen at different time points following infection with *L.mexicana*. At week 8 following infection, not only did susceptible male unlike resistant female mice exhibit significant serum titres of *Leishmania*- specific IgG1, but they also showed a significant increase in B cell number in their draining lymph nodes. Interestingly, high serum immunoglobulin (Ig) levels have been observed in patients suffering from non-healing visceral and cutaneous leishmaniasis (reviewed by Turk and Bryceson, 1971). Furthermore in a recent experimental study in susceptible BALB/c mice, it has been reported that B-cell lymphopoiesis is significantly increased in the spleen and draining lymph nodes following *in vivo* IL-7 treatment (Gessner *et al.*, 1995). This led to disease exacerbation following *L. major* infection indicating an association or correlation between B cell expansion and disease progression.

Several recent *in vivo* and *in vitro* studies have strongly suggested a critical role for

IFN-γ, derived from IL-12 activated NK cells, in the development of Th1 associated immune responses and the subsequent control of disease progression following *L.major* infection in susceptible mice (Sypek *et al.*, 1993; Afonso *et al.*, 1994; Heinzel *et al.*, 1993; Scharton-Kersten *et al.*, 1995; Laskay *et al.*, 1993). However, poly I:C treatment a stimulator of NK cell derived IFN-γ production conferred only transient protection in male DBA/2 mice following infection with *L. mexicana* and failed to control subsequent disease progression once the treatment was stopped after 4 weeks infection.

Previous experimental studies have shown that in DBA/2 mice resistance against *L. mexicana* may be oestrogen dependent (Khamis, 1990) while susceptibility to visceralizing *L. major* was testosterone-dependent (Mock and Nacy, 1988). In this study we observed that subcutaneous DHT implants in oophorectomised female DBA/2 mice significantly enhanced disease progression following infection with *L. mexicana*. This would imply that susceptibility of DBA/2 mice to *L. mexicana* was also testosterone dependent. *In vitro* studies have shown testosterone to enhance the activity of IL-2 which has been implicated in promoting under certain conditions disease progression in susceptible mice during *L.major* (Heinzel *et al.*, 1993) and *L. mexicana* infections (Mazingue *et al.*, 1989; Lezama-Davila *et al.*, 1992).

While the present study confirms the importance of IFN-γ in protection against *L. mexicana* it emphasises again that this parasite is under different immunoregulatory and genetic controls to those influencing *L.major* infection (Roberts *et al.*, 1990; Alexander and Kaye, 1985). The DBA/2 *L.mexicana* disease model presents not only a useful tool to study these immunological pathways and the mode of action of the *Scl-2* gene but a means of studying how these pathways are influenced by sex hormones.

Chapter 4

Sex differences in susceptibility to murine visceral leishmaniasis caused by *Leishmania donovani.*

4.1 Summary

Gender-related differences in susceptibility to visceral leishmaniasis have been reported in humans. In order to determine whether similar differences exist in murine visceral leishmaniasis, non-cure BALB/c and cure C57BL/10ScSn (or B10) male and female mice were infected with *L.donovani* and parasite burdens in the liver, spleen and bone marrow were assessed at different time points during the course of infection. Following infection with *L. donovani* BALB/c male mice displayed significantly higher liver parasite burdens at day 15 and 30 than their female counterparts. Although no differences were observed in the spleen parasite loads between male and female BALB/c mice throughout the course of infection, male mice displayed significantly higher bone marrow parasite burdens than females at day 15 post-infection. Furthermore, no *in vitro* differences were noted in either the ability of *L.donovani* promastigotes to infect or amastigotes to multiply within resting peritoneal macrophages from male or female BALB/c mice. On the other hand, following *L.donovani* infection ,male B10 mice, not only developed significantly higher liver parasite loads at day 15 and 30 , but also displayed significantly higher bone marrow parasite burdens at these time points than female B10 mice. Although, no differences were observed in splenic parasite loads between male and female B10 mice during the early phase of disease male B10 mice had significantly higher parasite burdens in their spleens at day 85 post infection. Therapeutic responses of *L.donovani* infected male and female BALB/c mice to sodium stibogluconate treatment as assessed by reduction in liver parasite multiplication were comparable in both sexes .

4.2 Introduction

Many clinical studies from different parts of the world have reported a higher incidence of visceral leishmaniasis amongst males than females. (Thakur, 1984 ; Hoogstraal and Heyneman, 1969; Ayele and Ali, 1984; Jahn *et al.*, 1986; De Beer *et al.*, 1990; Leng Yan-Jia, 1982). Although social or epidemiological factors have been implicated in some of the gender-related differences observed in human visceral and also cutaneous leishmaniasis (Southgate & Oriedo 1962; Wijers, 1963 ; Southgate, 1974; Marsden, 1986; Arias, 1988), sex hormone-associated differences have also been noted in murine leishmaniasis following *L. major* (Giannini, 1986; Mock and Nacy, 1988) and *L. mexicana* infections (Alexander, 1988). However, experimental studies investigating the effects of sex hormones in murine leishmaniasis have as yet to be extended to studies using *L. donovani* , *L. mexicana* and *L. major* are clinicopathologically different diseases from visceral leishmaniasis caused by *L. donovani* (Peters and Killick-Kendrick, 1987). Hence, in the present study we compared the visceral growth of *L. donovani* in male and female, susceptible BALB/c and resistant B10 mice. The effect of sex influences on the success of chemotherapy was also assessed.

4.3 Materials and Methods

Mice and infection

Eight to 10 weeks old age matched male and female BALB/c (H-$2^{d/d}$) or C57BL/10ScSn(H-$2^{b/b}$) mice were used in all the experiments.

Parasites

Leishmania donovani (L82) was maintained in hamsters as described in Chapter 2. The amastigotes were isolated and purified from the spleens of infected hamsters as described in Chapter 2. Promastigotes were obtained by *in vitro* culture of amastigotes in Schneider's insect culture medium .

Infection protocol

Twenty male and 20 female BALB/c or B10 mice were infected with 1 x 10^7 *L. donovani* amastigotes via tail vein injections. Disease progression was monitored by sacrificing 4 to 6 mice from each group and assessing parasite burdens in the liver , spleen and bone marrow at days 15, 30 and 50 post infection in BALB/c mice and at days 15, 30, 50 and 85 post infection in B10 mice as described in Chapter 2.

Isolation of resting peritoneal macrophages

Resting peritoneal macrophages were obtained from age matched male and female BALB/c mice as described in Chapter 2. Following enumeration of viable cells by trypan blue exclusion, the peritoneal cells were adjusted to 1 x 10^6 cells/ ml in complete RPMI 1640. Aliquots (500µl) containing 5 x 10^5 cells were added to the wells of 24-well flat bottomed tissue culture plates containing pre-sterlised (13mm diameter) cover slips and were incubated at 37°C for 2 hours to facilitate adherence of macrophages to glass cover slips.

In vitro infection of peritoneal macrophages

Following incubation, non adherent cells were removed by three gentle washes with complete RPMI. *L. donovani* promastigotes at a ratio of 4:1 parasites:macrophage

were added to each well in aliquotes of 200µl and cultures were further incubated at 37°C for 4 hours. Extracellular parasites were removed by three washes in complete RPMI and plates were further incubated under the same conditions for a further 48 hours. The level of infection in macrophages was assessed at 12, 24 and 48 hours by examining formalin fixed, Giemsa stained monolayers (Scott *et al.*, 1983; Liew and Dhaiwal, 1987).

Procedure for Giemsa staining

The wells containing infected macrophages were washed three times in PBS (pH 7.4). The glass coverslips with adherent macrophages were air dried and fixed in methanol (BDH, Poole, UK) for 10 min prior to their staining with 10% (v/w) Giemsa Stain for 10 minutes.Stained coverslips were washed vigorously in running tap water to remove excess stain, air dried and were mounted in green euparal for further examination by light microscopy.

Chemotherapy Experiments

Following infection with *L. donovani*, infected mice were treated with 200µl of either different concentrations of sodium stibogluconate solution (0.2, 0.4, 0.6, 0.8 and 1 mg/ml) (kindly provided by Dr. Chris Carter) or sterile PBS via tail vein injections on days 7 and 8 post-infection. Mice were sacrificed on day 14 post infection and parasite burdens in liver, spleen and bone marrow were determined. The data was expressed as the % parasite suppression (Carter, *et al.*, 1993).

Statistical analysis

The significance of differences between the groups was determined by the Student's unpaired *t* test.

4.4 Results

Growth of L. donovani in male and female BALB/c mice

Following intravenous inoculation with *L. donovani*, the parasite burdens in the viscera of male and female BALB/c mice were assessed at different time points. Male BALB/c mice exhibited significantly higher ($p<0.025$) liver parasite burdens than female mice at day 15 and 30 post infection (Fig.1). No significant differences were noted in spleen parasite burdens between *L. donovani* infected male and female BALB/c at any time points examined (Fig.2). However, at day 15 post-infection male mice had significantly higher ($p<0.025$) bone marrow parasite loads than their female counterparts (Fig.3).

Growth of L. donovani in male and female C57BL/10 mice

Male C57BL/10 mice displayed significantly higher ($p<0.025$) liver parasite burdens than their female counterparts at day 15 following infection with *L. donovani* (Fig. 4). No significant differences were noted in spleen parasite burdens between male and female C57BL/10 during the early course of disease. However, at day 85 post infection male mice had significantly higher splenic parasite loads ($p<0.05$) than female mice (Fig.5). Although, male mice displayed higher bone marrow parasite burdens than female mice throughout the course of infection, the differences were statistically significant only at day 30 post infection (Fig.6). However, in repeat experiments, although similar differences were observed in liver parasite burdens between *L. donovani* infected C57BL/10 male and female mice, no significant differences were noted in spleen and bone marrow parasite burdens at any of the time points examined.

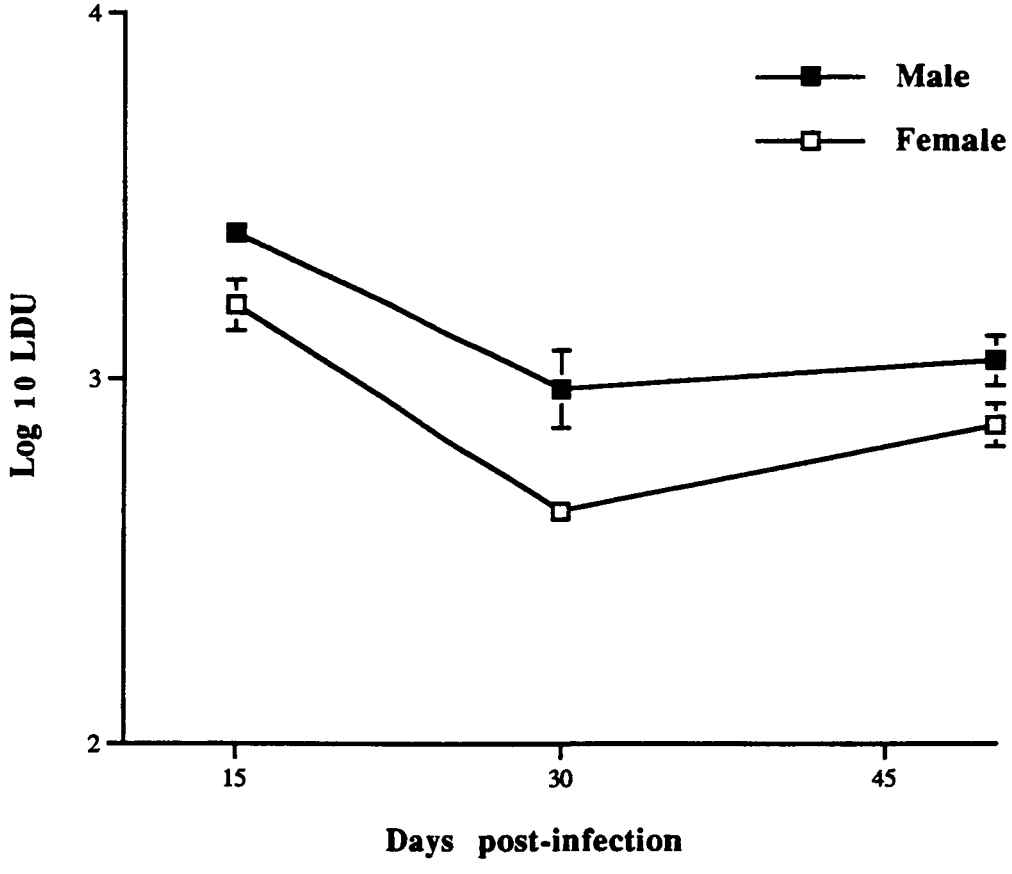

Fig.1: Liver parasite burdens in male (■) and female (□) BALB/c mice infected with *L. donovani*. Data expressed as Mean Log $_{10}$ LDU (Leishman Donovan Units) ± SE.

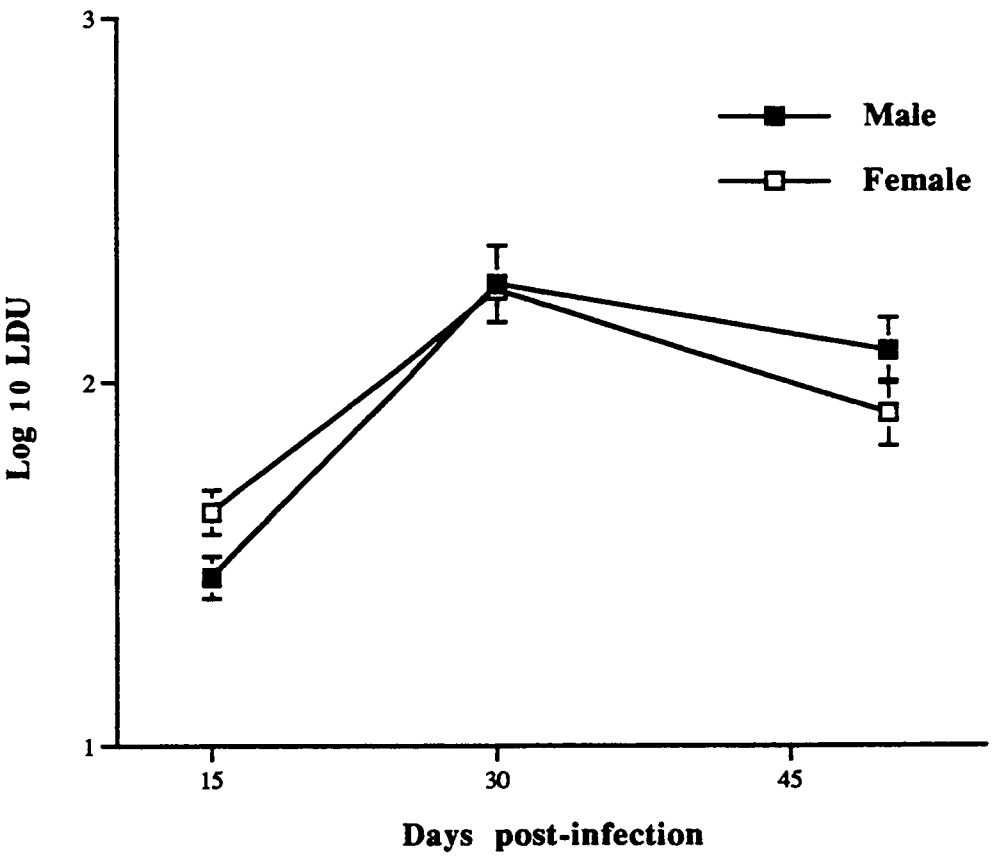

Fig.2: Spleen parasite burdens in male (■) and female (□) BALB/c mice infected with *L. donovani*. Data expressed as Mean Log $_{10}$ LDU (Leishman Donovan Units) ± SE.

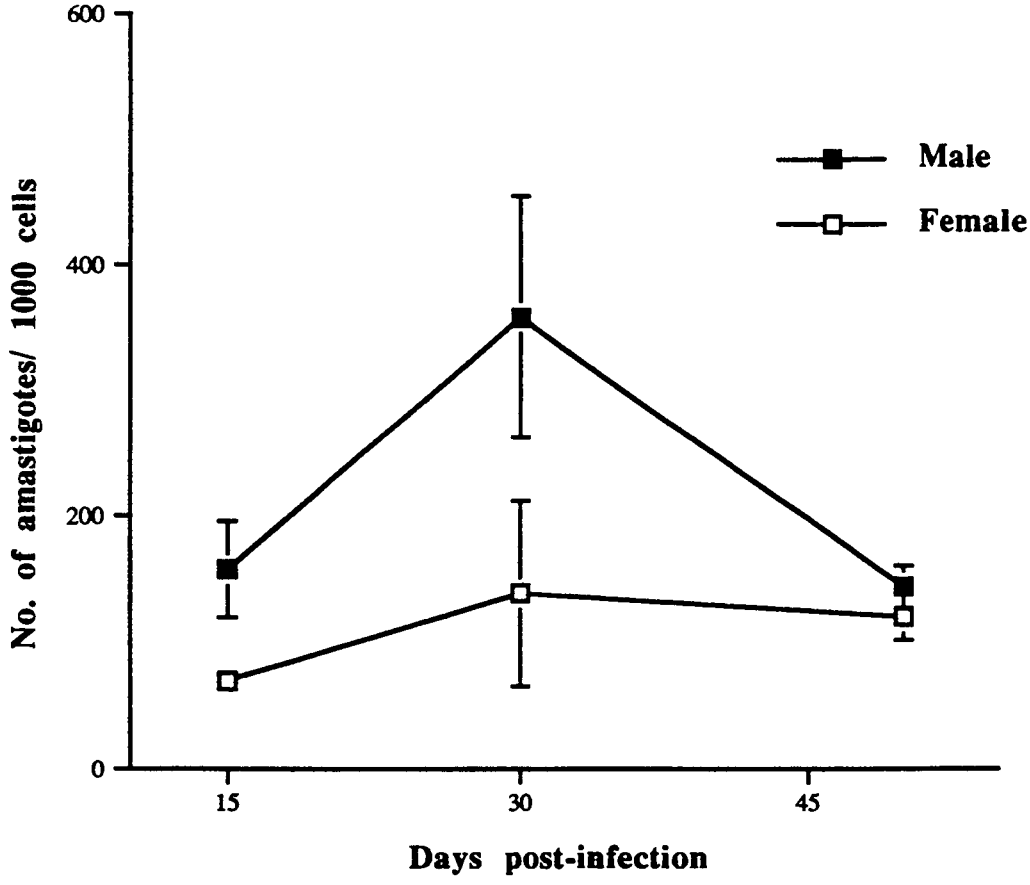

Fig.3: Bone marrow parasite burdens in male (■) and female (□) BALB/c mice infected with *L. donovani*. Data expressed as number of amastigotes/1000 cells ± SE.

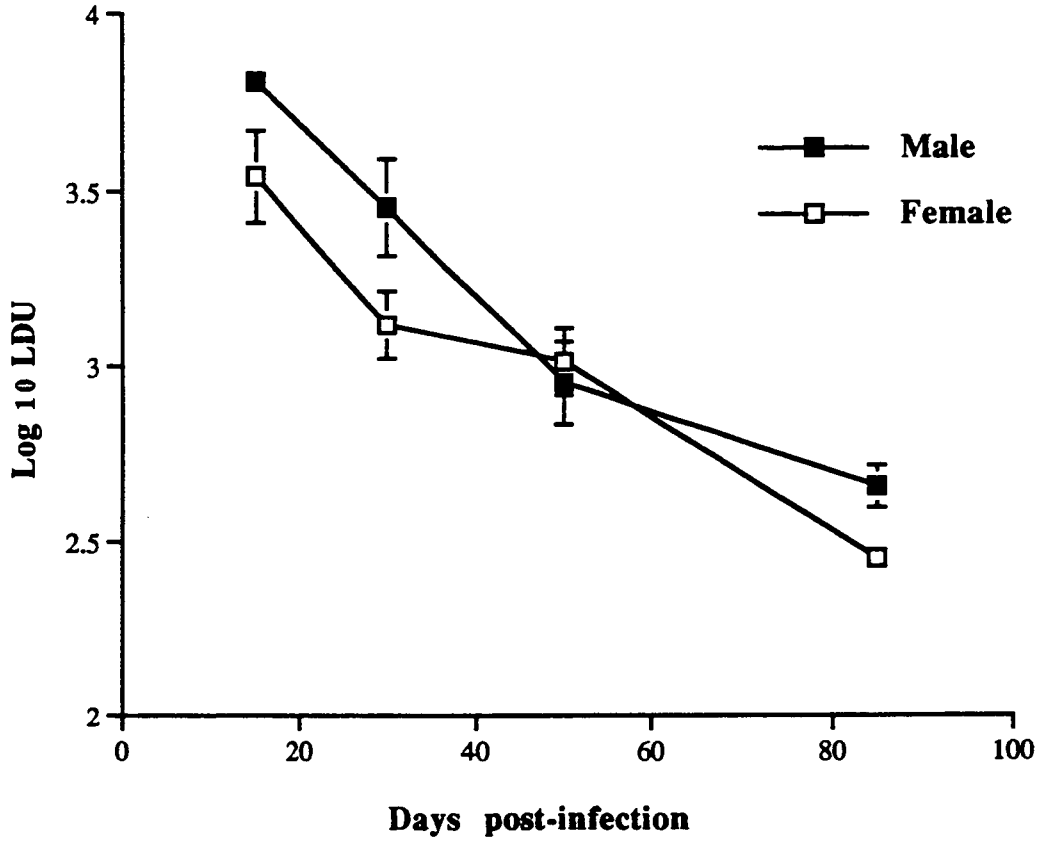

Fig.4: Liver parasite burdens in male (■) and female (□) C57BL/10 mice infected with *L. donovani*. Data expressed as Mean Log $_{10}$ LDU (Leishman Donovan Units) ± SE.

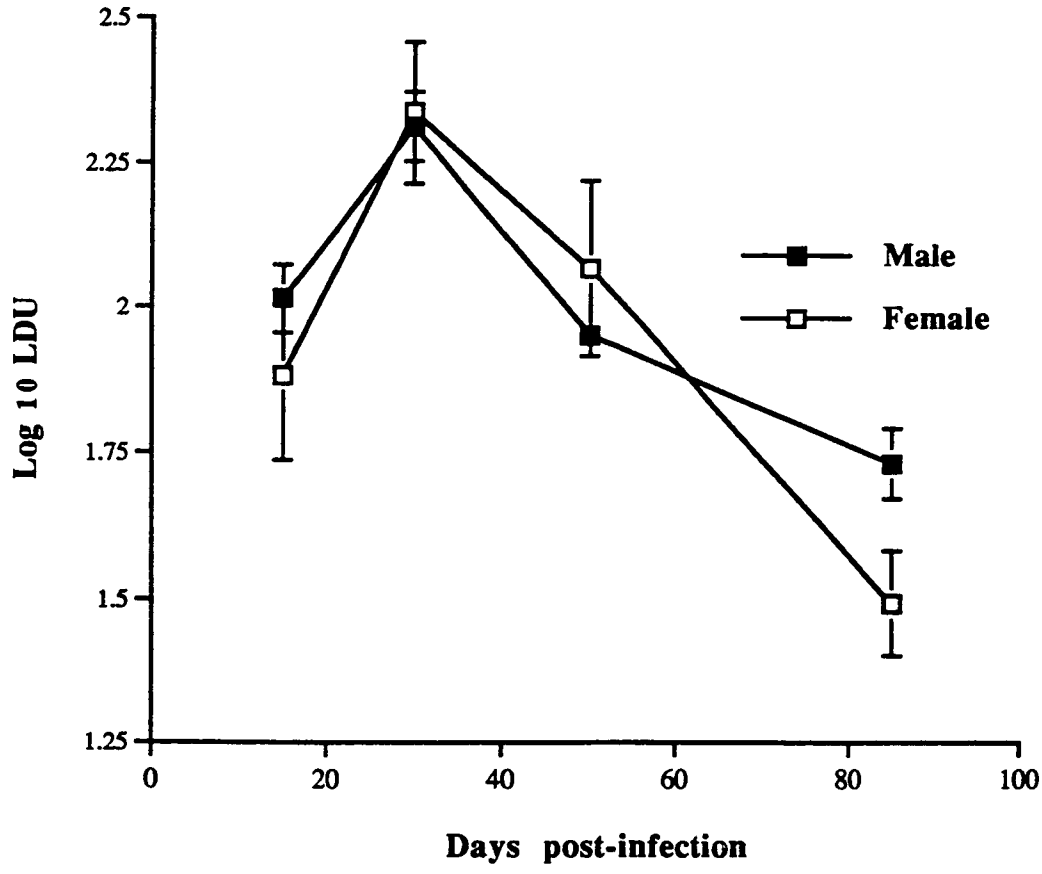

Fig.5: Spleen parasite burdens in male (■) and female (□) C57BL/10 mice infected with *L. donovani*. Data expressed as Mean Log $_{10}$ LDU (Leishman Donovan Units) ± SE.

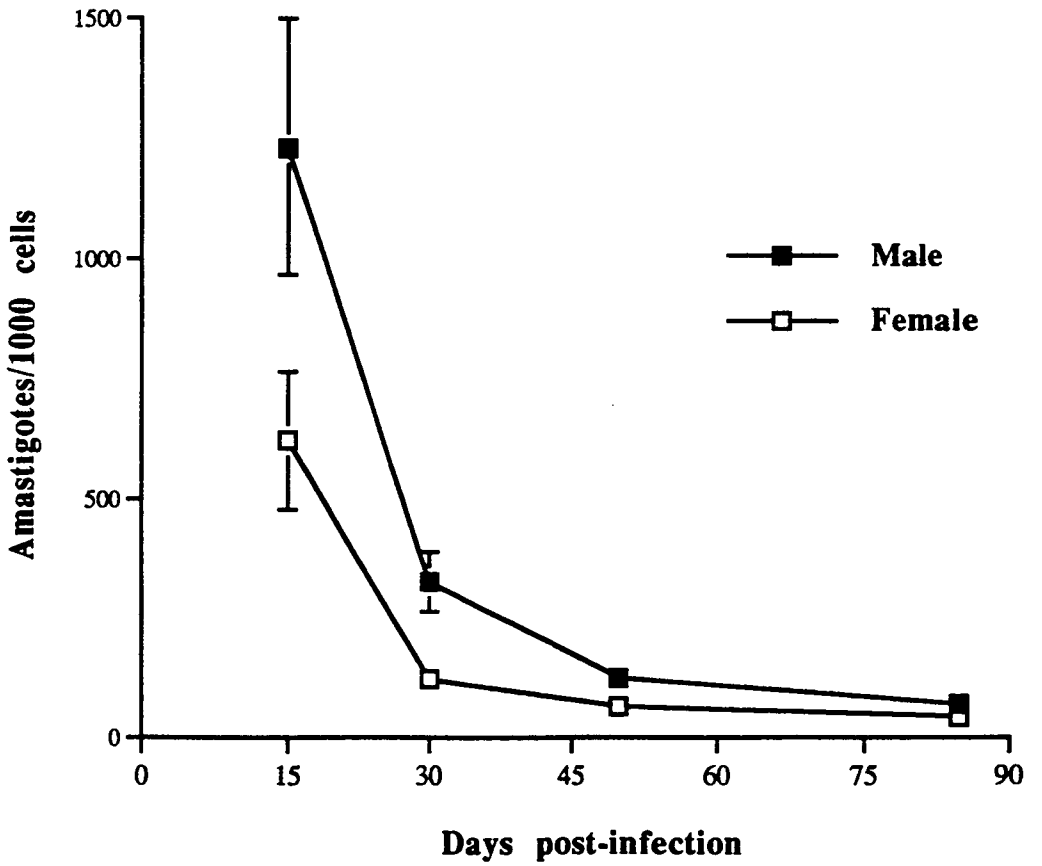

Fig.6: Bone marrow parasite burdens in male (■) and female (□) C57BL/10 mice infected with *L. donovani*. Data expressed as number of amastigotes/1000 cells ± SE.

Growth of L. donovani in macrophages from male and female BALB/c mice

No significant differences were observed in either infectivity or intracellular growth of parasites between male and female BALB/c macrophages (Fig.7).

Sodium stibogluconate therapy and parasite suppression in L. donovani infected male and female BALB/c mice.

At all the therapeutic doses examined, no significant differences could be noted in % parasite suppression between the viscera of male and female BALB/c mice (Fig.8).

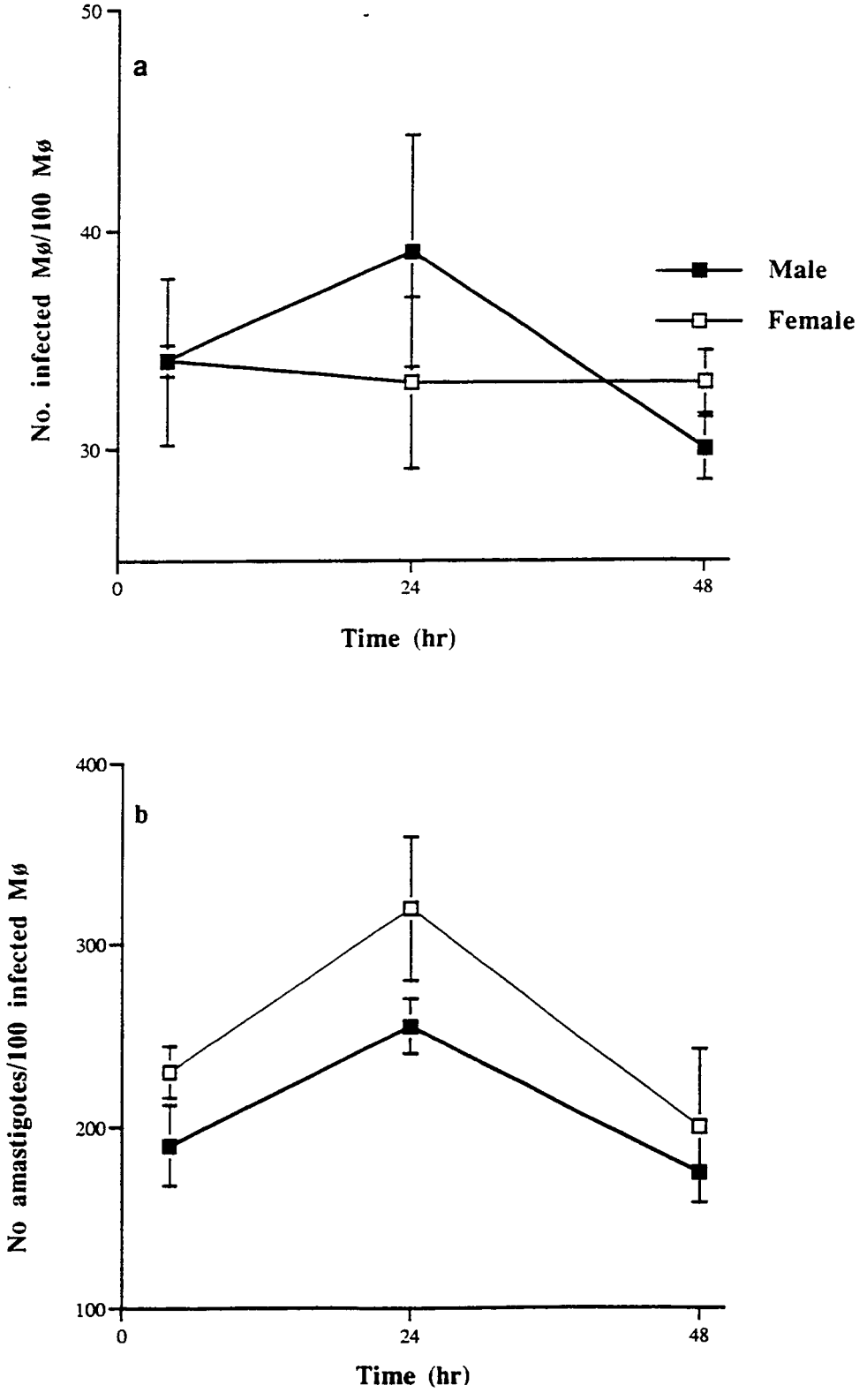

Fig.7: *In vitro* infectivity of *L. donovani* in resting peritoneal macrophages from male (■) and female (□) BALB/c mice. Data expressed either as Mean number of amastigotes/infected macrophage± SE or Mean number of infected macrophages/100 cells ± SE.

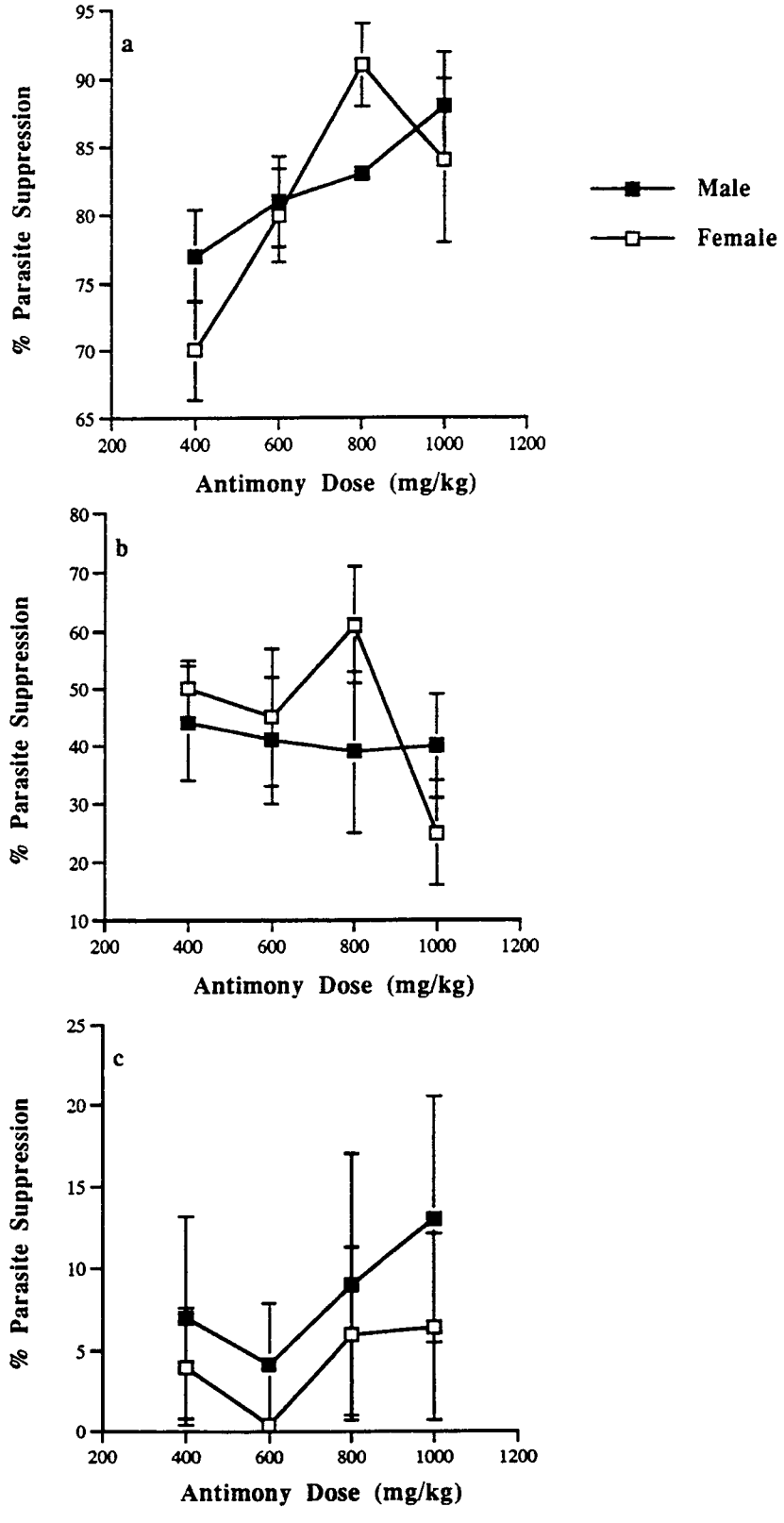

Fig.8: Effect of sodium stibogluconate therapy on liver (Fig.8a), spleen (Fig.8b) and bone marrow parasite (Fig.8c) burdens in male (■) and female (□) BALB/c mice infected with *L. donovani*. Data expressed as Mean % parasite suppression ± SE.

4.5 Discussion

Clinico-epidemiological studies done in the past from different parts of the world have reported significantly higher incidences of visceral leishmaniasis in males than females (Goble and Konopka, 1973; Hoogstraal and Heyneman, 1969; Ayele and Ali, 1984; Jahn *et al.* 1986; De Beer *et al.*, 1990; Leng, 1982). Although social and epidemiological factors, at least in part, were believed to be responsible for these gender related differences (Balasubramaniam, 1920-1921; Cunnigham and Pundit, 1924-1925, McKinnon and Fendall, 1956, Southgate, 1974), other clinical studies have cast a doubt on the role of such factors in mediating sex related differences in human visceral leishmaniasis (McKinnon, 1963; Ho *et al.*, 1982).

A number of experimental studies have implied a significant role for hormonal factors in determining the outcome of cutaneous leishmaniasis. Thus, male DBA/2 mice developed significantly more rapidly growing lesions than their female counterparts following subcutaneous inoculation with *L. mexicana* (Satoskar and Alexander, 1995; Alexander, 1988). On the other hand, following *L. major* infection, female but not male DBA/2 mice developed rapidly progressive non-healing ulcerating lesions (De Tolla et al., 1981; Giannini, 1986; Alexander, 1988). Later it was shown that oestrogen treatment of male DBA/2 mice rendered them significantly resistant to *L. mexicana* infection (Alexander and Stimson, 1988). In a study by Mock and Nacy (1988), male BALB/c mice developed significantly higher liver parasite burdens than female mice following intravenous inoculation with *L. major*. In the same study, treatment of female BALB/c mice with testosterone prior to intravenous inoculation with *L. major* exacerbated the disease and resulted in a significant increase in liver parasite burdens. Furthermore, Arcay (1985) showed that treatment with testosterone significantly exacerbated *L. mexicana amazonensis* infection in hamsters. In the present study, we have observed clear *in vivo* sex mediated differences in susceptibility to *L. donovani* in susceptible BALB/c and resistant B10 mice. The results obtained in our study mirror sexual dimorphism reported in human visceral leishmaniasis.

It is well established that macrophages play a pivotal role in the host defence against *Leishmania* parasites not only by killing intracellular parasites (reviewed by Alexander and Russell,1992), but also by their ability to present antigen and modulate cell mediated immune responses (reviewed by Liew and O'Donell,1993). Significantly, not only have receptors for sex steroid been detected on macrophages (Gulshan *et al.*, 1990), but sex hormones have also been shown not only to enhance macrophage phagocytic (Morahan *et al.*, 1984; Loose and Diluzio, 1976, Nicol *et al.*, 1965) and microbicidal activity (Morahan *et al.*, 1984; Warr and Slijivic, 1973) but also their capacity to process antigen (Fruhman, 1973). Oestrogens, in particular, have been reported to increase the number of liver macrophages (Warr and Slijivic, 1973) and enhance their microbicidal activity (Morahan *et al.*, 1984). Thus, it is perhaps not surprising that in the present study, following infection with *L. donovani*, female BALB/c and B10 mice had significantly less liver parasite burdens than their male counterparts.

In vitro, physiological concentrations of oestrogens have been shown to activate macrophages (Berak *et al.*, 1986) and enhance their IL-1 production (Kuang et al., 1988). Thus, *in vitro* treatment of male DBA/2 mouse peritoneal exudate macrophages with physiological concentrations of oestrogen resulted in enhancement of their leishmanicidal activity against *L. mexicana* (Khamis, 1990). However, in our study, no clear differences in infectivity and growth of *L. donovani* were, observed in resting peritoneal macrophages derived from BALB/c mice from either sex.

Innate susceptibility to murine visceral leishmaniasis following *L. donovani* infection has been shown to be under the control of a single gene, originally designated as *Lsh.* (Bradley *et al.*, 1977) now designated as *Nramp* for Natural resistance associated macrophage protein. The *Lsh* gene is believed to act at the macrophage level (Crocker *et al.*, 1984) and is expressed within 2 -3 days following *in vivo* or *in vitro* infection (Crocker *et al.*, 1984; Crocker *et al.*, 1987). *Nramp* has

been shown to play a role, either directly or as an additional pleotropic effect, in the Interferon-γ/LPS upregulated L-arginine transport across the macrophage membrane required for NO production (Blackwell *et al.*, 1994). It is possible that sex differences observed in the present study may be due to sex hormone mediated differential expression of *Nramp* gene products as hormones have been shown to regulate transcription of a number of genes involved in immunological responses (Beato,1989; Rories and Spelsberg, 1989).

Clinical studies in the past have indicated that the host response to drug treatment may be influenced by the sex of the host (Goble and Konopka, 1973; Aikat *et al.*, 1979). In Indian kala-azar, the preponderance of post kala-azar dermal leishmaniasis (PKDL) in males has indicated that the response to chemotherapy was poorer in males (Sen Gupta, 1956; Napier and Das Gupta, 1931-1932; Aikat *et al.*, 1979). However, we failed to observe such diferences between *L. donovani* infected male and female BALB/c mice in their ability to clear parasites following treatment with sodium stibogluconate.

Gender- related differences reported in murine visceral leishmaniasis mirror the situation observed in human visceral leishmaniasis. Hence the murine *L. donovani* model is a potential tool for investigating the role of sex hormones in the modulation of immunological responses during *L. donovani* infection.

Chapter 5

Disruption of the murine Interleukin-4 gene inhibits disease progression during *Leishmania mexicana* but does not increase the control of *Leishmania donovani* infection.

5.1 Summary

The growth of both cutaneous leishmaniasis and visceral leishmaniasis caused by *L.mexicana* and *L.donovani* respectively, was measured in the female IL-4 "knockout" mice (IL-4-/-) and compared with similarly infected wild-type (IL-4+/+) control mice. While IL-4+/+ mice developed large, non healing, cutaneous lesions when subcutaneously infected with $5x10^6$ *L.mexicana* into the shaven rump, no lesions whatsoever developed in IL-4-/- mice. In contrast IL-4-/- mice infected with $1x10^7$ *L.donovani* amastigotes intravenously, were found to have consistently higher parasite burdens in their livers throughout infection than their wild-type counterparts. However, these differences were only significant at 15 days post-infection. While the results reported here pertaining to *L. donovani* largely support previous studies, those related to *L. mexicana*, provide new observations. The immunological responses of IL-4-/- and IL-4+/+ mice infected with *L. mexicana* were, therefore, examined both *in vivo* and *in vitro*. Although neither IL-4-/- nor IL-4+/+ mice, infected with *L. mexicana* produced parasite specific IgG2a antibodies, IL-4+/+ mice, unlike IL-4-/- mice, developed significant IgG1 antibody titers as infection progressed, indicating a Th2 influenced response in wild-type mice. In addition IL-4-/- mice, unlike IL-4+/+ mice, developed a significant delayed-type-hypersensitivity (DTH) response, indicating a Th1 influenced response in IL-4-/- mice. Following *in vitro* stimulation splenocytes from IL-4+/+ mice infected with *L.mexicana* displayed significantly higher antigen-specific proliferative responses than IL-4-/- mice. However, IFN-γ production as measured from the supernatants of these *in vitro* splenocyte cultures, was significantly higher from IL-4-/- as opposed to IL-4+/+ mice. This again would indicate a predominantly Th1 influenced response in the absence of a Th2 response in IL-4-/- mice infected with *L.mexicana* . On the other hand, IL-4-/- mice displayed significantly higher *in vitro* antigen-specific proliferative responses in their draining lymph nodes than IL-4+/+. Analysis of these culture supernatants from *in vitro* lymph node cell cultures revealed significantly higher levels of IL-5 in IL-4+/+ mice as compared with IL-4-/- mice. Flow cytometric analysis of lymphocytic populations in draining lymph nodes from *L. mexicana* infected mice showed significant B cell

expansion in IL-4+/+ mice as compared with IL-4-/- mice. Immunohistochemical staining of the site of infection with an inducible oxide synthase (iNOS)-specific antiserum showed this enzyme present in substantial amounts in IL-4-/- but not IL-4+/+ mice. Despite the absence of obvious lesion development in IL-4-/- mice following infection with *L. mexicana*, viable parasites could be demonstrated following *in vitro* culture from cutaneous inoculation sites as well as the draining lymph nodes of infected IL-4-/- mice 16 week post-infection.

These results clearly demonstrate that, whereas IL-4 and/or Th2 lymphocyte expansion is necessary for disease progression as measured by lesion growth and the inhibition of a protective response in cutaneous infection caused by *L.mexicana*, such a role for Th2 cells in visceral leishmaniasis caused by *L.donovani* cannot be demonstrated.

5.2 Introduction

American cutaneous leishmaniasis resulting from *L.mexicana* infection is mainly a localised disease often associated with chronic infections of the ear whereas visceral leishmaniasis caused by *L.donovani* is characterised by the dissemination of parasites into the spleen, liver, lymph nodes and bone marrow (Blackwell, 1988). Although the clinical outcome of infection is undoubtedly due in part to the parasite species initiating infection, genetically controlled immunoregulatory factors operating within an individual host also influence disease progression (Alexander and Russell, 1992). Furthermore, studies on murine models of disease have shown that different genetic controls can operate within a single host to control the growth of different species of *Leishmania* even when these species may infect the same tissue sites (Mock *et al.*, 1985). Thus, while the visceral growth of *L.major* is totally independent of *Lsh* gene involvement, which controls the early growth of visceral *L.donovani* (Blackwell, 1988), the cutaneous growth of the former parasite has been shown to be under different genetic and immunoregulatory controls to those associated with the cutaneous growth of *L.mexicana* (Alexander and Kaye, 1985; Blackwell *et al.*, 1984; Roberts *et al.*, 1990).

Although numerous recent studies have clearly established the immunological mechanisms governing the growth of experimental cutaneous leishmaniasis caused by *L.major* (Liew and O'Donell, 1993), those immunological mechanisms controlling the visceral growth of species such as *L.donovani* and the cutaneous growth of species such as *L.mexicana* await elucidation. The control of *L.major* lesion growth in genetically resistant mice has been clearly associated with the expansion of the Th1 lymphocyte subset of the CD4+ T cell population and the production of the cytokines IFN-γ and IL-2 (Heinzel *et al.*, 1991). Conversely, non-healing responses in susceptible mice have been related to the expansion of the Th2 subset and production of cytokines such as IL-4 and IL-10 (Heinzel *et al.*,1989; Locksley *et al.*, 1991). The few experimental studies to date on the immunological control of *L.donovani* (Kaye *et al.*, 1991) and parasites of the *L.mexicana* complex, which includes *L.*

amazonensis(Afonso and Scott, 1993; Satoskar and Alexander, 1995), have indicated that the inability to generate a Th1 cell response, rather than the presence of a Th2 response, may be responsible for disease susceptibility.

The recent demonstration that disruption of the murine IL-4 gene blocks Th2 cytokine responses (Kopf *et al.*, 1993), has provided a tool by which to determine the precise role, if any, of Th2 cells in the growth of *Leishmania* species. Therefore, in the present study we have compared the visceral growth of *L.donovani* and the cutaneous growth of *L.mexicana* in (129/SvxC57BL/6) mice homozygous for the disrupted IL-4 gene (IL-4-/-) with their age and sex matched wild- type (IL-4+/+) counterparts.

5.3 Materials and Methods

Mice

IL-4-/- (129/Sv x C57BL/6) F2 mice were generated as described previously (Kopf *et al.*, 1993). Sex and age matched wild type mice (IL-4+/+) of the same strain combination were used as controls in all the experiments. Only female mice were used in all the experiments.

Parasites and Infection Protocol

Leishmania mexicana (MNYC/BZ/62/M379) and *Leishmania donovani* (L82) were maintained as described in Chapter 2.

L.mexicana

In a typical experiment groups of six, 8-10 weeks old sex matched IL-4-/- and wild type control (IL-4+/+) mice were infected with $5x10^6$ *L.mexicana* amastigotes by subcutaneous inoculation into the shaven rumps. Disease progression was monitored by the measurement of lesion diameters at 2 week intervals up to 16 weeks post infection.

L.donovani

In a typical experiment, groups of twenty, 8 to 10 week old, IL-4-/- and IL-4+/+ mice were infected with $1x10^7$ *L.donovani* amastigotes intravenously via tail vein injections. At 15, 30, 50 and 110 days post infection, 5 mice from each group were sacrificed and parasite burdens in the liver were assessed as described previously (Stauber, 1958; Bradley and Kirkley, 1977).

General Experimental procedures

Isotype specific ELISAs, T cell proliferation assays, cytokine assays, flowcytometric and histopathological analysis were carried out as described in detail in Chapter 2.

Induction and measurement of DTH responses

DTH responses were examined in IL-4+/+ and IL-4-/- mice at week 16 following

subcutaneous infection. 50µl of 10^6 formalin (5%) fixed, PBS washed *L. mexicana* promastigotes were inoculated into the right hind foot pad and the increase in foot pad thickness measured 48 hours later using a dial-gauge micrometer. Left hind foot pads were injected with sterile PBS as a negative control.

Preparation of tissues for Immunohistochemistry

Following infection with *L. mexicana* , spleen , draining inguinal lymph nodes and skin lesions were excised in IL-4+/+ and IL-4-/- mice at week 8 and 16 post infection and snap-frozen in liquid nitrogen. The tissues were embedded in OCT compound (Miles Scientific) and serial cryostat sections were cut at -23°C. The sections were air-dried, dipped in cold acetone for 1-2 seconds and were stored frozen at -20°C for further processing.

Immunohistochemical staining for CD4+ T cells and B cells

For immunocytochemical staining, the sections from spleen and draining lymph nodes from *L. mexicana* infected IL-4-/- and IL-4+/+ mice were fixed in cold acetone for 10 min, and rehydrated with PBS (pH 7.4). Non specific binding sites were blocked with 5% normal rabbit serum (NRS) in PBS for 30 min at room temperature prior to incubation with hybridoma supernatants containing either rat anti-mouse-CD4 antibody (clone:GK1.5) or rat anti-mouse-B220 antibody (clone:RA3-6B2). Following incubation with primary mAb at room temperature for 60 min , the slides were washed in PBS and then incubated with a biotinylated rabbit anti-rat Ig (2µg/ml) (Vector Laboratories, USA) which had been adsorbed against 1% normal mouse serum in PBS for 30 min at room temperature. The sections were then rinsed in PBS and endogenous peroxidase activity was quenched by placing the tissue in 0.3% hydrogen peroxide in PBS for 45 min at room temperature. After quenching, the sections were washed in PBS and were overlayed with preformed avidin-biotin-horseradish peroxidase complex solution (Vectastain Elite ABC, Vector) according to the manufacturer's instructions. Following three final washes in PBS, the enzyme activity in the bound complex was detected using ImmunoPure® Metal Enhancement

DAB substrate kit (Pierce, Rockford,IL,USA) as per manufacturer's instructions.

Immunohistochemical staining for cytokines

Cryostat sections from the spleens of *L. mexicana* infected IL-4-/- and IL-4+/+ mice were stained using hybridoma supernatant containing rat mAb against mouse IFN-γ (XMG1.2) to detect IFN-γ producing cells. Spleens from infected IL-4+/+ mice were also studied for IL-4 producing cells using hybridoma supernatant containing rat anti-mouse IL-4 mAb (BVD6.24G). The specificity of these anti-cytokine antibodies in immunostaining has been previously established (Bogen *et al.*, 1991; Bogen *et al.*, 1993).The staining was performed as described before (Bogen *et al.*, 1993) .

Briefly, the sections were fixed in cold acetone for 1 minutes and rehydrated in PBS with 1%FCS prior to their incubation with the hybridoma supernatants containing primary mAb for 45 minutes at room temperature. The slides were rinsed in PBS/ 1% FCS for 15 minutes and biotinylated rabbit anti-rat Ig, mouse Ig adsorbed (5μg/ml) in 1% normal mouse serum (Vector Laboratories, Burlingame, CA, USA) was added. Following this stage endogenous peroxidase activity was quenched by incubating sections with 0.3% (v/v) hydrogen peroxide in PBS for 45 minutes. The slides were washed in PBS for 15 minutes and the antibody-biotin conjugates were detected with an avidin-biotin-horseradish peroxidase complex (Vectastain Eliet ABC, Vector Laboratories, Burlingame, CA, USA) as per manufacturers instructions. Following three final washes in PBS, the enzyme activity in the bound complex was detected using ImmunoPure® Metal Enhancement DAB substrate kit (Pierce, Rockford,IL,USA) as per manufacturer's instructions.

Immunohistochemical staining for inducible nitric oxide synthase (iNOS)

Expression of iNOS was studied in spleens , draining lymph nodes and cutaneous lesions from IL-4-/- and IL-4+/+ *L. mexicana* infected mice at week 8 and 16 post infection. The sections were rehydrated in Tris-Saline (pH7.6) and were incubated

overnight at 4°C in 5% normal human serum (NHS) in PBS to block non specific binding sites prior to the addition of rabbit iNOS specific anti-serum (60 min, room temperature). Following incubation with primary antibody, the sections were washed twice for 15 min in Tris (pH 7.6), incubated with affinity purified, ALP conjugated donkey anti-rabbit IgG (60 min,room temperature), and washed again in Tris. The label was visualised by adding substrate. The sections were counter stained with Harris haematoxylin and examined by light microscopy for iNOS expression. Serial sections from the cutaneous inoculation sites and the draining lymph nodes were stained by Non-specific estrase (NSE) staining to detect macrophages (Hudson and Hay, 1989)

Isolation of parasites and estimation of parasite loads

The draining lymph nodes were excised aseptically in *L. mexicana* infected IL-4+/+ as well as IL-4-/- mice at week 16 post infection and single cell suspensions were prepared by gentle homogenisation in Schneider's insect culture medium (Sigma, Poole , Dorset, UK) supplemented with 20% FCS, 2mM L-glutamine, 100 units/ml penicillin and 100µg/ml streptomycin. For estimation of relative parasite load, 200 µl aliquots of the cell suspension (in tenfold dilutions) were added to the wells of a 96 well flat-bottomed tissue culture plate in triplicate and were incubated at 28°C for 72 hours. After incubation for 72 hours the cultures were examined for the presence of promastigotes using an inverted microscope.

For isolation of parasites from cutaneous infection sites of *L. mexicana* infected IL-4-/- and IL-4+/+ mice, skin biopsies were cultured in Schneider's insect culture medium (Sigma, Poole, Dorset, UK) supplemented with 20% FCS, 2mM L-glutamine, 100 units/ml penicillin and 100µg/ml streptomycin in tissue culture flasks at 28°C. The cultures were examined weekly for the presence of viable parasites using an inverted microscope.

Adoptive cell transfers of splenocytes

At 16 weeks post-infection with *L. mexicana*, the spleens from IL-4-/- and IL-4+/+ mice were excised aseptically and cell suspensions prepared in RPMI 1640 supplemented with 10% foetal calf serum, 2mM L-glutamine, 100 units/ml penicillin, 100μg/ml streptomycin by passage through a stainless steel mesh sieve. The cells were centrifuged at 200 g for 5 min and erythrocytes were lysed by resuspending and incubating the cells at 37°C for 3 min in 3 ml of Boyle's solution (0.17M Tris,0.16M ammonium chloride:BDH Ltd., Dorset, UK) (Boyle,1968). Following two washes in RPMI 1640 supplemented with 2mM L-glutamine, 100 units/ml penicillin, 100μg/ml streptomycin, viable cells were counted by trypan blue exclusion using an improved Nuebaer haemocytometer. Naive IL-4-/- and IL-4+/+ mice were inoculated with 2 x 10^7 splenocytes each from infected IL-4+/+ and IL-4-/- mice respectively by intraperitoneal injection prior to infection with *L. mexicana.*

Statistical significance

The Students' unpaired *t* test was used to determine the statistical significance.

5.4 Results

Growth of L.mexicana and L.donovani in IL-4+/+ and IL-4-/- mice

Following subcutaneous inoculation with $5x10^6$ *L.mexicana* amastigotes into the shaven rump, no lesions were found to develop at the site of inoculation or elsewhere in any IL-4-/- mice at any time during the study (Fig.1). By contrast all similarly infected wild-type control mice developed progressively growing non-healing lesions throughout the period of study (Fig.1). These results were completely reproducible in 5 separate experiments. Following intravenous inoculation of 10^7 *L.donovani* amastigotes into IL-4+/+ and IL-4-/- mice, the parasite growth in the liver was monitored from day 15 through to day 110 post-infection. Surprisingly, at all time points, less *L.donovani* amastigotes were observed in the IL-4+/+ wild type control mice than the IL-4 deficient mice. However differences in liver parasite burdens between the 2 groups was significant only during the early stage of infection, at day 15 ($p<0.05$) (Fig.2).

Antibody response to L.mexicana and L.donovani in IL-4+/+ and IL-4-/-mice

The specific antibody response measured during *L.mexicana* infection was barely detectable until week 8 post-infection in any of the mice (Fig.3a). At this time point and thereafter, neither IL-4+/+ nor IL-4-/- mice produced any measurable quantities of *L.mexicana* specific IgG2a. However, IL-4+/+ wild-type control mice produced significant quantities of *L.mexicana* specific IgG1 in the absence of any antibody production by IL-4-/- mice. At week 16 following infection, although IL-4+/+ mice had significantly higher titres of antigen specific IgG1 than at week 8 ($P<0.05$), IL-4-/- mice still had no detectable antibody response (Fig.3b). By contrast, *Leishmania* specific IgG2a, though not IgG1, was detected in plasma samples from both IL-4+/+ and IL-4-/- mice at day 50 following infection with *L. donovani* (Fig.4a). However, at day 85 following infection with *L. donovani* , both *Leishmania* specific IgG2a and IgG1 could be detected in IL-4+/+ as well as IL-4-/- mice (Fig.4b).

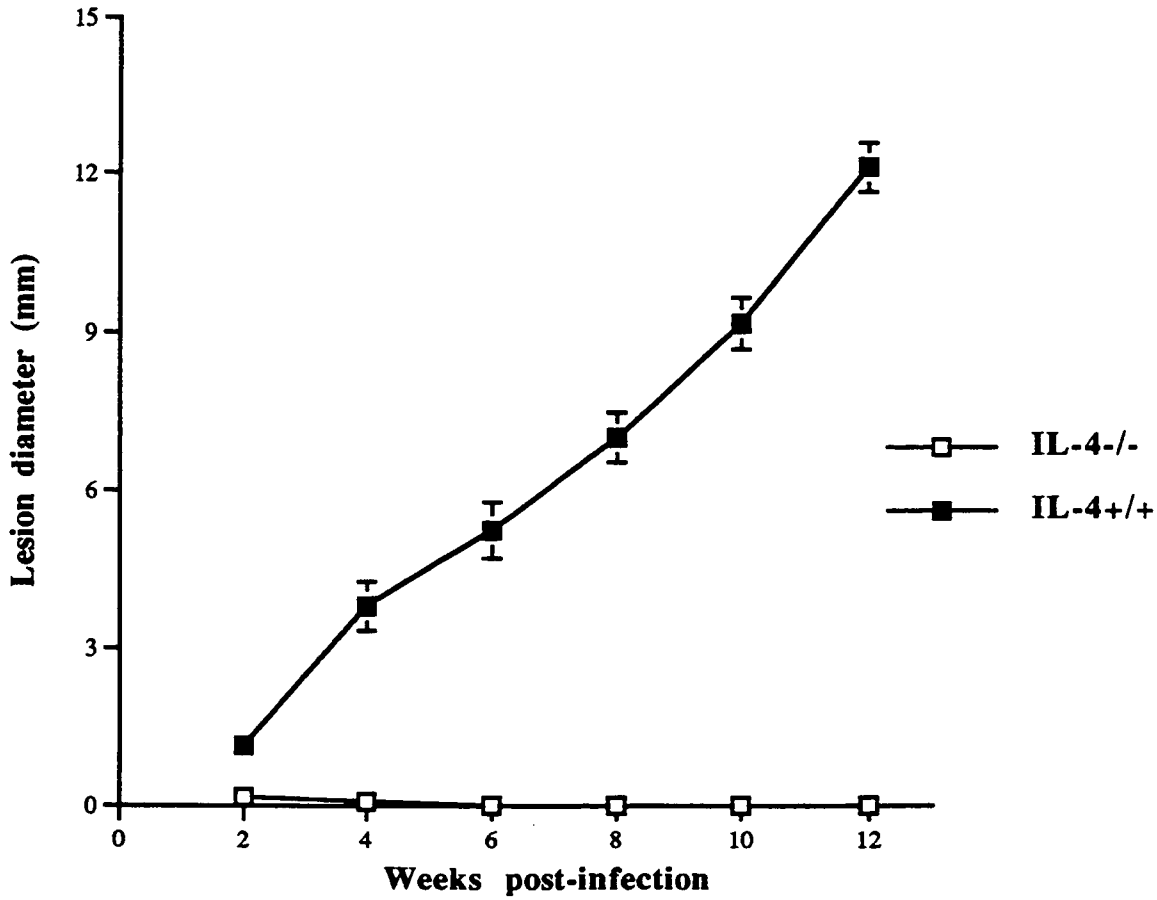

Fig.1: Mean ± SE lesion growth in the shaven rumps of IL-4+/+ (■) and IL-4-/- (□) mice infected with *L. mexicana*. Six animals were used in each group.

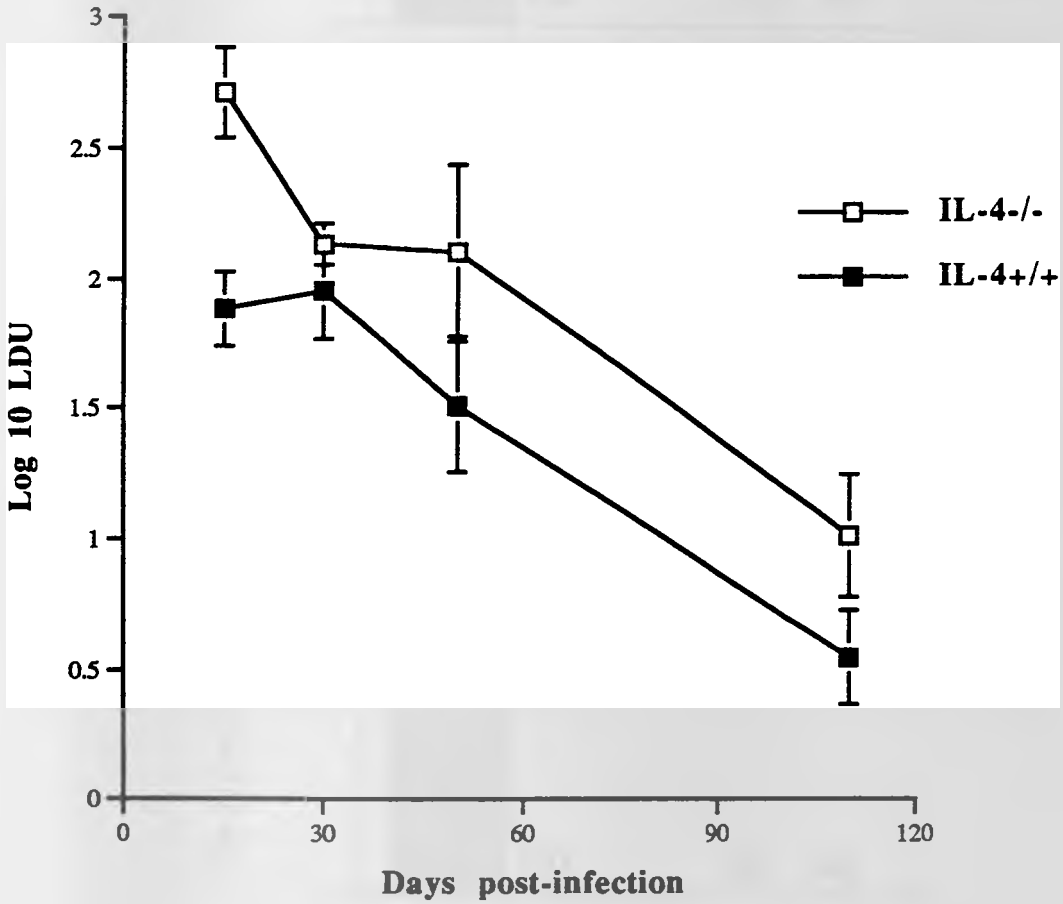

Fig.2: Liver parasite burdens in IL-4+/+ (■) and IL-4-/- (□) mice infected with *L. donovani*. Data expressed as Mean Log $_{10}$ LDU (Leishman Donovan Units) ± SE.

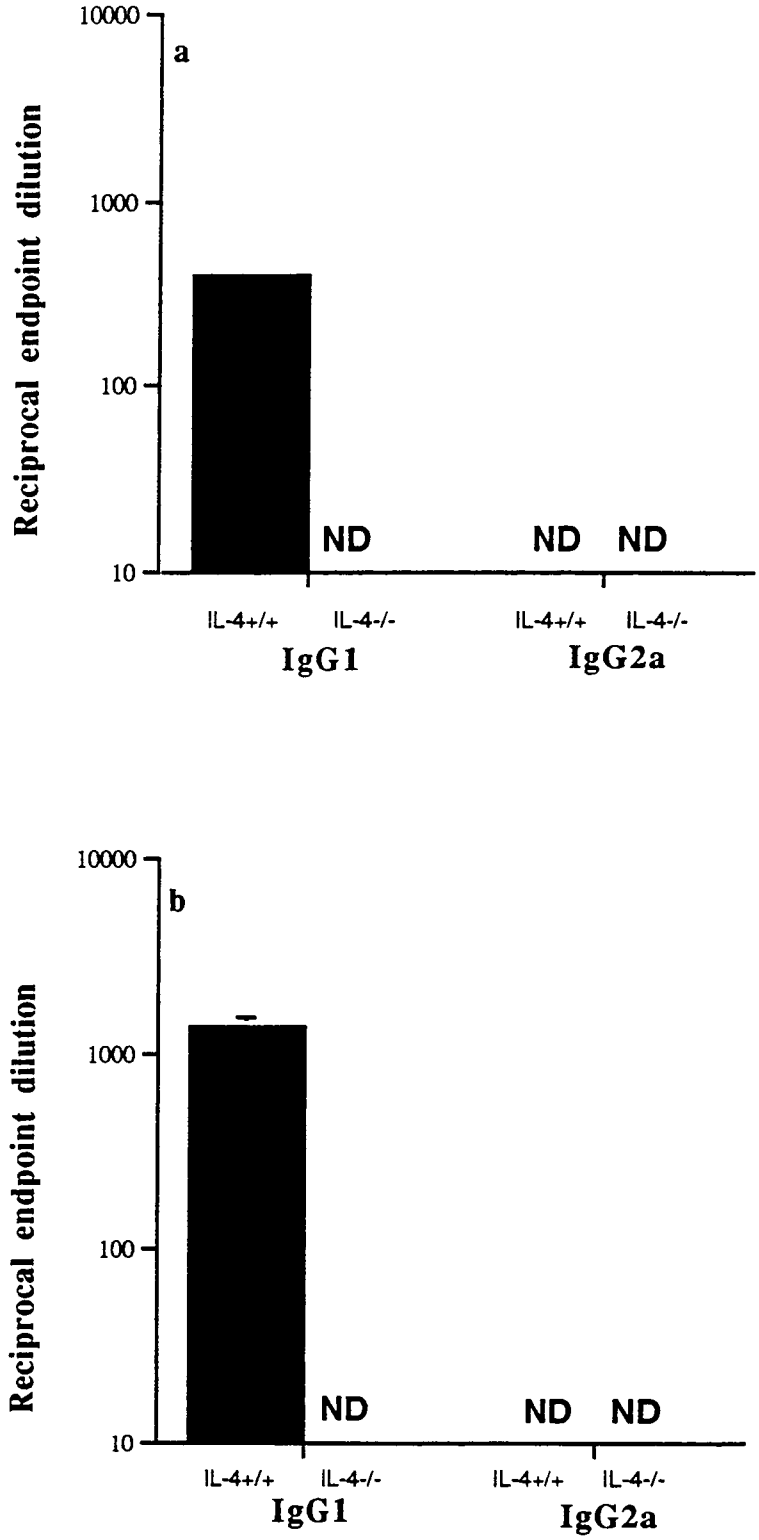

Fig.3: *L. mexicana* specific IgG1 and IgG2a production in IL-4+/+ (closed column) and IL-4-/- (open column) mice at week 8 (Fig. 3a) and week 16 (Fig. 3b) post-infection presented as Mean Reciprocal end point dilution ± SE (ND= no antibody detected at 1:100 dilution).

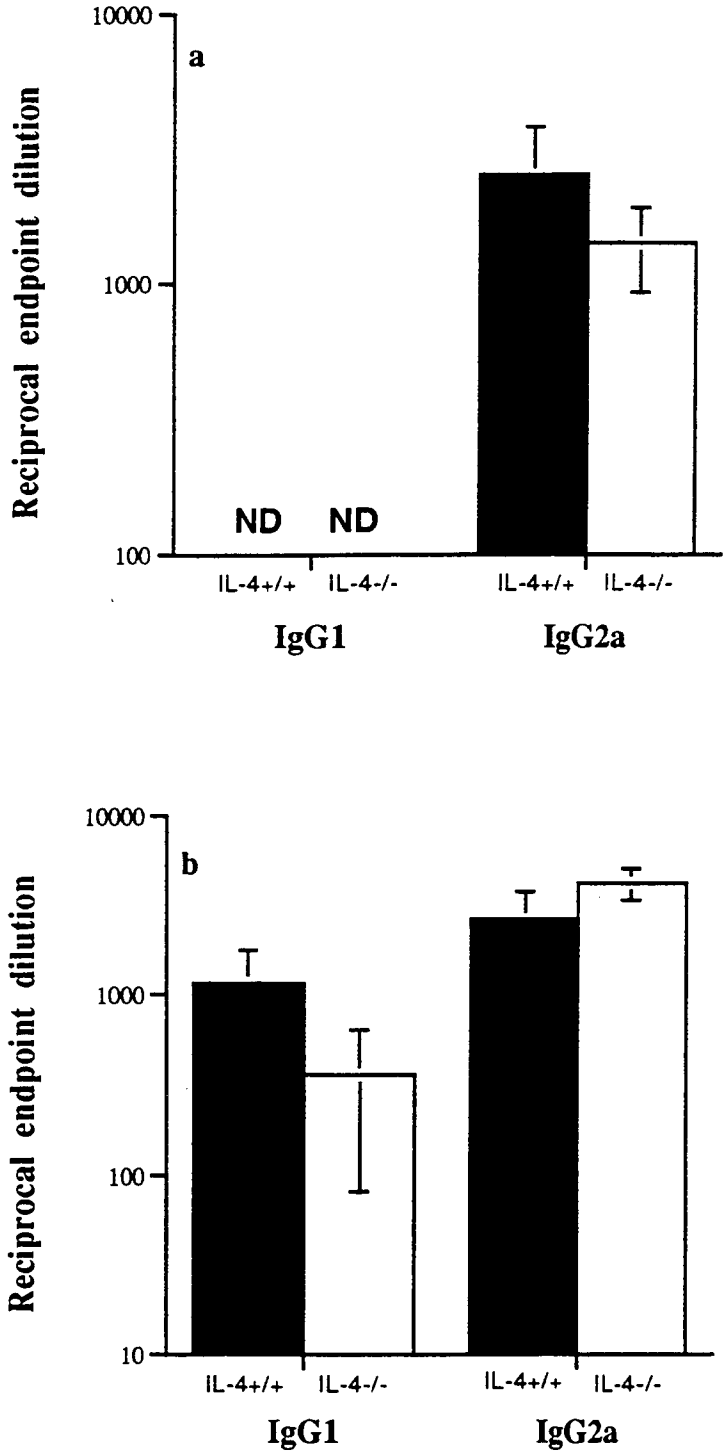

Fig.4: *Leishmania* - specific IgG1 and IgG2a production in *L. donovani* infected IL-4+/+ (closed column) and IL-4-/- (open column) mice at day 50 (Fig. 4a) and day 85 (Fig. 4b) post-infection presented as Mean Reciprocal end point dilution ± SE (ND= no antibody detected at 1:100 dilution).

In vitro T cell responsiveness

As IL-4 deficiency did not confer increased resistance to *L.donovani* which is in agreement with previous reports (Kaye *et al.*, 1991), the following studies were confined to *L. mexicana* infected mice where disruption of the IL-4 gene induced resistance. At weeks 8 and 16 post-infection splenocytes as well as draining lymph node cells from *L.mexicana* infected susceptible IL-4+/+ mice and resistant IL-4-/- mice displayed significant ConA induced ($P<0.025$) and *L.mexicana*-specific proliferative responses ($P<0.025$) (Fig.5 and 6). At week 8 post-infection Con A induced splenocyte proliferative responses were significantly higher in IL-4+/+ mice than IL-4-/- mice (Fig. 5a). As infection progressed, at week 16, splenocytes from *L. mexicana* infected IL-4+/+ mice displayed significantly greater ConA induced and *L.mexicana* antigen-specific proliferative responses than IL-4-/- mice (both $P<0.025$) (Fig. 5b). At this time point, draining lymph node cells as opposed to splenocytes from IL-4-/- mice displayed significantly higher Con A induced and antigen specific proliferative responses than those from IL-4+/+ mice (Fig.6b).

IFN-γ and IL-5 assays

The supernatants from the above splenocyte and draining lymph node cells assays were analysed for the presence of the Th1 associated cytokine, IFN-γ, and Th2 associated cytokine, IL-5 by ELISA (Fig.7 - 10).

Week 8 post-infection

At week 8 post-infection, spleen cells from both *L. mexicana* infected IL-4-/- and IL-4+/+ mice showed no significant differences in IFN-γ and IL-5 production following stimulation with *L. mexicana* antigen (Fig.7b). No differences in IFN-γ production could be detected from Con A stimulated splenocyte cultures from either IL-4+/+ or IL-4-/- *L. mexicana* infected mice (Fig.7c). On the other hand, at this time point, the draining lymph node cells from infected IL-4+/+ mice produced significantly higher quantities of IL-5 ($p<0.005$) than those from IL-4 -/- mice following Con A stimulation (Fig.8c).

Week 16 post infection

At week 16 , spleen cells from *L.mexicana* infected IL-4-/- mice produced significant quantities of IFN-γ ($P<0.025$) following stimulation with *L.mexicana* antigen, while no IFN-γ could be detected in the supernatants from antigen-stimulated IL-4+/+ spleen cells above basal level (Fig.9b) . No differences in IFN-γ nor IL-5 production could be detected from unstimulated splenocyte cultures from either IL-4+/+ or IL-4-/- *L.mexicana* infected mice (Fig.9a). Similarly, no increased IL-5 production above basal levels could be detected in the supernatants from antigen stimulated splenocytes from either IL-4+/+ or IL-4-/- *L.mexicana* infected mice (Fig.9b). However, the supernatants from ConA stimulated splenocytes from *L.mexicana* infected IL-4+/+ mice contained significantly higher quantities of IL-5 ($P<0.025$) than those derived from IL-4-/- mice (Fig.9c). At this time point, draining lymph node cells from *L. mexicana* infected IL-4+/+ mice also produced significantly higher amounts of IL-5 ($p<0.025$) than those from IL-4-/- mice following stimulation with Con A as well as *L. mexicana* antigen (Fig.10b and 10c). No differences in IFN-γ nor IL-5 could be detected in culture supernatants from unstimulated cells (Fig. 10a). However, the supernatants from ConA and antigen stimulated lymph node cells from infected IL-4-/- as well as IL-4+/+ mice contained comparable levels of IFN-γ.

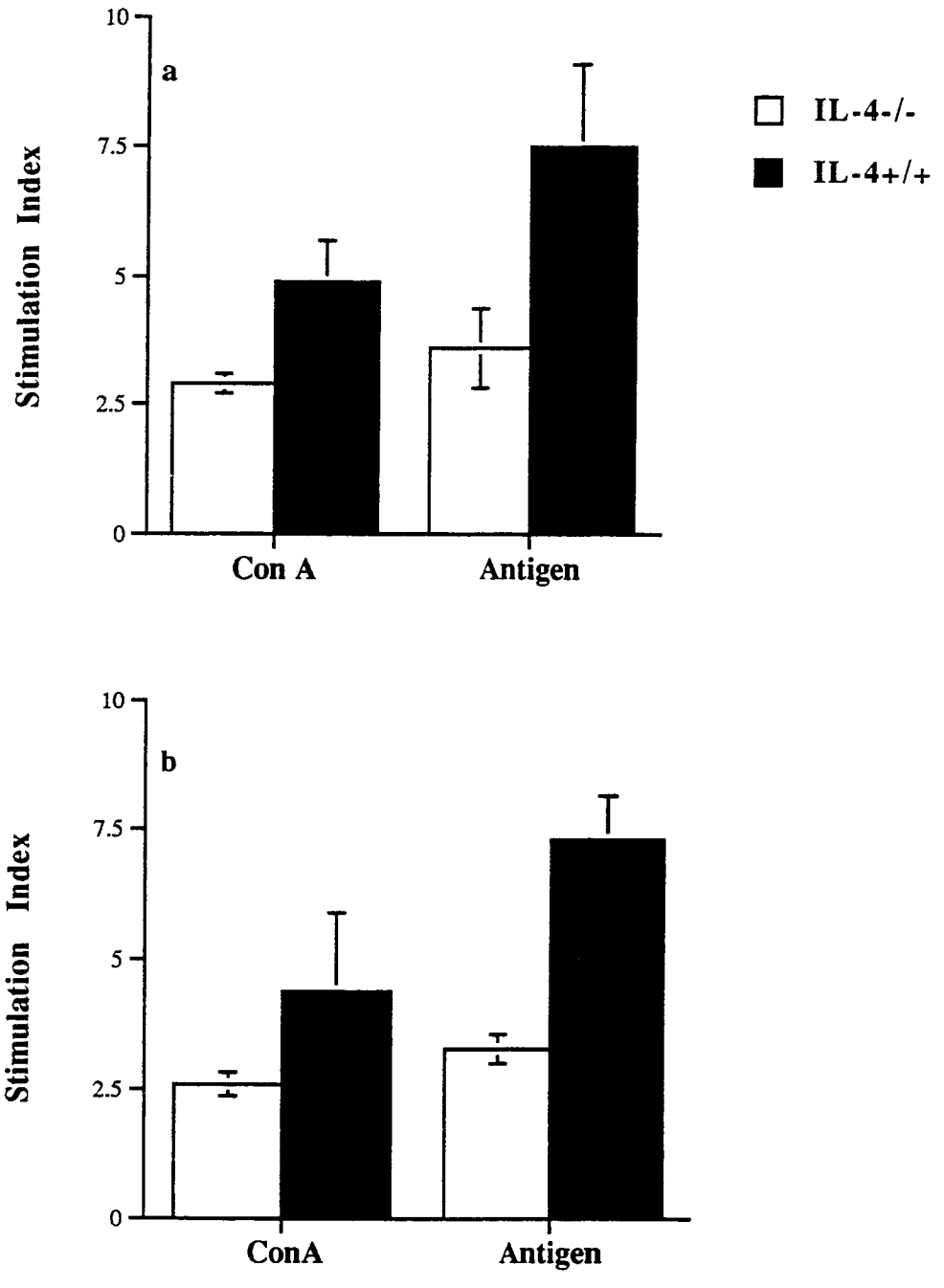

Fig.5: Con A (5μg/ml) and *L. mexicana* soluble antigen (50μg/ml) induced splenocyte proliferation responses in *L. mexicana* infected IL-4+/+ (closed column) and IL-4-/- (open column) mice at week 8 (Fig.5a) and 16 (Fig.5b) post-infection expressed as Mean Stimulation Index ± SE.

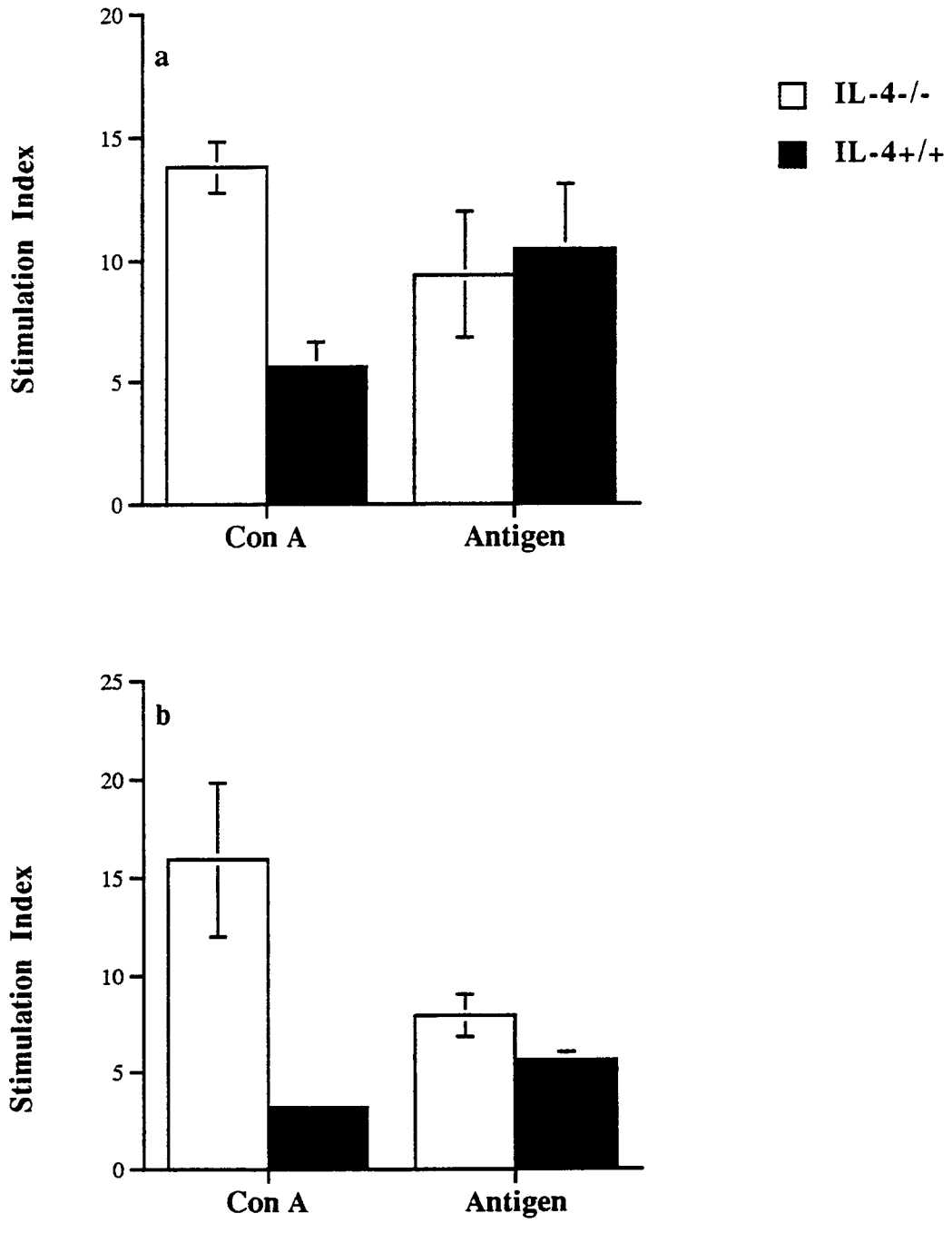

Fig.6: Con A (2.5µg/ml) and *L. mexicana* soluble antigen (25µg/ml) induced splenocyte proliferation responses in *L. mexicana* infected IL-4+/+ (closed column) and IL-4-/- (open column) mice at week 8 (Fig.6a) and 16 (Fig.6b) post-infection expressed as Mean Stimulation Index ± SE.

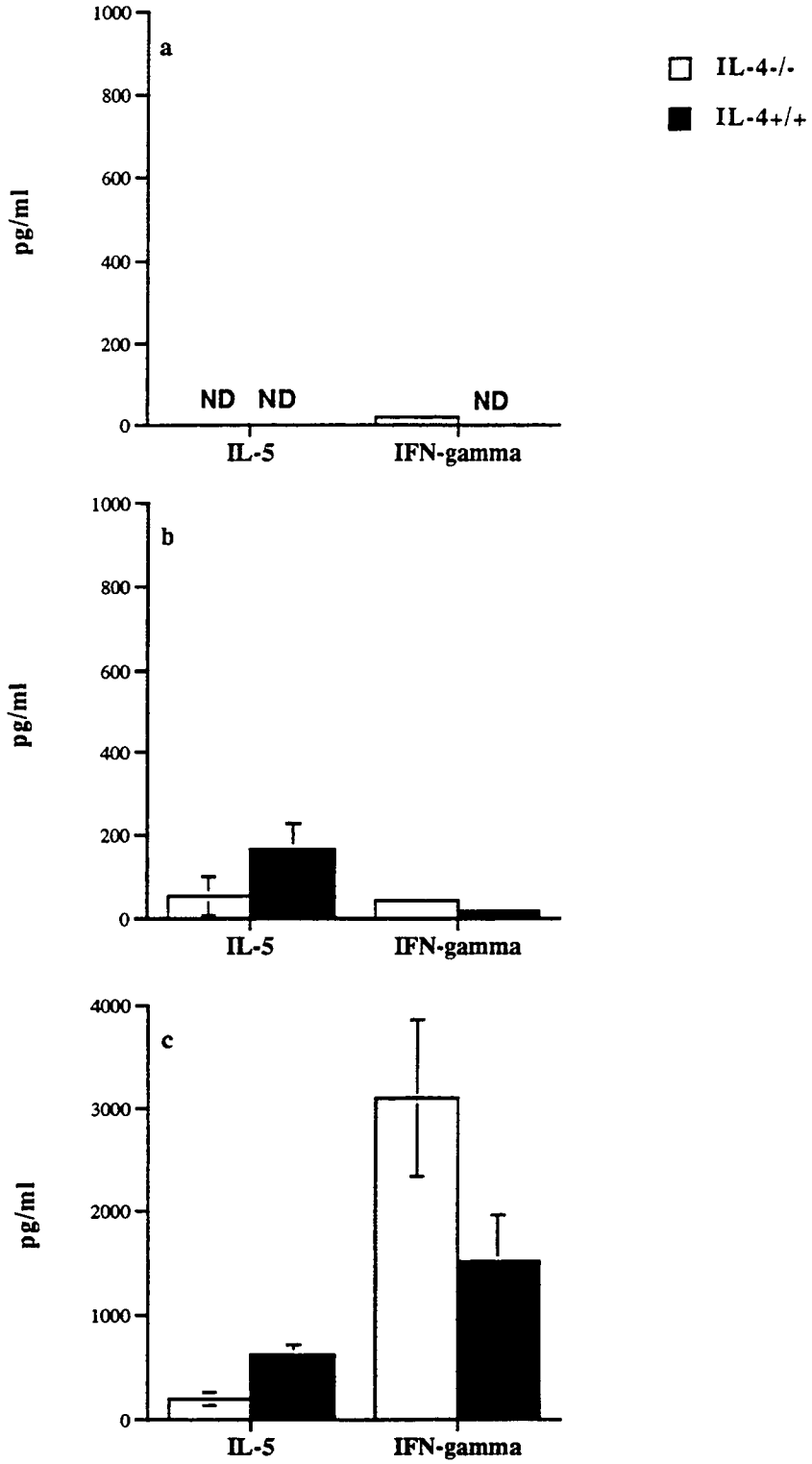

Fig.7: Interleukin-5 and IFN-γ production by splenocytes from *L. mexicana* infected IL-4+/+ (closed column) and IL-4-/- (open column) mice at week 8 post-infection in the absence of antigen (Fig. 7a), in the presence of 50μg/ml *L. mexicana* soluble antigen (Fig. 7b) and in the presence of 5μg/ml Con A (Fig. 7c). (ND= no cytokine detected).

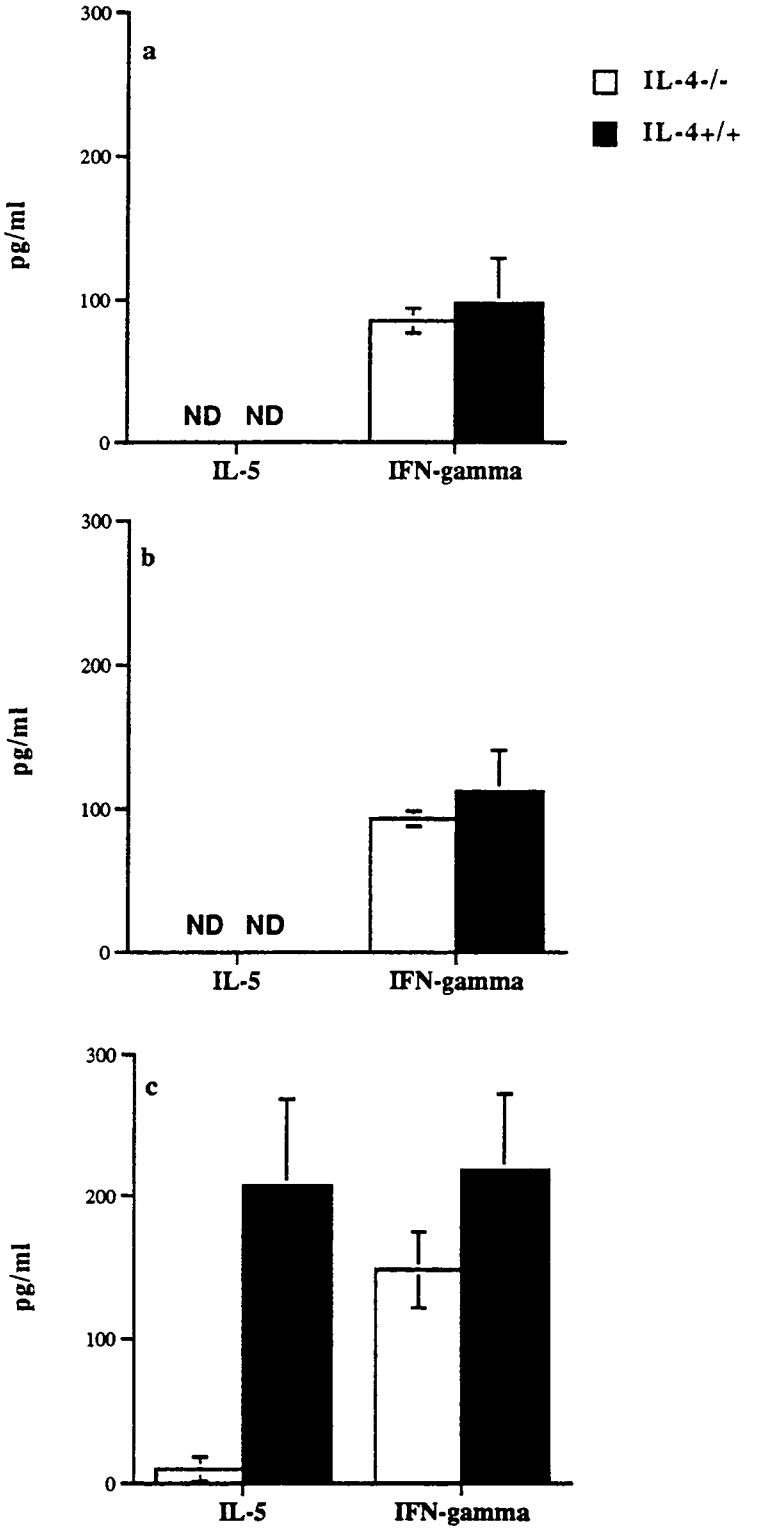

Fig.8: Interleukin-5 and IFN-γ production by draining lymph node cells from *L. mexicana* infected IL-4+/+ (closed column) and IL-4-/- (open column) mice at week 8 post-infection in the absence of antigen (Fig. 8a), in the presence of 25μg/ml *L. mexicana* soluble antigen (Fig. 8b) and in the presence of 2.5μg/ml Con A (Fig. 8c). (ND= no cytokine detected).

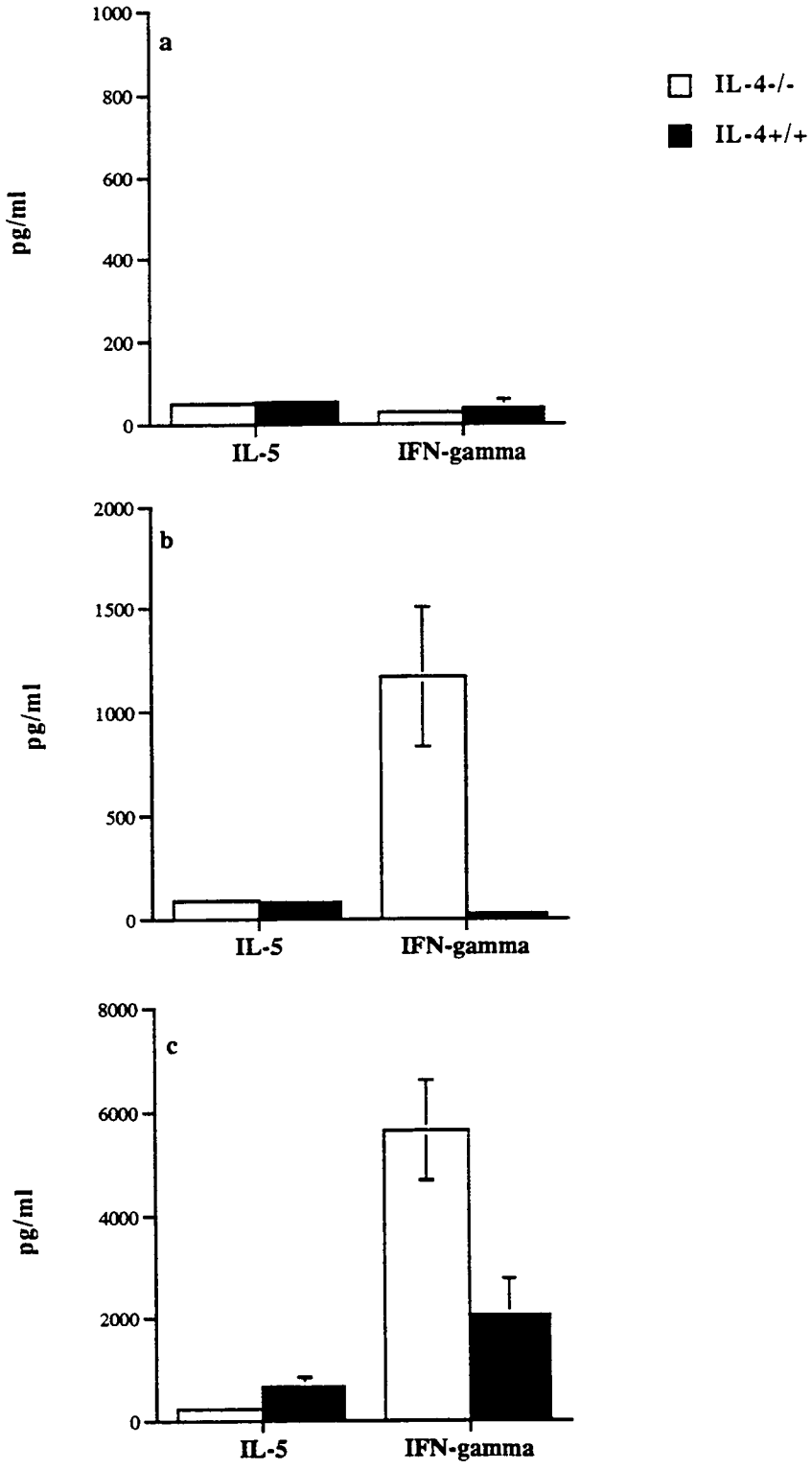

Fig.9: Interleukin-5 and IFN-γ production by splenocytes from *L. mexicana* infected IL-4+/+ (closed column) and IL-4-/- (open column) mice at week 16 post-infection in the absence of antigen (Fig. 9a), in the presence of 50μg/ml *L. mexicana* soluble antigen (Fig. 9b) and in the presence of 5μg/ml Con A (Fig. 9c).

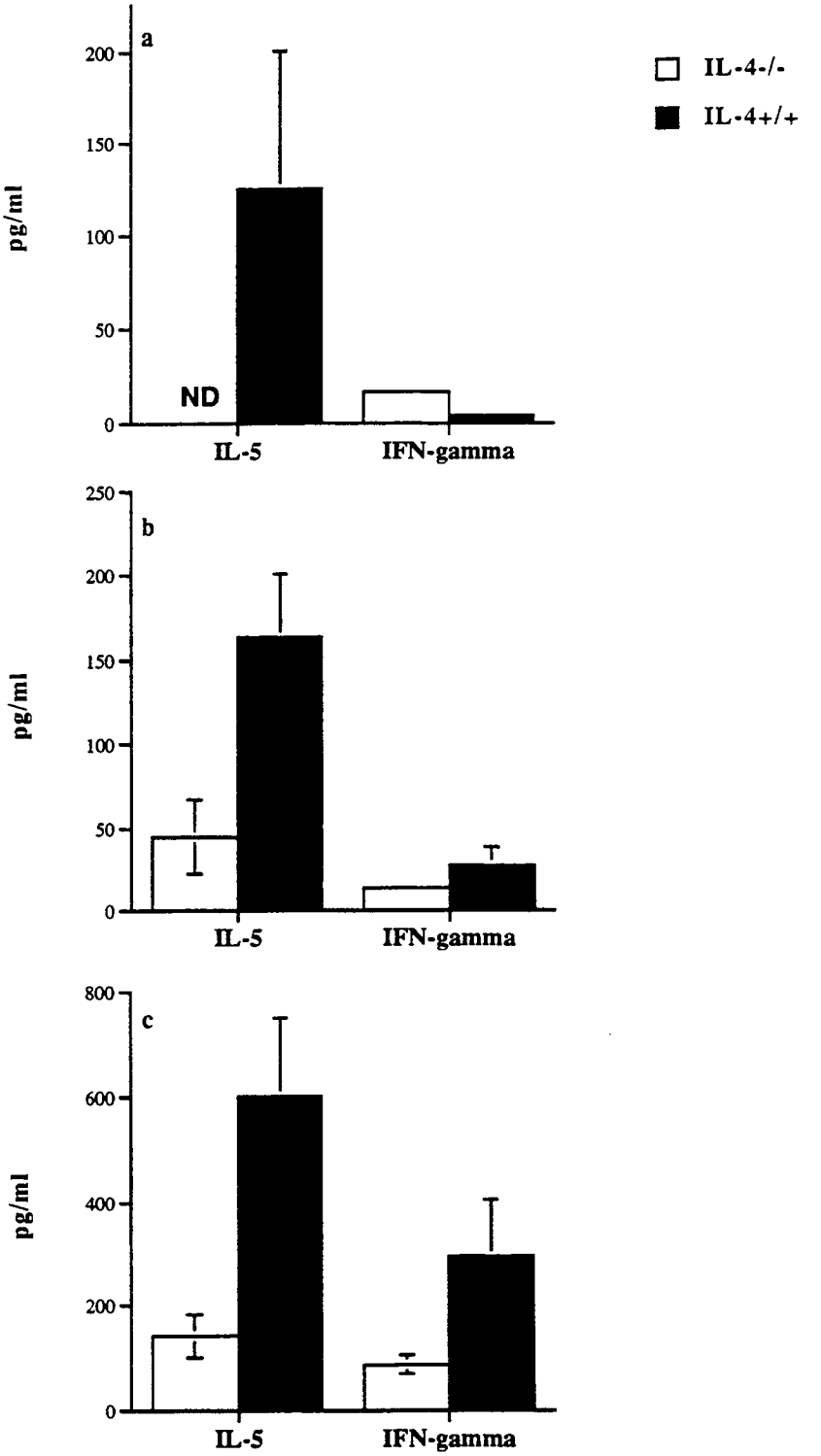

Fig.10: Interleukin-5 and IFN-γ production by draining lymph node cells from *L. mexicana* infected IL-4+/+ (closed column) and IL-4-/- (open column) mice at week 16 post-infection in the absence of antigen (Fig.10a), in the presence of 25μg/ml *L. mexicana* soluble antigen (Fig.10b) and in the presence of 2.5μg/ml Con A (Fig.10c). (ND= no cytokine detected).

Delayed type hypersensitivity (DTH) responses in L. mexicana infected IL-4-/- and IL-4+/+ mice

At week 16 following infection with *L. mexicana*, resistant IL-4-/- mice displayed a significant DTH response to antigen at 48 hours post inoculation (Fig.11). No significant DTH response was detected in IL-4+/+ mice at this time (Fig.11).

Parasite loads in L. mexicana infected IL-4+/+ and IL-4-/- mice

As IL-4-/- mice failed to develop lesions following infection with *L. mexicana* and parasites could not be detected in draining lymph nodes by light microscopical examination the presence of viable parasites in the draining lymph nodes, cutaneous infection sites and other viscera was determined by culturing them in Schneider's insect culture medium as described above.

At week 24 post-infection promastigotes could be cultured from the draining lymph nodes, liver , spleen and cutaneous infection sites from both *L. mexicana* infected IL-4-/- as well as IL-4+/+ mice. However susceptible IL-4+/+ mice displayed significantly higher parasite loads in their draining lymph nodes than IL-4-/- mice (Fig. 12).

Analysis of draining lymph node and spleen cell phenotypes in IL-4+/+and IL-4-/- mice infected with L. mexicana

Following infection with *L. mexicana* , at week 16, the draining lymph nodes and spleens from IL-4-/- and IL-4+/+ mice were excised and were analysed for CD4+ T cells, CD8+ T cells and B cells. Whereas both tissues were studied by immunocytochemistry, the lymph nodes were also analysed by flowcytometry (Fig. 16 ,18, 20 and 21).

At this time, *L. mexicana* infected IL-4-/- as well as IL-4+/+ mice showed an expansion of CD4+ T cell , CD8+ T cell and B cell populations in their draining lymph nodes (Table 1). However, the increase in B cell number was significantly

higher in *L. mexicana* infected highly susceptible IL-4+/+ mice than similarly infected IL-4-/- mice (Table 1).

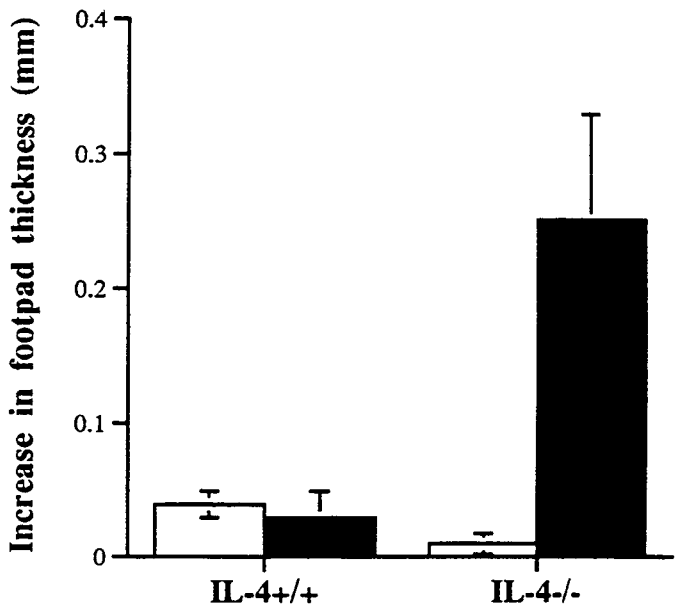

Fig.11: Delayed-type hypersensitivity responses at week 16 post-infection with *L. mexicana* in IL-4+/+ and IL-4-/- mice. Foot pad thickness was measured in infected (closed column) and control (open column) mice 48 hrs after inoculation of formalin fixed parasites. Data expressed as Mean increase in foot pad thickness (mm) ± standard error of the mean for animals per group.

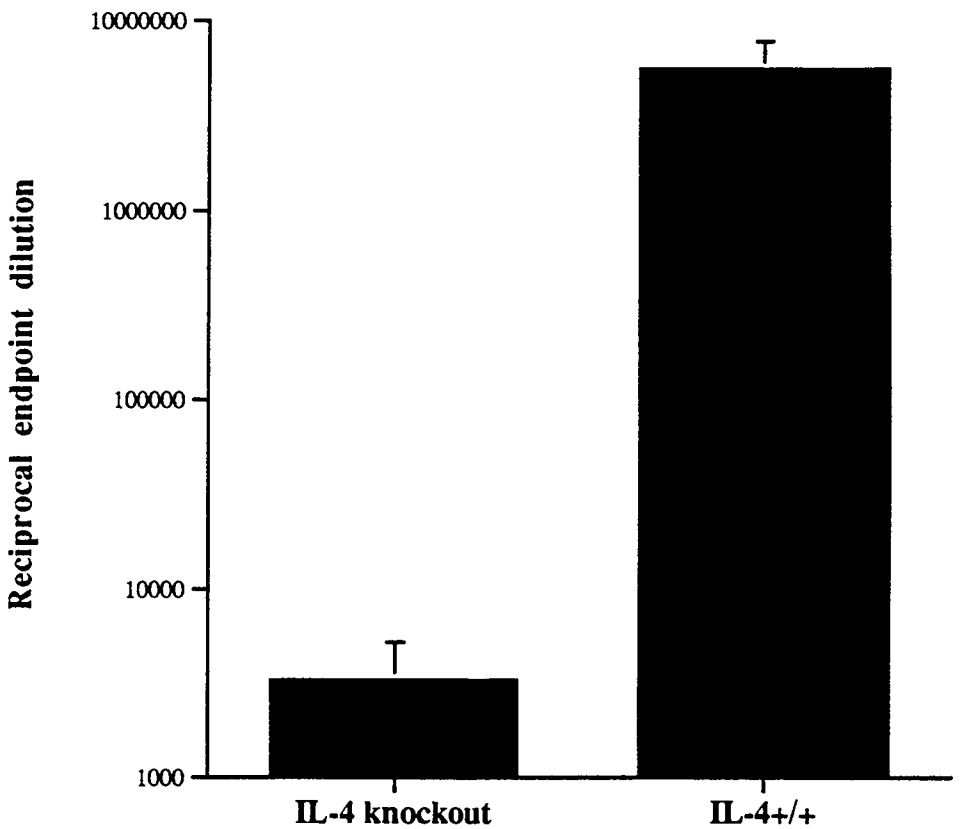

Fig.12: Quantitation of parasites in draining lymph nodes from *L. mexicana* infected IL-4+/+ and IL-4-/- mice at week 16 post-infection. Each bar represents mean ± standard error of the mean for animals per group.

Table 1: Lymphocyte populations in the draining lymph nodes of *L. mexicana* infected IL-4-/- and IL-4+/+ mice as determined by flowcytometry at day 0 and week 16 post-infection.

Mice	Weeks postinfection	CD4+T cells	CD8+T cells	B cells
IL-4 -/-	0	0.50±0.11	0.28±0.09	0.45±0.11
	16	0.94±0.19	0.67±0.12	0.95±0.27
IL-4 +/+	0	0.77±0.24	0.31±0.09	1.04±0.25
	16	1.83±0.28	0.73±0.04	4.37±0.46*

*p<0.0005

Data expressed as a mean of total number of cells in both inguinal lymph nodes /10^6 ± standard error of the mean for animals per group.

Histopathological analysis of skin, draining lymph node and spleen

Skin/Inoculation site

At both time points, skin lesions from IL-4+/+ mice displayed extensive subcutaneous tissue damage marked by inflammatory infiltrate which predominantly consisted of parasitised macrophages, neutrophils, eosinophils and lymphocytes (Fig.13a). On the other hand, IL-4-/- mice showed markedly less inflammatory infiltrate and virtually no parasites could be detected at the site of inoculation (Fig.14a).

Lymph nodes

The draining lymph nodes from IL-4+/+ mice showed a complete collapse of normal lymph node architecture which was accompanied by the distention and engorgement of lymphatic sinusoids with histiocytes (Fig.15a). Although, a similar pattern was observed in the draining lymph nodes from IL-4-/- mice, the engorgement of lymphatic sinusoids with histiocytes was less marked (Fig.17a). No parasites could be be detected in draining lymph nodes from either strain by light microscopy.

Spleens

Spleens from both mouse strains showed follicular and reticuloendothelial hyperplasia at both the time points examined (Fig. 19a and 20a).

Tissue expression of iNOS

Skin

IL-4 -/- mice displayed significantly stronger expression of iNOS in their skins as compared with IL-4+/+ mice (Fig. 13b and 14b). Non specific esterase (NSE) staining of serial sections from IL-4-/- mice revealed that the expression of iNOS was confined to areas which predominantly consisted of macrophages (Fig. 13c).

Lymph node

At both time points the draining lymph nodes from IL-4-/- mice showed clusters of cells stained positive for iNOS (Fig.17b). No such staining could be observed in the draining lymph nodes from IL-4+/+ mice (Fig.15b).

Immunocytochemistry

Serial cryostat sections from the above spleens and lymph nodes were analysed by immunocytochemistry using anti-B220 and anti-CD4 monoclonal antibodies as described previously in Chapter 2. At both time points, lymph nodes as well as spleens from both IL-4+/+ and IL-4-/- mice showed prominent B cell follicles and infiltration of CD4+ cells into B cell areas (Fig.16, 18, 19 and 20).

At week 16 post-infection ,spleens from IL-4+/+ and IL-4-/- mice were stained using anti-IFN-γ mAbs as described previously. IL-4-/- mice but not IL-4+/+ mice showed the presence of IFN-γ producing cells in periarteriolar areas (Fig.21a and 21b). On the other hand, at this time point, IL-4 producing cells could be detected in periarteriolar areas in spleens from IL-4+/+ mice (Fig.21c).

Adoptive transfer of splenocytes from L. mexicana infected IL-4-/- mice confers partial protection against L. mexicana in IL-4+/+ mice

As cell mediated immunity has been shown to play a paramount role in determining the ultimate disease outcome in cutaneous leishmaniasis, it was decided to investigate whether splenocytes from infected IL-4-/- mice could transfer resistance and reverse the non healing response of IL-4+/+ mice following infection with *L. mexicana* . Conversely, I also investigated whether IL-4-/- mice could be made susceptible to *L. mexicana* by transfer of splenocytes from infected IL-4+/+ mice.

Whereas the transfer of immune splenocytes from *L.mexicana* infected IL-4-/- mice conferred partial protection to the IL-4+/+ mice during the early course of disease following *L. mexicana* infection (Fig.22a), transfer of splenocytes from *L. mexicana* infected IL-4+/+ mice to IL-4-/- mice did not result in any disease exacerbation whatsoever following infection with *L. mexicana* (Fig.22b).

Fig.13: Histopathological examination of inoculation sites from *L. mexicana* infected IL-4+/+ mice at week 8 post-infection. (a) Section stained with hematoxylin-eosin showing extensive inflammatory infiltrate comprising of parasitised macrophages (arrows), eosinophils (E), neutrophils and lymphocytes. (b) Section stained for inducible nitric oxide synthase using anti-iNOS polyclonal antibody. No iNOS expression was identified despite the presence of parasitised macrophages. (c) Serial section stained for non specific estrase (NSE) showing absence of NSE staining in parasite laden macrophages. Similar histopathological findings were observed at week 16 post-infection. Magnification x 960.

Fig.14: Histopathological examination of inoculation sites from *L. mexicana* infected IL-4-/- mice at week 8 post-infection.(a) Section stained with hematoxylin-eosin showing inflammatory infiltrate predominantly comprising of macrophages, lymphocytes and a few neutrophils. (b) Section stained for inducible nitric oxide synthase using anti-iNOS polyclonal antibody showing strong localised expression of iNOS (arrows) confined to clusters of macrophages. (c) Serial section stained for NSE showing NSE staining of macrophages confined to areas showing iNOS expression. Similar histopathological findings were observed at week 16 post-infection. Magnification x 960.

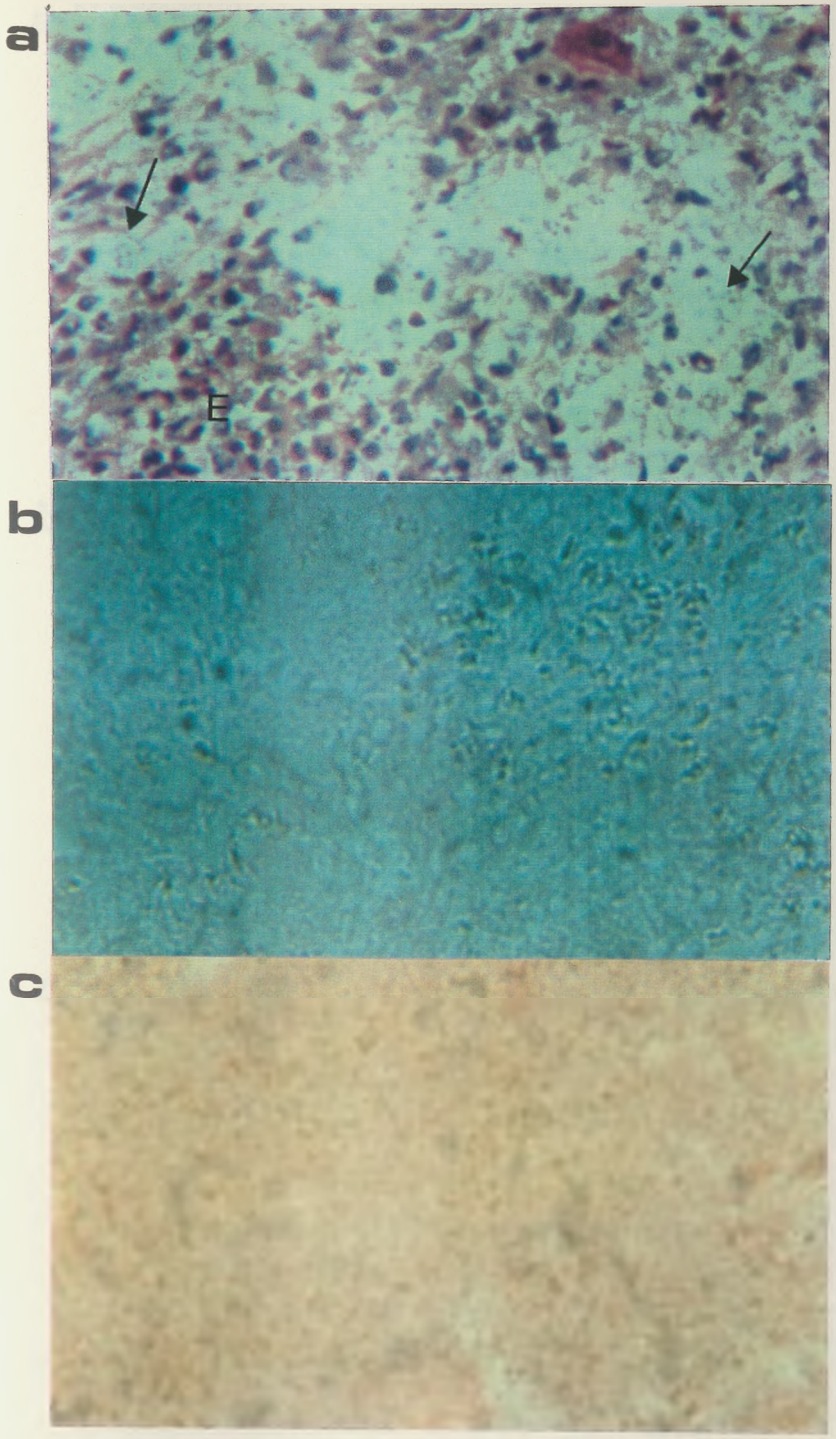

Fig.13: Histopathological analysis of inoculation sites from *L. mexicana* infected IL-4+/+ mice at week 8 post-infection.

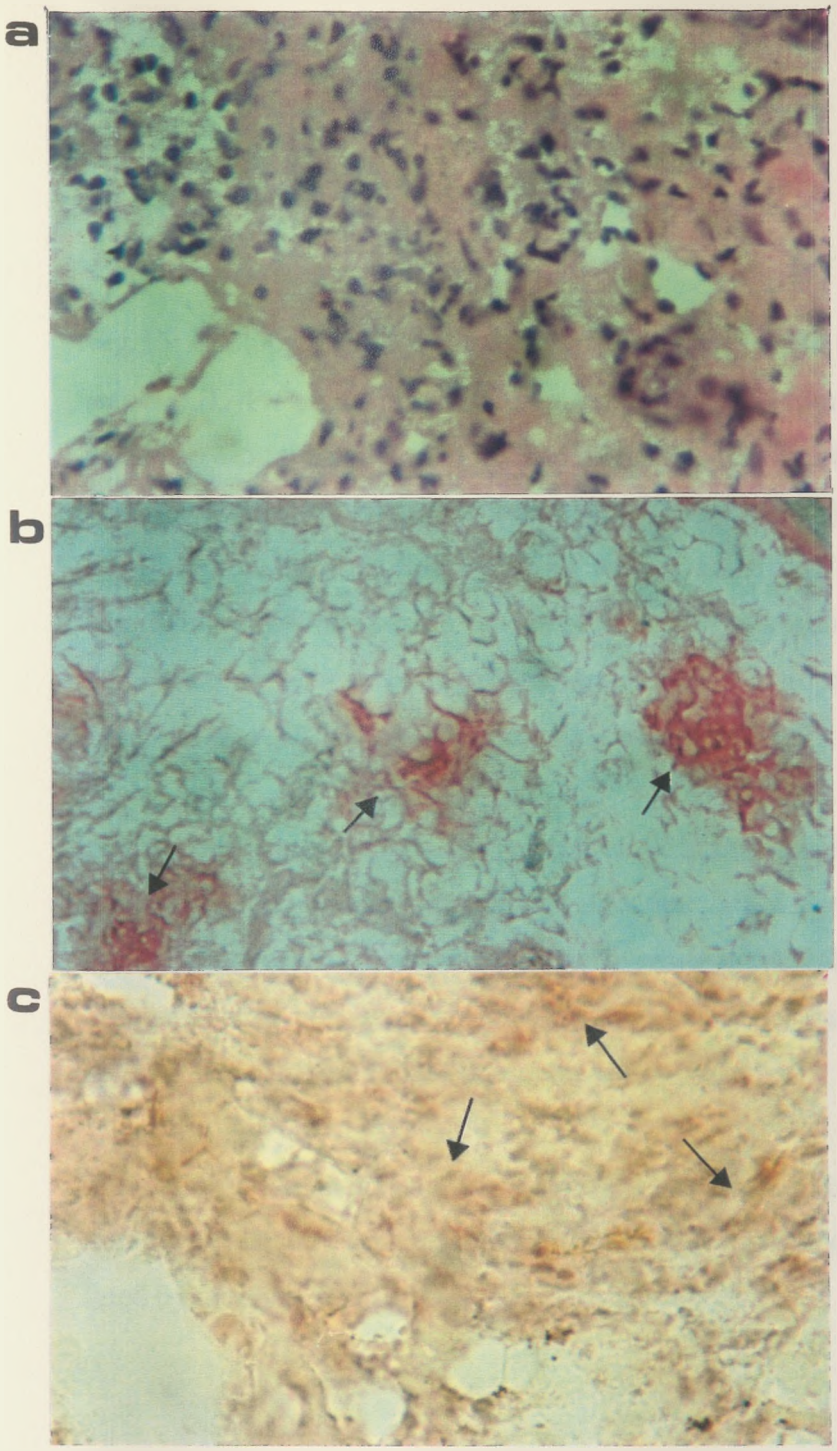

Fig.14: Histopathological analysis of inoculation sites from *L. mexicana* infected IL-4-/- mice at week 8 post-infection.

Fig.15: Histopathological analysis of draining lymph nodes from *L. mexicana* infected IL-4+/+ mice at week 8 post-infection. (a) Section stained with hematoxylin-eosin showing the collapse of normal lymph node architecture with marked distention and engorgement of lymphatic sinusoids with histiocytes (arrows). (b) Section stained for inducible nitric oxide synthase using anti-iNOS polyclonal antibody. No significant iNOS expression was detected (c) Serial section stained for NSE showing NSE staining of macrophages (arrows). Similar histopathological findings were observed at week 16 post-infection. Magnification x 960.

Fig.16: Immunocytochemical analysis of draining lymph nodes from *L. mexicana* infected IL-4+/+ mice at week 8 post-infection. Serial sections stained with either (a) anti-B220 mAb or (b) anti-CD4 mAb showing B cell follicles (B) and CD4 T cells (T4) infiltrating into B cell areas. A similar immunocytochemical pattern was observed at week 16 post-infection. Original magnification x 240.

The draining lymph nodes from age matched uninfected IL-4+/+ control mice showed normal architecture with prominent primary follicles in the cortex and normal sinuses lined by mononuclear cells.

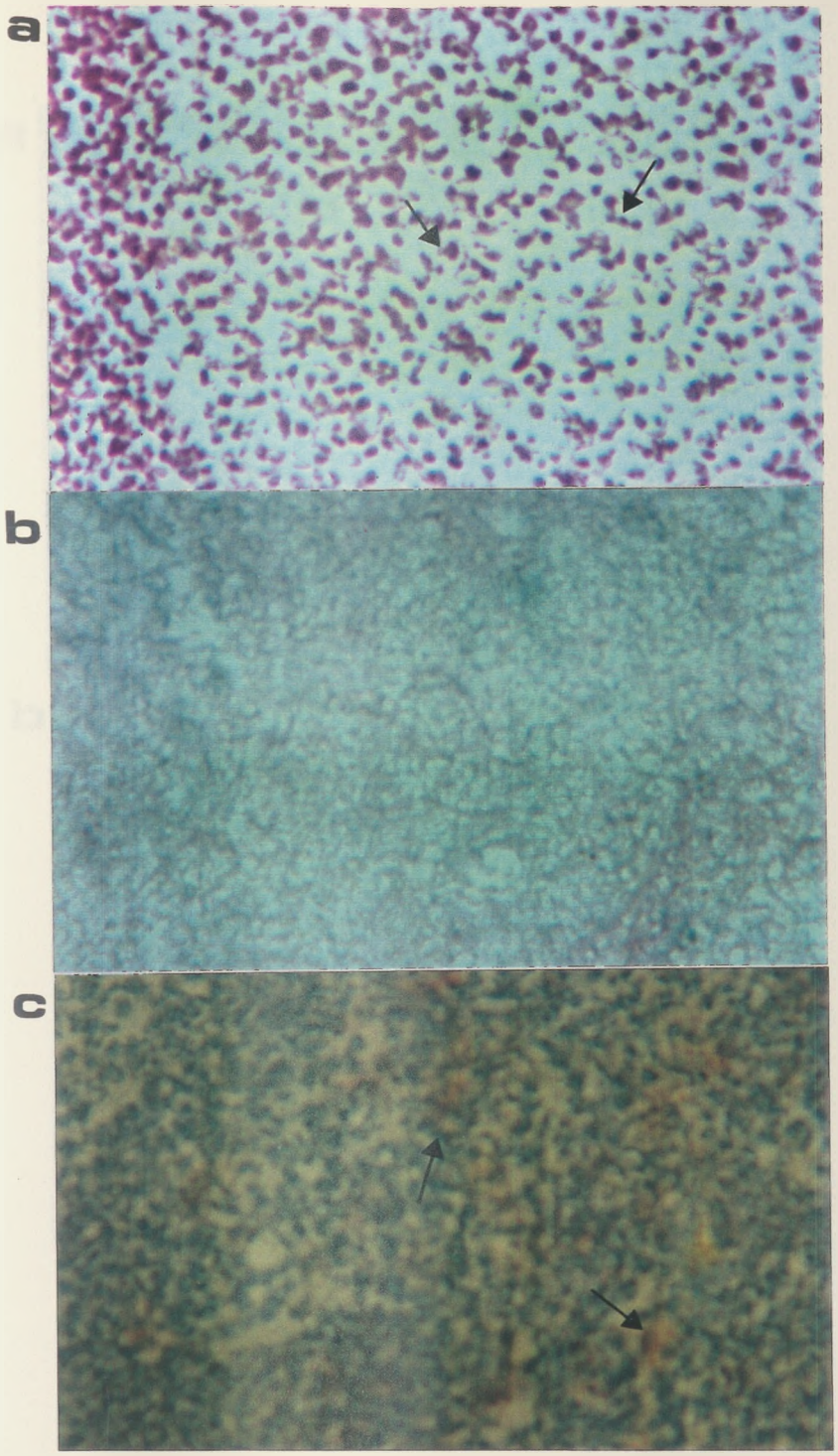

Fig.15: Histopathological analysis of draining lymph nodes from *L. mexicana* infected IL-4+/+ mice at week 8 post-infection.

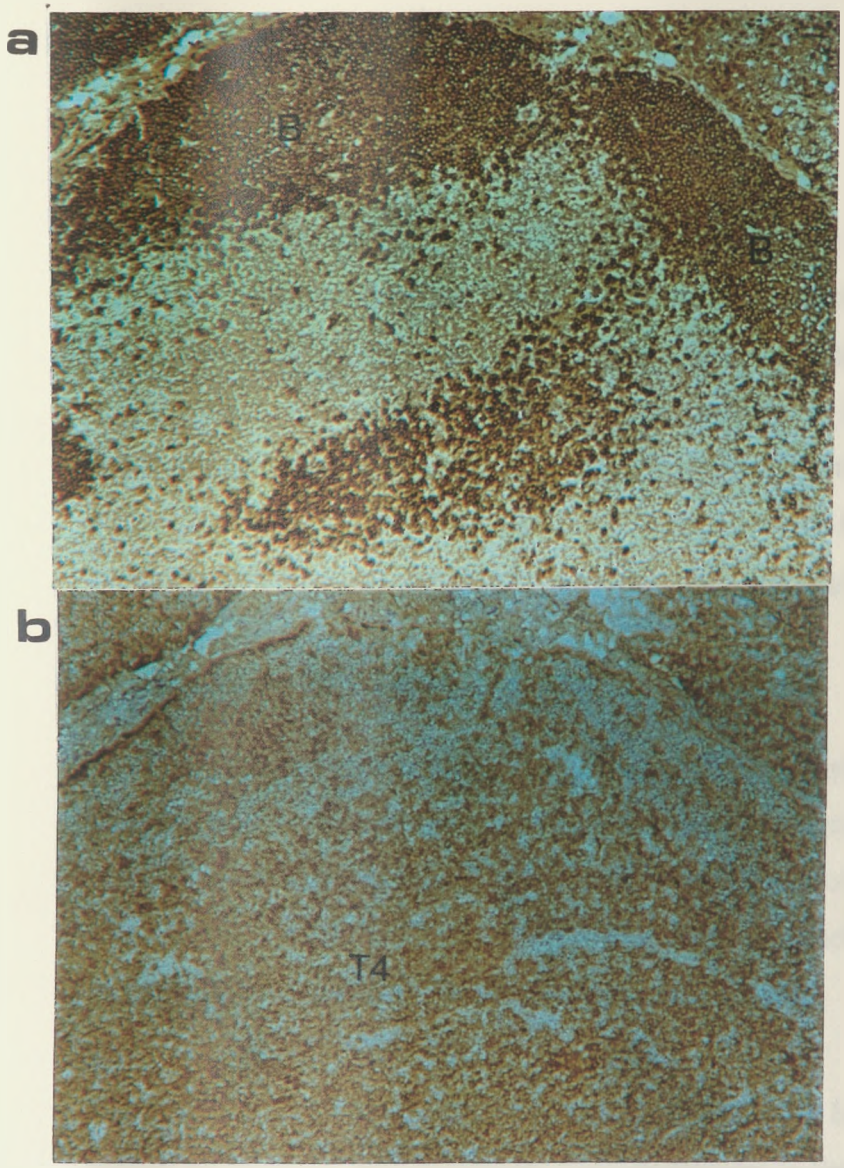

Fig.16: Immunocytochemical analysis of draining lymph nodes from *L. mexicana* infected IL-4+/+ mice at week 8 post-infection.

Fig.17: Histopathological analysis of draining lymph nodes from *L. mexicana* infected IL-4-/- mice at week 8 post-infection. (a) Section stained with hematoxyline-eosin showing collapse of normal lymph node architecture with distention and engorgement of lymphatic sinusoids with histiocytes. (b) Section stained for inducible nitric oxide synthase using anti-iNOS polyclonal antibody showing localised expression of iNOS (arrows). (c) Serial section stained for NSE showing scattered macrophages (arrows). Similar histopathological findings were observed at week 16 post-infection.Original magnification x 960.

Fig.18: Immunocytochemical analysis of draining lymph nodes from *L. mexicana* infected IL-4-/- mice at week 8 post-infection. Serial sections stained with either (a) anti-B220 mAb or (b) anti-CD4 mAb showing B cell follicles (B) and CD4 T cells (T4) infiltrating into B cell areas. Similar immunocytochemical pattern was observed at week 16 post-infection. Original magnification x 240.

The draining lymph nodes from age matched uninfected IL-4-/- control mice showed normal architecture with prominent primary follicles in the cortex and normal sinuses lined by mononuclear cells.

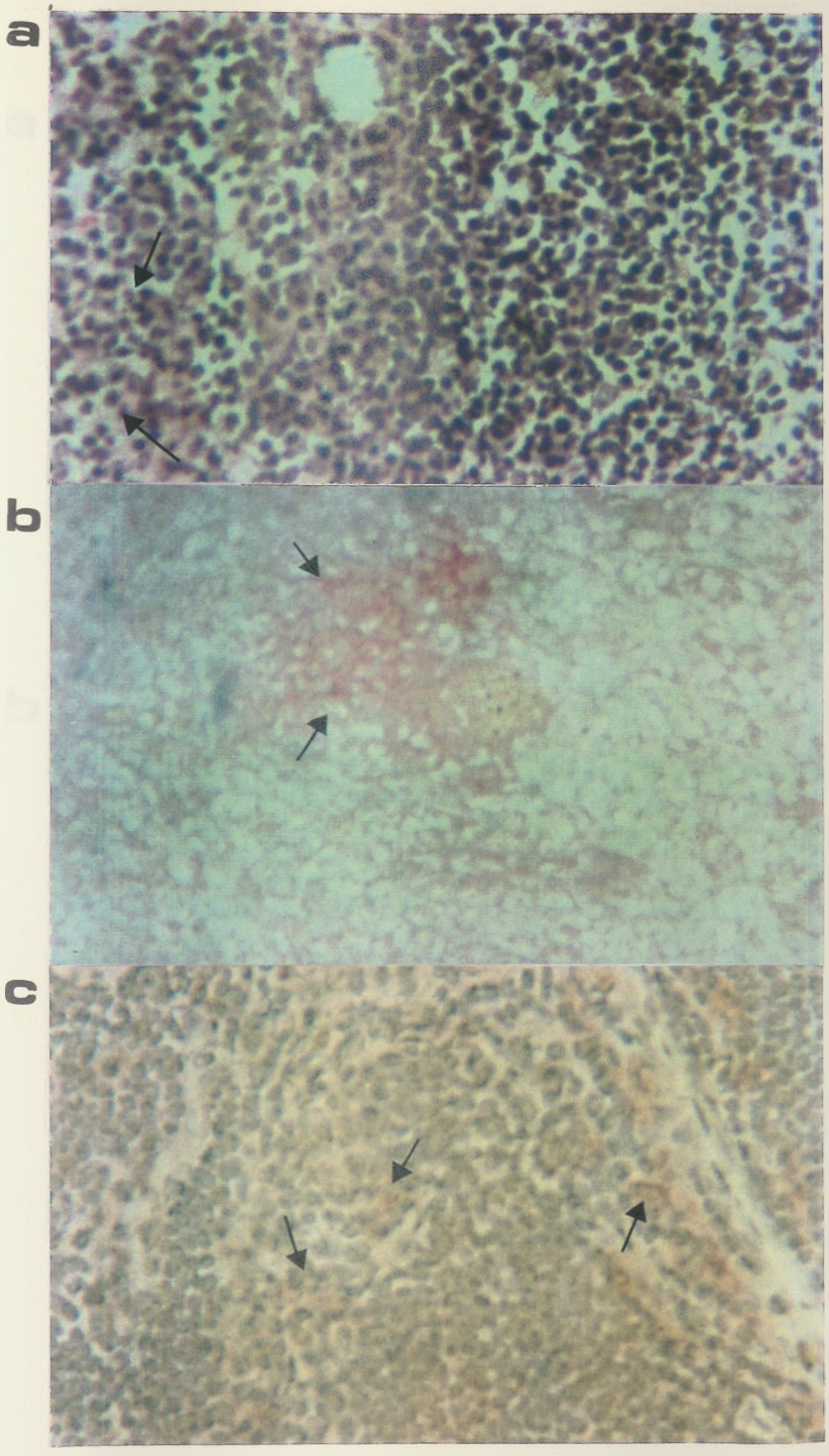

Fig.17: Histopathological analysis of draining lymph nodes from *L. mexicana* infected IL-4-/- mice at week 8 post-infection.

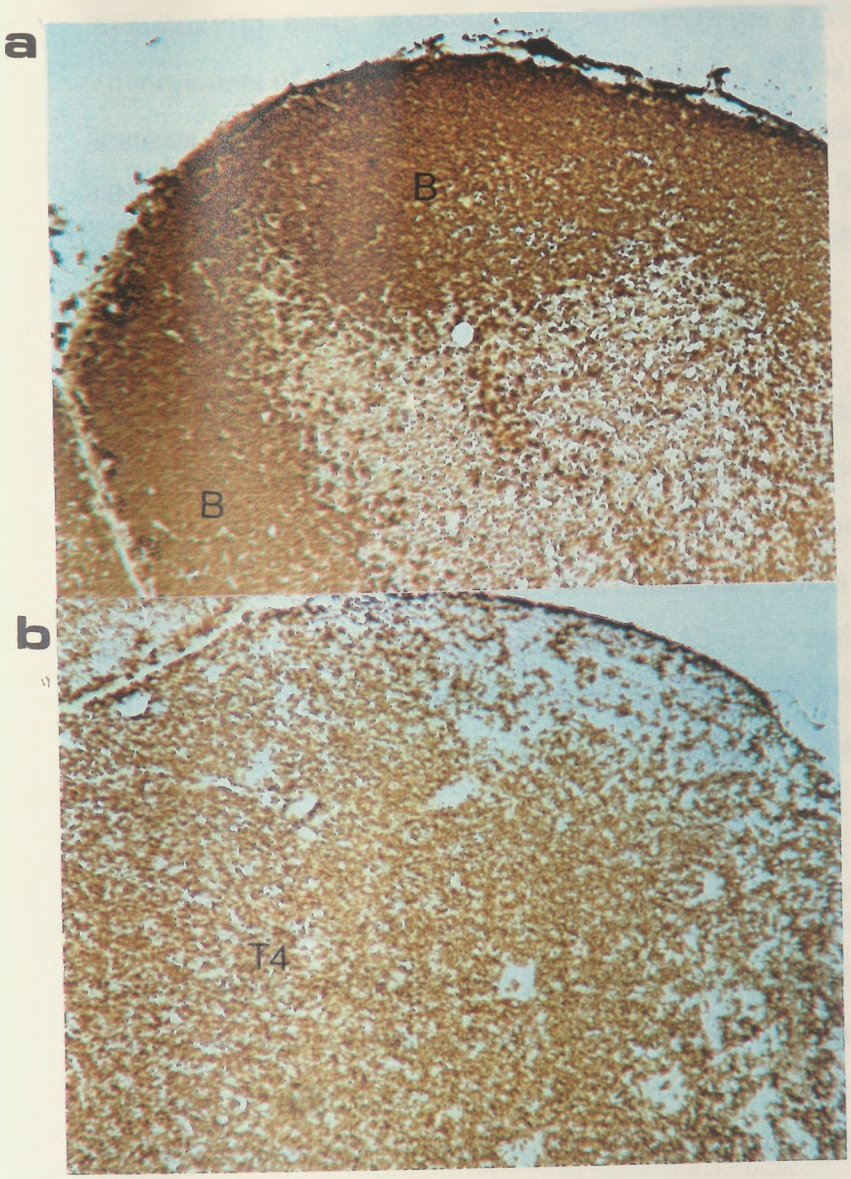

Fig.18: Immunocytochemical analysis of draining lymph nodes from *L. mexicana* infected IL-4-/- mice at week 8 post-infection.

Fig.19: Histopathological and immunocytochemical analysis of spleen from *L. mexicana* infected IL-4+/+ mice at week 8 post-infection. (a) Section stained with hematoxylin eosin showing reticuloendothelial hyperplasia characterised by engorgement of splenic sinusoids with histiocytes. CA is the central arteriole. Serial sections stained with either (b) anti-B220 mAb or (c) anti-CD4 mAb showing B cell (B) and CD4 T cell (T4) areas. Original magnification x 960 for (a) and x 240 for (b) and (c). Similar histological and immunocytological profiles were observed at week 16 post-infection.

Fig.20: Histopathological and immunocytochemical analysis of spleen from *L. mexicana* infected IL-4-/- mice at week 8 post-infection. (a) Section stained with hematoxylin eosin showing reticuloendothelial hyperplasia characterised by engorgement of splenic sinusoids with histiocytes. CA is the central arteriole. Serial sections stained with either (b) anti-B220 mAb or (c) anti-CD4 mAb showing B cell (B) and CD4 T cell (T) areas. Original magnification x 960 for (a) and x 240 for (b) and (c). Similar histological and immunocytological profiles were observed at week 16 post-infection.

Fig.21: Immunocytochemical analysis of cytokine producing cells in spleen from *L. mexicana* infected IL-4-/- and IL-4+/+ mice at week 16 post-infection. (a) Spleen from *L. mexicana* infected IL-4-/- mice stained with anti-IFN-γ mAb showing the presence of IFN-γ producing cells predominantly in the periarteriolar area in the vicinity of the central arteriole (CA). (b) Spleen from infected IL-4+/+ mice stained using anti-IFN-γ mAb showing the absence of IFN-γ producing cells in the periarteriolar area. (c) Spleen from infected IL-4+/+ mice stained with anti-IL-4 mAb showing IL-4 producing cells in the periarteriolar area in the vicinity of central arteriole (CA). Original magnification x 960.

The spleens from uninfected IL-4+/+ and IL-4-/- mice showed normal red and white pulp architecture.

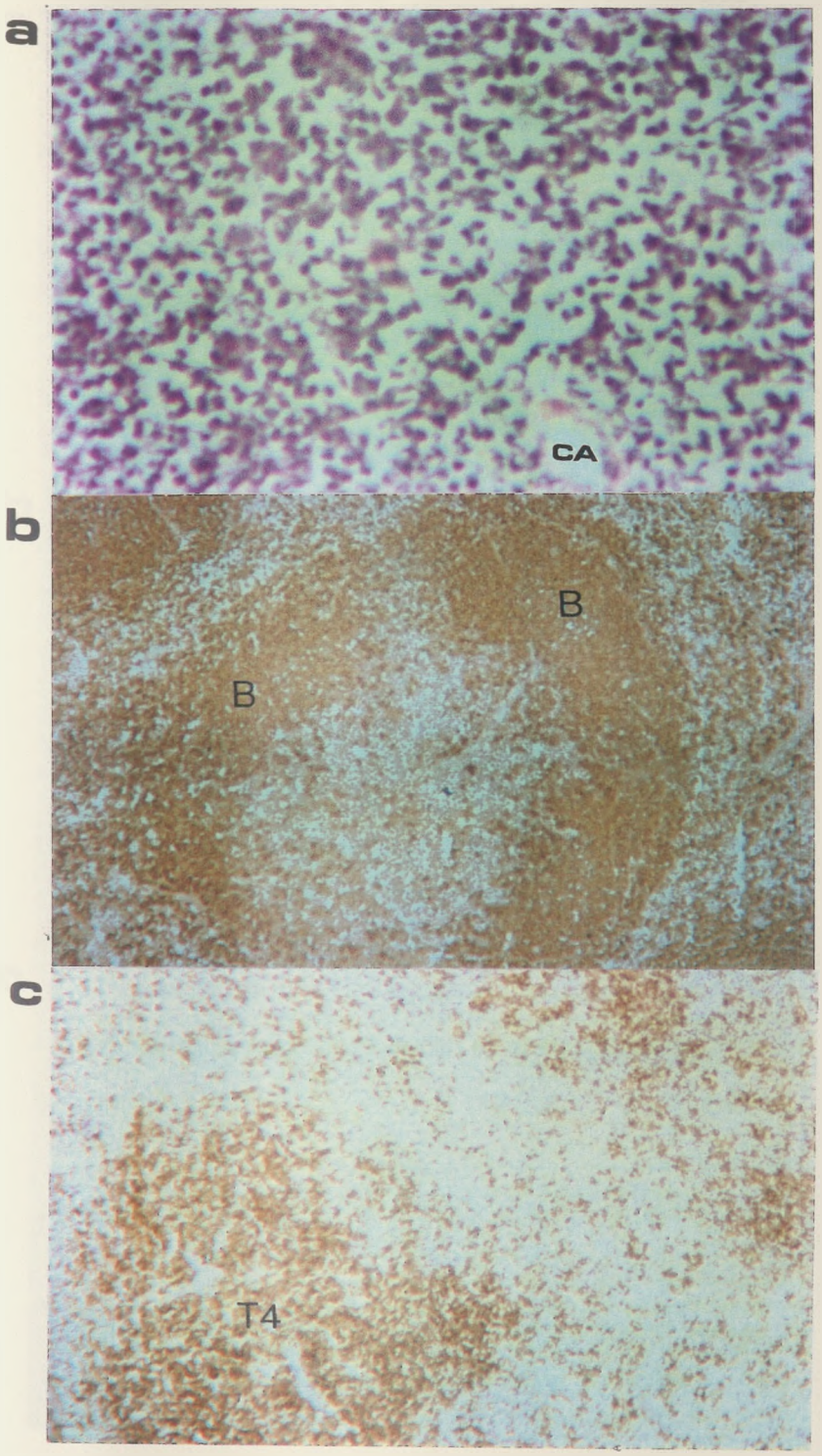

Fig.19: Histopathological and immunocytochemical analysis of spleen from *L. mexicana* infected IL-4+/+ mice at week 8 post-infection.

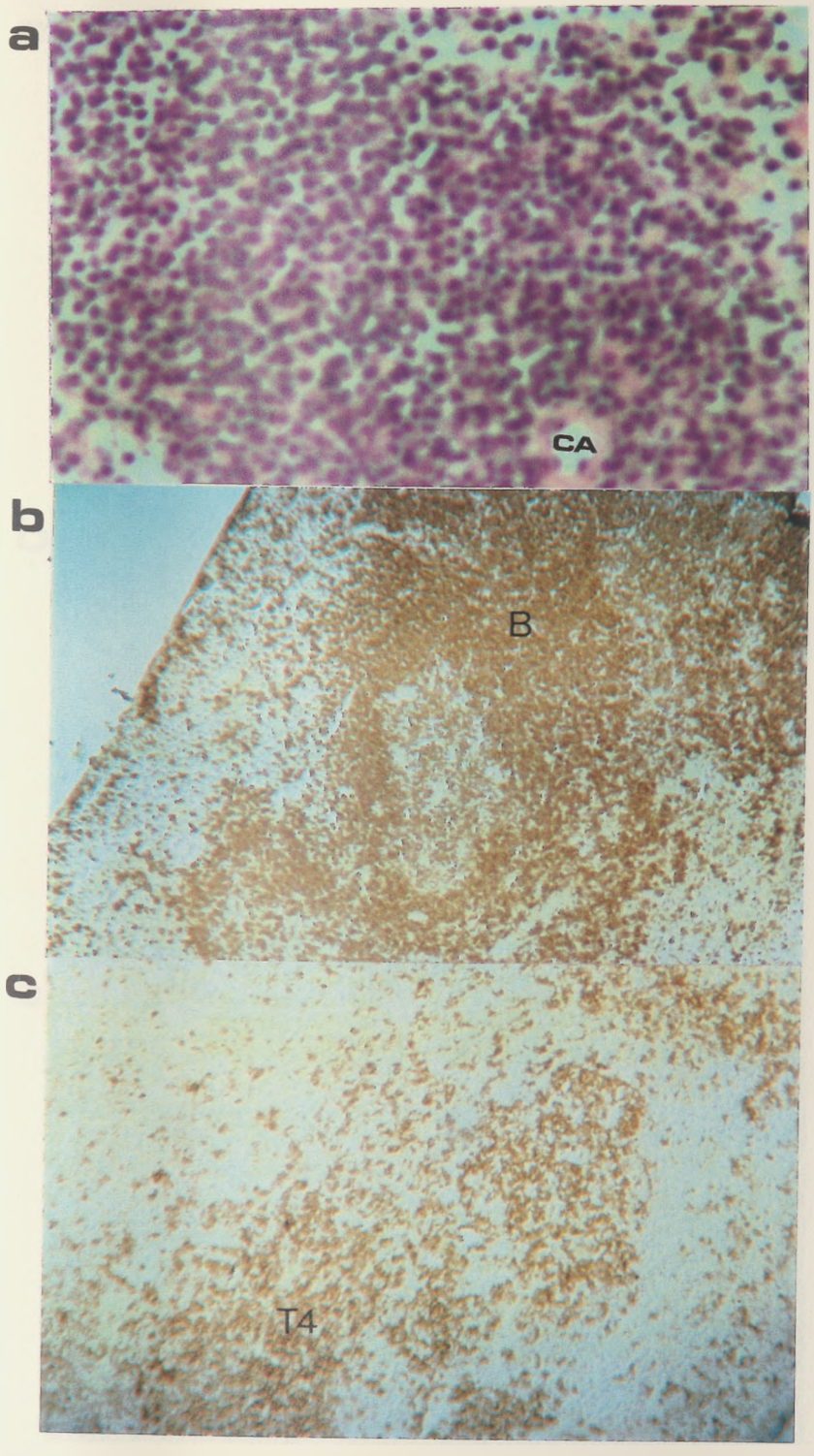

Fig.20: Histopathological and immunocytochemical analysis of spleen from *L. mexicana* infected IL-4-/- mice at week 8 post-infection.

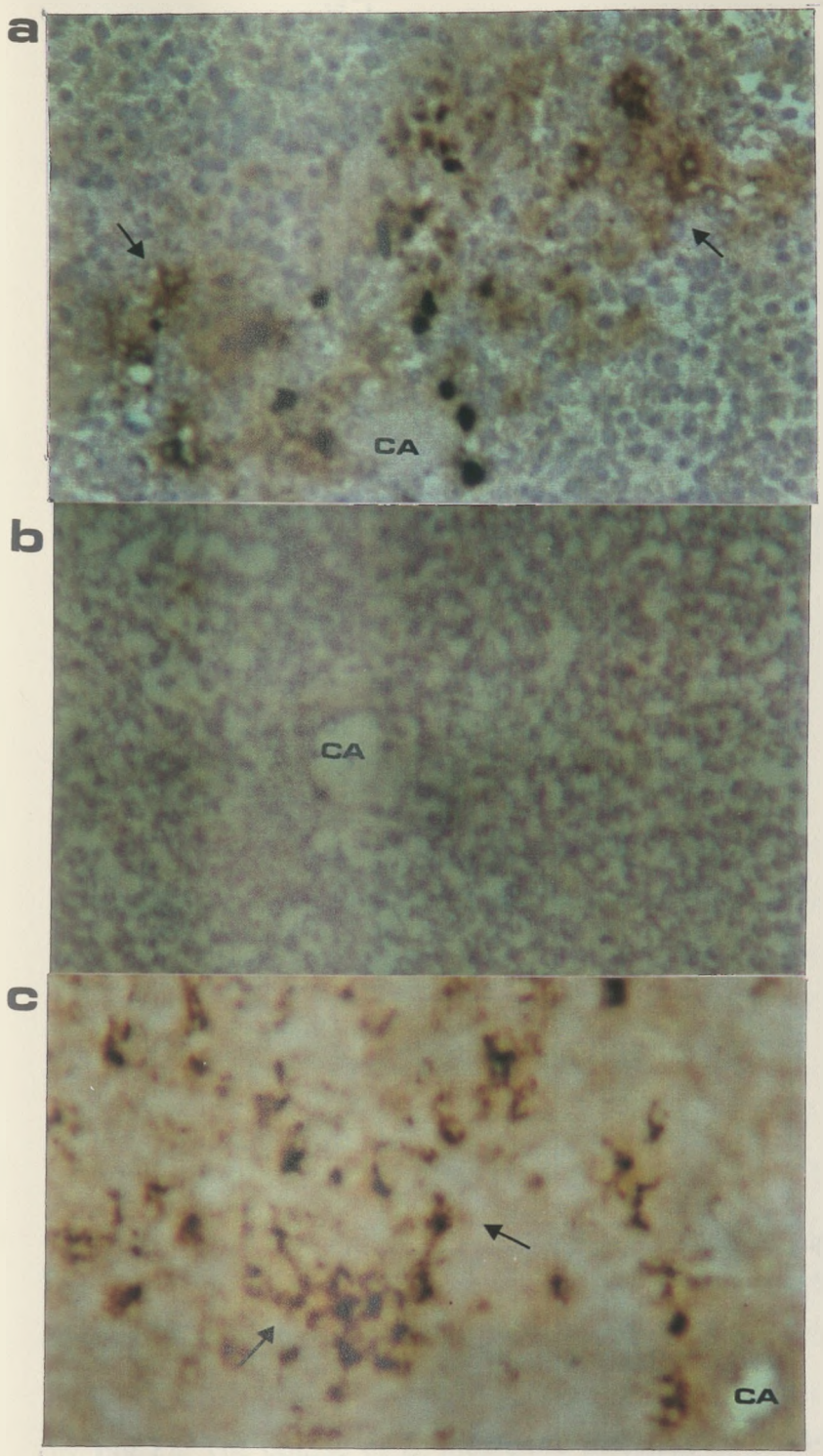

Fig.21: Immunocytochemical analysis of cytokine producing cells in spleen from *L. mexicana* infected IL-4-/- and IL-4+/+ mice at week 16 post-infection.

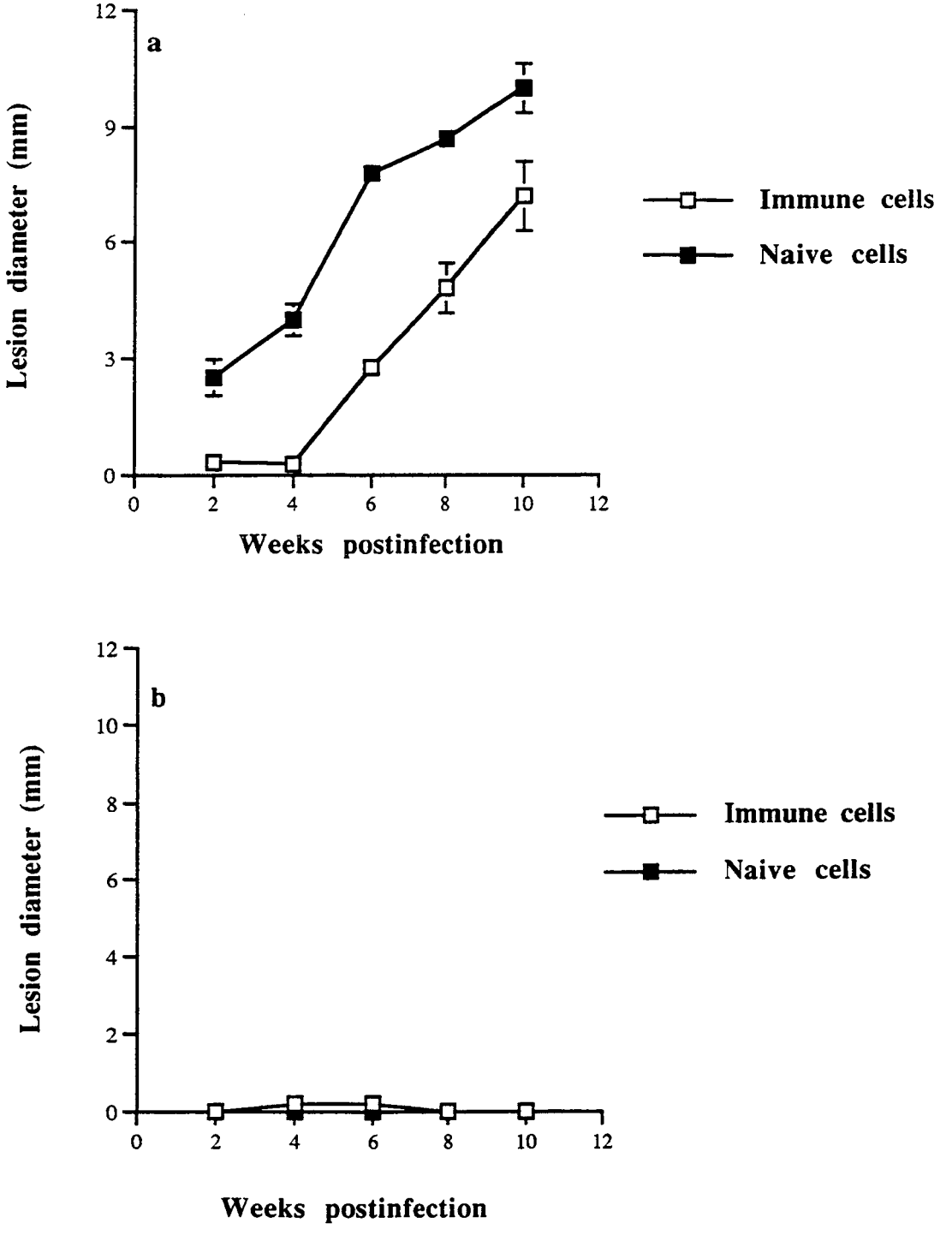

Fig.22: The course of *L. mexicana* infection in IL-4+/+ mice after the adoptive transfer of 10^7 splenocytes from infected IL-4-/- mice (Fig.23a) and in IL-4-/- mice after the adoptive transfer of 10^7 splenocytes from infected IL-4+/+ mice (Fig.23b). Control mice were inoculated with cells from uninfected donors.

5.5 Discussion

The present study quite clearly demonstrates that either the presence of IL-4, and perhaps as a consequence, the ability to generate Th2 responses is essential for the rapid lesion growth and non-healing responses of 129/Sv x C57BL/6 mice following cutaneous infection with *L.mexicana*. This indicates that although *L.mexicana* has been shown to be under different host genetic and immunoregulatory controls to *L.major* (Alexander and Kaye, 1985; Roberts *et al.*, 1990 ; Satoskar and Alexander, 1995) the non-healing response to both parasites is ultimately dependent on the down regulatory effect of Th2 products on protective immunity which has been shown to be mediated through IFN-γ production (Afonso and Scott, 1993; Heinzel *et al.*, 1991; Scott *et al.*, 1988; Stauber, 1988). However, the results also show that the Th2 subset does not play a disease exacerbating role during visceral *L.donovani* infections which fully supports previous studies on *L.donovani* (Kaye *et al.*, 1991).

Although it has provided valuable information on the genetic control of disease (Alexander and Russell, 1992), a major disadvantage of the murine model of *L.donovani* infection is that the direct inoculation of parasites intravenously effectively by-passes the peripheral lymphoid tissue. Hepatic antigen presenting cells are known to preferentially stimulate Th1, but not Th2 cells (Malgilavy *et al.*, 1989), a phenomenon clearly demonstrated by the successful vaccination of BALB/c mice against cutaneous *L.major* infection by the intravenous, but not the subcutaneous route of administration (Liew, 1989). It is perhaps not surprising, therefore, that lymphocytes from mice with even large *L.donovani* burdens failed to produce the Th2 cytokines IL-4 and IL-5 following *in vitro* stimulation (Kaye *et al.*, 1991). Nevertheless, the lack of a role for Th2 cells in the disease progression of visceral leishmaniasis in mice demonstrated in this and other studies (Kaye *et al.*, 1991) is mirrored in some recent studies in humans. Thus, not only have parasite reactive Th1 and Th2 clones been isolated from individuals recovered from visceral leishmaniasis (Kurtzhals *et al.*, 1994), but both IL-4 and IFN-γ have been shown to be produced by peripheral blood monocytes from these individuals (Kemp *et al.*,

1993b). Furthermore, in untreated patients, both IL-10 and IFN-γ mRNA's were greatly upregulated as measured in bone-marrow aspirates (Karp *et al.*, 1993). These observations clearly emphasise the apparent lack of a clear cut role for Th1 or Th2 cell activation in either disease cure or non-cure in visceral leishmaniasis. In fact, IL-4-/- mice were slightly, though significantly, more susceptible to *L.donovani* in the early stages of infection than their wild-type counterparts indicating a potentially protective role for IL-4. Under certain circumstances IL-4 may act synergistically with IFN-γ to induce leishmanicidal activity (Belsovic *et al.*, 1989; Bogdan *et al.*, 1991).

Previous studies by ourselves and others (Afonso and Scott, 1993; Scott *et al.*, 1988) have clearly demonstrated that acquired protective immunity against the *L.mexicana* complex, as with *L.major*, is ultimately dependent on the generation of a Th1 response and IFN-γ production. However, while many studies indicate a disease exacerbating role for IL-4 and Th2 cells in cutaneous infections produced by *L.major* (Heinzel *et al.*, 1989; Locksley *et al.*, 1991), the evidence for a similar role for this T cell subset and its products in cutaneous *L.mexicana* infections is limited. For example, mRNA transcripts for IL-4 and other Th2 cytokines have been shown to be present in both resistant female and susceptible male DBA/2 susceptible mice infected with *L.mexicana* (Satoskar and Alexander, 1995). In addition, neutralising IL-4 monoclonal antibodies, had little effect on the cutaneous growth of the closely related parasite *L.amazonensis* in C57BL/10 mice (Afonso and Scott, 1993). However, in the same study neutralising IL-4 antibodies did limit the growth of this parasite in BALB/c mice (Afonso and Scott, 1993). This and other studies (Alexander and Kaye, 1985) would suggest that perhaps the susceptibility of BALB/c mice to parasites of the *L.mexicana* complex is under different immunoregulatory pathways to those operating in other mouse strains susceptible to these parasites. Furthermore, while mouse strains such as CBA/Ca, C3H/He, C57BL/6, C57BL/10 ScSn, A, ATL, as well as BALB/c mice, go on to develop non healing lesions when infected subcutaneously with *L.mexicana*, all these strains, unlike the BALB/c mouse, develop small lesions which cure following subcutaneous infection with *L.major* (Blackwell,

1988).

Nevertheless, it is quite evident from the present study that IL-4 and/or Th2 cells are also of paramount importance in mediating susceptibility to cutaneous *L.mexicana* infection in 129/Sv x C57BL/6 mice. The results obtained in the present study clearly indicate that the presence of Th1 response in the draining lymph nodes in the absence of Th2 response confers protective immunity in IL-4-/- mice against *L. mexicana*. Moreover it also shows that the Th2-like response even in the presence of Th1-like response exacerbates the disease in IL-4+/+ mice. In addition, in a recent experimental study *L. major*- parasitised macrophages were shown to preferentially augment Th2-type T cell activation (Chakkalath and Titus,1994).

In addition to cutaneous growth, *L.mexicana* has been shown to disseminate systematically to the viscera and further cutaneous sites (Roberts *et al.*, 1989) to a degree independent of the size of the initial lesion. While parasite dissemination was observed in IL-4+/+ mice, no parasites were detected microscopically in any tissue site other than the site of parasite inoculation in IL-4-/- mice which also showed significantly stronger expression of iNOS in their skins than susceptible IL-4+/+ mice. In the past, viable parasites have been repeatedly isolated from cured mice following *L. major* infection (Hill *et al.*, 1983; Titus *et al.*, 1985; Aebischer *et al.*, 1993; Stenger *et al.*, 1994). Moreover, persistence of *Leishmania* parasites has also been reported in humans (Peters *et al.*, 1990; Gramiccia *et al.*, 1992). It has been suggested that one possible mechanism by which a memory immune response is maintained is the continuous presence of a particular antigenic stimulus (Gray and Matzinger, 1991). In addition it has been indicated that persistent expression of iNOS in a resistant mouse strain may be necessary for maintaining the low levels of parasitic infection required to maintain long lasting host immunity (Stenger *et al.*, 1994). Susceptible IL-4+/+ mice showed a significant infiltrate of eosinophils in their skin lesions as compared to IL-4-/- mice. Interestingly, in a recent study mast cells which are often associated with eosinophil activity were shown to be responsible

for parasite persistence and increase in lesion size following *L. major* infection (Wershil *et al.*, 1994). Although, no parasites could be identified in the draining lymph nodes from *L. mexicana* infected IL-4-/- mice on histological examination, promastigotes could still be cultured from tissue samples from this and other sites although in smaller numbers than those grown up from lesions or tissue samples from IL-4+/+ mice. Thus disruption of the IL-4 gene may control the cutaneous growth of *L.mexicana* in 129/SvxC57BL/6 mice but may not limit systemic spread of the parasite at subclinical levels. Total resistance to the growth of *L.mexicana* in IL-4-/- mice as measured by lesion growth was observed in both sexes and not confined to females as observed for the *Scl*-2 gene controlled resistance to *L.mexicana* in the DBA/2 mouse strain (Roberts *et al.*, 1990).

At week 16 post-infection susceptible IL-4+/+ mice showed a significant increase in B cells in their draining lymph nodes as compared with IL-4-/- mice. These experimental findings were similar to those reported in *L. major* infected BALB/c mice (Gessner *et al.*,1995) and *L. mexicana* infected male DBA/2 mice as reported in Chapter 3. In both these studies as with the present one B cell expansion in draining lymph nodes was associated with disease susceptibility.

Surprisingly, although IL-4-/- mice developed a positive DTH skin reaction, the classical *in vitro* correlate of delayed-type hypersensitivity, antigen specific lymphocyte proliferation, as well as mitogen induced proliferation was suppressed in splenocytes from IL-4-/- mice compared with those from IL-4+/+ animals. Interleukin -4, however, is an important T cell growth factor (Locksley *et al.*, 1991) and the value of the lymphocyte proliferation assay as a measure of protective immunity must be questioned. What is perhaps more relevant to the disease state or outcome of infection is that cytokine production is measured. The present study also clearly demonstrates that in a susceptible mouse strain the disruption of the IL-4 gene can, by polarising the immune response during *L.mexicana* infection to a Th1 type as characterised by a positive delayed-type hypersensitivity skin test response and IFN-γ

production at least by splenocytes, confer protective immunity. Conversely, the non-healing response in wild type IL-4+/+ mice is associated with IL-4 mediated Th2 cell activation as measured by the production of IL-5 and the IgG1 antibody isotype. It is, perhaps, significant that the correlation between disease severity and the presence of IL-4 demonstrated in mice in this study is mirrored in various forms of human American cutaneous leishmaniasis where large amounts of mRNA for IL-4 have been associated with disease progression (Caceres-Dittmar *et al.*, 1993).

Chapter 6

Course of *Leishmania mexicana* and *Leishmania donovani* infections in IL-6, IFN-γR, TNFR1 and MHC class II gene deficient mice.

6.1 Summary

Disease development following both *L. mexicana* and *L. donovani* infection was studied in female interleukin-6 (IL-6-/-), interferon gamma receptor (IFN-γR-/-), tumour necrosis factor -receptor 1(TNFR1 -/-) and MHC class II (B6.Aa-/-) gene deficient mice and compared with similarly infected appropriate control female mice. Following subcutaneous inoculation with 1 x 10^6 *L. mexicana* amastigotes into the shaven rump MHC class II deficient mice showed significantly slower disease progression than the control mice and no lesions developed until week 10 post-infection. Following subcutaneous infection with 5 x 10^6 *L. mexicana* amastigotes IFN-γR -/- mice developed non healing lesions that were significantly larger than their control counterparts. On the other hand, following intravenous infection with 1 x 10^7 *L. donovani* amastigotes no significant differences could be detected in liver parasite burdens between IFN-γR -/- mice and control mice. However, at day 30 post-infection IFN-γR-/- displayed significantly lower liver parasite loads than the control mice. No significant difference could be noted in the cutaneous growth of *L. mexicana* between TNFR1-/- mice and the control mice until week 10 post-infection. However, at 4 months post-infection the lesions healed in TNFR1-/- mice but still persisted in the control mice. Following *L. donovani* infection both TNFR1-/- mice and their control counterparts developed comparable liver parasite loads at both the time points examined. No significant differences were observed in disease progression of cutaneous and visceral leishmaniasis between IL-6-/- and control 129Sv mice following infection with *L.mexicana* and *L.donovani* respectively.

6.2 Introduction

Over recent years, gene-knockout mice generated by homologous recombination have become an important research tool in the understanding of *in vivo* immunological mechanisms involved in the control of bacterial (Rothe *et al.*, 1993; Dalton *et al.*, 1993; Aud *et al.*, 1995), fungal (Aud *et al.*, 1995), viral (Huang et al.,1993; Kundig *et al.*, 1993) as well as parasitic diseases (Wang *et al.*, 1994; Amiri *et al.*, 1994; Wang *et al.*, 1993; Overath and Harbecke, 1993; Kopf *et al.*, 1993).

Most experimental studies reported so far using gene knock out mice are principally confined to *L. major* (Wang *et al.*, 1994; Overath and Harbecke, 1993; Swihart *et al.*, 1995; Chakkalath *et al.*, 1995; Swier *et al.*, 1995).

In the present study, to elucidate the *in vivo* immune responses and investigate the role of cytokines during *L. mexicana* and *L. donovani* infection, we have compared the cutaneous and visceral growth of these two *Leishmania* species in IL-6, TNFR1, IFN-γR and MHC class II gene disrupted mice with their age and sex matched control counterparts.

6.3 Materials and Methods

Mice

Inbred IL6-/- , IFN-γR -/-, TNFR1 -/- on a C57BL/10.129 genetic background and MHC class II -/- on a C57BL/6 background mice were generated as described previously (Kopf *et al.*, 1994; Huang *et al.*, 1993; Rothe *et al.*, 1993; Kontgen *et al.*, 1993). Sex and age matched mice of appropriate inbred strain were used as controls in all the experiments. Only female mice were used in all the experiments.

Parasites and Infection Protocol

Leishmania mexicana (MNYC/BZ/62/M379) and *Leishmania donovani* (L82) were maintained as described in Chapter 2.

L.mexicana

In a typical experiment groups of five 8-10 weeks old sex matched IL-6-/-, IFN-γR -/-, TNFR1-/- and their corresponding control mice were infected with $5x10^6$ *L.mexicana* amastigotes by subcutaneous inoculation into the shaven rumps. In the experiments with MHC class II-/- mice $1x10^6$ parasites were used to infect the mice. Disease progression was monitored by the measurement of lesion diameters at 2 week intervals up to 12 weeks post infection.

L.donovani

In a typical experiment, groups of ten, 8 to 10 week old, IL-6 -/-, IFN-γR-/- , TNFR1-/- and their corresponding control mice were infected with $1x10^7$ *L.donovani* amastigotes intravenously via tail vein injections. At 15 and 30 days post infection, 5 mice from each group were sacrificed and parasite burdens in the liver were assessed as described previously (Stauber, 1958; Bradley and Kirkley, 1977).

Statistical analysis

The significance of differences between the groups was determined by the Student's unpaired *t* test.

6.4 Results

Growth of L.mexicana and L. donovani in IFN-γR-/- and IFN-γR+/+mice

Following subcutaneous inoculation with $5x10^6$ *L.mexicana* amastigotes into the shaven rump, IFN-γR -/- mice developed rapidly growing significantly large non-healing lesions that were significantly larger than IFN-γR+/+ mice (Fig. 1). In contrast to IFN-γR+/+ mice, IFN-γR-/- mice developed metastatic lesions in their extremities by 4 months following infection. Following intravenous inoculation of 10^7 *L.donovani* amastigotes into IFN-γR+/+ and IFN-γR-/- mice, the parasite growth in the liver was assessed on days 15 and 30 post infection. Interestingly, at both time points, IFN-γR -/- mice displayed lower parasite burdens in their livers than IFN-γR+/+ mice (Fig.2). However the differences were significant only at day 30 post-infection ($p<0.05$) (Fig.2).

Growth of L.mexicana and L. donovani in TNFR1-/- and TNFR1+/+mice

Following infection with *L. mexicana* as described above, both TNFR1-/- and TNFR1+/+ mice showed comparable lesion growth until week 10 post infection (Fig.3). However, at 4 months post-infection the lesions had healed in the majority of TNFR1 -/- mice although they still persisted in their control counterparts. On the other hand following infection with *L. donovani*, TNFR1-/- mice displayed significantly higher liver parasite burdens than TNFR1+/+ at both time points (Fig.4).

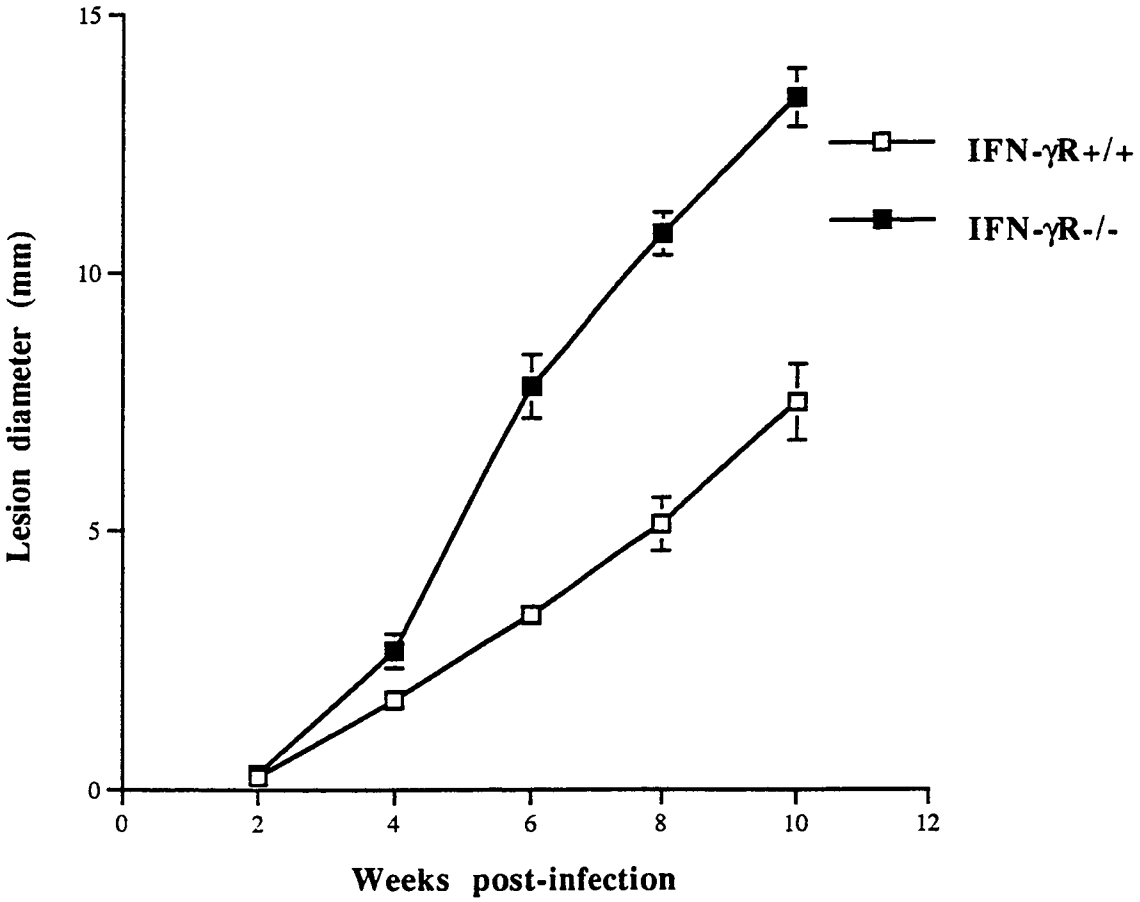

Fig.1: Mean ± SE lesion growth in the shaven rumps of IFN-γR +/+ (□) and IFN-γR-/- (■) mice infected with *L. mexicana*. Six animals were used in each group.

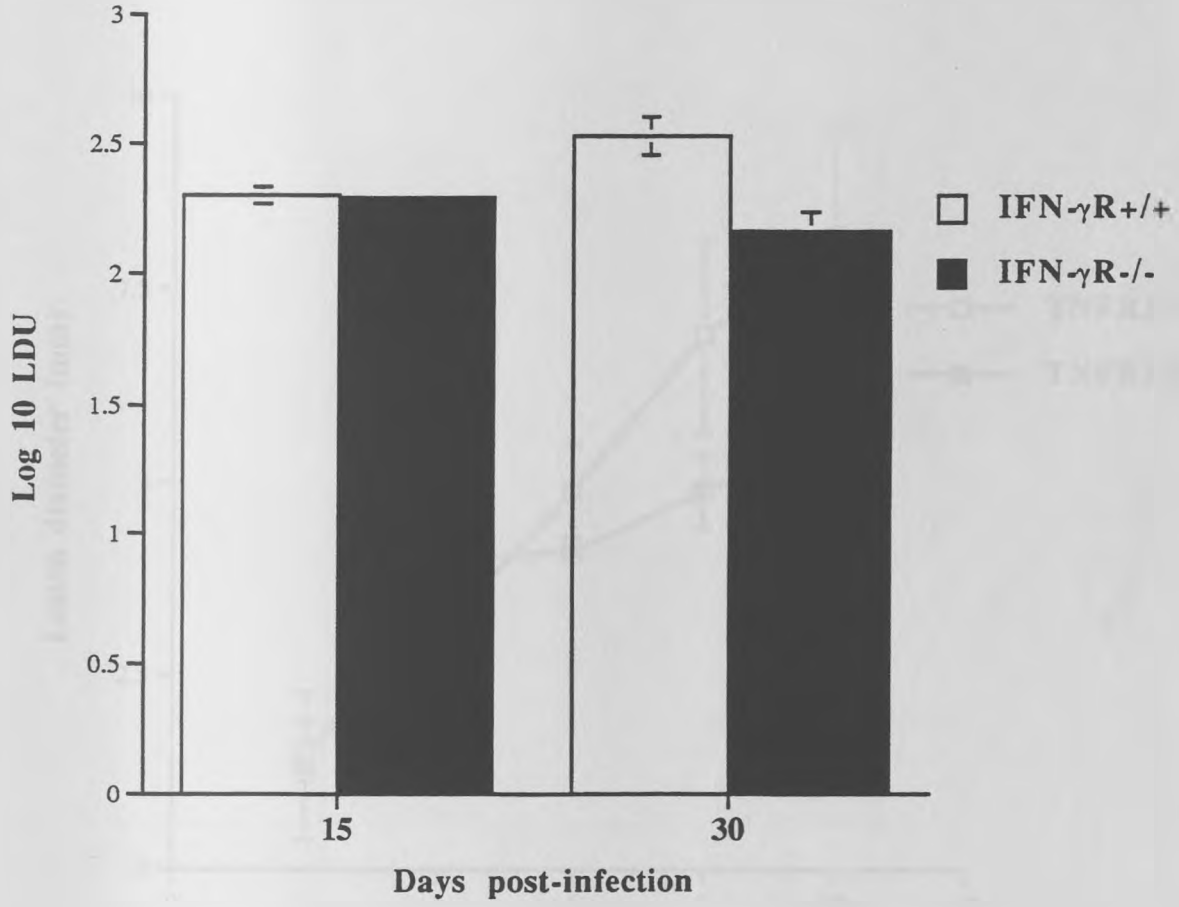

Fig.2: Liver parasite burdens in IFN-γR+/+ (open columns) and IFN-γR-/- (closed columns) mice infected with *L. donovani*. Data expressed as Mean Log $_{10}$ LDU (Leishman Donovan Units) $\pm$ SE.

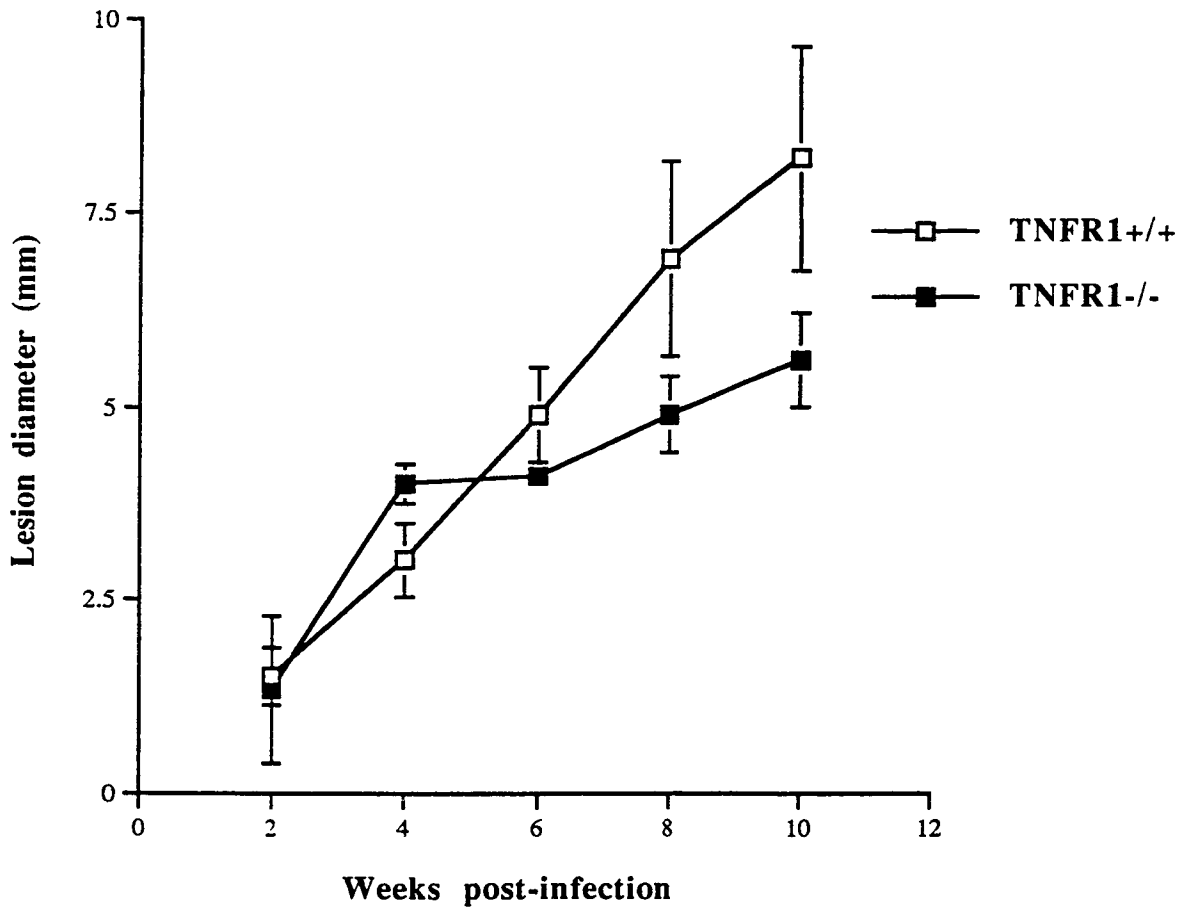

Fig.3: Mean ± SE lesion growth in the shaven rumps of TNFR1 +/+ (□) and TNFR1-/- (■) mice infected with *L. mexicana*. Six animals were used in each group.

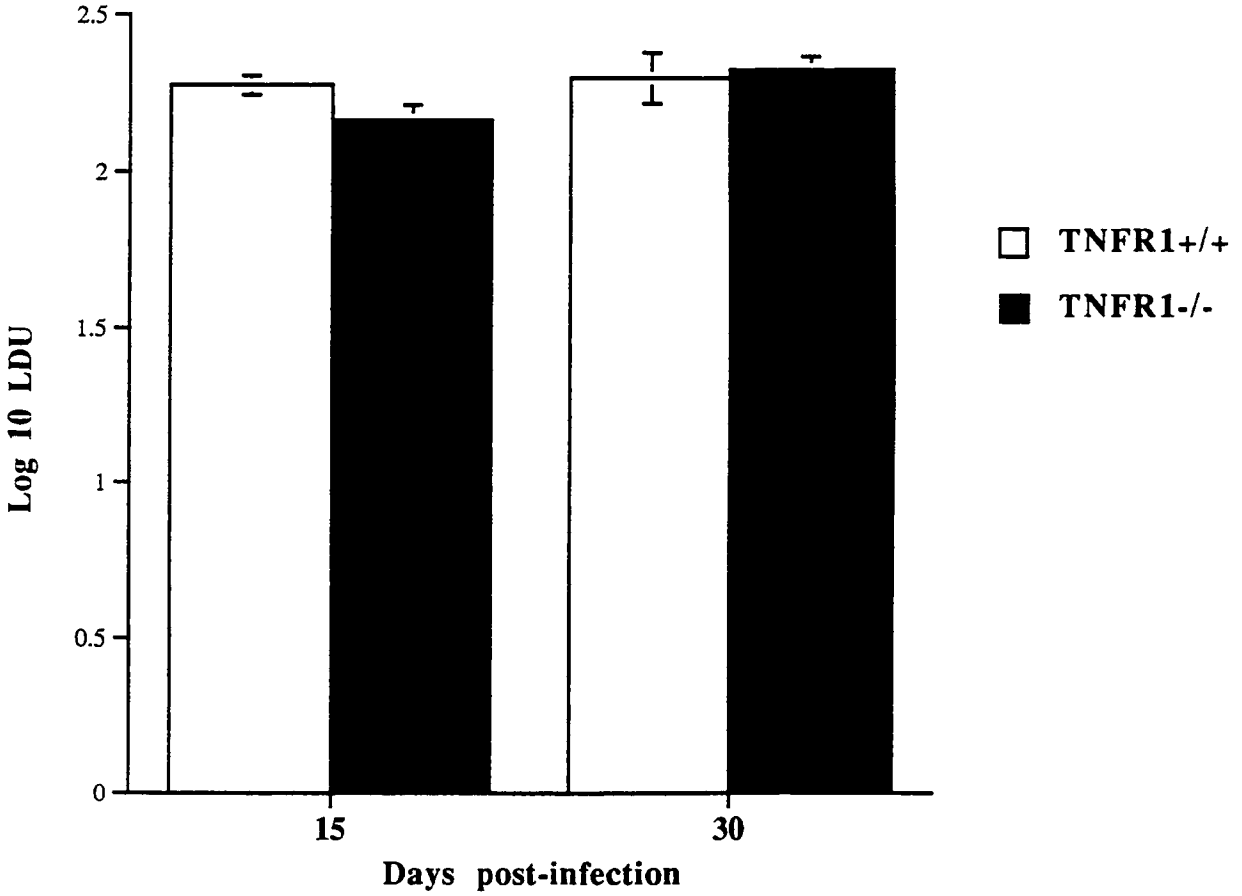

Fig.4: Liver parasite burdens in TNFR1+/+ (open columns) and TNFR1-/- (closed columns) mice infected with *L. donovani*. Data expressed as Mean Log $_{10}$ LDU (Leishman Donovan Units) $\pm$ SE.

Growth of L.mexicana and L. donovani in IL-6-/- and IL-6+/+ mice

No significant differences were noted in either lesion growth or liver parasite burdens between IL-6-/- and IL-6+/+ mice following infection with *L. mexicana* and *L. donovani* (Fig. 5 and 6 respectively).

Growth of L.mexicana in MHC class II -/- mice and MHC class II +/+ mice

In contrast to their control counterparts, MHC class II -/- mice failed to develop any lesions whatsoever until week 10 following subcutaneous inoculation with 10^6 *L. mexicana* amastigotes (Fig. 7).

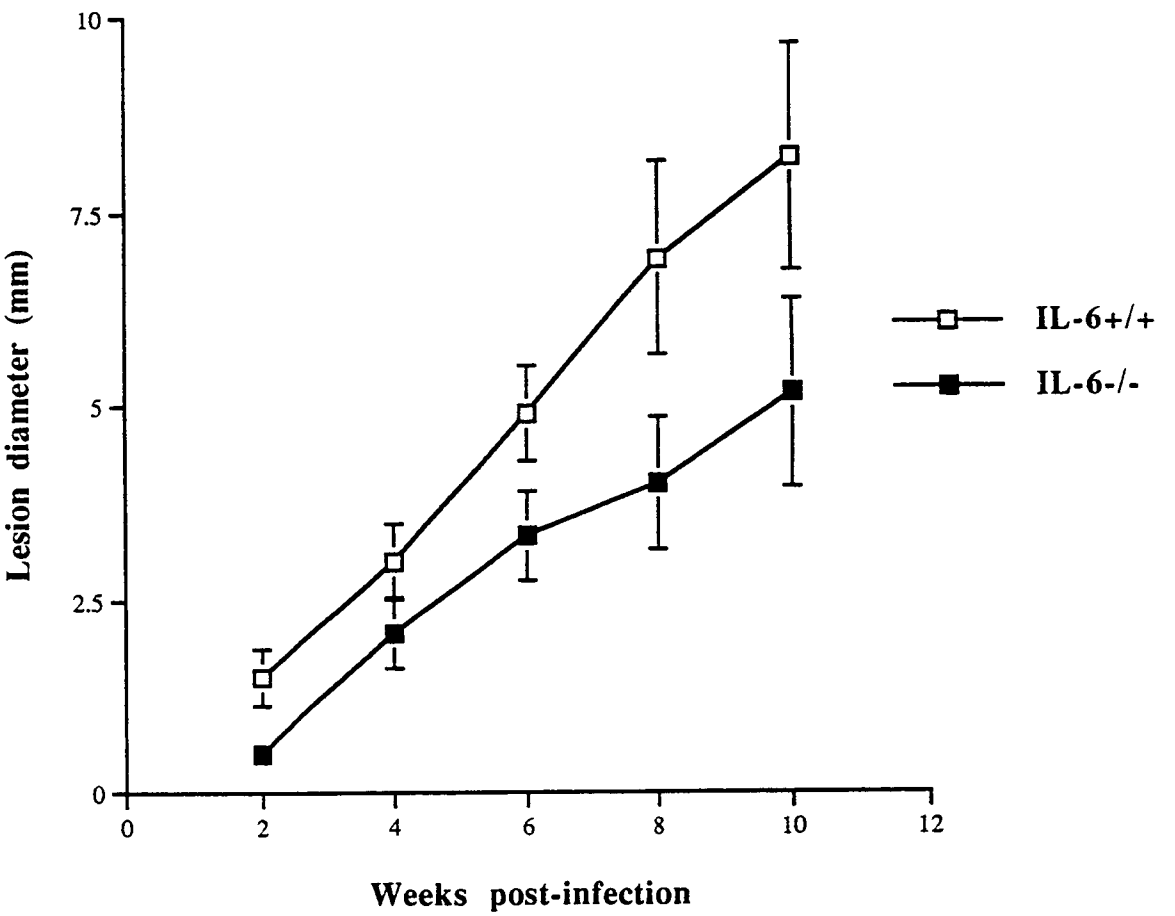

Fig.5: Mean ± SE lesion growth in the shaven rumps of IL-6+/+ (□) and IL-6-/- (■) mice infected with *L. mexicana*. Six animals were used in each group.

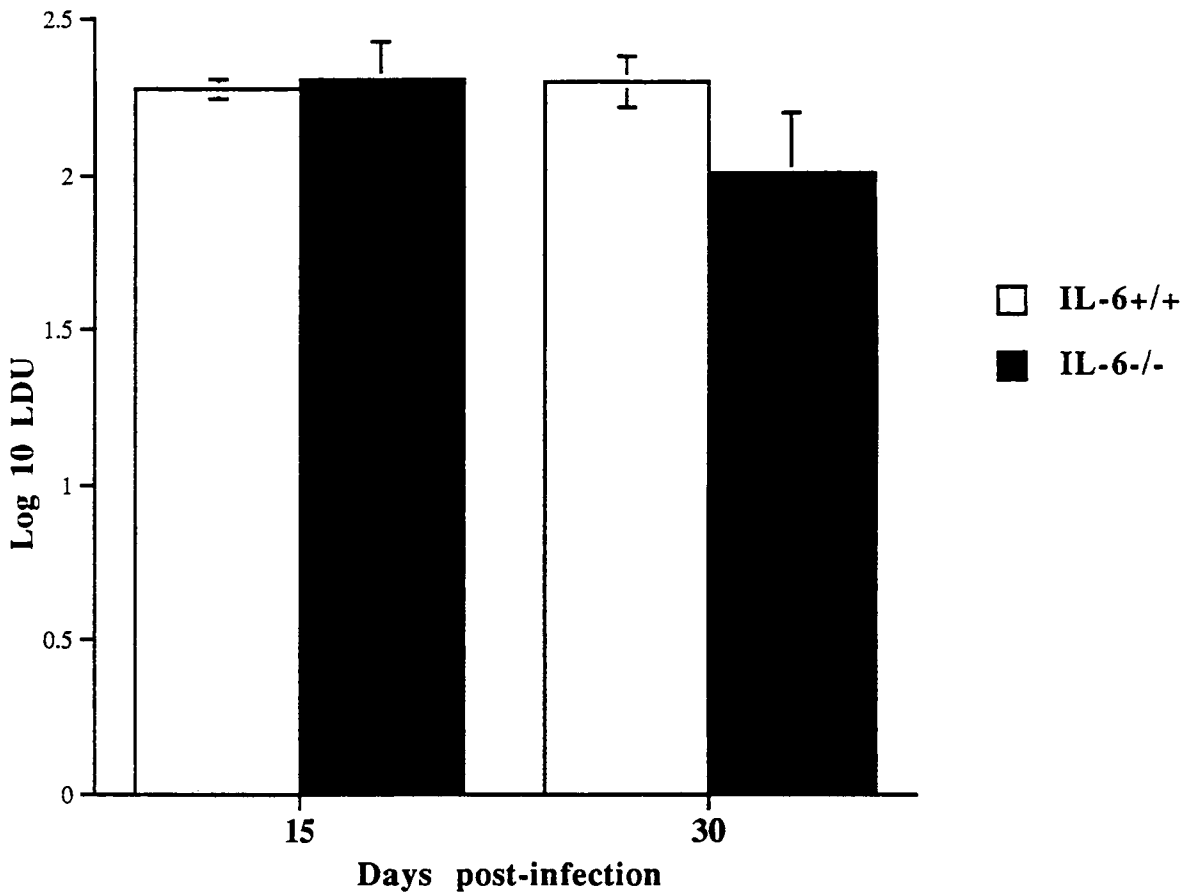

Fig.6: Liver parasite burdens in IL-6+/+ (open column) and IL-6-/- (closed column) mice infected with *L. donovani*. Data expressed as Mean Log $_{10}$ LDU (Leishman Donovan Units) ± SE.

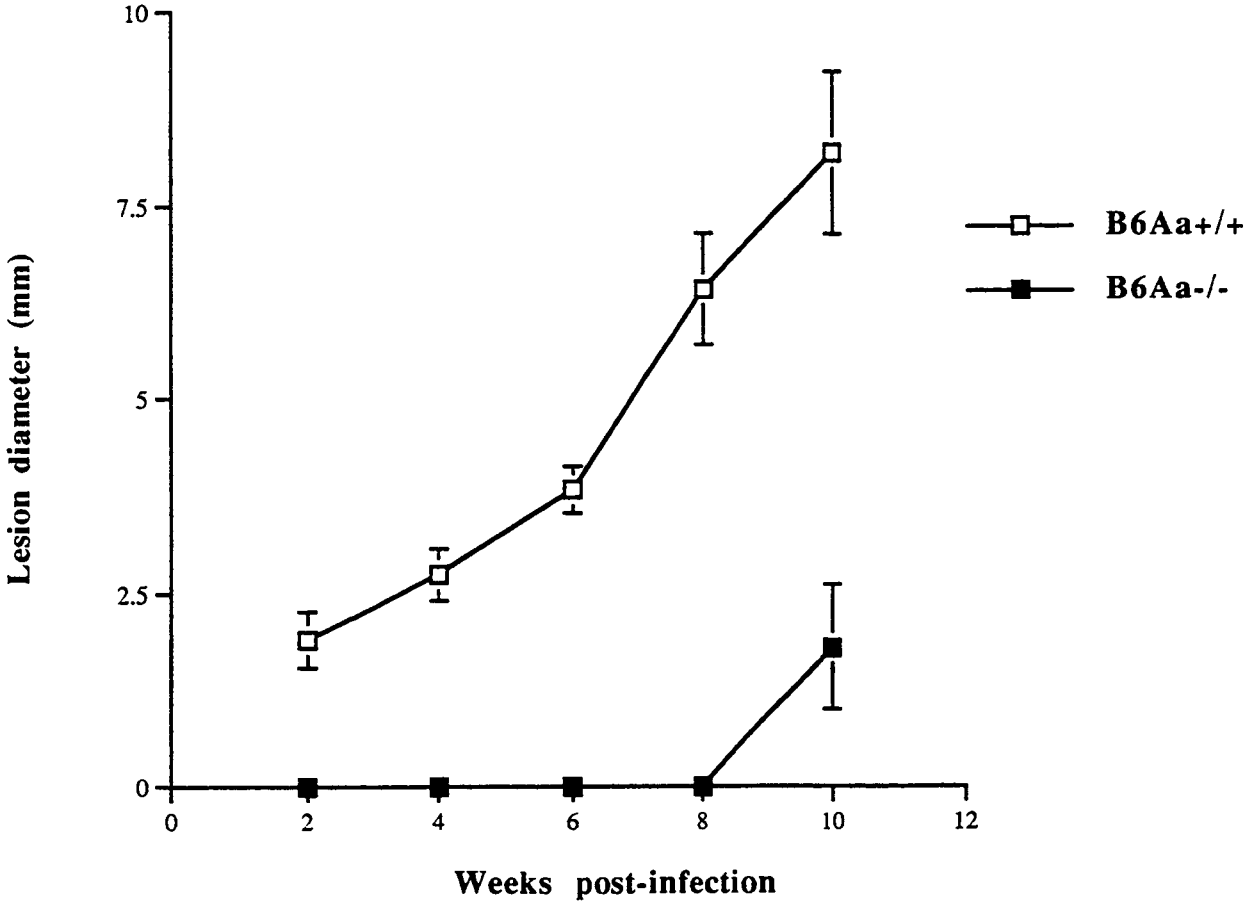

Fig.7: Mean ± SE lesion growth in the shaven rumps of MHC class II+/+ (□) and MHC class II-/- (■) mice infected with *L. mexicana*. Six animals were used in each group.

6.5 Discussion

A wealth of documented evidence, almost all derived from studies on *L. major*, implies that resistance or susceptibility to *Leishmania* infection is dependent on the preferential induction of different Th cell subsets (reviewed by Liew, 1992; Liew and O'Donnell, 1993; Milon *et al.*, 1995).

The recent availability of different gene knock-out mice has provided an important tool to precisely investigate *in vivo* the immunological mechanisms operating in cutaneous leishmaniasis caused by *L. major* (Wang *et al.*, 1994; Overath and Harbecke, 1993; Swihart *et al.*, 1995; Chakkalath *et al.*, 1995; Swier *et al.*, 1995). However, it is also becoming increasingly evident that other *Leishmania* species such as *L. amazonensis* (Afonso and Scott, 1993) and *L. donovani* (Kaye *et al.*, 1991; Satoskar *et al.*, 1995) do not fall into the clear Th1/Th2 pattern of disease development observed in *L. major* infection.

Previous studies by ourselves and others have clearly demonstrated a protective role for IFN-γ following infection with *L. mexicana* complex (Afonso and Scott, 1993; Scott *et al.*, 1988; Satoskar and Alexander, 1995; Chan, 1993). Hence it was hardly surprising that following *L. mexicana* infection IFN-γ R-/- mice developed rapidly growing, non-healing significantly larger lesions than immunocompetant controls. Similarly, IFN-γ has been also shown to be instrumental in host defence against *L. donovani* (Murray *et al.*, 1983; Murray *et al.*, 1985; Murray *et al.*, 1987; Squires *et al.*, 1989; Murray, 1990) and has even been used clinically in combination with chemotherapy to treat patients suffering from visceral leishmaniasis (Badaro and Johnson, 1993; Squires *et al.*, 1993; Sundar *et al.*, 1994; Murray, 1994). Interestingly, following *L. donovani* infection IFN-γR -/- mice displayed lower liver parasite burdens as compared with IFN-γR +/+ mice at day 15 and 30 post-infection, although the difference was significant at day 30 only. While IFN-γ has been shown to activate human and murine macrophages to kill *L. donovani* (Murray *et al.*, 1983; Murray *et al.*, 1985), it has also been shown to exert direct cytostatic effects against *L.*

donovani (Bhattacharya *et al.*, 1993). The lower parasite burdens observed in IFN-γR-/- mice may be either due to either other compensatory mechanisms or as a consequence of direct cytostatic effect of IFN-γ on parasites.

Tumour necrosis factor α (TNF-α) has been shown to play an influential role in the control of murine cutaneous *L. major* infection (Titus *et al.*, 1989; Liew *et al.*, 1990a,b,c). TNF-a appears to exert its leishmanicidal activity by activating macrophages, rather than by its direct effect on the parasites. Further studies have shown that TNF-α alone is unable to induce leishmanicidal activity of macrophages and requires the presence of either IFN-γ or LPS as a second signal (Bogdan *et al.*, 1990a; Liew *et al.*,1990a,b,c). In addition TNF-α has been shown to be the principal cytokine involved in the recruitment of NK cells to the liver (Pilaro *et al.*,1994). However, in the present study no significant differences were observed in the cutaneous growth of *L. mexicana* between TNFR1-/- and TNFR1+/+ mice until week 10 post-infection. However, by 4 months post-infection the lesions healed in the majority of TNFR1-/- mice but still persisted in the control mice. In contrast to its role in macrophage activation, excessive production of TNF-α has also been shown to cause localised tissue damage in some diseases (KhanolkarYoung *et al.*, 1995; Hernandez Pando and Rook, 1994; Bresse *et al.*, 1994). Furthermore, previous results from our study imply that while IFN-γ production alone is enough to control the cutaneous growth of *L. mexicana*, TNF-α production in the absence of IFN-γ is insufficient to control the disease (Satoskar and Alexander, 1995). The role of TNF-α in *L. donovani* infection appears complex. Although, treatment of mice with anti-TNF-α mAbs abrogated the resistance against *L. donovani*, exogenous administration of TNF-α in high dose exacerbated the disease (reviewed by Murray, 1994). However, we observed no significant difference in liver parasite burdens between TNFR1-/- and TNFR1+/+ mice following *L. donovani* infection.

Recently, IL-6 has been shown to down regulate the IFN-γ and TNF-α induced anti-leishmanial activity of human macrophages against *L. amazonensis* (Hatzigeorgio *et*

al., 1993). Furthermore in murine chronic visceral leishmaniasis this cytokine has been readily detected in low-density splenocytes even in the absence of *in vitro* stimulation (Kaye, 1994). However, in the present study there were no differences in growths of *L. mexicana* and *L. donovani* between IL-6+/+ and IL-6-/- mice.

It is well established that CD4+ T cells play undoubtedly the most important role in determining the disease outcome following *Leishmania* infection (reviewed by Liew and O'Donnell, 1993; Liew, 1992; Reed and Scott, 1993; Milon *et al.*, 1995). CD4 T cells belonging to the Th1 subset produce IFN-γ which plays the major protective role in cutaneous leishmaniasis whereas those belonging to Th2 subset produce IL-4, IL-5, IL-6, IL-10 and IL-13 which are responsible for disease exacerbation (Heinzel *et al.*, 1989; Boom *et al.*, 1990; Moll *et al.*, 1990; reviewed by Milon *et al.*, 1995). Although CD8 T cells have been shown to play an important role in mediating resistance against reinfection during cutaneous leishmaniasis, their role during primary infection is still unclear (Farrell *et al.*, 1989; Muller *et al.*, 1991; Muller , 1992; Wang *et al.*, 1993; Muller *et al.*, 1994; Overath and Harbecke, 1993). Furthermore *in vitro* studies have failed to demonstrate a role of CD8 T cells in killing of *Leishmania mexicana* -infected macrophages (Lopez *et al.*, 1993). On the other hand, other experimental studies suggest that cytotoxic CD8 T cells and the IFN-γ produced by activated cytotoxic CD8+ T cells may play an important role in protection against *Leishmania* infection (Pham and Mauel, 1987; McElrath *et al.*, 1988; Smith *et al.*, 1991; Chan, 1993).

MHC class-II gene knockout mice generated by targeted mutation of the MHC class II gene Aa have been shown to be deficient in class-II restricted CD4+CD8- T cells in their peripheral lymphatic organs (Kontgen et al., 1993). Interestingly in the present study following *L. mexicana* infection, CD4+ T cell-deficient-MHC class-II -/- mice developed no lesions before week 10 post-infection unlike their control counterparts which developed rapidly growing non-healing lesions. However, by week 10 post-infection some of the MHC class-II -/- mice developed lesions which enlarged as the

infection progressed. Recently, similar results were reported by another group following infection of MHC class II -/- mice with *L .major* (Chakkalath *et al.*,1995). In their study although MHC class II -/- mice were initially able to control the *L. major* infection, they eventually developed lesions and were unable to control the growth of the parasite. However in our study, as the infection progressed as opposed to all the control mice only 2 out of 5 MHC class II-/- mice developed large lesions after 3 months following infection. These results would imply that in the absence of functional CD4 T cells other cell phenotypes such as NK cells, CD8+ T cells can control the disease progression following *L. mexicana* infection. This is reminiscent of the immunological control of *Toxoplasma gondii* infection (Parker *et al.*, 1991).

In future studies it would be interesting to further characterise these models to elucidate the precise role of these cytokines, cytokine receptors and cells other than that are CD4+ T cells in murine cutaneous and visceral leishmaniasis.

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Sex-determined susceptibility and differential IFN-γ and TNF-α mRNA expression in DBA/2 mice infected with *Leishmania mexicana*

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SUMMARY

Female DBA/2 mice have been shown to be relatively resistant to infection with *Leishmania mexicana* when compared with male mice. In order to determine the immunological basis behind this difference the draining lymph nodes from male and female DBA/2 mice were excised and the RNA extracted at different time-points following infection. Following reverse transcription, the polymerase chain reaction (PCR) was used to identify mRNA transcripts for interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), interleukin-4 (IL-4), IL-10 and IL-12. The evolution of cytokine mRNA production was slow in both male and female mice as no newly synthesized transcripts were identified 5 weeks after infection. IL-10 was expressed constitutively in non-infected mice and was present throughout the experiment in all animals. By week 8, a clear dichotomy in cytokine mRNA expression was emerging between the resistant female and susceptible male mice. Whereas all females expressed IFN-γ and one also expressed TNF-α only two out of five males expressed IFN-γ and four out of five expressed TNF-α. The greatest lesion sizes at this time were recorded from those mice expressing TNF-α but not IFN-γ. No differences in IL-4 or IL-12 were noted with transcripts for both cytokines present in both sexes at week 8. By week 12 males had developed large non-healing nodules and in females lesions had either disappeared or were slow growing. At this time only transcripts for TNF-α were present in males and only those for IFN-γ were detected in females. Treatment of female mice following infection with IFN-γ neutralizing antibody resulted in lesion growth equivalent to male mice. IFN-γ production would, therefore, appear sufficient to limit the growth of *L. mexicana* in female DBA/2 mice while TNF-α production in the absence of IFN-γ confers no protection to DBA/2 male mice.

lthough, in the past, there have been numerous observations n age- and sex-determined patterns of infection, there has been tle analysis to determine the functional basis behind these bservations. Nevertheless, it is broadly accepted that, though no means without exception, males are more susceptible to arasitic infection than females.[1] This phenomenon is clearly emonstrated in DBA/2 mice; while the females are generally esistant to cutaneous lesion growth following infection with *ishmania mexicana*, males develop rapidly growing non-ealing lesions.[2] Resistance comes under single gene control and has been designated *Scl-2*.[3] Interestingly a similar henomenon has been observed in patients presenting with ons resulting from *L. mexicana* infection where females eloped the strongest skin test reactivity [delayed-type persensitivity (DTH)] and consequently a rapid healing sponse.[4] Total and specific IgE levels in these patients were sex dependent and inversely correlated with DTH. This is adly in agreement with murine studies of cutaneous leishmaniasis resulting from *L. major* where DTH-eliciting interferon-γ (IFN-γ)-producing Th1 cells are associated with resistance and IgE-promoting Th2 cells are associated with susceptibility.[5] Although preliminary studies indicate that oestrogen and testosterone can influence *L. mexicana* lesion growth in DBA/2 mice,[1] the immunological mechanisms underlying differences in disease progression between the sexes is still unclear. The following report is, therefore, part of a study to examine the different immunological pathways generated in male and female DBA/2 mice and to determine how sex hormones influence these pathways and ultimately to determine the mode of action of the *Scl-2* gene.

Eight- to 12-week-old DBA/2 mice of both sexes, bred and maintained at the University of Strathclyde Animal Unit were used in this study. *Leishmania mexicana* (MNYC/BZ/62/M379) was maintained by serial passage of amastigotes inoculated subcutaneously into the shaven rumps of BALB/c mice. *Leishmania mexicana* amastigotes were isolated from the cutaneous lesions of BALB/c mice as described by Alexander & Vickerman[6] and DBA/2 mice of both sexes were inoculated with 5×10^6 amastigotes subcutaneously into shaven rumps. All the animals were individually ear marked. Disease progression was monitored in individual mice by measuring

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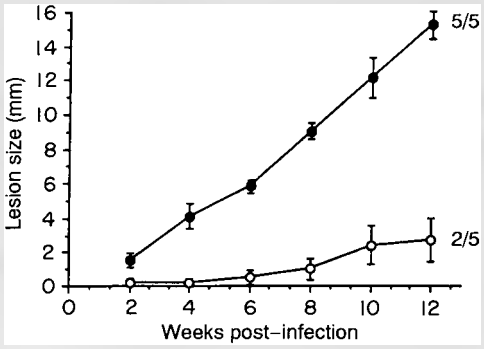

Figure 1. Mean ± SE lesion growth in the shaven rumps of male (●) and female (○) DBA/2 mice infected with *L. mexicana*. The numbers represent animals with lesions at week 12.

lesion diameter weekly up to 12 weeks. The draining inguinal lymph nodes were excised at 2, 5, 8 and 12 weeks post-infection. Total RNA was extracted using a modified acid–guanidium thiocyanate–phenol–chloroform method[7] and was precipitated at −70° using ethanol. For each sample, approximately 5 μg of RNA was amplified by reverse transcription–polymerase chain reaction (RT–PCR) using appropriate primers for β-actin, IFN-γ, tumour necrosis factor-α (TNF-α), interleukin-4 (IL-4), IL-10 and IL-12.[7,8] The PCR products were analysed using ethidium bromide-stained agarose gel electrophoresis and ultraviolet illumination. The R4-6A2 rat–mouse hybridoma secreting monoclonal antibody capable of neutralizing murine IFN-γ was kindly provided by Dr E. A. Havell (Trudeau Institute, New York). The antibodies were purified by affinity chromatography using a protein G column (Pharmacia, Uppsala, Sweden) and the concentration was determined by measuring optical density (OD) at 280 nm. Female DBA/2 mice received 0·4 mg purified R4-6A2 monoclonal antibody (mAb) in 1 ml phosphate-buffered saline (PBS) intraperitoneally 12 hr before infection and weekly thereafter up to 8 weeks. Control mice received equivalent amounts of purified rat IgG (Sigma Chemicals, Poole, UK). Statistical significance ($P < 0{\cdot}05$) was analysed by Student's unpaired *t*-test.

Male DBA/2 mice were extremely susceptible to *L. mexicana* when compared with female mice and developed significantly ($P < 0{\cdot}05$) larger non-healing lesions (Fig. 1). Female mice produced no lesions, small lesions which healed or slow-growing non-healing lesions (Fig. 1). Production of IFN-γ and TNF-α mRNA transcripts was slow in both sexes, and no transcripts for IFN-γ or TNF-α could be detected in either sex before week 5 (Fig. 2). By week 8 clear differences were observed between susceptible male and resistant female mice. All female mice (5/5) expressed mRNA transcripts for IFN-γ in their draining lymph nodes and one also expressed TNF-α (Fig. 3). However, although transcripts for TNF-α were detected in four out of five males, only two expressed transcripts for IFN-γ. At this time, mice expressing TNF-α but no IFN-γ had the largest lesion size (Table 1). No differences in IL-4, IL-10 or IL-12 expression were noted in either sex. IL-10 mRNA transcripts were detectable in the draining lymph nodes of both sexes at all time-points as well as in uninfected controls. Transcripts for IL-4 and IL-12 mRNA were expressed in all mice of both sexes by week 8. By week 12 all males had large non-ulcerating nodules, and of four mice examined none had transcripts for IFN-γ and two had transcripts for TNF-α mRNA. At this time many females did not have lesions and of three examined two had transcripts for

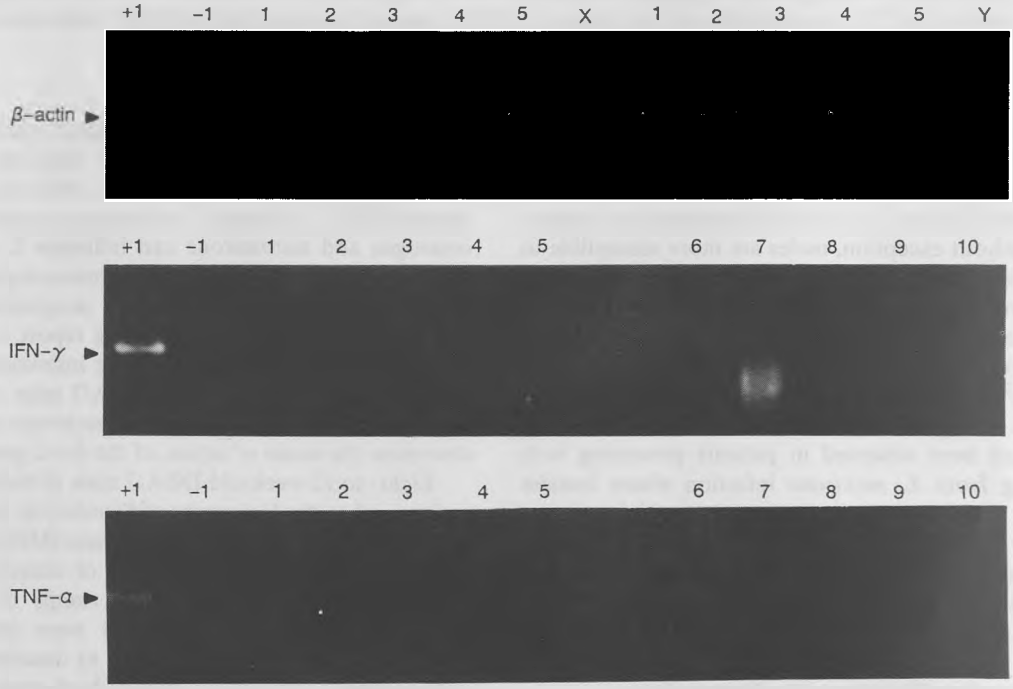

Figure 2. Computer-scanned prints of ethidium bromide-stained agarose gels showing PCR products for β-actin, IFN-γ and TNF-α 5 weeks post-infection. A positive control (+1) and a negative control (−1) are also included. Lanes 1–5 are female mice; lanes 6–10 male mice. Lanes X and Y are the actin controls for uninfected female and male mice respectively.

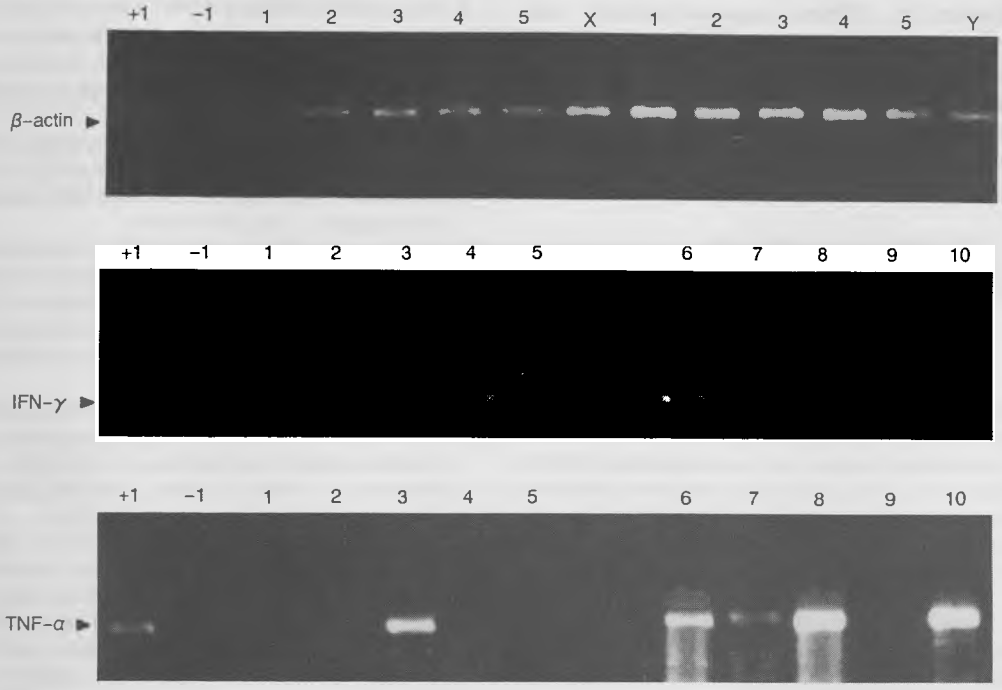

Figure 3. As Fig. 2 but 8 weeks post-infection.

IFN-γ in the draining lymph nodes but TNF-α mRNA could not be detected. A repeat experiment revealed a similar dichotomy between male and female mice in the preferential expression of TNF-α and IFN-γ mRNA.

Female DBA2 mice receiving intraperitoneal injections of IFN-γ neutralizing antibodies at weekly intervals during infection developed rapidly growing lesions comparable to those found in their untreated male counterparts.

The evolution of the immune response during *L. mexicana* infection is slow compared with *L. major*. While the cytokine profiles, reflecting the progress and outcome of disease in the draining lymph nodes, are well established by 4 weeks during infection in resistant and susceptible mice infected with *L. major*.[9] cytokine mRNA production could not be identified from the draining lymph nodes of *L. mexicana*-infected DBA/2 mice 5 weeks after infection. It is well established that *L. mexicana* is under different genetic and immunological controls from *L. major*.[3,10] The lesions tend to be slower growing and non-ulcerating with minimal lymphocyte infiltration.[11] This may be a direct result of this parasite's ability to inhibit the release of antigen from macrophages.[12] Nevertheless, by 8 weeks post-infection transcripts for IFN-γ were found in all resistant female DBA/2 mice but rarely in susceptible male mice where transcripts for TNF-α were present. Treating female mice with IFN-γ neutralizing antibodies resulted in lesion development equivalent to that observed in males.

Table 1. Lesion size (mm) and the presence of mRNA transcripts for TNF-α and IFN-γ in male and female DBA/2 mice at 8 weeks post-infection

Lesion size (mm)	Sex	Transcript present	
9	Male	TNF-α	
8	Male	TNF-α	
8	Male	TNF-α	
6	Male		IFN-γ
4	Male	TNF-α	IFN-γ
4	Female	TNF-α	IFN-γ
4	Female		IFN-γ
3	Female		IFN-γ
3	Female		IFN-γ
0	Female		IFN-γ

It is, perhaps, not surprising that IFN-γ was found in the resistant sex as this cytokine has been shown to be of paramount importance not only in helping to promote a Th1 response but in inducing macrophage leishmanicidal activity[5,13] via the generation of nitrogen intermediates. Whereas several studies have strongly suggested that IFN-γ and TNF-α can synergize to increase macrophage leishmanicidal capabilities,[5] other studies have cast doubt on a protective role for TNF-α independently.[14] The results from this study seem to imply that while IFN-γ production alone by female DBA/2 mice is enough to control the cutaneous growth of *L. mexicana* lesions, TNF-α production by male mice is insufficient to control disease.

Surprisingly, no differences were noted between the resistant and susceptible sexes in the generation of IL-12 and IL-4 mRNA. These cytokines have been shown to promote Th1 expansion and disease protection, and Th2 expansion and disease susceptibility respectively in mice infected with *L. major*.[15] However, it is becoming increasingly evident that other *Leishmania* species do not fall into the clear Th1/Th2 pattern of disease development demonstrated for *L. major*. Thus, for example, in non-curing visceral leishmaniasis[16] and *L. amazonensis* infections[17] an active Th2 component has been difficult to demonstrate. While the present study confirms the importance of IFN-γ in protection, it also emphasizes that *L.*

mexicana is influenced by different immunoregulatory and genetic controls from *L. major* infection.[3,10] The DBA/2 *L. mexicana* disease model presents not only a useful tool to study these immunological pathways and the mode of action of the *Scl-2* gene, but a means of studying how these pathways are influenced by sex hormones.

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Disruption of the Murine Interleukin-4 Gene Inhibits Disease Progression during *Leishmania mexicana* Infection but Does Not Increase Control of *Leishmania donovani* Infection

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The growths of both cutaneous leishmaniasis and visceral leishmaniasis caused by *Leishmania mexicana* and *Leishmania donovani*, respectively, were measured in interleukin-4 (IL-4) knockout mice (IL-4−/−) and compared with those of similarly infected wild-type (IL-4+/+) control mice. While large, nonhealing, cutaneous lesions containing large numbers of parasites developed in IL-4+/+ mice subcutaneously infected with 5×10^6 *L. mexicana* amastigotes in the shaven rump, in IL-4−/− mice no lesions whatsoever developed and parasites were difficult to detect. Systemic spread and metastasis were also noted in IL-4+/+ but not IL-4−/− mice. In contrast, IL-4−/− mice infected intravenously with 10^7 *L. donovani* amastigotes were found to have consistently higher parasite burdens in their livers throughout infection than did their wild-type counterparts. However, these differences were only significant at 15 days postinfection. While the results reported here pertaining to *L. donovani* largely support previous studies, those related to *L. mexicana* provide new observations. The immunological responses of IL-4−/− and IL-4+/+ mice infected with *L. mexicana* were, therefore, examined both in vivo and in vitro. Although neither IL-4−/− nor IL-4+/+ mice infected with *L. mexicana* produced parasite-specific immunoglobulin G2a antibodies, IL-4+/+ mice, unlike IL-4−/− mice, developed significant immunoglobulin G1 antibody titers as infection progressed, indicating a Th2-influenced response in wild-type mice. In addition, IL-4−/− mice, unlike IL-4+/+ mice, developed a significant delayed-type hypersensitivity response, indicating a Th1-influenced response in IL-4−/− mice. Following in vitro stimulation, splenocytes from IL-4+/+ mice infected with *L. mexicana* displayed significantly higher antigen-specific proliferative responses than did IL-4−/− mice. However, gamma interferon production as measured from the supernatants of the in vitro splenocyte cultures of IL-4−/− mice was significantly higher than that from IL-4+/+ mice. This again would indicate a predominantly Th1-influenced response in the absence of a Th2 response in IL-4−/− mice infected with *L. mexicana*. On the other hand, at the same time point, draining lymph node cells from IL-4+/+ mice produced significantly higher quantities of IL-5 than did those from IL-4−/− mice following in vitro antigenic stimulation. These results demonstrate that, whereas IL-4 and/or Th2 lymphocyte expansion is necessary for disease progression and the inhibition of a protective response in cutaneous infection caused by *L. mexicana*, such a role for Th2 cells in visceral leishmaniasis caused by *L. donovani* cannot be demonstrated.

The leishmaniases comprise a number of diseases with a wide spectrum of clinical manifestations. Thus, for example, cutaneous leishmaniasis caused by the *Leishmania mexicana* complex and visceral leishmaniasis caused by *Leishmania donovani* are clinicopathologically quite different diseases of humans in the New and Old Worlds respectively (24). While American cutaneous leishmaniasis resulting from *L. mexicana* infection is mainly a localized disease often associated with chronic infections of the ear, visceral leishmaniasis caused by *L. donovani* is characterized by the dissemination of parasites into the spleen, liver, lymph nodes, and bone marrow (24). Although the clinical outcome of infection is undoubtedly due in part to the parasite species initiating infection, genetically controlled immunoregulatory factors operating within an individual host also influence disease progression (3, 6). Furthermore, studies of murine models of disease have shown that different genetic controls can operate within a single host to control the growth of different species of *Leishmania* even when these species may infect the same tissue sites. Thus, while the visceral growth of *Leishmania major* is totally independent of *Lsh* gene involvement (23), which controls the early growth of visceral *L. donovani* (6), the cutaneous growth of the former parasite has been shown to be under genetic and immunoregulatory controls different from those associated with the cutaneous growth of *L. mexicana* (2, 7, 26). In addition, while the vast majority of mouse strains develop healing lesions when infected subcutaneously with *L. major*, virtually all develop nonhealing lesions full of parasites when infected subcutaneously with *L. mexicana* (6).

Although numerous recent studies have clearly established the immunological mechanisms governing the growth of *L. major* in experimental cutaneous leishmaniasis (20), those immunological mechanisms controlling the visceral growth of species such as *L. donovani* and the cutaneous growth of species such as *L. mexicana* await elucidation. The control of *L. major* lesion growth in genetically resistant mice has been clearly associated with the expansion of the Th1 lymphocyte subset of the $CD4^+$ T-cell population and the production of the cytokines gamma interferon (IFN-γ) and interleukin-2 (IL-2) (12, 28). Conversely, nonhealing responses in susceptible mice have been related to the expansion of the Th2 subset and the production of cytokines such as IL-4 and IL-10 (11, 21). The few experimental studies to date on the immunological control of *L. donovani* (15) and parasites of the *L. mexi-*

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cana complex, which includes *Leishmania amazonensis* (1, 27), have indicated that the inability to generate a Th1 cell response rather than the presence of a Th2 response may be responsible for disease susceptibility.

The recent demonstration that disruption of the murine IL-4 gene blocks Th2 cytokine responses (17) has provided a tool by which to determine the precise role, if any, of Th2 cells in the growth of *Leishmania* species. Therefore, in the present study we have compared the visceral growth of *L. donovani* and the cutaneous growth of *L. mexicana* in 129/Sv × C57BL/6 mice homozygous for the disrupted IL-4 gene (IL-4−/−) with those of their age- and sex-matched wild-type (IL-4+/+) counterparts.

MATERIALS AND METHODS

Mice. IL-4−/− (129/Sv × C57BL/6)F_2 mice were generated as described previously (17). Mice used in these experiments were bred and maintained at the University of Strathclyde. Sex- and age-matched wild-type mice (IL-4+/+) of the same strain combination were used as controls in all the experiments.

Parasites. *L. mexicana* (MNYC/BZ/62/M379) was maintained by serial passage of amastigotes inoculated subcutaneously into the shaven rumps of BALB/c mice. Amastigotes for use in experimental studies were isolated from the lesions and enumerated as described previously (4). *L. donovani* (L82) was maintained by serial passage of amastigotes into hamsters. The parasites were isolated and purified from the spleens of infected hamsters as described elsewhere (14) and were used for infecting mice.

Infection protocols. (i) *L. mexicana*. In a typical experiment, groups of six sex-matched IL-4−/− and control IL-4+/+ mice, 8 to 10 weeks old, were infected with 5×10^6 *L. mexicana* amastigotes by subcutaneous inoculations into their shaven rumps. Disease progression was monitored by the measurement of lesion diameters at 2-week intervals up to 16 weeks postinfection. At regular intervals up to week 24 following infection, serial sections of the skins from incubation sites were analyzed by routine histopathological examination and parasite burdens in the spleen and liver were assessed as described by Stauber (30).

(ii) *L. donovani*. In a typical experiment, groups of 20 8- to 10-week-old IL-4−/− and IL-4+/+ mice were infected intravenously via tail vein injections with 10^7 *L. donovani* amastigotes. At 15, 30, 50, and 110 days postinfection, five mice from each group were sacrificed and parasite burdens in the liver were assessed as described previously (30).

Preparation of soluble leishmanial antigen. Soluble leishmanial antigen was prepared from stationary-phase promastigotes of *L. mexicana*. Promastigotes were washed twice in ice-cold phosphate-buffered saline (PBS) and resuspended in hypotonic buffer consisting of 10 mM Tris-HCl, 2 mM EDTA, pH 7.8, with 50 mM *N-p*-tosyl-L-lysine chlormethyl ketone (TLCK) and 15 mM leupeptin (Sigma, Poole, United Kingdom). Following 15 min of incubation on ice, the promastigotes were disrupted in a Braun homogenizer and centrifuged at 10,000 × *g* for 60 min at 4°C. The supernatant containing soluble leismanial antigen was collected and dialyzed against PBS (pH 7.4) overnight at 4°C, and the protein concentration was determined by a Bradford assay (9).

Isotype-specific enzyme-linked immunosorbent assay (ELISA). Blood was collected with heparinized capillary tubes from tail snips from *L. mexicana*-infected IL-4 knockout and wild-type control mice at various time points after infection. Plasma samples were obtained following centrifugation at 200 × *g*, and these samples were used to determine the titers of Th2-dependent immunoglobulin G1 (IgG1) and Th1-dependent IgG2a *Leishmania*-specific antibodies (29). Briefly, each well of a 96-well microtiter plate was coated with 1 μg of soluble leishmanial antigen in PBS (pH 9.0). Following a 1-hr incubation at 37°C with serially diluted test plasma (starting dilution, 1:100), plates were washed three times and either rat anti-mouse IgG1 horseradish peroxidase conjugate (dilution, 1:8,000; Southern Biotechnology Associates, Birmingham, Ala.) or rat anti-mouse IgG2a horseradish peroxidase conjugate (1:2,000 dilution; Jackson Laboratories) was applied. After a further 1 h of incubation at 37°C and three washes, tetramethyl benzidine in a sodium acetate buffer (pH 5.5) containing H_2O_2 was added. The reaction was stopped after 20 min by adding 10% H_2SO_4, and the A_{450} was measured on a Titertek Multiscan plate reader.

Induction and measurement of DTH responses. Delayed-type hypersensitivity (DTH) responses in IL-4+/+ and IL-4−/− mice were examined at week 16 following subcutaneous infection. Fifty microliters of 10^6 formalin (5%)-fixed, PBS-washed *L. mexicana* promastigotes was inoculated into the right hind footpad of each mouse, and the increase in footpad thickness was measured 48 h later with a dial-gauge micrometer. Left hind footpads were injected with sterile PBS as a negative control.

T-cell proliferation assay. IL-4+/+ and IL-4−/− mice were killed at week 16 following infection with *L. mexicana*. Their spleens and draining inguinal lymph nodes were removed aseptically, and cell suspensions were prepared by gentle teasing in RPMI 1640 supplemented with 10% fetal calf serum (heat inactivated), 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.05 mM β-mercaptoethanol (Gibco, Paisley, United Kingdom). The cells were centrifuged at 200 × *g* for 5 min, and eythrocytes were lysed by resuspending spleen cells in 3 ml of Boyle's solution (0.17 M Tris–0.16 M ammonium chloride; BDH Ltd., Dorset, United Kingdom) and incubating at 37°C for 3 min. Boyle's lysis was not carried out for lymph node cells. Following two washes, the viable cells were counted by trypan blue exclusion with an improved Nuebaer hemocytometer and cell suspensions were adjusted to either 5×10^6 cells/ml for spleen cells or 3×10^6 lymph node cells in complete RPMI medium. Aliquots (100 μl) containing either 5×10^5 splenocytes or 3×10^5 lymph node cells were added in triplicate to the wells of 96-well flat-bottomed tissue culture plates (Costar, Cambridge, Mass.) containing 100 μl of soluble *L. mexicana* antigen per well in different concentrations (50, 10, 5, and 0 μg/ml for splenocyte proliferation; 25 and 0 μg/ml for lymph node cell proliferation) or concanavalin A (ConA) (5 μg/ml for splenocyte proliferation; 2.5 μg/ml for lymph node cell proliferation). Following incubation at 37°C in 5% CO_2 for 60 h, 100 μl of the culture supernatant was removed from each well (stored at −70°C for cytokine assays) and replaced with 100 μl of complete medium containing the appropriate amount of antigen or ConA. At the same time, the cells were pulsed with 0.25 μCi of tritiated thymidine (specific activity, 35 Ci/mmol; ICN/Flow, High Wycombe, United Kingdom) and, following a further 12 h of incubation at 37°C, were harvested onto filter paper (ICN/Flow) with a cell harvester (Skatronas, Lier, Norway). Thymidine uptake was measured by liquid scintillation on a β-counter (Pharmacia LKB Biotech., Milton Keynes, United Kingdom) with 1 ml of Optiscint (Pharmacia Biosystems) added to the filter discs in vials (Hughes and Hughes, Somerset, United Kingdom) and counted for 5 min each.

IFN-γ and IL-5 assays. IL-5 production and IFN-γ production by stimulated (antigen or ConA) and nonstimulated cells from *L. mexicana*-infected IL4−/− and IL-4+/+ mice were measured by capture ELISA. The supernatants, at a 1/2 dilution for IFN-γ and undiluted for IL-5, were analyzed in duplicate. The culture supernatants and standards, consisting of murine recombinant IL-5 (0 to 10,000 pg/ml) or IFN-γ (0 to 7,000 pg/ml) (Cambridge Bioscience, Cambridge, United Kingdom), were added in duplicate to the wells of a microtiter plate which had been precoated 12 h previously with capture antibody (rat anti-mouse/human IL-5, clone TRFK-5, or rat anti-mouse IFN-γ, clone R4-6A2; Cambridge Bioscience, Cambridge, United Kingdom) in PBS, pH 9.0, at 4°C. Following incubation at 37°C for 120 min, the wells were washed three times with PBS-Tween (pH 7.4; 0.05% Tween 20) and biotinylated anti-IFN-γ or anti-IL-5 antibodies were added. After a further incubation at 37°C for 60 min, the plates were washed as described above and 100 μl of detection complex (streptavidin-linked alkaline phosphatase, 1:2,000 dilution) was added to each well. The plates were further incubated at 37°C for 45 min in the dark, and the wells were washed three times with PBS-Tween before the addition of 100 μl of *p*-nitrophenylphosphatase prepared in glycine buffer (pH 10.1). After a further 30 min of incubation at 37°C, the A_{405}s were measured on a Titertek Multiskan plate reader. IFN-γ and IL-5 concentrations were determined from the standard curve (regression coefficient $r = 0.99$).

Statistical significance. Student's unpaired *t* test was used to determine the statistical significance of values obtained.

RESULTS

Growth of *L. mexicana* and *L. donovani* in IL-4+/+ and IL-4−/− mice. Following subcutaneous inoculation with 5×10^6 *L. mexicana* amastigotes into the shaven rump, no lesions were found to develop at the site of inoculation or elsewhere in any IL-4−/− mice at any time during the study (Fig. 1). By contrast, all similarly infected wild-type control mice developed progressively growing nonhealing lesions throughout the period of study (Fig. 1). These results were completely reproducible in five separate experiments (results not shown), *and* there were no sex-influenced differences in the growth of this parasite in these mice. Only occasional parasites could be detected histologically at the site of infection in IL-4−/− mice and no visceralization could be detected (results not shown) up to 24 weeks postinfection with *L. mexicana*. On the other hand, the systemic spread of parasites and the development of metastatic lesions at the extremities were often observed in IL-4+/+ mice as infection progressed. Following intravenous inoculation of 10^7 *L. donovani* amastigotes into IL-4+/+ and IL-4−/− mice, the parasite growth in the liver was monitored from day 15 through day 110 postinfection. Surprisingly, at all time points more *L. donovani* amastigotes were observed in the IL-4+/+ wild-type control mice than in the IL-4-deficient mice. However, the difference in parasite burdens between the

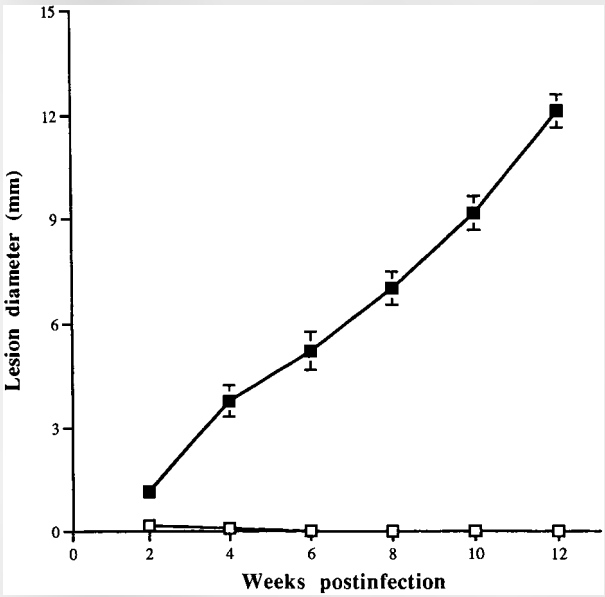

FIG. 1. Mean lesion growths in the shaven rumps of IL-4+/+ (■) and IL-4−/− (□) mice infected with *L. mexicana*. Six animals were used in each group. Bars show standard errors of the mean.

two groups was significant only during the early stage of infection, at day 15 ($P < 0.05$) (Fig. 2).

Antibody response to *L. mexicana* and *L. donovani* in IL-4+/+ and IL-4−/− mice. The specific antibody response measured during *L. mexicana* infection was barely detectable in any of the mice until week 8 postinfection (Fig. 3). At this time point and thereafter, neither IL-4+/+ nor IL-4−/− mice produced any measurable quantities of *L. mexicana*-specific IgG2a. However, IL-4+/+ wild-type control mice produced significant quantities of *L. mexicana*-specific IgG1 in the absence of any antibody production by IL-4−/− mice. At week 16 following infection, although IL-4+/+ mice had significantly higher titers of antigen-specific IgG1 than at week 8 ($P < 0.05$), IL-4−/− mice still had no detectable antibody response (Fig. 3). By contrast, *Leishmania*-specific IgG2a, though not IgG1, was detected in plasma samples from both IL-4+/+ and IL-4−/− mice at day 50 following infection with *L. donovani* (data not shown).

In vitro and in vivo T-cell responsiveness. As IL-4 deficiency did not confer increased resistance to *L. donovani* as previous reports would suggest (15), the following studies were confined to *L. mexicana*-infected mice in which disruption of the IL-4 gene induced resistance. At week 16 postinfection, resistant IL-4−/− mice displayed a significant DTH response ($P < 0.05$) to antigen at 48 h postinoculation with formalin-fixed parasites (Fig. 4). No significant DTH response was detected in IL-4+/+ mice at this time (Fig. 4). Splenocytes from *L. mexicana*-infected susceptible IL-4+/+ mice as well as resistant IL-4−/− mice displayed significant ConA-induced ($P < 0.025$) and *L. mexicana*-specific proliferative responses ($P < 0.025$) (Fig. 5). However, ConA-induced and antigen-specific proliferative responses were significantly greater ($P < 0.025$) in IL-4+/+ mice than in IL-4−/− mice (Fig. 5).

IFN-γ and IL-5 assays. The supernatants from the splenocyte assays described above were analyzed by ELISA for the presence of the Th1-associated cytokine IFN-γ and the Th2-associated cytokine IL-5 (Fig. 6). While spleen cells from *L. mexicana*-infected IL-4−/− mice produced significant quantities of IFN-γ ($P < 0.025$) following stimulation with *L. mexicana* antigen, no IFN-γ above basal level could be detected in the supernatants from antigen-stimulated IL-4+/+ spleen cells (Fig. 6b). No differences in IFN-γ or IL-5 production could be detected from unstimulated splenocyte cultures from either IL-4+/+ or IL-4−/− *L. mexicana*-infected mice (Fig. 6a). Similarly, no increased IL-5 production above basal levels could be detected in the supernatants from antigen-stimulated splenocytes from either IL-4+/+ or IL-4−/− *L. mexicana*-infected mice (Fig. 6b). However, the supernatants from ConA-stimulated splenocytes from *L. mexicana*-infected IL-4+/+ mice contained significantly higher quantities of IL-5 ($P < 0.025$) than did those derived from IL-4−/− mice (Fig. 6c). As splenocytes from *L. mexicana*-infected IL-4+/+ and IL-4−/− mice failed to show significant differences in IL-5 production following in vitro antigenic stimulation, the supernatants from assays with draining lymph node cells were analyzed for the presence of IL-5. The draining lymph node cells from IL-4+/+ mice produced significantly higher quantities of IL-5 ($P < 0.05$) than did those from IL-4−/− mice following in vitro stimulation with ConA and *L. mexicana* antigen (Fig. 7).

DISCUSSION

The present study quite clearly demonstrates that the presence of IL-4 and, perhaps as a consequence, the ability to generate Th2 responses are essential for the rapid lesion growth and nonhealing responses of 129/Sv × C57BL/6 mice following cutaneous infection with *L. mexicana*. This indicates that although *L. mexicana* has been shown to be under host genetic and immunoregulatory controls different from those of *L. major* (2, 26), the nonhealing response to both parasites is ultimately dependent on the downregulatory effect of Th2 products on protective immunity, which has been shown to be mediated through IFN-γ production (1, 12, 27, 28). However, the results also show that the Th2 subset does not play a disease-exacerbating role during visceral *L. donovani* infections, which fully supports previous studies of *L. donovani* (15).

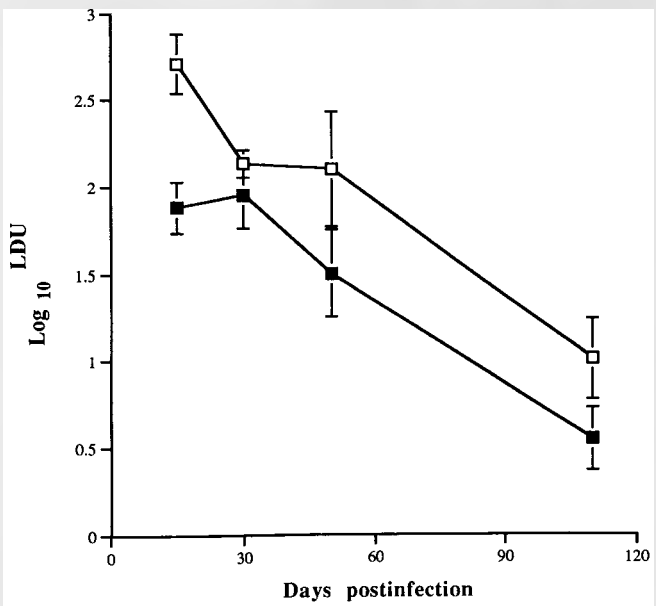

FIG. 2. Liver parasite burdens in IL-4+/+ (■) and IL-4−/− (□) mice infected with *L. donovani*. Data are expressed as mean $\log_{10}$ Leishman Donovan units (LDU) ± standard errors of the mean (bars).

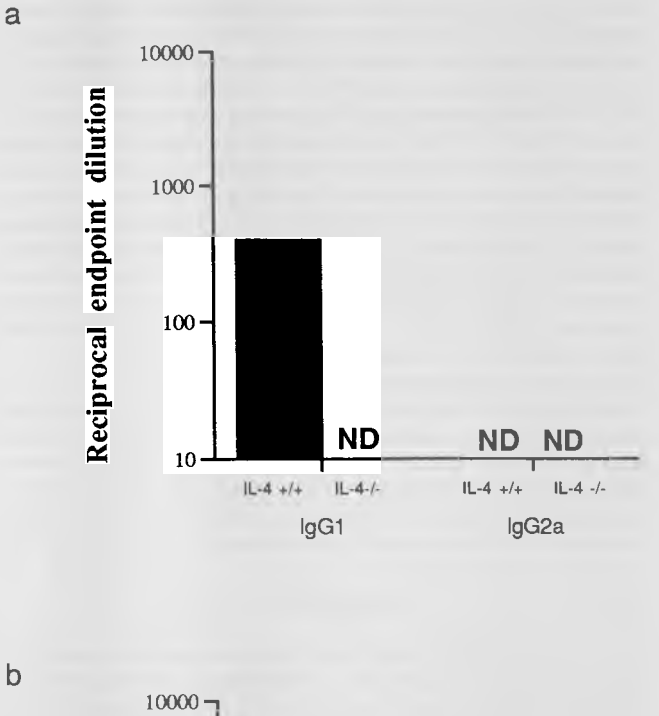

FIG. 3. *L. mexicana*-specific IgG1 and IgG2a production in IL-4+/+ and IL-4−/− mice at week 8 (a) and week 16 (b) postinfection presented as mean reciprocal endpoint dilutions. Bar in panel b shows ± standard error of the mean. ND, no antibody detected at the 1:100 dilution.

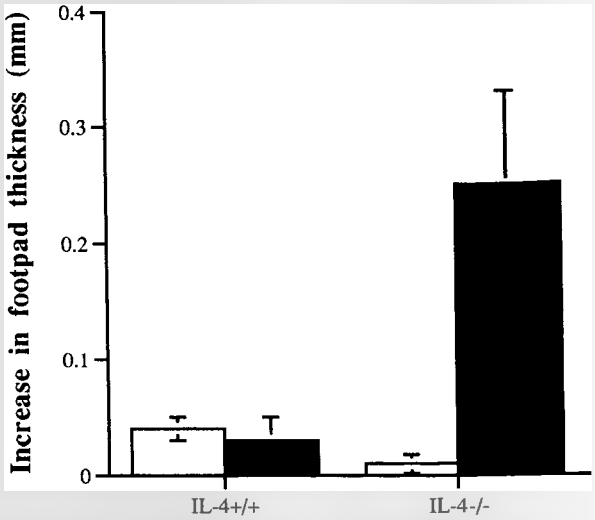

FIG. 4. DTH responses at week 16 postinfection with *L. mexicana* in IL-4+/+ and IL-4−/− mice. Footpad thickness was measured in infected mice (closed columns) and control mice (open columns) 48 h after inoculation with formalin-fixed parasites. Data are expressed as means ± standard errors of the mean (bars).

Although the murine model of *L. donovani* infection has provided valuable information on the genetic control of disease (3), a major disadvantage of this model is that the direct inoculation of parasites intravenously effectively bypasses the peripheral lymphoid tissue. Hepatic antigen-presenting cells are known to preferentially stimulate Th1 but not Th2 cells (22), a phenomenon clearly demonstrated by the successful vaccination of BALB/c mice against cutaneous *L. major* infection by the intravenous but not the subcutaneous route of administration (19). It is perhaps not surprising, therefore, that lymphocytes from mice with even large *L. donovani* burdens failed to produce the Th2 cytokines IL-4 and IL-5 following in vitro stimulation (15). Nevertheless, the lack of a role for Th2 cells in the disease progression of visceral leishmaniasis in mice demonstrated in this and other studies (15) is mirrored in some recent studies with humans. Thus, not only have parasite-reactive Th1 and Th2 clones been isolated from individuals who have recovered from visceral leishmaniasis (18), but both IL-4 and IFN-γ have been shown to be produced by peripheral blood monocytes from these individuals (16). Furthermore, in untreated patients, both IL-10 and IFN-γ mRNAs were greatly upregulated as measured in bone marrow aspirates (13). These observations clearly emphasize the apparent lack of a clear-cut role for Th1 or Th2 cell activation in either disease cure or noncure in visceral leishmaniasis. In fact, IL-4−/− mice were slightly, though significantly, more susceptible to *L. donovani* in the early stages of infection than were their wild-type counterparts, indicating a potentially protective role for IL-4. Un-

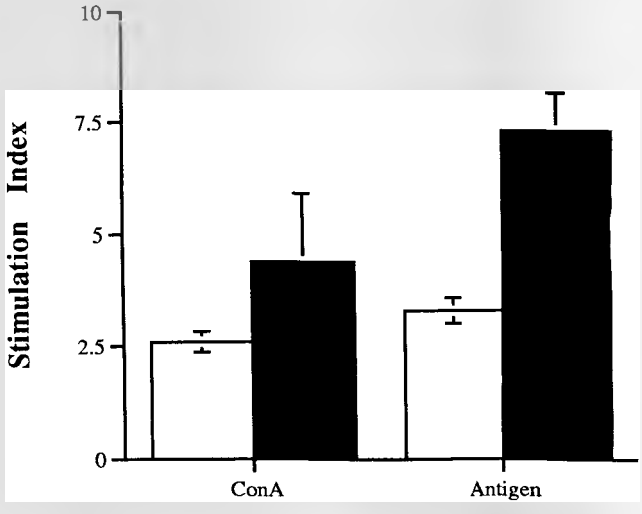

FIG. 5. ConA (5 μg/ml)-induced and *L. mexicana* soluble antigen (50 μg/ml)-induced splenocyte proliferation responses in *L. mexicana*-infected IL-4+/+ mice (closed columns) and IL-4−/− mice (open columns) at week 16 postinfection. Data are expressed as means ± standard errors of the mean (bars).

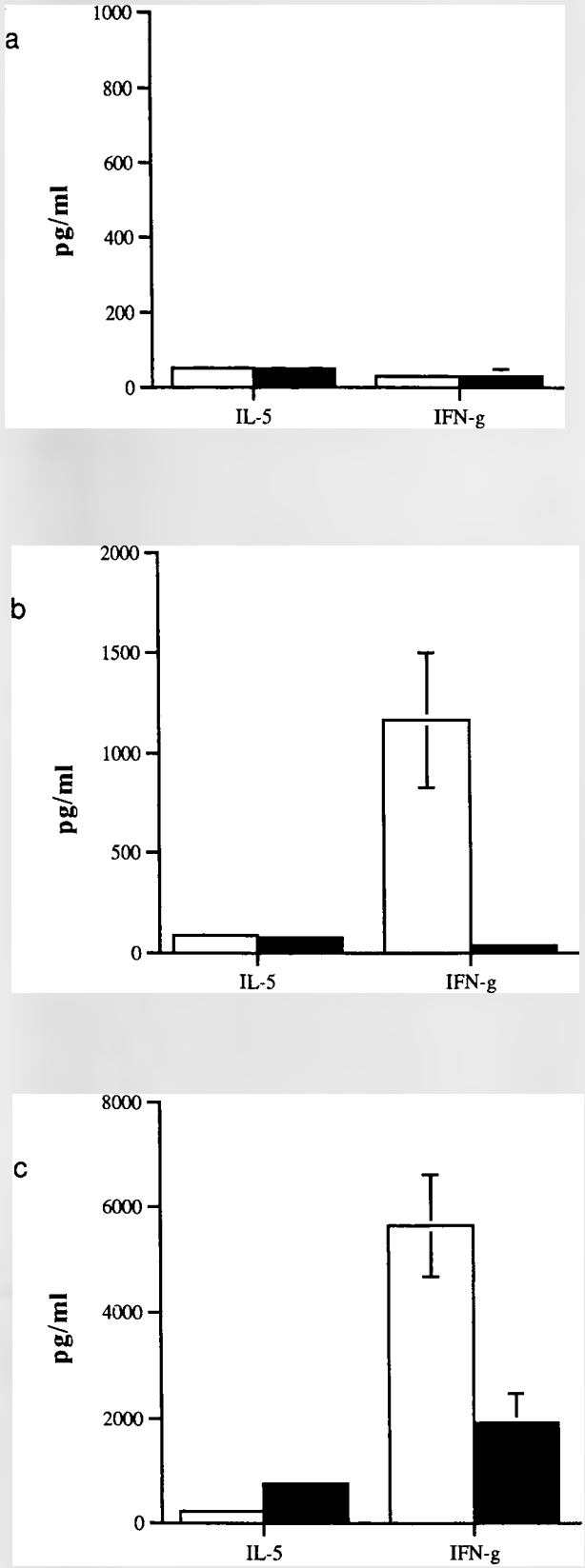

FIG. 6. IL-5 and IFN-γ (IFN-g) production by splenocytes from *L. mexicana*-infected IL-4+/+ mice (closed columns) and IL-4−/− mice (open columns) at week 16 postinfection. Results in the absence of antigen (a), in the presence of *L. mexicana* soluble antigen (50 μg/ml) (b), and in the presence of ConA (5 μg/ml) (c) are shown.

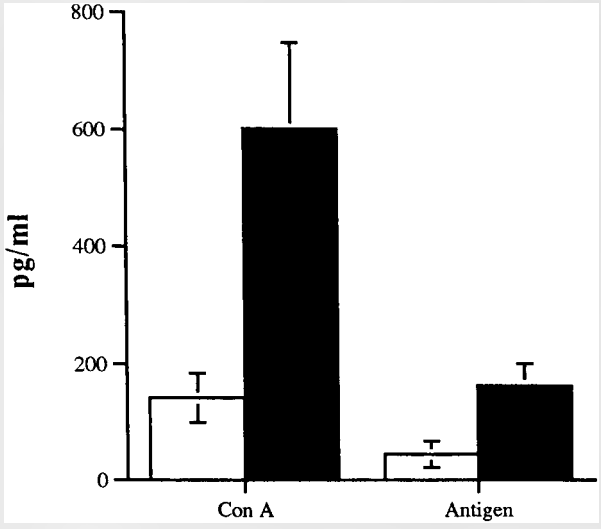

FIG. 7. In vitro IL-5 production by inguinal lymph node cells from *L. mexicana*-infected IL-4+/+ mice (closed columns) and IL-4−/− mice (open columns) at week 16 postinfection. Results in the presence of ConA (2.5 μg/ml) or *L. mexicana* soluble antigen (25 μg/ml) are shown.

der certain circumstances IL-4 may act synergistically with IFN-γ to induce leishmanicidal activity (5, 8).

Previous studies by us and others (1, 27) have clearly demonstrated that acquired protective immunity against the *L. mexicana* complex, as with *L. major*, is ultimately dependent on the generation of a Th1 response and IFN-γ production. However, while many studies indicate a disease-exacerbating role for IL-4 and Th2 cells in cutaneous infections produced by *L. major* (11, 21), the evidence for a similar role for this T-cell subset and its products in cutaneous *L. mexicana* infections is limited. For example, mRNA transcripts for IL-4 and other Th2 cytokines have been shown to be present in both resistant female and susceptible male DBA/2 mice infected with *L. mexicana* (27). In addition, neutralizing IL-4 monoclonal antibodies had little effect on the cutaneous growth of the closely related parasite *L. amazonensis* in C57BL/10 mice (1). However, in the same study neutralizing IL-4 antibodies did limit the growth of this parasite in BALB/c mice (1). This and other studies (2) would suggest that perhaps the susceptibility of BALB/c mice to parasites of the *L. mexicana* complex is under the control of immunoregulatory pathways different from those operating in other mouse strains susceptible to these parasites. Furthermore, while mouse strains such as CBA/Ca, C3H/He, C57BL/6, C57BL/10 ScSn, A, and ATL, as well as BALB/c mice, go on to develop nonhealing lesions when infected subcutaneously with *L. mexicana*, all these strains, unlike the BALB/c mouse, develop small lesions which heal following subcutaneous infection with *L. major* (6). Nevertheless, it is quite evident from the present study with IL-4−/− mice of the 129/Sv × C57BL/6 background, that IL-4 and/or Th2 cells are also of paramount importance in mediating susceptibility to cutaneous *L. mexicana* infection.

In addition to demonstrating cutaneous growth, *L. mexicana* has been shown to disseminate systematically to the viscera and further cutaneous sites (25) to a degree independent of the size of the initial lesion. While parasite dissemination was observed in IL-4+/+ mice, no parasites were detected in any tissue site other than the site of parasite inoculation in IL-4−/− mice. Thus, disruption of the IL-4 gene not only controls the cutaneous growth of *L. mexicana* in 129/Sv × C57BL/6

mice but also inhibits the metastatic spread of the parasite. Total resistance to the growth of *L. mexicana* in IL-4−/− mice was observed in both sexes and not confined to females as observed for the *Scl-2* gene-controlled resistance to *L. mexicana* in the DBA/2 mouse strain (26).

Surprisingly, the classical in vitro correlate of DTH, antigen-specific lymphocyte proliferation, as well as mitogen-induced proliferation, was suppressed in IL-4−/− mice compared with that in IL-4+/+ mice. IL-4, however, is an important T-cell growth factor (21), and the value of the lymphocyte proliferation assay as a measure of protective immunity must be questioned. What is perhaps more relevant to the disease state or outcome of infection is that cytokine production is measured. The present study also clearly demonstrates that in a susceptible mouse strain, the disruption of the IL-4 gene can confer protective immunity by polarizing the immune response in *L. mexicana* to a Th1 type, as characterized by a positive DTH skin test response and IFN-γ production. Conversely, the non-healing response in wild-type IL-4+/+ mice is associated with IL-4-mediated Th2 cell activation as measured by the production of IL-5 and the IgG1 antibody isotype. It is, perhaps, significant that the correlation between disease severity and the presence of IL-4 demonstrated for mice in this study is mirrored in various forms of human American cutaneous leishmaniasis for which large amounts of mRNA for IL-4 have been associated with disease progression (10).

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