Vaccination against experimental cutaneous leishmaniasis using attenuated *Salmonella typhimurium*

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by

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Abbreviations

Ag	Antigen
AP	Alkaline phosphatase
APC	Antigen presenting cell
ConA	Concanavelin A
CPM	Counts per minute
CSDM	Complete schneiders Drosophila medium
CTL	Cytotoxic T lymphocyte
DCL	Diffuse cutaneous leishmaniasis
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
GM-CSF	Granulocyte/macrophage-colony stimulating factor
gp	Glycoprotein
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducable nitric oxide synthase
kB	Kilobase
LDU	Leishman-Donovan units
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MCL	Mucocutaneous leishmaniasis
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
NK	Natural killer

NO	Nitric oxide
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PSP	Promastigote surface protease
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
SEM	Standard error of the mean
SLA	Soluble leishmania antigen
TcR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TNF	Tumour necrosis factor

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2. Xu, D., **McSorley, S.J.** & Liew, F.Y. (1995) Protection against *Leishmania major* infection in genetically susceptible BALB/c mice by GP63 delivered orally in attenuated *Salmonella typhimurium* ($AroA^-$ $AroD^-$). Immunology, 85: 1-7.

3. **McSorley, S.J.**, Proudfoot, L., O'Donnell, C.A. & Liew, F.Y. (1995) Experimental models of leishmaniasis. Clin. Dermatol. (in press).

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Abstract

The *Salmonella typhimurium* vaccine construct, GID101, which constitutively expresses *L. major* gp63 was used as an oral vaccine against experimental cutaneous leishmaniasis in the susceptible BALB/c mouse. After 2 oral immunisations with GID101, lesion development was reduced following *L. major* infection. This vaccine therefore represents a considerable improvement over the previous attenuated *S. typhimurium* vaccine SL3261-gp63 which was unable to confer any protection to susceptible mice. After two oral doses of GID101, spleen and mesenteric lymph node (MLN) cells from BALB/c mice were found to proliferate *in vitro* to parasite antigens and produce substantial amounts of IFN-γ and IL-2 but no IL-4 or IL-5. The production of these cytokines was $CD4^+$-cell dependant. Serum taken from immunised mice was found to contain parasite specific IgG2a but no parasite specific IgG1. In addition, no delayed type hypersensitivity (DTH) response to *L. major* was detected in vaccinated mice. These data are consistent with the notion that GID101 administration results in the induction of a protective Th1-like response to *L. major*.

L. major gp63 was also expressed in the attenuated *S. typhimurium* strain, BRD509, under the control of either the *nirB* promoter which responds to anaerobic conditions (GID105) or the *osmC* promoter which is induced under high salt conditions (GID106). The construct GID105 displays more stable plasmid retention during colonisation *in vivo* when compared with the constitutive construct GID101. After oral administration, the construct GID106 does not colonise spleen or liver at all although it is found in the MLN. In addition, this construct loses plasmid more quickly *in vivo* compared to the GID101 strain. An antibody response to gp63 is generated in BALB/c mice after one oral dose of GID101, GID105 or GID106, while only the anaerobic construct GID105 is able to induce a significant cellular response to antigen. Spleen and MLN cells from mice administered GID105 respond *in vitro* to parasite antigen in a Th1-like manner. A single oral delivery of GID105 or GID106 is sufficient to reduce lesion swelling in susceptible mice after *L. major* infection, while

a single dose of GID101 does not confer any protection. Two doses of GID105 also reduces liver parasite burdens during *L. donovani* infection, while GID101 or GID106 provide no protection. Therefore construct GID105 can confer a degree of protection to *L. major* infection after a single oral dose and to *L. donovani* after two doses, representing a further improvement of the gp63 construct.

A plasmid was generated which contains the cDNA for *L. major* p24/LACK under control of the inducable *NirB* promoter. This plasmid was introduced into the attenuated *S. typhimurium* strain BRD509. Expression of p24 by this construct (GID202) was demonstrated under anaerobic conditions by western blotting. After oral administration of GID202 to BALB/c mice bacteria were detected in the spleen, liver and MLN from around 3-7 days post administration until 21-28 days. Plasmid stability of the construct during colonisation was found be around 100% in all organs. BALB/c mice which were orally administered one or two doses of GID202 before *L. major* infection developed exacerbated disease. CBA mice given two oral doses of GID202 also generated an exacerbated infection although CBA mice which received a single dose were slightly protected. Spleen and MLN cells from mice administered GID202 responded *in vitro* in a Th1-like manner to both recombinant p24 and the immunodominant peptide of p24. These data demonstrate a disease-promoting effect of vaccination with p24 expressed in *S. typhimurium* correlating with a normally protective Th1 response *in vitro*.

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Chapter 1

General Introduction

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1.1 Leishmaniasis

Leishmaniasis is a disease of global importance as at present there are around 12 million infected individuals and much of the world's population live in endemic areas (1). At least 2 major epidemics of leishmaniasis, in Sudan (2) and India (3) are currently taking place. There is no reliable vaccine against any form of this disease and treatment of infection relies principally on the use of pentavalent antimonial compounds which are costly, highly toxic and not always effective (4). However, recent advances in understanding the immunology of leishmaniasis have elucidated the factors responsible for protection and susceptibility to infection, providing a foundation for the future development of a vaccine in addition to the rational design of effective therapeutic agents.

1.1.1 Clinical disease

There are three major clinical forms of leishmaniasis which together create a spectrum of disease ranging from self healing cutaneous lesions to fatal visceral disease (5).

Cutaneous leishmaniasis (also called Oriental Sore), develops as a skin lesion at the site where a sandfly has previously take a blood meal. These lesions are usually self-limiting, although a chronic condition can develop caused by certain parasite species known as Diffuse Cutaneous Leishmaniasis (DCL). In this case, the injected organism spreads to other subcutaneous tissues, resulting in extensive swelling at many sites in the skin. While the majority of infected individuals will heal a cutaneous lesion, there is often an unsightly scar left at the injection site.

Visceral leishmaniasis (or Kala Azar) also begins with lesion development around the site of inoculation but the infection slowly spreads to involve visceral

tissues, such as spleen, liver and bone marrow. Hepatosplenomegaly, diarrhoea, weight loss and severe immunosuppression occur as the disease progresses which ultimately leads to death unless adequate treatment is provided (5).

Muco-cutaneous leishmaniasis (MCL) (or Espundia) results from the reactivation of infection in individuals who have cured an initial cutaneous lesion many years previously. However, this reactivation does not take place at the initial infection site but is restricted to the mucous membranes of nose, mouth and pharynx. Spontaneous recovery from this condition is rare.

1.1.2 Species classification and global distribution

The parasites responsible for these diverse clinical diseases all belong to the genus *Leishmania*. These protozoan organisms belong to the class Zoomastigophorea and order Kinetoplastida, suborder Trypanosomatina (6). Their inclusion within this order is based primarily on the possession of a kinetoplast which is a round aggregation of DNA outside the nucleus which is readily observed under light microscopy using a DNA stain. This kinetoplast DNA is contained within a single, large mitochondrion and the position this occupies within the flagellate stage of the parasite allows easy differentiation of *Leishmania* from other Trypanosomatina (6).

The various *Leishmania* species can be grouped according to the geographical area in which they are found and the clinical disease they produce. This generates four distinct species groupings.

Two species which cause visceral leishmaniasis in northern and east Africa, southern Europe and parts of Asia are collectively known as agents of "Old World Visceral Leishmaniasis". The main species in this group is *L. donovani* which is the most widely studied of all visceral species (5). *L. infantum* (sometimes referred to as

L. donovani infantum) is a minor species which is endemic in parts of southern Europe and is thought normally to produce an asymptomatic infection in healthy adults (7).

"New World Visceral Leishmaniasis" is an identical disease to that caused by *L. donovani* . However, this disease is found in Mexico, Central and South America and is caused by *L. chagasi* infection.

The group of species responsible for "Old World Cutaneous Leishmaniasis" is more diverse and comprises three distinct species (6). *L. tropica* and *L. major* are found in Asia, Africa, the Middle East and parts of southern Europe. *L. aethiopica* is found only in east and north-east Africa and is known to be an agent of DCL.

A larger species group which causes "New World Cutaneous Leishmaniasis" can be subdivided, as only some of these can later produce MCL (5). *L. mexicana* does not cause MCL and has several distinct sub-species (sometimes treated as individual species) called *L. m. mexicana* , *L. m. garnhami* , *L. m. venezuelensis* , *L. m. amazonensis* and *L. m. pifanoi* . These are found in Mexico, Central and South America as well as in the southern states of the USA. *L. m. amazonensis* , *L. m. pifanoi* and *L. m. mexicana* can cause DCL while other *L. mexicana* species cause only cutaneous lesions. *L. braziliensis* has a number of sub-species some of which can cause MCL. *L. b. braziliensis* and *L. b. panamensis* are known to cause MCL while *L. b. guyanensis* is thought to only cause cutaneous infections. These species are found in Central and South America.

Although each parasite species will commonly cause the diseases outlined above, there are reports of certain species causing clinical disease outwith the normal groupings. There are reports of cutaneous disease caused by *L. chagasi* which will usually only transmit visceral infection (8). Conversely there are reports of visceral disease caused by *L. tropica* in Gulf War veterans with no indication of any cutaneous lesions (9). This particular disease is termed viscerotropic leishmaniasis. These data

indicate that parasite species are not always restricted to the classifications outlined above. The factors responsible for disease outside the normal species restrictions are not known but the immune status of the host may be critical in this regard (10).

1.1.3 Life cycle in sandfly

Leishmania species are all symbiotic, inhabiting the macrophages of vertebrates and the gut of sandflies during their lifecycle (6). They exist as two morphologically distinct forms, the promastigote and amastigote, corresponding to which of the two hosts they happen to be in. The promastigote is the parasite form found in the sandfly and is an extracellular, long, thin, flagellate protozoan. The amastigote, within a host macrophage, is smaller, round and has no flagellum.

The promastigote stage of the *Leishmania* spp. inhabit female sandflies of the genus *Phlebotomus* (Old World) or *Lutzomyia* (New World). The parasite exists within the sandfly gut, attached to midgut epithelial cells via the surface glycoconjugate Lipophosphoglycan (LPG), which has affinity for lectins expressed on the surface of these cells (11). These parasites divide rapidly, are non-infective and are termed procyclic promastigotes (12). In addition to providing a means of attachment to gut cells, LPG, which forms a dense glycocalyx over the entire promastigote surface may protect the parasite from enzymatic damage within the insect gut (13). A dense carbohydrate gel is also secreted from the promastigote, which may provide a physical barrier between the parasite and the gut environment (14).

LPG has four defined structural domains, consisting of a phosphatidylinositol lipid anchor, a phosphosaccharide core, a number of repeating oligosaccharide units and a short oligosaccharide cap. The lipid and core structure are conserved between species (15) whereas the repeating and cap oligosaccharides vary in size and number (16). In a process termed metacyclogenesis (17), the LPG on the surface of *L. major* undergoes a biochemical transformation which involves a doubling of the number of

repeating oligosaccharide units and a change in the terminal oligosaccharide side-chains from β-galactopyranose to α-arabinopyranose (18). This maturation allows the procyclic to detach from the lectins on the gut cell surface. *L. donovani* procyclics undergo a similar conformational change which allows detachment from the gut cell but without any alteration in the terminal saccharide units (19).

After detachment from the mid-gut, the mature, metacyclic forms migrate to the sandfly proboscis, a process which may involve parasite-mediated damage to the cardiac valve which then allows entry into the pharyngeal pump (20). The multiplication and maturation process of the parasite within the sand fly takes around 7-10 days as parasites harvested from infected flies are non-infective until around this time (12).

1.1.4 Mammalian life cycle

1.1.4.1 Infectivity

Metacyclic promastigotes do not divide and are highly infective (12). The expression of the mature form of LPG correlates well with the infectivity of promastigotes (21), and parasites devoid of surface LPG recover their virulence with the addition of purified LPG molecules (22). While other surface molecules such as the promastigote surface protease (PSP or gp63) have also been implicated in parasite virulence (23), this depends on the species of parasite studied. In New World species gp63 levels correlate well with infectivity (23) whereas in Old World species such as *L. major* no such correlation is detected (21).

LPG is thought to confer infectivity to the promastigote by nature of its dense elongated capsule-like structure which covers the entire parasite surface (16). This has been shown to prevent complement mediated damage to the parasite surface membrane (24). Procyclic promastigotes are extremely sensitive to complement lysis via the

alternative pathway when placed in the presence of serum (25). Metacyclic promastigotes fix complement using the classical pathway of activation but are not lysed even by the formation of the membrane attack complex C5b-C9 (26).

Sandfly salivary gland substances, when co-injected with L. major promastigotes, can also increase the infectivity of the parasite (27). How this is mediated is unknown but constituents within sand fly salivary gland material have been shown to inhibit macrophage activation *in vitro* (28).

1.1.4.2 Macrophage entry

The mechanism of promastigote entry into macrophages is not clearly understood and a number of diverse receptor-ligand interactions between the promastigote and macrophage have been implicated in the process (29). PSP and LPG on the parasite surface, and Complement Receptors 1 and 3 (CR1 and CR3), the mannose-fucose receptor (MFR), the receptor for advance glycosylation end products (AGE), the fibronectin receptor (FNR) and the Fc receptor (FcR) on the macrophage have all been suggested to mediate parasite entry.

Complement binding of C3 by LPG or other surface molecules is thought to be important in targeting promastigotes to the CR1 receptor which will allow macrophage entry in the absence of a respiratory burst. In the presence of serum C3b binding to CR1 was shown to be the major interaction involved in binding of metacyclic promastigotes of *L. major* to human macrophages (30). C3b binding on the surface of *L. major* metacyclics also enhanced the subsequent intracellular survival of the amastigote compared with uptake in the absence of serum (31). While other receptor events have been implicated in parasite uptake, this work has not always involved the use of the infective, metacyclic stage and thus CR1 is the most likely mechanism of entry.

Another complement receptor, CR3, has been implicated in macrophage entry but this pathway is believed to activate the respiratory burst and therefore is unlikely to be involved (32). In the initial cloning of gp63 there was predicted to be an RGD sequence (33) which is a sequence known to be used in the recognition of ligands by integrin receptors (34). However, a corrected gp63 sequence contained no RGD motif (35), making the interpretation of experiments describing the involvement of an RGD sequence in the binding to CR3 difficult (36). It is clear, however, that in the absence of serum, gp63 can participate in macrophage binding, as gp63 coated liposomes were engulfed by macrophages ten times more efficiently than uncoated liposomes (37).

In addition to complement receptors, a number of other candidate surface molecules on the macrophage have been examined. Experimental evidence suggests that the mannose-fucose receptor (MFR) may mediate parasite entry due to the ability of excess mannose to inhibit parasitisation of macrophages *in vitro* (37). The glycosylated structures on the promastigote surface do contain considerable quantities of mannose. However, the MFR elicits an oxidative burst after ligation and therefore would seem unlikely to offer a safe means of entry to the promastigote (38).

The fibronectin receptor is able to mediate parasite attachment to macrophages (39). However, regulation or blocking of this receptor failed to affect parasite binding to macrophages (36).

Other receptors such as the receptor for advanced glycosylation end products (40) and the Fc receptor (41) have been suggested as macrophage receptors for leishmania entry but are less well studied. A role for the FcR in a primary leishmania infection would require the presence of parasite specific antibodies which seems unlikely. There are a number of other parasite membrane proteins with unknown functions, such as PSA-2 (42) and gp46 (43), which may also have an as yet unrecognised involvement in macrophage parasitisation by *Leishmania*.

1.1.4.3 Intracellular survival

After internalisation, the promastigote transforms into an amastigote within the phagolysosome. Here, the amastigote is able to resist enzymatic degradation and an acidic pH of around 4.7-5.2 (44). The LPG molecule on the amastigote surface may provide protection against this environment, as LPG deficient strains of *L. major* are killed within 18 hours after internalisation (22). However, unlike the promastigote, the LPG structure present on amastigotes does not form a dense glycocalyx (45) and therefore it may be that other surface molecules are important in allowing survival under these conditions. It has been postulated that gp63 might degrade lysosomal enzymes due to its protease activity (46). This is unlikely, as gp63 is substantially down-regulated during the amastigote stage of the parasite (47). The amastigote, within the phagolysosome, divides by binary fission, is released when the macrophage lyses and in turn enters other macrophages to multiply. The receptors used for entry of the amastigote stage into macrophages are not well understood. Amastigotes, purified from lesions contain surface bound immunoglobulin and complement component C3b on the surface, suggesting that they may utilise the FcR or CR1 on the macrophage in order to gain entry (48). The parasite life cycle in the vertebrate host is completed when a subsequent sandfly ingests an infected macrophage or free amastigote, in the process of taking a blood meal.

1.1.4.4 Persistence of infection

There is a considerable amount of evidence which suggests that *Leishmania* parasites can establish a life-long persistent infection, which is usually asymptomatic, after cure of a primary infection. Persistence of parasites after successful treatment of visceral leishmaniasis in humans has been reported (49). A detailed study of 23 different mouse strains detected viable parasites in mice one year after cure of cutaneous disease (50). The increasing incidence of leishmaniasis in HIV-infected patients may represent, in part, a reactivation of latent infection as the host becomes

immunosuppressed (51). The host cell involved in this chronic parasitisation may not necessarily be the macrophage, as recent reports have documented the presence of *L. major* in lymph node dendritic cells during persistent infection (52). The ability of Leishmania parasites to persist in recovered patients may have considerable implications with regard to the generation of a vaccine against disease, as disease-cured individuals may represent a reservoir of infection.

1.2 Genetic regulation of leishmaniasis

1.2.1 Human infection

Little is known of the genetic control of leishmaniasis in human infection but it is clear that genetic factors are involved in the determination of susceptibility and resistance to disease. In a study of mucocutaneous leishmaniasis within two racially distinct communities in Bolivia, the severity of pathology associated with infection and the immune responses to parasites were different (53). The native Indian population had mainly chronic slow-growing, non-destructive lesions with low DTH responses to parasite antigens, whereas the Negro population had rapidly growing, mucocutaneous lesions which resulted in severe damage to the face, in the presence of strong DTH responses. Much of the data on the genetic control of *Leishmania* infection comes from studies of experimental models of infection in mouse models.

1.2.2 Murine *L. donovani* infection

The genetic factors responsible for resistance and susceptibility to *L. donovani* infection are well understood. There are two major stages of disease with different genes involved in the regulation of each (54).

During the early stages of *L. donovani* infection, resistance to infection is controlled by a non-MHC linked gene called *Lsh* which is found on mouse

chromosome 1 (55). The early control of *S. typhimurium* and *Mycobacterium* infections in mice is thought to depend on the same gene although in these models it is termed *Ity* or *Bcg* (56, 57). The *Lsh* gene product may therefore represent a common innate factor involved in the defence against intra-macrophage infection. Mouse strains expressing the Lsh^s allele are profoundly deficient in the control of initial parasite replication within the macrophage, which takes place in Lsh^r mice and is T-cell independent (58). The *Lsh* gene was recently cloned (59) and the encoded product was found to be structurally similar, but not identical, to a nitrate transporter encoded by the *CrnA* gene of *Aspergillus nidulans* (60). As resolution of *Leishmania* infection is dependent upon the production of reactive nitrogen intermediates (61, 62, 63), the function of the *Lsh* gene product may be in the transport of nitrates into the phagolysosome which mediates the control of infection. Mouse strains which are Lsh^s have a single base pair change from the Lsh^r gene which may cause the generation of a non-functional gene product, thus preventing *Leishmania* killing (59).

The later phase of murine *L. donovani* infection (> 2 weeks) in mice which are Lsh^s is controlled mainly by MHC-associated genes (64) and also by genes at the H-11 (65) and Ir-2 (66) positions. Three different disease profiles controlled by MHC-associated genes are detected in Lsh^s mice, according to the parasite burdens in the liver (64, 67). Mice of $H\text{-}2^s$ or $H\text{-}2^f$ haplotypes cure disease early, mice of $H\text{-}2^b$ haplotype cure eventually, while mice of $H\text{-}2^d$, $H\text{-}2^q$ or $H\text{-}2^f$ haplotype do not cure. Alleles at the H-11 or Ir-2 locus can also determine the outcome of *L. donovani* infection. Expression of one allele at H-11 converts the normally cure strain, B10, to a non-cure strain (65). Expression of this allele of H-11 has a similar effect on *L. major* infection (65), making H-11 the only gene known to affect both *L. donovani* and *L. major* infection. In a similar manner to H-11, expression of an alternative allele of Ir-2, called $Ir\text{-}2^b$, can convert B10.LP-$H\text{-}3^b$ mice from cure to early cure phenotype (66).

1.2.3 Murine *L. major* infection

Unlike *L. donovani* , murine *L. major* infection has no discernible innate and acquired phase of immunity and is completely unaffected by the expression of *Lsh* gene alleles (68). MHC-associated effects are also difficult to demonstrate in *L. major* infection as mice of similar H-2 haplotypes can be resistant or susceptible depending upon the background strain of the animal (69). BALB/c and BALB/b mice are very susceptible to experimental cutaneous leishmaniasis and will ultimately succumb to fatal disease. This extreme susceptibility maps to a single gene (70), sometimes refereed to as the "Susceptibility to Cutaneous Leishmaniasis" gene (Scl-1), which has been mapped to chromosome 11 (71).

1.3 Immunology of leishmaniasis

Many of the immune events involved in the resistance and susceptibility of mouse strains to *Leishmania* infection have been elucidated in the past few years (69). The immunology of leishmaniasis is the best understood of all parasitic infections and experimental murine leishmaniasis is a recognised model system for the study of the differential activation of T-cell subsets in infectious disease.

1.3.1 Human infection

It has long been known that individuals who recover from natural *Leishmania* infection are immune to further challenge (72, 73). In a large study of *L. tropica* reinfection, the success rate of experimental infection was reduced from 64% to 6% if the patient had experienced a previous natural infection (74). However, if rechallenge with parasite is attempted as a primary lesion is actively progressing, an exacerbation of disease is noted (74). Previous natural infection with one parasite species also provides protection against other species of *Leishmania* parasites (75). The mechanism of protective immunity in these individuals is not clear. What is known is that cell-

mediated and humoral responses can be demonstrated in infected and cured patients and that these responses vary according to disease type.

1.3.1.1 Cutaneous leishmaniasis

Recovery from cutaneous Leishmaniasis in humans is characterised by the development of a strong cell-meditated immune response to parasite antigens as well as a more variable humoral response. A strong DTH responses is generated to killed parasites injected into the skin (76) and peripheral blood lymphocytes proliferate *in vitro* to leishmania antigens (77). Where parasite specific antibody has been detected, the titre has been directly linked to the disease severity (78, 79).

Diffuse cutaneous leishmaniasis (DCL) is characterised by the absence of a DTH response to parasite antigens, which is antigen specific, and low numbers of lymphocytes within the lesions (80, 81). It has been suggested that the development of DCL may be due to a failure of antigen presenting cells to deliver necessary accessory signals to T-cells during the early stages of infection (82). This may account for the parasite specific anergy observed in this disease and the inability to contain parasite replication.

1.3.1.2 Mucocutaneous leishmaniasis

The lesions of MCL patients contain very few parasites and there is a strong DTH response to parasite antigens which is elevated above levels seen in cutaneous patients (83). The factors responsible for the presence of a strong cellular response in the virtual absence of parasites are unknown.

1.3.1.3 Visceral leishmaniasis

L. donovani infection in humans is associated with strong humoral responses to the parasite and a defective cell-mediated response. High titres of specific antibody can be detected in *L. donovani* infected individuals (84, 85) and elevated levels of non-specific IgM due to polyclonal B-cell activation (85, 86). Patients with *L. donovani* infection do not develop a DTH response to parasite antigens (10, 85) and peripheral blood lymphocytes from these patients are only weakly responsive to parasite antigens *in vitro* (87). Indeed, T-cell responses to mitogens is also impaired in visceral patients (86), suggesting that a wide-ranging immunosuppression occurs. Responsiveness to mitogens and *Leishmania* antigens is recovered after successful drug treatment (73)

1.3.1.4 Human T cell subsets

Murine T-cells have been shown to polarise into distinct T-cell subsets defined by differential cytokine production (88). Th1 cells secrete IFN-γ and IL-2 upon T cell receptor (TcR) ligation and are thought to control cell-mediated functions such as DTH which are important in the control of intracellular pathogens (89). Th2 cells which secrete IL-4, IL-5 and IL-10 upon antigen stimulation control resistance to extracellular pathogens by stimulating humoral immune functions and eosinophilia (89).

It is clear that human T-cells can polarise under certain conditions to generate T-cell subsets analogous to those found in the murine system (90). A polarised T-cell response can be demonstrated to occur *in vivo* in chronic disease states such as leprosy (91). It has become increasingly apparent that this model of polarised T-cell differentiation can be found during human leishmaniasis.

Peripheral blood lymphocytes from patients who have cured an *L. major* infection produce IFN-γ but no IL-4 in response to parasite antigens (92). The

production of IFN-γ *in vitro* by human lymphocytes can activate macrophages to kill parasites *in vitro* (93).

The absence of IFN-γ production by lymphocytes from seroconverted children living in an endemic region of Brazil was a reasonable predictor for the development of visceral disease (94). In addition, patients suffering from visceral leishmaniasis have elevated levels of IL-4 detectable in serum (95). Thus, during visceral disease a predominant Th2 response is manifest. However, lymphocytes from cured visceral patients produce both IFN-γ and IL-4 *in vitro* in response to parasite antigens (96) and human T-cell clones from cured visceral patients were shown to secrete IFN-γ, IL-4 or both when stimulated *in vitro* with antigen (97).

Thus, the presence of IFN-γ correlates with cure of both cutaneous and visceral disease while IL-4 is predominant during active visceral disease. This polarised Th1 and Th2-like cytokine production bears striking similarity to the murine model of *L. major* infection.

1.3.2 Experimental murine leishmaniasis

As mentioned above, most mice infected with *L. major* will develop an ultimately self-healing lesion around the inoculation site and will remain refractory to further challenge with this parasite. Mice on a BALB background are extremely susceptible to *L. major* infection and develop a non-healing lesion and ultimate visceral infection. The factors involved in the resistance and susceptibility of different mouse strains to cutaneous infection with *L. major* and *L. tropica* have been studied in detail as a model of the spectrum of disease seen in human infection with leishmania.

1.3.2.1 Cellular v's humoral immunity

A vast amount of data would indicate that recovery from *L. major* infection in the mouse is dependant upon the development of cell-mediated immunity, while humoral responses are of little importance (69, 98, 99, 100).

Anti-leishmanial antibodies can participate in complement mediated lysis of *L. donovani* promastigotes grown *in vitro* (101) and can also enhance parasite uptake by macrophages *in vitro* (102). F_{ab} fragments of anti-LPG monoclonal WIC-79.3, which bind to the parasite surface, have been shown to prevent the establishment of *L. major* infection in susceptible BALB/c mice when coadministered with a low parasite dose (103). Similarly, other leishmanial-specific monoclonal antibodies could protect against *L. mexicana* and *L. major* infection if administered with a low dose inoculum (104, 105). In addition to these data demonstrating a protective effect mediated by monoclonal antibodies, protective immunity mediated by vaccination with promastigote surface protein gp46 was shown to correlate with levels of antibody to this protein (106)

While these data would suggest a protective role for humoral responses against leishmaniasis there is much evidence to the contrary. Antibody levels or isotype differences in murine cutaneous infection of different mouse strains do not correlate with resistance to disease (107). Passive transfer of serum or antibody fractions from immune mice do not affect infection of BALB/c mice (108). In addition, Biozzi AB/L mice which have defective antibody responses are resistant to *L. major* infection (109). B-cell depletion in resistant mice was reported to exacerbate disease which suggests a functional role for B-cells in the development of protective immunity (110). However, resistance to infection could be restored in this model by T-cells alone, implying that T-cells are critical in determining disease outcome. Furthermore, later attempts at B-cell depletion have failed to influence the outcome of infection in resistant mice (111).

Depletion of B-cells in BALB/c mice reduced lesion sizes suggesting that B-cells are in fact counter-productive to the development of protective immunity (112).

From these data there would appear to be little role for antibody in determining the outcome of cutaneous leishmaniasis in the murine model. The mechanism by which monoclonal antibodies can mediate protection *in vivo* is unclear. It has been suggested that the monoclonal antibodies or F_{ab} fragments to surface molecules on the promastigote may simply hinder the promastigote from attaching to macrophages and in doing so prevent infection. In accord with this, a lowering of antibody dose, or raising of parasite numbers administered, was shown to ablate the protective effect observed with this treatment (103).

Cellular immune responses have been demonstrated to mediate protection against primary infection in resistant mice and vaccinated susceptible mice and also in secondary infection of susceptible mice cured by immunological intervention.

Athymic nude mice (nu/nu), which are T-cell deficient, are susceptible to *L. major* or *L. tropica* infection even on a resistant CBA or C57BL/6 background (113, 114). Adoptive transfer of syngeneic T-cells to these mice restores the ability to resist infection (114). Resistant mice, rendered T-cell deficient by thymectomy and irradiation also demonstrate a delay in cure of infection (115). Protective immunity can be transferred to syngeneic naive mice by T-cells from resistant mice which have cured a primary infection (116). Similarly, T-cells from susceptible mice which have been immunised against infection can transfer protection to naive recipients (117). As mentioned previously, the exacerbation of infection in resistant C3H mice by anti-μ antibody treatment is reversed by transfer of T-cells alone (110). These data suggest a critical requirement for T cells in the generation of immunity to experimental leishmaniasis.

1.3.2.2 $CD4^+$ T-cells in resistance

The expression of CD4 or CD8 molecules on the surface of T-cells after thymic development separates most T-cells into two separate lineages. Reconstitution of T-cell deficient mice with $CD4^+$ T-cells alone is sufficient to restore the resistance to disease observed with unfractionated T-cell transfer, as outlined above (114, 118). $CD4^+$ cells from resistant mice which have resolved a primary infection can also transfer immunity when administered to syngeneic recipients (116, 117, 119).

The susceptibility of BALB/c mice can be overcome by sub-lethal whole body γ-irradiation (120), injection with anti-CD4 monoclonal antibody (121) or cyclosporin A treatment (122). After the infection resolves, these mice are refractory to further leishmania challenge and mount a strong DTH response when injected with killed parasites (123). $CD4^+$ T-cells from these mice can transfer this DTH response and resistance to *L. major* infection if administered to naive susceptible mice (117, 123).

Resistance to infection can also be conferred to BALB/c mice by certain immunisation protocols. Repeated intravenous or intraperitoneal injections of irradiated, heat-killed or sonicated promastigotes enables these mice to survive subsequent challenge infection (124). This immunity from infection could be transferred to naive BALB/c mice by transfer of the $CD4^+$ T-cell population only (117). However, these cells are distinct from the population which transfer protection after cure of infection as they do not mediate DTH responses to parasite antigens. Indeed, these cells can suppress the induction of DTH responses in cured mice (125). In summary, it appears that $CD4^+$ T cells are required for resistance to cutaneous leishmaniasis and for the induction of protective immunity in susceptible mouse strains.

1.3.2.3 $CD4^+$ cells in susceptibility

The intrinsic susceptibility of BALB/c mice to *L. major* infection can also be attributed to $CD4^+$ T-cells. Early work in this area demonstrated that sublethally irradiated resistant mice are not able to clear infection if they are reconstituted with haematopoietic cells derived from naive susceptible mice (126). As already discussed, BALB/c mice are not intrinsically unable to mount an effective immune response as they can be induced to cure infection through various immunological interventions (120, 121, 122). The protective effect of such therapy can be overcome by transfer of naive susceptible T-cells (120). Therefore, not only do T-cells from susceptible mice fail to mediate cure but they can also suppress an ongoing protective response. These suppressive T-cells were also found to reside within the $CD4^+$ compartment of the T-cell population (119). In addition, $CD4^+$ T-cell lines derived from BALB/c mice were shown to be able to suppress the generation of protective immunity in resistant (127) and immunised susceptible (128) mice.

Certain immunisation protocols can also cause disease exacerbation. Injection of killed *L. major* parasites by intravenous or intraperitoneal routes provide BALB/c mice with protection from challenge infection (124), as previously discussed. However injection of the same antigen preparation by the subcutaneous route caused exacerbation of infection (129). T-cells from such immunised mice could also suppress the protective immunity induced in mice immunised intravenously or intraperitonealy (130). When analysed further the cells mediating this anti-protective effect were found to be $CD4^+$ T-cells which had the ability to mount a Jones-Mote DTH response, which peaks around 12-15 hrs rather than the classical 24-48hr, to parasite antigens (130). Thus both susceptibility and resistance are determined by a subset of T-cells which are $CD4^+$. These T-cells were thought to represent different T-cell lineages which either mediated effector function or suppressor function. The induction of either subset of $CD4^+$ T-cells was critically important in determining the outcome of disease.

1.3.2.4 $CD4^+$ T cell subsets

It was demonstrated by Mossman *et al* (88) that $CD4^+$ T-cells can be divided into at least two subsets according to lymphokine secretion. T-cells which respond to antigen to secrete IL-2, IFN-γ and TNF-β (Lymphotoxin) are labelled Th1 cells while cells which release IL-4, IL-5 and IL-10 are termed Th2 cells (89). In addition to the non-overlapping secretion of the products mentioned above, a number of lymphokines are also secreted by both subsets, including IL-3, GM-CSF and TNF-α (89). Th1 cells are thought to mediate cellular immune functions such as DTH (131), while Th2 cells provide efficient help for the generation of humoral responses (132). Th1 cells can in fact participate in the provision of B-cell help for antibody production but differ from Th2 cells in the selection of IgG isotypes as Th1 cell responses induce IgG2a responses, while Th2 cells select for IgG1 isotypes and IgE (133).

The demonstration that T-cell subsets exist *in vitro* was shortly followed by data implicating these in the cure or progression of experimental *L. major* infection (134, 135). In infected, resistant C57BL/6 mice, $CD4^+$ T-cells in the spleen and lymph node produce elevated levels of mRNA for IFN-γ and IL-2 compared to susceptible BALB/c mice (135). Conversely, $CD4^+$ T-cells from BALB/c mice during infection produced IL-4 and IL-10 mRNA in the spleen and lymph nodes while C57BL/6 mice had undetectable levels of these cytokines (135). Serum levels of IgE were also elevated in infected BALB/c mice (134), as expected from data implicating IL-4 in the control of IgE isotype switching (136). Thus, $CD4^+$ T-cells were demonstrated to develop polarised Th1 or Th2 responses *in vivo* in response to *L. major* infection, accounting for the ability to transfer protection and suppression with a $CD4^+$ population as discussed previously.

That Th1 and Th2 cells can control the response to experimental *Leishmania* infection has been well documented by the transfer of T-cell lines and clones that express functional differences in cytokine secretion after TcR ligation. Transfer to

BALB/c mice of T-cell lines which responded to *L. major* antigen *in vitro* to secrete either IL-2 and IFN-γ or IL-4 resulted in protection or exacerbation respectively (137). Similar T-cell transfer experiments in severe combined immunodeficient (SCID) mice resulted in an identical transfer of protection with Th1 lines or exacerbation with Th2 lines (138). In addition, a T-cell clone which recognises a soluble *L. major* antigen to secrete IL-2 and IFN-γ but not IL-4 or IL-5 can transfer protection to BALB/c mice (139). However, a further T-cell clone which can transfer exacerbation of infection to BALB/c mice was found to secrete IFN-γ and IL-2 in response to parasite antigen (127). Therefore, while much of the available data would point to a protective role for Th1 cells in leishmaniasis there is evidence to suggest that at least some Th1 cells may in fact participate in the development of progressive disease.

1.3.2.5 Cytokines

In addition to the cells themselves, the role of the various cytokines secreted by T-cell subsets in protection and exacerbation of disease has received much attention.

The importance of IFN-γ in resistance to *L. major* infection has been demonstrated by cytokine depletion *in vivo* using monoclonal antibodies. C3H/HeN mice which normally resolve *L. major* infection are unable to control infection after weekly administration of a neutralising IFN-γ monoclonal (140). A similar result was obtained by infection of resistant mice which have a targeted disruption in the IFN-γ gene (141). These animals are unable to control lesion development after *L. major* infection, indicating that IFN-γ is necessary for cure of experimental leishmaniasis. Interestingly, these mice, in the absence of IFN-γ spontaneously generate a Th2 response to infection, suggesting that Th2 responses in these mice can occur but are regulated by IFN-γ. While it is clear therefore, that IFN-γ is required to resolve infection in resistant mice, the administration of IFN-γ to susceptible BALB/c mice is insufficient to generate protective immunity (142, 143). Delivery of IFN-γ at the time of infection can modulate T-cell subset development but does not affect the eventual

outcome of disease (143). Sustained IFN-γ delivery to macrophages *in vivo* by *L. major* parasites themselves is also unable to change the course of infection (144).

The role of another Th1 cytokine, IL-2, in protection and susceptibility to infection has also been examined. While IL-2 is not produced by Th2 cells, its presence is required for the development of Th2 responses *in vitro* (145) and *in vivo* (146). Neutralisation of IL-2 in susceptible BALB/c mice at an early stage in infection is sufficient to prevent progressive disease (147). This neutralisation of IL-2 correlated with a reduction in mRNA for IL-4 and an upregulation in IFN-γ in the draining lymph node. Thus the protection mediated by anti-IL-2 therapy may be due to the control of IL-4 production at disease initiation. Administration of anti-IL-2 to resistant, C57BL/6 mice did not affect lesion development or cure from disease (147) implying that IL-2 has little functional significance in resistant mice during infection. Administration of exogenous IL-2 to resistant mice during infection was reported to exacerbate disease which was mediated by direct effects of IL-2 upon parasite growth (148).

While IFN-γ is necessary but is not sufficient for protective immunity to *L. major* there is evidence for a more substantial role for IL-4 in the generation of non-healing responses. Susceptible BALB/c mice, administered with a monoclonal antibody to IL-4 during early infection cure from disease completely (142). This cure correlated with the production of Th1-like cytokines in the draining lymph node. However, the intraperitoneal injection of recombinant IL-4 to resistant C3H/HeN mice was reported to be insufficient in the generation of susceptibility although the normal Th1 response was curtailed (149). Delivery of recombinant IL-4 to the lesion site in BALB/c mice was shown to exacerbate disease progression when administered at an early stage of infection (150) while delivery of IL-4 after lesion development resulted in the reduction of lesions (150, 151). While these data are inconclusive in supporting the sufficiency of IL-4 in the generation of Th2 responses, the role of IL-4 has been further explored using transgenic animals. Transgenic mice on a resistant, 129/Sv, background which over-express IL-4 in the B-cell compartment were shown to be

difficient in controlling *L. major* infection (152). Thus IL-4 is necessary and sufficient to generate a non-healing response in experimental leishmaniasis. In addition, a study of several mouse strains which exhibited varying degrees of resistance to *L. major* infection, the expression of IL-4 mRNA correlated well with the severity of lesion development (153). Conversely, the ability to express IFN-γ mRNA in response to infection did not correlate well with resistance. Thus, the Th2 cytokine IL-4 is dominant over IFN-γ production and seems to control lesion development.

IL-10, another Th2 cytokine may be involved in the regulation of immunity to *L. major in vivo* . As already mentioned, mRNA for IL-10 is found in the draining lymph nodes of susceptible but not resistant mice during infection (135). However, the neutralisation of IL-10 production during *L. major* infection of BALB/c mice, did not affect the progression of disease (154).

In summary, it has been demonstrated that the generation of resistance or susceptibility to *L. major* infection is largely dependant upon the production of Th1 or Th2 cytokines with a particular requirement upon the level of IFN-γ in the cure of infection and the production of IL-4 in mediating susceptibility.

1.3.2.6 Killing of *Leishmania*

T-cell derived cytokines directly control parasite replication due to their macrophage activating and deactivating properties. IFN-γ mediated macrophage activation causes the upregulation of surface molecules such as MHC II, as well as the synthesis and secretion of a number of new gene products. It has been demonstrated that IFN-γ can participate in the activation of parasitised macrophages to kill *L. major* (155) and *L. donovani* (156) *in vitro* . Killing requires the production of reactive nitrogen intermediates (RNI), such as nitric oxide (NO), via a newly-synthesised cytosolic protein called inducible nitric oxide synthase (iNOS). The production of NO is catalysed by the iNOS enzyme in a reaction involving the conversion of L-arginine

and molecular oxygen to NO and L-citrulline (63). The ability of activated macrophages to kill amastigotes *in vitro* and *in vivo* correlated with the production of NO (157, 158). The importance of NO in macrophage killing of *Leishmania* was demonstrated by the inhibition of iNOS using arginine analogues, such as L-N^G-monomethyl-arginine (L-NMMA) (158).

Killing of parasites after induction of NO by IFN-γ involves macrophage TNF-α production as an antibody to TNF-α can abrogate such leishmanicidal mechanisms (159). Furthermore, TNF-α can synergise with IFN-γ to induce NO production (160, 161). Indeed, cytokines which cause TNF-α production by macrophages during activation with IFN-γ, such as IL-3 (162), IL-4 (163) and IL-7 (164) can all synergise for the induction of parasite killing *in vitro* . The importance of TNF-α in mediating parasite destruction *in vivo* was confirmed by studies demonstrating that delivery of recombinant TNF-α to infected mice reduced lesion development (165), while anti-TNF-α caused an exacerbation of disease (166), although the final phenotype of disease was not altered. It should be noted, however, that TNF-α in the absence of IFN-γ cannot activate murine macrophages to clear infection (160) and it thus plays a secondary role to IFN-γ. Mice with a genetic disruption in the p55 TNF-α receptor gene are unable to clear *L. major* and develop a chronic low-level infection (167). Therefore TNF-α is involved in parasite destruction *in vivo* and provides a synergistic upregulation of IFN-γ macrophage killing via NO induction.

Th2 cytokines such as IL-4 and IL-10 can down-regulate macrophage activation caused by IFN-γ and so restrict parasite killing. IL-4 can inhibit the expression of the iNOS enzyme and NO production in IFN-γ activated macrophages (168) although, as already discussed, the timing of IL-4 administration is critical in this process. IL-10 can also downregulate iNOS induction caused by IFN-γ stimulation (169) and has been shown to prevent the killing of *T. gondii* by activated macrophages via this mechanism (170). Other cytokines such as TGF-β have also been shown to inhibit macrophage activation (171) and intracellular killing of *L. major* (172) but the role of such

mediators in experimental infection is not clear. Thus, Th2 cytokines can participate in macrophage deactivation caused by the Th1 cytokine IFN-γ and so restrict parasite killing by inhibiting macrophage induction of NO. Th1 and Th2 cytokines also cross-regulate the growth and function of the opposing subset, providing another level of interface which determines the outcome of *L. major* infection.

1.3.2.7 Crossregulation of T cell subsets

Th1 cytokines have been shown to inhibit certain Th2 cell functions. In *in vitro* experiments, IFN-γ can inhibit the proliferation of Th2 cells to IL-2 or IL-4 (173, 174), but does not affect Th1 cells in this manner. This is now thought to derive from the differential expression of the IFN-γ receptor β-subunit on the surface of these cells (175), allowing Th2 cells to receive negative signals from IFN-γ. BALB/c mice, injected with both IFN-γ and *L. major*, had reduced production of IL-4 in draining lymph node cells compared to mice administered parasites alone (143) although this was insufficient to cause cure from disease. As previously noted, resistant mice, unable to produce IFN-γ make a functional Th2 response to infection (141). This default to the Th2 phenotype suggests that in normal mice, the presence of IFN-γ suppresses the induction of these cells. However, IFN-γ is insufficient to cause Th1-cell differentiation in TcR transgenic mice on a BALB/c background (176) implying that other factors may be important in this process.

Conversely, IL-10 is known to inhibit cytokine secretion by Th1 cells (177) and can also limit Th1 cell proliferation by indirect action on antigen presenting cells (178, 179). Thus, cytokines produced by Th2 cells can inhibit Th1 cell function, while Th1 cells can have a similar effect on Th2 cells. Therefore, the initial generation of either cell subset will inhibit the generation and function of the other, causing a polarised response to infection. The initial events which cause T-cell subset generation are therefore critical in determining the outcome of disease.

1.3.2.8 Induction of T cell subsets

Th1 and Th2 cell subsets are thought to derive from a common precursor cell, called Th0, which is present early during *in vitro* cultures (180) and can secrete a wide range of cytokines of both Th1 and Th2 types (181, 182). The generation of a Th1 or Th2 response from this initial precursor cell is thought to be determined by a number of factors (98), including the nature of the antigen, the lineage of antigen presenting cell involved, the local cytokine environment created by other cells and by the interaction of various costimulatory molecules between T-cell and APC. Recent developments in this field will be discussed later in more detail (See General Discussion).

In addition to the ability of IL-4 to modulate IFN-γ mediated macrophage activation, and participate in the induction of humoral responses, it can also select for the induction of Th2 responses. Thus, the presence of IL-4 in culture can cause uncommitted $CD4^+$ cells from pigeon cytochrome c-specific TcR transgenic mice to progress along the Th2 pathway (183). As already discussed, resistant mice with B-cells which overexpress IL-4 develop a Th2 response to *L. major* infection and fail to cure (152). When the early T cell response to *L. major* was examined in resistant and susceptible mice, both strains developed a mixed Th0 like cytokine profile (184). In resistant mice, IL-4 production was quickly reduced while in BALB/c mice this remained high (184). All immunological interventions which cause cure of *L. major* infection in a BALB/c mouse, such as sub-lethal irradiation, cyclosporin A treatment, anti-CD4, anti-IL-4 or anti-IL-2 monoclonal antibody administration are only effective if used during the first few days of infection, coinciding with the uncontrolled IL-4 peak (98). All are also thought to mediate cure from disease by the ability to limit this production of IL-4 during early disease, implying that the generation of a deleterious Th2 subset requires the presence of IL-4. In addition to these observations, mice with disruptions in the IL-4 gene are unable to mount a functional Th2 response and clear infection with *Nippostrongylus braziliensis* (185). Together these data suggest a functional role for IL-4 in the development of Th2 responses.

IL-12, a recently cloned heterodimeric cytokine (186) which causes T-cell and NK cells to proliferate (187) and secrete IFN-γ (188), has also been shown to be important in the generation of the induction of the Th1 cell subset (189). IL-12 is produced by macrophages in response to inflammatory stimuli and by certain B cell lines (190). When used *in vitro* , IL-12 can select for Th1 cell growth and suppress Th2 cell development from uncommitted murine T-cells (189). *In vivo* administration of IL-12 is sufficient to cure *L. major* infection of BALB/c mice when it is delivered early in infection (191, 192). As previously mentioned, administration of IFN-γ alone is not sufficient to cause cure from disease, suggesting that IL-12 mediates it's protective effects only partly through the induction of IFN-γ. The protective effect of IL-12 may be due to its effects upon IL-4 synthesis, as IL-4 production in these mice was also reduced during the course of treatment (192). The importance of IL-12 in experimental infection has been demonstrated in experiments where the neutralisation of IL-12 production in resistant, C57BL/6 mice rendered them unable to control the progression of disease (192). Thus in *L. major* infection, IL-12 is critically important in the induction of Th1 cells and subsequent protective immunity.

1.3.2.9 Other cell types

While there is much evidence supporting the role of $CD4^+$ T-cells in the control of experimental leishmaniasis, there is data to suggest that other cell types may also be involved during this process.

$CD8^+$ T-cells are thought to play a role in protective immunity to *L. major* infection, particularly in the resistance to a secondary infection. An increase in the number of lymph node $CD8^+$ T-cell occurs at the onset of lesion cure in resistant mice (193) and depletion of $CD8^+$ cells using monoclonal antibody treatment resulted in lesion exacerbation in both susceptible and resistant mice (194). However, the phenotype of these mice was unchanged, as resistant mice eventually cured infection

and susceptible mice failed to resolve lesions. In *L. major* reinfection of resistant CBA mice, depletion of $CD4^+$ and $CD8^+$ T-cells exacerbated disease markedly whereas depletion of either subset alone did not (195). Thus, there is evidence for a more substantial involvement of $CD8^+$ T cells in limiting secondary *L. major* infection. $CD8^+$ T-cells have been demonstrated to be able to mediate parasite killing *in vitro* via the production of IFN-γ (196) and subsequent macrophage activation to produce NO (197). *In vivo*, $CD8^+$ T-cells were shown to participate in the production of IFN-γ in responses to a secondary *L. major* infection (198). Although these data imply a protective role for $CD8^+$ cells in the control of lesion development, $CD8^+$ cells are unable to transfer protective immunity to nude mice (118). In addition, targeted activation of $CD8^+$ T-cells by MHC class I peptide vaccination and infection of β2-microglobulin-deficient mice, which have no functional $CD8^+$ T-cell responses, failed to substantiate a role for $CD8^+$ T-cell mediated protection (199).

A minor T-cell population which expresses a rearranged γδ T-cell receptor instead of an αβ receptor has also been implicated in protective immunity to experimental leishmaniasis. At least a proportion of these gamma/delta (γδ) T-cells are though to respond to restricted set of antigens (200) possibly through antigen presentation via the CD1 molecule, although the precise nature of antigen recognition by these cells is unclear. These cells have been shown to accumulate in the lesions of individuals with cutaneous leishmaniasis (201) and are elevated in the peripheral blood of patients suffering from cutaneous, mucocutaneous and visceral leishmaniasis (202). An increase in γδ cell numbers was observed in both BALB/c and CBA/J mice during infection which, if depleted using a monoclonal antibody, resulted in the development of larger lesions and increased parasite numbers (203). It has been recently demonstrated that these γδ cells can polarise to produce Th1 and Th2 like cytokine secreting subsets during *Listeria monocytogenes* or *Nippostrongylus braziliensis* infection (204). However, these subsets have not been investigated in *L. major* infection to date. As the majority of these γδ cells do not express CD4 or CD8 molecules (205) (although a proportion do express the CD8α molecule), and resistance

to experimental cutaneous leishmaniasis is transferable by $CD4^+$ cells alone (117, 118), they would appear to be of secondary importance.

Another cell population, the natural killer (NK) cell has received some interest with regard to its function in experimental *L. major* infection. Mice deficient in NK cell cytotoxicity, bg/bg mice, display no differences in the course of *L. major* infection compared to normal resistant mice (206). NK cell production of IFN-γ early during infection has been recorded and may be important, in addition to IL-12, in the development of Th1 cells (184). While NK cells, γδ T-cells and $CD8^+$ T-cells may participate in protection against *L. major* infection there is a crucial requirement for the $CD4^+$ T-cell in the generation of protective immunity.

1.3.2.10 *L. donovani* infection

The course of *L. donovani* infection in different mouse strains depends largely upon the expression of alleles of the *Lsh* gene, as previously discussed. While this model of infection differs in many respects to experimental *L. major* infection, the mechanisms of protective immunity are similar, with some notable exceptions. Cell mediated immunity is required for disease cure rather than humoral responses, as T cell depletion experiments show (207). T cells from mice which have resolved infection can also transfer resistance to naive hosts (208). However, the ability of nude mice to control *L. donovani* infection was only achieved by the adoptive transfer of both $CD4^+$ and $CD8^+$ T-cells and not either subset alone, implicating a more fundamental role for $CD8^+$ T-cells in cure of infection (209). In addition, differential Th1 and Th2 cytokine production is not believed to be responsible for cure and susceptibility in experimental murine *L. donovani* infection (210, 211). While there is a strong correlation between parasite killing and the induction of IFN-γ production (210, 211) there is no converse production of Th2-like cytokines in mice unable to clear infection (210). Rather T-cells from chronically infected mice develop an inability to produce Th1 cytokines as the disease progresses, with no concurrent rise in Th2 responses. This infection model

involves the intravenous injection of the amastigote stage of the parasite. The lack of functional Th2 responses may therefore be attributed to the route of challenge, as in *L. major* infection use of this route in vaccination preferentially stimulates Th1 responses (124). That Th1 responses are important in cure of disease in this model is seen by data demonstrating cure of established *L. donovani* infection in BALB/c mice by IL-12 treatment (212). Thus, although in murine *L. donovani* infection a clear Th1/Th2 paradigm is not observed treatment with a cytokine which induces Th1 responses is sufficient to cure disease.

1.3.3 T cell subsets in other parasitic infections

The understanding of experimental leishmaniasis as a disease caused by diverse T-cell responses to infection has led to the extrapolation of this concept to other parasite models with interesting results. Th1 and Th2 cell polarisation can be observed in other chronic parasitic infections, however, the nature of the parasite and it's localisation within the host determine which T cell subset provides protective immunity.

Another group of kinetoplastidid organisms, the Trypanosoma spp., some of which inhabit macrophages, cells of the central nervous system and muscle cells (6) also require the presence of Th1 cytokines to be killed. Cure from *Trypanosoma cruzi* infection is dependent upon a $CD8^+$ and a $CD4^+$ T cell responses (213), perhaps reflecting the additional requirements for non-macrophage parasite killing by $CD8^+$ cells. IFN-γ can mediate killing of *T. cruzi in vivo* (214) and *in vitro* (215) via macrophage induction of NO (216) which is inhibited by IL-10 or TGF-β (217). Susceptible, B6 mice have increased IL-10 production during *T. cruzi* infection compared to resistant mice, which can partially inhibit IFN-γ mediated macrophage activation (218). Thus the polarised Th1/ Th2 paradigm seems also to hold true in aspects of murine trypanosomiasis although a more defined requirement for $CD8^+$ T cells is observed.

Other protozoan infections such as malaria and toxoplasmosis, which have intracellular life-cycle stages (219, 220), also show evidence of Th1 mediated protection during experimental infection. Th1 cells are reported to be involved in the generation of immunity (221) and also in the generation of pathology during murine malaria (222). In *Plasmodium chabaudi adami* infection, a Th1 cell line can transfer immunity to nude mice (221), while in *P. chabaudi chabaudi* infection, transfer of a Th1 or Th2 clone can reduce parasitaemia (223). The protective role of these different T cell subsets may represent a requirement for diverse responses in the killing of different life cycle stages of the parasite. In *Toxoplasma gondii* infection in mice, both $CD8^+$ and $CD4^+$ cells are responsible for protective immunity and production of IFN-γ (224). IFN-γ can activate macrophages to kill resident parasites *in vitro* (225) which can be inhibited by IL-10 (170). Thus in other protozoan infections, there is seen to be an involvement of Th1 cells and/or Th1 cytokines in parasite clearance similar to that seen in *L. major* infection.

Chronic infection of mice with the trematode, *Schistosoma mansoni* cause the induction of a dominant Th2 response (226), particularly to the parasite egg (227). Protective immunity, due to vaccination with irradiated cercariae is mediated by a Th1 response (226). Inhibition of Th2 responses using anti-IL-4 and anti-IL-5 failed to affect immunity to *S. mansoni* (228) indicating that the Th2-type effector mechanisms which can kill *S. mansoni in vitro* (229) are not required *in vivo* to clear infection. However, there is evidence that a Th1 response, TNF-α production and macrophage activation can also cause pathology in the form of granulomas during schistosomiasis (230). It is worth noting that murine infection with *S. mansoni* does not mirror human schistosomiasis, as correlates of protective immunity in human studies suggest the involvement of Th2-like mechanisms (231). However, it is clear that in experimental murine schistosomiasis Th1 mechanisms can mediate protective immunity.

The murine model of human trichuriasis involves infection with *Trichuris muris*, a nematode which lives in the large intestine (232). The resistance of inbred mouse strains to *T. muris* infection correlates with the ability to generate a Th2 response to parasite antigens (233, 234). Resistance is associated with the expansion of $CD4^+$ cells which can secrete IL-4, IL-5 and IL-9 (233) and this resistance can be inhibited by anti-IL-4 monoclonal antibodies (235). In susceptible mice which maintain a chronic infection, there is elevation of IFN-γ production while resistance can be achieved by administration of IL-4 or a neutralising antibody to IFN-γ (235). Thus, in response to this extracellular, multicellular organism a Th2 response mediates effective clearance. The involvement of a protective Th2 response has also been documented in response to other worms such as *Nippostrongylus braziliensis* (236), *Heligmosomoides polygyrus* (236) and *Trichinella spiralis* (237).

Thus, the regulation of immunity by Th1 and Th2 cells is not limited to models of leishmaniasis but has a wider relevance in the control of many parasitic infections, although the nature of a protective response may vary between parasite species.

1.3.4 T cell subsets in bacterial and viral infections

It is not only in chronic parasite infections that T-cell polarisation is observed to occur, as Th1 cell responses have been demonstrated to be of importance in intracellular bacterial and some viral infections. Mice with targeted disruptions in IFN-γ or IFN-γ receptor genes demonstrate exacerbated infection with the intracellular bacteria *Listeria monocytogenes* (238), *Mycobacterium bovis* (239) and *M. tuberculosis* (240). These experiments implicate a functional requirement for IFN-γ mediated macrophage activation in the response to intra-macrophage microbial pathogens which is thought to be mediated by Th1 cells (241). In human tuberculoid and lepromatus leprosy Th1 or Th2 cytokines can be detected in lesions (91) and Th1 and Th2-like clones can be found in the peripheral blood (242). The polarisation of these T cell subsets is thought to be involved in the spectrum of pathology associated

with this infection. In viral infection of mice with Respiratory Syncytial Virus (RSV), T-cell clones have been generated from infected mice which display distinctive T cell polarisation (243). These Th1 or Th2-like cell clones can transfer pathology to naive, resistant mice upon infection with RSV (244). While certain bacterial and viral pathogens do therefore elicit T cell subsets during infection the importance of these in mediating protection or pathology and the requirement for other cellular responses, such as antibody or $CD8^+$ T cells varies between organisms.

1.4 Vaccination against leishmaniasis

The generation of a vaccine against leishmaniasis seems theoretically possible, as patients recovering from infection remain refractory to further challenge (72, 73). However, the creation of a safe, effective vaccine against this parasite has to date proved elusive, although a number of important developments suggest that this may still be achieved. Much of the information regarding vaccination against leishmaniasis has been gathered from studies in the murine model of *L. major* infection, as outlined above, although there have been attempts to vaccinate human populations.

1.4.1 Human vaccination

The only vaccination strategy which has provided effective protection against leishmaniasis in humans is termed "leishmanization" and involves the low dose injection of a live cutaneous species (245) . Leishmanization has been carried out for many years in the Middle East and the southern Soviet Republics (74) and formed the basis of a large vaccination program in Iran in 1982 (246). This method of vaccination is certainly effective, providing protection to around 90% of those injected (74, 246), but is extremely unsafe. Some vaccinated individuals develop large, painful, slow healing lesions which may become sites of secondary infection or may adversely affect other skin conditions (247). The ability to resolve cutaneous lesions can be lost in immunosuppressed individuals, such as HIV infected patients (51), and can depend

also upon the nutritional state of the person at the time of infection (248). This process has been curtailed in the Soviet Republics and Israel due to these safety issues.

The use of an attenuated strain of *L. donovani* as a live vaccine did not provide any detectable resistance to further infection as the incidence of infection was similar in the treated and untreated populations (249). Some investigators have suggested that the failure of this vaccine resulted from the inability of the strain to develop an initial cutaneous lesion which usually correlates with the generation of immunity (247).

A killed vaccine preparation containing five strains of *Leishmania* was used to vaccinate a population in Brazil but the outcome of this programme is uncertain as the incidence of disease dropped in this area during the period after immunisation, making evaluation of protective immunity impossible (250). However, the majority of vaccinated patients did demonstrate positive skin tests to injected antigen. More recent trials with killed parasites co-injected with live bacille Calmette-Guerin (BCG) resulted in a reduction of established cutaneous lesions (251) indicating that, with an appropriate adjuvant, protective responses can be generated in humans using a killed vaccine.

Despite the failure of these attempts in vaccinating human populations, data from murine experimental leishmaniasis suggests that the generation of a safe, effective vaccine will be possible.

1.4.2 Murine vaccination

There have been a number of vaccination procedures used against experimental murine cutaneous leishmaniasis including the use of live attenuated parasite strains, killed parasites and undefined antigen preparations, purified and recombinant antigens, proteins expressed in live vectors and peptide vaccination. Similar to human leishmanization, cure of murine *L. major* infection in a resistant mouse confers

protection from secondary challenge demonstrating that protective immunity can be induced.

An avirulent *L. major* clone created by chemical mutagenisis which is deficient in the synthesis of the surface molecule LPG was used to vaccinate susceptible BALB/c mice (252). Mice administered intravenously with this clone demonstrated reduced lesion development upon virulent challenge, although complete cure of infection was not observed. $CD4^+$ cell lines from immunised mice, when transferred to naive BALB/c mice, were able to inhibit lesion development to a similar degree after virulent challenge (253). This T cell population produced more IFN-γ , TNF-α and IL-2 and less IL-4 than lines generated from non-immunised BALB/c mice, indicating that Th1 polarisation had occurred after vaccination.

Vaccination with killed *L. major* has been studied in detail and is referred to above. Immunisation with parasites killed by sonication or with soluble *L. major* antigen is effective at preventing disease if administered intravenously or intra-peritoneally (124). However, significant exacerbation is observed with subcutaneous immunisation of the same antigen preparation (129). These experiments demonstrate the importance of the route of immunisation and may be explained by presentation of antigen to naive T cells via different antigen presenting cells. B cells have been demonstrated to preferentially select for Th2 cells reponses during *L. mexicana amazonensis* infection while macrophage antigen presentation preferentially induces Th1 cells (254). The mechanism by which different cell populations select for the development of Th1 or Th2 cell development is not clear but may depend upon differential secretion of co-stimulatory cytokines during antigen presentation (98) or the expression of diverse cell surface stimulatory molecules such as B7-1 and B7-2 (255). IL-12, as previously mentioned, is thought to be critical in the development of protective Th1 responses. Indeed, subcutaneous vaccination of BALB/c mice with a soluble antigen extract of *L. major* and IL-12 provides complete protection from infection (256).

While considerable success has been achieved in vaccinating mice using parasite antigen preparations or killed parasites, the use of purified or recombinant antigens has not proved as effective. A number of purified and recombinant parasite molecules have been used to immunise resistant and susceptible mice against *L. major* infection, including LPG, gp46 and gp63.

Immunisation with purified LPG reduced lesion development in resistant, C3H/He mice and susceptible, BALB/c mice although immunisation did not confer the ability to cure disease (103). Liposomes containing LPG proved to be an efficient vaccine against *L. mexicana* infection and protection was transferred to naive mice by T cells (257). Further studies have demonstrated that the carbohydrate fraction of LPG will actually exacerbate disease (258) and that T cell responses to purified LPG *in vitro* by lymphocytes from cutaneous patients can be inhibited by treating LPG with proteinase K (259). These data suggest that the protection achieved with LPG immunisation is due, in fact, to LPG-associated proteins which have been subsequently described (260).

The membrane protein gp46 (M-2 protein) is a promastigote stage specific surface glycoprotein, with unknown function (261). The gene sequence for gp46 contains substantial homology to the Promastigote Surface Antigen-2 (PSA-2) gene family (262) which contain a group of at least three distinct proteins (263). Immunisation with purified gp46 and various adjuvants reduced lesion development caused by *L. m. amazonensis* infection in susceptible mice (106). The level of protective immunity provided by gp46 vaccination in different strains of mice correlated with the gp46 specific antibody response although cellular responses were not examined. Recombinant vaccinia viruses, engineered to express gp46, conferred some protection against *L. m. amazonensis* infection after immunisation of BALB/c mice (264). Although 45% of mice were reported to be completely cured 8 months after challenge, this was only effective at a relatively low dose parasite infection. The

protective response was shown to correlate with a mixed Th1/Th2 cytokine response to parasite antigens, although these responses were extremely low and were examined a long time after infection, and are perhaps indicative of the memory response rather than the response required to cure initial infection (264).

Gp63 has received much attention as a potential vaccine candidate as it is the most abundant surface membrane protein on the promastigote stage of *Leishmania* parasites with around 500 000 copies per cell, accounting for 0.5-1% of the total cell protein content (265). Gp63 is encoded by a multigene family containing single dispersed gene copies as well as tandem repeats that give rise to polycistronic mRNA (266). The genes are constitutively transcribed in the promastigote and amastigote stage (266), although there is some debate as to whether amastigotes express the translated protein (47, 267). Gp63 has been shown to function as a protease *in vitro* on a number of proteins (46, 268, 269), although the natural substrate is not known.

Serum from patients suffering from cutaneous and visceral disease contain high antibody titres to gp63 (270). Strong T cell responses to purified or recombinant gp63 have also been observed using lymphocytes from patients with active or cured cutaneous, mucocutaneous or visceral disease (271). In mice, native gp63 has been used successfully to immunise resistant mice against cutaneous disease, either alone as a purified protein (272) or incorporated into liposomes (257). However, vaccination with recombinant gp63 did not provide any protection to resistant or susceptible BALB/c mice upon infection with *L. major* (273).

Peptides from gp63 can immunise for Th1 or Th2 cell responses *in vivo* depending upon the sequence chosen. Initial studies using seven peptides, predicted to be T cell epitopes by algorithm analysis of the gp63 sequence, found one peptide capable of inducing Th1 responses and protective immunity to *L. mexicana* infection after subcutaneous immunisation (274). An examination of 24 peptides from gp63 found a number which were immunogenic in mice, induced DTH and Th1 responses

A recently described Leishmanial antigen, p24/LACK has been shown to function as an effective vaccine in susceptible BALB/c mice when administered with recombinant IL-12. A dominant immune response is detected to p24 during the first week of infection in both susceptible and resistant mice indicating that this antigen may be important in determining the early Th1 or Th2 response. In addition, a protective T cell clone which can transfer resistance to infection is known to recognise this molecule. Why this antigen provokes a strong immune response is unclear. It has homology to another eukaryotic protein, Receptor for Activated C Kinase (RACK) which is a protein kinase C docking molecule present in the cytoplasm of many cells. The function of p24/LACK in the parasite is unknown but it is present in all species studied so far.

and conferred protective immunity against *L. major* in resistant, CBA mice (275). A detailed study of overlapping peptides spanning the entire length of *L. major* gp63 found that the most immunodominant peptides generated a Th1-like response in vivo while peptides which generated a less efficient proliferative response showed a Th2-like profile (276).

Gp63 has also been expressed in both BCG (277) and *Salmonella typhimurium* (278) as candidate live vaccines. A single vaccination of the BCG clone expressing gp63 reduced lesion development in CBA mice after challenge with *L. mexicana* or *L. major* (277). Susceptible BALB/c mice were protected from *L. mexicana* infection after receiving this construct and also demonstrated a reduction in lesion development upon low dose *L. major* challenge (277). Gp63 expressed in *S. typhimurium* was efficient at protecting resistant mice from lesion development and this correlated with a Th1-like response to antigen *in vitro* by spleen cells from immunised mice (278). However, no protection was observed when this construct was used to vaccinate susceptible, BALB/c mice. Thus, of the available candidate antigens used to vaccinate against murine cutaneous leishmaniasis, gp63 is the most completely studied and has given the most encouraging responses.

1.5 *Salmonella* vectors

The use of live delivery systems in vaccine development has advanced the frontiers of vaccine research. Several attenuated organisms which have lost the ability to cause disease or have been engineered genetically to be avirulent have been studied with a view to the development of vaccines against natural infection (279). Live vaccines allow the generation of strong, cellular, long-lasting immune responses which many killed or subunit vaccines do not provide (280). Agents studied as live vaccine vectors include vaccinia virus, avipoxviruses, adenoviruses, polioviruses, salmonella, herpesviruses and BCG, among others (281). These organisms also allow the insertion of foreign genes into the genome which can be expressed *in vivo* as the vaccine

establishes it's limited life-cycle. It is therefore possible to envisage the generation of a live attenuated organism which, while protecting against infection with the wild-type pathogen, also provides effective immunity to diverse species (280).

There are, however, potential problems associated with the use of live attenuated vaccines. Reversion to virulence is a major problem which can, in part, be overcome by recent developments in the understanding of the molecular nature of bacterial and viral pathogenesis (282). Multiple, defined genetic disruptions in genes essential for virulence can render agents safe for human use. There are also problems relating to vector immunogenicity, as increased attenuation can often lead to decreased ability to induce immune responses (282).

Some of the best candidates for use as live vaccine vectors are attenuated strains of *Salmonella* which have been shown to function effectively as vaccines against human typhoid fever and have the additional benefit that they can be administered orally (283). Although this is obviously an inherent advantage in vaccinating against any pathogen, it is particularly useful when one considers the development of vaccines against diseases in the Developing World, as oral administration would reduce the costs associated with large scale vaccine delivery (284). It has also been observed that "the majority of infectious diseases are initiated by pathogens entering the body via exposed mucosal surfaces" and vaccination by a similar route may confer better protection against such diseases. (282) Live *Salmonella* also have advantages when contemplating large scale vaccine preparation due to the stability of the organisms during storage and the ease with which they can be grown in bulk cultures (285).

1.5.1 *Salmonella* life cycle and pathogenesis

An understanding of the pathogenesis of human typhoid fever caused by *S. typhi* and S. *paratyphi* comes from experimental murine infection with *Salmonella* strains which cause a very similar disease. These include *S. typhimurium* and *S.*

enteritidis which cause enterocolitis in humans but typhoid fever-like symptoms in mice and many domestic animals (285)

After oral inoculation of *S. typhimurium* to a susceptible mouse and survival of the stomach environment, *S. typhimurium* penetrate the wall of the small intestine through specific areas called Peyer's patches (286). In human typhoid fever, these areas represent the position of perforated ulcers which are the major cause of mortality during infection (287, 288). Previously thought to have little functional significance, Peyer's patches represent lymphoid follicles at which the immune system can sample the gut environment for immunesurveilence (289).

Entry into a Peyer's patch is controlled by specialised epithelial cells, termed M cells (due to the presence of "microfolds" on the luminal surface) which overly the basement membrane covering the lymphoid area (290). These cells allow the controlled flow of particles from the lumen into contact with lymphoid cells (291). In addition, these specialised cells are often tightly associated with lymphoid cells which sit within an invagination between M cells and adjacent enterocytes (286). While phagocytosis of particles by enterocytes leads to transport to lysosomes, particles or organisms which are ingested by M cells are delivered to lymphoid cells (292).

A number of enteropathogens such as *S. typhi* , *S. typhimurium* , *Yersinia enterocolotica* and reoviruses selectively attach to M cells in the lumen and then subvert the nature of this cell in order to gain access to tissues (289). A comparison of an invasive and non-invasive strain of *S. typhimurium* found that the non-invasive strain was deficient in M cell attachment, emphasising the necessary nature of this interaction for infection (293). Invasive strains induce membrane ruffling at the surface of epithelial cells which mediates entry (294). Although the attachment and induction of membrane ruffling is specific to certain invasive organisms, the uptake is not, as non-invasive bacteria or even latex beads can be ingested by epithelial cells if membrane ruffling is first induced (294). The relative membrane interactions between

the bacteria and the epithelial cell in the internalisation process are not known (295), although it is clear that invasion by *S. typhimurium* requires protein synthesis by the bacteria (296, 297) and both actin polymerisation (298) and an inositol phosphate flux (299) by the epithelial cell.

After invasion, *Salmonella* quickly kill the M cell before infecting neighbouring enterocytes (286). The bacteria can now gain entry into the deeper tissues but also continue to develop a destructive ulcer at the site of infection. If this ulcer progresses to a perforated state this allows other opportunistic gut organisms to gain entry to the tissues with often fatal consequences (300). *Salmonella* organisms, after gaining entry to the Payer's patch via M cells then translocate to the lamina propria and mesenteric lymph nodes where they can invade and survive within macrophages (285). Further colonisation of macrophages within the spleen and liver then takes place.

The ability to survive within macrophages after phagocytosis is critical to the pathogenesis of this organism (301). *Salmonella* enter macrophages via a membrane ruffling event similar to that observed with epithelial cells although in this case the bacteria induce ruffling of a larger membrane area (302). The organisms survive within a large vesicle similar in size to macropinosomes which are large endocytic vesicles inducible by phorbol ester treatment and distinct from smaller phagosomes (303). Existence within such a large vacuole may cause a dilution of any induced microbicidal compounds and a delay in acidification, allowing the bacteria to survive initial antibacterial defences (302). The acidification of the phagosome may also be actively delayed by the *Salmonella* themselves, as killed organisms induce a quicker reduction in vacuolar pH if ingested by macrophages (304).

Little is known of the mechanisms by which *Salmonella* survive in macrophages after initial invasion, although it is clear that acidification of the vacuole does eventually occur (304). The transcription of the bacterial phoP-activated gene (*pag*) promotes intra-macrophage survival and is switched on within hours of

phagocytosis (304, 305) although it is not clear how this virulence factor operates. The initiation of *pag* transcription is controlled by two other proteins, PhoQ and PhoP which allow the bacteria to detect acidification of the phagosome (306). Replication of the bacteria occurs within macrophages before release and invasion of other cells. Limited experiments on human volunteers suggest that a similar course of infection to the one described above in mice also takes place during human typhoid fever (307).

1.5.2 Immunity to *Salmonella* infection

In a manner similar to murine *L. donovani* infection, mice infected with *S. typhimurium* divide into susceptible and resistant according to the presence of different alleles of the *Lsh/Ity/Bcg* gene. Challenge of *Ity*^{r} mice results in the initial control of infection which is not T cell mediated (308) and further T cell mediated bacterial clearance (309). As with *L. donovani* this initial control may be due to the functional activity of a nitrogen transport protein encoded at the *Ity* locus (59). BALB/c mice, however, cannot control infection with virulent salmonellae and succumb to fatal infection.

Immunisation of BALB/c mice with live attenuated strains of *S. typhimurium* can induce protective immunity to further virulent challenge (310). There is a period of non-specific immunity which occurs as the initial replication of the attenuated organisms reaches a plateau, conferring resistance to infection with *Listeria monocytogenes* as well as virulent *Salmonella* (311). This non-specific protection is mediated primarily by activated macrophages and NK cells (312), can be transferred to naive mice by macrophages (313) and is inhibited by anti-IFN-γ monoclonal antibodies (314). This coincides with a period of non-specific T and B cell immunosuppression mediated by activated macrophages which can be inhibited by exogenous IL-4 (315).

Resistance to virulent infection after this initial phase is antigen specific and requires the presence of T cells (316). Surprisingly, T cells and/or macrophages are not

sufficient in the transfer of this immunity to naive susceptible animals (317) although transfer of T cell lines is sufficient to protect against strains of low virulence (318). For protective immunity to virulent challenge, serum transfer from immunised animals is required in addition to T cells (319). These antibodies are thought to opsonise extracellular organisms and so help to contain infection. Macrophage activation and recruitment by IFN-γ and TNF-α are critical in the killing of intracellular organisms (316) and can cause *S. typhimurium* killing if administered *in vivo* before infection (320). Lysis of infected cells by cytotoxic T cells is also suggested to contribute to salmonella clearance (321) as infection elicits a strong $CD8^+$ CTL response (322). As intracellular salmonella are retained within a vacuolar membrane it is difficult to explain antigen delivery to the class I pathway. An attenuated *Salmonella* strain expressing influenza A nucleoprotein did not deliver viral antigen to the MHC class I pathway during infection of CHO cells (323) suggesting that antigen presentation for the generation of $CD8^+$ T cell responses *in vivo* may derive from an unconventional class I pathway which is only found in macrophages (324).

1.5.3 Attenuated *Salmonella*

Various *S. typhi* and *S. typhimurium* mutants have been studied as possible vaccine candidates against human typhoid fever and the murine equivalent. Germanier and co-workers described a mutant *S. typhimurium* strain that was deficient in the production of lipopolysaccaride due to mutations at the *gal E* locus (325). The bacteria were defective in the enzyme UDP-galactose epimerase and were both highly attenuated and immunogenic. A similar mutant of *S. typhi* was generated called Ty21a which has now been used in a number of large-scale human vaccine trials (326, 327, 328) and has proved to be as effective as the killed typhoid vaccine in vaccinating against typhoid fever (327). While this strain is now licensed as a vaccine in a number of countries the genetic factors responsible for attenuation remain undefined (282). There are a number of differences between the Ty21a strain and the wild-type Ty2 strain which cannot be explained by disruption of the *galE* locus. In addition, a

genetically defined strain with a *galE* mutation remained virulent (329) in humans emphasising the undefined nature of Ty21a attenuation. The lack of information concerning the genetic attenuating factors involved and the observation that three doses of this vaccine are required for effective immunity (327) has resulted in further manipulation of *S. typhi* and *S. typhimurium* strains to create a defined, attenuated mutant with greater immunogenicity.

A *S. typhimurium* strain with a defined disruption of the *aroA* gene was reported to be severely attenuated and highly immunogenic *in vivo* as a single oral dose could protect BALB/c mice against virulent challenge (310). The *aroA* gene encodes the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase which is required for synthesis of aromatic rings in the chorismate pathway (330). This attenuation leaves the bacteria unable to synthesise the compounds p-amino-benzoic acid (pAB) and 2,3-dihydroxybenzoate (DHB) among others (310). These compounds are used in the generation of iron binding proteins and for DNA synthesis and repair. The lack of such compounds in the mammalian host severely attenuate the bacteria *in vivo*. Although the generation of other products such as the aromatic amino acids is also disrupted in these mutants it is the lack of available pAB and DHB *in vivo* that cause attenuation of the bacteria, as injection of these compounds during colonisation results in virulent infection (310). A *S. typhi* strain with a similar disruption generated a strain attenuated for human infection (331).

Further strains have been developed based on the *aroA* mutant, which contain other mutations to render the possibility of reversion to virulence extremely remote. *S. typhi* strains with mutations at both *aroA* and *purA*, a locus required for purine synthesis, resulted in the generation of a strain which was extremely safe but poorly immunogenic when given to humans (332). A similar *S. typhimurium* strain was also extremely attenuated, developing a persistent low level infection in BALB/c mice which was insufficient to generate immunity (333). Therefore, while the targeted disruption of two diverse pathways does increase attenuation, it leaves the bacteria

unable to achieve the level of colonisation required for the induction of effective immunity. Double mutants within the chorismate pathway such as *aroA* $^{-}$, *aroC* $^{-}$ or *aroA* $^{-}$, *aroD* $^{-}$ are severely attenuated yet retain immunogenicity. *S. typhimurium* mutants with two aro mutations were shown to be effective single dose vaccines in cattle (334) although similar *S. typhi* mutants, while being highly immunogenic, retained the ability to cause fever in human volunteers (335).

Many other *S. typhimurium* and *S. typhi* mutants which are genetically defined have been produced as knowledge of salmonella virulence factors has progressed. *S. typhimurium* strains with disruptions at the *cya* locus or *crp* locus are unable to make cyclic AMP or respond to cAMP (336). Similar *S. typhi* mutants were tested in humans and one of twelve recipients developed fever (335) indicating that this strain was not sufficiently attenuated for human use. Other strains which have been examined in animal models include those with mutations at *phoP* (306), *ompR* (337), *htrA* (338) and *hemA* (339). A strain of *S. cholerae-suis* with a mutation in the cdt (colonisation of deep tissue) gene were unable to mount a systemic infection after oral delivery (340) and may be useful as a heterologous vaccine carrier against mucosal pathogens. An investigation as to the balance between immunogenicity and safety of these strains may lead to the generation of those suitable for human use.

1.5.4 Attenuated *Salmonella* as heterologous carriers

In addition to the potential of attenuated *S. typhimurium* and *S. typhi* strains to vaccinate against typhoid fever, they have also received much attention regarding their potential as carriers of diverse antigens from a number of other pathogens (284, 285, 341). *Salmonella* mutants induce strong humoral and cellular immune responses and therefore can be manipulated to induce such immune responses to foreign antigens. Antigens of bacterial, viral and parasite origin have been expressed in *S. typhimurium* as prospective vaccines against a number of diseases.

Ty21a has been used to express a number of antigens in an attempt to generate a multivalent vaccine against various enteric bacterial pathogens. The introduction of a plasmid into the Ty21a strain generated a strain which express the β-subunit of *E. coli* heat labile enterotoxin (LT-B) (342). Ty21a has also been used to develop a construct expressing *Shigella sonnei* surface antigens (343) and a construct expressing *Vibrio cholerae* antigens (344). While these bivalent vaccines have been generated in *S. typhi*, most attempts to generate dual vaccines against diverse pathogens have concentrated on the use of attenuated *S. typhimurium* strains, as experimental vaccines.

Experimental vaccines against bacterial pathogens have been constructed using *S. typhimurium* mutants expressing *E. coli* fimbrial adhesin K88 (345), or LT-B (346), *Bordetella pertussis* P69 (347), a membrane constituent of *Brucella abortus* (348) *M. leprae* antigens (349) and Tetanus toxin fragment C (350). *AroA* mutants expressing β-galactosidase induced strong antibody and DTH responses to β-galactosidase in immunised mice, indicating that antigens delivered by this method are immuogenic (351). The construct expressing the P69 antigen of *B. pertussis* in the double mutant strain BRD509 was able to induce protective immunity to challenge with *B. pertussis* (352). Reduction in disease correlated with the induction of substantial cellular responses to P69.

A number of viral antigens have also been expressed in *S. typhimurium*. An initial report describing expression of *P. berghei* circumsporozoite antigen in *S. typhimurium* suggested that the protective immunity induced by this strain was due to the induction of CTL responses (353). Further work has demonstrated the induction of strong CTL responses to antigens expressed in salmonella (322, 354, 355) indicating that *Salmonella* may function as effective vectors for the delivery of viral antigens. However, influenza nucleoprotein (NP) expressed in attenuated *S. typhimurium* were unable to stimulate class I restricted T cell responses *in vitro* after macrophage infection, while class II restricted T cell clones were efficiently stimulated (356). *In vivo* studies confirmed these data, as infection of mice with *S. typhimurium* expressing

NP generated NP-specific class II restricted responses in the absence of class I responses (357). While some class II-restricted cytotoxicity was detected, there was efficient induction of Th1-like $CD4^+$ T cell responses to antigen indicating that this is the primary response induced to heterologous antigens delivered using *Salmonella*. As previously mentioned, *S. typhimurium* were unable to deliver antigens to the class I pathway during infection of CHO cells (323). Therefore the mechanism of CTL induction by antigens expressed in *S. typhimurium* strains is unclear, while the ability to deliver antigens to the class II pathway and induction of $CD4^+$ T cells has been repeatedly demonstrated (278, 356, 357). The prospective role of Salmonella mutant strains in the induction of $CD8^+$ T cell responses and protective immunity against viral infection requires further investigation.

As protective responses during primary *L. major* infection are mediated by $CD4^+$ cells, *Salmonella* seem ideal vectors to deliver *Leishmania* antigens. The gene encoding *L. major* gp63 was expressed in a single *aroA* mutant of *S. typhimurium* and this construct used to immunise mice (278). Resistant CBA mice after two oral doses of this construct had reduced lesion development upon *L. major* challenge. However, similar vaccination of BALB/c mice provided no protection to challenge infection. Immunisation with this construct induced Th1-like $CD4^+$ T cell reponses to gp63 which correlated with protective immunity (278). In addition to *L. major* gp63, a number of other antigens of parasite origin have been expressed in *S. typhimurium*, including *P. berghei* circumsporozoite antigen (353), *P. falciparum* blood stage antigens (358), and a *Schistosoma mansoni* glutathione S-transferase peptide (359).

S. typhimurium have also been engineered to express murine cytokines which are bioactive *in vivo*. *S. typhimurium* expressing murine IL-1β confer protection against lethal gamma irradiation due to functional IL-1β activity, when administered to mice (360). Murine IL-4 has also been expressed in *S. typhimurium* and shown to have bioactivity *in vivo* (361). While the use of recombinant cytokines with adjuvant

activity in vaccination has been documented (362) the use of cytokine expressing *S. typhimurium* in vaccination has not been addressed.

In conclusion, attenuated *S. typhimurium* can be used as carriers of heterologous antigens to the immune system in animal models and future progression from a single attenuated typhoid fever vaccine to a multivalent vaccine against diverse pathogens may be possible.

1.5.5 Other live vaccine vectors

While *S. typhimurium* are considered effective vaccine vectors there are a number of other live vectors which are just as efficient in the generation of immunity to expressed antigens, although some of these also have inherent problems.

Attenuated BCG have been used to deliver a number of heterologous antigens to the immune system (277, 363) including *L. major* gp63 (277). BCG offers a safe, well tested vector, as over 2.5 billion people have been immunised with this organism (363). It has also been well studied *in vitro* and can be manipulated to express foreign proteins (364). However, like many live vaccines there are concerns about the ability of mycobacteria to replicate in immunocompromised patients (363). In addition, BCG grows very slowly in culture which can make manipulation of strains difficult and time consuming. The number of individuals already immunised with BCG could also, in itself, represent a considerable disadvantage as strong anti-vector immune responses may reduce the efficacy of BCG to deliver foreign antigens. Attenuated strains of other bacterial pathogens such as *Listeria* and *Shigella* have also been studied as vaccine vectors (363).

Vaccinia virus has also been shown to be effective in functioning as a live vaccine vector, delivering a number of diverse antigens to the immune system (280, 281, 363). Unlike many other viruses, vaccinia allows incorporation of large amounts

of DNA into the genome (280). Antibody and CTL responses to foreign antigens have been demonstrated using vaccinia virus vectors. (363). However, there are problems concerning reversion to virulence after immunisation with vaccinia and attempts have been made to further attenuate the virus for human use (365). Other viral vectors such as avipoxviruses which are incapable of spreading infection in human cells and attenuated polioviruses are also being considered as possible viral vectors for the safe delivery of foreign antigens (281).

There are therefore a number of live attenuated vaccine vectors which can be utilised for the expression of foreign genes. With most of these organisms the problems of reversion to virulence and immunogenicity remain to be resolved.

1.6 Aim of thesis

Successful vaccination against experimental murine leishmaniasis is known to depend critically upon the induction of a protective Th1 response. The capacity of *S. typhimurium* to induce Th1 responses *in vivo* to heterologous antigens suggests that attenuated *Salmonella* may be ideal vectors for vaccination against leishmaniasis. A *S. typhimurium* construct (SL3261/gp63) which expressed *L. major* gp63 was shown to reduce lesion development in resistant CBA mice and induce a Th1 response to gp63. However, this construct failed to provide any protection to the susceptible, BALB/c, strain.

The aim of the work described in this thesis was to improve upon the vaccine strain SL3261/gp63 in order to provide protection against susceptible BALB/c mice. This was attempted by:

(a) the use of the attenuated *S. typhimurium* strain BRD509, which contains two deletions in Aro genes and has a lower frequency of reversion to virulance, to express gp63. The effects of this upon immunogenicity and protection were investigated.

(b) the use of inducable promoters to express gp63 in the vaccine strain, BRD509, with a view to the construction of a strain which provides protection with a single dose. The promoters *NirB* and *OsmC* were examined in this respect. In addition, this work sought to examine the protective immune responses involved in the induction of immunity after oral administration with attenuated *S. typhimurium* constructs expressing gp63. The relationship between plasmid stability of these constructs and immunogenicity was also investigated.

This work also describes the construction and use of a *Salmonella* construct expressing a recently described *L. major* antigen, p24/LACK. This antigen is known to provide complete protection to susceptible BALB/c mice after coadministration with IL-12. This vaccine protocol also causes the induction of a Th1-like response to p24. Therefore the combination of this antigen and oral salmonella vectors, which preferentially induce Th1 responses, was thought to provide an excellent vaccine against experimental leishmaniasis. The use of this construct as a vaccine against *L. major* in resistant and susceptible mice and an investigation of plasmid stability and immunogenicity was attempted.

The work described in this thesis, therefore, represents an attempt to advance an existing vaccine against *L. major* infection in the mouse model by the use of a different *Salmonella* strain, inducable promoters and new antigens. These investigations may aid the generation of a live attenuated oral vaccine against human leishmaniasis.

Chapter 2

Materials and Methods

Contents

2.1 Plasmids and oligonucleotides

Plasmids

Details of all plasmids used in this study, with their relevant properties and sources, are shown in Table 2.1.

Table 2.1: Plasmids

Plasmids	Relevant properties	Source or reference
pkk-Imm63-67	*L. major* gp63 cDNA, ampicillin resistance, *tac* promoter	Dr. W. R. McMaster, University of British Colombia, Canada.
pNirB/gp63	*L. major* gp63 cDNA, ampicillin resistance, *NirB* promoter	Dr. D. Xu, Dept. of Immunology, Western Infirmary, Glasgow, U.K.
pOsmC/gp63	*L. major* gp63 cDNA, ampicillin resistance *OsmC* promoter	Dr. D. Xu
pET-LACK.δ1	L.major p24 LACK cDNA	Dr. N. Glaichenhaus, Universite de Nice, France.
pNirB/p24	L. major p24 LACK cDNA, ampicillin resistance, *NirB* promoter	This study

Oligonucleotides

All oligonucleotides were synthesised by Genosys Biotechnologies, Inc. (Cambridge, U.K.)

The following primers were used for cloning of cDNA into pNirB:

L. major p24

5' GGCATATGGAGTTCCTGCGCGACGGTCAC

3' CCGGATCCTTACTCGGCGTCGGAGATGGACCA

2.2 Bacterial strains

Bacteria strains *Escherichia coli* and *Salmonella typhimurium* were routinely cultured in Millers-modified Luria Broth (L-broth) (Gibco BRL, Life Technologies, Paisley, U.K.) consisting of 1% SELECT Peptone 140, 0.5% Yeast Extract and 1% NaCl, or on Millers-modified Luria Agar (L-Agar) (Gibco) consisting of 1% SELECT Peptone 140, 0.5% Yeast Extract, 1% NaCl and 1.2% Agar. Both L-broth and L-agar were sterilised before use by autoclaving on liquid cycle (121°C for 2 hours). Bacterial strains containing plasmids were routinely cultured in the presence of ampicillin (100 μg/ml) (Pharmacy, Western Infirmary, Glasgow, U.K.) to select for transformed bacteria.

E. coli and *S. typhimurium* strains

All *E. coli* and *S. typhimurium* strains used are listed in tables 2.2 and 2.3 respectively.

Table 2.2: ***E. coli*** **strains**

Strain	Relevant properties	Source and/or reference
TG-1	supE, hsdΔ5, (lac-proA-B), F' [tra D36, pro AB^+, $lacI^q$, lacZΔM15]	Dr. D. Xu, Dept. of Immunology, Western Infirmary, Glasgow, U.K. (366)
TG1/NirB/p24	pNirB/p24 in TG-1	This study

Table 2.3: ***S. typhimurium*** **strains**

Strains	Relevant properties	Source and/ or reference
BRD509	SL1344 $AroA^-$ $AroD^-$	Dr. G. Dougan, Biochemistry Dept., Imperial College of Science, Technology and Medicine, London. (352)
GID101	pkk-Imm63-67 in BRD509	Dr. D. Xu, Dept of Immunology, Glasgow University.
GID105	pNirB/gp63 in BRD509	Dr. D. Xu
GID106	pOsmC/gp63 in BRD509	Dr. D. Xu
GID202	pNirB/p24 in BRD509	This study

2.3 Mice, parasites and infection

2.3.1 Mice

BALB/c and CBA mice were obtained from Harlan Olak (Bicester, U.K.) and maintained in the Joint Animal Facility, University of Glasgow, Glasgow. BALB/c and CBA mice in some experiments were bred and maintained in the animal facilities at Strathclyde University, Glasgow. Female mice aged 6-10 weeks were used in all experiments.

2.3.2 Growth and maintenance of *Leishmania*

Leishmania major

Leishmania major strain MRHO/SU/59/P, also known as LV39, was used throughout. The isolate was maintained in the laboratory by continuous passage in BALB/c mice to maintain virulence, as previously described (117). Non-ulcerated lesions of mice, infected four to five weeks previously in the right, hind footpad with $1x10^6$ parasites in 50μl Phosphate Buffered Saline (PBS [1.9mM NaH_2PO_4, 8.1mM Na_2HPO_4, 154mM NaCl, pH 7.2-7.4]), were removed and cut into small pieces in a petri dish (90mm) containing 10ml PBS. Parasites were further dispersed by forcing the suspension through a 5ml syringe placed against the bottom of the petri dish. The suspension was filtered into a universal using Monofilament Nylon Filter Cloth (NITEX) (Cadisch and Sons Ltd, London, U.K.) to remove remaining debris and washed twice by centrifugation at 1300g for 10 minutes at 4°C . After the final wash the pellet was resuspended in 5ml of Complete Schneiders Drosophila Medium (CSDM), consisting of Schneiders Drosophila Medium (SDM) (Gibco) containing 20% FCS (Gibco), 0.05% Gentamycin Sulphate (Gibco) and 2.5 % L-glutamine (Gibco). The parasites were then incubated for 3-4 days at 28°C in $25cm^3$ tissue culture flasks (Corning, New York, U.S.A.) to transform amastigotes. For storage, parasites were

then centrifuged at 1300g and resuspended in 2mls of CSDM containing 10% Dimethyl Sulfoxide (DMSO) (Sigma Chemical Co., Dorset, U.K.). A 10μl aliquot of this suspension was removed, diluted 1: 100 in PBS containing 2.5% Formalin (Sigma Chemical Co.) and the number of parasites counted using an Improved Neubauer Haemacytometer (Merck Ltd, Dorset, U.K.). Parasites were diluted to 2 x 10^7/ml in CSDM containing 10% DMSO, aliquoted into 1ml freezer vials and kept at -70°C overnight before storage in liquid nitrogen at -198°C.

For growing *L. major in vitro* a vial was thawed from liquid nitrogen at room temperature and the contents cultured for three days in 5mls CSDM at 28°C. Parasites were then passaged *in vitro* in CSDM, and every subsequent 7-10, days by adding 250μl of culture to 40mls of CSDM in a 80cm^3 tissue culture flask (Corning) and culturing at 28°C. Parasites were not maintained *in vitro* for longer than 4 passages for fear of loss of virulence.

L. donovani

L. donovani was maintained by continuous passage in Syrian hamsters, as previously described (367). Spleens were harvested from infected hamsters and cut into small pieces in 10mls RPMI 1640 (ICN Biomedicals Inc., Costa Mesa, CA., U.S.A.) and homogenised using a 15ml ground glass homogeniser (Jencons [Scientific] Ltd., Leighton Buzzard, U.K.). Debris was removed from the homogenate by centrifugation at 80g for 5 minutes at 4°C. The resulting supernatant was centrifuged at 1300g for 10 minutes at 4°C to pellet amastigotes and resuspended in 20 mls 0.05% saponin (Sigma Chemical Co.) to lyse remaining red blood cells. Cells were immediately centrifuged at 1300g for 10 minutes at 4°C to limit amastigote exposure to saponin, before being resuspended in 20mls RPMI. Amastigotes were further washed twice in RPMI and the pellet resuspended in 2mls RPMI. An aliquot was diluted 1:100 or 1:1000 in RPMI and amastigotes counted using an Improved Neubauer Haemacytometer, after allowing the parasites to settle for 10 minutes. Parasites were diluted to 1x10^8/ml and 1-2mls injected into each hamster intraperitoneally. Parasites

were passaged again after animals began to show pronounced weight loss and abdomen swelling which occurred approximately 6-8 weeks after infection.

2.3.3 Infection of mice with *Leishmania*

L. major

L. major was grown *in vitro* as described above. Parasites at day 7-10 after passage were centrifuged at 1300g and washed twice in sterile PBS. Promastigotes were counted as described above and resuspended at the appropriate concentration before being injected into mice subcutaneously in the left, hind footpad in a volume of 50μl. Footpads were monitored for thickening using a spring-dial calliper (Schnelltaster, Kroeplin, Surrey, U.K.). BALB/c mice typically received $5x10^4$-$1x10^6$ parasites per injection whereas CBA mice received $1x10^7$.

L. donovani

L. donovani amastigotes were harvested and purified from hamster spleens as described above. For infection of mice, amastigotes were resuspended at $5x10^7$ /ml in RPMI and 200μl injected intra-venously into the tail vein. At various time points after infection mice were monitored for parasite loads by sacrificing animals and obtaining organ imprints on glass slides of liver and spleen as previously described (368). These slides were fixed in 100% methanol (Fisons, FSA Laboratory Supplies, Loughborough, U.K.) for 20 minutes followed by staining in 10% Giemsa solution (BDH Laboratory Supplies, Poole, U.K.) for 30 minutes. Parasites and cell nuclei were counted from organ imprints using oil immersion light microscopy. The LDU (Leishman-Donovan Units) of each organ was calculated by multiplying the organ weight by the number of observed amastigotes per 1000 nuclei.

2.4 DNA manipulation

2.4.1 Quantitation of DNA, RNA and oligonucleotides by spectrophotometric analysis

In order to determine the concentration of DNA or RNA in solution, the optical density was determined at wavelengths 260nm and 280nm in 10mm quartz cuvettes (Jencons) using an Ultrospec 4050 spectrophotometer (LKB Biochrom Ltd, Cambridge, U.K.). The optical density at 260nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 50μg/ml of double-stranded DNA, 40μg/ml of single stranded DNA or RNA, or 20μg/ml of single stranded oligonucleotides. The ratio between 260nm and 280nm provides an estimate of the purity of nucleic acid in solution. Pure preparations of DNA and RNA have OD260/OD280 values of 1.8 and 2.0 respectively.

2.4.2 Restriction enzyme digestion

Restriction enzymes were purchased from Boehringer Mannheim (Boehringer Mannheim U.K. Ltd, East Sussex, U.K.) or Promega (Promega Corporation, Madison, WI, U.S.A.) as were the buffers used to create optimal digestion conditions. All reactions were performed according to manufacturers instructions; typically 1Unit of enzyme per μg of DNA was incubated at 37°C for 1-2 hours in the appropriate buffer. Table 2.3 details the restriction enzymes used, cleavage sites and buffers.

Table 2.4: Restriction enzymes

Restriction enzyme	Recognition site	Optimal buffer
Nde I	5'-CA • TATG-3' 3'-GTAT • AC-5'	Buffer B (10mM Tris-HCl, 5mM $MgCl_2$, 100mM NaCl and 1mM 2-Mercaptoethanol)
BamH I	5'-G • GATCC-3' 3'-CCTAG • C-5'	Buffer B
Pst I	5'-CTGCA • G-3' 3'-G • ACGTC-5'	Buffer H (50mM Tris-HCl, 10mM $MgCl_2$, 100mM NaCl and 1mM Dithioerythritol)
Ava I	5'-C • (T,A)CG(A,G)G-3' 3'-G(A,G)GC(T,A) • C-5'	Buffer B
EcoR I	5'-G • AATTC-3' 3'-CTTAA • G-5'	Buffer H

2.4.3 Agarose gel electrophoresis

An appropriate amount (typically 1%) of Ultra pure Agarose (Electrophoresis Grade, Gibco) was added to a measured volume of electrophoresis buffer (0.5 x TBE, [0.045M Tris-borate, 0.001M EDTA]) and boiled by heating in a microwave oven (Proline Powerware 800, Comet, Glasgow, U.K.). The agarose was allowed to cool to approximately 60°C and ethidium bromide (Sigma Chemical Co.) added to a final concentration of 0.5μg/ml. The agarose was then poured into a gel mould (Pharmacia, LKB Biotechnology AB, Uppsala, Sweden) and allowed to set. The comb was carefully removed and the gel mounted in a horizontal "submarine" electrophoresis

tank (Pharmacia Gel Electrophoresis Apparatus GNA-100, Pharmacia) filled with electrophoresis buffer. The DNA samples or 1μg of DNA 1Kb ladder (Gibco) were mixed with loading buffer (0.25% bromophenol blue [Sigma Chemical Co.], 0.25% xylene cyanole FF [Sigma Chemical Co.] and 15% Ficoll 400 [Sigma Chemical Co.] in water) and loaded into the gel. The gel was run under constant voltage at 1-5V/cm for 30-60 minutes and examined for DNA bands stained with ethidium bromide under ultra-violet illumination (Ultraviolet Transilluminator, UVP Inc., Cambridge, U.K.) before photography (Polaroid DS-34 Direct Screen Instant Camera, Sigma Chemical Co.).

2.4.4 DNA ligation

DNA ligation was performed according to suggested manufacturers conditions in a final volume of 20μl. For cohesive ligation, vector and insert DNA were mixed at a ratio of 1 : 3 in 14μl of distilled water and heated at 50°C for 5 minutes in a water bath before being placed on ice. One Unit of T4 DNA ligase (Gibco), and 5μl of Ligation buffer (0.25M Tris-HCl [pH 7.6], 50mM $MgCl_2$, 5mM ATP, 5mM DTT, 25% w/v Polyethylene glycol-8000, Gibco) were then added and the ligation mix incubated overnight at 4°C.

2.4.5 Bacterial transformation

Plasmid DNA was introduced into *E. coli* using $CaCl_2$ (369). Bacteria were grown in 3mls of L-broth overnight at 37°C from colonies on L-agar . This suspension was diluted 1:20 in L-broth and grown for a further 2 hours in order to obtain a mid-log phase culture (OD600 of 0.5). Bacteria were then cooled on ice for 5 minutes, centrifuged at 800g and washed once in ice cold 0.1M $CaCl_2$. The resulting pellet was resuspended in 1ml of 0.1M $CaCl_2$ and stored on ice. 10μls of ligation mix (see 2.4.4) or 0.5μg of plasmid DNA was mixed with 200μl of bacteria and stored on ice for 30-40 minutes. Bacteria were then subjected to heat shock at 42°C for exactly 2 minutes and

placed immediately on ice for 1 minute. 1ml of L-broth was then added and bacteria incubated at 37°C for 1 hour. 120μl of this broth was plated onto L-agar plates in the presence or absence of ampicillin and incubated at 37°C overnight. Plasmid was prepared from clones growing on ampicillin plates and the plasmid DNA verified by restriction enzyme digestion or by PCR.

2.4.6 Electroporation of bacteria

Bacteria from an L-agar plate colony were grown overnight in a volume of 3mls of L-broth. A small amount (250μl) of this bacterial suspension was added to a further 30mls of L-broth and incubated in an orbital incubator (INR-250 Orbital Incubator, Sanyo Gallenkamp Plc., Loughborough, U.K.) for 2-4 hours, achieving a log-phase culture. This culture was centrifuged at 800g for 10 minutes, resuspended in 25mls of sterile, ice-cold 10% glycerol (Fisons) in water before being centrifuged again. The pellet was resuspended in 500μl of ice-cold 10% glycerol/water and bacteria stored on ice. To 60μl of bacteria, plasmid DNA (approximately 200ng, resuspended in water) was added in a volume of 5μl. This suspension was added to a chilled 0.2cm Gene Pulser® cuvette (BioRad Laboratories, Richmond, California, U.S.A.) and electroporated (GenePulser®, BioRad Laboratories) under the following conditions:

E.coli	*S. typhimurium*
1.25 Kv	1.75Kv
25μF	25μF
600 Ohms	600 Ohms

Immediately after poration, 1ml of L-broth was added to the cuvette after which this broth was transferred to a 1.5ml eppendorf tube and incubated for 2 hours at 37°C in a water bath. The bacteria were then plated onto L-agar plates in the presence and absence of ampicillin (100μg/ml) and incubated overnight at 37°C. Bacteria growing on ampicillin L-agar plates were further grown overnight in L-broth and plasmid was extracted to confirm successful poration.

2.4.7 Quick plasmid preparation

This method was adapted from that of Brinboim and Doly (370). Bacteria were grown overnight at 37°C in 3mls L-broth containing 100μg/ml ampicillin from L-agar plate cultures. The suspension was centrifuged at 13,800g in a bench-top microfuge (Biofuge 13, Heraeus Equipment Ltd, Brentwood, Essex, U.K.) for 1minute in 1.5ml eppindorf tubes and the pellet resuspended in 100μl of Solution I (2mg/ml lysozyme (Boehringer Mannheim) in 50mM glucose (Fisons), 10mM EDTA (Sigma Chemical Co.), 25mM Tris (BDH)-HCl pH 8.0). After 5 minutes at room temperature, 200μl of Solution II (0.2M NaOH [Fisons], 1% SDS [BDH]) was added, the solution mixed well and incubated on ice for 5 minutes before 150μl of Solution III (3M Na Acetate pH 4.8 [Sigma Chemical Co.]) was added, mixed and the samples incubated for 10 minutes on ice. Samples were then centrifuged in a microfuge at 13,800g for 10 minutes and the supernatant poured into another eppendorf tube. An equal volume of Tris-HCl (pH 8.0) saturated phenol/chloroform (equilibrated phenol, chloroform, isoamyl alcohol [25:24:1]) was added, the samples mixed gently and centrifuged for 5 minutes at 13,800g. The upper aqueous phase was removed and DNA precipitated by adding 2.5 volumes of 100% ethanol (BDH) and storing at -20°C for 15 minutes or by adding 1 volume of isopropanol (Sigma Chemical Co.) and storing at 4°C for 15 minutes. DNA was pelleted by centrifugation at 13,800g for 10 minutes and washed with 1ml of ice-cold 70% ethanol at 13,800g for 7 minutes. The ethanol was decanted and the pellet dried under vacuum in a Vacuum Drier (Gyrovap, V. A. Howe & Co. Ltd, Banbury, Oxon., U.K.) before being resuspended in an appropriate volume of TE buffer (10mM Tris-HCl [pH 7.6], 1mM EDTA) or distilled water, as required. DNA concentration was estimated using OD measurements (2.4.1) and visualised using ethidium bromide agarose gel electrophoresis. As necessary, RNA was degraded by incubating plasmid DNA with 1-2μl of RNAse (DNAse-free) (Boehringer Mannheim) at 37°C for 1 hour. Plasmid DNA was then stored at -20°C.

2.4.8 Large scale preparation of plasmid DNA

Bacteria containing plasmid for purification were grown from L-agar plates containing 100μg/ml ampicillin. Colonies were inoculated into 30mls of L-broth containing 100μg/ml ampicillin and grown overnight at 37°C. This culture was added to 1.25 litres of L-broth containing ampicillin and grown at 37°C for 6-8 hours while shaking in an orbital incubator. To improve the yield of low copy number plasmids, 160μg/ml of chloramphenicol (Western Infirmary Pharmacy) was added and the culture incubated for a further 16 hours at 37°C while shaking. The bacteria were pelleted by centrifugation at 6370g for 7 minutes at 4°C in a High-Speed Centrifuge (J2-MC Centrifuge, Beckman - RIIC Ltd, High Wycombe, Buckinghamshire, U.K.) using a JA10 rotor (Beckman). The pellet was resuspended in 50mls of Buffer P1 (50mM Tris/HCl, 10mM EDTA, pH 8.0, Qiagen Ltd., Dorking, Surrey, U.K.) before 50mls of Buffer P2 (0.2M NaOH, 1% SDS, Qiagen) was added, mixed and the solution stored at room temperature. After 5 minutes, 50-75mls of ice cold Buffer P3 (3M Sodium acetate[pH 4.8], Qiagen) was added, mixed and the suspension stored on ice for 10 minutes. This bacterial lysate was then centrifuged at 17,700g for 20 minutes at 4°C and the supernatant filtered through NITEX. If the supernatant did not appear clear at this point it was subjected to a further centrifugation at the same speed. The plasmid DNA was purified from this supernatant in caesium chloride-ethidium bromide gradients (2.4.9) or by anion-exchange chromatography (2.4.10) using a Qiagen plasmid kit (Qiagen).

2.4.9 Purification of plasmid DNA using Caesium Chloride-Ethidium Bromide Gradients

Plasmid DNA in the supernatant from 2.4.8 was precipitated by adding an equal volume of isopropanol (as described in 2.4.7) and was then resuspended in TE buffer. Solid CsCl was added to the suspension (1g/ml) in addition to 0.8mls of ethidium

bromide solution (10mg/ml) for every 10mls of suspension. The solution was mixed and sealed into Beckman Quick Seal tubes (Beckman). The tubes were centrifuged at 250,000g for 36 hours in a Beckman Ultracentrifuge (Ti70.1 rotor, Beckman) at room temperature. After visualisation under ultra-violet light, the lower band of DNA, corresponding to closed circular plasmid DNA, was collected using an 18-gauge needle. The ethidium bromide was removed from the DNA by 4-6 extractions with equal volumes of isoamyl alcohol until the solution appeared clear. The DNA was mixed with an equal volume of isoamyl alcohol (Fisons) and centrifuged at 300g for 3 minutes at room temperature. The lower phase was extracted using a pasteur pipette and the process repeated. The CsCl was removed from the DNA by precipitation with 2 volumes of ethanol at 4°C for 15 minutes, followed by centrifugation at 10,000g for 15 minutes at 4°C. The DNA pellet was washed once with cold 70% ethanol and resuspended in 1ml of TE buffer. The concentration of the DNA was calculated from OD260 readings and the DNA stored at -20°C until required.

2.4.10 Purification of plasmid DNA using a Qiagen plasmid kit

The Qiagen plasmid purification procedure uses a Qiagen Resin compacted into a small column which allows selective purification of super coiled plasmid DNA. Qiagen Resin is an anion exchanger with a hydrophilic surface coating containing Diethylaminoetyl (DEAE) groups. The large pore size of the resin combined with the high density of anion exchange groups provide a wide separation range when eluting with varying salt concentrations. The separation range of Qiagen resin extends from 0.1M to 1.6M salt, allowing plasmid purification at high salt concentrations. The Qiagen-tip 2,500 (Qiagen) was first equilibrated using 35mls of QBT buffer (750mM NaCl, 50mM MOPS, 15% ethanol, pH 7.0, 0.15%Triton x-100, Qiagen) and the supernatant from section 2.4.8 applied to the column. The column was then washed with 200ml of QC buffer (1M NaCl, 50mM MOPS, 15% ethanol, pH 7.0, Qiagen) and the DNA eluted by adding 35mls of QF buffer (1.25M NaCl, 50mM Tris-HCl, 15% ethanol, pH 8.5, Qiagen). The DNA was precipitated from the QF buffer with 0.7

volumes of isopropanol (Sigma Chemical Co.) and centrifuged at 20,000g for 30 minutes at 4°C in a JA17 rotor (Beckman). The pellet was washed once with 5mls cold 70% ethanol, air dried for 10 minutes and then resuspended in 2-3mls of TE buffer or distilled water.

2.4.11 "Freeze-squeeze" purification of DNA fragments (371)

DNA bands for purification were cut from an ethidium bromide stained agarose gel illuminated using an Ultraviolet Transilluminator and placed in a 1.5ml eppindorf tube. 1ml of "freeze-squeeze" buffer (0.3M Na acetate [pH 4.8], 1mM EDTA [pH 7.0]) was added and the tube placed in the dark for a period of time according to the size of the DNA fragment. For 100-2Kb fragments the gel was placed in the dark for 10 minutes; for fragments greater than 2Kb this was increased to 30 minutes. The "freeze-squeeze" buffer was removed and the tube placed in a dry ice/ethanol bath for 30 minutes. The frozen gel was removed from the tube and placed into a Centrifuge Filter Unit (Costar, Cambridge, Massachusetts, U.S.A.) without thawing. The tube was centrifuged in a bench-top microfuge (Heraeus) at 13,800g for 10 minutes and the liquid volume increased to 400μl with TE buffer if necessary. An equal amount of Phenol/chloroform was added, the sample mixed well and centrifuged at 13,800g for 2 minutes. The upper, aqueous layer was carefully transferred to another 1.5ml eppindorf tube. Chloroform, in equal volume was added, mixed and the sample centrifuged again at 13,800g for 1 minute. The chloroform extraction was then repeated. To this extract, 0.1 volumes of 3M Na acetate (pH 7.0) and 2.5 volumes of 100% ethanol were added and the sample left at -20°C for 10 minutes. The precipitated DNA was pelleted by centrifugation at 13,800g for 30 minutes and washed with 70% ethanol before drying in a vacuum dryer. The DNA fragment was resuspended in a small volume (10-15μl) of TE buffer and a 1μl sample visualised using an ethidium bromide stained agarose gel to verify purification.

2.4.12 Polymerase Chain Reaction (PCR)

All buffers and enzymes used for PCR were purchased from Promega and used according to the manufacturers instructions. Template DNA (0.1μg/ml) or reverse-transcribed cDNA was placed in a 0.5ml eppindorf tube and the following reagents added: 10μl of 10x PCR buffer (100mM Tris-HCl, 500mM KCl [pH 9.0], and 1.0% TritonX-100); 5μl of $MgCl_2$ (25mM); 4μl of 2.5mM dNTP's; 1μl of each primer (0.2-0.3μM), and the volume made up to 99.5μl with sterile distilled water. The mixture was boiled in a water bath for 5 minutes and allowed to cool before 0.5μl of Taq polymerase (2.5 Units) was added to each tube. The reaction mixture was overlaid with one drop of mineral oil (Sigma Chemical Co.) and placed overnight on a Thermal Cycler (PHC-3 Dri-Block® Cycler, Techne [Cambridge] Ltd, Cambridge, U.K.) programmed with the following cycles; 94°C for 1 minute (denaturation), 55°C for 2 minutes (annealing), 72°C for 3minutes (polymerisation). This cycle was repeated 30-40 times and the final amplified mixture stored at 4°C. After amplification, 15μl of PCR product was visualised on an ethidium bromide- agarose gel as described.

2.5 Protein manipulation

2.5.1 Sodium Dodecyl Sulphate -PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to check foreign protein expression by *E. coli* and *S. typhimurium* constructs *in vitro* and from bacteria harvested *in vivo*. Glass plates were washed in 70% ethanol and allowed to air dry before assembly of the SDS-PAGE apparatus (Mighty Small™ II Vertical Slab Unit SE250, Hoefer Scientific Instruments U.K., Newcastle-under-Lyme, U.K.). The lower gel was prepared and consisted of; 5mls of (4x)lower gel buffer (1.5M Tris-HCl [pH 8.8], 0.4% SDS [BDH]), 15 mls of a mix of water and AC/BIS (30% Acrylamide [BDH], 0.8% N, N' -Methylene-bis-Acrylamide [Sigma Chemical Co.] in water), according to the desired percentage of

acrylamide in the final gel, and 0.3mls of 10% ammonium persulphate solution (BDH). To this mixture, 45μl of Tetra-methyl-ethylene diamine (TEMED) (Pharmacia Biotech, St. Albans, U.K.) was added and the gel poured immediately before polymerisation occurred. The top of the gel was overlaid with 1ml of distilled water and allowed to set for 1-2 hours. The water was removed and the top gel prepared, containing 6mls water, 1.5mls AC/BIS, 50μl 10% ammonium persulphate, 2.5 mls of (4x)upper gel buffer (0.5M Tris-HCl [pH 6.8], 0.4% SDS) and 10μl of TEMED. The upper gel was poured on top of the lower gel and allowed to set around a plastic comb. After the upper gel had set, the gel was placed into the electrophoresis tank which contained running buffer (25mM Tris-HCl, 200mM Glycine [BDH], 1% SDS) and placed under constant current at 25mA for 5-10 minutes.

Preparation of bacterial samples for electrophoresis

A 1ml sample of bacterial suspension (after induction of expression if appropriate [see 2.5.3]) was centrifuged at 13,800g for 1 minute and the supernatant discarded. The pellet was resuspended in 25μl of PBS using a whirlimixer (Fisons). To this sample, 25μl of 2x-sample buffer was added (50mM Tris-HCl [pH 6.8], 100mM dithiothreitol [Sigma Chemical Co.], 2% (w/v) SDS, 0.1% (w/v) bromophenol blue [Sigma Chemical Co.] and 10% (V/V) glycerol [Fisons]) and the sample boiled for 10 minutes in a water bath. Samples (15μl) were then loaded onto the gel along with one lane containing 3-5μl of Rainbow Protein Molecular Weight Markers (Amersham International Plc, Little Chalfont, Buckinghamshire, U.K.) and the gel run under constant current of 50mA for 1-2 hours.

2.5.2 Western blotting and dot blots

Proteins separated by SDS-PAGE were transferred electrophoretically onto nitro-cellulose membrane (BioRad Trans-blot® Transfer Medium, BioRad Laboratories Ltd, Hemel Hempstead, Hertfordshire, U.K.) at 100mA constant current overnight

using a Mini Trans-blot® Electrophoretic Transfer Cell (BioRad). The buffer used for transfer contained 1 volume of 10xtransfer buffer (250mM Tris-HCl, 150mM glycine), 2 volumes of methanol and 7 volumes of water.

The nitro-cellulose membrane was washed twice in PBS before blocking in 3% milk powder for 1 hour at room temperature (1% FCS was occasionally used in addition to 3% milk powder for blocking). The membrane was washed twice in PBS/Tween-20(0.05%)(Sigma Chemical Co.) and incubated for 2 hours with an appropriate antibody (see Table 2.5) diluted in 1.5% milk powder in PBS/Tw at room temperature. After two washes in PBS/Tw the membrane was incubated for 2 hours at room temperature with an appropriate antibody conjugate (see Table 2.6) diluted in 1.5% milk powder in PBS/Tw. After two washes in PBS/Tw and one final wash with PBS, the membrane was exposed to an insoluble horseradish-peroxidase substrate solution (10mg of 3,3'-Diaminobenzidine Tetrahydrochloride [Sigma Chemical Co.] dissolved in 15ml Tris Buffered Saline [pH 7.6] with 12μl of fresh 30% Hydrogen Peroxide [BDH]) and allowed to develop. The reaction was stopped by immersing the membrane in distilled water.

Dot blots were performed by plating cultures onto nitro-cellulose filters (Biosupport Membranes, Pall Process Filtration Ltd., Portsmouth, U.K.) which were in contact with L-agar plates containing 100μg of ampicillin. After overnight growth at 37°C the open plates were placed face-down over a chloroform (BDH) bath for 30 minutes. The nitro-cellulose filters were then washed in PBS and subjected to the blotting procedure described above.

Table 2.5: Primary antibodies for western blots

Antibody	Concentration used	Source
Anti-*L.major* gp63 (mouse monoclonal [mAb No. 235])	1:100 dilution	Dr. W. R. McMaster, University of British Colombia, Vancouver, Canada.
Anti-*L. major* p24 (mouse polyclonal)	1:500 dilution	Dr. N. Glaichenhaus, Universite de Nice, France.

Table 2.6: Antibody conjugates

Antibody	Concentration	Source
Rabbit anti-mouse IgG, HRP conjugated	1:1000 dilution	BioRad
Goat anti-mouse IgG, HRP conjugated	1:1000 dilution	Sigma Chemical Co
Rabbit anti-mouse IgG, AP conjugated	1:1000 dilution for ELISA 1:10,000 dilution for blotting	Sigma Chemical Co.
Rat anti-mouse IgG1, Biotin conjugated	1:16,000 dilution for ELISA	Pharmingen
Rat anti-mouse IgG2a, Biotin conjugated	1:1000 dilution for ELISA	Pharmingen

2.5.3 Inducing protein expression in bacteria

Inducing *NirB* promoter (372)

Overnight broth cultures of bacteria were diluted 30 fold into 7ml screw-cap bijou containers with 4mg/ml Glucose (BDH), 100μg/ml ampicillin in L-broth. The tubes were filled as full as possible to limit the supply of oxygen to the bacteria. The bacteria were then incubated for 4-6 hours before being analysed for protein expression.

Inducing *OsmC* promoter

Overnight cultures of bacteria were diluted 20 fold in 0.3M NaCl solution in L-broth containing 100μg/ml ampicillin and cultured for 2-4 hours before analysis of protein expression was performed.

2.6 Immunisation with Salmonella constructs

2.6.1 Bacterial culture and immunisation

A single colony of *S. typhimurium* from an L-agar plate was grown in 0.5-1 litre of L-broth overnight at 37°C without shaking. The optical density at 600nm of this suspension was measured and the bacteria centrifuged at 8670g for 7 minutes at 4°C using a JA-10 rotor(Beckman) in a High Speed Centrifuge (Beckman). The pellet was resuspended in sterile PBS to obtain a bacterial concentration of approximately 2.5 x 10^{10}/ml. The volume of PBS was calculated from the OD600 reading x 20, per litre of suspension. A typical one litre overnight culture has an OD600 of around 0.2 and would therefore be resuspended in 4ml of sterile PBS. The bacterial concentration of this suspension was checked by plating out dilutions of the suspension on L-agar plates after administration.

Mice received 0.1mls of sterile 5% $NaHCO_3$ (Fisons) solution orally using a gavage needle, immediately before *Salmonella* in order to neutralise stomach pH. The *Salmonella* suspension was administered orally by gavage needle in a volume of 0.2-0.4mls. Penetration of internal organs by the bacteria was assessed by killing one animal per group, one week after administration, and culturing Mesenteric Lymph Node (MLN) homogenates on L-agar plates (as described in 2.6.2) or by measuring anti-salmonella antibody responses in the serum of mice 2 weeks after administration (2.7.3).

2.6.2 Bacterial growth and plasmid stability *in vivo*

Bacterial colonisation and plasmid stability*in vivo* was measured by counting bacteria recovered from spleens, livers and mesenteric lymph nodes of immunised mice as previously described (333). Mice were administered 0.2mls of *Salmonella* as described in 2.6.1 and killed at various time points later. Spleens, livers and mesenteric lymph nodes from three mice per time point were placed in 10mls of sterile PBS and homogenised using a 15ml glass homogeniser. Ten fold dilutions of this homogenate were plated, in triplicate, in a volume of 100μl onto L-agar plates in the presence or absence of 100μg/ml ampicillin (Western Infirmary Pharmacy) and plates grown overnight at 37°C. The number of colonies which grew in the absence of ampicillin was used to calculate *Salmonella* colonisation of organs and the number growing with ampicillin was used to calculate plasmid stability.

2.7 Immunological analysis

2.7.1 Preparation of parasite antigens

A number of different parasite antigen preparations were used for immunological analysis. Parasites were grown *in vitro* as described in 2.3.2 and

harvested at 7-10 days after passage. In experiments where live parasites were used, these were merely washed twice with sterile PBS, counted and resuspended at the required concentration in Complete RPMI, consisting of RPMI 1640 containing 10% heat-inactivated FCS, 2mM L-glutamine, 100U/ml penicillin (Gibco), 100μg/ml streptomycin (Gibco) and 50μM 2-mercaptoethanol (Sigma Chemical Co.). Formalin-fixed parasites were obtained by fixing parasites ($2x10^8$/ml) in 0.5% formaldehyde (Sigma Chemical Co.) in PBS for 5 minutes at room temperature followed by three washes in sterile PBS before resuspending in Complete RPMI. Soluble Antigen was prepared by freezing and thawing parasites ($2x10^8$/ml in PBS) 5 times followed by centrifugation at 13,800g for 10 minutes. The resulting supernatant was filtered using a 0.45μm filter (Millipore S.A., Molsheim, France) and diluted in Complete RPMI. Parasites were also sonicated (20kHz, 1 minute) using a Branson Sonifier 250 (Branson Ultrasonics Corporation, Danbury, CT, USA) in PBS ($2x10^8$/ml) and resuspended in Complete RPMI. *Salmonella* lysate was prepared from an overnight culture of BRD509. 1ml of suspension was centrifuged at 13,800g and the pellet resuspended in 1ml of sterile distilled water and left at room temperature for 10 minutes. The lysate was centrifuged again and the supernatant stored at -20°C until used.

2.7.2 Parasite quantitation in footpads

The number of parasites present in infected footpads was quantified as previously described (278). At various time points after infection, footpads were removed from mice by cutting the foot above the ankle. Footpads were cut into small pieces in CSDM in a petri dish (90mm) and then filtered through NITEX. The resulting suspension was cultured for 48 hours at 28°C. Plates were then pulsed with ^{3}H-thymidine at 1μCi per well for 24 hours and harvested using an automatic cell harvester. Incorporated radioactivity was counted using a ß-counter (LKB, Betaplate, Turku, Finland).

2.7.3 Induction and measurement of Delayed-Type Hypersensitivity (DTH)

Immunised mice were injected in the left-hind footpad with $1x10^6$ live *L. major* promastigotes in 50μl of PBS. Both footpads were measured at regular intervals for up to 96 hours using a spring dial-calliper (Kroeplin). Change in footpad thickness was assessed as the footpad thickness of injected footpad minus footpad thickness of uninjected.

2.7.4 Measurement of specific antibody responses

Antibody responses were measured in mice immunised 2 weeks previously with 0.2 mls of *S. typhimurium* expressing *L. major* gp63. Blood was obtained by bleeding from the retro-orbitalplexus using Heparinised Capillary tubes (Hawksley & Sons Ltd., Lancing, Sussex, U.K.) and serum obtained by centrifugation at 350g. An ELISA method was used as previously described (278). Soluble *L. major* antigen ($4x10^7$/ml), recombinant gp63 (Dr. W. R. McMaster, University of British Colombia, Vancouver, Canada) or *Salmonella* lysate diluted in 0.1M Na_2CO_3 (pH 8.2) was coated onto Immulon-4 96-well plates (Dynatech Laboratories Ltd., Billingshurst, West Sussex, U.K.) in a volume of 50μl/well and incubated overnight at 4°C. Plates were washed twice in PBS containing 0.05% Tween-20 and blocked with 100μl/well of 20% FCS in PBS and incubated at 37°C for 1 hour. Plates were again washed twice using PBS/Tw, and serial dilutions of serum samples added to wells in a volume of 50μl, before plates were incubated for 2-3 hours at 37°C. Serum from infected mice and a monoclonal antibody against gp63 (Dr. W. R. McMaster) were used as control serum against gp63. Plates were washed 6-8 times in PBS/Tw and then conjugated antibodies added to detect IgG, IgG2a or IgG1 (Table 2.6) in a volume of 100μl and incubated at 37°C for 2 hours. ELISA's for IgG2a or IgG1 were washed a further 6-8 times and 100μl of Extravidin-Peroxidase (Sigma Chemical Co.) added at a concentration of 3μg/ml. All ELISA's were then washed 8 times in PBS/Tw before developing using TMB

Microwell Peroxidase Substrate (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, U.S.A). The colour change on the plates was measured using a MR5000 Microplate Reader (Dynatech) at 630nm.

2.7.5 Antigen specific proliferation *in vitro*

Antigen was prepared as described in 2.7.1 and diluted in Complete RPMI to the required concentration before adding 100μl/well to 96-well tissue culture plates (Costar). Where Concanavalin A (Sigma Chemical Co.) was used in proliferation assays, this was dissolved in Complete RPMI at 1mg/ml and aliquots stored at -20°C until used. Aliquots were then added to Complete RPMI at the required concentration and added to 96-well tissue culture plates in 100μl volumes.

Spleen and MLN cells were removed from mice and single cell suspensions obtained by gentle homogenisation using a 15ml glass homogeniser. Cells were filtered to remove debris using NITEX and washed 3 times in RPMI. Cells were resuspended in 2mls of Complete RPMI and again filtered before counting using an Improved Neubauer Haemacytometer, under phase-contrast microscopy. The cell concentration was adjusted to $4x10^6$/ml and 100μl added to each well in quadruplicate cultures. Cells were cultured at 37°C in 5% CO_2, pulsed with ^{3}H-Thymidine (1μCi per well), harvested on an automatic cell-harvester and radioactivity counted in a β-counter (LKB).

2.7.6 Antigen specific cytokine responses *in vitro*

Cultures were set up exactly as described in 2.7.4 and supernatants collected at various times after incubation to assess antigen-specific cytokine production. The levels of IL-2, IL-4 and IFN-γ were measured by ELISA using paired antibodies obtained from PharMingen (Cambridge Bioscience, Cambridge, U.K.). Immulon-4, 96-well plates were coated with 50μl of the appropriate coating antibody at the

concentration shown in Table 2.7 diluted in 0.1M Na_2CO_3 (pH 8.2). Plates were then washed twice in PBS/Tw and blocked with 20% FCS in PBS (200μl/well) for 1 hour at 37°C. Plates were again washed in PBS/Tw before samples (in quadruplicate) and standard recombinant cytokine dilutions (in duplicate) were added in a volume of 50μl. IFN-γ was a kind gift from Dr. G. Adolf, Vienna and IL-2 and IL-4 were obtained from Genzyme. Plates were incubated for 2 hours at 37°C. Plates were then washed 6-8 times in PBS/Tw and the appropriate detecting antibody added in a volume of 100μl, diluted to the concentration shown in Table 2.7 in 5% FCS in PBS before incubation for 1 hour at 37°C. For the IFN-γ ELISA, plates were washed a further 6-8 times in PBS/Tw before alkaline-phosphatase-conjugated goat anti-rabbit IgG (Sigma Chemical Co.) was added in a volume of 100μl/well. Plates were washed 8 times in PBS/Tw before 100μl/well of the substrate added (1mg/ml 3,3'-diaminobenzidine tetrahydrochloride in 1M Tris, 3mM $MgCl_2$) and the colour allowed to develop for 20-40 minutes. The colour change was measured at OD405nm using a Microplate reader. For all other ELISA's, after the biotinylated detecting antibody, plates were washed 8 times in PBS/Tw before Extravidin-Peroxidase (Sigma Chemical Co.) was added (100μl/well) at a concentration of 3μg/ml in 5% FCS in PBS followed by incubation at 37°C for 1 hour. Plates were again washed 8 times with PBS/Tw before 100μl of TMB Microwell Peroxidase Substrate was added and the colour change detected using a Microplate reader at 630nm.

Table 2.7: Antibody concentrations for ELISA

Cytokine	Primary antibody (coating conc.)	Source	Secondary antibody (conc.)	Source
IFN-γ	R46A2 (10μg/ml)	DNAX Corporation	Rb anti-IFN-γ (5μg/ml)	DNAX Corporation
IL-4	Anti-IL-4 MAb (2μg/ml)	Pharmingen	Biotinylated anti-IL-4 MAb (1μg/ml)	Pharmingen
IL-2	Anti-IL-2 MAb (2μg/ml)	Pharmingen	Biotinylated anti-IL-2 MAb (1μg/ml)	Pharmingen

2.7.7 Cell depletion *in vitro*

The effect of CD4 or CD8 cell depletion *in vitro* on cytokine production was assessed by adding anti-CD4 (rat hybridoma YTS 191) or anti-CD8 (YTS 169) (19) monoclonal antibodies to cultures. Anti-CD4 and anti-CD8 were grown as ascites in pristane-treated nude mice and partially purified by precipitation with 45% saturated ammonium sulphate. Cultures were set up as described in 2.7.5 and antibodies were added at 10μg/ml.

2.8 Statistical analysis

Statistical significance of the data was analysed using Student's t-test. A p value of less than 0.05 was considered significant.

Chapter 3

Immunisation with gp63 delivered orally in attenuated *Salmonella typhimurium* (aroA⁻ aroD⁻)

Contents

3.1 Introduction

An attenuated *S. typhimurium* construct (SL3261/gp63) expressing *L. major* gp63 has been described previously (278). This construct, when administered orally to CBA mice, which are genetically resistant to *L. major* infection, reduced lesion development upon challenge infection. Protective immunity in this model correlated with a Th1-like response to parasite antigens when spleen cells were cultured *in vitro*. While this *S. typhimurium* vaccine reduced lesion development in mice which eventually resolve *L. major* lesions it provided no protection to genetically susceptible, BALB/c mice after *L. major* infection.

The aroA⁻ aroD⁻ vaccine strain BRD509 has been described in detail previously (352). This attenuated *S. typhimurium* strain has two defined genetic disruptions in the genes *aroA* and *aroD*. Disruption of these genes at the aro locus render the bacteria deficient in pathways of aromatic amino acid biosynthesis. The bacteria are therefore unable to synthesise a number of products derived from this pathway including 2,3 Di-hydroxy-benzoic acid (DHBA), Para-amino benzoic acid (PABA) and the essential amino acids (330). The *S. typhimurium* strain used in the previous vaccine, SL3261, has a single deletion at this locus while BRD509 contains two. While this double deletion results in increased attenuation of the bacteria and a decrease in the chance of reversion to virulence, the effects of this upon the immunogenicity of foreign proteins expressed in this strain is unknown. This chapter details the construction of a *S. typhimurium* construct (GID101), based on the strain BRD509, which expresses *L. major* gp63. This construct provides protection to susceptible, BALB/c mice after live challenge with *L. major*. The immune responses induced in mice after oral delivery of this construct are also described.

3.2 Factors affecting oral *S. typhimurium* delivery

There are a number of parameters which can influence the ability of *S. typhimurium* to penetrate epithelial cells *in vitro*, such as bacterial growth state (373), and the expression of a number of bacterial virulence factors (374). An attempt was made to ascertain the optimal conditions for BRD509 colonisation *in vivo* with regard to bacterial growth state and stomach pH neutralisation before delivery.

3.2.1 Bacterial growth state

To monitor the effect of bacterial growth state upon colonisation, BALB/c mice were immunised orally with 5 x 10^9 attenuated *S. typhimurium* (BRD509) which had been grown *in vitro* for different periods of time. Eleven days after administration, mice were killed, and the number of viable bacteria present in the spleen, liver and mesenteric lymph nodes (MLN) determined by plating organ homogenates onto L-agar plates, as described in Chapter 2. The results of this experiment are shown in Table 3.1.

There was efficient colonisation of spleen, liver and mesenteric lymph nodes at 11 days post administration with cultures of BRD509 grown for both 16 and 24 hours. The cultures grown for 12 hours did not contain sufficient bacteria to allow an assessment of colonisation *in vivo*. While mice administered 16 or 24 hour cultures did not differ significantly in the number of colonies recovered from the spleen, there was considerably more bacteria recovered from the liver and MLN of mice administered the 16 hour culture. These data confirm that bacterial growth state does impact upon the ability to colonise *in vivo*. All future experiments are based on cultures grown for 16 hours only.

Table 3.1: Effect of culture times on BRD509 colonisation

Time of culture (hours)	No. of colonies per **SPLEEN** (SEM)	No. of colonies per **LIVER** (SEM)	No. of colonies per **MLN** (SEM)
12	N.D.	N.D.	N.D.
16	320 (35)	2900 (346) *	3100 (404) *
24	690 (283)	490 (162)	1300 (173)

Cultures of BRD509 were grown for 12, 16 or 24 hours before being orally administered to BALB/c mice. After 11 days mice were killed and organs assessed for bacterial colonisation. Results show means and SEM from three mice per group. N.D. - Not Determined. (insufficient bacteria to administer) * $p < 0.05$ compared to 24 hour culture.

3.2.2 Pre-dosing with carbonate solution

Some researchers pre-dose animals with sodium bicarbonate before orally administering *S. typhimurium* as a means to neutralise stomach pH and thus prevent bacterial killing. Other researchers deliver *S. typhimurium* directly with no neutralisation step. Experiments were conducted to determine if the neutralisation of stomach pH using sodium bicarbonate had any effect on the colonisation and survival of BRD509 *in vivo*. BALB/c mice were administered orally 5 x 10^9 attenuated *S. typhimurium* (BRD509) with or without oral pre-dosing with 0.1mls of 5% Na_2CO_3 solution and numbers of viable bacteria in spleen, liver and MLN determined at various time points later. The results of this experiment are shown in Figure 3.1.

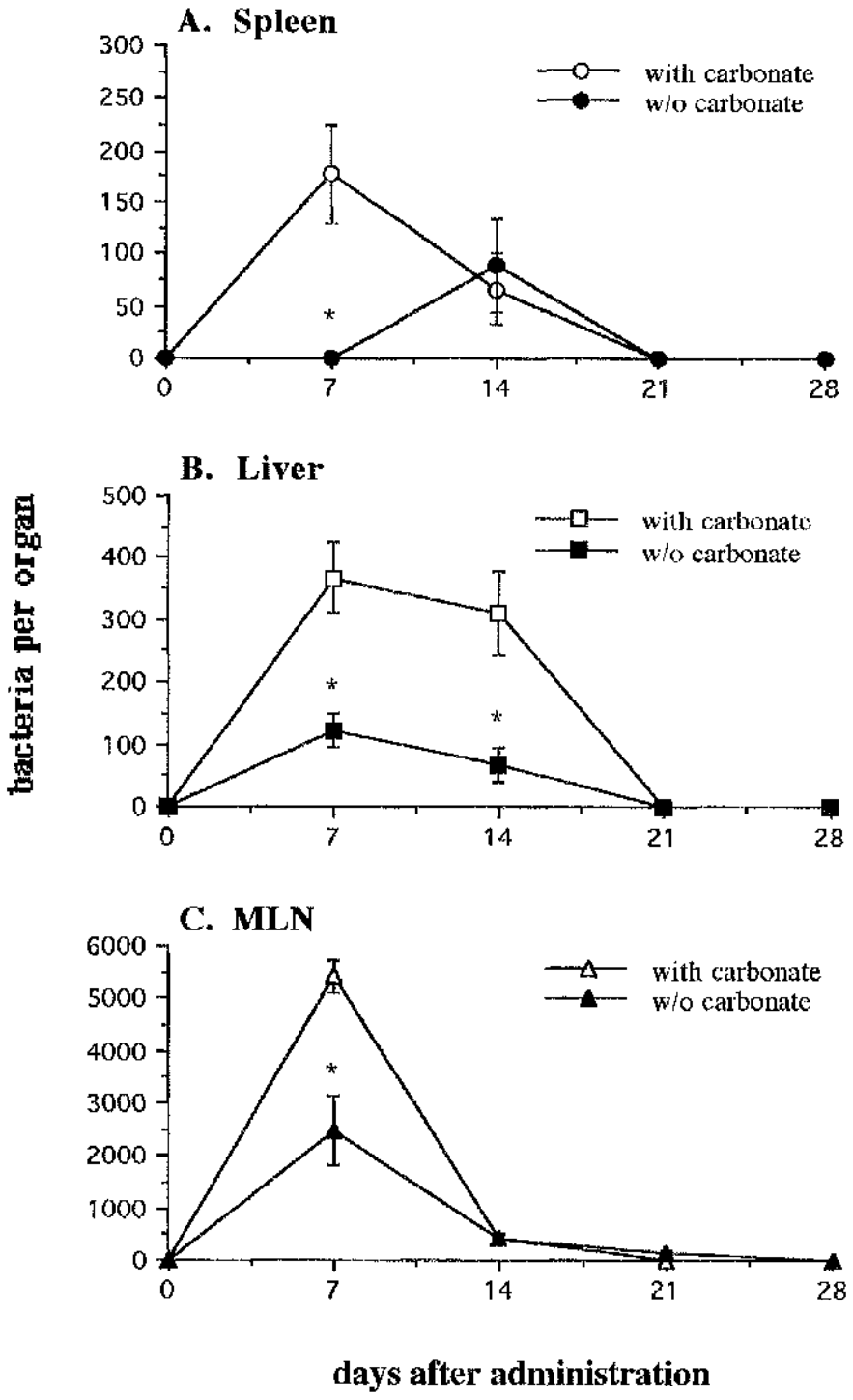


Figure 3.1: Effect of carbonate solution on colonisation of spleen, liver and MLN by BRD509. Each point represents mean and SD of three mice. * $p < 0.05$ compared to with carbonate data.

This experiment demonstrates that administration of sodium bicarbonate prior to BRD509 increases the bacterial colonisation of spleen, liver and MLN. In all future experiments mice received sodium bicarbonate before receiving *S. typhimurium.*

These data also show the colonisation of this attenuated strain *in vivo* over time. BALB/c mice administered 5 x10^9 BRD509 harbour bacteria in the spleen and liver until they are essentially eliminated by 2-3 weeks after the dose is given. The MLN usually contain considerably more bacteria than spleen or liver and these survive for up to 3-4 weeks after administration

3.3 Construction of *S. typhimurium* expressing *L. major* gp63 (GID101)

3.3.1 Construction of GID101

The construction of a *S. typhimurium* construct, based on BRD509, which expressed *L. major* gp63 was accomplished by Dr. D. Xu (Immunology Dept., Western Infirmary, Glasgow, U.K). and is described here only briefly. The plasmid pkk-Imm63-67 containing the cDNA for *L. major* gp63 was transferred to BRD509 using p22 transduction (375) and transductants selected by growth in L-broth containing ampicillin. Purified plasmids from colonies which grew on ampicillin were verified by restriction enzyme digestion. The *S. typhimurium* strain BRD509, containing the plasmid pkk-Imm63-67, was named GID101.

3.3.2 Expression of gp63 by GID101

Western blotting was performed as described in Materials and Methods to assess gp63 expression by the strain GID101. As the plasmid pkk-Imm63-67 contains a tac promoter which is constitutively transcribed (276), it was not necessary to induce

expression. Constitutive expression of gp63 was evident by a band at approximately 60kDa (Fig. 3.2). The native gp63 is glycosylated.

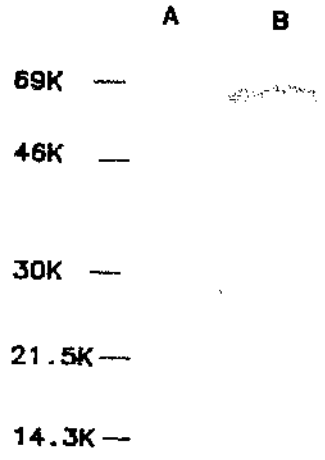


Figure 3.2: Western blot detecting gp63 expression in GID101 using mAb no. 235. Lane A: BRD509 control. Lane B: GID101.

3.4 Plasmid stability of GID101

3.4.1 Plasmid stability *in vitro*

Plasmid stability of GID101 was assessed *in vitro* by culturing the construct in the absence of antibiotic selective pressure. This experiment was performed by Dr. D.

Xu. The plasmid was quickly lost *in vitro* and was not maintained beyond three passages in the absence of selective pressure (Dr. Xu, personal communication).

3.4.2 Plasmid stability *in vivo*

BALB/c mice were orally administered 5 x 10^9 of GID101 and plasmid stability was assessed at various time points later in spleen, liver and MLN. Plasmid stability was assessed by counting the number of recoverable *S. typhimurium* which were not sensitive to the presence of ampicillin in L-agar plates, as described in Materials and Methods. Figure 3.3 shows the growth curve of GID101 in the spleen, liver and MLN of BALB/c mice. The plasmid in GID101 is relatively stably inherited by bacteria colonising the MLN but bacteria recoverable from the spleen and liver showed pronounced plasmid loss throughout the course of the experiment.

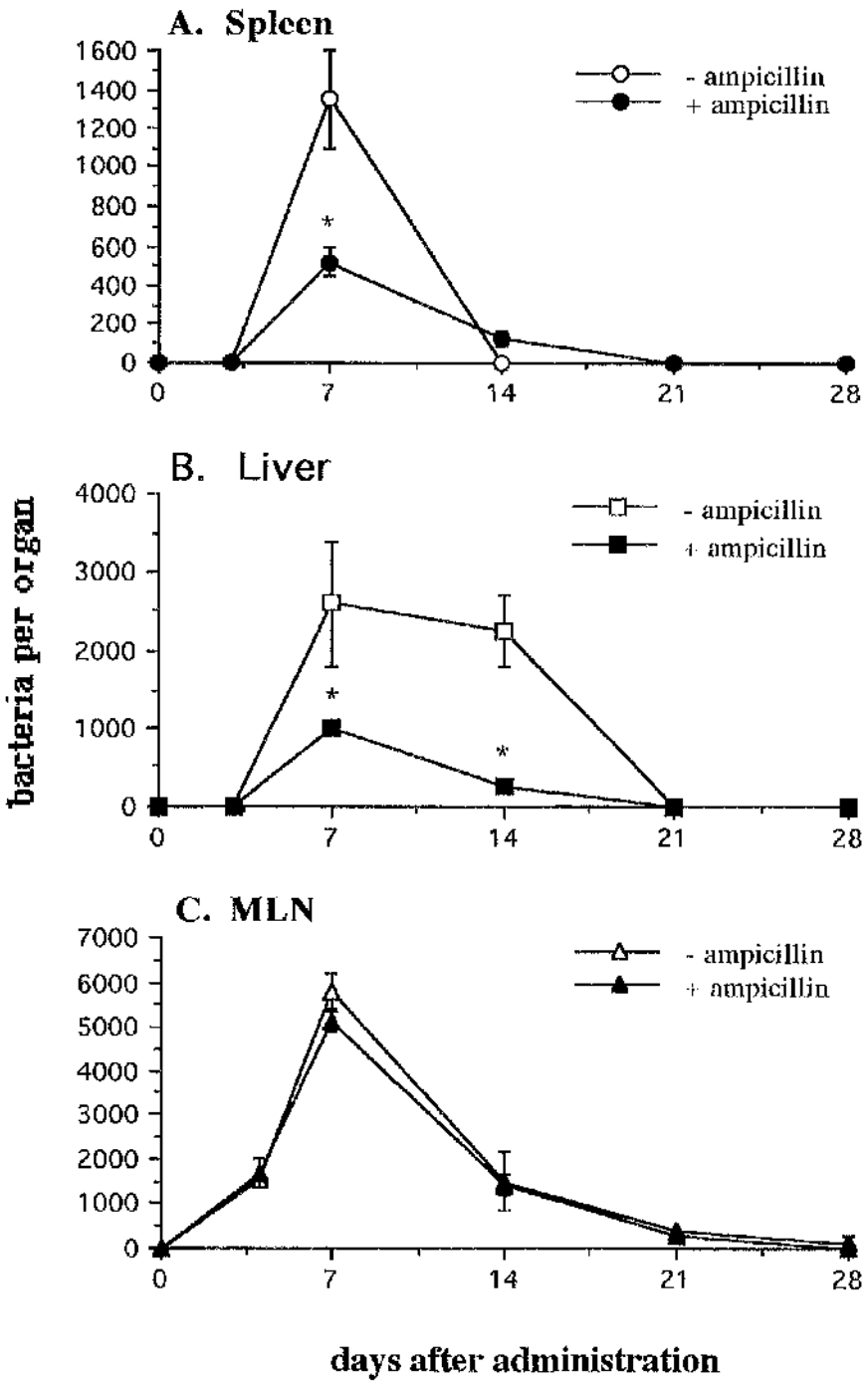


Figure 3.3: Plasmid stability of GID101 during BALB/c colonisation. Data show mean and SEM of three mice per time point. Data are representative of two individual experiments. * $p < 0.05$

3.5 Protection against *L. major* infection

BALB/c mice were immunised orally with 2.5-10x10^9 of GID101 or BRD509. Control mice received 0.2 mls of PBS orally. Two weeks later, this vaccination procedure was repeated and mice challenged in the footpad with *L. major* a further two weeks later. Mice which received two doses of GID101 developed significantly smaller lesions and showed a marked delay in the onset of disease compared to mice administered BRD509 (Fig. 3.4). Mice administered PBS developed lesions similar to those of mice which received BRD509. The lesion development paralelled the parasite load in the infected footpads as assessed by serial dilution analysis of the infected footpad tissue, as described in Materials and Methods (Fig. 3.5). Mice administered GID101 showed less parasite growth in footpad homogenates.

These data demonstrate significant protection against lesion development in susceptible BALB/c mice, conferred by GID101. However, it should be noted that this protection was insufficient to completely protect against challenge infection. Mice eventually developed lesions that necessitated their being killed, complying with Home Office guidelines. Notwithstanding, these experiments do clearly show a protective response induced by GID101 at the early stages of infection and indicate that gp63 expression in the strain BRD509 confers advantages over the previous strain, SL3261.

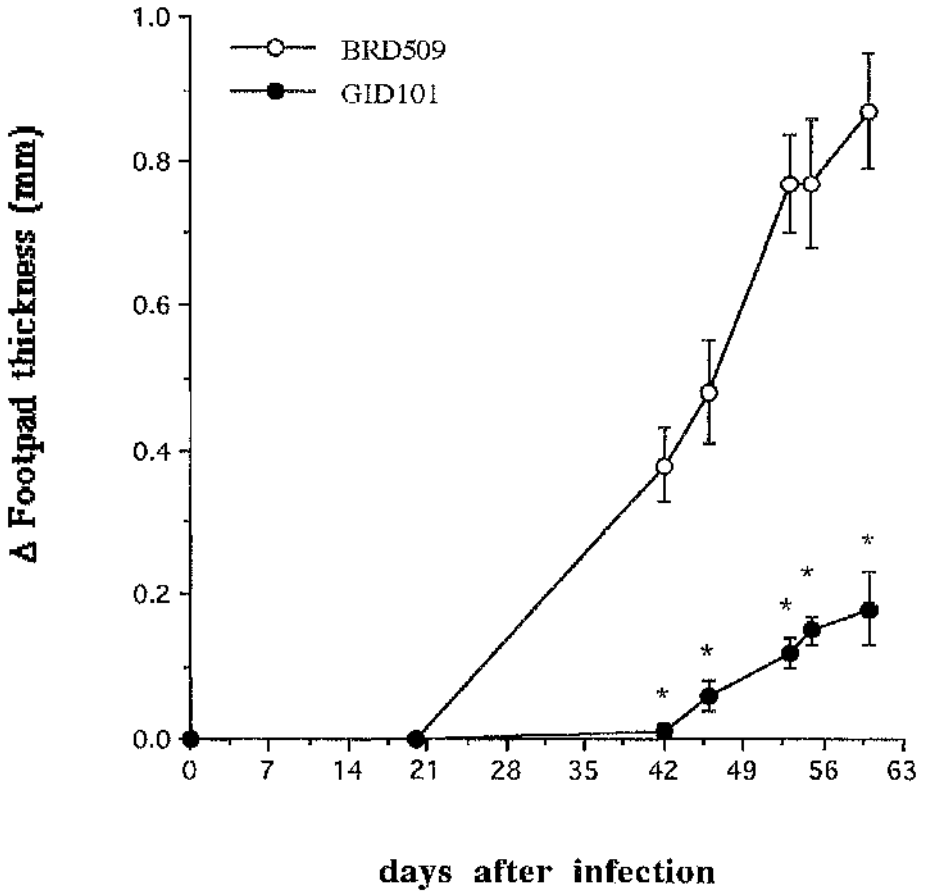


Figure 3.4: Protection against *L. major* infection by GID101. PBS administered mice develop lesions similar to those of BRD509 and are not shown for clarity of figure. Data show mean and SEM of six mice per group.

This experiment was repeated twice with similar results and these are the data from the first experiment. *$p<0.05$

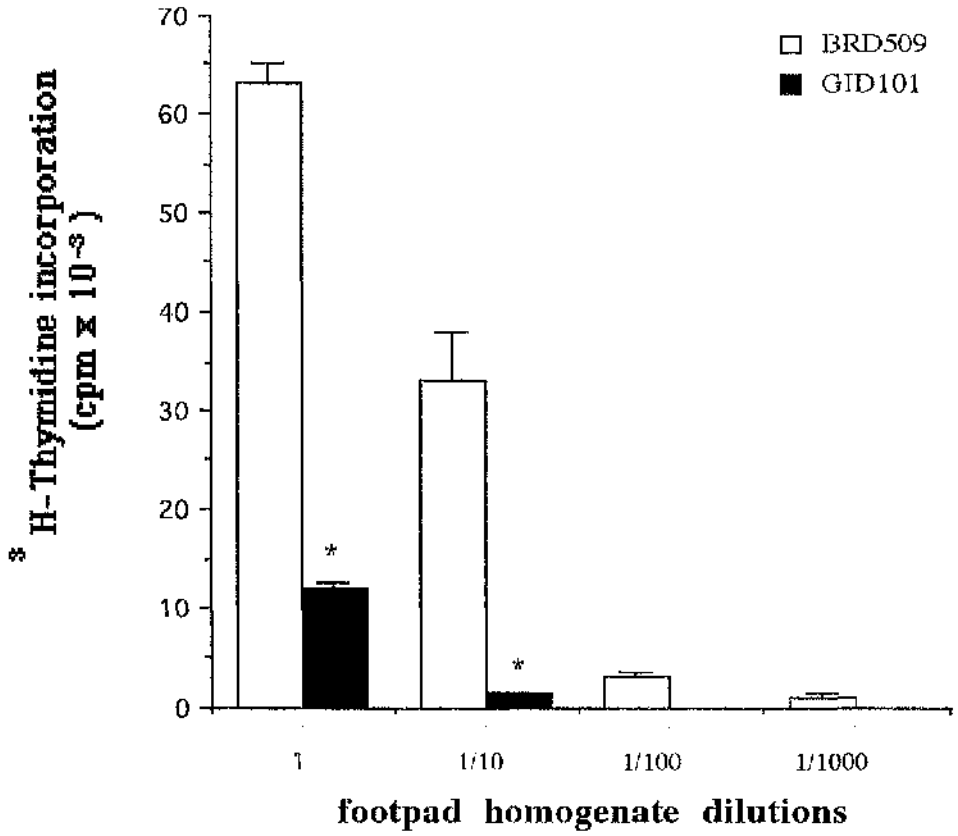


Figure 3.5: Parasite quantitation in infected footpads of mice administered GID101 or BRD509. Data show mean and SD of three mice per group. * $p < 0.05$

3.6 Immune response to *L. major*

The immune response to *L. major* induced by GID101 was analysed *in vitro*. BALB/c mice were orally administered twice with either GID101, BRD509 or PBS, as described in Materials and Methods. Spleen and MLN cells were removed from these mice 3-5 weeks later and stimulated *in vitro* with parasite antigens. Serum was also collected 3-5 weeks after immunisation and antibody responses to *L. major* antigens were assessed, as described in Materials and Methods. In parallel experiments, mice were injected with *L. major* in the footpad in order to measure parasite specific DTH responses.

3.6.1 DTH response

BALB/c mice receiving two oral doses of GID101 did not develop a detectable DTH response to footpad challenge with *L. major* promastigotes (data not shown).

3.6.2 Antibody response

Serum from mice vaccinated with two doses of GID101 contained significant levels of IgG to both recombinant *L. major* gp63 and Soluble *L. major* Antigens (SLA) (Fig. 3.6). When further analysed, the serum was found to contain significant levels of IgG2a to both rgp63 (data not shown) and SLA (Fig. 3.7A) yet no IgG1 to rgp63 (data not shown) or SLA (Fig. 3.7B) was detected. There was also a significant antigen specific IgG and IgG2a response in the serum of mice which had been administered the control *S. typhimurium* strain, BRD509, although this was much less than that of mice administered GID101.

3.6.3 Proliferative response *in vitro*

BALB/c mice were immunised with GID101, BRD509 or PBS twice, orally. Spleen and MLN cells were harvested 3-5 weeks later and cultured *in vitro* with formalin-fixed, sonicated or live *L. major* promastigotes or SLA, as described in Materials and Methods. Cells obtained from mice vaccinated with GID101 showed a strong proliferative response, at three days of culture, to live or sonicated parasites in a dose-dependent manner (Fig. 3.8A-B). There was also substantial parasite-specific proliferation detected at 5 days of culture (data not shown), although this was somewhat reduced in magnitude. These cells also proliferated when cultured with formalin-fixed promastigotes or SLA, but the level of response was much lower (Fig. 3.8C-D). No significant response to any of the above antigens was detected in the cell cultures from mice which received PBS or the control *S. typhimurium* strain, BRD509.

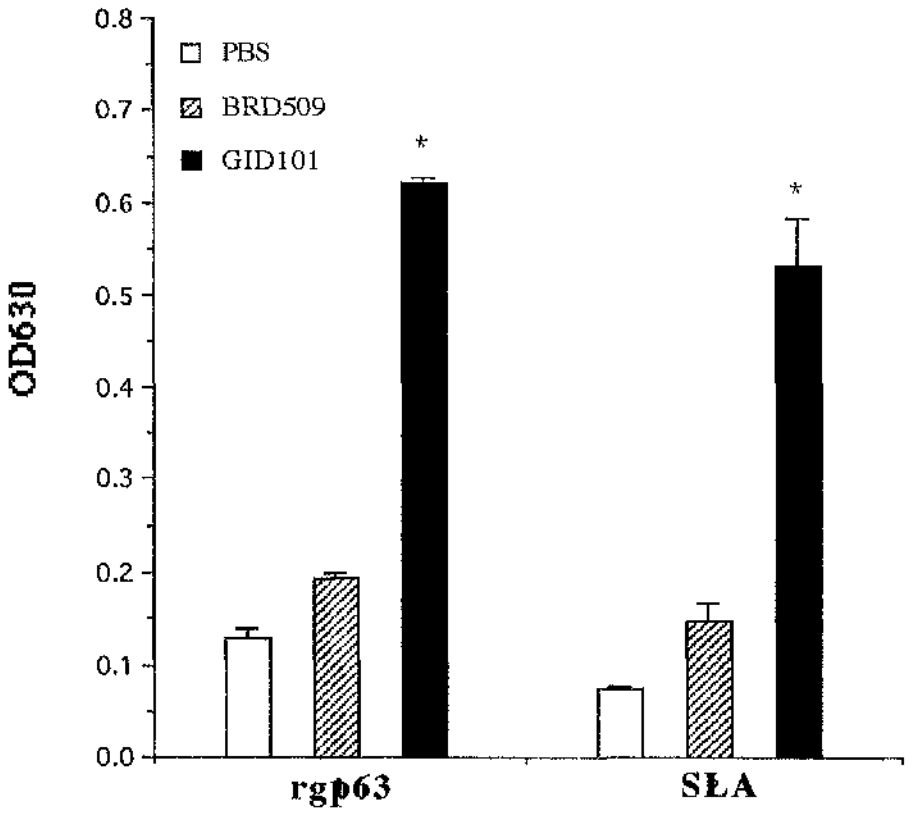


Figure 3.6: Parasite specific IgG after 2 oral doses of GID101. Serum from immunised BALB/c mice was diluted 1:400 and assessed for IgG to rgp63 and SLA. Data show mean and SD for three mice per group and is representative of three individual experiments. *p<0.05

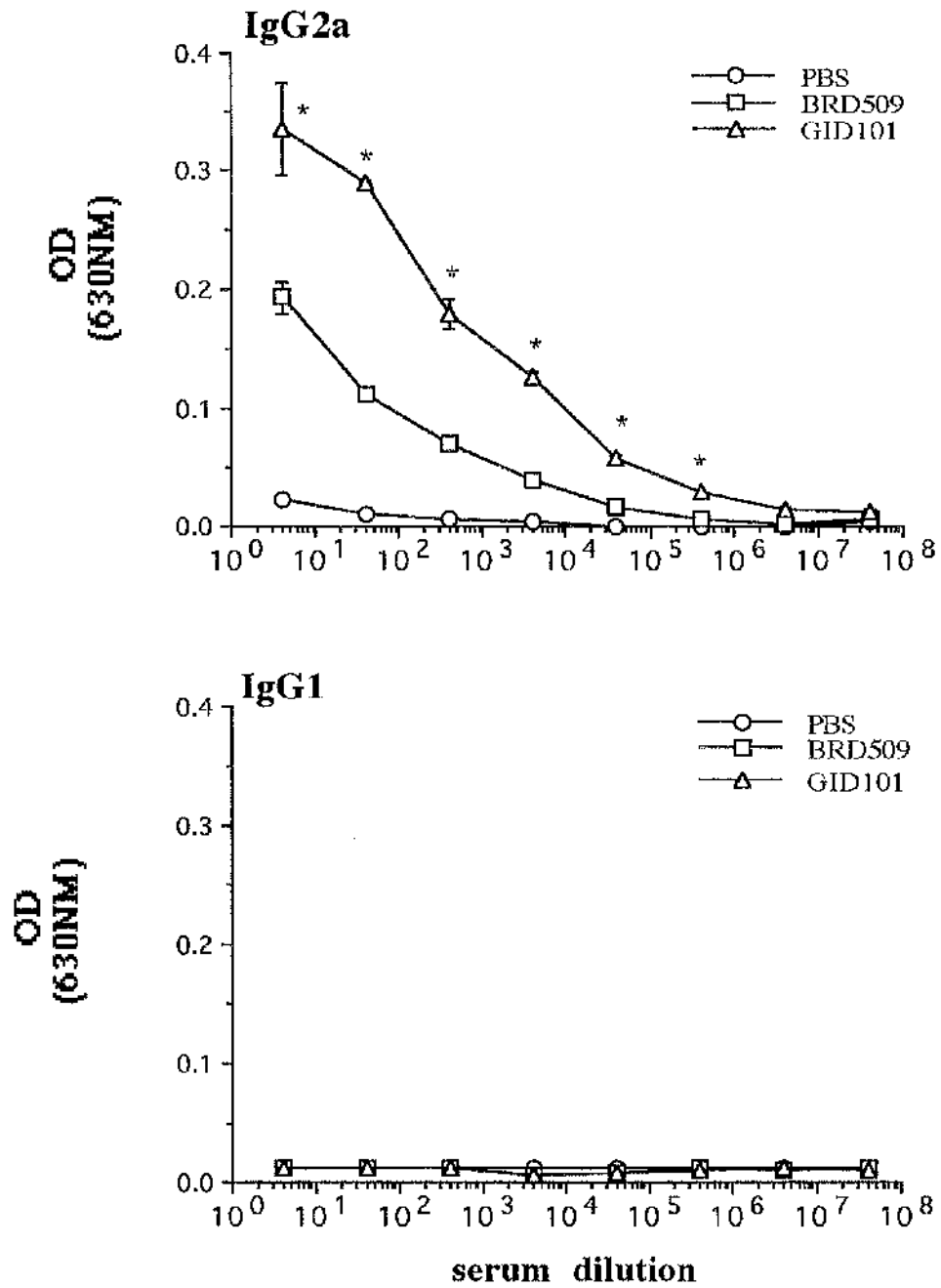


Figure 3.7: Parasite specific IgG2a and IgG1 after two oral doses of GID101. Serum from BALB/c mice was analysed at various dilutions for IgG isotype responses to SLA. Data show mean and SD for three mice and are representative of three individual experiments. * $p < 0.05$

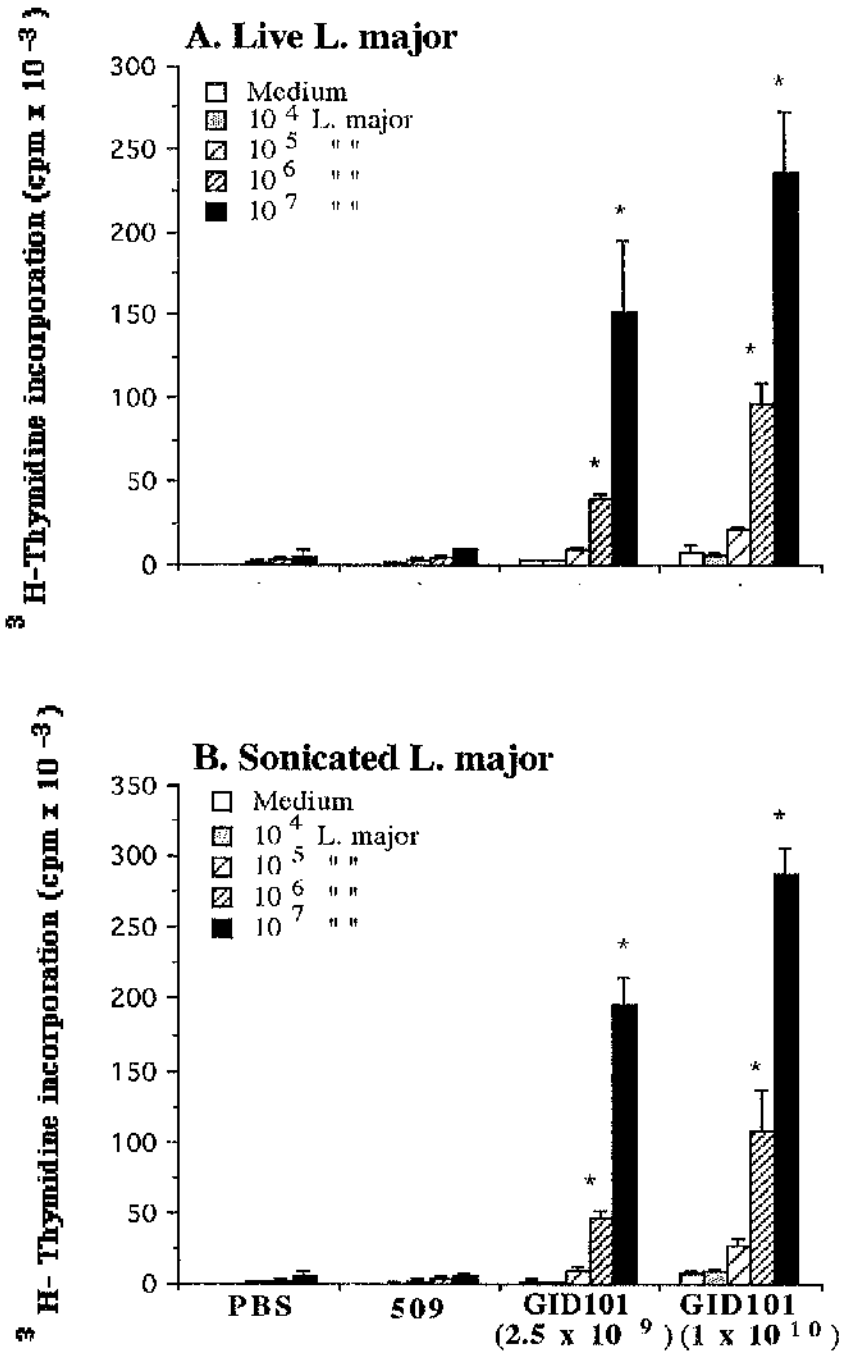


Figure 3.8 (A-B): Proliferative response of GID101 immunised cells to *L. major* antigens. Data show mean and SD of pooled cells from three mice. Data are representative of three individual experiments. * $p < 0.05$

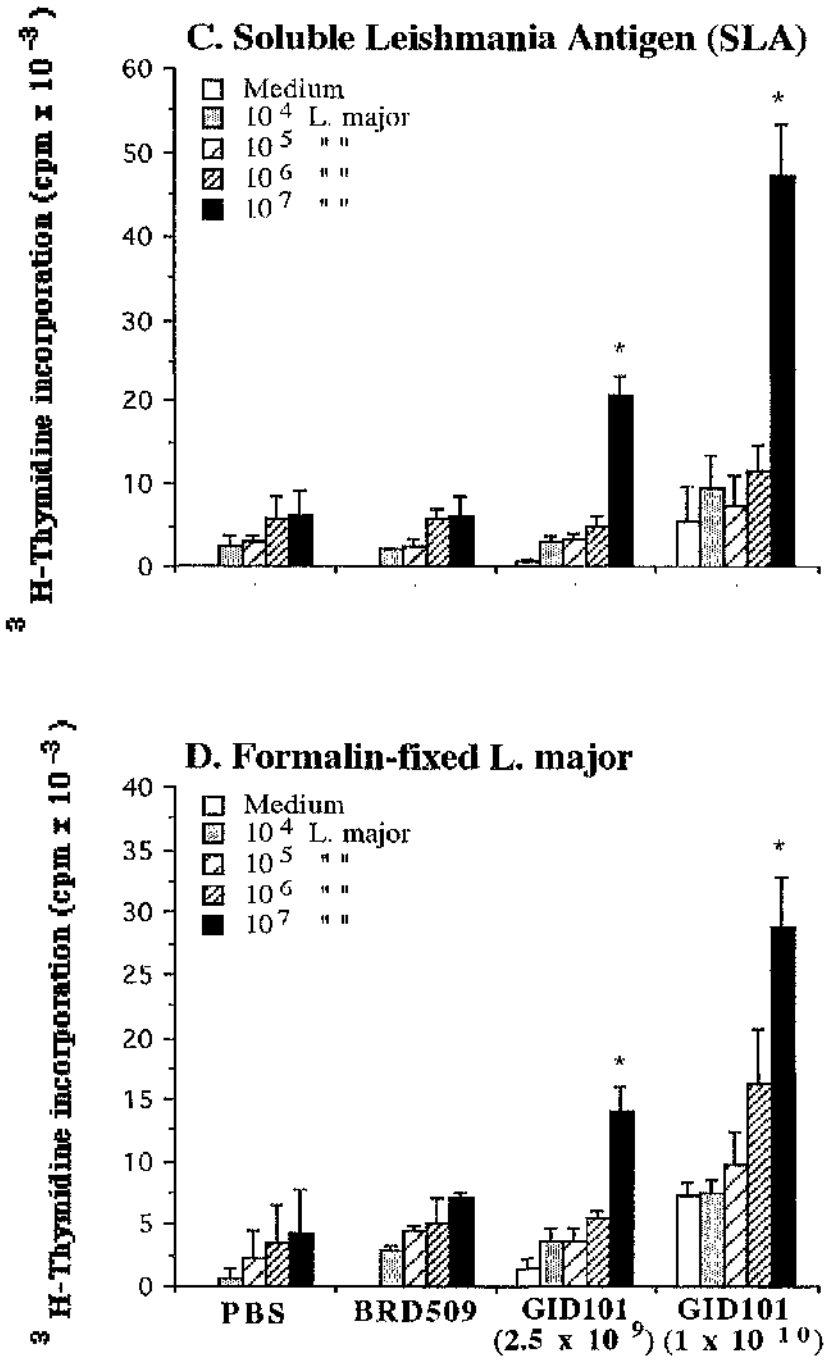


Figure 3.8 (C-D): Proliferative response of GID101 immunised cells to *L. major* antigens. Data show mean and SD of pooled cells from three mice and are representative of three individual experiments. * $p < 0.05$

3.6.4 Cytokine production *in vitro*

Spleen and MLN cells from mice immunised orally with GID101, BRD509 or PBS were cultured *in vitro* with SLA, as described in Materials and Methods. The SLA antigen preparation has been shown to be optimal in inducing cytokine production in this system (278). Cells from mice vaccinated with GID101 produced significant amounts of IL-2 and IFN-γ in response to antigen in a dose dependent manner (Fig. 3.9A-B). Both the production of IFN-γ and IL-2 peaked at 48 hours of culture with antigen (Fig. 3.10A-B). In cell cultures from mice immunised with PBS or the control *S. typhimurium* strain, BRD509, there was no detectable production of IL-2 or IFN-γ in response to antigen at any time point. No antigen specific IL-3, IL-4 or IL-5 was detected at any time point in any of the cell cultures (data not shown).

3.6.5 Cell depletion *in vitro*

Antigen specific IL-2 and IFN-γ production was $CD4^+$-cell dependent. Addition of anti-CD4 antibody (YTS169), at 10μg/ml, completely abolished IL-2 and IFN-γ production in cell cultures from mice immunised with GID101 (Fig. 3.11A-B). Addition of anti-CD8 antibody (YTS191) at the same concentration did not significantly alter cytokine production.

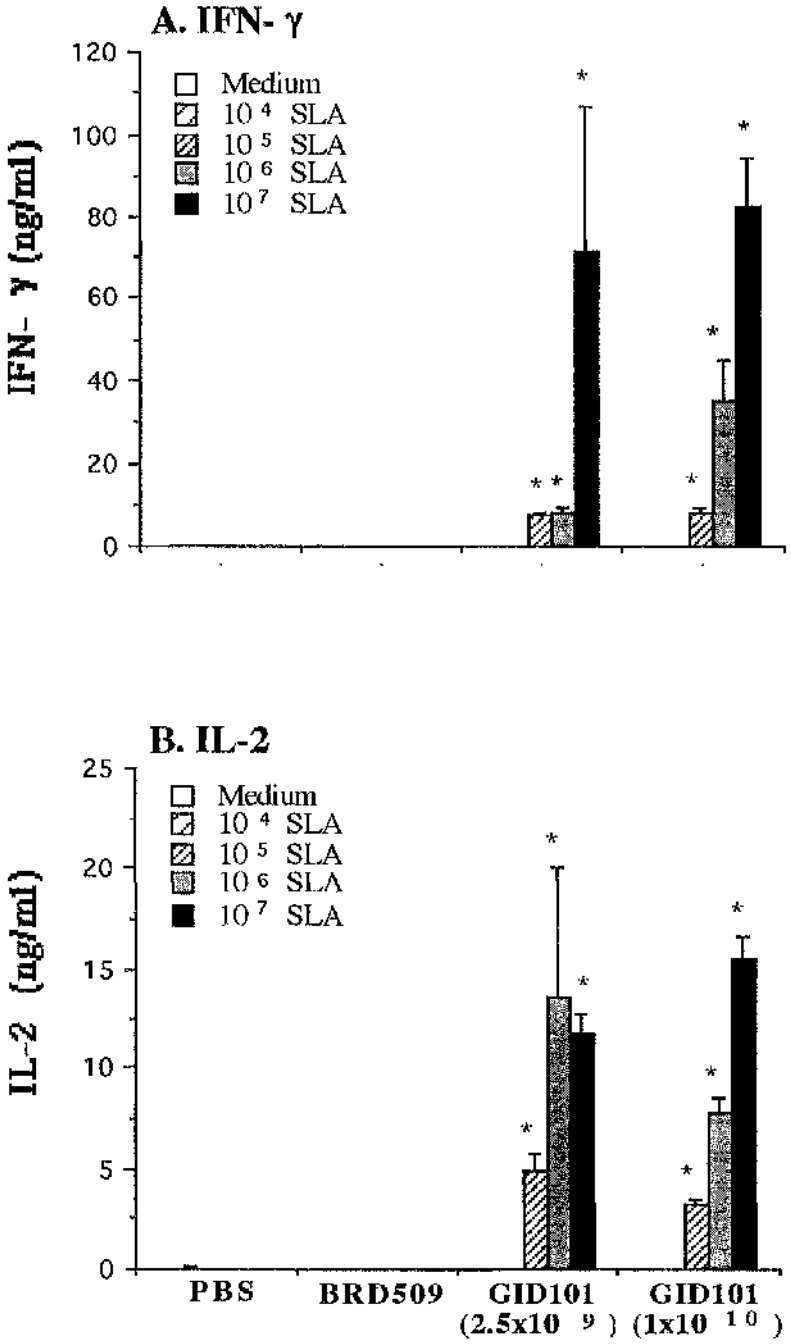


Figure 3.9: Cytokine production by cells from GID101 immunised mice to SLA *in vitro*. Data show mean and SD of cells from three pooled mice.
Data are representative of four individual experiments.

*$p<0.05$ compared to appropriate BRD509 group.

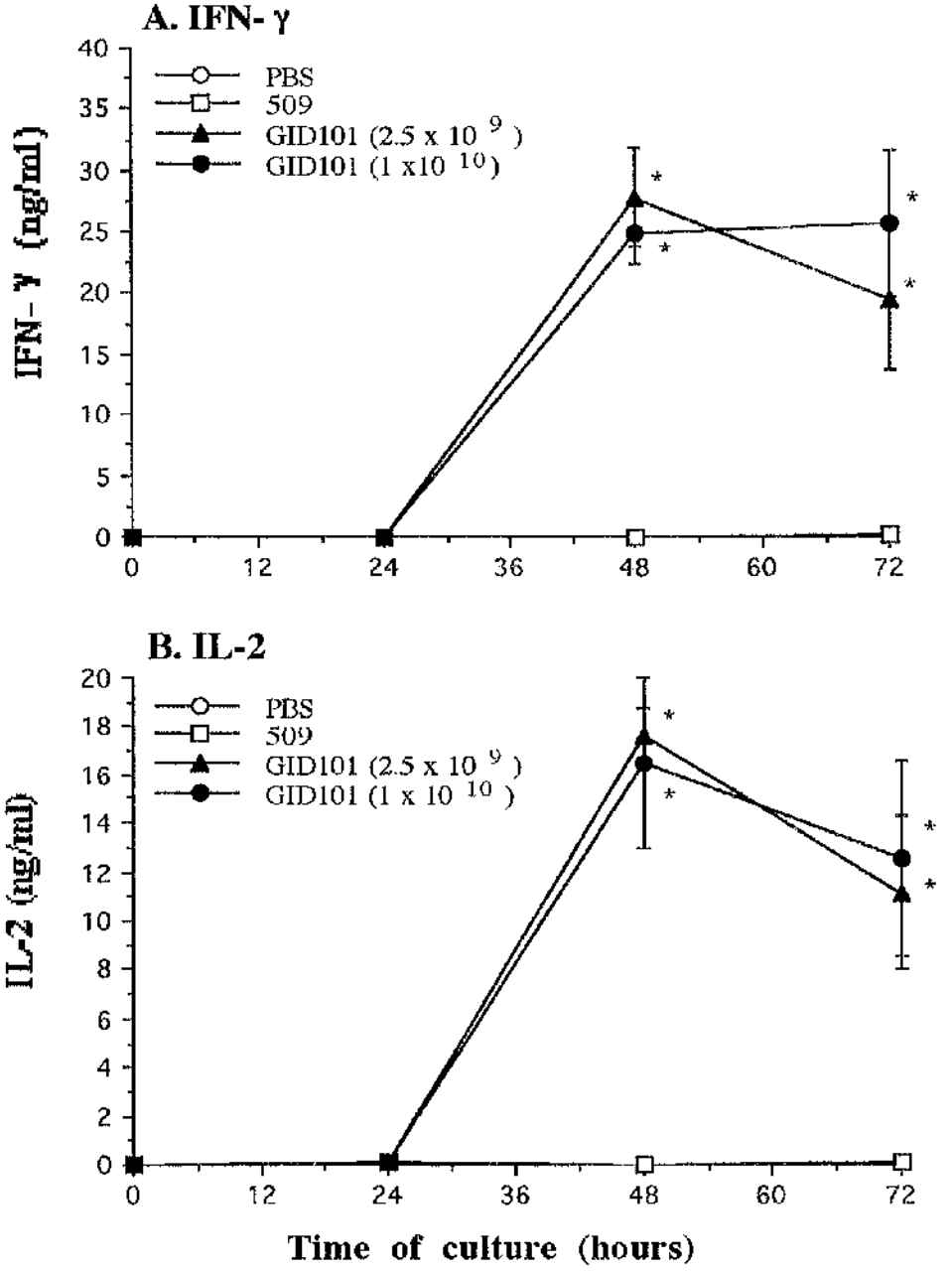


Figure 3.10: Time course of cytokine production to SLA (1 x 10^6/ml). BALB/c mice were orally administered two doses of either PBS, BRD509 (5x10^9) or GID101 (5x10^9 or 1x10^{10}) and stimulated in vitro with SLA 3-5 weeks later. Data show mean and SD of pooled cells from three mice per group and are representative of two individual experiments. * $p < 0.05$

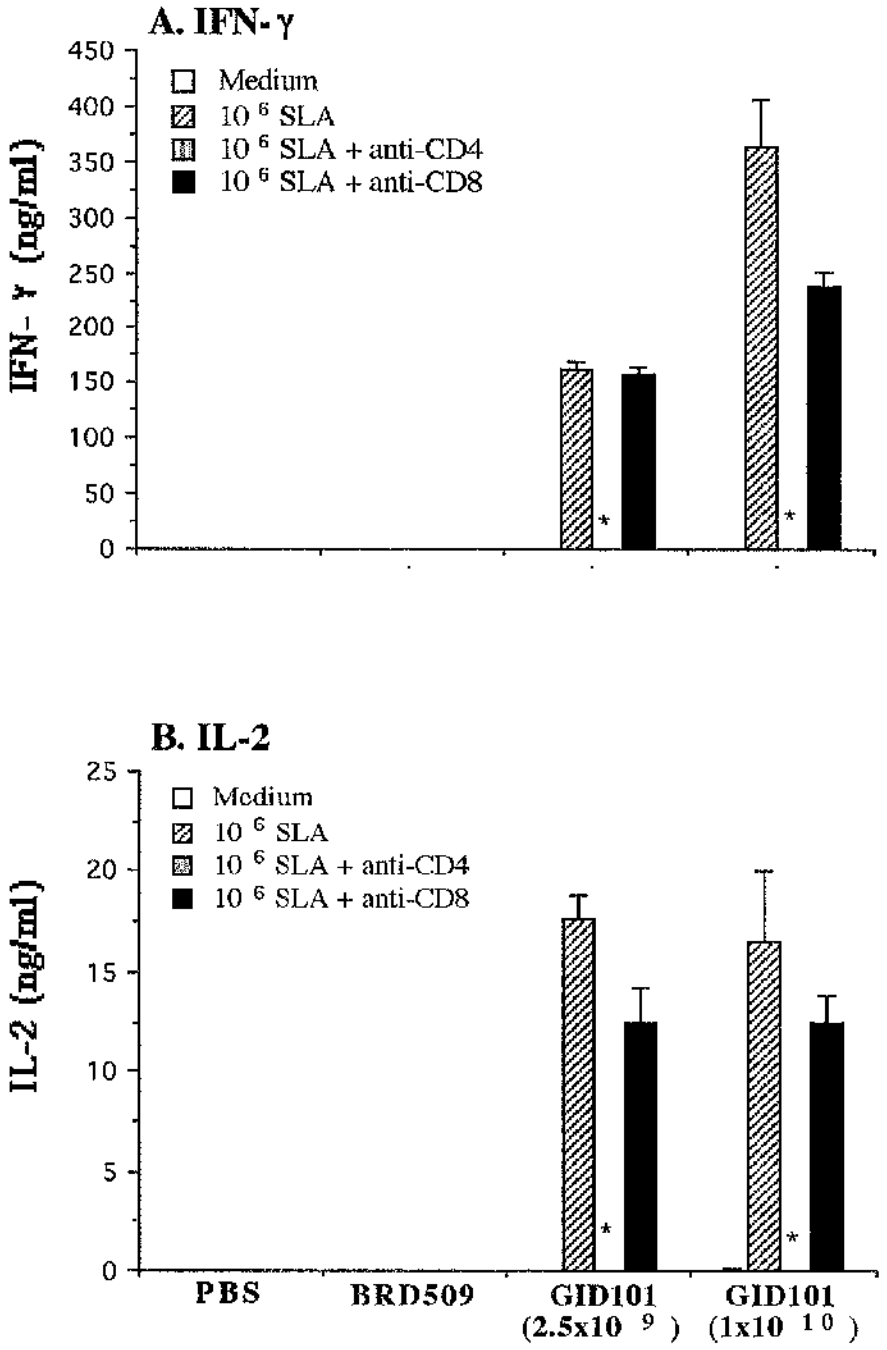


Figure 3.11: Effect of anti-CD4 (YTS169) and anti-CD8 (YTS191) on cytokine production. Data show mean and SD of pooled cells from three mice. Data are representative of two individual experiments. * $p < 0.05$

3.7 Immune response to *S. typhimurium*

3.7.1 Antibody response

Serum from mice administered BRD509 or GID101 contained significant levels of antibody against *S. typhimurium* (Fig. 3.12). Mice vaccinated with GID101 contained slightly higher serum IgG responses to *S. typhimurium*. No anti-*S. typhimurium* antibody was detectable in the serum of mice administered PBS alone.

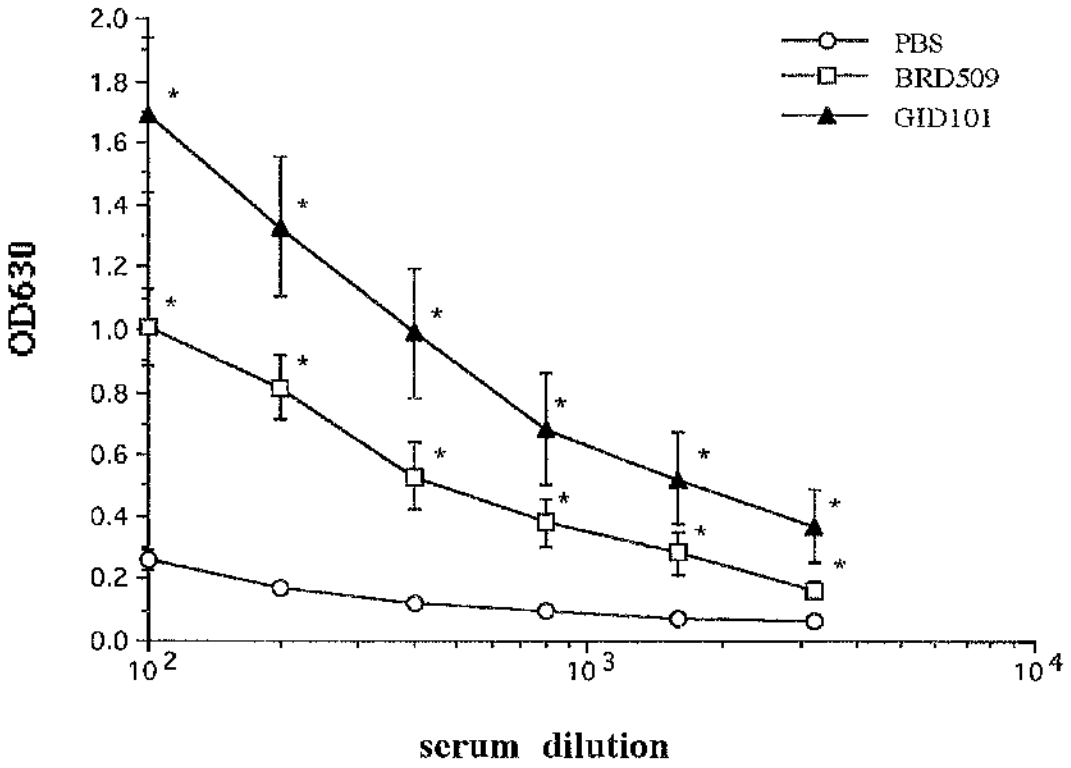


Figure 3.12: *S. typhimurium* specific IgG from serum of mice immunised with BRD509 or GID101. Each point represents mean and SD of pooled serum from three mice. Data are representative of three individual experiments. * $p < 0.05$

3.7.2 Proliferative response *in vitro*

Spleen and MLN cells from mice administered BRD509 or GID101 did not proliferate *in vitro* to a *S. typhimurium* lysate above background levels detected in cultures of cells from PBS administered mice in two individual experiments (data not shown).

3.7.3 Cytokine production *in vitro*

Spleen and MLN cells were cultured *in vitro* with a lysate from *S. typhimurium* and cytokine levels in supernatants were measured. Cells from mice administered GID101 or BRD509 produced a substantial level of IFN-γ that was greater than the background level of IFN-γ produced by PBS administered mice (Fig. 3.13).

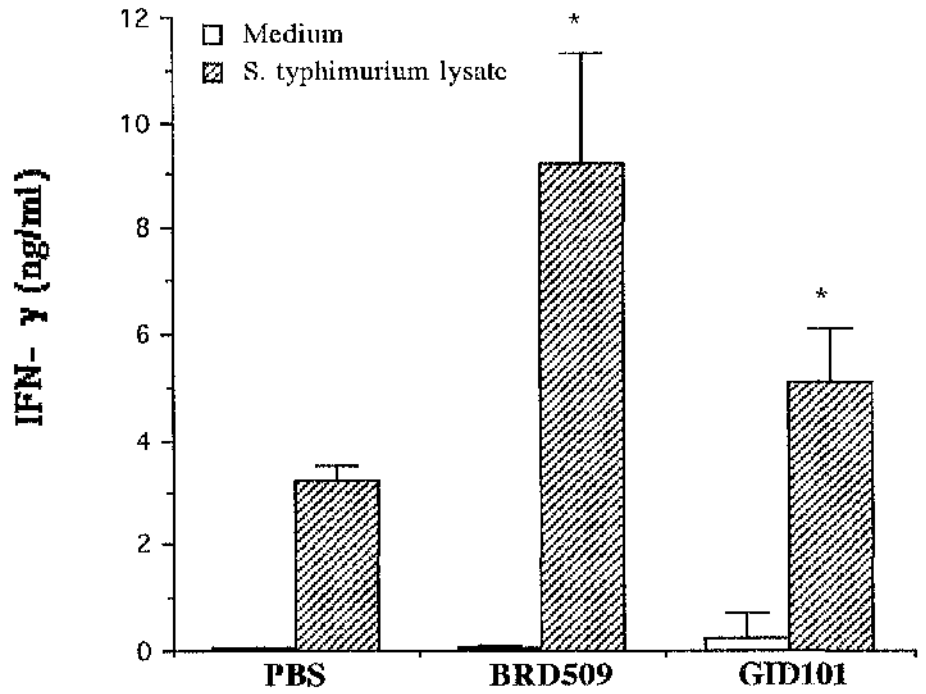


Figure 3.13: IFN-γ production in response to *S. typhimurium* lysate at 72 hours of culture. Data show mean and SD of pooled cells from three mice and are representative of two individual experiments. * $p < 0.05$

3.8 Discussion

The data presented above demonstrate that when *L. major* gp63 is expressed in the attenuated *S. typhimurium* strain BRD509, this construct provides protection to susceptible BALB/c mice upon live challenge. The protection, while not complete, is a substantial improvement upon the previous vaccine, SL3261/gp63, which did not afford susceptible mice any protection against infection.

Why gp63 expression in the double deletion mutant, BRD509, provides better protection than the same protein expressed in SL3261 is not clear. The double deletion causes increased attenuation of the bacteria so that they do not persist *in vivo* as long as SL3261 (Fig. 3.3, 278). While BRD509 are not readily detectable after day 21 *in vivo*, SL3261 persists until around day 42 after administration. The factors responsible for immunogenicity of recombinant proteins expressed in *S. typhimurium* are unknown. Previous work would suggest that increased attenuation of *S. typhimurium* reduces the immunogenicity of antigens they deliver (332, 333), while the data presented here would suggest that the opposite may be true. A more detailed comparative study on immunogenicity of proteins expressed in various *S. typhimurium* strains would be needed to address this issue.

The immune response of BALB/c mice after GID101 immunisation provide data upon the mechanisms involved in the protection from infection observed. There was no detectable DTH response in immunised mice to *L. major* promastigotes. DTH responses have been thought to be a good indicator of protective immunity against *L. major* (123). There are however reported incidences of protective immunity in the absence of any DTH response (125) and thus the protection observed with GID101 occurring in the absence of a DTH response is not unusual. In as much as DTH responses have been associated with Th1 cells (131), this data would indicate the involvement of other cell types in this protective response.

Mice immunised orally with GID101 produced significantly higher levels of leishmania-specific IgG than control groups. The immunised group also contained high titres of IgG2a but no IgG1 to parasite antigens. These results are consistent with the notion that GID101 preferentially induced a Th1-like response to gp63. The detection of antigen specific IgG and IgG2a responses in BRD509 immunised mice may represent polyclonal B-cell stimulation by *Salmonella* LPS.

Spleen and MLN cells from immunised mice produced substantial levels of IFN-γ and IL-2 *in vitro* when stimulated with parasite antigens. These cells also proliferated in culture with various parasite antigen preparations, to varying degrees. Cells responded best to live or sonicated parasites but less well to SLA or formalin-fixed promastigotes. These differences are not due to differences in the kinetics of response, as all responses peaked at 2 days of culture, but may represent modification of gp63 by different antigen preparation protocols. IL-2 and IFN-γ production to antigen was $CD4^+$- cell dependant, further suggesting that there is a predominant Th1-like response to gp63 in vaccinated mice.

Taken together these data suggest the involvement of a Th1-like response in mediating the protection observed using this construct. The absence of DTH response may represent the further heterogeneity of T-cell subsets, as has been previously suggested (377).

Spleen and MLN cells from mice immunised with GID101 or BRD509 produce more IFN-γ *in vitro* to a Salmonella lysate than mice administered PBS. Serum from these mice also contained high levels of *S. typhimurium* specific IgG. There is therefore a strong antibody and cellular response to salmonella antigens induced after delivery of these constructs, indicating that the GID101 construct may function as a bivalent vaccine against both *L. major* and virulent *S. typhimurium* infection. Challenge of immunised mice with a virulent strain of *S. typhimurium* would be required to confirm this.

These data also confirm previous reports using other *S. typhimurium* strains which detail the effects of various factors upon the ability of the bacteria to colonise mice *in vivo* . As shown above, altering the growth state of *S. typhimurium* and the use of a carbonate solution can significantly alter the numbers of recoverable bacteria from immunised mice. The fact that these factors can have a marked effect upon colonisation underline the importance of optimising live vaccine delivery conditions, although the effects of poor delivery conditions upon immunogenicity have not been addressed directly by this study.

3.9 Summary

The vaccine construct GID101 represents a considerable improvement over the previous vaccine strain SL3261/gp63. GID101, after 2 oral immunisations confers reduced lesion development to susceptible, BALB/c mice after *L. major* challenge infection. Spleen and MLN cells from immunised BALB/c mice respond *in vitro* to parasite antigens and produce substantial amounts of IFN-γ and IL-2, while no IL-4 or IL-5 was detected. The production of these cytokines was $CD4^+$-cell dependant. Serum from immunised mice contain parasite specific IgG2a but no parasite specific IgG1. No DTH to *L. major* was detected in vaccinated mice. These data are consistent with the notion that GID101 administration results in a protective Th1-like response to *L. major*.

Chapter 4

Gp63 expression in BRD509 using different promoters

Contents

4.1 Introduction

A significant obstacle in the induction of immune responses *in vivo* to heterologous antigens expressed in attenuated *S. typhimurium* is plasmid loss, as the bacteria multiply within the host (378). As there is no selective pressure to maintain the plasmid *in vivo* and as the production of a foreign protein in large amounts confers a considerable disadvantage in terms of energy expenditure, many plasmids are only retained transiently in the mammalian host after administration (Chapter 3, 278). Therefore, many *S. typhimurium* strains which express foreign antigens are poorly immunogenic and require repeated dosing to ensure antigen specific immune responses are induced (378).

Inducible promoters, which only allow gene transcription under certain environmental conditions have been used as a means of conferring greater plasmid stability and therefore better immunogenicity to foreign antigens delivered by *S. typhimurium* . The promoter, *NirB*, which responds to anaerobic conditions has been used to express tetanus toxin fragment C (TetC) in an attenuated *S. typhimurium* strain (372). The resulting construct induced strong immune responses to TetC after a single oral dose (378). This increased immunogenicity was thought to result from the stable retention of plasmid by this bacteria *in vivo* .

Another inducable promoter, *OsmC*, has recently been cloned from *E. coli* and is known to respond to changes in environmental osmolarity (D. Xu, Department of Immunology, Glasgow).

Both *NirB* and *OsmC* were used to express *L. major* gp63 in the attenuated *S. typhimurium* strain, BRD509, to address whether changes in promoter control would allow protection from *L. major* infection after a single oral dose.

This chapter describes a comparison of three different *S. typhimurium* constructs which express *L. major* gp63 under the control of either a constitutive, anaerobic or osmotic promoter. The effects of altered promoter control are examined with regard to the stability of plasmid *in vivo* , immune response generated to gp63 and protection against challenge infection.

4.2 Construction of *S. typhimurium* expressing *L. major* gp63 under different promoter control.

4.2.1 Plasmid construction

Plasmids pNirB/gp63 and pOsmC/gp63 were constructed by Dr. D Xu (Dept. of Immunology, Western Infirmary, Glasgow) and consisted of gp63 cDNA inserted downstream of promoters *NirB* and *OsmC* respectively (Fig. 4.1).

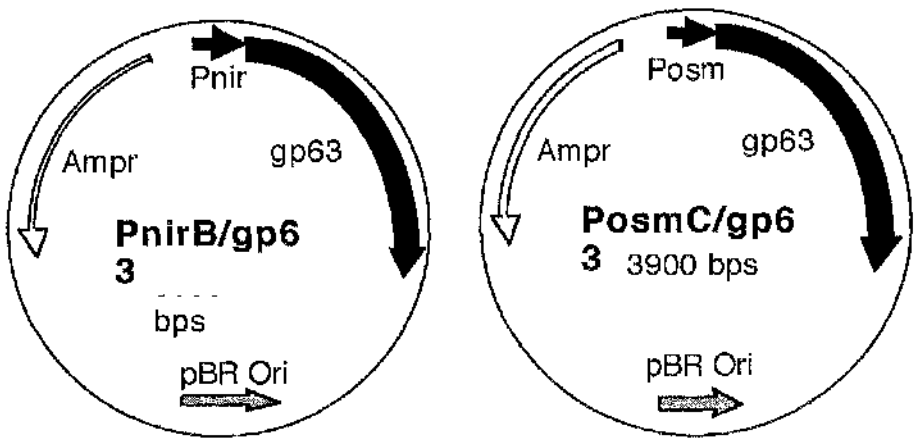


Figure 4.1: Construction of plasmids pNirB/gp63 and pOsmC/gp63. The cDNA for *L. major* gp63 was inserted downstream of promoters *NirB* or *OsmC*. An ampicillin resistance gene allows selection for bacteria retaining these plasmids *in vitro*. These plasmids were constructed by Dr. D. Xu (Department of Immunology, Western Infirmary, Glasgow).

4.2.2 Expression of gp63 *in vitro*

After transformation of the attenuated *S. typhimurium* strain, BRD509, with either pNirB/gp63 or pOsmC/gp63, bacteria strains were termed GID105 (pNirB/gp63) or GID106 (pOsmC/gp63). Expression of gp63 in GID105 was induced under anaerobic conditions, as described in Materials and Methods, and detected by western blot using a monoclonal antibody (mAb No. 235). Strain GID105 did not express any gp63 under non-inducing conditions but a product was detected, at the correct molecular weight after induction under anaerobic conditions (Fig. 4.2). Expression of gp63 by GID106 was also assessed using western blot, with and without induction using high salt conditions, as described in Materials and Methods (Fig. 4.2). In this strain, there is detectable gp63 expression before induction which is further upregulated under inducing conditions.

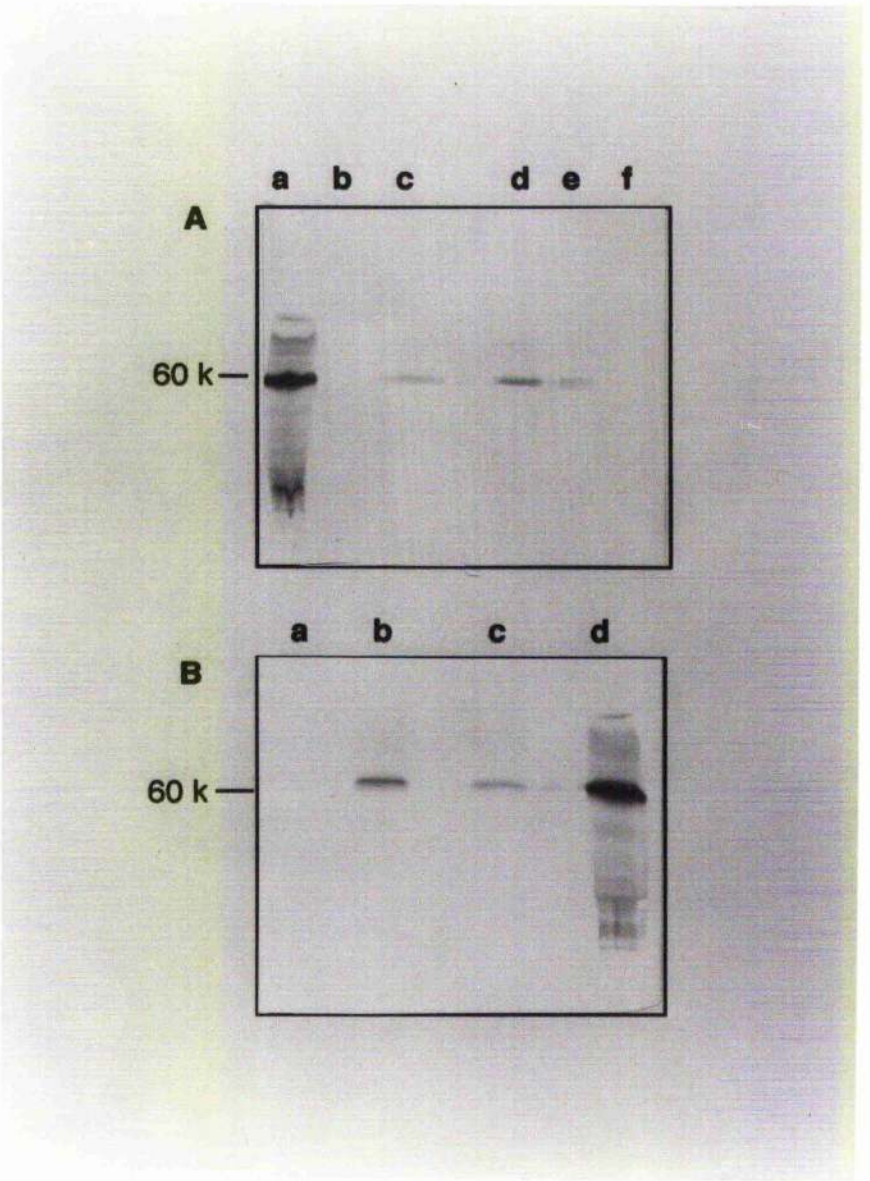


Figure 4.2: Western blot for gp63 in *E coli* (A) and *S. typhimurium* (B) constructs using mAb No. 235. A. gp63 expressed in *E. coli* **a**: pkk/gp63; **b**: NirB/gp63 (aerobic); **c**: NirB/gp63 (anaerobic); **d**: Osm/gp63 (high salt); **e**: Osm/gp63 (low salt); **f**: control. B. gp63 expressed in *S. typhimurium* **a**: BRD509; **b**: GID106 (high salt); **c**: GID105 (anaerobic); **d**: GID101.

4.2.3 Plasmid stability *in vitro*

Plasmid stability of GID105 and GID106 was monitored *in vitro*, in the absence of antibiotic selective pressure, by repeated culture on L-Agar, as described in Materials and Methods. This experiment was carried out by Dr. D. Xu. As shown in Table 4.1, while GID105 showed no plasmid loss *in vitro* after 6 days in the absence of ampicillin, GID101 and GID106 experienced significant plasmid loss.

Day of culture	GID101 (pkk)	GID105 (NirB)	GID106 (Osm)
1	85	100	84
2	82	100	82
3	78	100	76
6	6	100	23

Table 4.1: Plasmid stability of GID101, GID105 and GID106 *in vitro* in the absence of ampicillin. Data show number of colonies, from an initial 100, which retain ampicillin resistance after culture without antibiotic selective pressure. Data are representative of three experiments.

4.2.4 Plasmid stability *in vivo*

Plasmid stability of GID105 and GID106 was assessed in the spleen, liver and MLN of BALB/c mice after oral delivery. Figure 4.3 shows that, similar to the *in vitro* results, GID105 shows virtually no plasmid loss *in vivo* throughout the period of colonisation in all these organs. There were, however, significant differences in the numbers of recoverable bacteria when compared with the control strain BRD509 (Fig. 3.1). Although mice administered GID105 did not harbour bacteria after 28 days, there was a markedly higher number of bacteria harvested from all organs during the period of colonisation when compared to BRD509. In the spleen and liver there was a 10-100

fold increase in bacteria while there was about a four fold increase in bacteria recovered from MLN.

GID106 showed very poor plasmid stability *in vivo* , with bacteria at around 7 days after administration showing approximately 50% plasmid loss (Fig. 4.4). There were also differences in the colonisation capacity of this strain when compared to the control strain BRD509. There was no colonisation of spleen or liver at all, although MLN colonisation levels did not differ from BRD509 controls.

Recovered colonies of GID105 and GID106 that were ampicillin resistant were also able to express gp63 under inducing conditions, as assessed by western blot (data not shown).

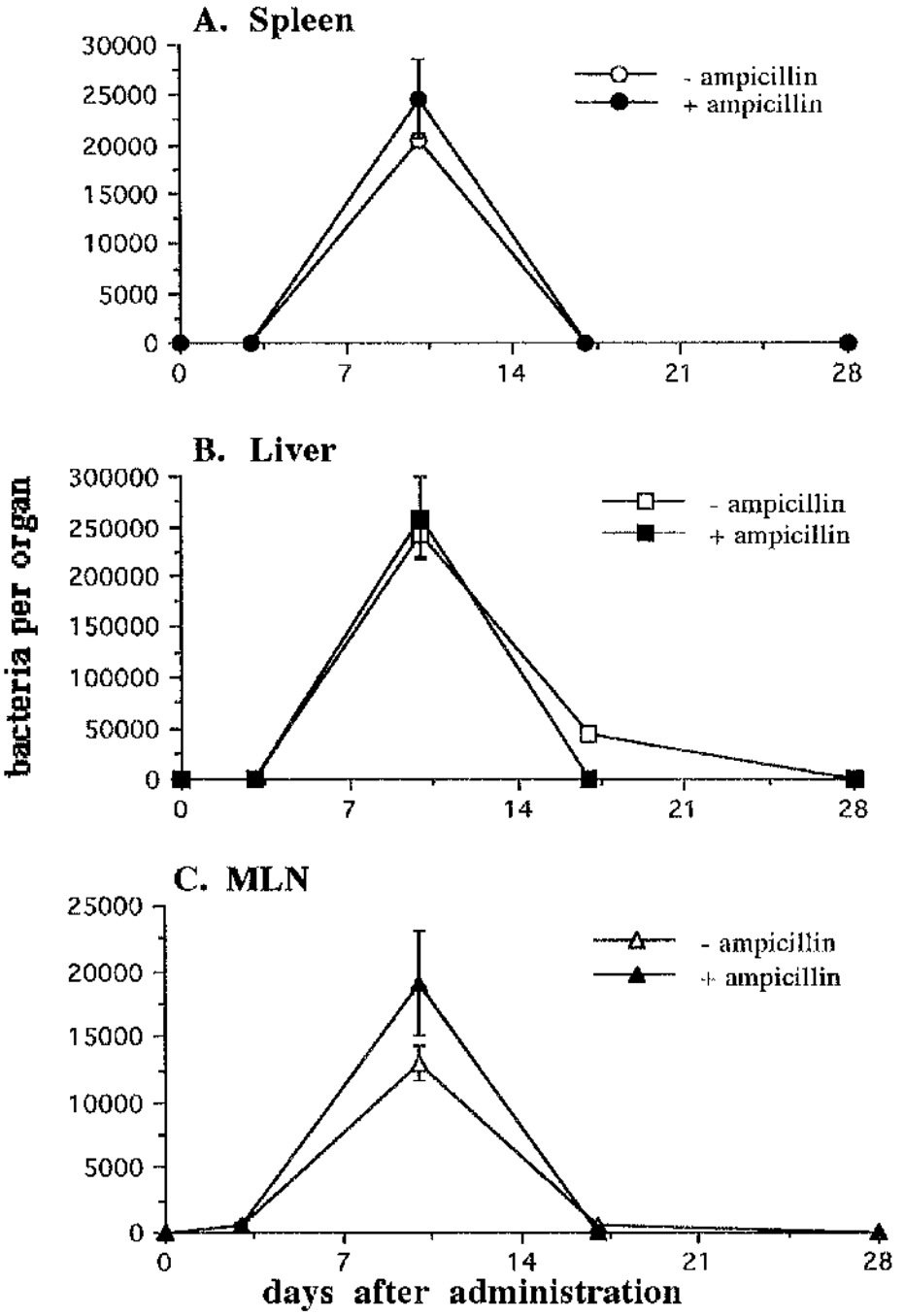


Figure 4.3: Plasmid stability of GID105 during colonisation of BALB/c mice. Data show mean and SEM of three mice per time point. Data are representative of two individual experiments.

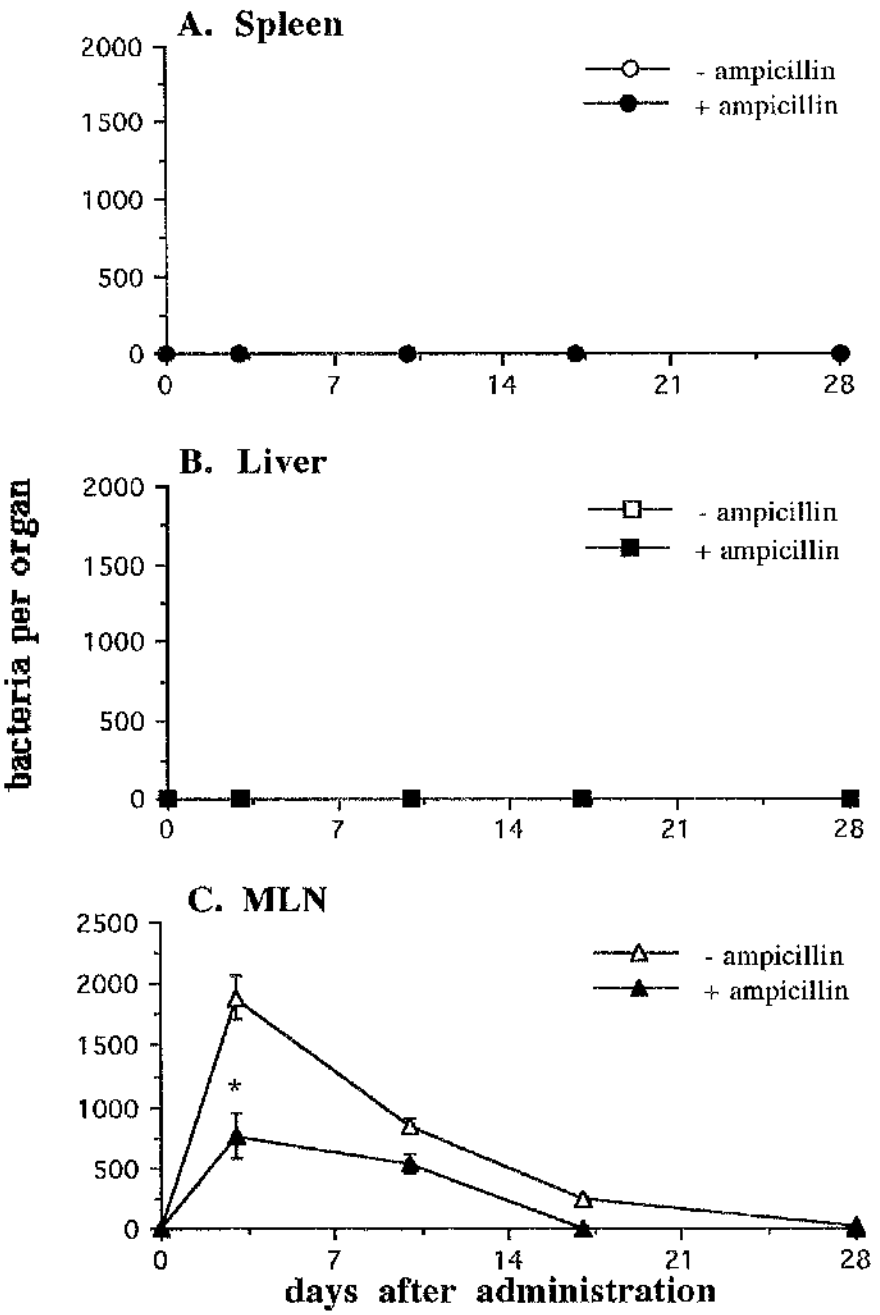


Figure 4.4: Plasmid stability of GID106 during colonisation of BALB/c mice. Data show mean and SEM of three mice per time point. Data are representative of two individual experiments.

* $p < 0.05$

4.3 Immune response to *L. major* gp63 after a single oral administration.

The immune response to gp63 after a single oral administration of either BRD509, GID101, GID105 or GID106 was analysed in BALB/c mice. Mice were orally administered $1x10^{10}$ bacteria, and spleen and MLN cells were harvested three weeks later to examine *in vitro* responses to antigen, as described in Materials and Methods. Serum was also collected at this time and used to measure IgG responses to gp63.

4.3.1 DTH response

BALB/c mice which received one oral dose of either GID101, GID105, GID106 or the control *S. typhimurium* strain, BRD509, did not develop a detectable DTH response to live *L. major* challenge in the footpad (data not shown).

4.3.2 Antibody response

Measurement of IgG to recombinant gp63 and soluble *L. major* antigen (SLA) in the serum of immunised mice was determined by ELISA, as described in Materials and Methods. Serum from BALB/c mice which were administered one dose of either GID101, GID105, GID106 or the control strain BRD509 three weeks previously, was diluted 1:40 and antigen specific IgG measured. As shown in Figure 4.5, there was a detectable response to SLA generated by all constructs which expressed gp63 while there was no specific response in the serum of BRD509 immunised mice. A similar pattern of IgG response was measured when recombinant gp63 was used as the antigen (data not shown). All constructs produced a low level of response compared to two dose administration of GID101 (Fig. 3.6) and there was no significant difference in magnitude of response between the constructs.

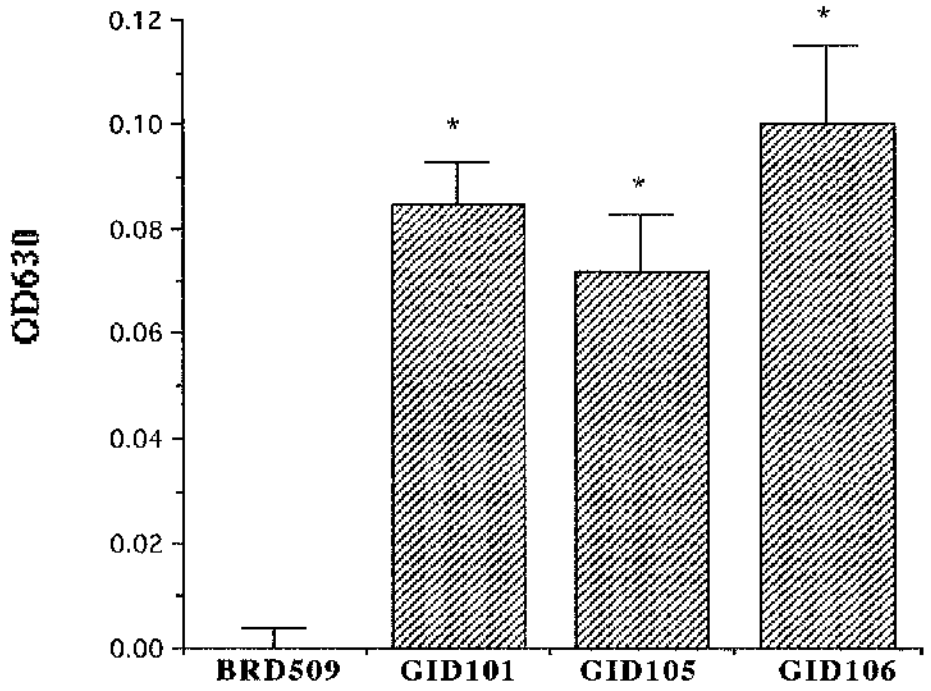


Figure 4.5: Parasite specific IgG after one oral dose of either GID101, GID105, GID106 or control strain BRD509 (5 x 10^9 bacteria). Data show IgG responses in 1:40 serum dilution against SLA. The background OD630 value of control mice administered PBS (0.107) has been subtracted from each value. Data show mean and SD for three mice per group and is representative of three individual experiments.
* $p < 0.05$ compared to BRD509 value.

4.3.3 Proliferative response *in vitro*

Proliferation of spleen and MLN cells from immunised mice to live *L. major* was determined, as described in Materials and Methods. Cells from mice administered one dose of GID105 showed substantial levels of proliferation to live *L. major* at five days of culture, while no antigen specific proliferation was detected at this time point from cells of mice administered one dose of GID101, GID106 or BRD509 (Fig. 4.6). Cells from all groups of mice did not proliferate to parasites at 3 days of culture (data not shown).

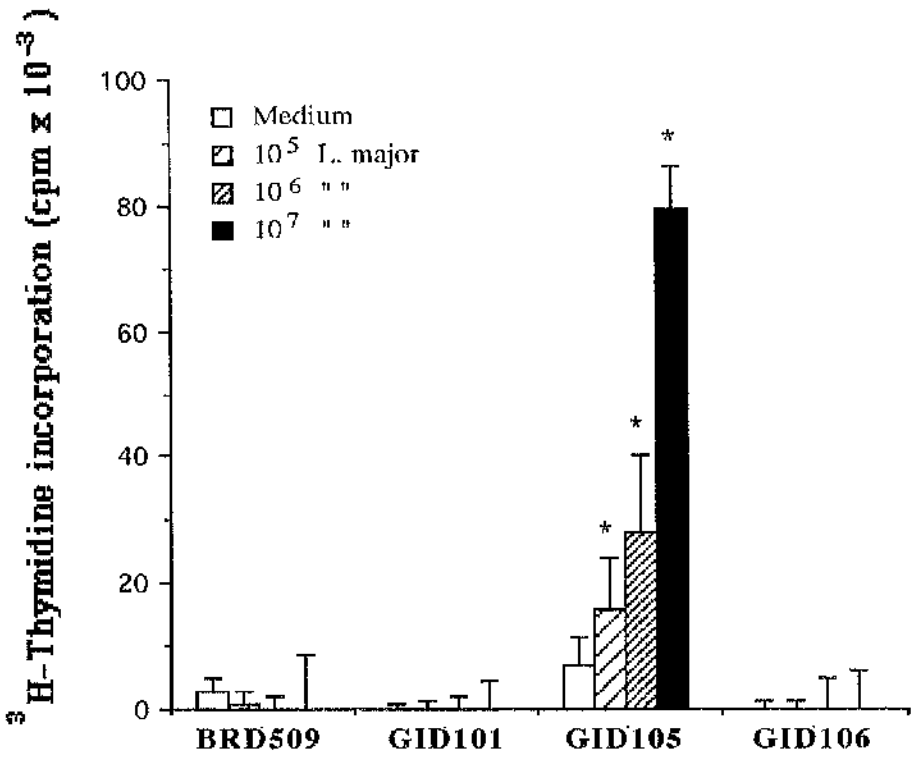


Figure 4.6: Proliferative response of cells from mice administered one oral dose of $1x10^{10}$ of BRD509, GID101, GID105 or GID106 when cultured *in vitro* with live *L. major*. Data show mean and SD of pooled cells from three mice after subtraction of background value in PBS control cultures, due to parasite proliferation (67,670 cpm). Data are representative of two individual experiments.
* $p < 0.05$ compared to BRD509 values.

4.3.4 Cytokine production *in vitro*

Levels of IFN-γ, IL-2 and IL-4 were measured in culture supernatants of parallel cultures to those above. Cells used in these cultures were incubated *in vitro* with SLA at various concentrations and supernatants harvested at different time points later. Cells from mice administered one dose of GID105 produced IFN-γ *in vitro* in response to antigen while mice administered PBS, BRD509, GID101 or GID106 did not (Fig. 4.8). There was no detectable IL-2 or IL-4 production in any of these cultures (data not shown).

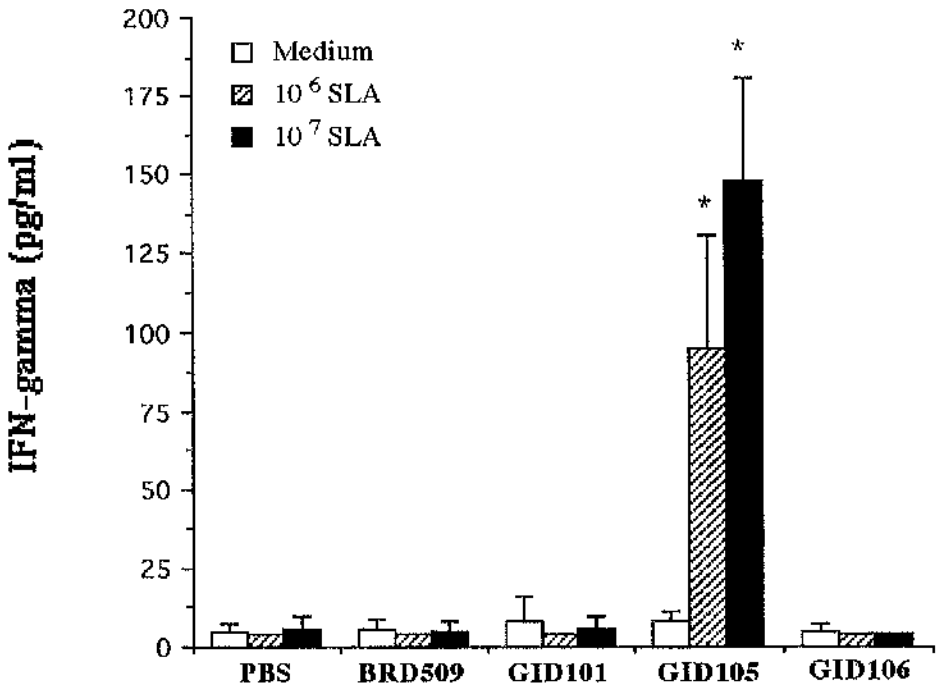


Figure 4.7: IFN-γ production *in vitro* from cells stimulated with SLA. Mice were immunised with one dose of PBS of $5x10^9$ BRD509, GID101, GID105 or GID106 before spleen and MLN cells were placed in culture 3 weeks later. Data show mean and SD of cells from three pooled mice and are representative of two individual experiments. * $p < 0.05$ compared to BRD509 values.

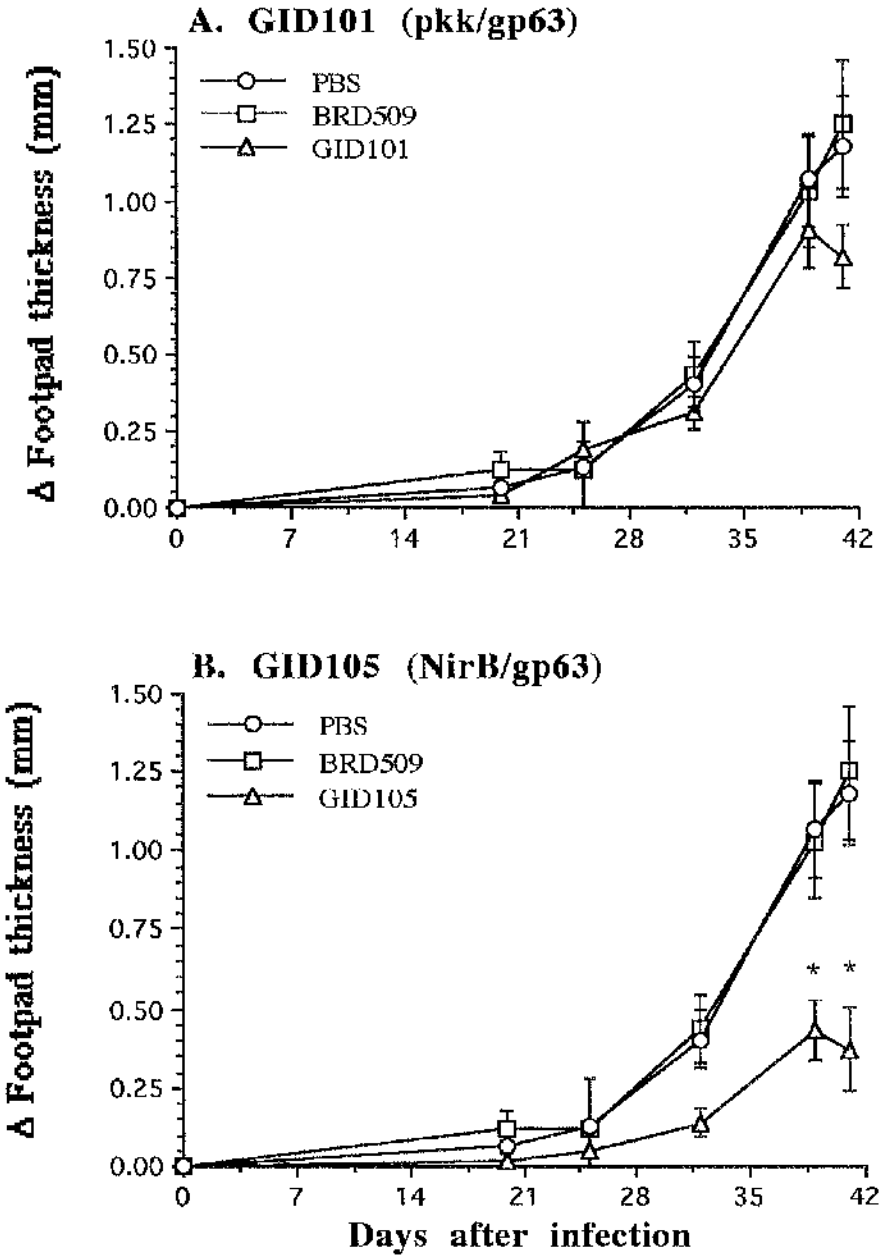
4.4 Protection against Leishmaniasis

The three constructs, GID101, GID105 and GID106 were compared for their ability to protect BALB/c mice against *L. major* and infection after a single oral dose of $5x10^9$ bacteria. Protection against *L. donovani* infection after two oral doses was also examined.

4.4.1 Protection against *L. major* infection

BALB/c mice were administered one oral dose of either BRD509, GID101, GID105, GID106 or were given PBS only. Two to three weeks after vaccination, mice

were challenged with $1x10^6$ *L. major* promastigotes in the footpad and lesion development was monitored to assess vaccine efficacy. Mice administered GID101 showed no protection against infection after a single oral dose (Fig. 4.8A). GID105 conferred significant protection against *L. major* infection as lesion swelling was reduced during the infection compared to control mice (Fig. 4.8B). GID106 also conferred significant protection against infection but to lesser degree than GID105 (Fig. 4.8C). Although lesion development was reduced using GID105 and GID106, mice did eventually succumbed to progressive infection at a later stage, similar to the results observed with two dose GID101 protection.


A. GID101 (pkk/gp63)
Δ Footpad thickness (mm)
PBS
BRD509
GID101
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
7
14
21
28
35
42
B. GID105 (NirB/gp63)
Δ Footpad thickness (mm)
PBS
BRD509
GID105
* *
Days after infection

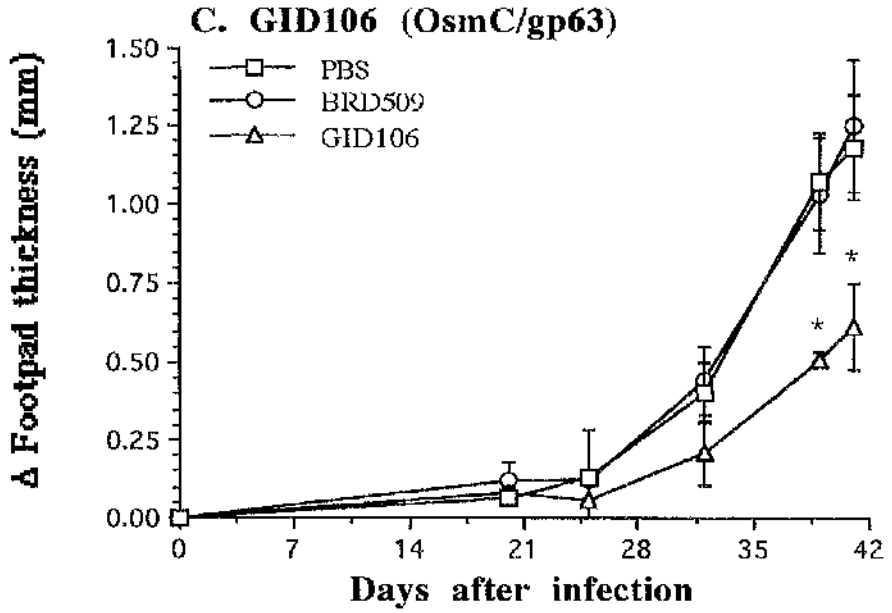


Figure 4.8: Protection against *L. major* infection after one oral dose of either GID101, GID105 or GID106. Mice were challenged with $1x10^6$ *L. major* promastigotes 2-3 weeks after receiving bacteria. Data show mean and SEM of six or seven mice per group and are representative of two individual experiments.
* $p < 0.05$ compared to BRD509 values.

4.4.2 Protection against *L. donovani* infection

BALB/c mice administered two oral doses ($1x10^{10}$) of either construct were challenged intravenously with $1x10^7$ *L. donovani* amastigotes, three weeks after the last dose. Five mice per group were sacrificed at 2 and 4 weeks after infection and liver impressions stained and counted to determine infection levels. LDU's were calculated for individual mice, as described in Materials and Methods. Mice vaccinated with GID105 showed considerable reductions in parasite burdens at 2 and 4 weeks after infection while GID101 or GID106 vaccinated mice did not differ significantly from controls (Fig. 4.9).

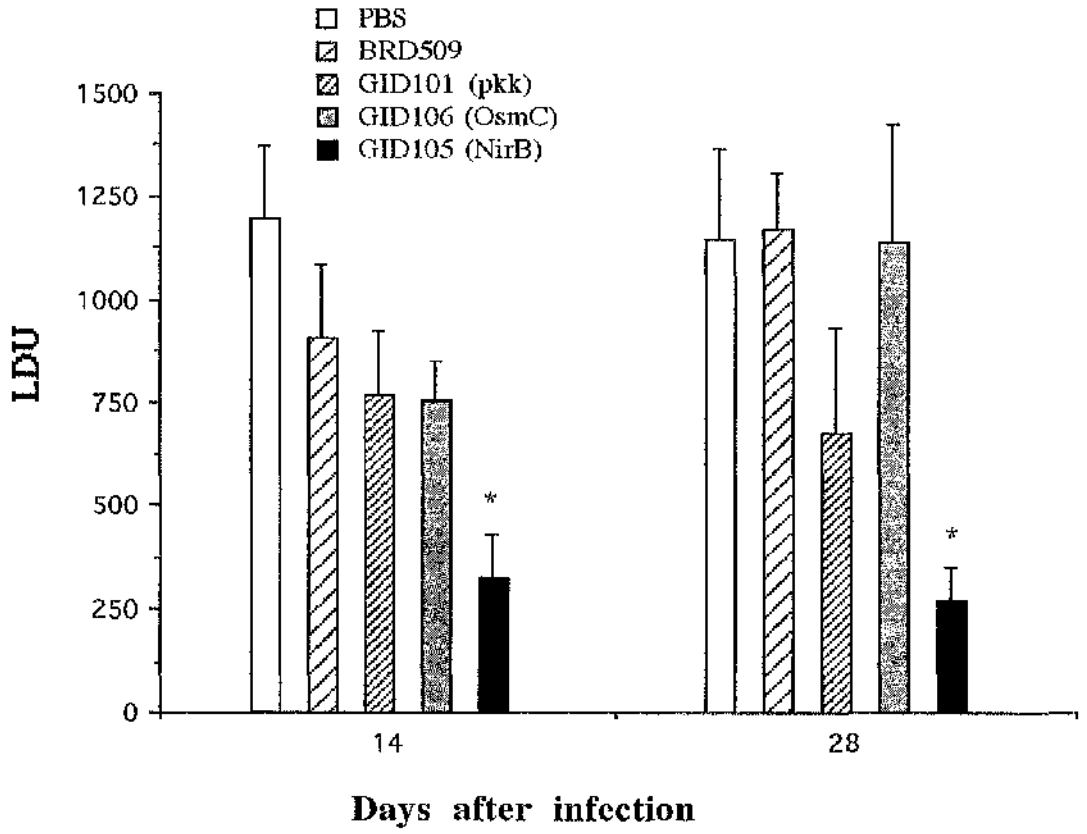


Figure 4.9: Protection against *L. donovani* infection after 2 oral doses with either GID101, GID105 or GID106 ($1x10^{10}$). Data show mean and SEM of 5 individual mice per group. * $p < 0.05$ compared to PBS controls.

4.5 Discussion

The ability to confer protective immunity to any pathogen after a single vaccine administration of is extremely advantageous. The requirement for multiple doses of certain current vaccines represents a impediment to large scale vaccine coverage of populations. A vaccine requiring only a single oral dose would be particularly advantageous when considering vaccinating against a major disease of the developing world, such as leishmaniasis, where considerations of cost are particularly important.

While the *S. typhimurium* construct, GID101, provides a degree of protective immunity after two oral administrations, this chapter describes two other BRD509

based constructs which use inducable promoters. There is evidence to suggest that the use of such promoters can allow the generation of strong immune responses to antigens delivered in *S. typhimurium* after a single dose (378).

The construct GID105, expresses gp63 under anaerobic conditions. The control of expression is extremely tight, with no product detected as the bacteria grow in aerobic conditions. This construct also shows stable plasmid retention even in the absence of antibiotic selective pressure both *in vitro* and *in vivo* , which may be due directly to the efficient promoter control of gp63 expression. This strain behaves differently to the control strain BRD509 *in vivo* in that it colonised at a much higher level in all organs examined, although was still cleared *in vivo* after 28 days. Why this increased colonisation occurs is unclear, but it is possible that the expression of gp63 by this construct provides some protection against adverse conditions within the host macrophage due to the proteolytic activity of gp63 (46).

The construct GID106 shows sudden plasmid loss in the absence of selective pressure *in vitro* and *in vivo* . This may be due to the incomplete control of expression associated with this construct, as even under non-inducing conditions a product is detectable by western blot. This construct also has a different colonisation pattern *in vivo* compared with BRD509 as there are no detectable bacteria present at any time point in the liver or spleen, although MLN colonisation occurs normally. As little is known of this promoter, it is difficult to speculate as to why its use should apparently increase the attenuation of the bacteria *in vivo*. It may be that the use of the *OsmC* promoter to express gp63 may interfere with necessary metabolic pathways within the *S. typhimurium* as it colonises *in vivo*. Further studies on both the *NirB* and *OsmC* promoter are required to determine why they have such profound effects upon BRD509 colonisation in BALB/c mice.

All constructs (GID101, GID105 and GID106) were able to induce a detectable antibody response to gp63 after a single oral delivery but only the anaerobic construct,

GID105 was able to generate a substantial cellular response to antigen. As was the case with two doses of GID101 (Chapter 3), this response was of a Th1-type in that IFN-γ but no IL-4 was produced in response to antigen *in vitro*, although the magnitude of the response was much reduced.

After a single oral delivery, GID105 was also able to reduce lesion swelling in susceptible BALB/c mice upon challenge infection whereas a single dose of GID101 conferred no protection. Therefore, the ability to generate protective immunity after a single oral administration correlated with a Th1-like response *in vitro* to parasite antigens and not with the ability to generate antibody responses to gp63. The ability to generate a protective cellular response by this construct after a single dose may be due to the stable plasmid retention *in vivo* or may be due to the increased capacity to colonise organs during administration, compared with the GID101 strain.

The osmotic construct, GID106, which did not produce demonstrable cellular responses to antigen *in vitro* was still able to confer a degree of protection to *L. major* challenge infection. The mechanism of protection in this system is not clear and requires further study.

As in the case of the protection associated with the administration of two doses of GID101 (Chapter 3), the protection conferred by a single oral dose of GID105 or GID106 is incomplete. Although vaccinated mice develop smaller lesions after infection, these lesions do not cure. While this is disappointing, it may reflect the maximum capacity for this single antigen to provide protection to susceptible mice. Previous vaccine work with gp63 in BALB/c mice has either demonstrated incomplete protection (257, 272, 277) or none at all (275, 278), depending upon the method of antigen delivery. Investigators have shown that gp63 expression by the parasite is down-regulated upon macrophage entry and may not be expressed at all on the amastigote stage of leishmania parasites (47). Recent work has shown that the vaccination of BALB/c mice with gp63 and IL-12 does not protect against infection

(379), while the delivery of SLA (256) or p24/LACK (379) under this regime provides complete protection. Therefore, the work detailed in this chapter may represent the optimisation of a live vaccine using a poor vaccine candidate and reflect the maximum capacity of gp63 to provide protective immunity to susceptible mice. The identification of better vaccine candidates and the use of these in this *S. typhimurium* system, either alone, or in combination with a gp63 construct may allow for the production of a vaccine which completely protects the susceptible BALB/c mouse against *L. major* infection.

While gp63 seems to be of limited use in protecting susceptible mice against cutaneous Leishmaniasis the use of the construct GID105 in vaccinating against *L. donovani* infection is more encouraging. When BALB/c mice were administered two oral doses of this construct, this resulted in a 70% reduction in LDU after parasite challenge. Although these studies did not examine parasite burdens at later time points it does suggest that this vaccine may have some potential against visceral leishmaniasis. A more complete study of this vaccine in *L. donovani* infection is required to address whether sterile immunity results at later time points.

4.6 Summary

L. major gp63 was expressed in *S. typhimurium* strain, BRD509, under the control of either the *NirB* promoter which responds to anaerobic conditions (GID105) or *OsmC* which is induced under high salt conditions (GID106). The construct GID105 shows extremely stable plasmid retention as it colonises *in vivo* when compared with the constitutive construct GID101. The construct GID106 does not colonise spleen or liver at all after oral delivery and loses plasmid more quickly than GID101 *in vivo* . An antibody response to gp63 is generated in BALB/c mice after one oral dose of GID101, GID105 or GID106, while only the anaerobic construct GID105 is able to induce a significant cellular response to antigen after a single oral dose. Spleen and MLN cells from mice administered with GID105 respond *in vitro* in a Th1-

like manner. A single oral delivery of GID105 or GID106 is sufficient to reduce lesion swelling in susceptible mice after *L. major* infection, while a single dose of GID101 does not confer any protection. Two doses of GID105 also provides protection against *L. donovani* infection, while GID101 or GID106 do not.

Chapter 5:
p24/LACK expression in BRD509
Vaccination against *L. major*

Contents

5.1 Introduction

The immune response at the very early stage of *L. major* infection is thought to be critically important in determining the outcome of disease. Therapeutic intervention in susceptible mice using IL-12 or anti-IL-4, allow the development of a Th1 response and eventual cure from disease (142, 191, 192). However, the timing of such intervention determines its eventual success in the highly susceptible, BALB/c mouse. If intervention is delayed until after the first two weeks after infection, when there is still no apparent clinical disease, the dominant Th2 response has already established and intervention is ineffective. Therefore, the polarisation of T-cell subsets occurs very early after infection and is not readily altered once established.

The factors responsible for the divergent polarisation of T-cells seen in susceptible and resistant mice in response to *L. major* infection are not known but there are a number of plausible suggestions. Among the factors which are known to affect T cell polarisation *in vitro* or *in vivo* are: the cytokine environment at the time of antigen challenge; the antigen type; and the route of immunisation used to deliver antigen (98). A recent study addressed the possibility that different antigens are responsible for T cell polarisation in *L. major* infection (380). This involved the analysis of T-cell receptor usage after infection in susceptible and resistant mice. It was shown that there is a restricted expansion of Vα8Vβ4 bearing T-cells in both susceptible and resistant mice early after infection, indicating that there may be a response to the same immunodominant antigen in both strains of mice. While this does not completely rule out the possibility that different antigens may be responsible for divergent T-cell subsets in some cases, it does suggest that T-cell polarisation during early *L. major* infection may result from altered responses to the same immunodominant antigen. Therefore, the induction of a Th1 response to this antigen in susceptible mice may be an effective vaccine strategy. Indeed, this antigen, p24/LACK, was recently cloned and used to vaccinate susceptible mice in combination with recombinant IL-12 (379). These mice were shown to be completely protected

against challenge infection and developed strong Th1 responses early after parasite challenge.

This chapter describes the construction of *S. typhimurium* strain GID202 which expresses p24/LACK under the control of the anaerobic *NirB* promoter. The use of this construct in vaccination against *L. major* infection is discussed as well as the immune responses induced in BALB/c mice after oral delivery of this construct.

5.2 Construction of GID202

5.2.1 Construction of plasmid pNirB/p24

The cDNA for p24/LACK was amplified by PCR from plasmid pET-LACK.δ1 (obtained from Prof. N. Glaichenhaus, University of Nice, Nice, France.). Primers were constructed which contained restriction enzyme sites for BamH1 and Nde1, as shown in Figure 5.1A. The amplified product was approximately 0.5 kB in size (Fig. 5.2) and was purified from an agarose gel by "freeze-squeeze" purification (see Materials and Methods). Both the plasmid, pNirB/gp63 and the p24/LACK PCR fragment were digested using BamH1 and Nde1 restriction enzymes (Fig. 5.3) and the digested DNA purified from agarose gels using the "freeze-squeeze" purification method. Digested p24/LACK cDNA and digested pNirB were ligated overnight, as described in Materials and Methods (Fig. 5.1B).

Fig 5.1A: PCR amplification of p24/LACK (showing restriction enzyme sites and start codon).

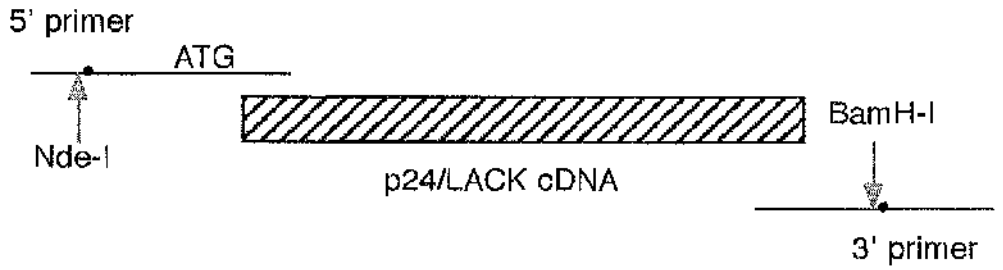
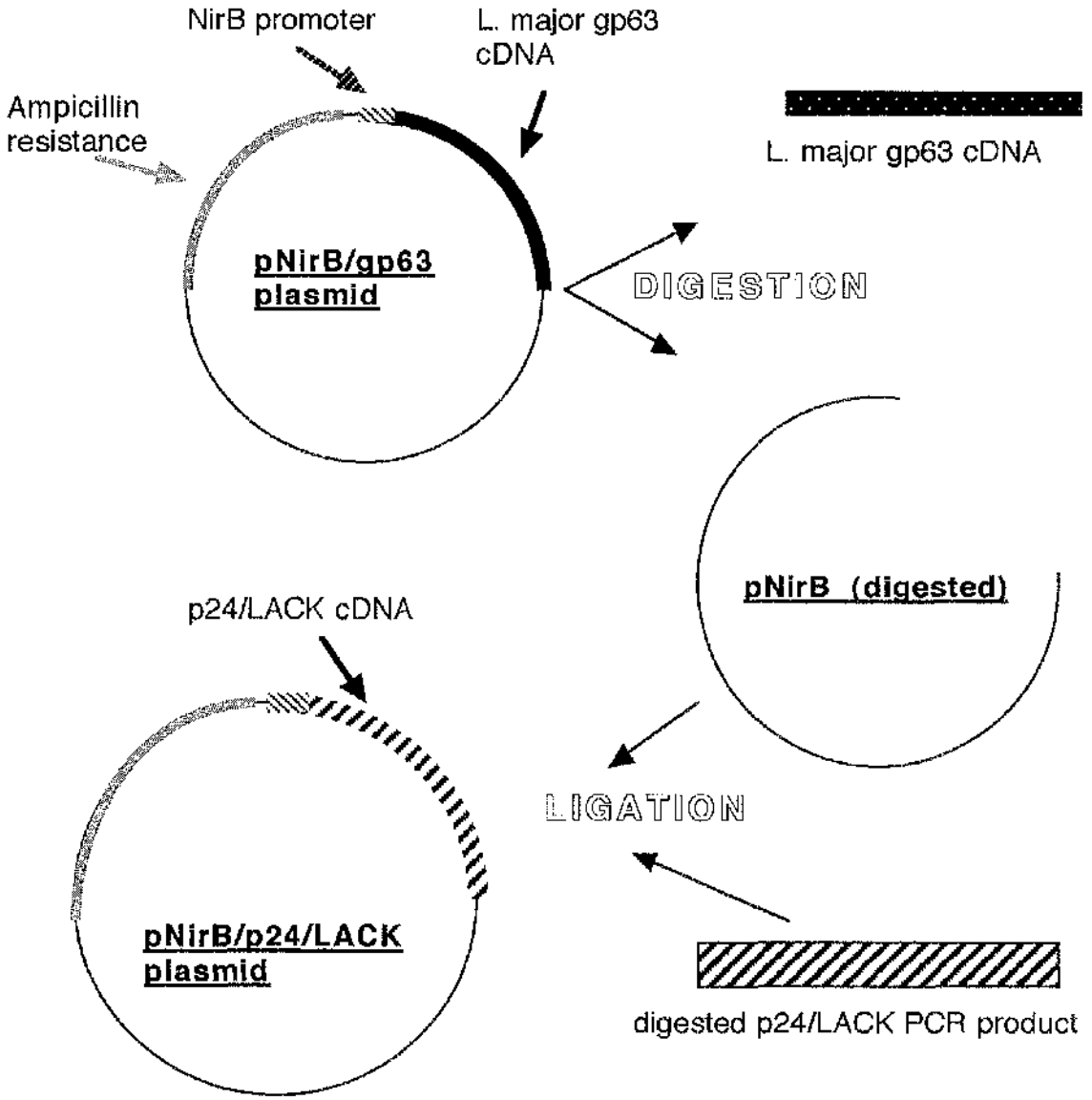


Fig 5.1B: Coloning of p24/LACK into pNirB/gp63 (Showing pNirB/gp63 digestion with Nde-1 and BamH-1 followed by ligation with digested p24/LACK PCR fragment).



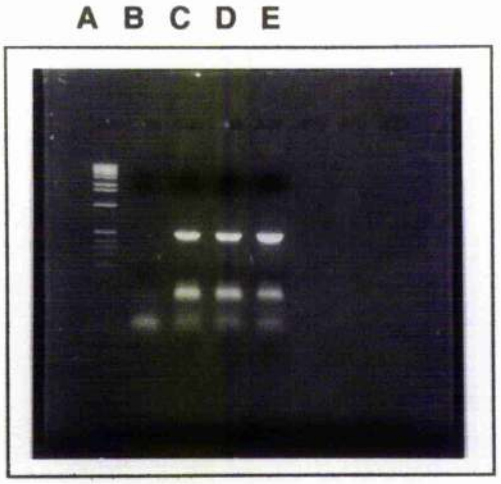


Figure 5.2: PCR amplification of cDNA for P24/LACK using pET-LACK.δ1 as a template. The product was approximately 0.5 kb in size. Lane A: no plasmid control. Lane B: 0.1μg plasmid. Lane C: 0.5μg plasmid. Lane D: 1.0μg plasmid.

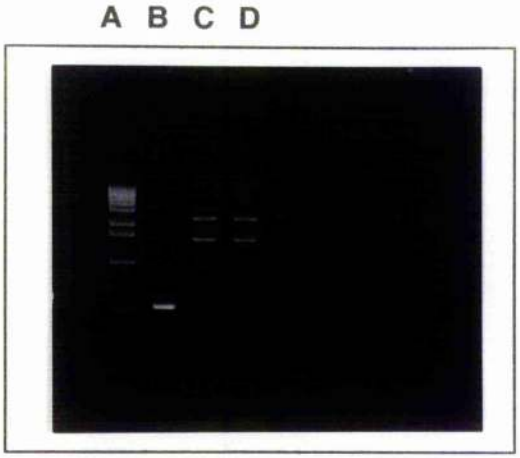


Figure 5.3: Agarose gel showing BamH1 and Nde1 digestion of both p24/LACK PCR product and the plasmid pNirB/gp63. Lane A: 1kb markers. Lane B: p24 PCR cDNA. Lane C and D: pNirB/gp63.

The resulting ligation mix was used to transform TG1 *E. coli* by heat-shocking in $CaCl_2$, as described in Materials and Methods. Plasmid was purified from clones which grew on L-agar in the presence of ampicillin and these plasmids were digested to determine if successful ligation had occurred (Fig. 5.4). A number of colonies which contained the correct pattern of restriction digestion were grown and analysed for protein expression by dot blot.

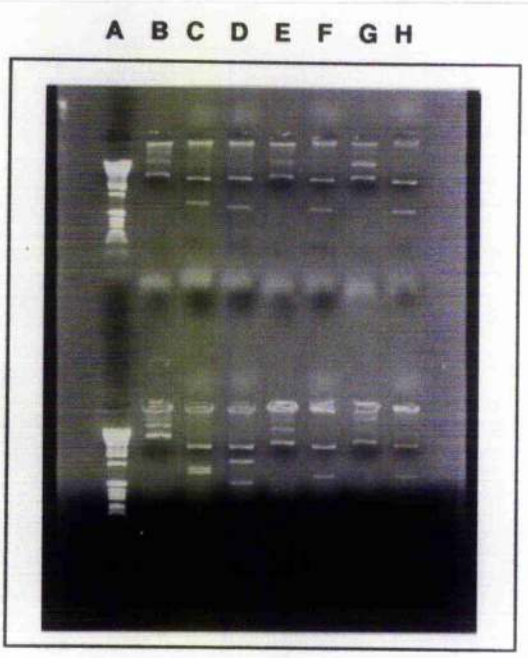


Figure 5.4: Digestion of plasmids from ligated clones grown on ampicillin L-Agar after bacterial transformation. Six clones were chosen and plasmid prepared and digested as described in Materials and Methods. (Top) A: 1kB markers; B: clone 1 undigested; C: clone 1, EcoR I and Pst I (0.8kB); D: clone 1, EcoR I and Ava I (0.6kB); E: clone 2, undigested; F: clone 2, EcoR I and Ava I; G: clone 3, undigested; H: clone 3, EcoR I and Ava I. (Bottom) A: 1kB markers; B: clone 4 undigested; C: clone 4, EcoR I and Pst I (0.8kB); D: clone 4, EcoR I and Ava I (0.6kB); E: clone 5, undigested; F: clone 5, EcoR I and Ava I; G: clone 6, undigested; H: clone 6, EcoR I and Ava I.

5.2.2 Dot blot of TG1/pNirB/p24

A number of colonies were screened by dot blotting, using a murine derived polyclonal antiserum against p24 (Fig. 5.5). A number of transformed TG1 colonies were stained using this antibody, and these were chosen for further westen blot analysis.

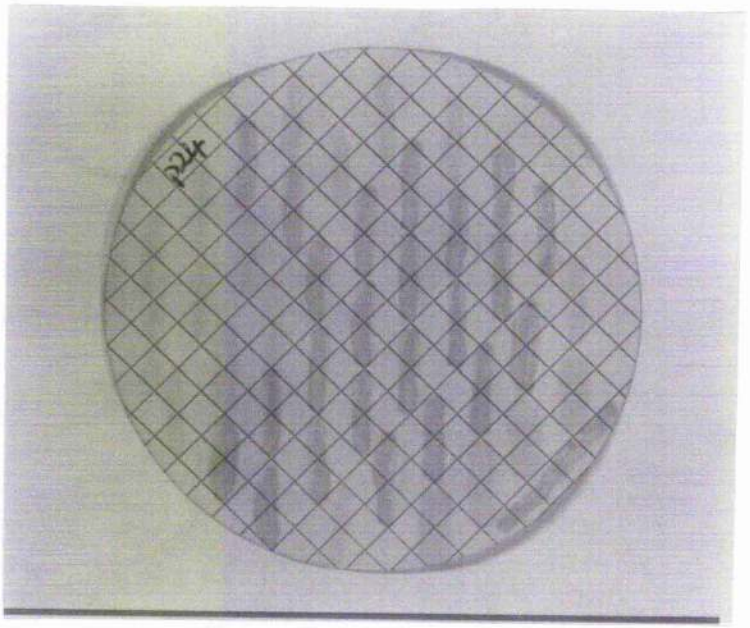


Figure. 5.5: Dot blot of TG1/pNirB/p24 using a polyclonal antiserum against p24/LACK. Colonies expressing p24/LACK were stained using a HRP conjugate.

5.2.3 Western blotting

A western blot of TG1/pNirB/p24 lysates also showed considerable cross-reactivity at a number of protein bands, although there was detection of a protein band at the correct molecular weight in the transformed bacteria that was not present in TG1 controls or uninduced bacteria (Fig. 5.6A).

After electroporation of pNirB/p24 into BRD509, further western blots were performed to detect expression of p24 by this construct (Fig. 5.6B). Again, there were a number of cross-reactive antigens detected by this antiserum although again there

was detection of an induceable protein that corresponded to the correct molecular weight. Various alterations to the blocking stage of the blotting procedure such as blocking with 10 or 20% FCS did not remove the majority of the cross-reactive proteins detected. Similar problems have been encountered using this antiserum to detect p24 expression in BCG (Prof. N. Glaichenhaus, unpublished observations). There is currently no monoclonal antibody against this protein as it has only been recently described. While the polyclonal antiserum shows considerable crossreaction with a number of bacterial antigens, the consistent detection of a protein band at the correct molecular weight which is only expressed under anaerobic stimulus suggest that p24/LACK is being expressed in this construct, which was termed GID202.

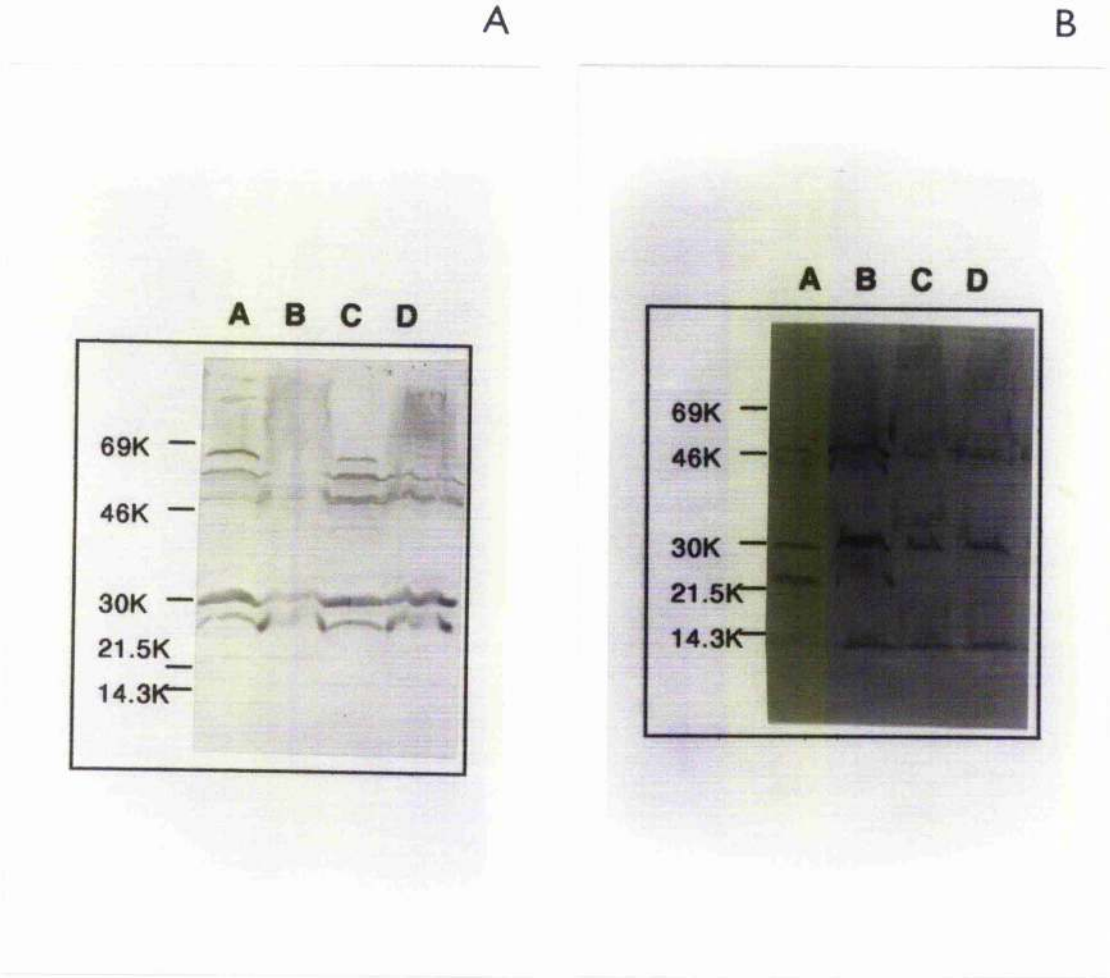


Figure 5.6: Western blot of p24/LACK expression in **TG1** and **BRD509**. Blotting was performed as described in Materials and Methods. **TG1** A: TG1/NirB/p24 (anaerobic); B: TG1/NirB/p24 (aerobic); C: TG1 (anaerobic); D: TG1 (aerobic). **BRD509** A: BRD509/NirB/p24 (anaerobic); B: BRD509/NirB/p24 (aerobic); C: BRD509 (anaerobic); D: BRD509 (aerobic).

5.3 Plasmid Stability

5.3.1 Plasmid Stability *in vivo*

After oral administration of BALB/c mice with 1 x 10^{10} GID202 bacteria spleen, liver and mesenteric lymph nodes were removed at various time points to determine bacterial growth *in vivo* and plasmid stability, as described in Materials and Methods. Stable inheritance of the plasmid was observed in all organs examined (Fig. 5.7).

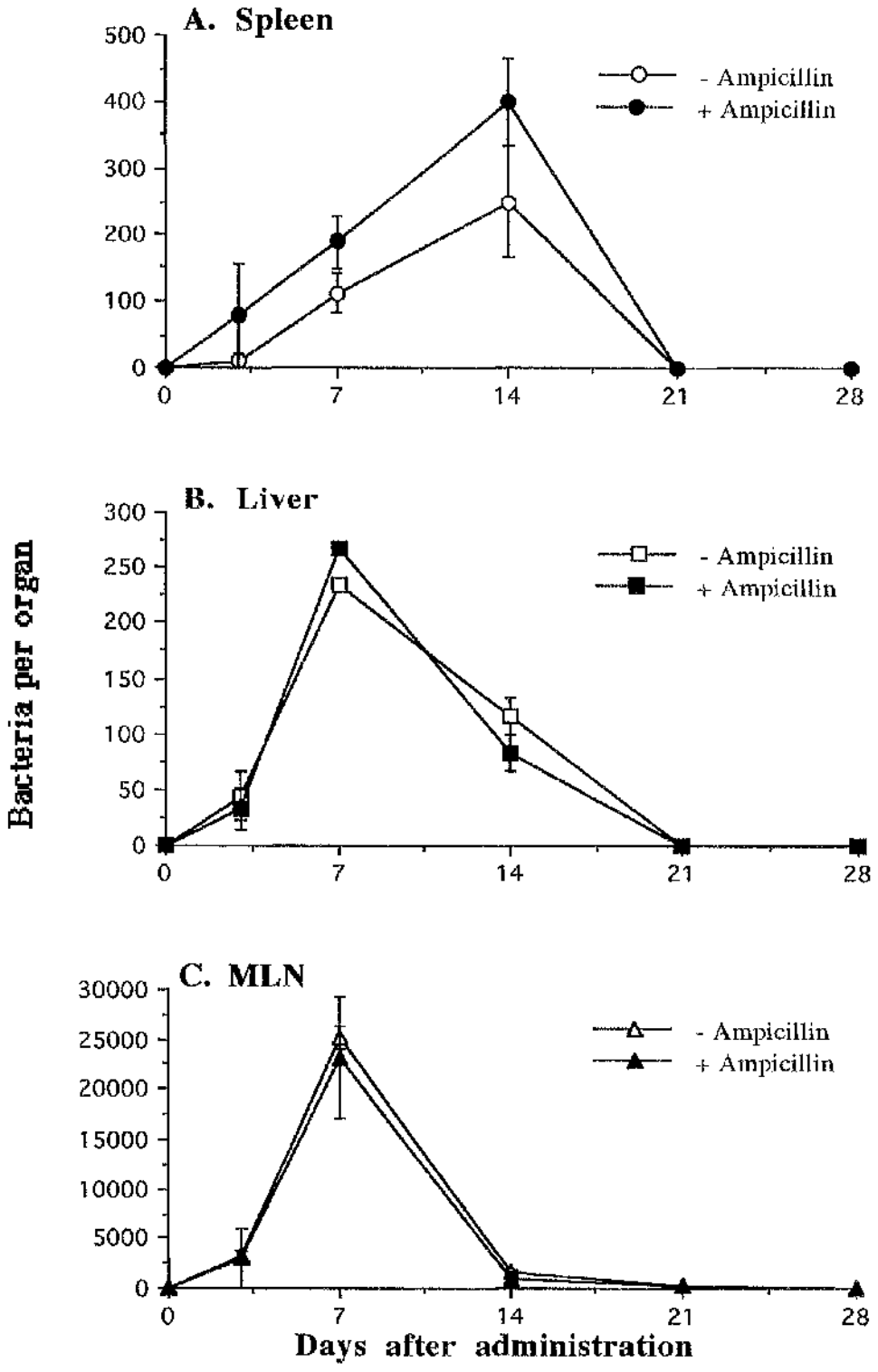


Fig. 5.7: Plasmid stability of GID202 *in vivo* . Bacteria (1 x 10^{10}) were administered orally and colonies in spleen liver and MLN detected at various time points after administration. Data represent mean and SEM of 3 pooled mice.

5.4 Vaccination against *L. major*

The construct GID202 was used to vaccinate resistant, CBA, and susceptible, BALB/c, mice against *L. major* infection. Mice were administered orally with one or two doses of 1 x 10^{10} bacteria (either BRD509 or GID202) or were given PBS only. Three weeks after the last dose, mice were challenged in the footpad with 5 x 10^4 *L. major* promastigotes and lesions monitored to assess infection. In some experiments, BALB/c mice were injected sub-cutaneously with recombinant p24/LACK in the footpad and also received a single intra-peritoneal injection of recombinant IL-12 (100ng) before infection with *L. major*.

5.4.1 Vaccination of CBA mice

CBA mice which received one oral dose of GID202 before infection demonstrated a slight decrease in lesion development compared with BRD509 treated mice (Fig. 5.8A). CBA mice which received two oral doses of GID202 developed significantly smaller lesions at the early stage of infection compared with control mice but then later developed a profoundly exacerbated infection (Fig. 5.8B). Mice given two oral doses of GID202 had a mean peak lesion size which was approximately 100% greater than controls (1.008 +/- 0.139 compared with controls of 0.489 +/- 0.041 [PBS] and 0.442 +/- 0.058 [BRD509].

5.4.2 Vaccination of BALB/c mice

BALB/c mice which were administered one or two oral doses of GID202 three weeks before infection with *L. major* also developed exacerbated lesions compared to mice administered BRD509 or PBS (Fig. 5.9A and B). Control mice administered two subcutaneous injections of recombinant p24 and IL-12 had reduced lesion sizes, as previously reported (379).

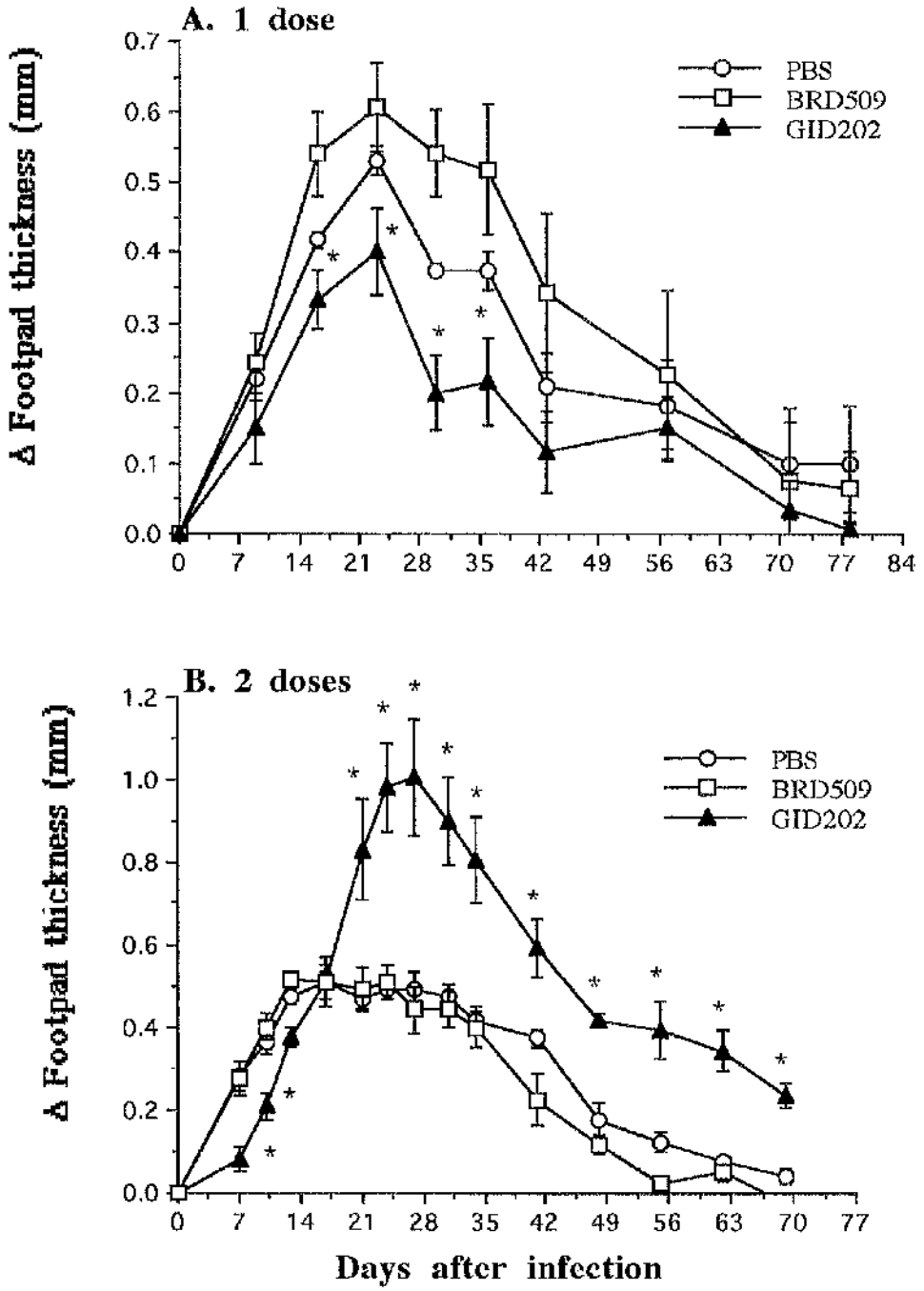


Figure 5.8: Exacerbation of *L. major* infection in CBA mice by GID202. Mice received one (A) or two (B) oral doses of GID202, BRD509 or PBS before challenge with $5x10^4$ *L. major* three weeks later. Each point represents mean +/- SEM of six mice per group. The data are representative of two individual experiments. * $p < 0.05$ compared to BRD509 control mice.

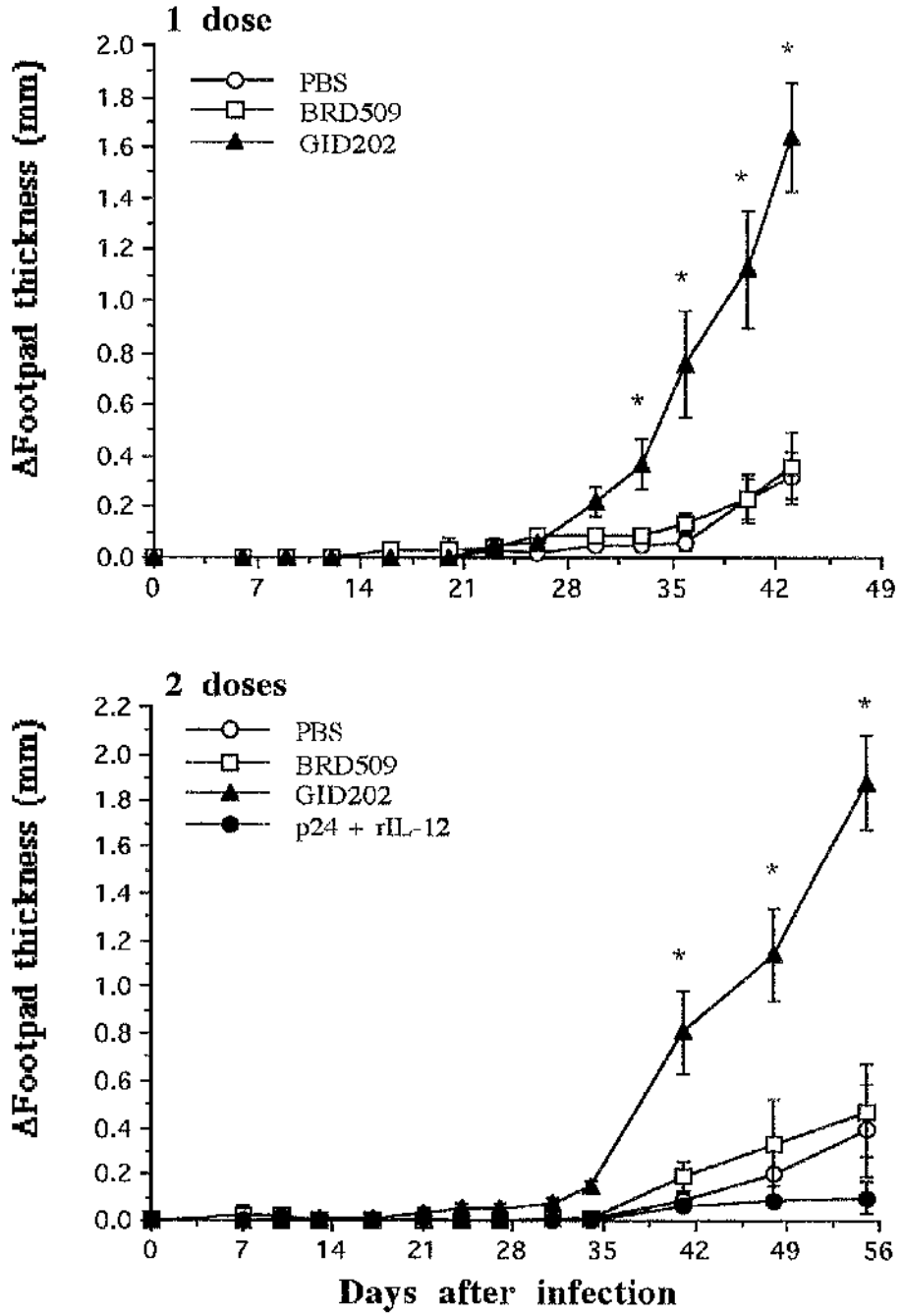


Fig. 5.9: Exacerbation of *L. major* infection in BALB/c mice by GID202. Mice received one or two oral doses of either GID202, BRD509 or PBS before infection with 5 x 10^4 L. major three weeks later. Each point represents the mean and SEM of six mice per group except the p24 + rIL-12 group which shows the mean and SEM of four mice. Data are representative of two individual experiments. * $p < 0.05$ compared to BRD509 controls.

5.5 Immune response in vaccinated BALB/c mice

5.5.1 DTH response

BALB/c and CBA mice administered one or two oral doses of GID202 did not develop a detectable DTH response when injected subcutaneously with live *L. major* in the footpad (data not shown).

5.5.2 Proliferative response *in vitro*

Spleen cells from BALB/c mice which received one oral dose of GID202 were cultured *in vitro* with the immunodominant peptide from p24 (previously demonstrated to account for the majority of class II, I-E^d restricted responses [Prof. Glaichenhaus, personal communication]) or with recombinant p24 to assess recall responses to this antigen *in vitro* . Antigen specific proliferation occurred in cultures of cells from GID202 administered mice in response to peptide while no proliferation was observed in control cultures (Fig. 5.10). Cells from mice immunised with GID202 also proliferated *in vitro* in response to recombinant p24, although control cultures also demonstrated a degree of proliferation presumably due to contaminants in the p24 preparation.

5.5.3 Cytokine response *in vitro*

Cells from GID202 administered mice also produced substantial amounts of IFN-γ in response to peptide *in vitro* (Fig. 5.11A) while there was no detectable IL-4 production in any of the cultures (data not shown). These cells also produced IFN-γ but no IL-4 in response to rp24 although at the highest concentrations of antigen, control cultures also produced IFN-γ (Fig. 5.11B).

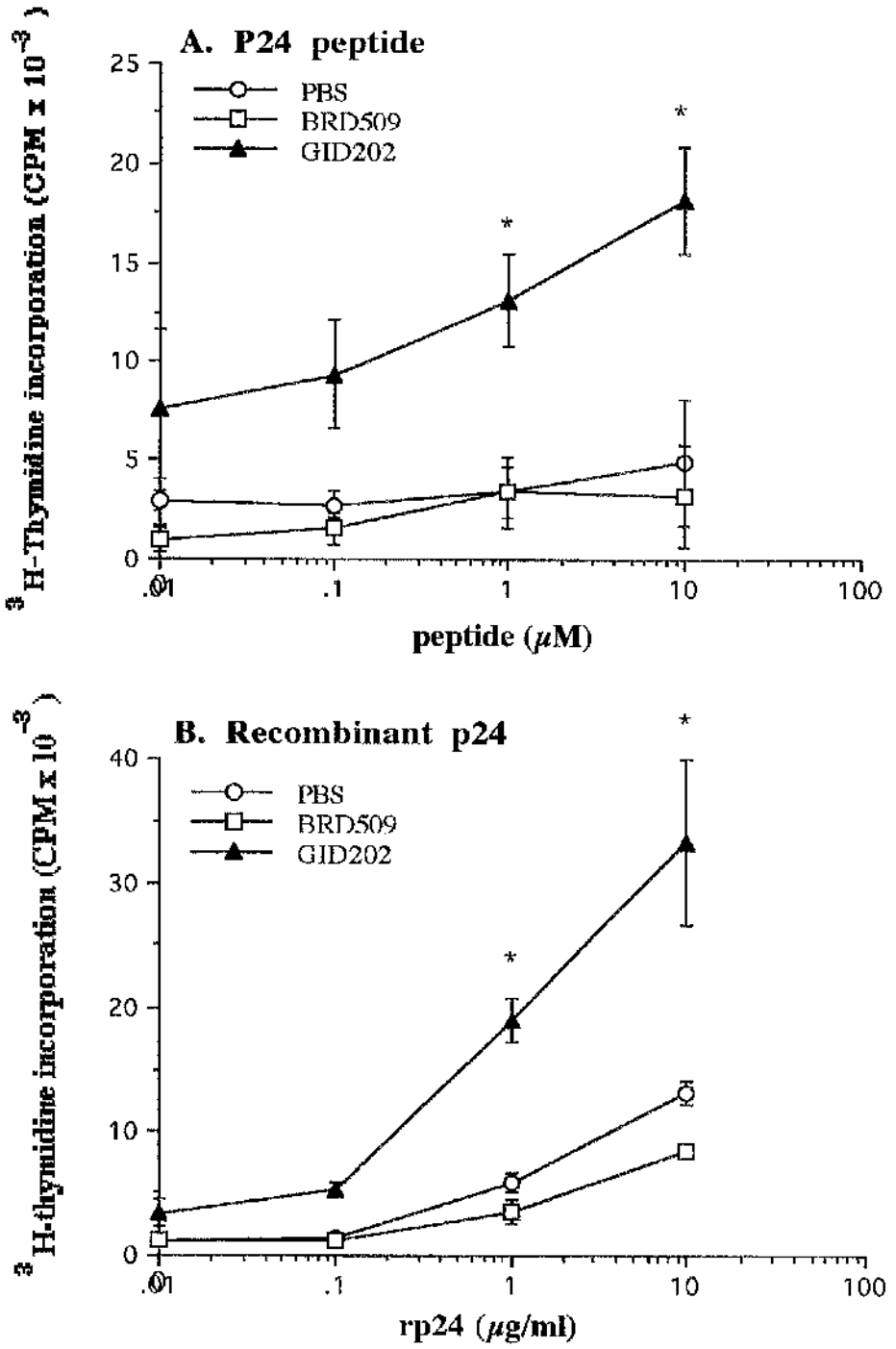


Fig. 5.10: Proliferation to the immunodominant peptide of p24 (FSPSLEHPIVVSGSWD) and recombinant p24 by spleen and MLN cells from BALB/c mice immunised with either BRD509, GID202 or PBS only. Data represent mean and SD of pooled cells from 3 mice. * $p < 0.05$ compared to BRD509 group.

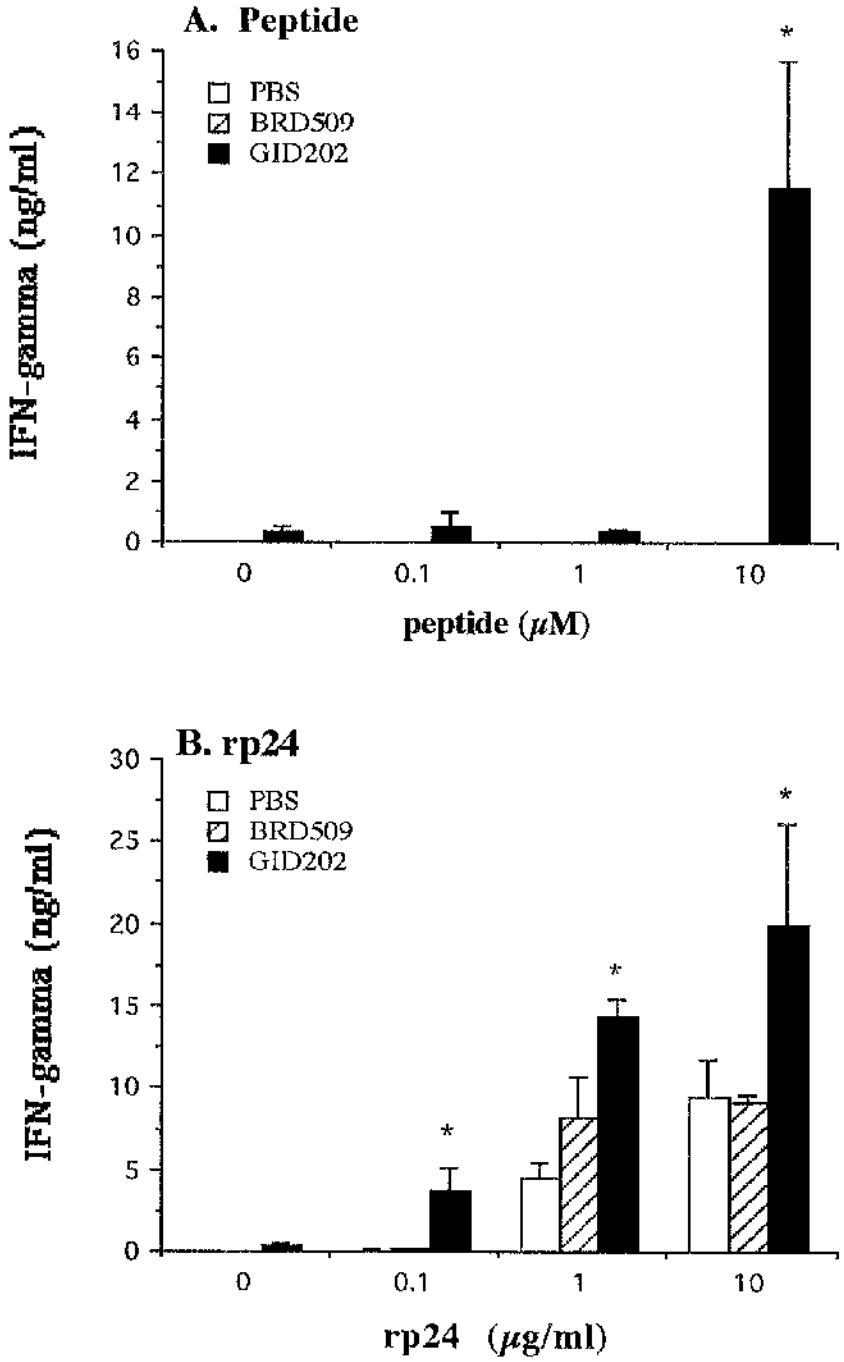


Fig. 5.11: Antigen specific IFN-γ production *in vitro* by spleen and MLN cells from immunised mice. Data represent mean and SD of pooled cells from 3 mice. * $p < 0.05$ compared to BRD509 group.

5.6 Discussion

A *S. typhimurium* construct was created which has the plasmid pNirB/p24/LACK, containing the cDNA encoding p24/LACK of *L. major* placed downstream of the inducible *NirB* promoter. This construct expresses a product of 23-24KDa under anaerobic conditions which can be detected by western blot using a polyclonal antiserum against p24/LACK. This product is not expressed in the control strain BRD509 in aerobic or anaerobic conditions. Although other bands were detected by this antiserum the 23-24KDa band is likely to represent p24 production. A monoclonal antibody against p24 is currently being developed to confirm this.

After oral administration of GID202 to BALB/c mice, the construct can be found in the MLN 3 days later and persists for up to 3 weeks. Spleen and liver colonisation also take place but at a much lower level, similar to that observed with the control strain BRD509 (Chapter 3). The numbers of bacteria which were grown on L-Agar and L-Agar containing ampicillin were very similar, indicating that little plasmid segregation had occurred *in vivo*, as previously noted using the NirB promoter (Chapter 4).

BALB/c mice which received one or two oral doses of GID202 displayed profound exacerbation of disease after *L. major* infection compared to control mice, while mice administered subcutaneously with recombinant p24 and IL-12 were protected from disease. CBA mice administered two doses of GID202 also developed exacerbated infection after challenge. Thus, subcutaneous immunisation with p24 generated a protective response, while oral immunisation with the same antigen expressed in *S. typhimurium* caused exacerbation of infection. The diverse course of infection after these immunisation protocols using p24 indicate that responses to this antigen may be critical in determining the outcome of disease. Therefore this antigen represents a good candidate antigen for vaccination against leishmaniasis although

these data indicate that attenuated *S. typhimurium* may not be appropriate vaccine vectors in this instance.

A weak protective response was induced in CBA mice which received a single oral dose of GID202 while mice receiving two doses exacerbated infection, suggesting that the induction of exacerbation and protective immunity using p24/LACK may depend on the dose of antigen administered. Further experiments with varying doses of GID202 will be required to examine the effects of antigen dose upon the generation of protective immunity or exacerbation.

Although CBA mice administered GID202 did eventually cure of infection the exacerbation seen after 2 oral doses of this construct demonstrate that vaccination can generate an inappropriate response to antigen which can impair the generation of natural resistance. If p24/LACK is to be used as a vaccine in the future care must be taken to avoid the generation of such inappropriate responses. It will be of interest to determine whether further doses of GID202 before challenge can generate a non-healing response in these animals.

A Th1 response is induced after subcutaneous immunisation with p24 and IL-12 which accounts for the protective response observed using this vaccination protocol (379). Surprisingly, cells from mice immunised with GID202 responded *in vitro* to p24 or a peptide from p24 by producing IFN-γ but no IL-4, similar to the response generated using the gp63 construct (Chapter 3 and 4). Thus exacerbation did not correlate with the presence of a Th2-like response to p24 as may be expected. The generation of a Th1 response which correlates with exacerbation suggests that further diversity of T cell subsets may exist, as previously suggested (377). A Th1-like cell clone which transfers exacerbation to resistant and susceptible mice has been described previously (127). However, exacerbation of infection is unlikely to be due directly to the production of IFN-γ, as IFN-γ can activate macrophages to kill resident parasites and can block Th2 cell induction (143, 155). Other cytokines such as IL-10 and TGF-β

which have been shown to inhibit protective responses may be involved in the exacerbation observed here. An examination of these possibilities will require further *in vitro* and *in vivo* studies.

These data demonstrate that delivery of antigens to the immune system using attenuated *S. typhimurium* may not always generate a protective response and may in fact have an adverse effect upon disease. A detailed examination of immune responses induced after vaccination with heterologous *S. typhimurium* is required in order to understand the relative merits and pitfalls associated with this system.

5.7 Summary

A plasmid was generated which contains the cDNA for p24/LACK under control of the inducible *NirB* promoter. This plasmid was introduced into the attenuated *S. typhimurium* strain BRD509. Expression of p24 by this construct (GID202) under anaerobic conditions was demonstrated by western blotting. After oral administration of GID202 to BALB/c mice bacteria were detected in the spleen, liver and MLN until 21-28 days later. Plasmid stability of the construct during colonisation was found be around 100%. BALB/c mice which were orally administered one or two doses of GID202 before *L. major* infection developed exacerbated disease. CBA mice given two oral doses of GID202 also generated an exacerbated infection although CBA mice which received a single dose were slightly protected. Spleen and MLN cells from mice administered GID202 responded *in vitro* in a Th1-like manner to both recombinant p24 and the immunodominant peptide of p24. These data demonstrate a disease-promoting effect of vaccination with p24 expressed in *S. typhimurium* which correlates with a Th1 response *in vitro*.

Chapter 6

General Discussion

Contents

6.1 Conclusions

A *Salmonella typhimurium* vaccine construct, GID101, was examined with respect to its protective effects upon *L. major* infection and the immune responses induced after immunisation. GID101 represents a considerable improvement over the previous vaccine strain SL3261/gp63 in that after 2 oral immunisations it reduced lesion development in susceptible, BALB/c mice after *L. major* challenge infection, while SL3261/gp63 does not. Spleen and MLN cells from immunised BALB/c mice respond *in vitro* to parasite antigens to produce substantial amounts of IFN-γ and IL-2, while no IL-4 or IL-5 was detected. The production of these cytokines was $CD4^+$-cell dependent and serum from immunised mice contain parasite specific IgG2a, but no parasite specific IgG1, indicating the generation of a Th1-like response to antigen *in vivo* and upon restimulation *in vitro*. While the cytokine profile and antibody response suggested a Th1-like profile, there was no detectable DTH response to *L. major* after immunisation.

L. major gp63 was expressed in *S. typhimurium* strain, BRD509, under the control of either the *NirB* promoter which responds to anaerobic conditions (GID105) or *OsmC* which is induced under high salt conditions (GID106). These constructs were compared to GID101 to determine if use of inducable promoters could confer protection after a single immunising dose. The construct GID105 displayed extremely stable plasmid retention as it colonised *in vivo* as compared to the constitutive construct GID101 which experienced significant plasmid loss. The construct GID106 was found not to colonise spleen or liver at all after oral delivery and loses plasmid more quickly than GID101 *in vivo* . An antibody response to gp63 is generated in BALB/c mice after one oral dose of GID101, GID105 or GID106, while only the anaerobic construct, GID105, is able to induce a detectable cellular response to antigen after a single oral dose. Spleen and MLN cells from mice administered with GID105 respond *in vitro* in a Th1-like manner but no DTH is conferred to immunised mice. A single oral delivery of GID105 or GID106 is sufficient to reduce lesion swelling in

susceptible mice after *L. major* infection, while a single dose of GID101 does not confer any protection. Two doses of GID105 also provides protection against *L. donovani* infection, while GID101 or GID106 do not. Thus it was possible to generate a protective response after a single administration of constructs using inducible promoters although these varied in terms of protective capacity and immune response elicited.

A further construct was made expressing a recently discovered *L. major* antigen which can elicit better protective responses than gp63 when used in combination with IL-12. A plasmid was generated which contains the cDNA for p24/LACK under the control of the inducible *NirB* promoter and was introduced into the attenuated *S. typhimurium* strain BRD509. Expression of p24 by this construct (GID202) under anaerobic conditions was demonstrated by western blotting. After oral administration of GID202 to BALB/c mice, bacteria were detected in the spleen, liver and MLN until 21-28 days later and plasmid stability of the construct during colonisation was found be around 100%. BALB/c mice which were orally administered one or two doses of GID202 before *L. major* infection developed exacerbated disease. CBA mice given two oral doses of GID202 also generated an exacerbated infection although CBA mice which received a single dose were slightly protected. Spleen and MLN cells from mice administered GID202 responded *in vitro* in a Th1-like manner to both recombinant p24 and the immunodominant peptide of p24. These data demonstrate a disease-promoting effect of vaccination with p24 expressed in *S. typhimurium* which correlates with a Th1 response *in vitro*.

6.2 What determines T cell subset induction in vaccination against leishmaniasis ?

A number factors responsible for the development of uncommitted $CD4^+$ T cells to polarised Th1 and Th2 subsets have been uncovered in recent years. The nature of antigen, lineage of antigen presenting cell (APC) and the presence of soluble

or membrane bound costimulatory molecules have all been demonstrated to affect the generation of T cell subsets (98). The relative importance of each of these factors in the development of T cell responses during leishmaniasis is not clear but an understanding of the process of lineage commitment may have consequences with regard to vaccination against leishmaniasis.

An analysis of TcR usage in BALB/c and C57BL/6 mice demonstrated the expansion of a Vα8Vβ4 population early during *L. major* infection of both strains, suggesting that responses to diverse antigens are not the primary cause of differential T cell polarisation (380). However, certain leishmanial antigens have been shown to preferentially induce Th1 or Th2 cells after immunisation of BALB/c mice (137). Immunisation of BALB/c mice with peptides from gp63 also suggests that certain sequences have an inherent tendency to drive Th1 or Th2 responses (276). In addition, a recently cloned antigen from *L. braziliensis* has the capacity to generate Th1 responses in PBMC's from patients with leishmanial infection whereas unstimulated cells or cells stimulated with parasite lysate produced a mixed Th1/Th2 profile of cytokine secretion (381). Therefore, certain antigens or peptides from *Leishmania* parasites have the capacity to generate a polarised T cell response. This phenomenom may account for the capacity of certain antigens such as p24/LACK to generate cure of disease in immunised susceptible mice while others such as gp63 cannot (379).

In agreement with the antigen-driven selection of T cell subsets in leishmaniasis, immunisation of mice with RSV proteins using identical protocols demonstrated that in this model induction of Th1, Th2 or CTL responses depended upon the antigen used (243). These experiments demonstrate that certain antigens do have the capacity to select for certain T cell subsets, although the relative importance of these in pathogenesis and the mechanism by which antigens can select for differing responses are not known.

In addition to the contribution of antigens themselves in selecting T cell subsets, the lineage of antigen presenting cell can influence polarisation. Injection of killed leishmanial antigens intravenously or intraperitonealy confers the ability to cure disease in the susceptible BALB/c strain while subcutaneous injection of the same preparation causes exacerbation of infection (124, 129). These diverse outcomes may result from antigen presentation by different cell populations selected by the route of immunisation. In agreement with this hypothesis, B cells have been found to generate leishmania specific Th2 cells while macrophages preferentially induced a Th1-like population from mice immunised with antigen from *L. m. mexicana* (254). The mechanism by which APC's can influence the generation of T cell subsets may involve specific membrane interactions between the T cell and the APC or may derive from the activities of APC secreted products.

Costimulation of T cells via membrane interactions with APC's during antigen presentation can determine the polarisation of $CD4^+$ T cell responses. *In vitro* and *in vivo* evidence suggest that ligation of B7-1 and B7-2 molecules on the APC membrane by T cell molecules during antigen presentation can select for Th1 or Th2 generation respectively (382). Interestingly, *L. donovani* infection of bone-marrow macrophages inhibits the membrane expression of B7-1 in response to activating stimuli, although whether this process can modulate subsequent T cell subset generation was not addressed (383).

Costimulation by soluble factors during TcR ligation can also affect subsequent T cell responses although these may derive from other cell sources in addition to the APC. Initial cytokine responses in susceptible and resistant mice after infection with *L. major* are of a mixed Th0 type with mRNA of both IFN-γ and IL-4 detected in the draining lymph node during the first week of infection (184). In resistant mice the initial IL-4 mRNA is rapidly controlled which may account for the development of a Th1 phenotype in response to infection. Conversely, the maintenance of elevated IL-4 production in BALB/c mice after the first week of infection may be required for the

generation of Th2 responses. Consistent with this, immunological interventions which restrict early IL-4 production in BALB/c mice also induce healing, and excess IL-4 production in a transgenic resistant strain render it deficient in the control of infection (98, 152). Thus a leishmanial specific Th2-cell response requires the presence of IL-4 at T cell priming, as has been observed in other disease models (185).

The source of IL-4 during early *L. major* infection may derive from $CD4^+$ cells themselves, as SCID mice and anti-CD4 treated BALB/c mice do not generate this early response (184). An unusual cell population which are $CD4^+$, $NK1.1^+$ has been described which accounts for early cytokine production hours after the injection of anti-CD3 monoclonal antibody (384). This cell may be the source of early IL-4 production after *L. major* infection. While mast cells and basophils have also been suggested to contribute to Th2 cell generation via the production of IL-4, the activating mechanisms for early cytokine production by these cell types is undefined.

The relative contribution of antigen, lineage of presenting cell and costimulation in the induction of T cell subsets after *L. major* infection is not clear. An increased understanding of these processes may lead to improved antigens for vaccination and the generation of defined adjuvants which can differentially select T cell subset differentiation.

With this in mind, the capacity of *Salmonella* vectors to initiate a restricted Th1-like response to gp63 after oral administration suggests that *Salmonella* may alter the cytokine environment during T cell priming. As purified gp63 is not intrinsically able to induce a Th1 response after subcutaneous immunisation (373), it is likely that the induction of strong Th-1 like responses after *Salmonella* delivery of gp63 is due to costimulatory effects mediated by the *Salmonella* themselves. It may be that salmonella alter membrane costimulatory molecule expression on infected macrophages to select for B7-1 expression, but it is more likely that they alter the local cytokine environment. BCG and *Toxoplasma gondii* induce the production of various

cytokines by the macrophage during entry, including TNF-α, IL-12 and IL-1 (98). It is therefore possible that *Salmonella* do likewise, and indeed preliminary *in vitro* experiments using attenuated salmonella (BRD509) to infect the macrophage cell line J774 also induced IL-12 production (Author's unpublished observations). There is little data available on the induction of cytokines during macrophage infection by *Salmonella in vitro* or *in vivo*. The secretion of IL-12 by infected macrophages *in vivo*, as they present *Salmonella* and heterologous antigens, will preferentially select for Th1 responses. Further work in this area is required in order to determine the factors involved in T cell subset selection during *Salmonella* infection but it seems likely that the non-specific induction of cytokines by *Salmonella* themselves may serve to polarise T cell responses.

6.3 Further T cell heterogeneity

While the involvement of Th1 and Th2-like $CD4^+$ T cells in the regulation of immunity in experimental cutaneous leishmaniasis is well documented, there are indications that further heterogeneity of the T cell compartment exists. BALB/c mice immunised intravenously with killed *L. major* recover from cutaneous disease and this immunity from infection can be transferred to naive BALB/c mice by transfer of the $CD4^+$ T-cell population (117). However, unlike the $CD4^+$ cells from mice which have recovered from infection, these cells do not confer DTH responses to injected parasite antigen. Indeed, transfer of these cells can suppress a DTH response which normally occurs in cured mice (125). In addition to these data, protection of BALB/c mice after oral administration of a *S. typhimurium* construct expressing *L. major* gp63 did not correlate with the induction of a DTH response to injected parasites (Chapter 3). Therefore at least two functionally distinct protective populations exist which differ in the ability to transfer DTH responses, although it remains to be determined whether these represent further distinct T cell subsets or are developmental stages of Th1 cells.

A Th1 like cell clone has also been described which transfers DTH responses to parasite antigens and an exacerbative effect to both resistant and susceptible mice upon *L. major* infection (127). Chapter 5 of this thesis describes a *S. typhimurium* construct expressing p24/LACK, which generates a Th1-like response to p24, causes exacerbation to L. major infection yet does not mediate DTH. Therefore the differential capacity to generate DTH responses while mediating exacerbation is also consistent with further diversity of T cell subsets.

These observations in the *L. major* model are supported by additional data from T cell lines and clones. Cytokine expression by individual T cells is thought to be independently regulated in that although populations of T cells may display a profound bias in terms of cytokine secretion, single cells within this population display considerable diversity in the degree of polarisation (386). Thus a "Th1 population" does not represent a homogeneous population and groups of cells may exist which can be further subdivided.

Further evidence suggests that other diverse T cell subsets may exist in addition to Th1 and Th2 cells. Foreign antigens delivered by attenuated *S. typhimurium* vectors tend to induce Th1-like recall responses *in vitro* (278, 356, 357) yet a feature of *Salmonella* infection is the production of antigen specific IgA which is characteristic of a Th2-like response (284). While this may be attributed to different localised T cell responses in the mucosal and systemic immune system compartments, the detection of elevated serum IgA in addition to IgA at mucosal surfaces suggests that a more general interplay of Th1 and Th2 responses occurs.

Thus, while murine *L. major* infection represents a clear model of differential activation of T cell subsets, there is evidence to suggest further $CD4^+$ T cell subset diversity. The further elucidation of the true nature of T cell responses after immunisation in the murine model may aid the generation of defined vaccines to stimulate protective T cell compartments.

6.4 Plasmid stability and immunogenicity

The sustained inheritance of plasmid by *Salmonella* vectors as they colonise *in vivo* is thought to determine the immune response to heterologously expressed antigens. This simple correlation between plasmid stability *in vivo* and increased immunogenicity is, however, not always observed. While the integration of foreign genes into the chromosome of attenuated salmonella strains increases the capacity to maintain antigen expression throughout the life cycle *in vivo* , it also reduces the level of protein expression and reduces immunogenicity (387).

The use of inducible promoters to control antigen expression *in vivo* can enhance plasmid stability while maintaining high levels of expression (372). However, sustained protein expression *in vivo* using inducible promoters does not always increase immunogenicity as the highly unstable construct GID106 described in this thesis confer protection to *L. major* infection while a more stable construct, GID101, does not (Chapter 4). Thus although plasmid stability and protein expression can alter immunogenicity, the factors responsible for the generation of these responses are not clearly defined.

The stage in *Salmonella* colonisation at which inducable promoters initiate transcription may be critical in the generation of immune responses, as this will determine how antigen presentation takes place. Antigen which is expressed after macrophage invasion by *Salmonella* in the visceral tissues may generate different responses from antigens expressed only in the local mucosal environment at the initiation of infection as these may mimic antigen delivery by different routes of immunisation.

In addition, the ability of salmonella strains to initiate the secretion of cytokines after infection of macrophages may also determine immunogenicity. Different attenuated strains of *Salmonella* may differ in the capacity to generate such responses

and therefore vary in the capacity to induce immune responses to foreign antigens as the cytokine environment is altered during T cell priming (see 6.1). The presence of these cytokines may aid or hinder the generation of immune responses to heterologously expressed antigens. With this in mind, it is of interest that *galE Salmonella* mutants which have defective LPS structures are poorly immunogenic and LPS is known to induce macrophage cytokine production. Thus the choice of bacterial strains which can elicit non-specific cytokine production by macrophages may allow the selection of better immunogenic *Salmonella*. Therefore, while it is clear that increased plasmid stability can alter the immunogenicity of antigens expressed in attenuated *Salmonella* other factors may also modulate the generation of immune responses and may contribute to the generation of an effective *Salmonella* vaccine against leishmaniasis.

6.5 *Salmonella* as vaccine vectors against diverse pathogens

The ability of many *Salmonella* constructs to generate Th1-like responses to expressed antigens suggests that *Salmonella* vectors may be useful in vaccine design against pathogens which require such responses to clear infection. The induction of Th1 responses after vaccination with gp63 or p24 in *S. typhimurium* (Chapter 3, 4 and 5) suggests that responses to heterologous antigens may be restricted to a Th1 phenotype. Therefore while attenuated *S. typhimurium* may be useful vectors for vaccination against intracellular pathogens they may not represent an ideal carrier for vaccination against extracellular infections which require protective Th2 responses.

This issue is important when considering attenuated *Salmonella* as a base for vaccination against diverse pathogens. While a vaccine construct has been described which can induce immune responses against *Salmonella*, Tetanus toxin fragment C and *Schistosoma mansoni*, the protective nature of these responses was not examined (359). The induction of immune responses to diverse pathogens by a single *Salmonella* construct does suggests that multivalent immunisation is possible, however the nature

of immune responses induced in multivalent vaccine constructs may do little to prevent disease against the respective pathogens and may in fact increase pathology. As demonstrated in this study, disease exacerbating responses can be induced following vaccination with leishmanial antigens (Chapter 5). Further studies on the nature of immune response induced following vaccination with attenuated *Salmonella* may give a clearer indication of the potential to vaccinate against diverse organisms. The restricted use of attenuated *Salmonella* to vaccinate against pathogens which require similar protective immune responses may be a rational approach to future vaccine design.

6.6 Factors affecting host response to vaccination

A further consideration in the generation of protective immune responses to leishmaniasis after vaccination is the predisposition of the host to generate Th1 or Th2 responses. The predisposition of BALB/c mice to generate Th2 responses maps to a region on chromosome 11 (71). This area contains the genes for IL-4, IL-12 and iNOS and it has been suggested that the tendency for BALB/c mice to develop Th2 responses results from a defect in the IL-4 gene (98). A vaccine which induces a protective Th1 response in BALB/c mice overcomes this inherent defect. However, in addition to genetic factors, there are also certain environmental factors which can affect the outcome of immune responses and should be taken into account when considering vaccination.

Pregnancy is associated with elevated Th2-like responses to antigen due to interactions between the endocrine and immune system (387). Pregnant mice develop exacerbated lesions during *L. major* infection due to the decreased ability to mount Th1 responses. Interestingly, such mice also demonstrate increase foetal rejection due to an elevated Th1 response to the parasite. Thus responses to the parasite and to the foetus can be adversely altered due to environmental influences, irrespective of the genetic tendency of the host. A similar effect may take place in during vaccination to

generate Th1 responses using *S. typhimurium*, although these issues have not been addressed to date.

As much of the population in developing countries have resident parasitic worm infestations in the gut which require Th2 responses for clearance, this may also alter immune reponses to vaccination. A dominant response to the vaccine or to the worm may have adverse effects upon the resident infection or limit vaccine immunogenicity. The effect of pregnancy and infection upon leishmanial vaccination have not been directly addressed although experimental models suggest that factors which cause the presence of Th2 cytokines during infection can lead to the generation of exacerbated disease in preference to cure of infection (98). Therefore environmental influences such as pregnancy and secondary infections may have considerable influence upon the induction of protective immunity against leishmaniasis. When considering vaccination against a pathogen such as *Leishmania* these factors must be considered and further study of the nature of environmental influences in vaccination is required.

6.7 Future directions

Gp63 *Salmonella* constructs

It is clear from the data presented in this thesis that gp63 can provide a degree of protection to susceptible mice when expressed in the *S. typhimurium* strain BRD509 and when inducible promoters are used. Thus the present system seems to be optimised to generate protective immunity using an antigen which has performed poorly in other vaccine studies. However, it is also clear that a reduction in lesion size after immunisation with these constructs does nothing to protect susceptible mice from fatal infection, as lesions are not ultimately controlled. Thus these constructs may represent the optimal protection that is possible using gp63. This may be due to the fact that the expression of gp63 is probably turned off or at least downmodulated following the transition from promastigote to amastigote stage. Thus a strong Th1-like

response to gp63 is not beneficial during later infection as there is little gp63 expressed at this stage. While these results are discouraging there are positive conclusions that can be drawn from these studies.

(1) These studies represent an optimisation of vaccination using an antigen that was thought to be important during disease as it is the major surface antigen of promastigotes. While use of this antigen in *Salmonella* does not allow mice to ultimately resist disease, other antigens used in this system may do so. Thus identification of further *L. major* antigens which are expressed on both amastigote and promastigote stage may aid the construction of an effective vaccine, using the strain BRD509 and the promoter NirB shown to be optimal in these studies.

(2) While the gp63 constructs do not in themselves constitute an effective vaccine, the use of these with other vaccine constructs such as GID202 (expressing p24) may be effective as inducing immune responses against gp63 should limit the initial parasite growth. It seems likely that an effective vaccine against leishmaniasis should include more than one antigen due to the success of whole parasite antigen vaccination and the relatively poor results using single antigens. Studies using both gp63 and p24 constructs to vaccinate susceptible mice are currently ongoing.

(3) The performance of the GID105 construct in reducing parasite burdens during experimental *L. donovani* infection indicate that this construct may be useful against this pathogen. A dog vaccination trial is currently underway in Portugal using the construct GID105 to assess immunogenicity and protection against *L. infantum* infection which is an endemic pathogen in this area.

P24 *Salmonella* construct

The exacerbative effect of the GID202 construct upon experimental cutaneous leishmaniasis suggests that responses to this antigen are critical in modulating disease.

The exacerbative effect of this construct may be due to clonal exhaustion of p24 reactive cells. Thus the exacerbation during disease may represent an inability to respond to p24/LACK during infection. Vaccination with lower doses of GID202 may define a critical inoculum which can induce immune responses but does not exhaust the cells. In agreement with an effect of dosage upon the exacerbative effect, CBA mice administered one dose of GID202 were slightly protected. Therefore further dose experiments and an examination of tolerance induction by the construct GID202 are underway.

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