In vitro Characterisation of the *Leishmania*:Dendritic Cell Interaction

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For Nanny

I declare that the work presented in this thesis is my own, except where otherwise stated

Clare Bennett

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Abstract

Leishmania sp. are protozoan parasites that cause a wide spectrum of diseases in man and other mammals. Resolution of *Leishmania* infections depends strictly on the activation of a type 1 T helper response, which must be initiated by activated antigen presenting cells displaying parasite antigens in the context of major histocompatibility (MHC) class II molecules. Macrophages (MΦ) and Dendritic cells (DC) are specialised antigen presenting cells capable of stimulating T cells. Primary T cell responses however, can only be initiated by DC.

MΦ are the principal host cells for *Leishmania* parasites and are ultimately responsible for parasite clearance, via the production of nitric oxide. However, MΦ do not become activated upon infection and do not efficiently present *Leishmania*-derived antigens to T cells, raising the question of how the response is initiated. As *Leishmania* also infect DC, it has been assumed that *Leishmania*-infected DC stimulate the primary anti-*Leishmania* T cell response. The specific interaction between *Leishmania* parasites and DC has not, however, been investigated in detail. This work examines the *Leishmania*:DC interaction through the use of defined *in vitro* systems and therefore evaluates the hypothesis that infection of DC by *Leishmania* parasites is sufficient to activate the primary anti-*Leishmania* T cell response. In particular, it addresses two aims: first to develop an experimental model to investigate the fate of *Leishmania* antigens in infected cells; second to examine the *Leishmania*:DC interaction in a defined *in vitro* model.

In initial experiments, lines of transgenic parasites were generated in which the MHC II-dependent T cell epitope Moth Cytochrome C (MCC) was expressed within a number of different fusion proteins, to provide a model in which the fate of parasite-derived antigens could be followed in infected DC. MHC II-MCC complex formation in DC and MΦ infected with these transgenic parasites was investigated using the complex-specific monoclonal antibody, D4, and a complex-specific T cell line. Although the fusion proteins were clearly demonstrated to be secreted at high levels, no cell surface staining could be detected with D4 and neither infected DC nor infected MΦ could stimulate T cell proliferation. *Leishmania*-infected DC were however, shown to efficiently process and present exogenous antigen to T cells *in vitro*.

Alternative strategies were therefore developed to probe the DC:*Leishmania* interaction in more detail. Investigation into the effect of uptake of EGFP-expressing *L. mexicana* parasites by different DC cultures *in vitro* demonstrated that, in the absence of exogenous factors, uptake of *L. mexicana* amastigotes did not cause activation of DC. Uptake of *L. mexicana* promastigotes resulted in activation of a small proportion of DC indicating that promastigotes do encode an activation signal, but that this is not sufficient to activate the entire DC population. However, neither *L. major* promastigotes nor *L. mexicana* promastigote mutants lacking surface lipophosphoglycan (LPG) activated DC *in vitro*. Therefore these data suggest that *L. mexicana* promastigotes encode an activation signal, but that this is not sufficient to stimulate all DC. As the promastigotes which did not activate DC either lacked, or expressed a modified version of, LPG, we propose that LPG is a *L. mexicana* pathogen-associated molecular pattern (PAMP).

The work presented in this thesis demonstrates that infected DC are capable of initiating the anti-*Leishmania* response *in vivo,* as they efficiently present antigen to T cells. However, infection *per se* is not sufficient to activate all DC. These data therefore suggest that during the initiation of an anti-*Leishmania* T cell response DC are likely to be activated by factors produced in response to injection of parasites by the insect vector, such as pro-inflammatory cytokines.

Contents

Chapter 1: Introduction

Vertebrates possess a highly evolved immune system that is rapidly activated upon encounter with invading organisms (see Abbas 1997; Parham 2000). The immune response is divided into two arms: the immediate, non-specific, innate response and the acquired, antigen-specific, adaptive response. The acquired immune response is characterised by the generation of immunological memory, which ensures a more rapid, amplified immune response on the second and subsequent encounters with a particular pathogen. Both innate and adaptive responses must be tightly controlled to maximise responses against harmful pathogens, while minimising immune responses to self- or harmless non-self proteins. Dendritic cells (DC) have recently been identified as the antigen presenting cells (APC) that initiate adaptive immune responses, thus providing a bridge between the innate and adaptive arms of the immune response, and may also play a more instructive role in shaping the outcome of the adaptive response depending on the nature of the invading organism.

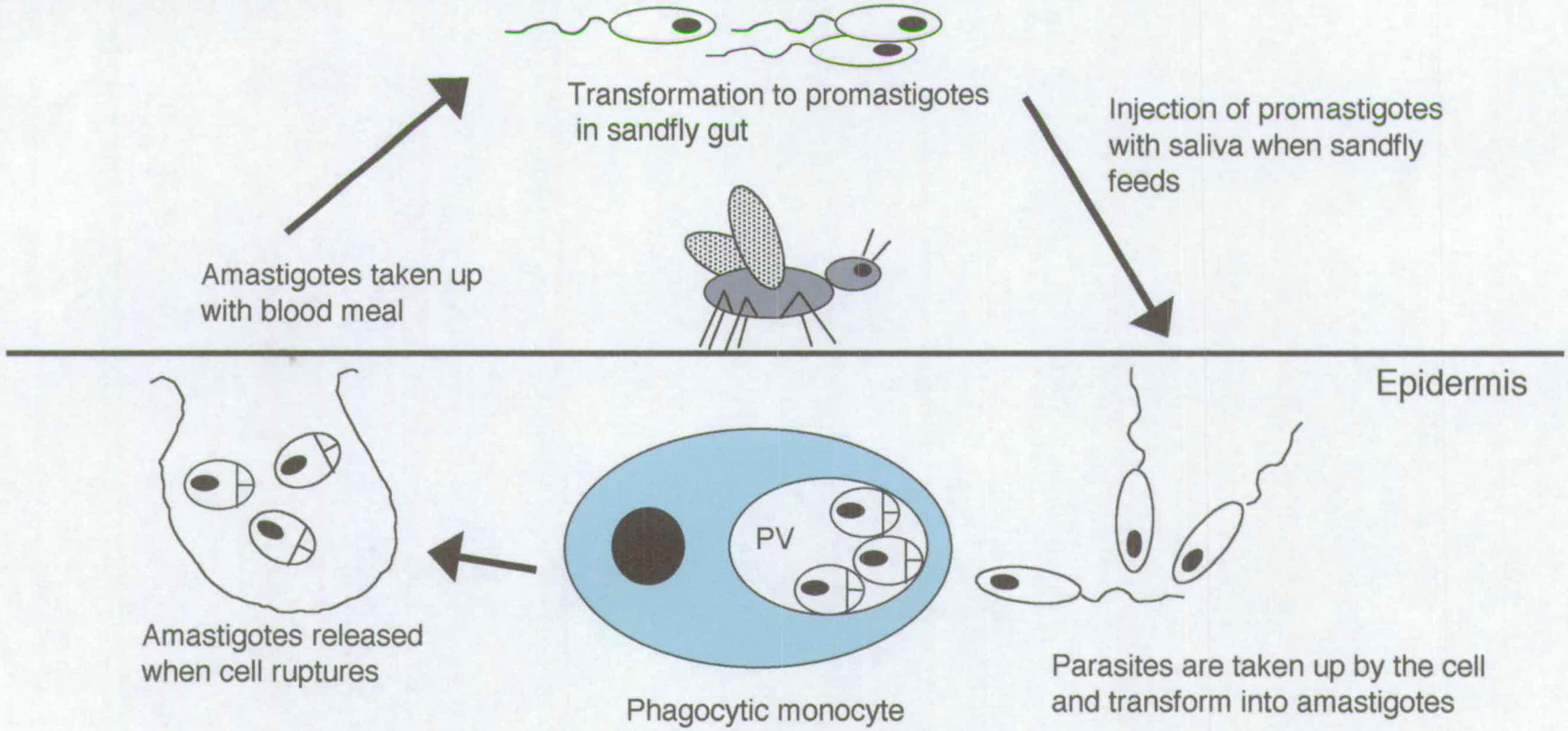
The immunobiology of *Leishmania sp.* has been extensively studied over the last 20 years, in particular due to the key role that T helper effector subsets play in the resolution of infection. Until recently, however less was known about the interaction of *Leishmania* with DC, and the role these cells play in initiating the anti-parasite immune response. This chapter reviews the literature on *Leishmania* infection, the role of DC in priming naïve T cells and the interaction between *Leishmania* and the immune system: section 1.1 describes *Leishmania* infection in mammals; section 1.2 introduces DC and discusses their role in initiating immune responses to pathogens; section 1.3 summarises the molecular mechanisms involved in presentation of *Leishmania*-derived antigens to T cells; section 1.4 describes T cell responses to *Leishmania* infection, and the role of infected macrophages (MΦ) and DC; finally, section 1.5 outlines the aims of this project and the experimental approach taken.

1.1 The Immunobiology of *Leishmania*

1.1.1 The *Leishmania* Life Cycle

Leishmania sp. are protozoan parasites. They have a digenetic life cycle, comprising a flagellated promastigote that resides in the sandfly vector and an aflagellated amastigote that multiplies in its mammalian host (for an outline of *Leishmania* biology see (Roberts 1996). A schematic representation of the *Leishmania* life cycle is depicted in Figure 1.1. All *Leishmania* species are transmitted by phlebotomine sandflies; promastigotes reside in the gut of the sandfly in the form of dividing procyclic promastigotes which are adapted for attachment to the gut epithelium and resistance to digestive enzymes. Metacyclic promastigotes then differentiate through two to three different stages as they migrate along the sandfly gut to the proboscis (Saraiva *et al.* 1995 and references therein), culminating in the infective metacyclic form which will be transmitted when the sandfly takes a blood meal. Metacyclic promastigotes are coated with a thick glycocalyx which protects them from immune-mediated lysis upon injection into the host. Once in the mammalian host promastigotes are taken up by phagocytic monocytes, principally MΦ, which have been recruited to the site of infection. *L. mexicana* promastigotes are initially contained in small individual phagosomes. These dilate to form large communal parasitophorous vacuoles (PV) (Courret *et al.* 2001), probably due to fusion of the phagosome with host endosomal compartments, and to vacuole: vacuole fusion (Antoine *et al.* 1998). Not all *Leishmania* species induce the formation of large communal PV, however; *L. major* and *L. donovani* parasites remain within individual vacuoles that do not fuse with other compartments in infected cells (Antoine *et al.* 1998). Once taken up by MΦ, metacyclic promastigotes transform into aflagellated amastigotes. This obligate intracellular form is well adapted to survival in the acidic phagolysosome. Amastigotes reside and multiply in the PV until they are released, probably upon rupture of the cell. They will subsequently be taken up by monocytes attracted to the site of infection, or by a sandfly as it feeds upon an infected host, thus perpetuating the cycle.

Figure 1.1: Schematic Representation of the *Leishmania* Life Cycle



Leishmania parasites are transmitted by sandflies when they feed on a mammalian host. *Leishmania* promastigotes are injected into the epidermis of the skin where they are taken up by phagocytic monocytes, principally MΦ. Within the monocyte promastigotes transform into amastigotes, the obligate intracellular form of the parasite. Amastigotes are contained within a phagolysosome known as the parasitophorous vacuole (PV). Parasites are released when the cell ruptures and are taken up by other monocytes at the site of infection, or by a sandfly feeding on an infected host, thus perpetuating the cycle.

A natural model of cutaneous *Leishmania* infection has recently been established which mimics transmission of the parasites by the sandfly (Belkaid *et al.* 1998; Belkaid *et al.* 2000). In this murine model, infection is imitated by injection of low numbers (one hundred) of *L. major* metacyclic promatigotes into the dermis of the ear. This reflects the low numbers of parasites transmitted by sandflies and the observation that exposed dermal areas in humans are most vulnerable to infection (Gaafar *et al.* 1999). Using this model, the course of *Leishmania* infection was divided into three phases (Belkaid *et al.* 2000): initially the parasites reside silently within cells of the dermis, in the absence of an anti-parasite immune response. This phase lasts four to five weeks, and is asymptomatic. After week five the second phase is initiated, characterised by a wave of cells invading the dermis. This cellular infiltrate comprises neutrophils, MΦ and eosinophils. At this point lesions develop, and there is a dramatic reduction (95%) in parasite numbers. Naïve and memory T cells now migrate into the site of the infection, and, in humans, were shown to make up fifty percent of the infiltrate (ElHassan *et al.* 1995). The final phase of the infection is characterised by the persistance of one hundred to ten thousand parasites at the site of the lesion. At this point the majority of infiltrating cells in the ear are MΦ. Virulent parasites were shown, in a different model of infection, to persist indefinitely in the host and disease can recur if the host becomes immuno-compromised (Aebischer *et al.* 1993).

In human cutaneous *Leishmania* infection, parasites have been identified in DC and MΦ at the site of the lesion (ElHassan *et al.* 1995; Gaafar *et al.* 1995). Parasites in various stages of degradation were observed within activated MΦ by microscopy (Gaafar *et al.* 1995). The most efficient mechanism for destroying parasites, however, is thought to be via necrosis of infected MΦ, which results in the development of the ulcerative lesions that are characteristic of the disease (Gaafar *et al.* 1995).

1.1.2 Leishmaniasis – The Disease

Leishmania sp. cause a wide range of diseases in humans, known collectively as Leishmaniases. World Health Organisation statistics estimate that up to 12 million

people are affected in 88 countries, mainly in the developing world. 1.5 to 2 million new cases are reported per year and there is growing concern as increases are seen in both the number of cases and the spread to previously non-endemic countries. The emergence of Leishmaniasis as a co-infection with HIV has lead to worry about the appearance of the disease in Southern Europe and the Southern states of America.

The severity of Leishmaniasis depends on the tissue tropism of the species involved; *L. major* and *L. mexicana* are restricted to the skin and cause mainly self-healing cutaneous lesions, while *L. braziliensis* causes a disfiguring mucocutaneous disease that can lead to destruction of nasopharyngeal tissue. *L. donovani* infects macrophages in the liver, spleen and bone marrow, resulting in visceral infections which are fatal if left untreated (Herwaldt 1999).

1.1.3 Therapeutic Agents

Treatment is available for Leishmaniasis, however the drugs are expensive and have toxic side-effects. Pentavalent antimony compounds are effective. However, they are difficult to administer as they require long regimes of therapy, in addition, their mode of action remains unclear. More recent approaches using interferon gamma (IFNγ) and topical application of paromomycin have only modest cure rates (Herwaldt 1999), and the isolation of parasites resistant to pentavalent antimonials from patients who are unresponsive to therapy suggests that the effectiveness of anti-*Leishmania* chemotherapy may be limited (Grogl *et al.* 1992; Ouellette 1993). In Leishmaniasis-endemic areas "Leishmanisation" is practised: in this custom, individuals deliberately infect themselves with parasites from an open lesion on another infected person, choosing an infection site where scarring will not be visible. They then develop an infected lesion which is generally resolved and subsequently develop solid immunity against further infection. A vaccine against Leishmaniasis should therefore be attainable and is highly desirable (Handman 1997).

1.2 Dendritic Cells and their Response to Infection

1.2.1 Introduction

DC were first identified as a novel population of spleen cells characterised by their adherence to glass upon culture (Steinman and Cohn 1973), and were subsequently shown to be potent stimulators of B and T cells responses *in vitro* and *in vivo*. Inaba and colleagues (Inaba *et al.* 1983) initially demonstrated that DC could activate B cell responses to sheep erythrocytes *in vitro* and that purified DC were extremely efficient at stimulating this response: purified DC numbers as low as 0.1-3% of the culture could stimulate antibody production by B cells to the same extent as unfractionated splenocytes. Purified DC were approximately one hundred times more potent than unseparated splenocytes in stimulating Host versus Graft reactions *in vivo* (Knight *et al.* 1983), and direct injection of antigen-pulsed DC into naïve recipients primed antigen-specific T cells responses (Inaba *et al.* 1990).

As DC represent less than one percent of splenocytes (Schuler 1999), protocols to enrich DC populations *in vitro* and *ex vivo* were crucial for the progression of the field. Culture of DC/Langerhans cells (LC) had been shown to be dependent on the presence of Granulocyte/MΦ Colony Stimulating Factor (GM-CSF) (Witmer-Pack *et al.* 1987). Using this cytokine, Inaba and colleagues (Inaba *et al.* 1992) demonstrated that DC could be generated *in vitro* from cultures of mouse blood leukocytes. These blood-derived DC expressed high levels of Major Histocompatibility (MHC) II on their surface, potently stimulated T cells in the Mixed Leukocyte Reaction (MLR) and migrated to the T cell areas of the draining lymph node (LN) upon injection into mouse footpads, indicating that they were *bona fide* DC. It was subsequently demonstrated that the culture of mouse bone marrow precursors with GM-CSF led to the development of bone marrow-derived DC (Inaba *et al.* 1992). These cells, termed BM-DC, also expressed high levels of MHC II and efficiently stimulated T cells in the MLR. DC are now also routinely generated from human blood-derived monocytes (Sallusto and Lanzavecchia 1994). *In vivo* elicitation and harvesting protocols arose in mice from the

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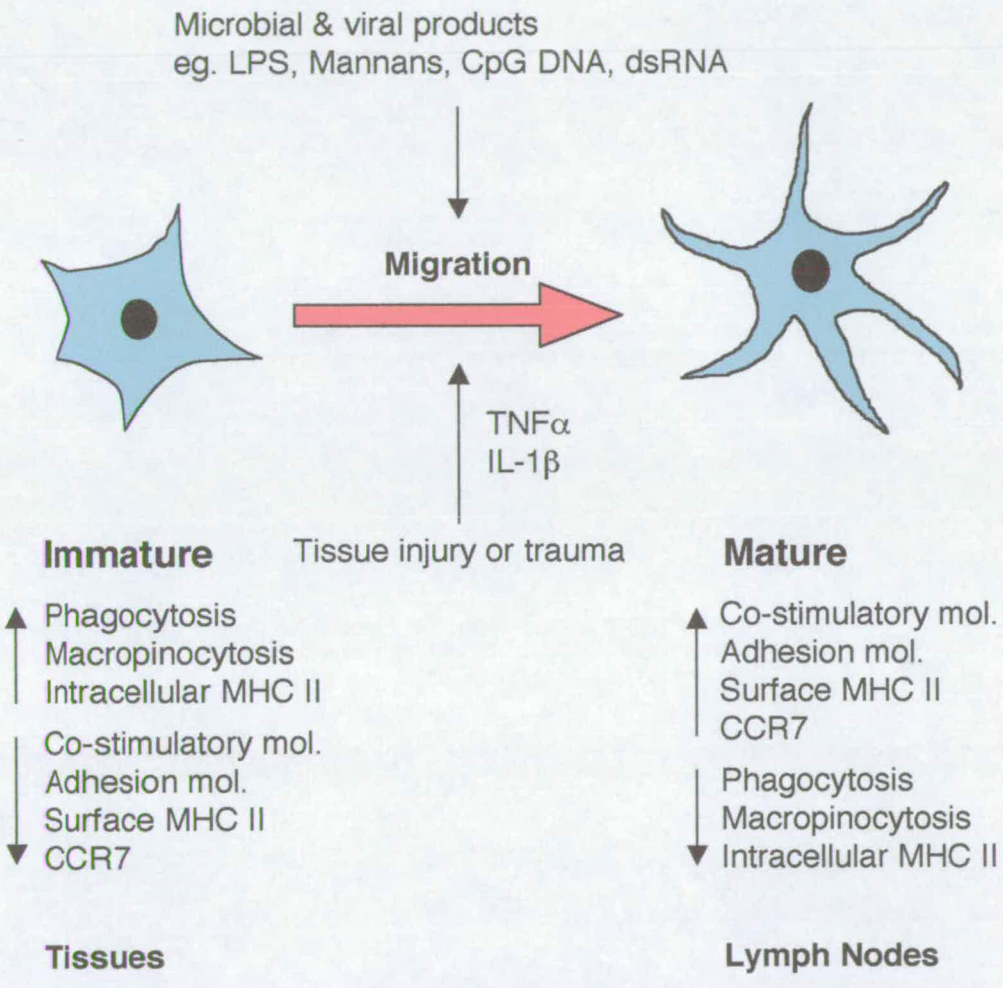
discovery that injection of Flt-3 ligand enhanced DC numbers in the spleen. Flt 3 is a haematopoetic growth factor (Rosnet *et al.* 1991) and administration of Flt3-ligand to mice resulted in an up-regulation of cells in the spleen that expressed markers characteristic of DC. These cells, when purified, could stimulate antigen-specific T cell responses *in vitro* and *in vivo* (Maraskovsky *et al.* 1996). Unlike other protocols, enhancement of DC numbers *in vivo* with flt3-ligand does not require GM-CSF. Thus, all DC subsets are expanded in these mice, whereas GM-CSF-based protocols favour the expansion of myeloid DC (see section1.2.2).

1.2.2 Functional Maturation and Migration of DC – Initiation of a Primary Immune Response

Activation of naïve T cells requires two signals: signal one is provided by the interaction between the T cell receptor (TCR) and peptides presented on the surface of APC in the context of MHC molecules; signal two is provided by ligation of co-stimulatory molecules on the surface of the APC with their receptors on T cells. Receipt of signal 1 by a T cell in the absence of signal 2 results in anergy, or unresponsiveness (Bretscher & Cohn 1970). DC are the only APC which constitutively express the co-stimulatory molecules needed to stimulate naïve T cells in the draining LN (Inaba *et al.* 1990), and therefore have been termed "professional APC" (Matzinger 1994; Mellman *et al.* 1998). In the tissues DC exist in an immature state, in which they are extremely efficient at sampling the local microenvironment and at retaining peptides intracellularly in an antigenic form. Receipt of specific activating signals results in the maturation of DC, which is associated with up-regulation of surface molecules necessary for the interaction with naïve T cells and concomitant migration of DC to the T cell areas of the draining LN (see Figure 1.2). Activated DC are characterized by high levels of cell surface expression of MHC I and II, the co-stimulatory molecules B7-1 (CD80), B7-2 (CD86) and CD40, the cell adhesion molecule ICAM-1 (CD54), and the chemokine receptor CCR7. Maturing DC are also induced to produce the pro-inflammatory cytokines

Figure 1.2: Activation of Immature DC Results in their Maturation and Migration to the Lymph Nodes



Abbreviations: LPS, lipopolysaccharide; dsRNA, double stranded RNA; TNF, tumour necrosis factor; IL, interleukin; CCR7, C-C chemokine receptor 7

interleukin (IL)-1β, IL-6, IL-12, and tumour necrosis factor (TNF)α (Banchereau *et al.* 2000).

1.2.2.1 The Sentinel Role of DC and their Activation in the Periphery

Immature DC have evolved a number of different mechanisms with which to capture antigens for delivery to processing and presentation pathways. The local micro-environment is constantly sampled due to high levels of constitutive macropinocytosis, which concentrates macrosolutes into putative peptide loading compartments (Sallusto *et al.* 1995), and uptake of particles via receptor-mediated endocytosis (Garrett *et al.* 2000). DC express the mannose receptor, which facilitates uptake of a broad range of sugars, including mannose and fucose. This receptor releases its ligand at low pH, for example in endosomes, and recycles to the cell surface, therefore enabling a limited number of receptors to accumulate high concentrations of antigen (Sallusto *et al.* 1995). DC also express several lectin receptors including DEC205 (Jiang *et al.* 1995) and DCIR (DC immuno-receptor) (Bates *et al.* 1999). DEC 205 was shown to deliver glycoprotein antigens to MHC II-rich compartments of DC (Jiang *et al.* 1995; Mahnke *et al.* 2000).

Immature DC are actively phagocytic and express high levels of Fc receptors (FcγRI, II and III) that mediate uptake of immune complexes and opsonised particles. Internalisation of antigen via Fc receptors significantly increased the efficiency of antigen presentation by DC; antigen-specific T cells were a hundred times more sensitive to DC that had been incubated with an antigen and antibody to that antigen than to DC given antigen alone (Sallusto and Lanzavecchia 1994). FcR-mediated up-take has also been shown to target antigen to the MHC II presentation pathway (Fanger *et al.* 1997; Maurer *et al.* 1998).

DC are activated by a number of pathogen-derived products (see section 1.2.4) and by cytokines produced in the tissue as a result of inflammation or tissue damage. In particular TNFα, IL-1β and IL-6 are potent activators of immature DC (Banchereau *et al.* 2000). IL-12 production by activated DC has been reported to act in an autocrine loop to stimulate increased production of IL-12 (Grohmann *et al.* 1998) and IFNγ

(Ohteki *et al.* 1999). IFNγ production by DC early during the immune response could ensure the rapid activation of other cells involved in the innate response, such as Natural Killer (NK) cells and MΦ. IL-4, which is generally associated with down-regulation of T helper type 1 (Th1) responses, has also been shown to induce production of the Th1 polarising cytokine, IL-12, in both murine (Fukao *et al.* 2000; Hochrein *et al.* 2000) and human DC (Kalinski *et al.* 2000). In both of these studies IL-4 was demonstrated to enhance production of the bioactive IL-12 p70 heterodimer, but inhibited formation of antagonistic p40 homodimers (see section 1.4.1.3.2).

Activating signals result in the down-regulation of antigen capture and processing and the concomitant migration of DC to the draining LN (Reis e Sousa *et al.* 1993; Sallusto and Lanzavecchia 1994). Antigens which have been captured in the periphery by DC in their immature form can be retained intracellularly for at least sixty hours, during which time the cells migrate to the T cell areas of the lymphoid organs (Inaba *et al.* 2000). In this way DC survey the tissues and provide T cells with a 'snap-shot' of the antigens present in the local microenvironment at the point when the DC were activated.

1.2.2.2 Migration of DC to the Draining LN

DC aquire antigen in the periphery but must migrate to the draining LN and spleen in order to interact with, and prime, naïve T cells bearing an appropriate cognate receptor. This spatial and temporal separation of the sentinel and antigen presenting cell functions of DC was demonstrated in studies of contact hypersensitivity responses, where LC were shown to take up large amounts of the sensitiser antigen and then migrate to the draining LN where they stimulated specific T cell responses (Macatonia *et al.* 1987). The molecular mechanisms that regulate the retention of immature DC in the tissues and their migration to the T cell areas of the LN upon activation are now understood in some detail, and are mediated by the expression of chemokines and chemokine receptors. Chemokines are small chemoattractive proteins that play a pivotal role in controlling tissue specific localisation of cells of the haematopoetic system, including DC. They are generally classified according to the number and spacing of cysteine residues at their

amino-terminus (Cyster 1999). Immature DC express receptors for inflammatory chemokines. These receptors include the CC chemokine receptors (CCR) 1, 2, 5 and 6, which bind to pro-inflammatory chemokines including MΦ inflammatory protein (MIP)-1α, MIP-1β and RANTES (regulated on activation, normal T cell expressed and secreted) (Dieu *et al.* 1998; Sozzani *et al.* 1998). These receptors are down-regulated upon receipt of an activating stimulus, thus abrogating responses to inflammatory chemokines and enabling DC to exit from the inflamed tissue. Maturing DC up-regulate expression of CCR7. This binds to MIP-3β and SLC (secondary lymphoid tissue chemokine), which are produced by endothelial cells and mature DC in the LN (Dieu *et al.* 1998; Sallusto and Lanzavecchia 2000). These chemokines are therefore thought to attract activated DC to the T cell areas of the draining LN, where naïve T cells, which also express CCR7, are localised (Sallusto and Lanzavecchia 2000). In support of this hypothesis, neither CCR7 nor SLC deficient mice were able to generate primary T cell responses, due to the inefficient migration of DC and naïve T cells into the T cell areas of the secondary lymphoid organs (Forster *et al.* 1999; Gunn *et al.* 1999). Activation and migration of LC is also associated with down-regulation of E-cadherin, allowing movement of cells from the epidermis and dermis of the skin into the lymphatics (Tang *et al.* 1993).

1.2.3.3 Final Maturation of DC in the Lymph Nodes

T cell-derived signals are required by activated DC to complete their maturation *in vivo*. Shreedhar *et al* (Shreedhar *et al.* 1999) reported that T cell-deficient mice ($RAG2^{-/-}$ or SCID) had significantly lower numbers of DC in the draining LN than littermate controls, and showed impaired contact hypersensitivity responses which could be rescued by transfer of T cells from wild type mice, indicating that T cells are required for the presence of mature DC in the LN. Several studies suggest that the interaction between T cells and DC is likely to be mediated by binding of CD40 on DC to its ligand on T cells: ligation of CD40 on DC resulted in up-regulation of surface activation markers and production of high levels of IL-12p70 (Cella *et al.* 1996; Koch *et al.* 1996); and the effects of microbial stimuli (Schulz *et al.* 2000) and IL-4 (Hochrein *et al.* 2000) on IL-12p70 production by DC were augmented in the presence of CD40 ligation. This

idea is attractive, since ligation of CD40 causes up-regulation of OX40 ligand on DC, which co-stimulates cytokine production by T cells (Chen *et al.* 1999). Indeed, it was thought that the OX40L:OX40 interaction may polarise T helper type 2 (Th2) responses (Flynn *et al.* 1998), although $OX40L^{-/-}$ mice were subsequently shown to be defective in DC-induced production of both IL-4 and IFNγ by T cells (Chen *et al.* 1999), and ligation of OX40 on activated T cells is now thought to promote survival of all $CD4^+$ T cells (Rogers *et al.* 2001). Activated T cells also express TRANCE (TNF-related activation-induced cytokine) that is recognised by a receptor expressed by DC (Wong *et al.* 1997). Incubation of DC with TRANCE resulted in the production of IL-1β and IL-12p40 but not Th2-associated cytokines such as IL-4, IL-5 or IL-10 (Josien *et al.* 1999). TRANCE-stimulated DC showed an enhanced capacity to stimulate T cells in the MLR and were also resistant to apoptosis due to up-regulation of Bcl-x_L (Wong *et al.* 1997). This indicates that the interaction between TRANCE on T cells and its receptor on DC may promote the survival of mature DC in the LN, and direct their function.

1.2.3 Heterogeneity of Dendritic Cells

1.2.3.1 Murine DC Subsets

Murine DC have been subdivided into increasingly defined subsets based on levels of different surface markers and their location in the body. At present, however, the extent to which these different subsets represent distinct DC populations with independent functions, or merely represent different maturation stages within a single population remains unclear. The overlap in markers for these subsets makes it very difficult to define a role of individual DC subsets *in vivo* and to assess the degree of redundancy between the functions of these different groups.

Early studies indicated that murine DC could be sub-divided into myeloid and lymphoid DC, which are phenotypically defined by expression of CD11c and MHC II, are $CD11b^+$ or $CD11b^-$ respectively, and were thought to arise from distinct precursors (reviewed by

Banchereau *et al.* 2000). Data from knock-out mice supports the existence of separate DC lineages: $RelB^{-/-}$ mice lack most myeloid cells, including DC, except LC, (Burkly *et al.* 1995). Wu and colleagues (1998), however, noticed a residual population of $CD8\alpha^+$ DC in the spleen of these mice, suggesting that these cells have a non-myeloid lineage. Mice which express the mutated dominant negative Ikaros protein are blocked in the generation of all cells of the lymphoid lineage (Georgopoulos *et al.* 1994; Wang *et al.* 1996). Most of the DC are missing from these mice indicating that the majority of DC *in vivo* are of the lymphoid lineage or that this mutation also effects the development of some myeloid cells. In mice expressing the more specific C-terminal null Ikaros mutation, B and NK cells are ablated but some T cell development leaks through. Importantly, this is accompanied by the appearance of $CD8^+$ DC in the thymus (Wu *et al.* 1997), indicating that T cell and DC development are related.

Epidermal LC may represent a third DC lineage: they have generally been considered the archetypal myeloid DC, but are present in $RelB^{-/-}$ mice. Furthermore, LC have been shown to acquire a lymphoid ($CD8\alpha^+$) phenotype upon migration to the draining LN (Anjuere *et al.* 1999).

The α-chain of the CD8 T cell co-receptor is present as a homodimer on a distinct subset of DC, and has provided a convenient marker for the identification of different DC populations. Identification of $CD8\alpha^+$ DC populations in the thymus led to the assumption that this was a marker for the lymphoid DC lineage since transfer of intrathymic $CD4^{lo}$ lymphoid precursors into irradiated mice led to the development of a thymic DC population of which sixty percent were $CD8\alpha^+$ (Ardavin *et al.* 1993). However, definitive proof of this relationship awaits clonal analysis of thymic precursors.

$CD8\alpha^+$ DC were originally believed to be less efficient than $CD8\alpha^-$ DC at stimulating T cell responses *in vitro* (Kronin *et al.* 1996; Suss and Shortman 1996) and express high surface levels of Fas ligand which might induce apoptosis of Fas-expressing $CD4^+$ T cells (Suss and Shortman 1996; Inaba *et al.* 1997). This led to the proposal that $CD8\alpha^+$

DC might mediate tolerogenic responses, and CD8α$^-$ DC initiate immunity to foreign antigens. However, DC derived from bone marrow precusors cultured with flt-3 ligand were recently shown to up-regulate CD8α upon activation with LPS (Brasel *et al.* 2000) indicating that myeloid DC can be induced to express CD8α, and the thymus is no longer thought to be the only origin for CD8α$^+$ DC (Brasel *et al.* 2000; Liu *et al.* 2001). Furthermore, a recent study has demonstrated that the transfer of bone marrow-derived common myeloid progenitors into irradiated mice led to the development of CD8α$^+$ DC in both the spleen and thymus of recipient mice (Traver *et al.* 2000). The significance of CD8α as a marker of different DC lineages, and whether it plays a role in the function of CD8α$^+$ DC, thus remains unclear. It is becoming obvious, however, that the convenient division of DC into 'myeloid' and 'lymphoid' lineages was too simplistic.

Murine splenic DC have now been divided into three populations: CD8α$^+$CD4$^-$, CD4$^+$CD8α$^-$ and double negative (CD8α$^-$CD4$^-$) (Kamath *et al.* 2000). The CD8α$^+$ cells are DEC205$^+$ and negative for the myeloid marker CD11b. Conversely, both CD8α$^-$ populations are DEC205$^-$ and CD11b$^+$. Experiments in which these populations were matured *in vivo* by injection of LPS have indicated that they do not represent activation stages of the same population of DC. BrdU labelling studies have also indicated that the three DC subsets represent three separate developmental streams, since the turnover of all populations was equivalent, and one did not lag behind another (Kamath *et al.* 2000).

DC populations in the LN may be more complex that in the spleen, probably reflecting the presence of epidermal Langerhans cells (LC) and dermal DC populations (Anjuere *et al.* 1999; Ruedl *et al.* 2000; Henri *et al.* 2001).

1.2.3.2 Human DC subsets

At present human DC can be divided into two subsets: myeloid (DC1) or plasmacytoid (pDC2) DC (Rissoan *et al.* 1999). DC1 express myeloid antigens such as CD11b, CD11c and CD14, whereas pDC2 are CD11c$^-$, CD4$^+$ and express other markers also found on lymphocytes (Liu *et al.* 2001). Plasmacytoid DC require IL-3 rather than GM-

CSF for survival, expressing high levels of the IL-3 receptor α chain and low levels of the GM-CSF receptor α chain, and are characterised by rapid production of type 1 interferons (IFNα, IFNβ) in an innate response to viral infection (Liu *et al.* 2001).

1.2.4 DC as a Link Between the Innate and Adaptive Immune Responses to Pathogens

The innate immune response is an evolutionarily conserved, rapid immune response to pathogens that is characterised by expression of germline-encoded receptors on effector cells. Vertebrates have also evolved a second arm, the adaptive response, in which receptors on effector cells are generated by gene rearrangement during development, the outcome of which is the ability to generate a memory response. Induction of the innate response must only be initiated in the presence of foreign or damaged cells, and therefore, has evolved to respond to specific pathogen-derived signals via conserved receptors.

1.2.4.1 The Danger Hypothesis

The immune system has been traditionally viewed as distinguishing between self and non-self molecules. Central tolerance to self antigens has long been known to be achieved via deletion of self-reactive T cells in the thymus, and is based on recognition of signal 1, the interaction between the TCR and MHC-peptide complex. Peripheral tolerance to antigens which would not be present in the thymus was partly explained by the two signal hypothesis, since presentation of antigens in the absence of signal 2 results in T cell anergy (Bretscher and Cohn 1970). T cell responses to peripheral antigens are also limited by the restriction of naïve T cells to the blood, lymph and secondary lymphoid organs. Using APC which could either provide the TCR cross-linking signal 1 or co-stimulatory signal 2, but not both, Lui and Janeway (Liu and Janeway 1992) showed that expansion of $CD4^+$ T cells is more efficient when both

signals are present on the same cell. This supported the earlier work by Bretscher and Cohn (Bretscher and Cohn 1970), demonstrating that cells that had taken up antigen could not activate a T cell response unless they also expressed co-stimulatory molecules on their surface. The inducibilty of the co-stimulatory signal and the nature of the signals that APC responded to could therefore provide a very specific mechanism for controlling activation of naïve T cells, and raised the question of how the induction of responses was controlled. Janeway (Liu and Janeway 1992) proposed that pathogen-derived molecules, but not harmless exogenous proteins, could provide an activating signal which would enable APC to distinguish between infectious and non-infectious non-self. This information would be relayed to the T cell via upregulation of co-stimulatory molecules (signal 2) only in the presence of an infection. This hypothesis was later modified by Ibrahim and colleagues (Ibrahim *et al.* 1995), who argued that the innate immune system must also respond to local microenvironmental damage, in order to explain immune responses to allergens or alloantigens when no pathogen was present.

These ideas became cemented into the "danger theory", proposed by Matzinger in 1994, which stated that the primary force driving development of the immune system was recognition of destruction and cell death (Matzinger 1994). Matzinger suggested that the ability of professional APC, i.e. DC, to respond to specific activation signals by up-regulation of expression of surface co-stimulatory molecules meant that the initiation of a primary T cell response would occur only in the presence of "danger". Danger, in this view, was defined as "tissue destruction", which included active signs of stress such as production of heat shock proteins (hsp), or passive signs such as the release of internal molecules that are not normally secreted. This presented, for the first time, the idea that DC could sense their environment and respond to infection or tissue damage by activating a T cell response. The danger hypothesis reinforces the significance of the presence or absence of signal 2 in establishing tolerance to peripheral antigens, and expands on this by describing up-regulation of co-stimulatory molecules as a response by DC to environmental stimuli. However, until recently the precise nature of the DC receptors which enabled DC to sense and respond to these stimuli was unknown.

1.2.4.2 PAMPs and PRRs

Janeway proposed that the innate immune system must encode receptors for conserved molecules which are components of pathogenic agents and are distinct from self molecules (Janeway 1989; Janeway 1992). These conserved molecules have been termed PAMP (Pathogen-Associated Molecular Patterns) and are recognised by PRR (Pattern Recognition Receptors) on immune cells (Medzhitov and Janeway 1997). Since the immune system will exert selective pressure against pathogens expressing molecules recognised by PRR, PAMP are likely to be molecules that are essential for the survival of the pathogen, e.g. virulence factors. They are also likely to be highly conserved, enabling limited numbers of germline-encoded PRR to recognise large groups of microbes.

In keeping with this prediction, all microbial PAMP identified to date are essential for the pathogen's survival, and include cell wall components of Gram negative (lipopolysaccharide (LPS)) and Gram positive (lipoteichoic acids (LTA), peptidoglycan (PDG), lipopeptides) bacteria; yeasts (Mannans); bacterial CpG DNA; and double stranded (ds)RNA of viruses (Medzhitov and Janeway 1997). PRR thus far identified include molecules such as CD14 which bind PAMP in association with other accessory proteins such as LBP (LPS-binding protein), receptors for lectin-type molecules such as the mannose receptor, DEC 205, and complement receptors involved in uptake of opsonised antigens (Medzhitov and Janeway 1997). The recent identification of the Toll-like receptor (TLR) family as receptors for specific PAMP has provided a molecular link between recognition of specific pathogen-derived signals and the activation of an immune response.

1.2.4.2.1 Toll-Like Receptors

The human TLR family was identified by their homology to the *Drosophila* receptor, Toll (Medzhitov *et al.* 1997). Toll was originally identified as a protein which is essential for establishing the dorso-ventral axis in early *Drosophila* embryos (St Johnston and Nusslein-Volhard 1992). Toll family members are characterised by an extracellular domain containing a number of Leucine-rich repeats and an intracellular

cytoplasmic signalling domain (reviewed by Medzhitov and Janeway 2000). There is little conservation between the extracellular domains of different Toll homologues, indicating that they may bind diverse ligands. In contrast, the intracellular domains are highly conserved and are homologous to the murine IL-1 receptor signalling domain. Conservation between Toll-like proteins involved in plant and mammalian immune responses, and the presence of NF-κB response elements in genes transcribed upon infection in insects lead to the discovery of a role for Toll and other related genes in *Drosophila* innate immune responses. Flies possess a primitive immune system, and respond to pathogens by the rapid and transient synthesis of anti-microbial peptides following injury. Toll gain-of-function mutants constitutively expressed the anti-fungal peptide drosomycin in the absence of challenge, while homozygous Toll null mutants rapidly succombed to fungal infection (Lemaitre *et al.* 1996). These flies, however, remained able to control infection with the bacteria *E. coli* via the production of specific anti-bacterial peptides. 18 Wheeler was subsequently identified as the receptor necessary for transcription of anti-bacterial peptides (Williams *et al.* 1997), illustrating that the *Drosophila* innate immune responses can recognise, and respond appropriately to, different classes of pathogens.

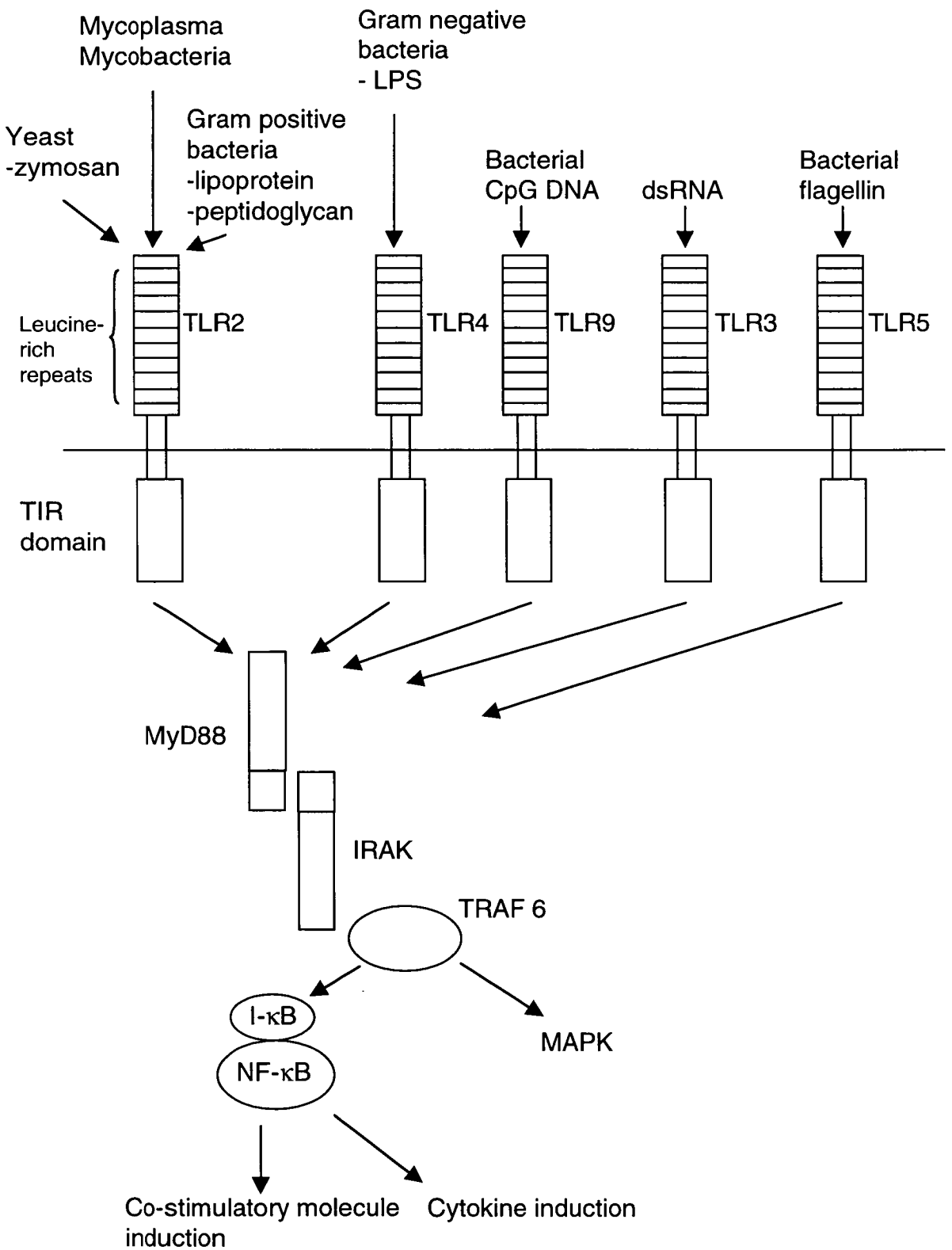
Human orthologues of Toll were first cloned in 1997 (Medzhitov *et al.* 1997) and were shown to contain conserved structures seen in the *Drosophila* proteins. mRNA for human Toll was found in monocytes, MΦ and DC. Constitutively active Toll mutants were found to induce activation of NF-κB regulated genes such as IL-1, IL-6 and IL-8 in transfected Jurkat cells, and also induced up-regulation of B7-1. This lead to suggestion that TLR could function as non-clonal PRR in the vertebrate immune system. The human Toll signalling pathway has been described, based on conservation with the *Drosophila* pathway (reviewed by Medzhitov and Janeway 2000). During Toll signalling an adaptor protein MyD88 (Myeloid Differentiation factor 88) is recruited and interacts with a domain at the C-terminus of the TLR, known as the TIR (Toll/IL-1R) domain. MyD88 in turn binds IRAK (IL-1 Receptor Activated Kinase) via its death domain. IRAK is activated by an unknown mechanism, becomes autophosphorylated, dissociates from MyD88, and then binds to another adaptor protein, TRAF 6 (TNF

Receptor Associated Factor), which interacts with MAP Kinase family members. This signalling pathway results in dephosphorylation of IκB, release of NF-κB, and its translocation into the nucleus to activate genes containing NF-κB response elements. These include a number of genes encoding proteins involved in immune responses such as IL-12, and other pro-inflammatory cytokines (Abbas 1997)(Figure 1.3).

1.2.4.2.2 TLR 2 and TLR 4

The first documented interaction between a defined TLR and PAMP was the binding of the Gram negative cell wall component, LPS, to TLR2. TLR2 was shown to be expressed on human monocytes and MΦ and expression increased upon activation with LPS (Yang *et al.* 1998). Transfection of TLR2 into human embryonic kidney 293 cells conferred responsiveness to LPS, however this was only efficient in the presence of LPS-binding protein (LBP) and CD14 (Kirschning *et al.* 1998; Yang *et al.* 1998). Positional cloning of the *lps* gene, which renders C3H/HeJ mice unresponsive to LPS-induced toxic shock, however, identified the mutated gene as *tlr4* (Poltorak *et al.* 1998); the gene encoding TLR4 in C3H/HeJ mice was shown to contain a mutation resulting in a switch from proline to histidine in a conserved region of the TLR4 intracellular domain (Poltorak *et al.* 1998). TLR4 null mice are completely unresponsive to LPS and recapitulate the phenotype of the *lps* mice (Hoshino *et al.* 1999); $tlr4^{-/-}$ MΦ were not induced to produce TNFα or NO_2^- upon activation with LPS, and $tlr4^{-/-}$ mice showed neither LPS-induced proliferation of B cells, nor upregulation of surface MHC II, although $TLR4^{-/-}$ B cells could still be activated by IL-4. Therefore, these data indicate that TLR4 is the principal receptor responsible for recognition of LPS by immune cells *in vivo*. The conflict between the TLR2 and TLR4 data was resolved by Hirshfeld *et al* (Hirschfeld *et al.* 2000) who showed that careful purification of LPS to remove contaminating endotoxins abrogated TLR2-mediated responses to LPS. Therefore, the original results reporting TLR2 responses to LPS are likely to be due to sensitivity of TLR2 to the presence of other endotoxin contaminants in LPS samples used.

Figure 1.3: Schematic Representation of Human Toll-like Receptors and their Ligands



Adapted from Kaisho and Akira (2001)

MyD88 knock-out mice showed a similar phenotype to the TLR4 mutants, and were resistant to LPS-induced endotoxic shock (Kawai *et al.* 1999). However, unlike *tlr4*$^{-/-}$ or C3H/HeJ mice, *myd88*$^{-/-}$ bone marrow-derived DC are activated by LPS to up-regulate surface markers such as MHC II and B7-2, and show enhanced T cell stimulatory capacity in comparison to immature DC. Furthermore, activation of MAPK and NF-κB remains intact in *myd88*$^{-/-}$ mice, although the kinetics are slowed (Kaisho *et al.* 2001). Interestingly, CpG DNA, (which binds to TLR9), is unable to induce activation of DC from *myd88*$^{-/-}$ mice (Kaisho *et al.* 2001). Therefore these data demonstrate the existence of two pathways: a MyD88-dependent pathway is activated through TLR2, 4 and 9; and a MyD88-independent pathway which is also activated by the interaction of LPS with TLR4. The adaptor proteins TIRAP ((TIR) domain-containing adaptor protein) and Tollip may provide a platform for MyD88-independent signaling from TLR4 (Fitzgerald *et al.* 2001; Granucci *et al.* 2001; Horng *et al.* 2001).

TLR2 has now been shown to transduce signals from a number of different classes of pathogens, including fungi and Gram positive bacteria. Expression of a dominant negative form of TLR2 by a MΦ cell line blocked production of TNFα in response to the yeast cell component, zymosan (Underhill *et al.* 1999), and TLR2 mutants also did not respond to *S. aureus* or Gram positive cell wall preparations (Takeuchi *et al.* 1999; Underhill *et al.* 1999). TLR2 was also subsequently shown to function as a receptor for Mycobacterial components (Takeuchi *et al.* 1999; Wang *et al.* 2000).

1.2.4.2.3 Other Members of the TLR Family

At present ten members are reported to belong to the Toll-like receptor family (Rock *et al.* 1998; Kaisho and Akira 2001) However ligands have only been identified for TLR 2, 3, 4, 5 and 9 (Figure 1.3).

TLR3 is unique among members of the TLR family because the proline which is mutated in LPS-unresponsive mice is replaced by an alanine (Kaisho and Akira 2001). This led to the suggestion that TLR3 was not a functional receptor. However,

Alexopoulou *et al* (Alexopoulou *et al.* 2001) recently reported that TLR3 was the receptor for viral dsRNA: 293T cells expressing TLR3 plus an NF-κB-dependent reporter responded specifically to the synthetic dsRNA analogue, poly(I:C), and this was confirmed by the generation of *tlr3*$^{-/-}$ mice, since MΦ from these mice produced neither pro-inflammatory cytokines nor anti-viral type I interferons (IFNα, IFNβ) in response to poly(I:C). Interestingly, while splenocytes from *myd88*$^{-/-}$ mice did not proliferate in response to poly(I:C), and DC from these mice were not induced to produce IL-12, incubation with poly(I:C) did induce up-regulation of MHC II and CD86 on the surface of *myd88*$^{-/-}$ DC. Therefore, as with TLR4, TLR3 signalling in response to poly(I:C) induces cytokine production via an MyD88-dependent pathway, but maturation of DC via an MyD88-independent pathway.

TLR5 was cloned by data base searching, due to the presence of its TIR domain, and is expressed by monocytes, immature DC and epithelial cells. Screening of putative pathogen-derived products using COS cells transfected with TLR5 linked to a luciferase reporter identified Gram positive and Gram negative bacterial culture supernatants as activators of TLR5-expressing cells. This activity was subsequently demonstrated to be due to bacterial flagellin (Hayashi *et al.* 2001), as activation of TLR5 was restricted to Gram positive and Gram negative flagellated bacteria and was inhibited on deletion of flagellin. Interestingly, on interstitial epithelial cells, TLR5 expression has been shown to be restricted to the basal membrane (Gewirtz *et al.* 2001), suggesting a mechanism to prevent flagellated commensal bacteria in the gut from activating an inflammatory response unless they cross the intestinal epithelia.

TLR9 was cloned and characterised as the mammalian receptor for CpG-containing DNA (Hemmi *et al.* 2000). Non-methylated CpG motifs are characteristic of bacterial DNA and have been shown to activate mammalian DC and drive Th1 responses (Jakob *et al.* 1998; Hartmann *et al.* 1999). This receptor is highly specific for CpG DNA, since *tlr9*$^{-/-}$ mice responded to LPS, zymosan, and peptidoglycan.

A lack of specific reagents has meant that questions such as whether binding of PAMP to TLR is direct or via interaction with other accessory proteins, and where TLR are localised in the cell, remain largely unanswered. Some TLR are, however, thought to be intracellular: HA-tagged TLR2 transfected into a MΦ cell line was recruited to phagosomes following ingestion of zymosan (Underhill *et al.* 1999); TLR9 is likely to be endosomal (Hemmi *et al.* 2000) since responsiveness to CpG was shown to require its internalisation (Krieg *et al.* 1995). Additionally, it has recently been suggested that TLR may form functional heterodimers thus broadening the specificity of the pathogenic components which are recognised: Ozinsky *et al* (2000) showed that the PAMP specificity of TLR 2 and 6 is partially overlapping and that both these receptors are recruited to phagosomes upon uptake of zymosan, or peptidoglycan derived from Gram positive bacteria. However, TLR2 and 6 also co-localised with phagosomes containing opsonised red blood cells, so it was not clear whether this recruitment was specific or merely reflected non-specific internalisation of the receptors on phagocytosis. Immunoprecipitation of epitope-tagged TLR2 co-precipitated TLR6 and deletion of the intracellular domain indicated that this interaction was mediated by the extracellular domains of the proteins. Chimeric proteins, in which the signalling domain of TLR molecules were fused to the extracellular domain of CD4, were used to investigate the function of TLR homo- and heterodimers. TLR4 homodimers were constitutively active, and induced NF-κB-dependent reporter gene expression in transfected 293 cells (Hoshino *et al.* 1999), suggesting that TLR4 forms functional homodimers *in vivo*. CD4-TLR1, 2 or 6 homodimers, however, did not induce constitutive cytokine expression when transfected into a MΦ cell line (Ozinsky *et al.* 2000), although CD4-TLR2/CD4-TLR6 or CD4-TLR1/CD4-TLR2 heterodimers activated production of TNFα. These data imply that TLR may form functional heterodimers which confer specificity to different pathogen-derived products, thus broadening their recognition spectrum. The inability of TLR2 homodimers to activate cytokine production indicates that TLR2-transfected cells which responded to signals from Gram positive bacteria must express additional unidentified TLR molecules (eg.Yang *et al.* 1998).

1.2.4.3.4 Expression of TLR by DC

In order to assess the potential physiological role of TLR in the activation of DC it is important to determine their expression patterns on immature and mature DC. TLR2 mRNA was most highly expressed on monocytes and was down-regulated as the cells were matured into DC (Thoma-Uszynski *et al.* 2000; Visintin *et al.* 2001). Production of IL-12 p40 by monocyte-derived DC, on activation by lipoprotein, was blocked on addition of an anti-TLR2 antibody supporting the role for TLR-dependent activation of DC as a link between innate and adaptive immune responses (Thoma-Uszynski *et al.* 2000). Lipopeptides induce classical activation of DC; CD80, CD86, CD54 and MHC II were upregulated, endocytosis was down-regulated and the DC showed an enhanced ability to stimulate T cells in an MLR (Hertz *et al.* 2001). All of these responses were inhibited on addition of an anti-TLR2 mAb (Hertz *et al.* 2001). TLR4 gene expression also decreased as monocytes mature into DC (Visintin *et al.* 2001), but was upregulated on DC activated by bacterial products and cytokines, in a mechanism which could be blocked by IL-10 (Muzio *et al.* 2000). However surface expression of TLR4 proteins was low on monocytes and dropped as the cells differentiated into immature and then mature DC (Visintin *et al.* 2001). Furthermore, LPS induced activation of the signalling molecule IRAK in immature not mature DC, supporting the idea that only immature DC are competent to respond to signalling through TLR. TLR3 was highly expressed on human DC, but levels decreased upon activation with LPS (Muzio *et al.* 2000).

Taken together DC expression and function data support the hypothesis that TLR function as PRR, enabling immature DC to act as a bridge between the innate and adaptive immune system. A recently published study on human DC demonstrated that distinct DC subsets expressed distinct TLR patterns; plasmacytoid (pDC2) were shown to specifically express TLR9 which correlated with responses to CpG but not other stimuli, whereas immature DC1 expressed TLR1, 2 and 3, and specifically responded to proteoglycan and poly(I:C) (Kadowaki *et al.* 2001). This raises the possibility that the pattern of TLR expression by different DC subsets may determine which DC populations are competent to respond to different pathogen-derived signals, which in turn could determine the outcome of the subsequent T cell response (see section 1.2.5).

Thoma-Uszynski *et al* (Thoma-Uszynski *et al.* 2000) reported that activation of TLR2 by lipoprotein results in preferential production of IL-12 rather than IL-10. Therefore, this indicates that activation of DC via TLR2 could result in the development of an IL-12-driven polarised Th1 response. However, it has not been shown whether signalling via other TLR leads to production of alternative cytokines.

1.2.5 Polarisation of the T cell Response by Activated DC

Recent studies have revealed that DC do not simply initiate the adaptive immune response in the presence of danger but also play a role in directing the shape of the effector T cell response (Moser and Murphy 2000). It is clear that resolution of infection by different pathogens requires different effector responses, e.g Th1-mediated cellular immune responses are generally required for the clearance of intracellular bacteria and viruses but Th2-mediated humoral responses are usually needed for protection against helminths (see section 1.4.1.2). Activation of inappropriate responses will result in an inability to control the infection and can lead to the development of immunopathologies that are harmful to the host. Two hypotheses have been put forward to explain the mechanisms by which DC polarise $CD4^+$ Th responses (Figure 1.4):

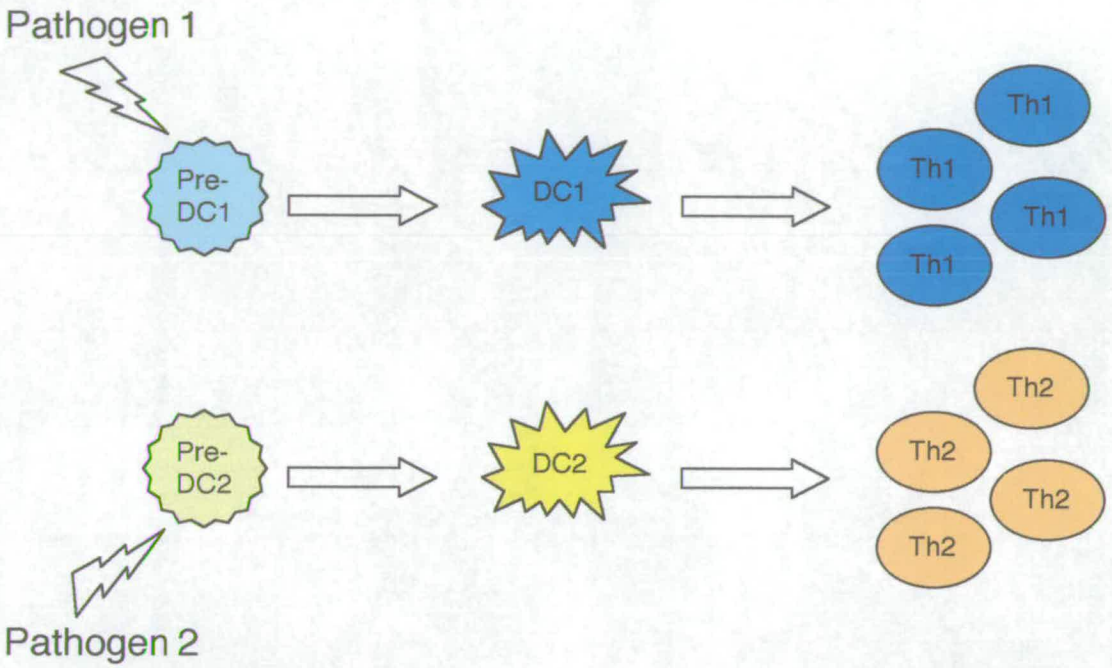
1) DC of different lineages elicit distinct T helper cell responses,
2) the ability of DC to regulate the T cell response is flexible and is shaped by signals from the pathogen and the microenvironment in which the cells are activated. This latter hypothesis requires the involvement of a "third signal" which instructs the DC as to the nature of the immune challenge (Kalinski *et al.* 1999).

1.2.5.1 Model One: Different DC Subsets are Polarised to Direct Different T Helper Cell Responses

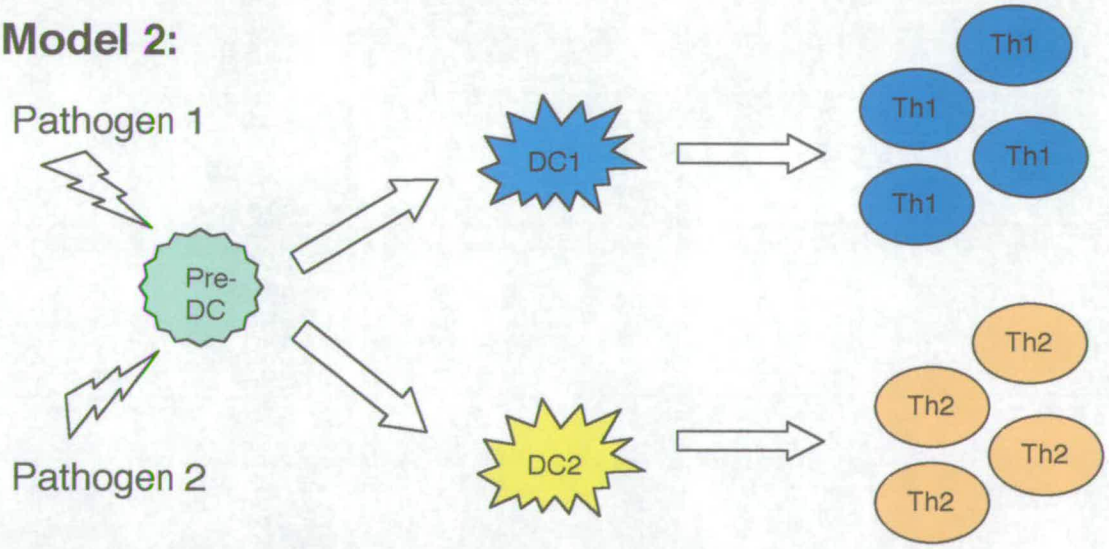
In this model, DC1 and DC2, which prime Th1 and Th2 responses respectively, constitute different DC subsets, which may be derived from separate DC lineages (Figure 1.4). Of the three murine splenic DC subsets,

Figure 1.4: Polarisation of the T Helper Cell Response by DC

Model 1:



Model 2:



Two different models to explain the polarisation of T helper cell development by DC. In model one DC1 and DC2 are derived from different DC lineages and are inherently different in their ability to respond to pathogen-derived signals. In model two, the precursor DC can develop into either a DC1 or DC2 effector cell depending on the nature of the pathogen, and cytokines present in the local microenvironment at the time of activation (Adapted from Kalinski *et al* 1999).

(Kamath *et al.* 2000, section 1.2.2.1) it is the $CD8\alpha^+$ population which has been repeatedly shown to produce IL-12 upon activation *in vitro* (Hochrein *et al.* 2001) and *in vivo* (Maldonado-Lopez *et al.* 1999; Schulz *et al.* 2000): splenic DC were incubated overnight with Keyhole Limpet Haemocyanin (KLH), then sorted into $CD8\alpha^+$ and $CD8\alpha^-$ populations and transferred into naïve hosts. LN cells from mice receiving either population of DC proliferated on restimulation with KLH *in vitro*, indicating that they had been primed by the injected DC. Analysis of cytokine release from these cells showed that injection with $CD8\alpha^+$ DC had primed an IL-2 and IFNγ-producing Th1 response while $CD8\alpha^-$ DC had primed a Th-2 like response (Maldonado-Lopez *et al.* 1999). In a separate set of experiments splenic DC were sorted into $CD11c^+/CD11b^{dull}$ "lymphoid" cells, which would contain the majority of the $CD8\alpha^+$ DC, and $CD11c^+/CD11b^{bright}$ "myeloid" DC, which would be predominantly $CD8\alpha^-$ (Pulendran *et al.* 1999). These subsets were incubated with OVA overnight and injected into BALB/c mice that had been reconstituted with OVA-specific T cells. The $CD11c^+/CD11b^{bright}$ DC primed IL-4 and IL-10 production in recipient mice, consistent with the $CD8\alpha^-$ DC transfer data of Maldonado-Lopez *et al* (Maldonado-Lopez *et al.* 1999). However, unlike the transfer of $CD8\alpha^+$ or $CD8\alpha^-$ subsets (Maldonado-Lopez *et al.* 1999), injection of both $CD11b^{dull}$ and $CD11b^{bright}$ subsets induced equal levels of IL-2 and IFNγ in these experiments. The reason for this discrepancy is unclear but may be due to fact that the DC populations were sorted using different criteria. Based on these data, it has been proposed that the response directed by activated DC will depend on the subset of DC which encounters antigen, whereby $CD8\alpha^+$ DC (DC1) direct Th1 responses and $CD8\alpha^-$ DC (DC2) direct Th2 responses. The converse is true in humans where myeloid "DC1" have been shown to prime Th1 primary allogeneic responses and plasmacytoid "DC2" prime Th2 responses (Rissoan *et al.* 1999). The work by (Maldonado-Lopez *et al.* 1999) strongly supports this, since in these experiments all the DC were incubated with antigen together. Thus, all the cells had been exposed to the same microenvironment and antigen, but primed different polarised responses *in vivo*. A recent publication describing the differential expression of different TLR on "myeloid" or plasmacytoid human DC supports the hypothesis that different lineages of DC could respond to different pathogens (Kadowaki *et al.* 2001).

1.2.5.2 Model two: Polarisation of DC is Driven by the Pathogen and the Microenvironment in which Challenge Occurs.

The lineage argument (model one) is limited by the fact that $CD8\alpha^+$ DC are localised to the T cell areas of the LN and spleen (de St Groth 1998) but all antigens will first encounter $CD8\alpha^-$ DC in the periphery before being taken to secondary lymphoid organs. It also seems inefficient since, in this scenario, activation of an appropriate response would depend on the correct DC meeting the correct pathogen. Kalinski and colleagues proposed that immature DC can adopt a Th1 or Th2-promoting function depending on pathogen-derived signals and the nature of the microenvironment in which the encounter occurs (Kalinski *et al.* 1999). This implies that DC1 and DC2 can develop from the same precusor (see Figure 1.4). Immature monocyte-derived DC stimulated with IFNγ *in vitro* produced IL-12. If subsequently matured in an IFNγ-containing environment, these cells become effector "DC1" which produced large amounts of IL-12 on CD40 ligation, irrespective of the culture conditions. Conversely, if the DC were stimulated and matured in the presence of prostaglandin (PG) E_2 they did not produce IL-12 even in the presence of IFNγ (Vieira *et al.* 2000). These data imply that the environment in which the DC are activated directs their maturation into DC1 or DC2, but that this becomes fixed and cannot be influenced by cytokines in the LN, away from the site of infection, in keeping with the 'snap-shot' idea of DC maturation.

Model 2 was supported by data obtained using pathogen-derived antigens, which directed the establishment of Th1 or Th2 responses. *Toxoplasma gondii* soluble antigen (STAg) primed a strong Th1 response *in vivo* and splenic DC from infected mice produced large amounts of IL-12 (Sousa *et al.* 1997). IL-12 production was principally localised to the $CD8\alpha^+$ subset, but $CD8\alpha^-$ DC could also be induced to secrete IL-12 when activated with STAg, or CpG DNA, in the presence of CD40 ligation (Schulz *et al.* 2000), or when incubated with heat killed *Brucella abortus* (Huang *et al.* 2001). Uptake of different forms of the fungus *Candida albicans* by DC can lead to distinct effector functions *in vitro* and *in vivo*: yeast stimulated IL-12 production and promoted a Th1 response, while hyphae directed Th2 priming via IL-4 production (d'Ostiani *et al.* 2000). However, these studies were performed using unsorted splenic DC, and it will be

interesting to define the respective roles of CD8α positive and negative populations in these responses.

It has been more difficult to demonstrate the ability of DC activated by pathogen-derived signals to direct a Th2 response, since IL-12 production seems to be the dominant response to infection. However, some parasite-derived products were clearly able to polarise DC to a "DC2" phenotype (Whelan *et al.* 2000; MacDonald *et al.* 2001). Bone marrow-derived DC cultured with OVA and the filarial nematode glycoprotein ES-62 induced production of IL-4 by OVA-specific T cells *in vitro*. Control DC given LPS not ES-62 induced the production of IFNγ but not IL-4 (Whelan *et al.* 2000). Likewise, DC pulsed with Schistosome soluble egg antigen (SEA) primed Th2 responses *in vivo*, as determined by production of IL-4 (MacDonald *et al.* 2001). Interestingly, levels of the DC surface markers MHC II, CD80, CD86 and CD40 were not up-regulated upon incubation with ES-62 or SEA, and these DC did not differentially up-regulate production of IL-10. Development of a Th2 response may therefore simply be a default pathway in the presence of antigen but relative lack of co-stimulatory molecules or it may result from an alternative form of activation involving up-regulation of as yet un-identified co-stimulatory molecules. Since low doses of antigen preferentially induce Th2 responses the polarisation of a Th2 response by SEA may also be a result of low levels of MHC II on the surface of these DC (Ruedl *et al.* 2000). The work of Fe d'Ostiani *et al* (d'Ostiani *et al.* 2000), however demonstrates that an antigen can stimulate IL-4 production by DC and this will drive a Th2 response *in vivo*.

Although $CD8\alpha^+$ DC have repeatedly been identified as the source of bioactive IL-12 (eg.Maldonado-Lopez *et al.* 1999; Schulz *et al.* 2000; Hochrein *et al.* 2001) it is clear that, under certain conditions, $CD8\alpha^-$ DC can also be polarised along Th1 pathways. Maldonado-Lopez and colleagues (2001) recently demonstrated that, while DC function appears to be set by its lineage, there is a certain amount of flexibility in the response, depending on the cytokines present in the local microenvironment. It is therefore likely that the polarisation of the Th response by DC will result from a combination of factors including lineage, the nature of the challenge and the microenvironment in which it

occurs, and models one and two should not be considered as exclusive. However, model two is more appealing than model one since it relies only on one precursor cell which can become either a Th1- or Th2-priming cell depending on the pathogen it encounters. A kinetic model of DC activation has also recently been proposed whereby progression from a DC1 to a DC2 phenotype depends on the length of time DC have been stimulated. Therefore, during activation DC will progress from a Th1-promoting DC which secretes IL-12 to a Th2-promoting DC which is "exhausted" and no longer secretes IL-12 (Langenkamp *et al.* 2000). This model would imply that priming of a Th2 response would depend on DC moving rapidly through the Th1-promoting phase to the exhausted Th2-promoting phase. This seems inefficient compared to the flexibility of model two.

1.3 Processing and Presentation of Exogenous Antigen by Antigen Presenting Cells

Antigens are presented to T cells by DC and other APC in the form of short peptides complexed to MHC Class I and II molecules. These complexes are recognised by cognate T cell receptors on $CD8^+$ and $CD4^+$ T cells respectively. Cytosolic proteins, or antigens from intracellular pathogens, are usually presented on the surface of cells in the context of MHC I. MHC class II molecules usually present antigens from extracellular proteins, bacteria and parasites. MHC I is expressed by all nucleated cells, whereas MHC II expression is restricted to B cells, MΦ and DC, and to thymic epithelial cells and activated keratinocytes (Abbas 1997). This discussion will be restricted to presentation of exogenous antigens by MHC II molecules, since the outcome of *Leishmania* infection is critically dependent on the presentation of *Leishmania* antigens to $CD4^+$ T cells in the context of MHC II.

1.3.1 Antigen Presentation by MHC II Molecules

Processing and presentation of exogenous antigens by APC requires newly synthesised MHC II molecules to meet antigens that have been brought into the cell via endocytosis. Thus, pathways leading to proteolytic degradation must integrate with those trafficking MHC II from the ER to the cell surface. To achieve this APC exploit pre-existing endosomal pathways.

1.3.1.1 Formation of MHC II : Peptide Complexes in APC

MHC class II molecules consist of an αβ heterodimer which forms in the endoplasmic reticulum (ER) and is stabilised by the binding of a protein known as Invariant Chain (Ii). Trimers of Ii associate with three class II heterodimers to form nonameric complexes. Ii is successively cleaved by cell proteases, resulting in release of a 24 amino acid peptide known as CLIP, (class II associated invariant chain peptide) which binds MHC II molecules via the antigen-binding groove, and which is replaced by exogenous peptides in peptide loading compartments. Ii plays a critical role in controlling MHC II function: the Ii-derived CLIP peptide binds the antigen-binding groove of the heterodimer, thus preventing binding of inappropriate self-peptides in the ER; sorting signals at the cytoplasmic tail of Ii mediate trafficking of the complex into appropriate cellular compartments; and an internalisation signal ensures that any unloaded MHC II dimers reaching the cell surface are rapidly endocytosed (Pieters 1997).

Mice express two different isoforms of Ii, Ii-p33 and Ii-p41. Both forms interact equally with newly folded αβ heterodimers (Fineschi *et al.* 1995), but the presence of Ii-p41 enhanced the processing of certain antigens (Peterson and Miller 1992), and inhibited the lysosomal protease Cathepsin L (Bevec *et al.* 1996) (see section 1.3.2.3).

H2-M, HLA-DM in humans, is a class II-like αβ heterodimer which catalyses the removal of CLIP from the peptide-binding groove and allows its replacement with antigenic peptides (Denzin and Cresswell 1995; Sloan *et al.* 1995). This activity has

been demonstrated by a number of studies which showed that in the absence of H2-M presentation of antigens to T cell hybridomas was inefficient, and MHC II molecules on the cell surface were exclusively loaded with CLIP (Martin *et al.* 1996; Miyazaki *et al.* 1996). Furthermore, H2-$M^{-/-}$ mice were deficient in the negative selection of $CD4^+$ thymocytes as indicated by their strong response in a syngeneic MLR, since in these mice all MHC II molecules in the thymus are loaded with CLIP rather than the appropriate negatively selecting ligands (Martin *et al.* 1996; Miyazaki *et al.* 1996): in H2-$M^{-/-}$ $Ii^{-/-}$ mice, which do not express CLIP, this phenotype was corrected (Swier *et al.* 1998).

H2-M dimers possess tyrosine-based sorting motifs in their cytoplasmic tails that differ from the di-isoleucine motifs which target the Ii:αβ complex. This suggests that Ii and H2-M are transported to the MHC II loading compartment via distinct pathways, such that H2-M cannot catalyse inappropriate loading of MHC II (Pieters 1997). H2-M is also thought to play a proof-reading function by catalysing removal of low-affinity peptides from the MHC binding groove, ensuring that only the most stable complexes reach the cell surface (Kropshofer 1996). H2-M is transported from the Golgi to lysosomes in association with the related molecule H2-O (HLA-DO), which may regulate H2-M function (Liljedahl *et al.* 1996).

1.3.2.2 Processing Compartments within APC

The delay in the appearance of MHC II-peptide complexes on the surface of APC reflects the need for the MHC II pathway to integrate with endocytosed antigen. The route of entry of antigens into APC, and the nature of the intracellular compartment to which antigens are delivered, will play an important role in determining degradation of proteins and the dominant T cell epitopes generated. Electron microscopy studies indicated co-localisation of the antigen import pathway (surface immunoglobulin (Ig)) and the MHC II export pathway in an endocytic compartment, thus demonstrating that the molecules involved in processing and presentation might meet via the endosomal pathway (Guagliardi *et al.* 1990). Compartments containing high levels of MHC II were subsequently identified in B cell lines and termed MIIC for MHC II-containing

compartments (Peters *et al.* 1991). These multi-laminar compartments have since been identified in DC and MΦ (Harding and Geuze 1993; Kleijmeer *et al.* 1995). MIIC are positive for the lysosomal markers LAMP-1 and CD63, negative for the transferrin receptor, but can be reached by endocytic tracers, identifying them as late endosomal/early lysosomal compartments (Peters *et al.* 1991; Kleijmeer *et al.* 1997). MIIC contain high levels of the exchange catalyst H2-M (Sanderson *et al.* 1994) and, in a human cell line, are also strongly positive for the mAb YAe (Kleijmeer *et al.* 1997), which recognises the MHC II I-A^b molecule bound to the endogenous HLA-DR α chain (Murphy *et al.* 1989). These data therefore implicated MIIC as the principal sites of MHC II-peptide loading. A second distinct MHC II-rich compartment was subsequently identified in B cells (Amigorena *et al.* 1994; Drake *et al.* 1997). These Class II containing Vesicles (CIIV) contained small amounts of transferrin receptor and lacked lysosomal markers, indicating that they were more closely related to early and recycling endosomes. CIIV contained SDS-stable MHC II αβ heterodimers, ie. peptide-loaded complexes (Amigorena *et al.* 1994), and were positive for MHC II αβ-Ii complexes and H2-M (Amigorena *et al.* 1995). This led to the proposal that they may play a role in the final steps of peptide loading before transport of MHC II-peptide complexes to the cell surface (Amigorena *et al.* 1995; Pierre *et al.* 1997).

An alternative MHC II loading pathway has been proposed that utilises the recycling of surface MHC II into early endosomal or recycling compartments (Salamero *et al.* 1990; Pinet *et al.* 1995). This pathway appears to be relatively unimportant compared to classical transport of newly synthesised MHC II molecules, however it may play a role in the presentation of peptides which are sensitive to degradation in acidic endosomal compartments. Early endosomes could function as peptide exchange compartments, resulting in the replacement of low affinity peptides with high affinity peptides on surface MHC II molecules (Salamero *et al.* 1990). Immature DC have also been reported to use a third "rogue pathway" in which MHC II-Ii complexes transiently appear on the plasma membrane before being internalised and loaded with peptide (Saudrais *et al.* 1998). The functional significance of this pathway is, however, unclear.

Transport of MHC II-peptide complexes from peptide-loading compartments to the cell surface is thought to be via direct transport from the loading compartment to the cell surface using vesicular carriers or fusion of MIIC with the plasma membrane. This is supported by experiments in which the transport of MHC II complexes was examined in Mel JuSo cells transfected with a MHC II β chain:GFP fusion protein (Wubbolts *et al.* 1996). These experiments demonstrated, using confocal microscopy, that MHC II:GFP was concentrated in MIIC-like late endosomal/early lysosomal compartments. MHC II:GFP-containing vesicles were visualised migrating from the region of these compartments to the plasma membrane independently of conventional endocytic routes. MIIC-derived vesicles have also been shown to fuse with the plasma membrane and to be shed by B cells (Raposo *et al.* 1996), and T cells were stimulated by MHC II-peptide complexes on B cells in which Transferrin-containing early endosomes had been ablated (Pond and Watts 1997). Collectively, these data support the direct transport of MHC II-peptide complexes to the plasma membrane, without interaction with the early endosomal/recycling pathway.

1.3.1.3 Generation of Antigenic Peptides and Control of Processing of Ii by Host Proteases.

Cleavage of antigens by endosomal proteases will determine the dominant T cell epitopes presented from exogenous proteins. The aspartate proteases Cathepsins D and E and the cysteine proteases Cathepsins B, L, S and H have all been implicated in the processing of exogenous antigens - either directly through modulation of the peptide or through interactions with Ii. Cathepsins D and E are both involved in the production of functional T cell epitopes whereas H and B appeared to play more redundant roles (Villadangos *et al.* 1999). Hewitt and colleagues (Hewitt *et al.* 1997) extensively analysed the ability of Cathepsins D and E to cleave the C fragment domain from tetanus toxin and produce functional T cell epitopes. This work revealed preferential cleavage sites for each epitope, allowing characterisation of putative amino acid consensus sequences. The similarity in sites cleaved by Cathepsins D and E suggested that they might have overlapping functions. Consistant with this, Cathepsin $D^{-/-}$ mice were able to

process and present four different antigens to T cell clones, indicating that Cathepsin D is not necessary for MHC II-mediated presentation (Deussing *et al.* 1998).

A novel protease, asparaginyl endopeptidase (AEP), has recently been identified which may be responsible for initial processing of native proteins (Manoury *et al.* 1998). Incubation of a tetanus toxin antigen with disrupted lysosomes from a transformed B cell line revealed that the majority of the processing activity was due to this protease (Manoury *et al.* 1998). AEP was demonstrated to cleave the tetanus toxin protein after asparagine (N) residues, indicating that the positioning of these residues may dictate cleavage of dominant epitopes by this protease. N-glycosylation of asparagine residues has been demonstrated to block processing by AEP (Manoury *et al.* 1998). Thus, bacterial proteins, which are non-glycosylated, may be more sensitive to degradation by AEP than self proteins, suggesting that cleavage by AEP may bias the repertoire of complexes generated away from self proteins.

Proteases also control the presentation of T cell epitopes via cleavage of the Ii protein. Cathepsin S catalyses cleavage of a 10KDa CLIP-containing fragment from Ii (Ii-p10), allowing dissociation of MHC II αβ:Ii nonamers into MHC II αβ:CLIP complexes that are receptive for peptide (Riese *et al.* 1996). The importance of this activity was demonstrated through inhibition of Cathepsin S, which resulted in the retention of MHC II αβ:Ii molecules within lysosomes and a lack of peptide-loaded MHC II on the plasma membrane (Pierre and Mellman 1998). Cathepsin L also cleaves Ii but, unlike Cathepsin S, is expressed in cortical thymic epithelial cells and not in DC or B cells (Nakagawa *et al.* 1998).

1.3.2 Control of the MHC II Processing and Presentation Pathway in DC

The maturation of DC as they migrate from the skin to the draining LN is reflected by changes in MHC II compartmentalisation: in immature bone marrow-derived DC, MHC II was shown to be abundant in intracellular MIIC, but as the cells matured MHC II localised to CIIV-like compartments, and in fully mature DC all MHC II molecules were found on the plasma membrane (Nijman *et al.* 1995; Pierre *et al.* 1997). Maturation of DC was accompanied by a change in the stability of MHC II-peptide complexes at the cell surface: in immature DC, MHC II complexes are rapidly re-routed to lysosomes for degradation, but as the cells mature, the half life of complexes on the surface increases from approximately ten to more than one hundred hours (Cella *et al.* 1997). This observation led to the hypothesis that control of surface expression of loaded MHC II molecules is due to rapid recycling of these molecules into degradative compartments. This was recently modified by Inaba and colleagues who, in the absence of a maturation stimulus, could not detect the generation of MHC II-peptide complexes using a complex-specific mAb (C4H3) (Inaba *et al.* 2000). In these experiments antigen appeared to be internalised and retained intracellularly for at least sixty hours, but C4H3 positive complexes were not observed unless LPS was added to the culture. C4H3 positive complexes appeared to be sorted into CIIV-like structures, along with other T cell co-stimulatory molecules, which were then transferred to the cell surface (Turley *et al.* 2000). These data imply that control of presentation in DC is at the level of antigen processing/peptide loading and not due to degradation of pre-formed complexes. However, the well characterised differences in the half life of MHC II-peptide complexes in immature versus mature DC make it unlikely that the model proposed by Inaba *et al* is the sole mechanism for controlling antigen presentation by DC. We have recently shown, using an alternative complex-specific mAb, that MHC II-peptide complexes form rapidly in immature DC, upon incubation with exogenous protein in the absence of a strong maturation signal (Colledge *et al.*). Furthermore, while both immature and activated DC generated MHC II-peptide complexes with equal kinetics, in immature, but not mature, DC these complexes were rapidly internalised from the

plasma membrane and degraded (Villadangos *et al.* 2001). Together, these data support the hypothesis that control of the rate of degradation of MHC II-peptide complexes is responsible for the regulation of their expression in immature versus mature DC (Cella *et al.* 1997).

Loading of MHC II molecules with peptides and transport to the cell surface is dependent on cleavage of Ii. Significant amounts of Ii-p10 accumulated in immature but not mature DC and this decrease as the cells were activated was paralleled by decreased levels of Cystatin C, an inhibitor of Cathepsin S (Pierre and Mellman 1998). A model has been proposed whereby control of cleavage of Ii restricts loading of MHC II molecules and transport to the cell surface in immature cells. This model is based on experiments which demonstrated that in immature DC, Cystatin C blocked Cathepsin S activity and MHC II αβ-p10 complexes were retained in lysosomes. As the DC matured, Cystatin C appeared to be sequestered in the Golgi, permitting Cathepsin S to degrade Ii-10 to CLIP, which will be replaced by antigenic peptides (Pierre and Mellman 1998). However, Villadangos and colleagues (Villadangos *et al.* 2001) could not detect a difference between localisation and trafficking of MHC II-peptide complexes in DC from Cathepsin S-dependent ($I\text{-}A^b$) and Cathepsin S-independent ($I\text{-}A^s$) allotypes of mice suggesting that Ii does not control loading of MHC II complexes in DC.

1.4 Initiation and Resolution of *Leishmania* Infection

The immunobiology of *Leishmania* infection has been extensively studied in mice due to the development of a well-characterised model of Leishmaniasis that reflects the spectrum of dieases found in man. The primary and effector T cell responses to *Leishmania* are dependent on priming of T cells by activated DC and MΦ. The literature discussed in this section describes the role of T cells in the balance between resistance and susceptibility to *Leishmania* infection, and highlights the importance of the interaction between *Leishmania* parasites and DC and MΦ for the development of this response. This chapter will focus on studies investigating the infection of mice with the

cutaneous species *Leishmania major* and *Leishmania mexicana* or *Leishmania amazonensis*.

Most inbred strains of mice are resistant to infection with *L. major*, and develop small, self-healing lesions. However a few strains, such as BALB/c, CB.17 or NZW mice, are unable to control the infection (Morris *et al.* 1993). Studies on immune responses generally focus on C3H/HeN or C57BL/6 mice, which are genetically resistant, and can control infection with *L. major*, and BALB/c mice, which are highly susceptible, and generally die due to uncontrolled dissemination of the parasites (Reiner and Locksley 1995). However, other *Leishmania* species that cause cutaneous Leishmaniasis do not induce such clear cut polarised responses in "resistant" or "susceptible" strains; whilst BALB/c mice are susceptible to infection with all *Leishmania* species, "resistant" C3H/Hen or C57BL/6 mice are unable to control infection with *L. amazonensis* (Afonso and Scott 1993; Soong *et al.* 1997).

1.4.1 The Role of T Cells in Controlling Infection with *Leishmania*

1.4.1.1 Control of *Leishmania* Infection Requires MHC II-Dependent CD4$^+$ T Cells

T cell-deficient BALB/c *nude* (Moll *et al.* 1988) or thymectomised BALB/c mice (Shankar and Titus 1995) could not resolve infection with *L. major*, and developed large ulcerating lesions. Adoptive transfer of CD8$^+$ (Ly-2$^+$)-depleted, but not CD4$^+$ (L3T4$^+$)-depleted, T cells from non-infected animals into BALB/c *nude* mice conferred an ability to heal the infection (Moll *et al.* 1988), indicating that CD4$^+$ T cells were essential for the generation of protective responses in infected mice. In subsequent experiments Locksley and co-workers (1993) demonstrated that C57BL/6/129 F1 MHC II-deficient mice were highly susceptible to infection with *L. major*, confirming that stimulation of CD4$^+$ T cells was critical for the resolution of the disease. Lesion development in *L. amazonensis*-infected C57BL/6 *nude* mice was delayed compared to wild type mice

(Soong *et al.* 1997), and lesion size directly correlated with the number of $CD4^+$ T cells transferred into these mice, implying that $CD4^+$ T cells play role in the immune-pathology, as well as the resolution, of infection with *L. amazonensis*.

Mice lacking MHC I molecules (due to a disruption in the β_2-microglobulin gene) have normal $CD4^+$ T cell numbers but lack MHC I-restricted $CD8^+$ T cells, and have disrupted NK cell activity. A number of different studies have shown that the course of infection with *L. major* or *L. mexicana* parasites in these mice on C57BL/6/129 F1, 129 or BALB/c backgrounds was equivalent to infection in matched wild type mice, indicating that $CD8^+$ T cells do not influence the outcome of the response to these parasites (Locksley *et al.* 1993; Overath and Harbecke 1993; Wang *et al.* 1993). BALB/c mice that had healed the infection did, however, show an increase in the production of IFNγ by $CD8^+$ T cells (Wang *et al.* 1993). Mice immunised with predicted MHC I epitopes from the *Leishmania* surface glycoprotein, gp63, developed specific cytotoxic T lymphocyte ($CD8^+$ T cell) responses to some peptides but were not protected against infection (Wang *et al.* 1993). Therefore, collectively these data indicate that $CD8^+$ T cells are not critical for controlling the outcome of infection with cutaneous *Leishmania* species but that IFNγ-production by $CD8^+$ T cells may enhance $CD4^+$-mediated cure of infection.

The presence or absence of B cells from non-infected mice, when transferred with T cells into C.B-17 *scid/scid* mice had no effect on the induction of a protective T cell response to *L. major*, even when given at a ratio of ten B cells to one T cell (Varkila *et al.* 1993). These data indicate that B cell responses neither control nor exacerbate the infection. BALB/c μMT mice, which lack B cells due to a disruption of the IgM locus (Kitamura *et al.* 1991), remained susceptible to *L. major* infection, while C57BL/6 μMT mice remained resistant (Brown and Reiner 1999), suggesting that B cells are also not required for the development of T helper subsets in *L. major* infected mice (see section 1.4.1.2). B cell-derived antibodies may play a role in the uptake of opsonised parasites by APC, since mice lacking Fc receptors were resistant to infection with *L. major* (Kima *et al.* 2000). Furthermore, mice lacking circulating antibodies, which had been injected

with serum from *Leishmania*-infected mice or mice immunised with amastigote membrane components, were more susceptible to infection than those receiving serum from non-immune mice (Peters *et al.* 1995; Kima *et al.* 2000). Thus, *Leishmania*-specific antibodies may mediate uptake of parasites by cells expressing FcR (see section 1.4.2.1) but B cell responses do not otherwise influence the outcome of infection.

1.4.1.2 The Role of Polarised T Helper Cell Subsets in Resistance or Susceptibility to Infection with *L. major*

1.4.1.2.1 Development of $CD4^+$ Th1 and Th2 Subsets

Murine $CD4^+$ Th cells can be divided into subsets based on the secretion of different cytokines upon restimulation. Mosmann *et al* (Mosmann *et al.* 1986) originally reported that a panel of $CD4^+$ T helper clones could be divided into two groups depending on the secretion of IL-2 and IFNγ or their IgG- and IgE-enhancing activities upon activation *in vitro* (Mosmann *et al.* 1986). These subsets have subsequently been well characterised and shown to have distinct, often antagonistic roles in the immune response (Abbas *et al.* 1996); Th1 cells secrete IFNγ, thus directing cell-mediated immune responses, and promote switching to IgG2a and IgG2b isotypes, while Th2 cells secrete the cytokines IL-4, IL-5 and IL-13 and promote switching to IgE, IgG1 and IgG4 antibody responses which characterise the development of humoral responses.

Naïve Th precursor cells, termed Th0 cells, can develop into either Th1 and Th2 effector cells depending on whether they are cultured in the presence of IL-12 or IL-4 respectively (Abbas *et al.* 1996). IL-12 induces activation of Th1 cells via the signalling molecule STAT (Signal Transducer and Activator of Transcription) 4 (Thierfelder *et al.* 1996; Kaplan *et al.* 1996a), while IL-4 induced responses are transduced via STAT 6 (Kaplan *et al.* 1996b) Stimulation of Th0 precusors, e.g. with anti-CD3 antibodies, induces the cells to proliferate and begin to produce effector cytokines (Bird *et al.* 1998; Gett and Hodgkin 1998). Recent studies using the fluoresecent dye CFSE (carboxyfluoroscein diacetate succinimidyl ester) have demonstrated that cytokine production is linked to the number of cell cycles undergone after stimulation (Bird *et al.* 1998; Gett and Hodgkin 1998). Thus, IFNγ is produced successively as the cells divide

whereas expression of IL-4 genes requires additional cell divisions (Bird *et al.* 1998; Gett and Hodgkin 1998). The transcription factor Gata-3 is thought to be the key mediator in development of Th2 cells (Zheng and Flavell 1997), while a second transcription factor, T-bet, has recently been identified as a potential inducer of Th1 development (Szabo *et al.* 2000). The differential expression of IL-4 and IFNγ by activated T cells was shown to be controlled by epigenetic constraints; chromatin structure and DNA methylation prevented transcription of cytokine genes before the cells had undergone the requisite number of divisions. This process was strictly related to the number of actual cell divisions and not the time taken for these divisions to occur (Bird *et al.* 1998). Once demethylation of IFNγ or IL-4 genes occurred it was stably inherited by daughter cells, ensuring that descendants of the original polarised precursor would maintain that effector T helper phenotype (Bird *et al.* 1998).

Individual cells within pools of naïve T cells were shown to have the potential to secrete both Th1- and Th2-associated cytokines, suggesting that the development of Th0 precusors into Th1 or Th2 effector cells was a stochastic event (Kelso and Gough 1988; Kelso *et al.* 1995). A more recent study has, however, indicated that there is also an inherent instructive component to this process: IFNγ- and IL-4-secreting cells developed from naïve precursors in cultures in which all polarising cytokines were neutralised (Farrar *et al.* 2001); furthermore, retro-viral expression of Gata-3 in polarised Th1 or Th2 cells resulted in the skewing of the majority of these cells to a Th2 phenotype, indicating that their phenotype had not been fixed by selective differentiation of progenitors in IFNγ or IL-4 (Farrar *et al.* 2001). This result, however, contradicts the observation of Bird *et al.* (1998), discussed in the previous paragraph, that polariastion of Th cells was stably inherited due to epigentic constraints on cytokine gene expression. It is therefore likely that the selective expansion of Th1 versus Th2 clones in response to stimulation will depend on both instructive and selective signals, whereby progenitors have a tendency towards different fates, but this can be influenced by the cytokine milieu in the LN at the time of activation.

Different T helper subsets are important in the elimination of different pathogens: Th1 responses are generally thought to be required for protection against intracellular pathogens, including viruses, while Th2 responses eliminate extracellular pathogens and mediate allergic responses. While this paradigm is useful for defining responses to different organisms, immune responses to pathogens usually develop as a balance between these two extremes (Allen and Maizels 1997). *Leishmania* infection has emerged as a model system in which the ability to cure infection is strictly related to the Th subset which predominates.

1.4.1.2.2 The Role of Th Subsets in Infection with *Leishmania*

Resistance to infection with *L. major* was originally shown to correlate with expansion of the IFNγ-producing Th1 subset, while Th2 responses predominated in susceptible BALB/c mice (Scott *et al.* 1988; Heinzel *et al.* 1989). The role of these two subsets in controlling the outcome of infection was further defined by studies showing that transfer of Th1 or Th2 cell lines conferred protection or exacerbation of disease respectively: T cell lines were generated from two fractions of soluble *Leishmania* antigens (SLA) which were known to be protective or not on immunisation of BALB/c mice. The T cell line specific for the protective fraction produced IL-2 and IFNγ upon restimulation with *Leishmania* antigen, whereas, the T cell line expanded with the non-protective SLA fraction produced Th2-type cytokines (Scott *et al.* 1988). This observation was supported by a study that showed that reconstitution of BALB/c *scid* mice with T cells from cured mice transferred the healing phenotype, an effect that was blocked on administration of anti-IFNγ antibodies, whilst adoptive transfer of T cells from chronically infected mice conferred IL-4-dependent susceptibility (Holaday *et al.* 1991).

The polarisation of responding T cells towards a type 1 or type 2 phenotype occurs very rapidly after infection by *L. major*. Three days post-infection a five to ten fold increase in cell numbers was observed in LN draining the site of infection, with a concomitant increase in the levels of IFNγ or IL-4 present in the draining LN of C3H/Hen or BALB/c mice respectively (Scott 1991).

Interestingly, transfer of low numbers of T cells into BALB/c mice (Mitchell *et al.* 1981; Moll *et al.* 1988; Varkila *et al.* 1993), or infection of these mice with low numbers of parasites (Doherty and Coffman 1996) resulted in the development of a healing Th1 response. This demonstrated that susceptible BALB/c mice do contain 'healer' Th1 cells, and suggests that they can control the infection when there is a reduction in the frequency of IL-4-producing cells early on in the infection (see section 1.4.1.4).

1.4.1.3 The Role of Th1-Derived Cytokines in Resistance to Infection

The correlation between expansion of the Th1 cell subset and resolution of infection strongly implicates the Th1-associated cytokines IFNγ and IL-12 in development of the protective anti-*Leishmania* immune response.

1.4.1.3.1 The Role of IFNγ in the Anti-*Leishmania* Response

IFNγ is produced by activated T cells and NK cells. Among other roles, IFNγ is a potent activator of mononuclear phagocytes, and promotes differentiation of Th cells to Th1 phenotypes while inhibiting development of Th2 cells. Activation of MΦ by IFNγ leads to enhanced microbicidal mechanisms such as production of nitric oxide (NO), and up-regulation of surface expression of phagocytic receptors (Boehm 1997). IFNγ will, therefore, play a dual role in the outcome of *Leishmania* infection; by promoting differentiation of Th1 cells and via direct effector functions on MΦ at the site of infection.

Treatment of resistant *L. major*-infected mice with anti-IFNγ mAb abrogated their ability to control the infection (Belosevic *et al.* 1989) and switched cytokine production in the draining LN from IFNγ to IL-4 and IL-5 (Scott 1991). Parasite burdens in treated mice were equivalent to those in susceptible BALB/c mice, indicating that ablation of IFNγ is sufficient to convert a resistant to a susceptible phenotype (Belosevic *et al.* 1989). This is supported by the observation that IFNγ-deficient mice were unable to control infection and produced significant amounts of Th2 cytokines (Wang *et al.* 1994). Significantly, a single dose of anti-IFNγ given at the time of infection, but not a week later, was sufficient to switch the response from healing to non-healing (Belosevic *et al.*

1989; Scott 1991). Thus, the timing of IFNγ production is important for its ability to control infection. Administration of IFNγ to BALB/c mice at the time of infection led to an increase in IFNγ production by LN cells and a decrease in IL-4 and IL-5. However, lesion development only showed a transient delay and the mice were not able to control the infection (Scott 1991). Therefore, while IFNγ appears to be critical in controlling infection with *Leishmania,* it is not sufficient to control the spread of *Leishmania* infection in susceptible mice, indicating that other factors must also be involved.

The early requirement for IFNγ implies that it is produced as part of the innate response to infection, before the adaptive response is initiated. Activated NK cells were present in the LN of infected mice three days post-infection (Scharton and Scott 1993). High NK cell activity in different inbred stains of mice inversely correlates with parasite burden, thus resistant mice with a low parasite burden tend to have higher NK cell activity (Scharton and Scott 1993). Ablation of NK cells in mice using a rabbit anti-asialo antiserum or an anti NK1.1 mAb led to reduced levels of IFNγ production in the draining LN and an initial enhancement in susceptibility, although the mice eventually recovered (Laskay *et al.* 1993). These data suggest that NK cells may play a role in control of *Leishmania* parasites via non-antigen-specific IFNγ production during the early stages of infection.

1.4.1.3.2 The Role of IL-12 in Initiating the Development of a Th1 Response to *Leishmania*

Introduction to IL -12

IL-12 was first identified in the supernatants of transformed B cells which had been stimulated with the phorbol diester PDBu (phorbol-12,13-dibutyrate). Supernatant from these cells induced high amounts of IFNγ in peripheral blood lymphocytes (PBL), augmented cytotoxic activity against target cell lines, and enhanced PBL proliferation in response to PDBu. All three of these activities were co-purifed with a 70KDa glycoprotein which was initially called Natural Killer cell Stimulatory Factor (NKSF) but was later renamed IL-12 (Kobayashi *et al.* 1989; Trinchieri 1995). IL-12 is produced

by DC, MΦ and, to a lesser extent, B cells. It is biologically active as a 70KDa heterodimer (p70) that is composed of two covalently linked subunits of 40 (p40) and 35 (p35) KDa respectively. IL-12 both induces NK cells and T cells to secrete IFNγ and functions as a T cell growth factor, thus contributing to optimal proliferation of, and IFNγ production by, differentiated Th1 clones in response to antigen (Trinchieri 1995). IL-12 production by activated DC or MΦ is critical for the priming of a type 1 Th cell response (Hsieh *et al.* 1993; Macatonia *et al.* 1995): mice in which the p40 gene was mutated (Magram *et al.* 1996) showed decreased NK cell activity and reduced IFNγ production in response to LPS, and consequently failed to generate normal Th1 responses. LN cells from $p40^{-/-}$ mice immunised with KLH also did not efficiently secrete IFNγ upon restimulation *in vivo*, although antigen-induced proliferation of LN cells was normal. Production of IL-4 was enhanced in these mice compared to wild type controls, suggesting that IL-12 may inhibit production of IL-4. IL-12 has therefore been proposed to act as a bridge between the innate and adaptive type 1-biased immune responses since production of IL-12 upon activation of APC directly induces the development of these T cell responses.

The p35 and p40 proteins are encoded by genes on separate chromosomes in humans and mice, indicating that they are differentially controlled (Trinchieri 1995). Differential regulation of both subunits by cytokines (Hochrein *et al.* 2000) can result in the production of an excess of the p40 protein which forms homodimers that can inhibit responses to IL-12 p70 by blocking its binding to the IL-12 receptor (Gillessen *et al.* 1995). $(p40)_2$ homodimers blocked IL-12-induced proliferation and IFNγ production by T cell blasts (Gillessen *et al.* 1995), and the presence of $(p40)_2$ homodimers suppressed rejection of myocytes which had been transplanted into the quadriceps of allogeneic mice (Kato *et al.* 1996).

IL-12 and Resolution of Infection with *Leishmania*

IFNγ is clearly required to control infection with *Leishmania*, but the inability of exogenous IFNγ to heal susceptible mice suggested that other factors were also involved

in the development of a resistant immune response. Treatment of BALB/c mice with recombinant IL-12 (rIL-12) during the first week of infection with *L. major* parasites rendered these mice resistant to infection in an IFNγ-dependent manner (Heinzel *et al.* 1993; Sypek *et al.* 1993). Lymphocytes from the draining LN of treated mice produced ten to fifty times less IL-4 than wild type mice in response to *Leishmania* antigen, indicating that the presence of IL-12 had resulted in a switch from a Th2 to Th1 response (Heinzel *et al.* 1993). Furthermore, immunisation with SLA and IL-12, but not with SLA alone, resulted in complete protection of mice against challenge with *L. major* promastigotes (Afonso *et al.* 1994). As had been shown previously for IFNγ (Belosevic *et al.* 1989; Scott 1991), IL-12 is required during the early stages of infection, since administration of rIL-12 during the first week elicited a durable healing response that lasted up to four months (Heinzel *et al.* 1993), whereas administration of rIL-12 after the first week could only delay lesion development (Sypek *et al.* 1993). These data imply that early production of IL-12 initiates the early IFNγ response that is needed to control the infection. Evidence for the role of endogenous IL-12 *in vivo* was demonstrated by the administration of anti-IL-12 antibodies to C57BL/6 mice. Infection of these mice with *L. major* resulted in a decrease in IFNγ production in the draining LN and an increase in parasite burden compared to non-treated animals (Sypek *et al.* 1993; Heinzel *et al.* 1995). IL-4 production in infected C3H/Hen mice treated with an anti-IL-12 antibody was equivalent to levels induced on infection of susceptible BALB/c mice (Scharton-Kersten *et al.* 1995), indicating that ablation of IL-12 is sufficient to switch the outcome of infection. Definitive evidence for the role of IL-12 in mediating resistance to infection with *Leishmania* came from infection of resistant 129 mice lacking either IL-12 p35 or p40. These mice were highly susceptible to infection with *L. major* and produced levels of IL-4 equivalent to those seen in infected wild-type BALB/c mice. This increase in IL-4 was associated with reduced levels of IFNγ. In this study there was no difference between p35 or p40 deficient mice (Mattner *et al.* 1996).

Three separate studies have shown a requirement for the interaction between CD40 on APC and CD40L on activated T cells for the resolution of infection by *L. major* or *L. amazonensis* (Campbell *et al.* 1996; Kamanaka *et al.* 1996; Soong *et al.* 1996). This

phenotype is also likely to be due to a lack of IL-12 since CD40 ligation enhances IL-12 production and Th1 responses are decreased in mice lacking either CD40 (Kamanaka *et al.* 1996) or CD40L (Campbell *et al.* 1996).

Regulation of Expression of the IL-12 Receptor Correlates with Resistance to *Leishmania* Infection

The IL-12 receptor (IL-12R) is composed of two chains, IL-12R β1 and β2 (Gately *et al.* 1998). Culture in Th1-promoting conditions induced up-regulation of IL-12R β2 expression on T cells, while Th2-promoting conditions induced a transient up-regulation of IL-12R β2 mRNA followed by down-regulation to undetectable levels (Szabo *et al.* 1997), suggesting that maintained expression of the IL-12R β2 subunit promotes development of a Th1 versus a Th2 response.

LN cells from C3H/HeN mice infected with *L. major* for two days bound more IL-12 than cells from BALB/c mice. This correlated with increased expression of IL-12R β2 in the draining LN, although expression of the β2 subunit was up-regulated on all cells, not just those which were antigen-specific (Jones *et al.* 1998). Administration of rIL-12 to *L. major*-infected BALB/c mice, or treatment with an anti-IL-4 antibody, increased IL-12R β2 expression in LN cells, while anti-IL-12 antibodies inhibited IL-12R β2 expression and decreased levels of IFNγ mRNA in the LN (Jones *et al.* 1998). Therefore, these data suggest that maintenance of IL-12R β2 expression is related to the ability of resistant mice to control infection with *Leishmania*.

Heath and co-workers (Heath *et al.* 2000) demonstrated that ectopic expression of the IL-12R β2 chain in Th2 cells did not induce IFNγ production upon restimulation, even though IL-12-induced STAT signalling was restored in these cells. BALB/c mice in which the IL-12R β2 chain was over-expressed under the control of a CD2 promoter and CD2 locus control region developed Th2 responses in the presence of IL-4, even if IL-12 was also present (Nishikomori *et al.* 2000). These mice were as susceptible as their wild type litter-mates to infection with *L. major*, and developed a polarised Th2 response in

the draining LN (Nishikomori *et al.* 2001). The observation that BALB/c mice developed a healing phenotype if given IL-12 at the time of infection, but not seven days post-infection (Heinzel *et al.* 1993; Sypek *et al.* 1993), was assumed to be due to the fact that the IL-12R β2 chain is no longer expressed by T cells at later time points. Administration of IL-12 seven days post-infection of the IL-12R β2 chain transgenic mice did not, however, result in an ability of these mice to control the infection (Nishikomori *et al.* 2001). Thus, these results demonstrate that maintenance of the IL-12R β2 chain is not sufficient to switch a Th2 response to Th1, and that expression of IL-12R β2 is not the primary factor determining the ability of resistant versus susceptible mice to control infection.

C3H/Hen mice cannot control infection with *L. amazonensis*, and this also correlated with a lack of expression of IL-12R β2 on $CD4^+$ T cells and an inability of these cells to respond to IL-12. This lack of response was independent of IL-4 and IL-10. Interestingly, the link between IL-12 responsiveness and the inability of C3H/HeN mice to resolve infection with *L. amazonensis* is different to that linking susceptibility of BALB/c mice to *L. major*, because administration of exogenous IL-12 failed to resolve infection with *L. amazonensis* (Jones *et al.* 2000).

1.4.1.4 The Role of Th2-Derived Cytokines in Susceptibility to Infection

1.4.1.4.1 The Role of IL-4 in Exacerbating Disease

IL-4 promotes the growth and differentiation of Th2 cells and antagonises production of IFNγ by Th1 cells. Eighty five percent of BALB/c mice infected with *L. major* recovered after administration of an anti-IL-4 antibody (Sadick *et al.* 1990) and anti-IL-4 antibodies switched the cytokine response in the draining LN from IL-4 to IFNγ (Holaday *et al.* 1991). Furthermore, a single injection of an anti-IL4 mAb was sufficient to switch the outcome of infection to a healing Th1-mediated response when it was given two days prior to infection. These mice were also resistant to re-infection (Chatelain *et al.* 1992). Reconstitution of SCID mice with splenocytes from wild type or $IL\text{-}4^{-/-}$ BALB/c mice showed that innate production of IL-4 by non-lymphocytes may

play a role in the development of lesions, but that lymphocyte-derived IL-4 was needed for exacerbation of the disease (Satoskar *et al.* 1997). Unexpectedly, IL-4$^{-/-}$ mice were initially reported to remain highly susceptible to infection with *L. major*, indicating that there is not a simple relationship between the presence of IL-4 and susceptibility of BALB/c mice (Noben-Trauth *et al.* 1996). However, other groups have subsequently reported that the same IL-4 deficient mice are resistant to infection with *L. major* (Kopf *et al.* 1996) and *L. mexicana* (Satoskar *et al.* 1997). *L. mexicana*-infected C57BL/6 IL-4$^{-/-}$ mice were resistant to infection and this was associated with the development of a Th1 response. Ablation of STAT6, which is an essential component of the IL-4 signalling pathway (Takeda *et al.* 1996), resulted in mice that resolved infection with *L. mexicana* (Stamm *et al.* 1998). Thus, together these data indicate that IL-4 plays a dominant role in exacerbating disease in susceptible mice.

Infection of BALB/c mice in the foot pad with *L. major* led to two waves of IL-4 production by CD4$^+$ T cells in the draining LN: a peak in IL-4 mRNA was first seen at sixteen hours post-infection, decreased and peaked again at five days (Launois *et al.* 1995). This peak of IL-4 was not seen in resistant strains of mice such as C57BL/6 or C3H/Hen and has been thought to correlate with the enhanced susceptibility of BALB/c mice to *L. major* (Launois *et al.* 1995). Anti-IL-4 antibodies given at the time of infection did not abrogate the initial peak of IL-4 production at sixteen hours but did prevent the second peak at five days post-infection, indicating that early IL-4 regulates the development of the effector Th2 response, and subsequent IL-4 production (Launois *et al.* 1997). CD4$^+$ T cells from infected BALB/c and C57BL/6 mice both expressed IL-12R β2 mRNA 24 hours post-infection, but by day five expression had decreased in BALB/c mice to below the level of detection. This decrease did not occur in BALB/c mice treated with anti-IL-4 antibodies (Himmelrich *et al.* 1998), thus correlating the early production of IL-4 in susceptible mice with down-regulation of the IL-12R β2 chain on T cells.

Screening of a *L. major* promastigote expression library with the protective Th1 cell clone 9.1-2 (Scott *et al.* 1988) identified an antigen which was subsequently cloned, and

characterised as a *Leishmania* homologue (LACK) of a mammalian Receptor of Activated C Kinase (RACK 1) (Mougneau *et al.* 1995). LACK is a 36KDa protein that is expressed by both promastigotes and amastigotes and is highly conserved between all *Leishmania* species tested (Mougneau *et al.* 1995). Infection with *L. major* had been previously shown to induce the clonotypic expansion of heterodimeric Vβ4, Vα8 TCR-expressing T cell clones in both susceptible and resistant mice (Reiner *et al.* 1993). Mougneau and colleagues (Mougneau *et al.* 1995) demonstrated that LACK-specific T cell hybridomas all expressed TCR composed of Vβ4 and Vα8 chains, suggesting that LACK induced expansion of these cells during natural infection with *L. major*. Launois *et al* (Launois *et al.* 1997) later confirmed this by demonstrating that deletion of the Vβ4 chain by infection of mice with mouse mammary tumour viruses (MMTV(SIM)) resulted in the ability to resolve infection with *L. major*, which was associated with ablation of the early peak of IL-4 and the concommitant up-regulation of Th1 responses in the draining LN. In order to determine whether the early LACK-induced peak of IL-4 was solely responsible for exacerbation of disease in BALB/c mice, LACK cDNA was expressed from the MHC II I-Eα promoter to generate mice that were tolerant to LACK. Infection of these mice did not induce production of IL-4 in the LN and LACK-tolerant mice were able to control infection (Julia *et al.* 1996), suggesting that development of a susceptible phenotype in BALB/c mice was dependent on early production of IL-4 by LACK-specific T cells. Recent studies have, however, indicated that LACK itself is not crucial for the development of a Th2 response; Vβ4-deficient mice on a BALB/c background could control infection with *L. major*, but uncontrolled lesions developed in the presence of exogenous IL-4 (Himmelrich *et al.* 2000). Non-infected BALB/c mice were shown to possess LACK-reactive T cells, which cross-reacted with microbial antigens in the gut (Julia *et al.* 2000), which would therefore explain the rapid production of IL-4 upon infection with *Leishmania*, before the primary effector response has been primed.

It has recently been shown that administration of IL-4 eight hours post-infection, before the T cell-derived peak of IL-4 has occurred, resulted in cure of *L. major* infection by BALB/c mice and the development of a Th1 response equivalent to that seen in resistant

C57BL/6 mice (Biedermann *et al.* 2001). This was associated with IL-12 production by DC in the LN, which inhibited the subsequent burst of IL-4. If IL-4, however, was given at the time of T cell priming, the mice developed a Th2 response and could not control the infection. These results indicate that the non-specific production of IL-4 very early on during infection could promote the development of a healing Th1 response. They do not, however, suggest a source for this early IL-4 if it does play a role during the natural infection.

1.4.1.4.2 The Role of IL-10 in Susceptibility to Infection

IL-10 is produced by Th2 $CD4^+$ T cells and indirectly down-regulates the development of Th1 responses by inhibiting expression of co-stimulatory molecules and pro-inflammatory cytokines by DC and MΦ (Moore *et al.* 2001).

IL-10 was shown to promote survival of intracellular *L. major* parasites, and this correlated with a decrease in production of NO by infected MΦ (Vouldoukis *et al.* 1997). Administration of anti-IL-10 antibodies to susceptible BALB/c mice, however, did not induce resistance to infection with *L. major*, although other IL-10-mediated responses were altered in these mice (Chatelain *et al.* 1999), and C57BL/6 mice engineered to over-express IL-10 remained resistant to infection with *L. major* (Hagenbaugh *et al.* 1997). These data suggested that IL-10 was not a key mediator in controlling susceptibility or resistance of inbred mouse strains to *Leishmania* infection. Other studies using transgenic mice indicate, however, that IL-10 does promote parasite survival: resistant mice in which IL-10 was expressed from the MHC II Eα promoter were susceptible to infection with *L. major* (Groux *et al.* 1999), possible due to the inhibition of both Th1 and Th2 responses in these mice; likewise, Kane and Mosser (2001) recently demonstrated that BALB/c IL-$10^{-/-}$ mice were relatively resistant to infection with *L. major*.

Wild type mice infected intradermally with a low dose of *L. major* promastigotes contained parasites that persisted at the site of infection and in the draining LN for the

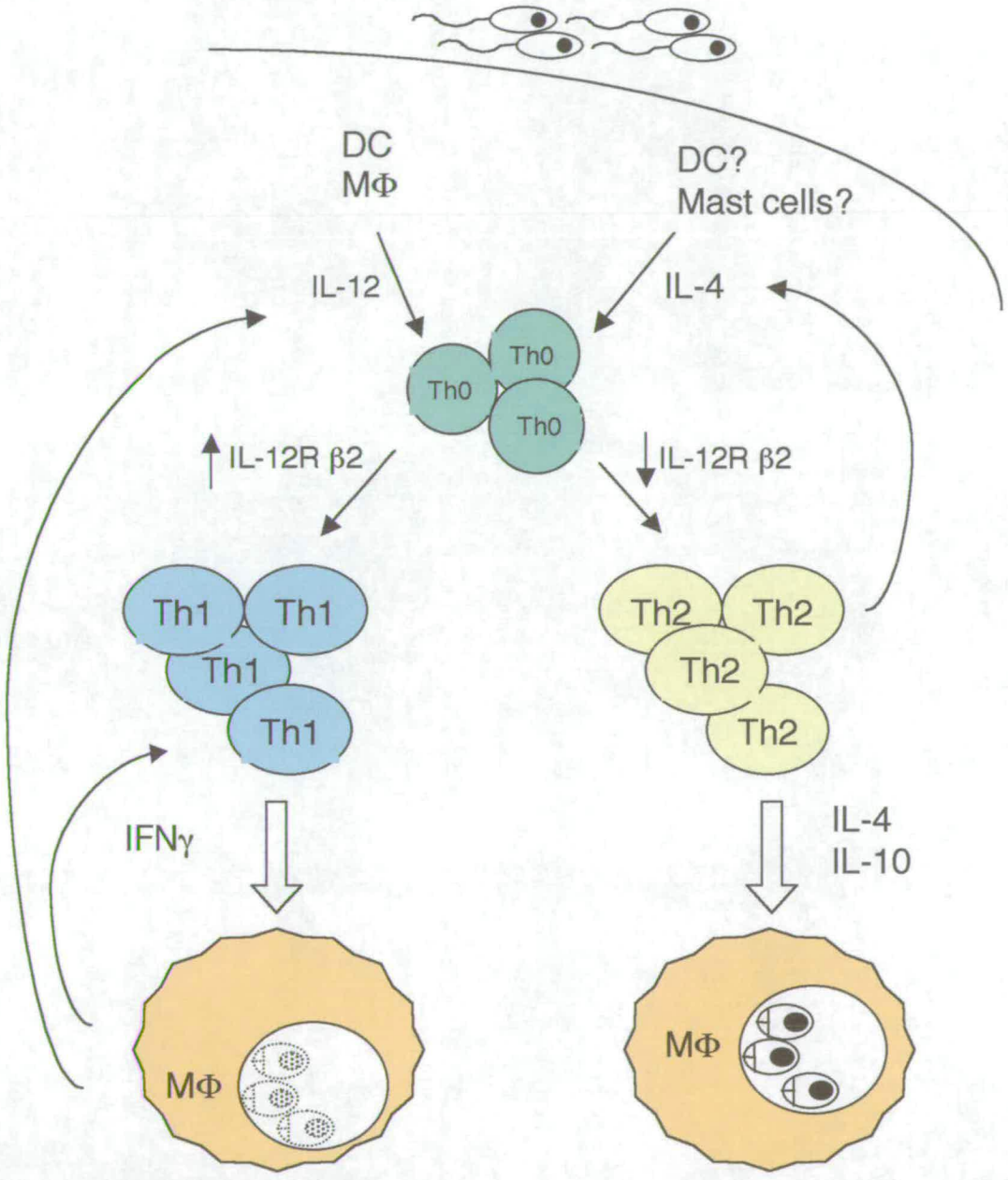
life-span of the animal. Persistent parasites were not, however, detected in C57BL/6 IL-$10^{-/-}$ mice (Belkaid *et al.* 2001) indicating that IL-10 also plays a role in the maintenance of a parasite reservoir in cured hosts.

1.4.2 Summary I: The Balance Between Th1 and Th2 Responses in *Leishmania* Infection

Resolution of infection with *Leishmania* is dependent on the activation of MHC II-dependent $CD4^+$ T cells. The outcome of the infection depends on the rapid activation of specific T helper cells, and represents a balance between the production and maintenance of Th1-or Th2-promoting cytokines. IL-12 production drives Th1 responses, leading to the production of IFNγ, which in turn activates the Leishmanicidal properties of MΦ. Conversely, IL-4 down-regulates expression of IL-12, IL-12R β2, IFNγ, inhibits NO production in MΦ and stimulates differentiation of the Th2 subset of $CD4^+$ T cells. This leads to an inability of the mouse to control infection. IL-4 production is clearly important in the outcome of disease since susceptible inbred mouse strains have a higher precursor frequency of antigen-specific IL-4-secreting T cells than resistant strains, and IL-4 but not IFNγ levels in the draining LN of *L. major*-infected mice correlate with susceptibility (Morris *et al.* 1993; Morris *et al.* 1993).

Based on the data reported in this section, the following model for infection of inbred strains of mice with *L. major* can be proposed: in resistant mice IL-12R β2 expression is up-regulated on $CD4^+$ T cells due to IL-12 production by activated DC and MΦ and IFNγ production by NK cells, and is maintained at high levels on these cells. IL-12 signalling inhibits the early IL-4 production by Vα8, Vβ4 T cells and induces development of IFNγ-producing Th1 cells. In susceptible mice, T cells do not maintain expression of the IL-12R β2 chain and its down-regulation is reinforced by the early LACK-induced peak of IL-4. These T cells develop into Th2 effector cells that produce more IL-4 and lead to exacerbation of disease (Figure 1.5).

Figure 1.5: Schematic Representation of the Role of T Helper Cell Responses in the Outcome of Infection with *Leishmania*



- Destruction of intracellular parasites via production of NO
- Cognate activation of effector Th1 cells via upregulation of MHC II-parasite antigen complexes
- Bystander activation of MΦ

- Suppression of Leishmanicidal properties

Data from mouse models of *Leishmania* infection demonstrate that the outcome of infection is the result of interplay between a number of different factors, none of which alone is sufficient to resolve or exacerbate the disease. Guler *et al* (1996) have elegantly shown that inherent genetic differences between resistant and susceptible mice control the differential ability of T cells from these mice to maintain their responsiveness to IL-12. Thus, data from infection of inbred strains of mice must be considered in the light of the genetic pre-dispositions of these mice. Natural infection in outbred hosts is likely to represent a less extreme polarisation of Th1 and Th2 responses, and the majority of humans are able to resolve infection with cutaneous species of *Leishmania* (Handman 1997). The model proposed in this summary highlights, however, the critical role of DC and MΦ from both resistant and susceptible strains of mice in controlling initial responses to infection and progression of the disease.

1.4.3 The Role of Infected Macrophages in the Anti-*Leishmania* Immune Response

Activated APC present *Leishmania*-derived antigens in the context of MHC II molecules. In most immunocompetent individuals, cell-mediated immune responses are efficiently activated and recovered patients are protected against subsequent infections (Handman 1997). However, MΦ are the principal host cells for *Leishmania* parasites, and low parasite numbers persist indefinitely in 'cured' hosts (Aebischer *et al.* 1993). The outcome of infection with *Leishmania* parasites will depend on a balance between the ability of the parasites to avoid activation of their host cells and the success of APC in presenting parasite antigens to, and activating, the appropriate T cell response. Therefore, a detailed understanding of the specific interaction between *Leishmania* parasites and their host antigen presenting cells is crucial to the understanding of the generation of protective immune responses to *Leishmania* infection.

1.4.3.1 Up-take of *Leishmania* by Macrophages

The receptors engaged by *Leishmania* promastigotes and amastigotes upon entry into host cells will have important consequences for their ability to avoid activation of these cells. Internalisation via different receptors will also determine the intracellular compartments in which the parasites initially reside, and therefore, access of the host processing and presentation machinery to *Leishmania* proteins.

Promastigotes and amastigotes at the site of infection will be exposed to serum proteins, including Complement components and Ig. Binding of antibodies and Complement components to invading microbes targets them for phagocytosis by mononuclear phagocytes via FcR and Complement Receptors (CR) respectively. The Complement system, which comprises a group of serum proteins that respond to infection via a system of enzymatic cascades, is composed of two converging pathways: the classical pathway is initiated by binding of complement proteins to antigen-antibody complexes; while the alternative pathway is initiated by microbial stimuli, without the need for specific antibodies. A third pathway, the lectin pathway is triggered on binding of the mannose-binding protein to mannose-containing components on the surface of pathogens (Parham 2000). The classical and alternative pathways both result in the proteolysis of a protein, C3 to C3b or iC3b by either the classical pathway or alternative pathway C3 convertase complex respectively. C3b coated molecules bind to Complement Receptor (CR)1, while iC3b binds to CR3. Binding of C3 proteolysis products to C3 convertase changes it to a C5 convertase which catalyses cleavage of C5 to C5b, this in turn triggers formation of a structure known as the membrane attack complex (MAC) by the soluble Complement components C5b to C9. The presence of the MAC on the surface of cells causes their lysis by osmosis through the formation of pores in the plasma membrane (Parham 2000). The consequence of Complement fixation on *Leishmania* infection is discussed below.

1.4.3.1.1 Uptake of *Leishmania* Promastigotes by Macrophages

The surface of *Leishmania* promastigotes is dominated by two molecules: lipophosphoglycan (LPG) and the metalloprotease gp63. *Leishmania* promastigotes bind

to mannose receptors (Blackwell 1985; Blackwell *et al.* 1985), and fibronectin receptors (Brittingham *et al.* 1999). However, uptake primarily occurs on binding to the Complement receptors CR1 and CR3 (Mosser and Edelson 1985; Da Silva *et al.* 1989; Mosser *et al.* 1992).

Promastigotes were originally thought to interact with components of the alternative Complement pathway since *L. major* promastigotes were shown to be bound predominantly by C3, which was then rapidly converted to iC3b (Mosser and Edelson 1985), probably due to the proteolytic activity of gp63 (Brittingham *et al.* 1995). A number of groups have documented the inhibition of uptake of *L. major*, *L. donovani* and *L. mexicana* promastigotes in the presence of anti-CR3 antibodies (Blackwell *et al.* 1985; Mosser and Edelson 1985; Talamas-Rohana *et al.* 1990; Mosser *et al.* 1992). In particular, Blackwell and colleagues (Blackwell *et al.* 1985) reported eighty percent inhibition of uptake of *L. donovani* promastigotes in the presence of anti-CR3 antibodies indicating that CR3 might play a dominant role in mediating their internalisation. Subsequent studies showed, however, that *L. major* promastigotes were also coated with C3b, a component of the classical pathway which mediates binding to CR1 (Puentes *et al.* 1988). Sub-division of the promastigote population into non-infective (peanut agglutinin (PNA) positive) log phase parasites and infective (PNA^-) metacyclic parasites demonstrated that it was infective metacyclic parasites which predominantly bound C3b, and that LPG appeared to be the acceptor for C3b molecules (Puentes *et al.* 1988). Procyclic parasites also fixed C3, which was, however, released on cleavage to iC3b. Thus, the different developmental stages of *L. major* promastigotes appear to bind different components of the Complement pathways, such that procyclic promastigotes interact with the alternative pathway while infective metacyclics interact with the classical pathway. Since different CR will mediate uptake of different forms of parasites, this might result in differential activation of host MΦ Leishmanicidal mechanisms. However, uptake by neither the C3b receptor nor the iC3b receptor induced the release of toxic oxygen from human cells (Wright and Silverstein 1983). Transformation of procyclics to metacyclics results in a thickening of the LPG glycocalyx (see Chaper 6, section 6.3). This transformation was associated with the shedding of membrane attack

complex components C5b-C9 from the surface of the parasites (Puentes *et al.* 1990), which supports data showing that infective, metacylic, promastigotes are more resistant to complement-mediated lysis than non-infective forms (Puentes *et al.* 1988). Therefore, *L. major* promastigotes have evolved so that the infective form of the parasite avoids Complement-mediated lysis, but can utilise binding by Complement components to facilitate their uptake by MΦ.

Leishmania promastigotes of all species bind directly to MΦ (Mosser 1994). Uptake of *L. major* promastigotes by MΦ in the absence of serum was blocked by anti-CR3 antibodies (Blackwell *et al.* 1985; Mosser and Edelson 1985). However, promastigotes incubated with MΦ acquired low amounts of MΦ-derived C3 on their surface, which may mediate this interaction (Wozencraft *et al.* 1986). Furthermore, Mosser and colleagues were unable to detect binding of *L. major*, *L. donovani*, or *L. mexicana* promastigotes to Mac-1 (CR3)-coated plates when incubated in serum depleted of C3 (Mosser *et al.* 1992). Gp63- or LPG-coated beads were taken up by MΦ and this could also be blocked by anti-CR3 antibodies (Russell and Wright 1988; Talamas-Rohana *et al.* 1990). Gp63 contains an RGD-like motif (Button and McMaster 1988; Miller *et al.* 1990) which is similar to that which mediates binding of iC3b to CR3 (Russell and Wright 1988), suggesting that a direct interaction could occur between gp63 and CR3. Uptake of *L. major* promastigotes was only inhibited by fifty percent on incubation of the MΦ cell line J774 with anti-gp63 F(ab)' fragments (Russell and Wilhelm 1986), however, and *L. amazonensis* promastigotes expressing a mutated form of the motif could bind MΦ as well as wild type parasites, indicating that other MΦ binding sites are also employed by the parasites (Brittingham *et al.* 1999).

The uptake of *Leishmania* promastigotes by MΦ is likely to involve multiple receptors. Sheep erythrocytes opsonised with Complement bound to MΦ, but were not phagocytosed, but cross-linking of gp63 to opsonised sheep erythrocytes led to an increase in the efficiency of binding and internalisation by MΦ (Brittingham *et al.* 1999). This suggests that receptors such as the fibronectin receptor (which binds to

gp63) may work in conjunction with Complement receptors to mediate internalisation of Complement-bound parasites.

1.4.3.1.2 Uptake of Amastigotes by Macrophages

In comparison to promastigotes, the amastigote surface is much less well defined, and is dominated by a small GPI-anchored glycolipid, EpiM3 (Winter *et al.* 1994). *L. amazonensis* amastigotes have some capacity for interacting directly with host cells via heparin-binding to cell surface heparin sulphate proteoglycans (Love *et al.* 1993) probably via a number of low affinity interactions with the MΦ glycocalyx (Peters *et al.* 1995). This may account for the ability of amastigotes to bind to a wide range of adherent cells, while promastigotes, which do not have this heparin-binding activity, are more restricted in the cells to which they adhere (Mosser 1994). *L. major* and *L. mexicana* amastigotes did not bind mannose receptors (Guy and Belosevic 1993; Peters *et al.* 1995), and neither *in vitro* cultured amastigotes, nor amastigotes isolated from SCID mice bound CR3 or FcR efficiently, indicating that the parasites do not bind these receptors in the absence of antibodies (Guy and Belosevic 1993; Peters *et al.* 1995). BALB/c-derived amastigotes are coated with Ig and C3, which mediates their uptake by FcR and CR3 respectively (Guy and Belosevic 1993). However, immunofluorescence analysis of lesion sections revealed that, *in vivo*, free *L. mexicana* amastigotes are bound by mouse IgG but are not coated with Complement (Peters *et al.* 1995). Mice lacking Ig, or the common γ chain of Fc receptors, were refractory to infection with *L. mexicana*, supporting the role for antibody binding of amastigotes and their consequent uptake by FcR during infection (Kima *et al.* 2000). Evidence that this was due to amastigote-specific antibodies came from experiments showing that large lesions developed in B cell-deficient mice that had been injected with serum from infected mice, or mice immunised with amastigote membrane components, but not in mice which had been given non-immune serum (Peters *et al.* 1995; Kima *et al.* 2000). Amastigotes must also bind receptors other than the FcR for their uptake, however, since amastigotes from SCID and B cell-deficient, mice are still internalised by MΦ (Guy and Belosevic 1993; Peters *et al.* 1995). A role has been proposed for *L. major* amastigote LPG in mediating binding to MΦ in a CR1- and CR3-independent manner (Kelleher *et al.* 1995). This was

via the phosphorylated oligosaccharide repeat region of *L. major* LPG and may be mediated by a lectin-like receptor (Kelleher *et al.* 1995).

1.4.3.2 Infection of Macrophages by *Leishmania*

Leishmania-infected MΦ play two important roles in determining the outcome of infection: first infected MΦ must be activated to kill intracellular parasites via the expression of microbicidal molecules in order to limit parasite numbers and ultimately to control the infection; second infected MΦ, or MΦ that have engulfed dead parasites, must present parasite-derived antigens to activated T cells at the site of infection. Survival of the parasites, however, will depend on their ability to interfere with both of these processes.

1.4.3.2.1 Inhibition of Activation of *Leishmania*-Infected Macrophages

Cytokine-activated MΦ are the principal Leishmanicidal cells; MΦ express inducible Nitric Oxide Synthase (iNOS) which produces NO from L-arginine. NO reacts with oxygen radicals to form toxic products that kill intracellular *Leishmania* (Green *et al.* 1990), and mice lacking iNOS are unable to control infection with *L. major* (Wei *et al.* 1995). MΦ production of IL-12 is also required for the development of IFNγ-producing Th1 cells that will in turn activate killing of *Leishmania* by infected MΦ. MΦ are not, however, activated simply by uptake of *Leishmania* parasites and low numbers of parasites persist in the LN and at the site of infection in an immune host (Aebischer *et al.* 1993; Schubach *et al.* 1998)

NO production is not triggered upon infection of MΦ by *Leishmania*, unless they are activated by an additional stimulus (Reiner *et al.* 1994; Carrera *et al.* 1996; Weinheber *et al.* 1998); and infection of MΦ by neither *L. major* promastigotes (Reiner *et al.* 1994; Carrera *et al.* 1996) nor *L. mexicana* amastigotes (Weinheber *et al.* 1998) induced production of IL-12. Indeed, infection with *L. mexicana* amastigotes and *L. major* metacyclic promastigotes suppressed production of IL-12 by MΦ (Belkaid *et al.* 1998; Weinheber *et al.* 1998). This inhibition of IL-12 is highly, selective since TNFα is secreted by infected MΦ upon activation *in vitro* (Carrera *et al.* 1996; Belkaid *et al.*

1998) and by infected MΦ *in vivo* (Gorak *et al.* 1998). The mechanisms leading to suppression of IL-12 production are not clear but may be related to receptor ligation on entry of *Leishmania sp.* into MΦ; ligation of FcγR or CR by erythrocytes opsonised with IgG or Complement respectively did not induce production of IL-12p40 and suppressed activation of MΦ by LPS (Sutterwala *et al.* 1997). Suppression of *L. mexicana* amastigote-infected MΦ was, however, independent of engagement of CR3 or FcγR (Weinheber *et al.* 1998), and phagocytosis of latex beads also resulted in inhibition of IL-12 production by MΦ in this study, indicating that this phenomenon is not specific to uptake of *L. mexicana* amastigotes (Weinheber *et al.* 1998). By comparison, IL-10 was produced upon ligation of FcR by lesion-derived, but not *in vitro*-derived axenic *L. major* amastigotes (Kane and Mosser 2001). Inhibition of IL-12 on ligation of the FcγR was shown to be due to specific suppression of the IL-12 p35 and p40 genes (Sutterwala *et al.* 1997). However, inhibition of IL-12 on infection of MΦ with *Leishmania* is controlled both at the transcriptional (Carrera *et al.* 1996; Piedrafita *et al.* 1999) and post-transcriptional (Weinheber *et al.* 1998) levels. The molecular interactions which result in the specific suppression of IL-12 thus remain unclear. However, purified LPG was shown to inhibit IL-12 production in the absence of infection (Piedrafita *et al.* 1999).

1.4.3.2.2 Stimulation of Primed T Cells by *Leishmania*-Infected Macrophages

A number of studies have shown that the kinetics of presentation of parasite antigens to $CD4^+$ T cells are impaired in MΦ infected with different *Leishmania* species (Wolfram *et al.* 1995; Kima *et al.* 1996; Prina *et al.* 1996), and that this deficiency extended to the processing of exogenously supplied antigens (Fruth *et al.* 1993; Prina *et al.* 1993). However, inefficient presentation of exogenous antigens could be overcome by increasing antigen concentration (Prina *et al.* 1993) and by using peptide antigens (Fruth *et al.* 1993), suggesting defects in loading of MHC II and processing of parasite proteins respectively. Presentation of endogenous parasite antigens can be enhanced by killing the parasites within MΦ (Wolfram *et al.* 1995), showing that compartmentalisation of proteins within the parasites plays an important role in the inefficient processing and

presentation of *Leishmania* antigens by infected MΦ. MΦ infected with *L. major* and *L. donovani* promastigotes transfected with an episomal vector, pX, carrying the *ovalbumin* and *lacZ* genes efficiently stimulated OVA- or β-galactosidase-specific T cells, respectively, *in vitro* (Kaye *et al.* 1993). Likewise, *L. mexicana* promastigotes over-expressing an endogenous protein, the membrane-bound acid phosphatase, also stimulated an efficient T cell response *in vitro* (Wolfram *et al.* 1996). Collectively, these studies showed that parasite-derived antigens localised to the PV could be processed and presented to T cells in the context of MHC II. Thus, there is no functional defect in MHC II presentation pathways in infected cells, and vesicular flow can occur from the PV to the plasma membrane. Promastigote-infected MΦ could stimulate T cells specific for the intracellular protein, LACK, but this ability was lost 48 hours post-infection, and amastigote-infected cells were never able to activate LACK-specific T cells (Prina *et al.* 1996). Furthermore, when promastigotes were sub-divided into procyclic and metacylic forms only the procyclic parasites could activate LACK-specific T cells (Courret *et al.* 1999). This indicates that the infective forms of the parasite have evolved so that immuogenic molecules are not easily accessible to the host processing and presentation machinery.

Infection of MΦ by *L. donovani* did not lead to up-regulation of the co-stimulatory molecule B7-1, and these cells were unresponsive to activation with LPS. Thus, rather than avoiding presentation to T cells, infected MΦ could also promote anergy of parasite-specific T cells by providing signal 1 in the absence of signal 2 (Kaye *et al.* 1994).

1.4.3.2.3 MHC II Loading Compartments in *Leishmania*-Infected Macrophages

Processing of *Leishmania* antigens in infected cells will require convergence of the PV with the endosomal pathway. PV are late endosomal/early lysosomal, LAMP-1$^+$ compartments (Russell *et al.* 1992; Antoine *et al.* 1998), which share similarities to MIIC: they contain the host proteases Cathepsins B, D, H and L (Prina *et al.* 1990); MHC II but not MHC I molecules (Antoine *et al.* 1991; Lang *et al.* 1994); and the

loading catalyst, H2-M (Antoine *et al.* 1999). Therefore, PV are potential sites for processing and loading of *Leishmania* antigens onto MHC II molecules. Biosynthesis and steady-state levels of MHC II are unaltered by infection, (Antoine *et al.* 1991; Lang *et al.* 1994) although immunohistochemical studies have documented the redistribution of MHC II molecules to the limiting membrane of the PV in IFNγ-activated MΦ (Antoine *et al.* 1991; Lang *et al.* 1994; De Souza Leao *et al.* 1995). MHC II molecules in the PV tend to be concentrated at the point at which amastigotes bind to the membrane, a phenomenon known as "capping" (Antoine *et al.* 1991; De Souza Leao *et al.* 1995). Parasites attach to this point at the site of the megasome, a lysosome-like organelle at the posterior pole of the parasite that contains abundant cysteine proteases. Treatment of infected MΦ with protease inhibitors resulted in an increase in PV-associated MHC II, Ii and amastigote-associated MHC II molecules (De Souza Leao *et al.* 1995). Parasites in infected cells were also shown to co-localise with MHC II and H2-M by confocal microscopy (Antoine *et al.* 1999; Courret *et al.* 2001). These observations suggest that *Leishmania* parasites interact with components of the host processing and presentation machinery in the PV of infected cells. Whether this is due to active mechanism on the part of the parasite, however, remains open to debate.

Parasite antigen-MHC II complexes have never been visualised in infected cells. Thus, it is not know whether parasite antigens complex with MHC II molecules in the PV, for subsequent transport directly to the cell surface, or whether *Leishmania* antigens are transported out of the PV to meet MHC II molecules at a different point along the endosomal pathway. The observation of Wolfram and colleagues (Wolfram *et al.* 1996) that degradation of intracellular parasites, or over-expression of a membrane-bound or secreted antigen, is sufficient to restore efficient presentation of *Leishmania* antigens to T cells strongly implies that the lack of presentation reflects inaccessibility of parasite-derived antigens, rather than an active block on the part of the parasite. In this case, co-localisation, and the implied sequestration, of MHC II molecules with *Leishmania* parasites would not necessarily represent an active mechanism on the part of the parasite to prevent presentation of parasite-derived antigens to $CD4^+$ T cells, but would simply

reflect non-specific transport of nutrients into the PV via the endocytic pathway, and general degradation of proteins in late endosomes/early lysosomes.

1.4.4 The Interaction Between DC and *Leishmania* Parasites

The requirement for DC to stimulate a primary T cell response, and the relatively poor presentation of parasite antigens by infected MΦ, has lead to the proposition that infected DC are responsible for initiating the anti-*Leishmania* T cell response. Moll *et al* demonstrated internalisation of *L. major* promastigotes by LC in the epidermis of the skin (Moll *et al.* 1993), and showed that LC incubated with promastigotes or amastigotes could stimulate T cells from infected mice (Will *et al.* 1992). Infection of mice with *L. major* promastigotes led to the loss of LC in the epidermis overlying the site of infection, (Blank *et al.* 1993) and the migration of fluorescently-labelled amastigote-infected epidermal cells from the skin to the LN was observed (Moll *et al.* 1993). Parasite-primed T cells were specifically stimulated *in vitro* by NLDC 145^+ cells from these LN, i.e. DC (Moll *et al.* 1993). *Leishmania*-infected LC are also found in the epidermis, dermis and draining LN of infected humans (ElHassan *et al.* 1995). Therefore it was proposed that DC are infected in the skin and migrate to the draining LN where they prime the primary anti-*Leishmania* T cell response (Moll *et al.* 1993). The DC:*Leishmania* interaction has not, however, been well characterised and initial studies have produced conflicting data on whether uptake of *Leishmania* parasites activated DC, possibly reflecting differences in the species of parasite used and the population of DC infected. Infection of LC with *L. major* parasites, or lysates, prevented the down-regulation in MHC II biosynthesis normally seen after LC culture *in vitro* (Flohe *et al.* 1997), indicating suppression of maturation of these cells. However this observation may be a general phenomenon related to non-specific phagocytosis (Scheicher *et al.* 1995). Uptake of lesion-derived *L. major* amastigotes, but not promastigotes, by foetal skin-derived DC induced the up-regulation of MHC II, CD40, CD54, CD86 and TNFα. IL-12 p40 was induced upon infection with these amastigotes but the bioactive p70 heterodimer could only be detected when cultures were co-stimulated with IFNγ or

CD40L (von Stebut *et al.* 1998; von Stebut *et al.* 2000). Infection of foetal skin-derived DC with *L. major* amastigotes down-regulated E-cadherin expression, which is associated with migration of LC out of the epidermis *in vivo* (Tang *et al.* 1993; von Stebut *et al.* 1998). In agreement with these studies, Konecny *et al* (Konecny *et al.* 1999) showed that uptake of *L. major* promastigotes by splenic DC did not affect surface expression levels of any of the activation markers tested. By comparison, uptake of both *L. amazonensis* promastigotes and amastigotes activated bone marrow-derived DC (Qi *et al.* 2001). Marovich *et al* (Marovich *et al.* 2000) used the promastigote surface molecule, LPG, to shown selective activation of infected cells, however, staining for the presence of LPG would not have distinguished between DC which had taken up live, intact or degraded parasites. $CD11c^+$ splenic DC were induced to produce IL-12 p40 upon infection with *L. major* promastigotes (Konecny *et al.* 1999), and, in agreement with the need for synergy between a microbial stimulus and CD40 ligation to induce optimal levels of IL-12 (Schulz *et al.* 2000), promastigote-infected human DC secreted high amounts of IL-12 p70 upon ligation of CD40 (Marovich *et al.* 2000).

DC infected with *Leishmania* parasites *in vitro* can clearly be used to generate a primary T cell response in mice. Vaccination of BALB/c mice with LC incubated with *L. major* promastigote lysate, but not with lysate alone, immunised mice against challenge with live parasites. This was associated with decreased footpad swelling and a bias towards a healing Th1 response (Flohe *et al.* 1998). Secondary challenge of healed, immunised mice with *L. major* promastigotes resulted in more rapid healing, indicating that a memory repsonse had been established. Likewise, injection of *L. major* amastigote-infected BALB/c-derived DC into syngeic mice resulted in an enhanced ability to resolve the infection (von Stebut *et al.* 2000). These studies do not, however, address the interaction between DC and *Leishmania* during the course of a natural infection.

In vivo, parasites and parasite-derived material were detected in the LN (Moll *et al.* 1993) and spleen (Gorak *et al.* 1998) of infected mice. IL-12 p40 was detected by immunohistochemistry in DC on sections of spleens from *L. donovani*-infected mice one day after infection (Gorak *et al.* 1998), although it was impossible to confirm whether

IL-12 was specific to cells containing whole amastigotes. This expression was transient and was down-regulated three days post-infection.

DC from BALB/c or C57BL/6 and C3H/HeJ mice showed no difference in their ability to ingest *L. major* or *L. amazonensis* parasites, and were equivalent in the upregulation of surface DC activation markers upon infection with lesion-derived amastigotes (von Stebut *et al.* 2000; Qi *et al.* 2001). However, BALB/c DC infected with *L. amazonensis* amastigotes secreted significantly more IL-4 than C3H/HeJ DC *in vitro* , and transfer of amastigote-infected BALB/c-derived DC into non-infected BALB/c recipients induced production of IL-4 from cells of the draining LN when restimulated with parasite lysate. This was not seen when C3H/HeJ DC or BALB/c IL-$4^{-/-}$ DC were infected and adoptively transferred. IFNγ was not induced in the LN of recipient mice. These data imply that infection of BALB/c DC with *L. amazonensis* amastigotes activates them such that they are polarised to stimulate a Th2 response *in vivo*. This study did not, however, distinguish between the effect of the activated DC, and the effect of the presence of *Leishmania* parasites in the recipient mice (Qi *et al.* 2001).

Thus, DC are clearly infected by *Leishmania* parasites *in vivo* and DC can efficiently stimulate the anti-parasite T cell response. However, whether the cells that initiate this response contain live, intact parasites and whether infection of DC by *Leishmania* at the site of infection is sufficient to activate this response remains unclear. Evidence from other systems that DC polarise the effector Th response (section 1.2.5) and the data from (Qi *et al.* 2001) suggest that uptake of *Leishmania* parasites by DC from susceptible and resistant strains of mice may play an important role in polarisation of the subsequent T cell response. However, further experiments are required to determine the ability of *Leishmania*-infected DC to polarise Th1 versus Th2 responses, and also whether receipt of *L. mexciana*-encoded or *L. major*-encoded signals by DC may explain the differential ability to C57BL/6 mice to control infection with these parasites.

1.4.5 Summary II: The Significance of Infection of APC by *Leishmania* to the Development of an Anti-*Leishmania* Immune Response

The expansion of parasite-specific IFNγ-secreting Th1 cells is critical for the ability of an infected host to resolve infection with *Leishmania* (Figure 1.5). *Leishmania* parasites reside within the cells that are ultimately responsible for their clearance, however, activation of infected cells depends on IFNγ secretion by Th1 $CD4^+$ T cells and evolutionary pressure will select parasites that minimise antigen presentation by infected cells.

Phagocytosis of opsonised particles in the absence of other exogenous stimuli does not result in the activation of MΦ, thus triggering of the activation of immune cells on uptake of cell debris in the absence of an infection or trauma is avoided. *Leishmania* promastigotes have apparently exploited this mechanism by using the same receptors to mediate their entry into host MΦ. It has recently been demonstrated, furthermore, that amastigotes bind phosphatidylserine (PS) which is normally displayed by apoptotic cells. Recognition of this molecule by MΦ induces transforming growth factor (TGF)-β and IL-10 production, resulting in the entry of amastigotes into cells without induction of an inflammatory response (de Freitas Balanco *et al.* 2001). Thus, infected MΦ are not stimulated by infection with *Leishmania* and become refractory to stimulation by exogenous stimuli. Lack of activation of Leishmanicidal activities on uptake of *Leishmania* parasites allows a delay in the initiation of the anti-parasite T cell response. During this time promastigotes will transform into amastigotes, which are better adapted for intracellular survival and display fewer potential antigens on their surface. The paucity of *Leishmania*-derived T cell antigens that are accessible to the host processing and presentation pathway apparently represents an additional strategy evolved by the parasites to avoid activating a deleterious T cell response.

DC containing *Leishmania* parasites have been shown to produce IL-12 *in vivo*. This will polarise the development of Th1 cells upon presentation of *Leishmania* antigens by DC to naïve T cells in the LN. IFNγ production by activated Th1 cells will activate destruction of intracellular parasites by MΦ, and will up-regulate surface expression of peptide-MHC II molecules, thus facilitating the interaction with activated Th1 cells, and augmenting the response. Degradation of intracellular parasites will result in an increase the pool of accessible antigens that can be presented by MHC II molecules (Overath and Aebischer 1999).

1.5 Aims and Experimental Approach

DC are critically important for the initiation of the primary T cell response against foreign organisms. Recognition of conserved determinants on the surface of pathogens results in the activation of DC to a mature form that expresses high levels of co-stimulatory molecules and other surface molecules necessary for the interaction with naïve T cells. Peptide fragments from phagocytosed organisms, which were taken up at the site of infection, are displayed on the surface of these DC in the context of MHC II molecules. It is clear from the literature that DC internalise *Leishmania* parasites at the site of infection, and that DC prime parasite-specific T cells in the draining LN of infected mice. The data reported in the literature, however, do not demonstrate whether uptake of the parasites *per se* is sufficient to activate immature DC, that is, whether *Leishmania* parasites express PAMP on their surface. Furthermore, it is not known whether the primary anti-*Leishmania* T cell response is primed by DC presenting antigens generated from intracellular parasites.

The aim of this thesis was to address the hypothesis that infection of DC by *Leishmania* parasites is sufficient to activate the primary anti-parasite T cell response. This hypothesis was addressed through two aims: first to develop an experimental model to investigate the fate of *Leishmania* antigens in infected cells; second to examine the *Leishmania*:DC interaction *per se* in a defined *in vitro* model.

Chapter three addresses the first aim and describes the development of a model system with which to follow the fate of *Leishmania*-derived antigens in infected DC. In this experimental model the MHC II-restricted T cell epitope MCC is transgenically expressed by *L. mexicana* parasites. A complex-specific mAb, or MCC-specific T cells, were used to detect the formation of MHC II-MCC complexes in infected cells. This chapter addresses the problems encountered in establishing this experimental model. Chapters four, five and six address the second aim. Chapter four describes the use of an experimental model in which *in vitro*-cultured *L. mexicana* parasites are incubated with bone marrow-derived DC. The effect of uptake of the parasites on the maturation status of the DC is examined. In chapter five the interaction between *L. mexicana* parasites and a splenic DC culture is investigated. The use of this DC culture allowed the effect of long-term infection of DC by *L. mexicana* parasites to be investigated. Finally, chapter six describes the use of this *in vitro* model to identify *L. mexicana* LPG as a putative *Leishmania* PAMP.

Chapter 2: Materials and Methods

Unless otherwise stated, analytical grade chemicals were obtained from either Sigma or BDH Laboratory supplies (Merck Ltd.). Analytical grade agarose was supplied by Biowhittaker Molecular Applications. All bacterial media components were supplied by DIFCO laboratories. Synthetic oligonucleotides were synthesised by OSWEL DNA service (University of Southampton, U.K.). Radioisotopes were supplied by Amersham International plc (Little Chafont, U.K.). All tissue culture media were bought from Sigma or GibcoBRL.

2 x Semi-defined medium (SDM) (all components from GibcoBRL or Sigma)

Components	**Amount for 10L**
Minimum essential medium (S-MEM) powder (with Earle's salt, L-glutamine, without sodium carbonate)	140g
M199 medium powder (with Hank's solution, L-glutamine, without sodium carbonate)	40g
MEM essential amino acids	160ml
MEM non essential amino acids	120ml
Glucose	20g
Hepes buffer	160g
Mops buffer	100g
$NaHC0_3$	40g
Sodium pyruvate	2g
L-alanine	4g
L-glutamine	6g
L-arginine	2g
L-methionine	1.4g
L-phenylalanine	1.6g
L-proline	1.2g
L-serine	1.2g

L-taurine	3.2g
L-threonine	7g
L-tyrosine	2g
Adenosine	0.2g
Guanosine	0.2g
Glucosamine-HCl	1g
Folic acid	0.08g
p-aminobenzoic acid	0.04g
Biotin	0.004g

The medium was made without $NaHCO_3$, the pH adjusted to 7.0, then $NaHCO_3$ added and the pH corrected to 7.3. Medium was filter sterilsed and stored at –20°C.
For use 2 x SDM was diluted to 1 x with sterile H_2O.

2.1 Molecular Biology Methods

General molecular biology techniques and the preparation of standard solutions was carried out according to (Sambrook *et al.* 1989) unless otherwise stated. Restriction digests were performed as recommended by the suppliers (New England Biolabs and Roche). Digestion products were routinely analysed by agarose gel electrophoresis with gels cast and run in 1 x TAE buffer at an appropriate concentration containing 0.5µg/ml ethidium bromide. 0.5µg of 1kb DNA ladder (GibcoBRL) was loaded on each gel as a size standard.

2.1.1 General Cloning Techniques

DNA fragments were routinely subcloned by restriction enzyme digestion and cloning into plasmids. Taq polymerase PCR products (which often have a deoxyadenosine added to the end) were subcloned using the TOPO TA Cloning® kit (Invitrogen), according to the manufacturer's instructions.

2.1.1.1 Gel purification of DNA

Restriction fragments for subcloning, plasmid vectors and PCR products were purified as follows. DNA fragments were run on 0.8% agarose gels until suitable separation had occurred to isolate single bands. Bands of interest were excised from the gel slice using the Geneclean® kit (Bio 101 inc., U.S.A.), according to the manufacturer's instructions.

2.1.1.2 Modification of Enzyme Sites with the Klenow Fragment

Restriction enzyme sites with cohesive ends were ablated when necessary using the Klenow fragment. DNA was digested and incubated with 1U Klenow (NEB) per µg DNA and 33µM dNTP in 1 x restriction enzyme buffer (NEB), for 15 minutes at 25°C. The enzyme was then heat inactivated at 75°C for 10 minutes. The digested DNA was then religated as a blunt end ligation.

2.1.1.3 Ligations

For ligations, plasmid vectors were digested and gel purifed. Restriction fragments were also gel purified. To prevent re-ligation of empty vectors, purified, linearised vectors were dephosphorylated by treatment with Shrimp Alkaline Phosphatase (SAP) (Amersham). The DNA was incubated with 1µl (1 unit) of SAP in 1 x SAP buffer for 90 minutes at 37°C, then at 65°C for 15 minutes to inactivate the SAP.

Ligations were set up with 1:1 and 1:10 vector:insert ratio of molar ends with 1µl (1 unit) of T4 DNA ligase (Roche) and 2µl of 10 x ligase buffer (660mM Tris.HCL pH7.5; 50mM $MgCl_2$; 10mM ATP; Roche) to a final volume of 20µl. Cohesive end ligations were performed at room temperature for at least 1 hour, blunt end ligations were incubated overnight at 18°C. To determine the background level of vector re-ligation, a control reaction containing vector alone was also set up.

2.1.1.4 Transformations

Competent DH5α bacteria were routinely used for chemical transformations. KCM-competent bacteria were prepared as described by (Chung and Miller 1988). A single bacterial colony from a freshly streaked plate was used to innoculate 5mls Luria Broth

(LB) culture media (1% w/v tryptone; 0.5% w/v yeast extract; 85mM NaCl) which was grown at 37°C overnight with shaking. The 5ml overnight culture was added to 500ml LB media and the culture grown to A_{600}=0.3-0.6 to ensure the cells were in log phase. The cell suspension was split between two tubes and centrifuged at 3000 rpm, in a Sorvall GSA rotor, for 10 minutes at 4°C. The cell pellet was resuspended in 1/20 volume ice cold, sterile LB pH6.1, supplemented with 10% PEG Mw 3350; 5% DMSO; 10mM $MgCl_2$; 10mM $MgSO_4$ and 10% glycerol, and incubated on ice for 10 minutes. Cells were dispensed into 100µl aliquots in pre-chilled eppendorf tubes and snap frozen in liquid nitrogen before storage at –80°C.

10µl of the ligation reaction was mixed with 20µl 5 x KCM (0.5M KCl; 0.15M $CaCl_2$; 0.25M $MgCl_2$) in a final volume of 100µl, and chilled on ice. Frozen competent bacteria were thawed on ice. 100µl of cells were added to the KCM-ligation mix, incubated on ice for 20 minutes then transferred to room temperature for 10 minutes. 1ml of LB was added and the sample incubated at 37°C for at least 30 minutes. 0.1ml and 0.9ml were plated out onto LB agar plates (1.5% w/v agar in LB) with 50µg/ml ampicillin or 50µg/ml kanamycin.

2.1.1.5 Screening Transformants

Recombinant bacterial colonies were screened for presence of inserts by plasmid DNA preparation (section 2.1.2.1) and subsequent restriction enzyme analysis, or by amplifying PCR products directly from picked colonies (section 2.1.7.1).

2.1.2 Isolation of Nucleic Acids

2.1.2.1 Plasmid Preparation

Plasmid isolation was either performed using Qiagen plasmid isolation kits (Qiagen) according to the manufacturers instructions, or by the following protocol taken from (Zhou *et al.* 1990)

A single colony was picked with a sterile yellow tip into 5mls of LB with the appropriate antibiotic and the culture incubated overnight at 37°C, with shaking. 1.5ml of overnight culture was centrifuged for 1 minute at 13 000 rpm (microfuge) to pellet the cells. The supernatant was tipped off and the pellet resuspended in the remaining 50-100µl by vortexing. 300µl TENS (10mM Tris.HCl pH8.0; 1mM EDTA pH8.0; 100mM NaOH; 0.5% SDS) was added, the solution vortexed for 4 seconds and placed on ice. To this tube was added 150µl of 3M NaOAc pH 5.2 and the tube vortexed for 3 seconds and put on ice again. Samples were then centrifuged at 13 000 rpm for 10 minutes. The supernatant was transferred to a fresh tube and the samples spun again to precipitate all particles. The supernatant from the second spin was transferred to fresh tubes and mixed with 900µl 100% ethanol (stored at –20°C). The DNA was pelleted by centrifugation at 13 000 rpm for 15 minutes. The DNA pellet was washed in 70% ethanol, air-dried and resuspended in 40µl of H_2O. DNA concentration was determined by absorbance at 260nm.

DNA prepared this way was of sufficient quality for diagnostic digest analysis. If DNA was to be used for sequencing reactions (section 2.1.6) it was prepared using Qiagen miniprep kits. For all midi and maxi preps DNA was prepared using Qiagen plasmid purification kits.

2.1.2.2 Isolation of *Leishmania* Genomic DNA

1ml of parasite culture was pelleted at 6000 rpm for 10 minutes. The cells were resuspended in 450µl P1 buffer (Qiagen: 50mM Tris-HCl, pH8.0; 10mM EDTA, 100µg/ml Rnase A) and 50µl 10% SDS, and incubated at room temperature for 5 minutes. 2µl of a stock solution of Proteinase K (20mg/ml; Sigma) was then added and the tubes incubated at 55°C for at least 3 hours. The DNA was purified by sequential extraction with 500µl (1 volume) phenol, 500µl 1:1 phenol/chlorofom and 500µl chloroform. The DNA was precipitated with 0.1 volumes 3M NaOAc and 2.5 volumes 100% ethanol, and resuspended in 20-30µl sterile H_2O.

2.1.2.3 Isolation of RNA

Total RNA was isolated from promastigotes using Trizol® reagent (Gibco). Approximately $1x10^7$ parasites were pelleted at 6000 rpm for 10 minutes. The pellet was resuspended in 1ml Trizol and incubated for 5 minutes at room temperature to allow complete lysis of the cells. RNA was isolated by adding 200µl chloroform, shaking for 15 seconds followed by centrifugation at 13 000 rpm for 15 minutes. The aqueous phase was transferred to a fresh tube and RNA was precipitated by addition of 500µl isopropanol. After incubation for 10 minutes at room temperature the samples were centrifuged at 13 000 rpm for 10 minutes at 4°C. The RNA pellet was washed in 1ml 75% ethanol, air-dried and resuspended in 20µl RNase-free H_2O (Qiagen). The RNA concentration was determined by absorbance at 260nm. Samples were stored at –80°C.

2.1.3 Northern Blotting

5µg total *Leishmania* RNA was separated on a denaturing formaldehyde gel as follows. 0.8g of agarose were dissolved in 77ml H_2O and boiled. The solution was allowed to cool slightly and 10ml 10 x MOPS (200mM MOPS; 50mM NaOAc; 10mM EDTA; pH 7.0) and 5.1 ml 37% formaldehyde were added to make a 0.8% agarose gel. The required volume of RNA was made up to 5µl with RNase-free H_2O and mixed with 25µl sample buffer (50% formamide; 2.2M formaldehyde; 1 x MOPS; 5% glycerol; 5% 2.5% bromophenol blue) and 0.125µl ethidium bromide (10mg/ml). Samples were loaded onto the gel and electrophoresis performed at 80V in 1 x MOPS.

The gel was washed in 0.1% DEPC-treated H_2O and photographed. A piece of Hybond N^+ nylon membrane (Amersham) was cut to the size of the gel, and a capillary blot was set up and left overnight according to the Hybond manufacturers instructions. The membrane was removed after blotting, rinsed in H_2O and baked at 80°C for 30 minutes.

2.1.4 Radio-labelling Probes

DNA PCR products were radiolabelled by random priming (Sambrook *et al.* 1989). PCR products were gel purified (section 2.1.1.1) and the concentration determined by absorbance at 260nm. Random priming was performed using High Prime (Roche) according to the manufacturer's instructions.

25ng of DNA was adjusted to a final volume of 11µl with H_20 and denatured by boiling for 10 minutes and chilling on ice. On ice, 4µl High Prime (containing 1U/ml Klenow polymerase; 0.125mM dATP; 0.125mM dGTP; 0.125mM dTTP) and 5µl (50µCi) [α^{32}P]dCTP were added to the DNA and the reaction incubated at 37°C for 10 minutes. The reaction was stopped by adding 2µl 0.2M EDTA (pH 8.0). Unincorporated nucleotides were removed by centrifugation of the reaction through a G-50 sephadex column at 2000 rpm (microfuge) for 2 minutes. Prior to hybridisation the probe was denatured at 100°C for 10 minutes, snap cooled on ice and added directly to the hybridisation mix.

2.1.5 Hybridisation Conditions

All hybridisations and washes were performed in Techne hybridisation bottles rotating in a Techne HB-1 oven using 20ml hybridisation buffer per filter. Prehybridisation, hybridisation and washes were all carried out at 65°C.

Filters were prehybridised for at least 15 minutes in Rapid-hyb buffer (Amersham). Denatured radiolabelled probe was added to the same hybridisation buffer for 2.5 hours. Following hybridisation, filters were washed briefly with 2 x SSC; 0.1%SDS at room temperature followed by 2 x 20 minute washes with the same wash solution and 2 x 20 minute washes with 1 x SSC; 0.1% SDS, all at 65°C. After washing, filters were rinsed in H_2O, wrapped in Saran Wrap and exposed to autoradiographic film at –70°C for the appropriate length of time (1-7 days) for a signal to appear.

2.1.6 DNA sequencing

The manipulation of raw sequence data was performed using Genejockey II for Macintosh.

2.1.6.1 Automated Cycle Sequencing

Automated cycle sequencing was performed using the Perkin-Elmer Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). The cycle sequencing reaction is a modification of the dideoxy-termination method of (Sanger *et al.* 1977). The four 2',3'-dideoxynucleoside 5'triphosphates (ddNTP) are covalently linked to different fluorescent dyes allowing the sequencing reaction to be carried out in a single tube.

Plasmid DNA was prepared using a Qiagen miniprep kit. The DyeDeoxy terminator cycle sequencing reactions were carried out according to the manufacturers instructions. To conserve reagents, half the amounts were used (200-500ng plasmid DNA, 1.6pM primer (section 2.1.6.2), 4µl terminator ready reaction mix and sterile H_2O to 10µl volume). The reactions were subjected to 25 PCR cycles of 96°C for 30 seconds; 50°C for 20 seconds; 60°C for 4 minutes. The PCR products were precipitated with 1µl 3M NaOAc, pH 5.2 and 100% ethanol on ice for 20 minutes. The DNA pellet was washed in 70% ethanol, air-dried and resuspended in 4µl loading buffer (5mM EDTA pH8.0; 10mg/ml Blue dextran in deionised formamide). The sequencing reactions were denatured at 95°C for 2 minutes and 2µl run on a denaturing polyacrylamide gel (7M urea; 5% acrylamide (29:1 Biorad); 1 x TBE; 0.06% ammonium persulphate; 15ml TEMED per 50ml mix) in 1 x TBE on the ABI PRISM 377 DNA Sequencer. Sequencing gels were run by the CGR sequencing facility.

2.1.6.2 Sequencing Primers

For all constructs PCR primers used for the cloning strategies were also used in sequencing reactions. Additional sequencing primers were:

a) GST-specific primers

GST-int F': 5' GCGTGCAGAGATTTCAATGC 3'

GST-int R': 5' GGATGGGTTACATGATCACC 3'

b) MBAP-specific primers

HA SEQ F': 5' CCTCTACCGGTGCGTGGTGGT 3'

1203F': 5' CCACCTGGCGAAACTGCTGGT 3'

1531R': 5' ACGTGTTGCCCGTCGCGTCC 3'

MBAP01 F': 5' GTGGTGTCGTGTGCACTGTACTCG 3'

c) Gp63-specific primers

Gp63-803F': 5' ACATCACGGAAGGCGTGACC 3'

Gp63-1462R': 5'ACAAGTTCCGACTCTCAGACG 3'

2.1.7 Polymerase Chain Reaction

All PCR reactions were carried in a GeneAmp 9700 thermal cycler (PE Applied Biosystems). dNTPs and Taq polymerase were supplied by Roche. PCR reactions to clone genes were performed using a proof-reading Taq polymerase, (Expand™; Roche) and a high concentration of template with a low number of cycles to reduce the frequency of Taq-induced mutations in the amplified product.

2.1.7.1 PCR to Screen Transformants

Single bacterial colonies were picked with a sterile yellow tip, then both mixed with 5µl H_2O in a thin walled 0.2ml PCR tube and smeared on a gridded bacterial agar plate. 5µl of a PCR mix was added containing 1µl forward primer (100ng/ml), 1 µl reverse primer (100ng/ml), 2µl 2mM dNTPs, 1 x PCR buffer (containing 50mM KCl, 2.5mM $MgCl_2$; Roche) and 0.1 units Taq. PCR reactions were performed using appropriate internal primers.

2.1.7.2 PCR on Genomic DNA

To 100ng genomic DNA was added 2µl 10 x PCR buffer; 2µl forward primer (100ng/µl); 2µl reverse primer (100µl/ml); 2µl 2mM dNTPs; 0.1units Taq and the volume adjusted to 20µl with sterile H_2O.

The PCR conditions were:

GST-L (GST-int-XhoI F' and GST-int-XbaI; PromR' and GST-int R'): 95°C for 5 minutes followed by 30 cycles of 95°C 20s; 58°C 30s; 72°C 2 minutes; followed by 72°C 7 minutes.

CLB clones (CLB2: MCC1 F' and 1531R'; HA SEQ F' and MCC-Hind R'): 95°C for 5 minutes followed by 25 cycles of 95°C 30s; 58°C 30s; 72°C 1.5 minutes; followed by 72°C 7 minutes.

GP63 (gp63-XhoI F' and gp63-XbaI R'): 95°C for 2 minutes followed by 30 cycles of 95°C 30s; 58°C 20s; 72°C 2 minutes; followed by 72°C 7 minutes.

Integration PCR (Prom R' and SL-pX): 95°C for 5 minutes followed by 95°C 30s; 55°C 30s; 72°C 2 minutes; followed by 72°C 7 minutes.

Control PCR on promoter region (Prom R' and Prom F'): 95°C for 1 minute followed by 30 cycles of 95°C 30s; 58°C 30s; 72°C 30s; followed by 72°C 7 minutes.

PCR products were analysed directly on a 0.8% TAE agarose gel.

2.1.7.3 Primers used for PCR

	5'	3'
GST-int XhoI-BsrGI F':	GGCTCGAGCCCTGTACATGTCCCCTATACTAGG	
GST-int XbaI R':	GGTCTAGATTCAGTCAGTCACGATGAATTCCC	
SL-Px R':	GCCTCTGAGCATCTCGAGGCTAGCCTCCAGGGC	
MCC-1 F':	GGGTGTACACCGCGAACGAGCGCGCGGACCTGATCGCGTACC TGAAGCAGGCGACGAAGCGGTACCGGG	
Hind-MCC R':	AGCTTGCCTTCGTCGCCTGCTTCAGGTACGCGATCAGGTCCG CGCGCTCGTTCGCGTTAACGA	
Gp63-XbaI R':	TCTAGATTCAGACGTCCTTGGCAGCTTTGACG	
PROM F':	TTGTGTGACAACGTGAGTCG	
PROM R':	CATGTTTTGTGTGTGCTGCC	

2.1.8 Generation of Constructs

2.1.8.1 MCC Peptide Sequences

The following MCC peptide sequences were used for the generation of MCC fusion proteins (Reay *et al.* 1994):

$MCC_{(88\text{-}103)}$: ANERADLIAYLKQATK

$MCC_{(95\text{-}103)}$: IAYLKQATK

For all MCC sequences the DNA sequence was corrected for optimal codon usage by *Leishmania* parasites according to (Langford *et al.* 1992).

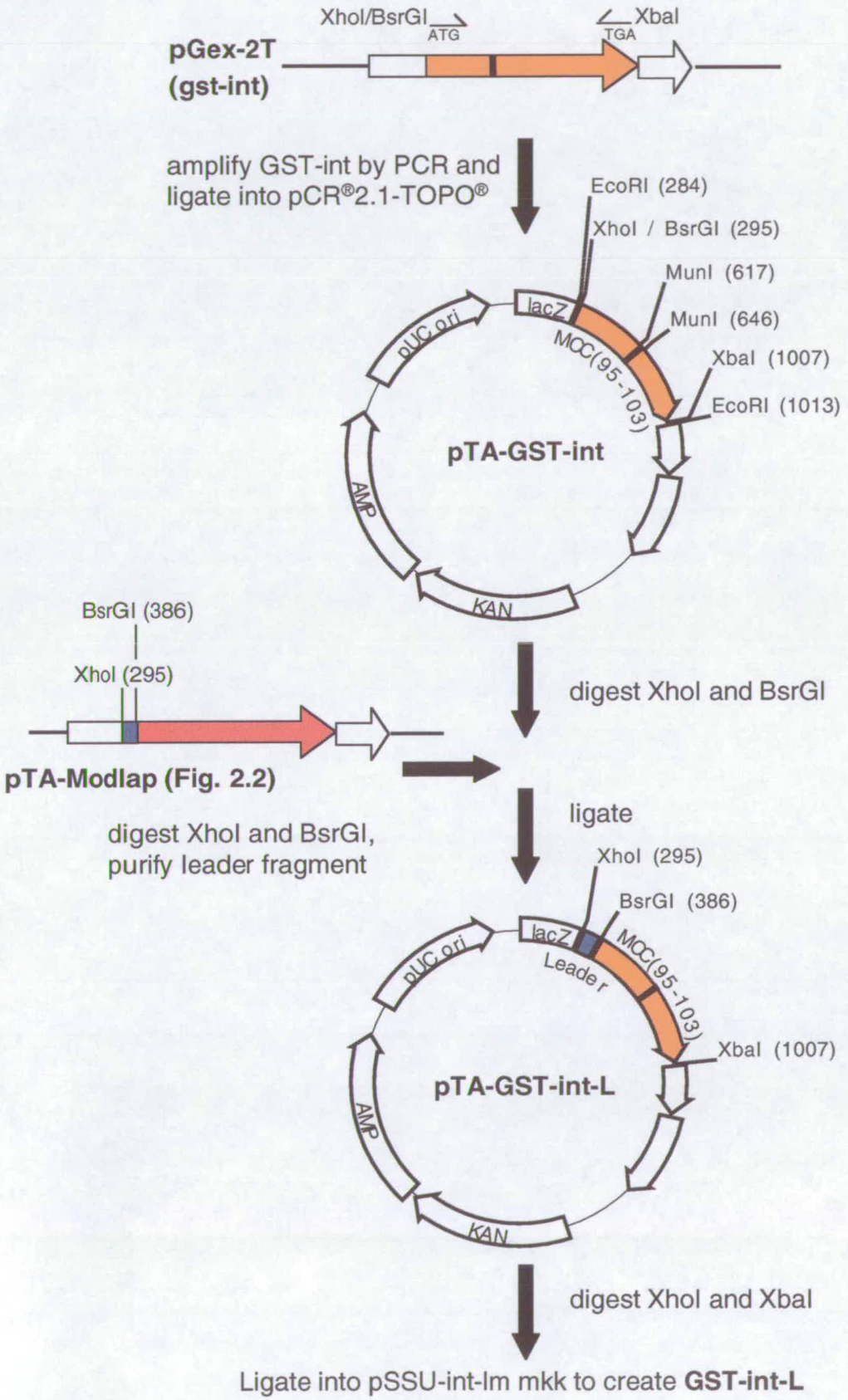
2.1.8.2 GST-int-L

For the following cloning strategies, a slightly modified form of the pSSU-int vector was used (kindly provided by Martin Wiese, Tübingen); *hygromycin*^{r} was replaced by *puromycin*^{r}, and the *CPB 2.8* intergenic region was cloned downstream of the multiple cloning site (see Appendix A-a).

The cloning strategy for the generation of pSSU-GST-int-L is depicted in Figure 2.1. GST-int was amplified from pGEX-2T (GST-int) (Appendix A-a) using GST-int-Xho-BsrGI F' and GST-int-Xba R' primers (section 2.1.7.3) that were complementary to the start ATG and the TGA stop codon respectively. XhoI and BsrGI sites were included at the 5' end of the GST-int-Xho-BsrGI F' primer and an XbaI site was included at the 5' end of GST-int-Xba R', so that the amplified product would be flanked by these restriction sites. The PCR conditions were: 94°C 5 minutes; then 20 cycles of 94°C 20s; 60°C 30s; 72°C 10s; followed by 72°C 10 minutes. The amplified PCR product was sub-cloned into a TA cloning vector (pTA-GST-int).

The 91bp leader peptide sequence from the *L. mexicana* membrane-bound acid phosphatase (*mbap*) gene was cloned onto the N-terminus of GST-int, immediately upstream of the start ATG, using the XhoI and BsrGI sites that had been introduced by PCR. The leader peptide was digested from pTA-Modlap (see Figure 2.2) with XhoI and BsrGI, and the 91bp fragment separated on a 6% polyacrylamide gel. The resulting

Figure 2.1: Cloning Strategy for the Generation of GST-int-L



bands were excised from the gel, eluted on incubation with 0.5M NH_4OAc, 1mM EDTA over-night at 37°C, and inserted into XhoI, BsrGI digested pTA-GST-int, generating pTA-GST-int-L.

The resulting construct was carefully sequenced to check it was in frame and then digested with XhoI and XbaI and directionally cloned into pSSU-int-lm mkk digested with the same enzymes, to generate pSSU-GST-int-L (Appendix A-b).

Prior to transfection pSSU-GST-int-L was linearised using the PacI (410) site at the 5' end of the integration cassette (Appendix A-a), gel purified to remove any non-digested DNA and electroporated into log phase *L. mexicana* promastigotes (see section 2.3.1.4). For sterilisation of purified DNA the sample was precipitated with 0.1 volumes 3M NaOAC and 2.5 volumes 100% ice cold ethanol for 20 minutes at –70°C, microfuged at 13 000 rpm for 5 minutes, washed with 70% ethanol and air dried in a laminar flow hood. The DNA pellet was resuspended in 20µl sterile water.

2.1.8.3 MBAP

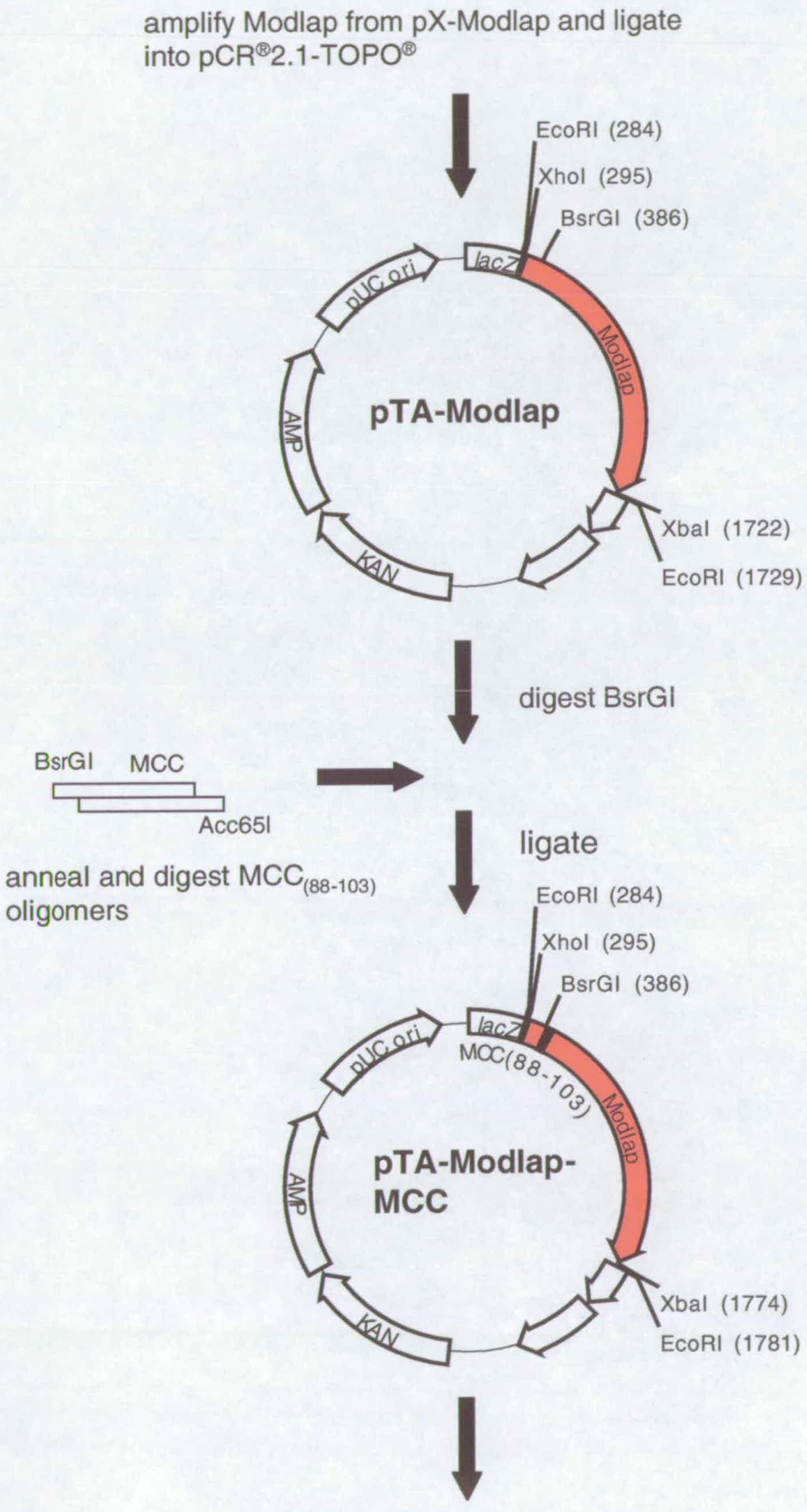
CLB1

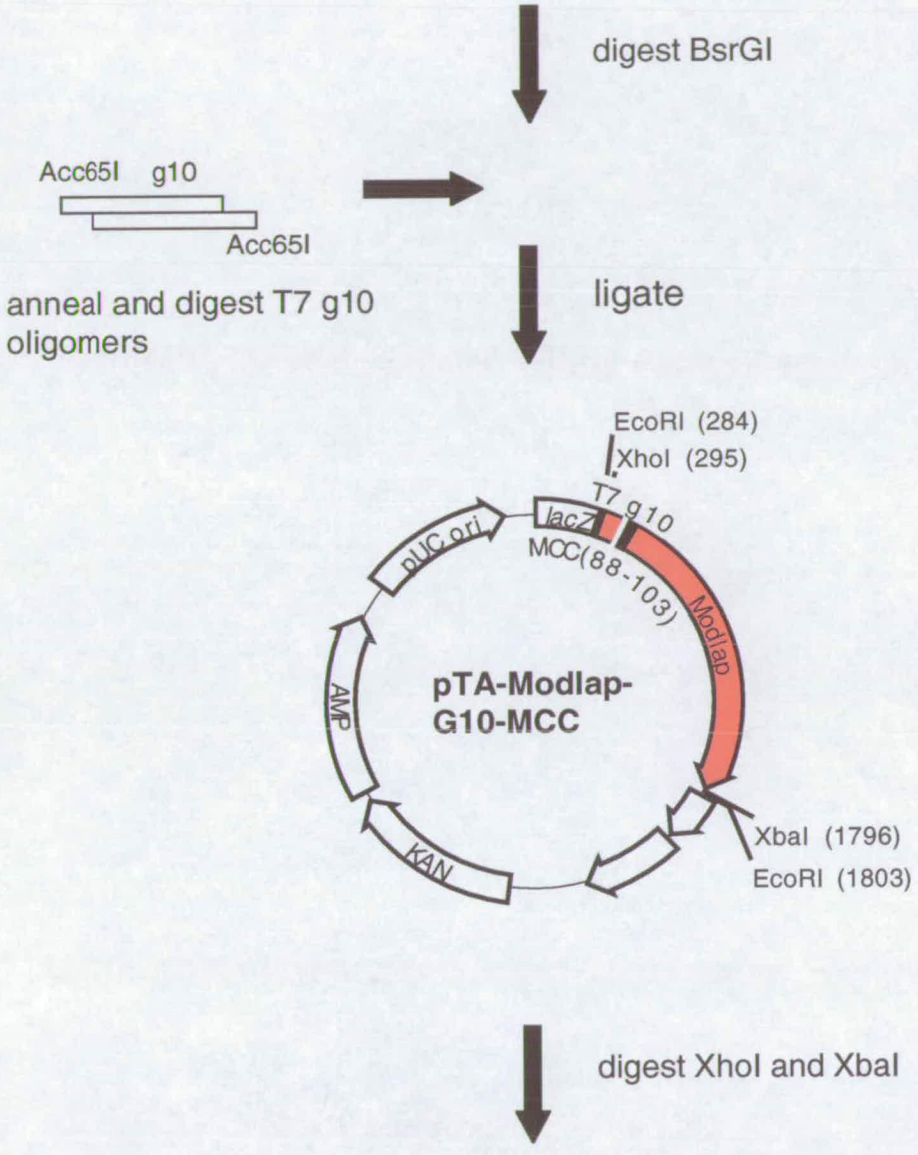
The cloning strategy for the CLB1 construct is depicted in Figure 2.2. *L. mexicana mbap* was amplified from the vector pX-Modlap (provided by Martin Wiese, Tübingen) using the following primers:

XhoI-lmmbap 5' CCCCTCGAGATGCTCAGAGGCATCGGTTTCCTC 3'
XbaI-lmmbap 5' GGGTCTAGATCAGATCTCGCCGCTGGACATGGG 3'

The amplified product was cloned into the TA cloning vector for subsequent cloning steps (pTA-Modlap). Two complimentary $MCC_{(88\text{-}103)}$ olignucleotides were designed flanked by sites for the restriction enzymes BsrG I and Acc 65I at the 5' and 3' ends respectively. These sites are compatible with the BsrGI (386) site immediately downstream of the *mbap* leader peptide. Acc65I and BsrGI have compatible cohesive ends, but this destroys the BsrGI site, resulting in loss of the BsrGI site at the 3' end of the MCC epitope.

Figure 2.2: Cloning Strategy for the Generation of CLB1





Ligate into pSSU-int-lm mkk to create **CLB1**

MCC-1: 5' GGGTGTACACCGCGAACGAGCGCGCGGACCTGATCGCGTACCTGAAGCAG-
GCGACGAAGCGGTACCGGG 3'

MCC-2: 5' CCCGGTACCGCTTCGTCGCCTGCTTCAGGTACGCGATCAGGTCCGCGCGC-
TCGTTCGCGGTGTACACCC 3'

The MCC oligomers were resuspended at a concentration of 1μg/ml, mixed at a ratio of 1:1 and annealed by heating to 65°C then cooling slowly to room temperature. The annealed oligomers were then digested with BsrGI and Acc65I, purified with phenol/choloform and inserted into BsrGI digested pTA-Modlap, to create pTA-Modlap-MCC. Olignucleotides specific for the T7 gene 10 epitope tag (Novagen) were designed, which also contained restriction sites for BsrGI, and inserted into the BsrGI site which was now 5' to the MCC epitope.

G10-1: 5' AAAGGTACCCCATGGCGTCGATGACGGGCGGCCAGCAGATGGGCCGGTACC-
GGG 3'

G10-2: 5' CCCGGTACCGGCCCATCTGCTGGCCGCCCGTCATCGACGCCATGGGGTACC-
TTT 3'

pTA-Modlap-G10-MCC was sequenced extensively to ensure that no mutations had been introduced during PCR amplification of *mbap*, that the gene 10 tag and MCC epitope were both inserted in frame with the initiation Methionine and that they were present in the correct orientation. The transgene was digested with XhoI and XbaI and inserted into XhoI, XbaI digested pSSU-int-lm mkk to generate CLB1 (see Appendix A-b). CLB1 was linearised using the PacI site at the 5' end of the integration cassette, as described in section 2.8.1.2, and electroporated into log phase *L. mexicana* promastigotes.

CLB2

CLB2 was generated as described for CLB1 but without insertion of the T7 gene10 tag (Appendix A-b).

CLB3

The cloning strategy to generate CLB3 is depicted in Figure 2.3. An AvrII restriction site was inserted into a site at the C-terminus of pTA-Modlap using the following primers:

AvrII F': 5' CCTAGGCGAGATCTGATCTAGA 3'

AvrII R': 5' CCTAGGGACATGGGCGACGATC 3'

AvrIIF' and AvrIIR' are homologous to a sequence at the C-terminus of Modlap and contain an overlapping region that contains the AvrII site. The PCR conditions were 94°C for 5 minutes followed by 94°C 15 seconds; 58°C 30 seconds; 72°C 4 minutes for 30 cycles, then 72°C for 10 minutes. The linear PCR product was digested with AvrII and ligated to create a circular plasmid (pTA-Modlap-AvrII.1) containing the unique AvrII (1716) site. pTA-Modlap-AvrII.1 was digested with HindIII, end-filled with Klenow fragment and religated, thus destroying the HindIII site and generating pTA-Modlap-AvrII.2. Complementary oligonucleotides encoding a multiple cloning site (MCS) flanked by AvrII restriction sites were annealed, digested with AvrII and inserted into AvrII digested pTA-Modlap-AvrII.2 to generate pTA-Modlap-MCS.

MCSI: 5' TTTCCTAGGTCCGGAATCGATGTTAACCCGTACGATATCAAGCTTCAATTG-
CCTAGGTTT 3'

MCSII: 5' AAACCTAGGCAATTGAAGCTTGATATCGTACGGGTTAACATCGATTCCGG-
ACCTAGGAAA 3'.

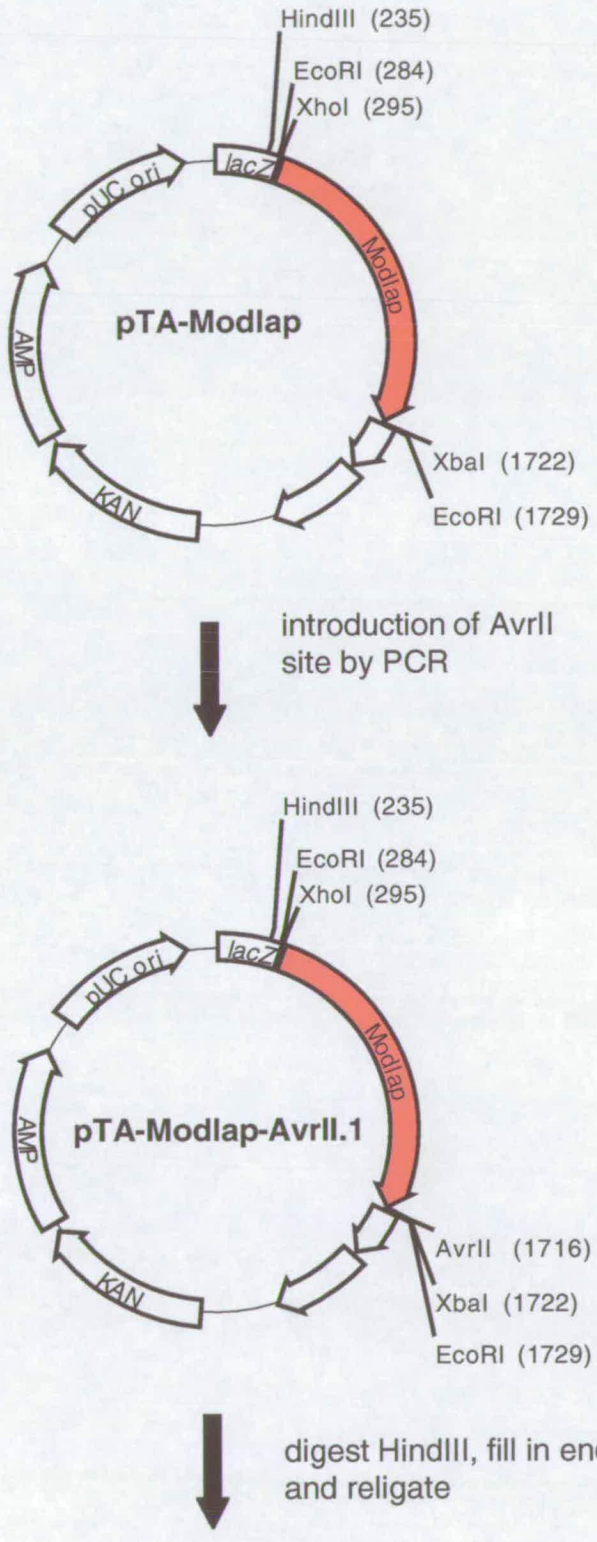
$MCC_{(88\text{-}103)}$ was inserted as two annealed oligomers which were flanked by HindIII sites.

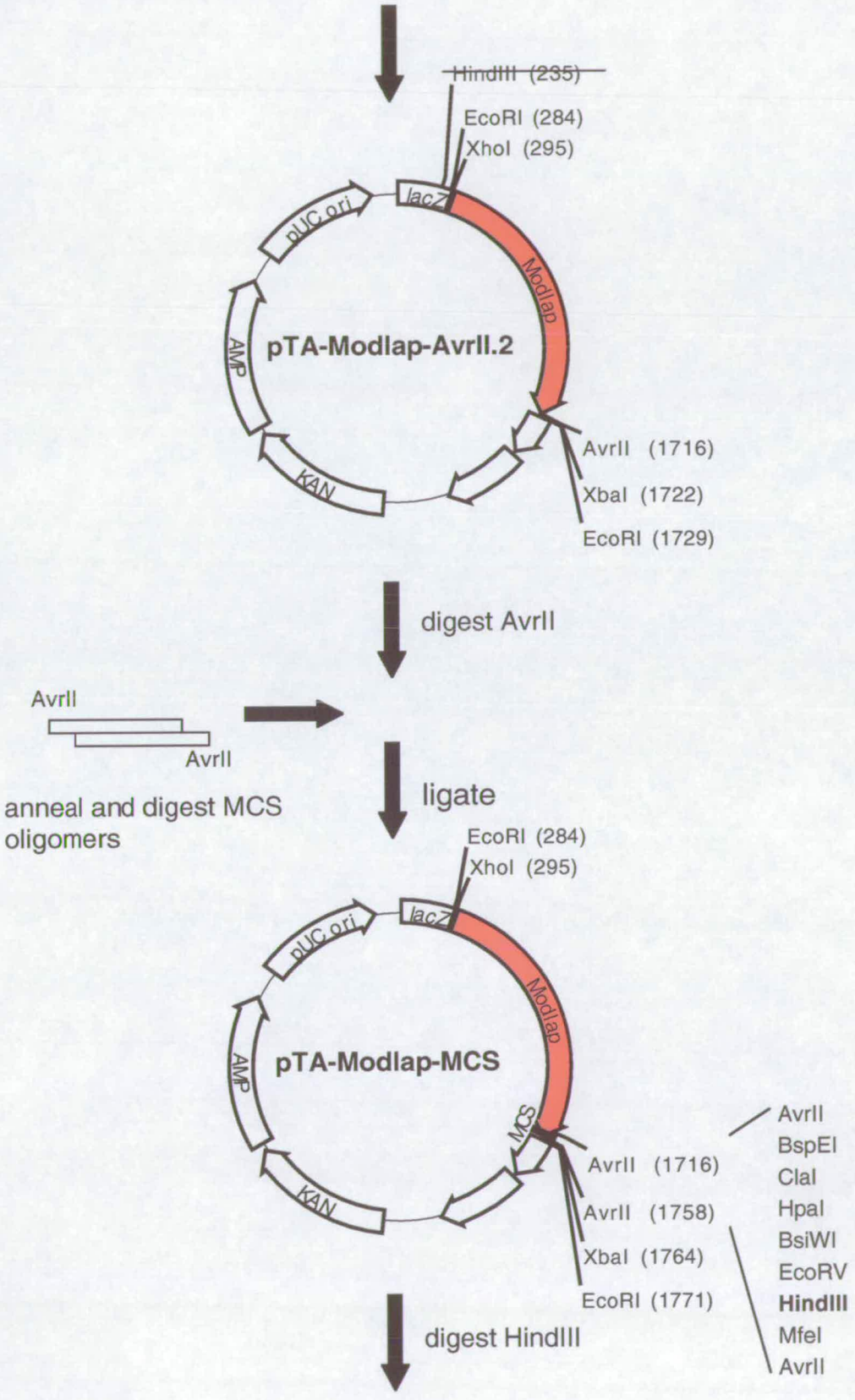
MCC-HindIIIF': 5' AGCTTGCTTAACGCGAACGAGCGCGCGGACCTGATCGCGTACCT-
GAAGCAGGCGACGAAGGCA 3'

MCC-HindIIIR': 5' AGCTTGCCTTCGTCGCCTGCTTCAGGTACGCGATCAGGTCCGCG-
CGCTCGTTCGCGTTAACGA 3'

The MCC oligonucleotides were annealed, digested with HindIII and inserted into the HindIII site of the MCS at the C-terminus of pTA-Modlap-MCS to create pTA-Modlap-MCS-MCC. Clones were analysed by sequencing to ensure that the MCC epitope was inserted in the correct orientation, and that it was in frame with the rest of the MBAP

Figure 2.3: Cloning Strategy for the Generation of CLB3




HindIII (235)
EcoRI (284)
XhoI (295)
lacZ
pUC ori
ModIap
AMP
KAN
pTA-ModIap-AvrII.2
AvrII (1716)
XbaI (1722)
EcoRI (1729)
digest AvrII
AvrII
AvrII
anneal and digest MCS oligomers
ligate
EcoRI (284)
XhoI (295)
lacZ
pUC ori
ModIap
AMP
KAN
MCS
pTA-ModIap-MCS
AvrII (1716)
AvrII (1758)
XbaI (1764)
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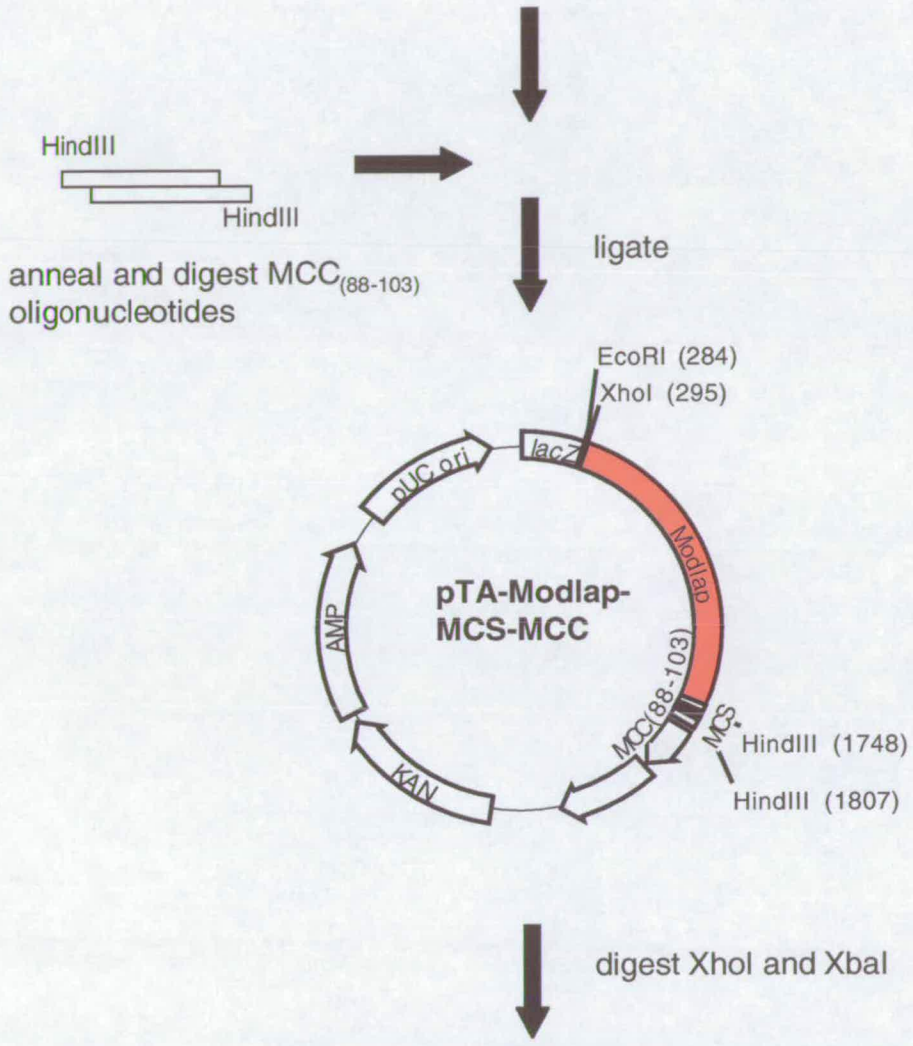
HindIII
MfeI
AvrII
digest HindIII



Ligate into pSSU-int-lm mkk to create **CLB3**

protein. Verified clones were digested with XhoI and XbaI and inserted into XhoI, XbaI digested to pSSU-int-lm mkk to generate CLB3 (Appendix A-b).

2.1.8.4 GP63-MCC

The cloning strategy for the generation of pSSU-int-gp63-MCC is depicted in Figure 2.4. The sequence for the *L. amazonensis gp63 C1* gene was obtained from Genbank, accession number X64394. The truncated gene was amplified from *L. mexicana* wild type genomic DNA using the following primers:

Gp63-XhoI F': 5' CTCGAGATGCCCGTCGACAGCAGCAGC 3'
Gp63-XbaI R': (see section 2.1.7.3).

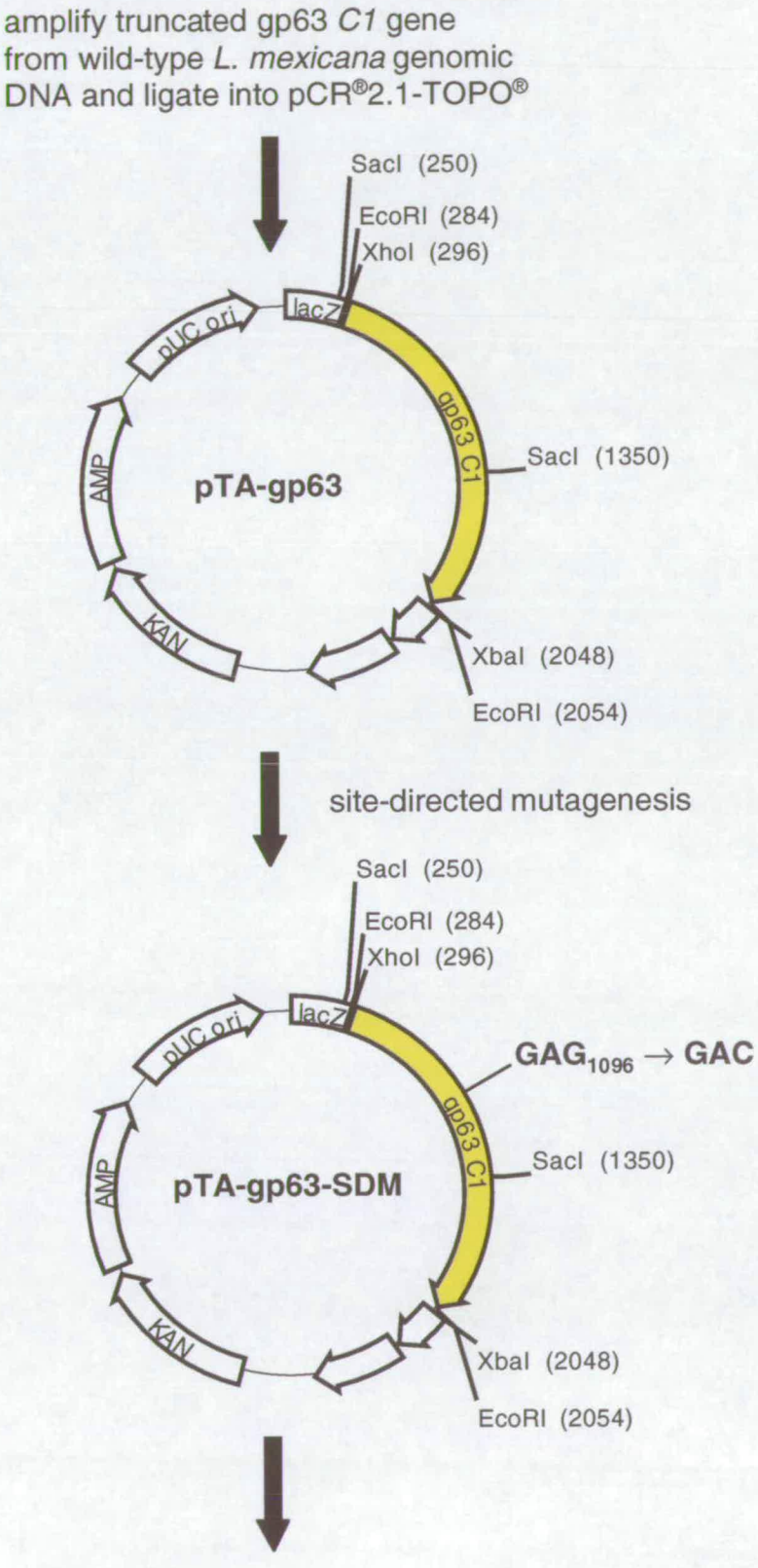
Gp63-XhoI F' contains the start ATG codon and Gp63-XbaI R' is complementary to a sequence at the 3' end of the gene, 5' of the GPI anchor addition site. Gp63-XbaI F' inserts a stop codon (TGA) immediately after Aspartate residue 1921. Therefore the amplified product would lack the GPI anchor addition site, mutation of which has been shown to result in a secreted form of the intact protein (McGwire and Chang 1996). The Xho I and Xba I sites included in Gp63-XhoI F' and Gp63-XbaI R' respectively were included to facilitate sub-cloning of the product into pSSU-int-lm mkk. The PCR conditions were: 94°C 5 minutes; then 20 cycles of 94°C 30s; 62°C 30s; 72°C 1 minute; followed by 72°C for 7 minutes. The amplified PCR product was sub-cloned into a TA cloning vector to generate pTA-gp63.

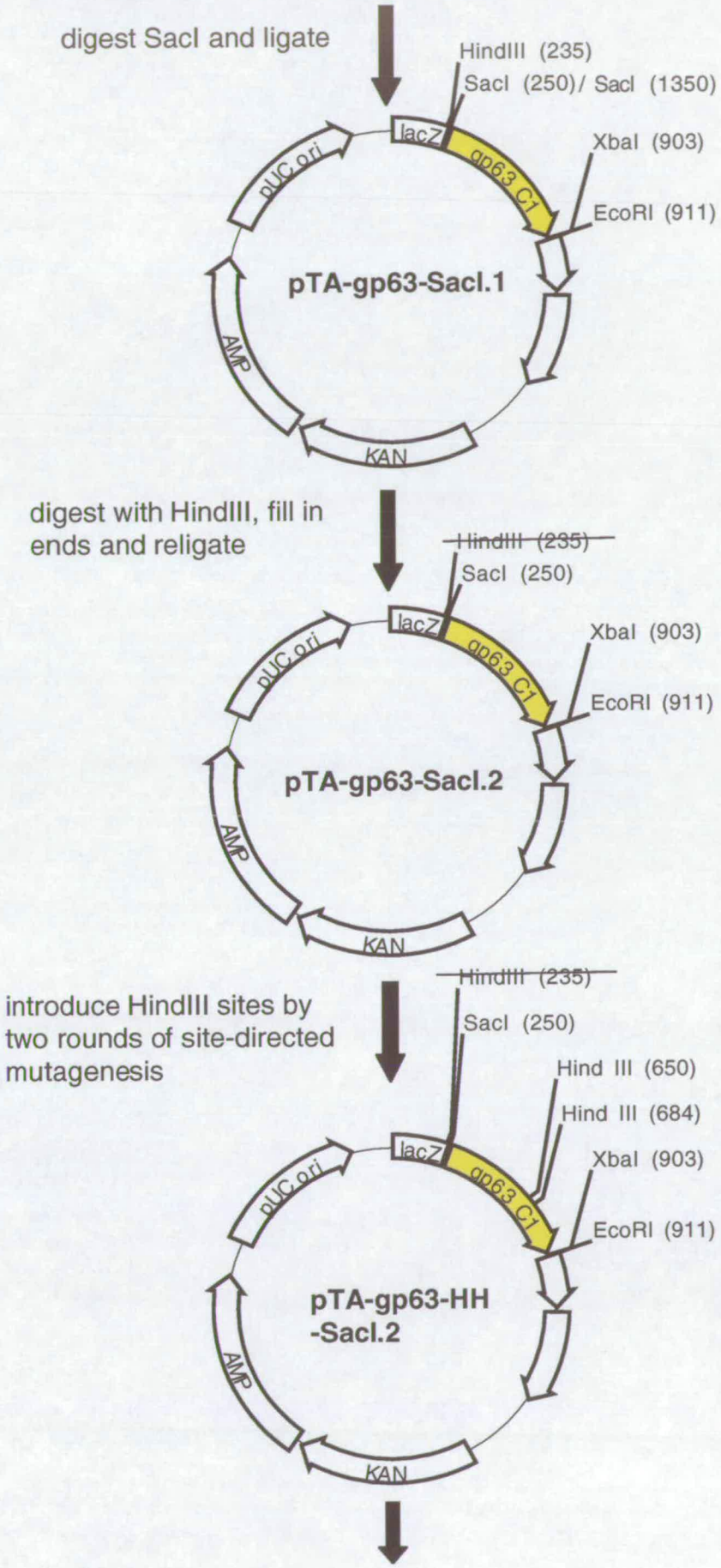
To mutate the active site of gp63 Glutamate 265 was converted to an Aspartate residue using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). Antisense primers were designed containing the mutagenised Glutamate→Aspartate (GAG→GAC) codon (mutated base pairs are underlined).

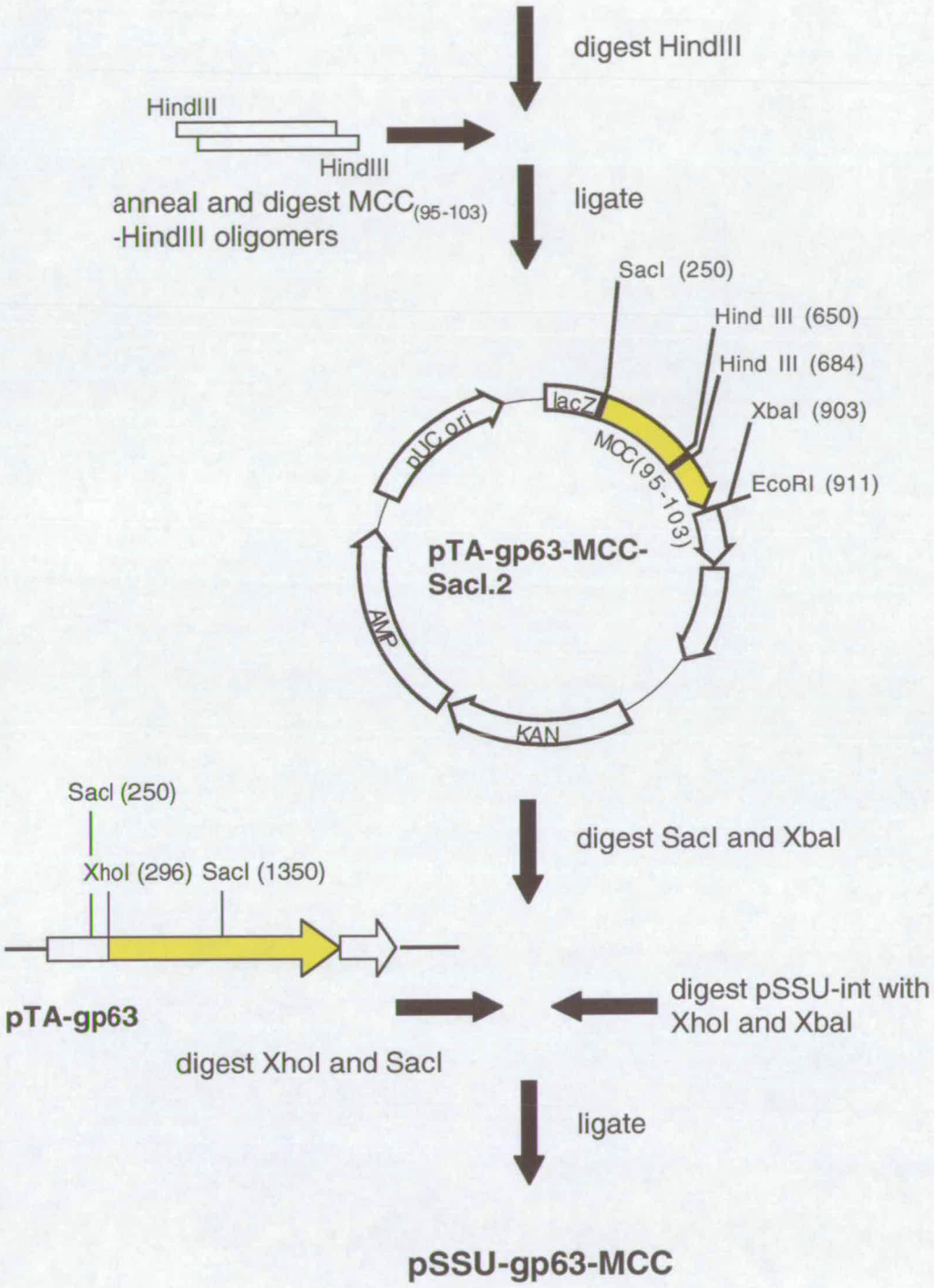
GLU-265 F': 5' GTCACGCACGACATGGCGCACG 3'
GLU-265 R'; 5' CGTGCGCCATGTCGTGCGTGACG 3'

pTA-gp63 was amplified with the mutagensis primers using *Pfu* polymerase (Stratagene) and the following PCR conditions: 95°C for 30 seconds then 12 cycles of 95°C 30 seconds; 55°C 60 seconds; 68°C 12 minutes; then 68°C for 7 minutes. The PCR

Figure 2.4: Cloning Strategy for the Generation of pSSU-gp63-MCC




digest SacI and ligate
HindIII (235)
SacI (250)/ SacI (1350)
XbaI (903)
EcoRI (911)
lacZ
gp63 C1
pUC ori
AMP
KAN
pTA-gp63-SacI.1
digest with HindIII, fill in ends and religate
HindIII (235)
SacI (250)
XbaI (903)
EcoRI (911)
pTA-gp63-SacI.2
introduce HindIII sites by two rounds of site-directed mutagenesis
HindIII (235)
SacI (250)
Hind III (650)
Hind III (684)
XbaI (903)
EcoRI (911)
pTA-gp63-HH -SacI.2


digest HindIII
HindIII
HindIII
anneal and digest MCC(95-103)
-HindIII oligomers
ligate
SacI (250)
Hind III (650)
Hind III (684)
XbaI (903)
EcoRI (911)
lacZ
MCC(95-103)
pUC ori
pTA-gp63-MCC-
SacI.2
AMP
KAN
SacI (250)
XhoI (296)
SacI (1350)
digest SacI and XbaI
pTA-gp63
digest pSSU-int with
XhoI and XbaI
digest XhoI and SacI
ligate
pSSU-gp63-MCC

reaction was then incubated with the restriction enzyme DpnI that digests the methylated template DNA but not the non-methylated PCR products. The PCR product, which is maintained as a nicked circular molecule is then transformed into competent cells, in

pTA-gp63-SDM was digested with SacI and religated to isolate the C-terminus of gp63 for subsequent cloning steps. pTA-gp63-SacI.1 was digested with Hind III, the ends filled in with the Klenow fragment and religated, thus destroying the Hind III site and generating pTA-gp63-SacI.2. Two Hind III sites were then introduced into pTA-gp63-SacI.2 by two rounds of site-directed mutagenesis. These sites flanked base pairs 1697-1729 (see Figure 3.19), which could subsequently be removed and replaced by the MCC epitope. The primers used for the site-directed mutagenesis were as follows:

SDM-Hind III 1: 5' CGCAGCGGTTCTAAGCTTCGGCGTGAAGG 3'
SDM-Hind III 2: 5' GCCTTCACGCCGAAGCTTAGAACCGCTCGC 3'
SDM-Hind III 3: 5' CGTTGGCGCAAAGCTTGGTGTAGTATCC 3'
SDM-Hind III 4: 5' GGATACTACACCAAGCTTTGCGCCAACG 3'

The following PCR conditions were used for both rounds of site-directed mutagenesis: 95°C for 30 seconds then 16 cycles of 95°C 30 seconds; 55°C 60 seconds; 68°C 9 minutes; then 68°C for 7 minutes. The resulting construct was called pTA-gp63-HH-SacI.2. Two complementary $MCC_{(95\text{-}103)}$ oligomers were designed flanked by Hind III sites:

MCC-gp63 F': 5' AGCTTAACATCGCGTACCTGAAGCAGGCGACGA 3'
MCC-gp63 R': 5' AGCTTCGTCGCCTGCTTCAGGTACGCGATGTTA 3'

These oligomers were annealed, and inserted into HindIII digested TA-gp63-HH-SacI.2 to generate TA-gp63-MCC-SacI.2. This construct was thoroughly sequenced to confirm the orientation of the inserted MCC epitope, and that it was in-frame with the rest of the gp63 protein. The pTA-gp63-MCC-SacI.2 SacI-XbaI fragment was ligated with the pTA-gp63 XhoI-SacI fragment and XhoI, XbaI digested pSSU-int-lm mkk to generated pSSU-gp63-MCC (Appendix A-b).

2.2 Protein Analysis

2.2.1 Preparation of Protein Samples

Parasite material was boiled in 1x reducing protein sample buffer for 10 minutes with vortexing to lyse the parasites. Samples were then placed on ice. Parasite supernatants were diluted in 5 x reducing protein sample buffer and boiled. To concentrate supernatants, samples were spun through a concentrating column (Vivaspin Concentrator, 5,000 Mw cut-off; Vivascience).

2.2.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Discontinuous SDS-PAGE was performed as described by (Laemmli 1970). Protein samples were separated by electrophoresis through acrylamide gels using a Bio-Rad mini gel apparatus. Stock acrylamide solutions for polyacrylamide gels were 30% acrylamide/ bis (37.5:1) (Bio-Rad). The lower resolving gel consisted of 10 or 12.5% acrylamide, 1.5M Tris-HCl pH8.8 and 0.4% SDS, while the upper stacking gel was 4% acrylamide, 0.5M Tris-HCl pH6.8 and 0.4% SDS. Reducing protein sample buffer (1 x concentration) consisted of 0.5% bromophenol blue, 2% SDS, 10% glycerol, 62.5mM Tris-HCl pH6.8 and 2.9M β-mercaptoethanol. Samples were run through the stacking gel at a constant current of 10mA per gel and through the resolving gel at 20mA per gel until the bromophenol blue dye ran off the end of the gel. Running buffer contained 25mM Tris-HCl pH8.3, 192mM glycine and 0.1% SDS.

Full range pre-stained molecular mass standards (Amersham) were routinely run as markers on SDS-PAGE gels.

2.2.3 Western Blotting

Proteins separated by SDS-PAGE were transferred to Hybond™ECL™ nitrocellulose membrane (Amersham) by electroblotting. Transfers were performed using a semidry blotter (Bio-Rad) at a constant voltage of 100V with 25mM Tris-HCl pH8.3, 192mM

Glycine, 0.1% SDS and 20% methanol as the transfer buffer. After blotting, membranes were blocked by incubation in blocking solution (PBS-T (PBS, 0.05% Tween-20) / 5% (w/v) skimmed milk powder with shaking overnight at 4°C. Membranes were washed three times with PBS-T before incubation with primary antibody diluted in blocking solution for 1 hour shaking at room temperature. Membranes were washed a further 3 times with PBS-T before incubation with a 1: 5, 000 dilution of an appropriate hydrogen peroxidase (HRP)-conjugated anti-species IgG+IgM (Sigma) for 1 hour at room temparature with shaking. After another round of washes the membranes were developed using the Enhanced Chemiluminescence (ECL™, Amersham) reagents and exposed to Hyperfilm™ECL™ (Amersham) for appropriate lengths of time.

2.2.4 Antibodies used for immunoblotting

The T7 gene 10 tag was detected with an anti-gene 10 mAb (Novagen) diluted 1:3000 and detected with a goat anti-mouse-HRP secondary at 1:5000. The primary anti-GST polyclonal antibody (Amersham) was diluted 1:1000 and detected with a mouse anti-goat-HRP secondary at 1: 160 000.

2.2.5 Acid Phosphatase Assay

Acid phospatase activity was assayed as described by (Menz *et al.* 1991). Amastigotes were cultured overnight in volumes of 1ml at $1x10^7$/ml in a 24 well plate. Supernatants were harvested the next day and stored at –80°C. For the phosphatase assay 10µl of supernatant was incubated with 90µl 5mM p-Nitrophenol phosphate (pNPP) (Sigma) in 100mM NaOAc, pH5.0 in triplicate wells of a 96 well plate. Samples were incubated for 30, 60 and 90 minutes at 37°C, and the reactions stopped with 10µl of 2M NaOH. Absorbance was read at 405nm, and a blank was subtracted from wells containing samples. The acid phosphatase activity was calculated in units, whereby 1 unit equals the amount of enzyme that hydrolyses 1µM of substrate per minute, using the equation:

Units = $\Delta OD_{405nm}(t_2-t_1)/\varepsilon_{405nm}$, $\varepsilon_{405nm} = 18500M^{-1}cm^{-1}$.

2.3 General Tissue Culture

All cell culture was performed in laminar flow sterile hoods using strict sterile technique which included wiping the hood down and spraying all items entering the hood with 70% industrial methylated spirits (IMS). All solutions were tested for sterility by incubation of aliquots at 37°C for at least 24 hours before use. Cells were examined using an inverted microscope (Nikon TMS).

2.3.1 Culture of *Leishmania* Parasites

L. mexicana (strain MNYC/BZ/62/M379) and *L. major* (LRC-L137 V121) were used for *Leishmania* experiments.

2.3.1.1 Thawing of Promastigotes Cultures

Vials of frozen promastigotes were taken directly from the liquid nitrogen storage and quickly thawed in a 37°C water bath. The cell suspension was transferred to a 20ml universal tube containing 10ml of pre-warmed promastigote media. The cell suspension was centrifuged immediately at 3000 rpm for 5 minutes. The supernatant was poured off and the pellet resuspended in 1ml pre-warmed promastigote culture medium, then transferred to a $25cm^3$ flask containing 9ml of the pre-warmed medium.

2.3.1.2 Passage and Expansion of Parasite Cultures

Promastigotes were cultured *in vitro* in SDM / 10% heat inactivated foetal calf serum (iFCS) at 26°C. Cultures were maintained in a volume of 10 ml in $25cm^3$ tissue culture flasks and passaged weekly. Amastigotes were cultured axenically (section 2.3.1.2) at 34°C in Schneider's *Drosophila* medium (GibcoBLR) supplemented with 20% iFCS and 3.9g/l 2-(N-morpholino)ethane-sulphonic acid (Sigma). Cultures were split 1:20 weekly. Amastigote clumps were dispersed by passing them up and down through a 23G needle (Beckton-Dickinson) before passage.

Parasites were passaged through mice to maintain virulence. Stationary phase promastigotes were washed 3 times in PBS and resuspended at $1x10^8$/ml. Approximately 30µl ($3x10^6$) parasites were injected sub-cutaneously at the base of the tail of BALB/c

mice. Mice were sacrificed after at least 4 weeks post-infection and the popliteal lymph nodes taken. Lymph nodes were cut up and cultured in 10 ml SDM / 10% iFCS with antibiotics (50U/ml Penicillin and 50µg/ml Streptomycin, Gibco) at 26°C.

2.3.1.2 Generation of Axenic Amastigotes

L. mexicana axenic amastigotes were generated as described by (Bates *et al.* 1992; Bates 1994). Stationary phase promastigotes were diluted 1:10 into amastigotes medium and cultured at 26°C until dense. Cultures were diluted 1:10 again into fresh medium and transferred to 34°C. Amastigotes were then passaged at least twice before use.

2.3.1.3 Freezing *Leishmania* Parasites

10ml log phase *L. mexicana* and *L. major* promastigotes were centrifuged at 3000 rpm for 5 minutes, the cell pellet resupended in 2.5ml of ice cold freezing solution (iFCS / 10% DMSO) and rapidly transferred in 500µl aliquots to Nunc cryotubes on ice. Cryotubes were wrapped in blue towel and transferred to –80°C for at least 24 hours, then moved to liquid nitrogen storage. Exposure of parasites to DMSO is kept to a minimum as it toxic to the cells.

2.3.1.4 Transfer of DNA to *Leishmania* Parasites

Exogenous DNA was routinely introduced into *L. mexicana* promastigotes by elecroporation as described by (Kapler *et al.* 1990). Briefly, log phase, actively dividing promastigotes were centrifuged at 3000 rpm for 5 minutes and the supernatant discarded. The cell pellet was washed in 10ml sterile elecroporation buffer (EPB; 21mM HEPES pH7.5, 137mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 6mM glucose), resuspended in 10ml EPB and counted in a haemocytometer. The cells were centrifuged again and resuspended at $1x10^8$/ml in EPB and chilled on ice. 400ul of cells were added to chilled 0.2cm elecroporation cuvettes (Bio-Rad) containing 20ul of sterile linearised DNA (section 2.1.8.2). Cells were elecroporated at 0.45V, 500µF in a Bio-Rad Genepulser, which would normally result in a time constant of 4.0. The cuvette was incubated on ice for 10 minutes before transfer of the cells to 10ml promastigote medium. The next day the parasite culture was made up to 24ml and Puromycin added at a final concentration of 40µM. The culture was split into a 24 well plate. Based on an transfection efficiency

of 1 in $1x10^6$ this will result in approximately one transfected parasite per well, and cultures grown up from each well were therefore be considered lines.

After approximately 5 days parasites were split 1:10 and the media replaced in the original plate. By 2 weeks positive wells were confluent with drug-resistant parasites that were passaged to be frozen, and harvested for analysis of genomic DNA (section 2.1.7.2).

2.3.2 Culture of Mammalian Cells

All mammalian cells were cultured at 37°C in a humidified incubator with 5% CO_2.

2.3.2.1 T Cell Hybridomas

The 2B4 T cell hybridoma was cultured in RPMI cell culture medium containing 10% FCS; 4mM Glutamine 2mM sodium pyruvate; 0.1% MEM non-essential amino acids; 0.1mM 2-mercaptoethanol. All stock solutions were prepared by at the CGR tissue culture facility. The cells were cultured in 6 well plates and split 1:10 into fresh medium every other day. Aliquots of $1x10^6$ cells were thawed and frozen, as described in sections 2.3.1.1 and 2.3.1.3.

2.3.2.2 IL-2-Dependent Cells (Swain *et al.* 1981)

The IL-2-dependent T cell line C.C3.11.75 was thawed as described in section 2.3.1.1 and resuspended in 11ml RPMI / 10% FCS in a $25cm^3$ tissue culture flask with 1ml CASM (section 2.3.2.3). Cells were passaged every 4 days or less by transferring 1ml into a new flask containing 10ml culture medium and 1ml CASM. Cells were monitored by microscopy to ensure that they grew as clumps. Once the cells started growing as single cell suspensions, the cultures were discarded and new aliquots thawed. $1x10^6$ cells were frozen in 500µl aliquots as described in section 2.3.1.3.

2.3.2.3 ConA-Stimulated Medium

ConA-stimulated medium (CASM) was prepared as follows. Rat spleen single cell suspensions were cultured at $2x10^6$/ml in R10 containing 2.5µg/ml Concanavalin A (Sigma) for 48 hours. The contents were then transferred to 50ml Falcon tubes and centrifuged at 1200 rpm for 5 minutes. Aliquots of the supernatant were transferred to 20ml Universal tubes containing 0.4g of α-methyl mannoside (Sigma) and stored at –20°C. Aliquots were filter sterilised before use, and tested for IL-2 activity using an IL-2-dependent cell line (see section 2.5.4.3).

2.3.2.4 Culture of D1 Cells

D1 DC were cultured as described by (Winzler *et al.* 1997). The cells were cultured in IMDM supplemented with 10% FCS, 2mM L-glutamine, 50µM 2-mercaptoethanol antibiotics (100U/ml Penicillin and 100µg/ml Streptomycin) and 30% supernatant from NIH/3T3 cells containing 10-20ng/ml GM-CSF.

Cells were harvested for passaging by removing the supernatant and incubating the cells in 2mM EDTA/PBS for 10 minutes at room temperature. Cells were dislodged by vigourous washing with PBS and harvested into 15ml Falcon tubes. Cells were centrifuged at 12000 rpm for 5 minutes and the supernatant discarded. Cells were resuspended in 2ml D1 cell media and counted on a haemocytometer. $2.5x10^6$ cells were plated in 10ml D1 cell medium in 10cm tissue culture dishes, or $5x10^5$ cells in 2ml in 35mm tissue culture dishes for infection with *Leishmania.*

2.4 Primary Tissue Culture

2.4.1 Animal Maintenance

Six to eight week old female CBA or BALB/c mice were obtained from colonies maintained at the Centre for Genome Research and Ann Walker animal facilities at the University of Edinburgh. All animals were housed and bred according to the provisions of the Animals (Scientific Procedures) Act (UK) 1986.

2.4.2 Isolation of Bone Marrow

Femurs and tibias were taken from CBA mice, sterilised in 70% ethanol and washed well in RPMI. The ends were cut off the bones and the bone marrow flushed out using RPMI in a 1ml syringe with a 26G needle (Beckton Dickinson). Cells were passed through a cell strainer (Beckton Dickinson) to remove any bone fragments and to create a single cell suspension.

2.4.3 Generation of Bone Marrow-Derived DC

BM-DC were cultured *in vitro* according to a method adapted from (Inaba *et al.* 1992). The bone marrow single cell suspension was centrifuged for 5 minutes at 1200 rpm and red blood cells lysed by incubation of the pellet in 5ml TBAC (144mM NH_4Cl, 17mM Tris pH7.2) for 5 minutes at 37°C, then washed 3 times in RPMI, 10% FCS, 4mM glutamine, 2mM sodium pyruvate (R10). The cell pellet was resuspended in 2ml R10 and cells counted on a haemocytometer. Bone marrow cells were diluted a final concentration of $3.75x10^5$/ml in R10, with 10ng/ml recombinant GM-CSF (Peprotech) or 20% supernatant from fibroblasts engineered to secrete GM-CSF (X63 cells; (Stockinger *et al.* 1995). Cells were plated out in 24 well plates at 1ml per well. The cultures were washed on days 3 and 6 to remove non-adherent granulocytes and lymphocytes. On day 7, loosely adherent BM-DC were removed by vigorous washing and replated to exclude firmly adherent MΦ from subsequent culture. Where necessary, DC were stimulated for 18 hours with 1μg/ml *E. coli*-derived LPS (Sigma) and 10^3 U/ml IFNγ (R&D systems), or with 10ng/ml TNFα (Peprotech).

For infection with *Leishmania* parasites, washed parasites were added to replated DC at a ratio of 8:1 and the cells cultured at 37°C for 18 hours. Promastigotes were taken from stationary phase cultures, in which parasites were not dividing but which did not contain debris indicative of cell death. The majority of stationary phase promastigotes were assumed to be metacyclic.

2.4.4 Generation of Bone Marrow-Derived MΦ

Single cell suspension bone marrow cultures were plated at a concentration of $5x10^5$/ml in MΦ medium (DMEM supplemented with 20% FCS, 4mM glutamine, 2mM sodim pyruvate, and 30% L929 cell supernatant). 12ml of cells were plated in 130mm bacterial petri dishes. After 6 days in culture, non-adherent cells were washed away with warm PBS. The adherent MΦ were removed by 5 minutes incubation at 37°C with PBS, 3mM EDTA and 10mM glucose, followed by vigourous washing with cold PBS.

For infection with *Leishmania* parasites, MΦ were replated at a concentration of $1x10^5$/ml in 2ml MΦ medium in 30mm bacterial petri dishes and incubated overnight at 37°C. Washed parasites were added at a concentration of 8:1 and the cells incubated at 34°C overnight, for 4 or 7 days. For long-term culture, the cells were give fresh medium every three days. MΦ were given low levels of IFNγ (200 U/ml) either overnight, with the overnight infection, or on days two, three and six of culture to up-regulate expression of MHC II without activating destruction of the parasites.

DC were also incubated with 3.0μm latex beads (Sigma).

2.5 Immunological Methods

2.5.1 Flow Cytometry

2.5.1.1 Preparation of Cells

Day 7 BM-DC were harvested as described in section 2.4.3 into 15ml Falcon tubes (one well per Falcon tube) and diluted with PBS. Alternatively D1 cells were harvested into Falcon tubes, as described in section 2.3.2.3. Cells were centrifuged for 5 minutes at 1200 rpm and cell pellets were resuspended in 100μl PBS / 10% FCS (FACS wash), transferred to a 5ml FACS tube (Beckton-Dickinson) and placed on ice.

2.5.1.2 FACS Analysis

Parasite-infected cells were fixed by addition of 20µl fixation medium A (Fix & Perm kit, Caltag) for 20 minutes at room temperature and then washed with FACS wash. Cells were resuspended in 50µl FACS wash and FcR were blocked by adding 50µl 2.4G2 antibody diluted to a 2 x concentration in FACS wash to the tube. Cells were mixed by agitation of the tube and incubated for 20 minutes at room temperature (if fixed) or at 4°C. Cells were centrifuged at 1200 rpm for 5 minutes and washed in FACS wash. Cells were resuspended in 50µl FACS wash and 50µl of the appropriate primary antibody or antibodies added, diluted to a 2 x concentration in FACS wash. Cells were incubated for 20 minutes at room temperature or on ice, centrifuged and washed. Where necessary cells were resuspended in 50µl FACS wash and incubated with 50µl of a 2 x secondary antibody.

For intracellular staining, the cells were fixed as described above and 20µl of permeabilisation solution B (Fix & Perm Kit, Caltag) was added to the cells with each round of antibody and the cells vortexed gently.

After the final staining, cells were resuspended in 300µl FACS wash containing 0.01% sodium azide for direct analysis or 300µl FACS wash containing 0.5% paraformaldehyde and 0.01% sodium azide if the cells had not already been fixed and were to be analysed more than 12 hours later. Cells were stored at 4°C in the dark until analysis, for no more than 96 hours. Stained cells were analysed on a Becton Dickinson FACSCalibur. FITC and PE labelled cells were identified in the FL-1 and FL-2 channels respectively. FACS data was analysed using CELLQuest version 3.2 (Beckton Dickinson). Dead cells were routinely excluded by gating. Statistical analysis on FACS data was performed using Student's T-Test.

2.5.1.3 Analysis of Dead Cells by Flow Cytometry

Dead cells were identified by staining with Propidium Iodide (PI) which distinguishes viable cells from non-viable cells. Cells were washed in cold PBS and resuspended in 1x binding buffer (10mM Hepes, NaOH pH7.4, 140mM NaCl, 2.5mM $CaCl_2$) at a

concentration of $1x10^6$ cells/ml. 50µl of the solution was incubated with 5µl PI (50µg/ml dissolved in PBS buffer; 8g NaCl, 0.2g KCl, 1.44g $Na_2HPO_4 \cdot 7H_2O$ 0.24g KH_2PO_4 in 1 litre H_20, pH7.2). The cells were vortexed and incubated for 15 minutes at room temperature in the dark. 200µl binding buffer was added to the tube and the cells analysed by flow cytometry.

2.5.1.4 FACS Sorting

Day 7 BM-DC cultures were incubated for 18 hours with EGFP-expressing *L. mexicana* promastigotes or amastigotes. The cells were harvested from multiple wells of the 24 well plate, pooled into 15ml Falcon tubes, then centrifuged at 1200 rpm for 5 minutes. The cell pellet was then washed two times in a large volume of PBS. Cells were resuspended in 1-2ml PBS and placed on ice. EGFP positive and negative fractions were isolated using a MoFlo® (Cytomation) cell sorter at 4°C, recovered in R10 and placed on ice. A sample of the sorted population was reanalysed to check the purity of the sort. FACS sorting was carried out by Steven LeMoenic (CGR).

2.5.2 Magnetic Cell Sorting (MACS)

Day 7 BM-DC cultures were enriched for $CD11c^+$ cells by MACS. Cells were harvested and incubated with a FITC-conjugated anti-CD11c antibody at a concentration of 0.2µg per $1x10^6$ cells for 20 minutes on ice. Cells were centrifuged for 5 minutes at 1200 rpm and washed with MACS wash (PBS/2mM EDTA with 0.5% BSA). $1x10^7$ cells were incubated with 90µl MACS wash and 10µl anti-FITC magnetic beads (Miltenyi Biotec) for 15 minutes at 4°C. After washing with MACS wash cells were loaded onto a pre-washed MS+ column held in a MACS magnet (Miltenyi Biotec). The column was washed thoroughly and the effluent discarded. The column was then removed from the magnet and the CD11c-$FITC^+$ cells pushed through with a syringe plunger. CD11c enriched cells were plated in 24 well plates overnight before analysis.

2.5.3 Cytokine Detection

2.5.3.1 Enzyme-Linked Immunosorbant Assays (ELISA)

Supernatants were collected from pelleted DC cultures and stored at –80°C. Supernatants were analysed for cytokines by ELISA using Pharmingen OptEIA™ Sets as follows. 96 well plates were coated overnight at 4°C with the appropriate capture antibody diluted to the recommended concentration in 0.06M Sodium Bicarbonate. The following day the wells were aspirated and washed 3 times with wash buffer (PBS / 0.05% Tween20). Plates were blocked with >200µl assay diluent (PBS / 10% FCS) for one hour at room temperature. Wells were then aspirated and washed 3 times with wash buffer. Following the last wash plates were blotted on absorbant paper to remove any residual buffer. Plates were then incubated with 50µl of the appropriate samples or recombinant standards diluted in assay diluent (section 2.5.3.2) for 2 hours at room temperature. The plates were sealed to prevent evaporation. Samples were then washed as before. 50µl of the Working Detector (biotinylated detection antibody + Avidin-HRP), diluted to the recommended concentration in assay diluent, was added to the wells, the plates sealed and incubated at room temperature for 1 hour. Plates were then washed again, as before. To detect bound antibody samples were incubated with 100µl Tetramethylbenzidine (TMB) and Hydrogen Peroxide (Pharmingen) for 30 minutes, or until a blue colour had developed, and the reaction stopped with 50µl 2M H_2SO_4. Absorbance was read at 450nm and a blank was subtracted.

2.5.3.2 ELISA Samples

Supernatants were diluted in assay diluent unit to an appropriate concentration for the standard curve. Each sample was tested in triplicate. Recombinant standards were diluted 1:2 down the plate from a 1000pg/ml standard, in triplicate. The last wells were incubated with assay diluent alone as the blank. Cytokine concentrations were determined by extrapolation from the standards. Statistical analysis was performed using Student's T-Test.

2.5.4 T cell Assays

2.5.4.1 2B4

Presentation of MCC was assayed using the MCC-specific T cell hybridoma 2B4. DC and MΦ were plated out in 96 well plates for T cell assays. As controls, DC or MΦ had been pulsed with 1μM or 10μM MCC or 50μM PCC for 4 hours and washed twice by centrifuging the plate for 4 minutes at 800 rpm then inverting it on blue towel, and refilling the wells with fresh medium. T cells were incubated with DC or MΦ at a ratio of 10 T cells (10^5) to 1 DC (10^4) or 1 T cell (10^5) to 1MΦ (10^5) in a total volume of 200μl of R10, for 18 hours. 150μl of supernatant was subsequently removed and snap frozen at –80°C to kill any cells. Each well was set up in triplicate.

2.5.4.2 IL-2 Assays

Activation of 2B4 cells was assayed by measuring IL-2 production using the IL-2-dependent cell line, C.C3.11.75 (Swain *et al.* 1981), as described by (Lawrence *et al.* 1994). 20μl supernatant from T cell assays was added to individual wells of round-bottomed 96 well plates (Nunc). A standard was also set up using recombinant IL-2 (Sigma) diluted 1:2 down the plate from 1000U/ml. IL-2-dependent cells were washed 3 times to remove all CASM and resuspended in R10 at a concentration of $1x10^5$/ml. To this was added an anti-IL-4 antibody (11B11) diluted 1:400, because the IL-2-dependent cells also proliferate in response to IL-4. 100μl cells was added to each well of the 96 well plate and cultured at 37°C for 24 hours. The following day 1μl [^{3}H]thymidine (1μCi) was added to each well in 10μl R10, to measure cell proliferation by thymidine incorporation, and the plates incubated for a further 18 hours. Cells were harvested and counted using a Top Count Microplate Scintillation Counter (Canberra Packard).

2.6 Antibodies

2.6.1 Primary Antibodies

Antigen	Specificity	Clone	Conjugation	Dilution	Company/ Cat. no.
CD11b	Rat α mouse	M1/70	FITC	1:100	Pharmingen 01710D
CD11c	Hamster α mouse	HL3	FITC	1:50	Pharmingen 09704D
DEC205	Rat α mouse	NLDC145		1:50	Serotec MCA949
CD16/CD32 Fc Block®	Rat α mouse	2.4G2		1:100	Pharmingen 01241D
CD40	Rat α mouse	3/23	PE	1:100	Pharmingen 09665B
CD54	Rat α mouse	KAT-1	PE	1:200	Southern Biotechnology 1700-08
CD54	Hamster α mouse	3E2	Biotin	1:100	Pharmingen 01542D
CD80	Hamster α mouse	1G10	PE	1:100	Pharmingen 09605B
CD86	Rat α mouse	2F7	PE	1:100	Pharmingen 09275B
MHC I	Mouse α mouse	AF6-88.5	PE	1:100	Pharmingen 06105A
MHC II	Mouse α mouse	14-4-4S	PE	1:100	Pharmingen 06025A
CD45R/B220	Rat α mouse	RA3-6B2	PE	1:100	Pharmingen 01125B
CD3	Rat α TCR^+ hybridoma	17A2	FITC	1:100	Pharmingen 28004D
F4/80	Rat α mouse		FITC	1:100	Caltag RM2901
H_2-E^k-$MCC_{(95-103)}$	Mouse α mouse	D4		1:1000	Reay *et al* (1994)
Leishmania parasites	Rabbit α prom/amas.			1:400	T. Aebischer

2.6.2 Isotype Controls and Secondary Reagents

Reagent	Description	Clone	Conjugation	Dilution	Company/ Cat. no.
Hamster IgG1 λ	Hamster isotype	A19-3	FITC	1:100	Pharmingen 11154C
Rat IgG2a κ	Rat isotype	R35-95	PE	1:100	Pharmingen 11025A
Rat IgG2b κ	Rat isotype	G28-5	FITC	1:100	Pharmingen 11184C
Mouse IgG2a κ	Mouse isotype	G155-178	PE	1:100	Pharmingen 03025A
Anti-rat	Goat IgG+IgM (H+L)		FITC	1:100	Jackson Immunoresearch 112-095-068
Anti-mouse	Goat IgG+IgM (H+L)		FITC	1:100	Jackson Immunoresearch 115-095-100
Streptavidin			PE	1:100	Pharmingen 13025D

Chapter 3:

Generation and Testing of a Model Experimental System Proposed for the Investigation of Processing and Presentation of *Leishmania*-Derived Antigens in Infected DC

3.1 Introduction

Several studies have addressed the capacity of infected MΦ to present parasite-derived antigens and have demonstrated that MΦ infected with *L. major* or *L. amazonensis* parasites and treated with IFNγ to up-regulate expression of MHC II did not efficiently stimulate antigen-specific T cells (Fruth *et al.* 1993; Prina *et al.* 1993). Since the killing of intracellular parasites resulted in the stimulation of T cells specific for parasite cysteine proteases (Wolfram *et al.* 1995), and MΦ infected with *Leishmania* which over-expressed parasite-derived or exogenous antigens could efficiently stimulate specific T cells *in vitro* (Kaye *et al.* 1993; Wolfram *et al.* 1996), this inefficient presentation of *Leishmania* proteins was proposed to reflect a general inaccessibility of parasite antigens to the antigen processing and presentation machinery. In infected MΦ, *L. mexicana* parasites reside in large phagolysosomes, known as parasitophorous vacuoles (PV), that develop as a result of fusion of the original phagosome with host endosomal compartments (Antoine *et al.* 1999). These compartments contain the molecules necessary for processing and presentation of *Leishmania* antigens, and have been compared to MIIC, the putative site of peptide loading onto MHC II molecules. Complexes of MHC II molecules loaded with parasite-derived antigens have, however, never been directly visualised in infected APC and therefore it is not known whether

these complexes can form in the PV, or whether parasite antigens exit the PV and meet MHC II molecules in alternative endosomal compartments. These studies, furthermore, have focused on infection of MΦ with *Leishmania sp.* and the presentation capacity of infected DC has not been directly addressed. Therefore, it is not known whether DC containing live parasites can efficiently present parasite antigens to T cells, or whether the kinetics of presentation are impaired, as in infected MΦ. The work discussed in this chapter aimed to develop a model experimental system with which to address the fate of parasite-derived antigens in infected DC and MΦ.

3.2 The Experimental Model

To follow the fate of parasite-derived antigens in infected cells, an optimal model system would require the uniform infection of DC and MΦ with *Leishmania* parasites expressing an antigen that can be detected when complexed with MHC II molecules *in vitro*. Ideally, the formation of MHC II: parasite antigen complexes would be monitored in infected cells using a detection system which could distinguish between MHC II complexes displayed on the surface of the cells and complexes which were sequestered intracellularly.

3.2.1 Detection of MHC II-Peptide Complexes *in vitro*

The formation of complexes between MHC II molecules and a defined T cell epitope can be monitored using a complex-specific mAb which recognises MHC II molecules, of a defined haplotype, only when bound by a relevant peptide. D4 is such an antibody that specifically recognises MHC II H2-E^k molecules when bound by the MHC II-restricted T cell epitope Moth Cytochrome C (MCC). Detailed characterisation of the binding of a series of peptides differing by a single amino acids from MCC, to a specific T cell receptor (TCR), identified $MCC_{(95\text{-}103)}$ as the core epitope recognised by T cells

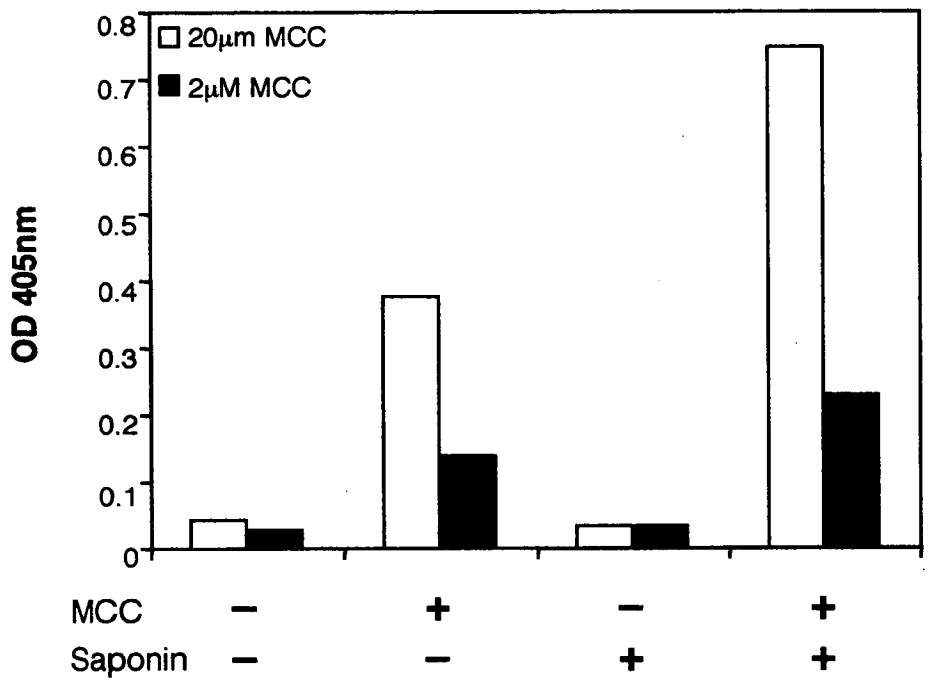
(Reay *et al.* 1994). D4 binds only to H2-E^k molecules complexed with this epitope (Figure 3.1) (Reay *et al.* 2000), which is derived from MCC protein, or with the equivalent epitope in Pigeon Cytochrome C (PCC), which differs by one amino acid from MCC, by APC (L. Colledge unpublished results). D4 binds to H2-E^k–MCC complexes with a rate constant of $1.25x10^{-8}M^{-1}$, indicating that it can detect complex formation with a sensitivity equivalent to that of a TCR (Reay *et al.* 2000). The T cell hybridoma, 2B4, also specifically recognises H2-E^k molecules bound to $MCC_{(95-103)}$ (Hedrick *et al.* 1982). Thus 2B4 T cells can also be used to follow the appearance of MCC-loaded MHC II molecules on the surface of cells.

3.2.2 Expression of Transgenic Proteins by *Leishmania sp.*

Transgenes are classically expressed in *Leishmania* parasites using the episomal expression vector, pX. pX was constructed using DNA from a circular region identified in methotrexate-resistant *L. major* parasites that encoded the dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene, and appeared to contain all the elements necessary for expression of this gene and replication of the DNA (LeBowitz *et al.* 1990). While pX has been successfully used in many different studies to over-express *Leishmania*-derived (e.g. Wolfram *et al.* 1996) or exogenous (e.g. Kaye *et al.* 1993) proteins in different *Leishmania* species, it has a number of limitations: transgene expression in pX-transfected cultures is extremely heterogeneous (Wolfram *et al.* 1996); transgenes are expressed much more highly by promastigotes than amastigotes (Misslitz *et al.* 2000); and continuous selective pressure is required to ensure that the episome is maintained at a high copy number, limiting the use of transgenic parasites *in vivo*.

An alternative vector has recently been developed that mediates the stable integration of the gene of interest downstream of a promoter that drives high level expression of that gene (Misslitz *et al.* 2000). Integration of the transgene is particularly efficient because homologous recombination in *Leishmania*, as in yeast, predominates over non-homologous recombination (Cappecchi 1990). The integration vector, pSSU-int, contains homology arms for sequences in the 18S small sub-unit ribosomal RNA

Figure 3.1: D4 only Detects MHC II Complexes in the Presence of MCC.

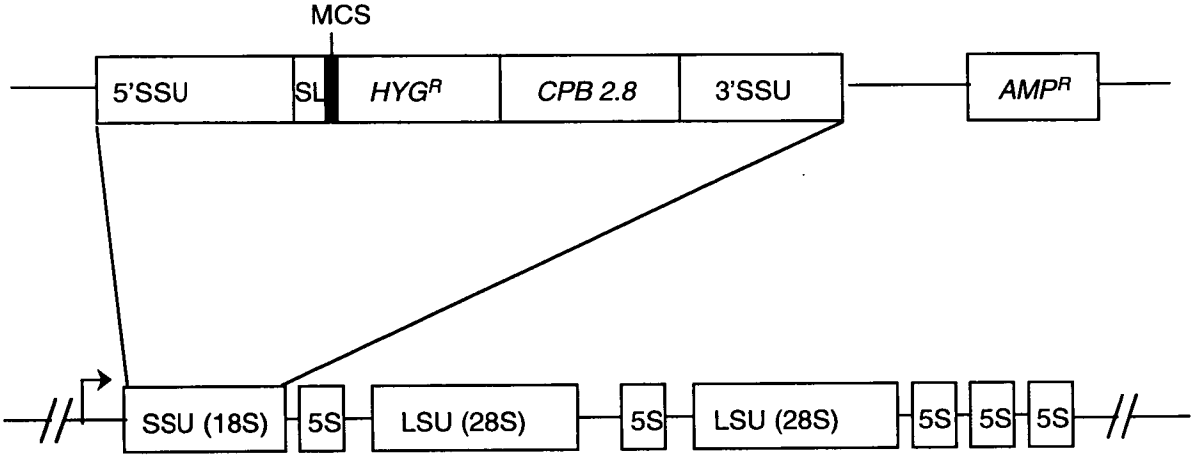


MCC peptide was incubated over-night with CHO-17 cells that had been engineered to express MHC II (H2-E^k) molecules and other components of the MHC II processing and presentation pathway (CHO-17 cells are described by Colledge *et al.* 2001). Formation of MHC II-MCC complexes in these cells was detected by ELISA using D4 with or without saponin to permeabilise the cells. Binding of D4 was visualised using an anti-mouse-alkaline phosphatase secondary antibody.

(rRNA) gene that is transcribed by both promastigotes and amastigotes (Figure 3.2 and Appendix A-a). Transcription of ribosomal genes from this locus is driven by an RNA polymerase I promoter, whereas protein-encoding genes are normally expressed from RNA polymerase II promoters. Post-transcriptional trans-splicing of a spliced leader transcript from the vector onto the transgene means, however, that genes expressed from the ribosomal locus will be expressed as genes transcribed by RNA polymerase II. Expression of *Leishmania* genes is controlled by undefined elements in 3' untranslated intergenic regions that couple both trans-splicing and polyadenylation of the upstream gene (LeBowitz *et al.* 1993). Sequences cloned into pSSU-int therefore require a 3' non-translated region to ensure correct expression of the transgene: the intergenic region from the cysteine protease B 2.8 gene cluster (*CPB 2.8*) is used since it is highly expressed in *L. mexicana* amastigotes (Mottram *et al.* 1997), ensuring high-level transgene expression in the intracellular form of the parasites. A selectable marker was also included in the cassette upstream of a second *CPB 2.8* intergenic region, to allow selection of targeted parasites.

The efficacy of pSSU-int was demonstrated through the characterisation of transgenic parasites expressing β-galactosidase or the enhanced green fluorescent protein (EGFP). *L. mexicana* parasites transfected with pSSU-int carrying lacZ or EGFP cDNA expressed these proteins as promastigotes and expression was up-regulated to high levels in amastigotes (Misslitz *et al.* 2000). Expression of EGFP was equivalent in *in vitro* cultured axenic amastigotes and lesion-derived amastigotes, even when isolated from animals that had been infected for several months, indicating that high levels of the protein were expressed in the absence of selection (Misslitz *et al.* 2000). A significant advantage of this vector compared to pX is that transfected parasites express highly homogeneous levels of the transgene in culture; flow cytometric analyses of EGFP production by promastigotes and amastigotes revealed a normally distributed single histogram for the culture (Misslitz *et al.* 2000). Therefore, pSSU-int was chosen as the optimal vector for these studies.

Figure 3.2: Integration of a Transgene into a 18S Small Subunit rRNA Locus using pSSU-int



Schematic representation of pSSU-int and integration into the rRNA small subunit locus by homologous recombination. 5' SSU and 3' SSU refer to the sequences required for the homology arms for recombination into the 18S rRNA gene. SSU= small sub-unit, LSU= large sub-unit; SL, splice leader; MCS, multiple cloning site; *HYG*^{r} hygromycin resistance gene; CPB 2.8 IR, *L. mexicana CPB 2.8* gene intergenic region; *AMP*^{r}, ampicillin resistance gene for selection in bacteria.

Taken from Misslitz *et al* (2000). For a plasmid map of pSSU-int see Appendix A-a.

3.2.3 The Model (Figure 3.3)

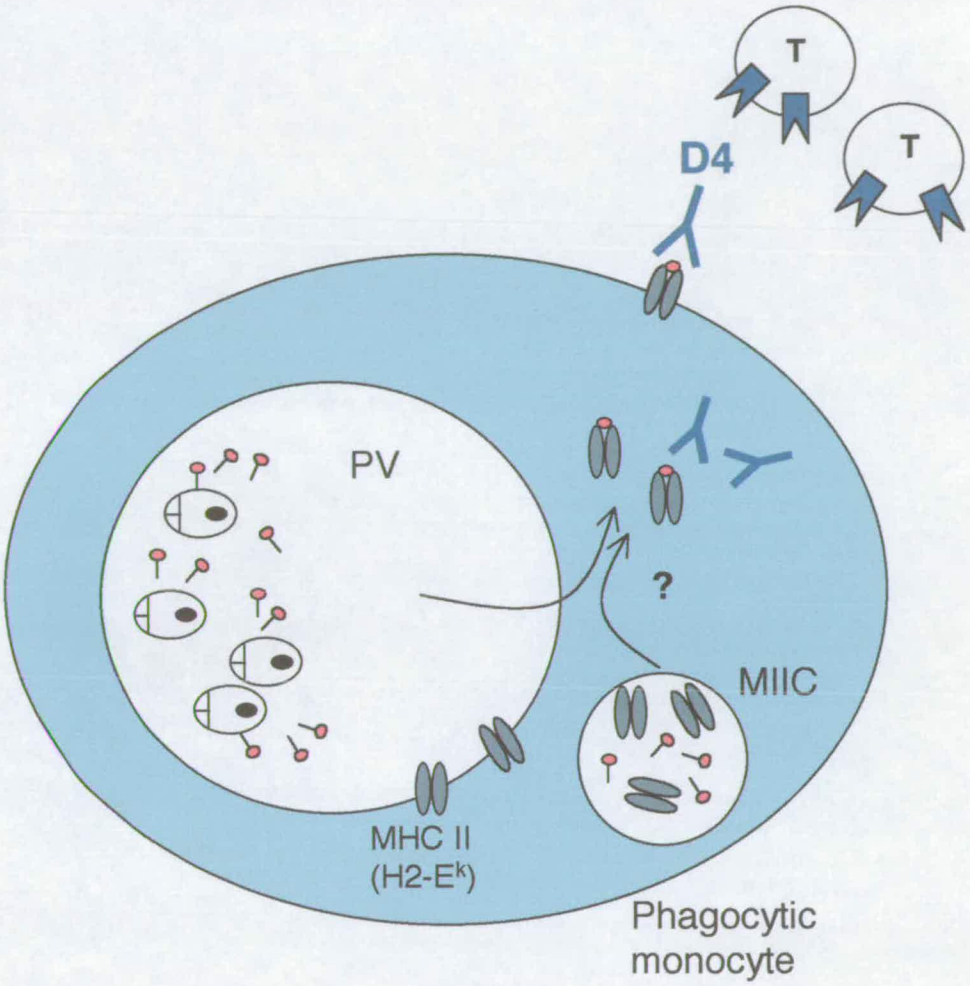
A model experimental system for the investigation of the fate of parasite-derived antigens in infected cells was designed based on the tools described above. This model proposed that transgenic expression of MCC as a fusion protein in *L. mexicana* parasites would permit the generation of complexes between MHC II molecules and parasite-derived MCC in infected DC and MΦ to be detected *in vitro* using D4 and the T cell hybridoma, 2B4. *L. mexicana* was chosen for this model since it can be cultured both as promastigotes and amastigotes *in vitro*, facilitating uniform infection of cultured cells, and the effect of infection of MΦ with these parasites has been studied in detail. Furthermore, it has been shown that over-expression of a *Leishmania* protein by transgenic parasites results in its detection by parasite-specific $CD4^+$ T cells (Wolfram *et al.* 1996), implying that complexes between *L. mexicana*-derived antigens and MHC II molecules do form in infected MΦ.

This chapter describes the derivation and characterisation of several lines of transgenic *L. mexicana* parasites in which different protein carriers were used as vehicles for the expression of the MCC epitope. These lines were used to infect BM-DC and MΦ in order to establish whether formation of MHC II-parasite-derived MCC complexes could be detected *in vitro* and, therefore, whether the experimental model described herein was suitable for investigating the formation of MHC II-parasite-derived antigen complexes in infected DC and MΦ.

3.3 Integration of GST-int into the rRNA Locus of *L. mexicana* Parasites

A proven processing-dependent MCC carrier protein, GST-int, was initially used for these experiments.

Figure 3.3: Schematic Representation of an Experimental Model with which to Detect the Fate of *Leishmania*-Derived Antigens in Infected Cells



Detection of parasite-derived antigen-MHC II complexes in *Leishmania*-infected DC and MΦ. Cells are infected with transgenic *Leishmania* parasites that secrete a fusion protein containing the T cell epitope Moth Cytochrome C (red circles). The complex-specfic mAb, D4, is used to detect the formation of H2-E^k-MCC complexes in infected cells, either intracellularly or on the cell surface. An MCC-specific T cell hybridoma can also be used to detect presentation of MCC epitopes on the surface of infected cells. PV, parasitophorous vacuole; MIIC, MHC II containing compartment; T, MCC-specific T cells.

3.3.1 GST-int

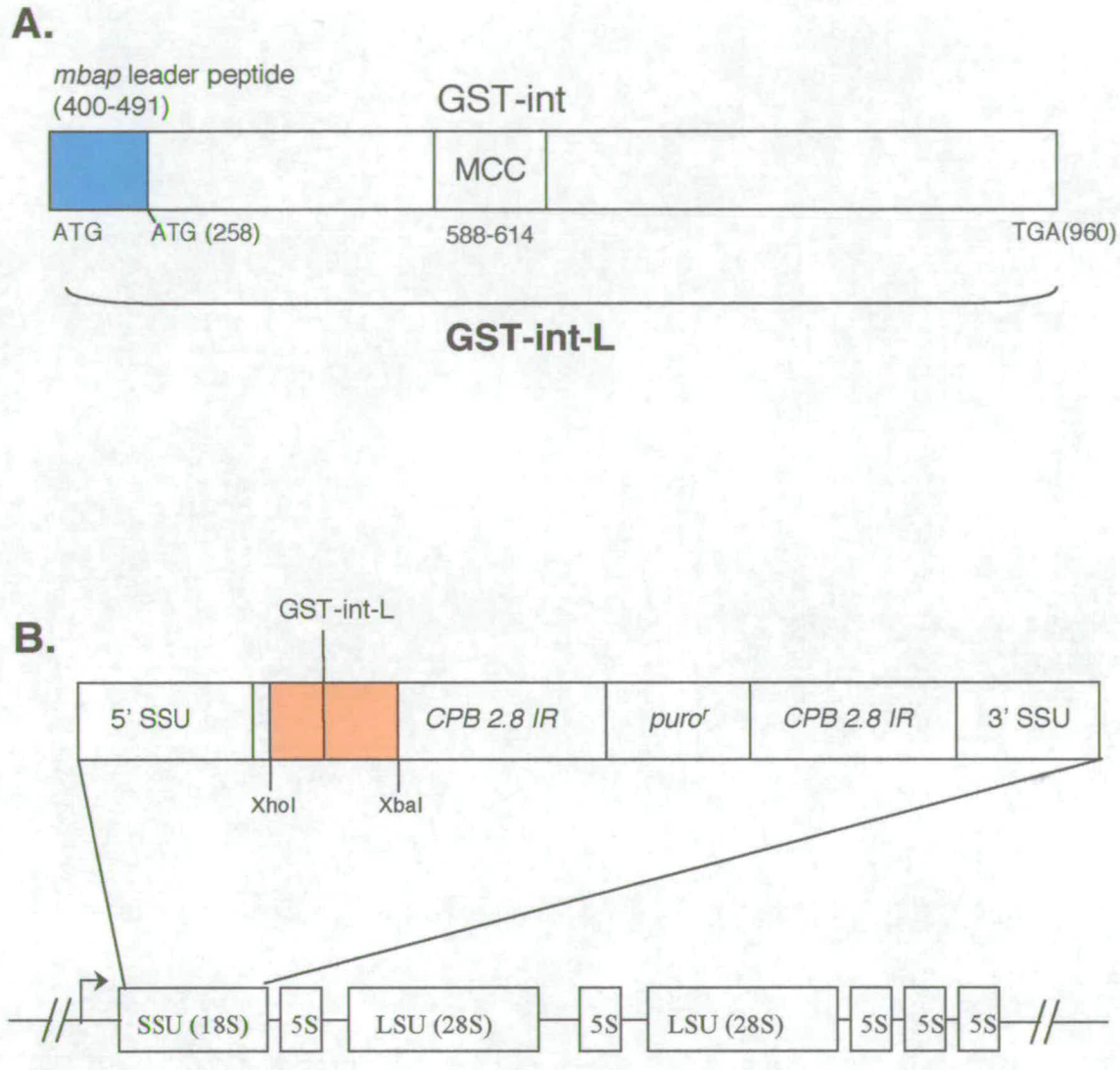
Glutathione S-transferase (GST) is a well-characterised enzyme, commonly used for fusion protein production. The core $MCC_{(95-103)}$ epitope was used to replace non-conserved residues in an internal loop of GST, generating the construct known as GST-int (kindly provided by Phil Reay, Oxford; (Colledge *et al.* 2001)). Two of the endogenous GST amino acids provided amino acids 93 and 94 of the MCC peptide. Processing and presentation of MCC from this carrier protein has been carefully characterised (Colledge *et al.* 2001), and MHC II-MCC complexes could be detected with D4 in DC infected with bacteria expressing this fusion protein (L. Colledge unpublished results). GST-int was therefore a potential carrier molecule from which to express the MCC epitope in *L. mexicana* parasites.

3.3.2 Generation of the Targeting Construct and Integration into *L. mexicana* Parasites

An initial cloning strategy was designed in which GST-int was cloned into pSSU-int-lm mkk (kindly provided by Martin Wiese, Tübingen; see Appendix A-a). The cloning strategy for the generation of the construct pSSU-GST-int-L is described in Chapter 2 (section 2.1.8.2). *Leishmania* antigens had previously been shown to be most efficiently presented to T cells if over-expressed on the surface of the parasites, or secreted into the PV (Wolfram *et al.* 1996). The leader sequence from the *L. mexicana* membrane-bound acid phosphatase gene (*mbap*) was therefore cloned onto the N-terminus of GST-int so that the transgene would be targeted to the plasma membrane and would be secreted by the parasite (Figure 3.4).

Log phase promastigote cultures were electroporated with the GST-int-L construct, passaged one day later into a 24-well plate and grown in Puromycin to select for

Figure 3.4: Schematic Diagram Showing Integration of GST-int-L into the 18S SSU rRNA Gene



A. Schematic diagram of GST-int-L. The blue box represents the 90bp leader peptide derived from the *L. mexicana mbap* gene. $MCC_{(95\text{-}103)}$ is represented in orange. Numbers are base pairs from the start of the transcribed GST-int DNA or *mbap* DNA sequences.

B. Integration of GST-int-L (orange) into the 18S small sub-unit rRNA gene.

transfectants. It was assumed, based on a transfection efficiency of 1 in $1x10^6$, that each well contained approximately one parasite. Parasites derived from each well were therefore considered clonal lines (see section 2.3.1.4). 10 out of the 24 wells contained parasites that grew in the presence of Puromycin. Positive wells were screened by PCR using a forward primer specific for the promoter of the 18S rRNA gene, outside the homology arms of the integration cassette, and a reverse primer specific for the integrated transgene. Of the lines tested, four were shown to contain GST-int-L correctly integrated into the rRNA 18S SSU gene by PCR. In a further line GST-int-L integrated into the genomic DNA but not into the correct locus (Figure 3.5).

3.3.3 Expression of GST-int-L by *L. mexicana* Parasites

Expression of GST-int-L mRNA by two of these transgenic lines of parasites (10 and 22) was confirmed by Northern blot analysis. The blot was probed with a labelled PCR fragment specific to GST-int-L. GST-int-L was transcribed by clones 10 and 22 (Figure 3.6A). However, Western blot analysis using an anti-GST polyclonal antibody was unable to detect the fusion protein in promastigote or amastigote pellets or supernatants (Figure 3.6B).

Despite the strong inference that no stable fusion protein was being produced by these parasites, further experiments were carried out to determine whether DC infected with GST-int-L parasites could stimulate MCC-specific T cells. BM-DC were incubated with parasites from GST-int-L lines 10 and 22 at a ratio of 1:8 overnight, washed and co-cultured with the MCC-specific T cell hybridoma, 2B4, at a ratio of 1 DC:10 T cells for 18 hours. Activation of T cells was followed by analysing IL-2 production in the culture supernatant. As expected, DC incubated with purified GST-int protein overnight efficiently stimulated the T cell hybridoma, confirming that MCC was processed and presented from this antigen by DC. However, DC infected with GST-int-L parasites did not stimulate MCC-specific T cells *in vitro* (Figure 3.7).

A. Genomic DNA from GST-int-L lines was analysed for integration into the ribosomal locus by PCR using a forward primer external to the 5' homology arm of the vector and specific to the promoter region, and a reverse primer specific to a sequence within the GST-int-L construct (red arrows). The predicted band size for this product was approximately 2kb. 4 clones showed correct integration of GST-int-L into the ribosomal locus. A PCR product was not seen when these primers were used with wild type (WT) genomic DNA.

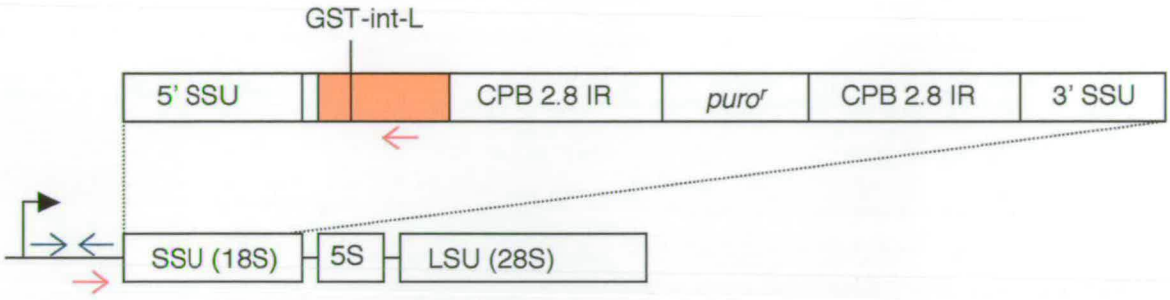
B. PCR with primers specific for the GST-int-L transgene to indicate the presence of the GST-int-L transgene. The predicted band size for this product was 700bp. In addition to the 4 lines identified in A., one line (4) contains the GST-int-L DNA but is not integrated into the correct locus.

C. Primers specific to the ribosomal promoter region external to the 5' homology arm of pSSU-int, which should be present in all samples, were used as a postive control (blue arrows).

Numbers represent the wells of a 24 well plate containing Puromycin-resistant parasites.

M = 1kb ladder.

Figure 3.5: PCR Analyses to Show Correct Integration of GST-int-L Lines into the 18S SSU Ribosomal Locus



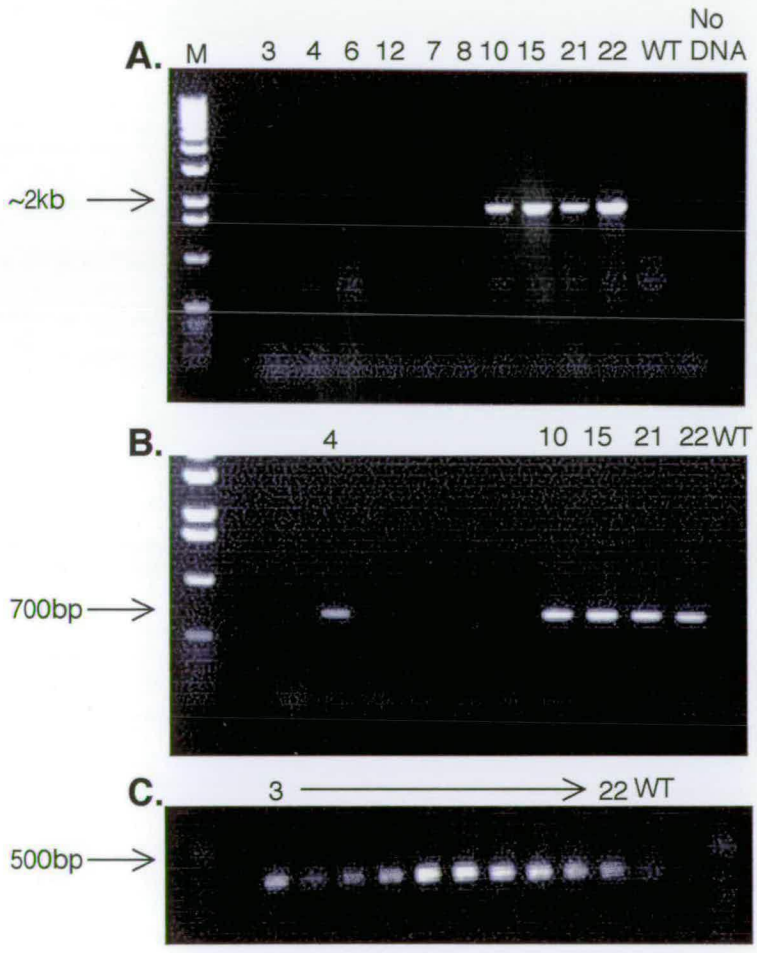
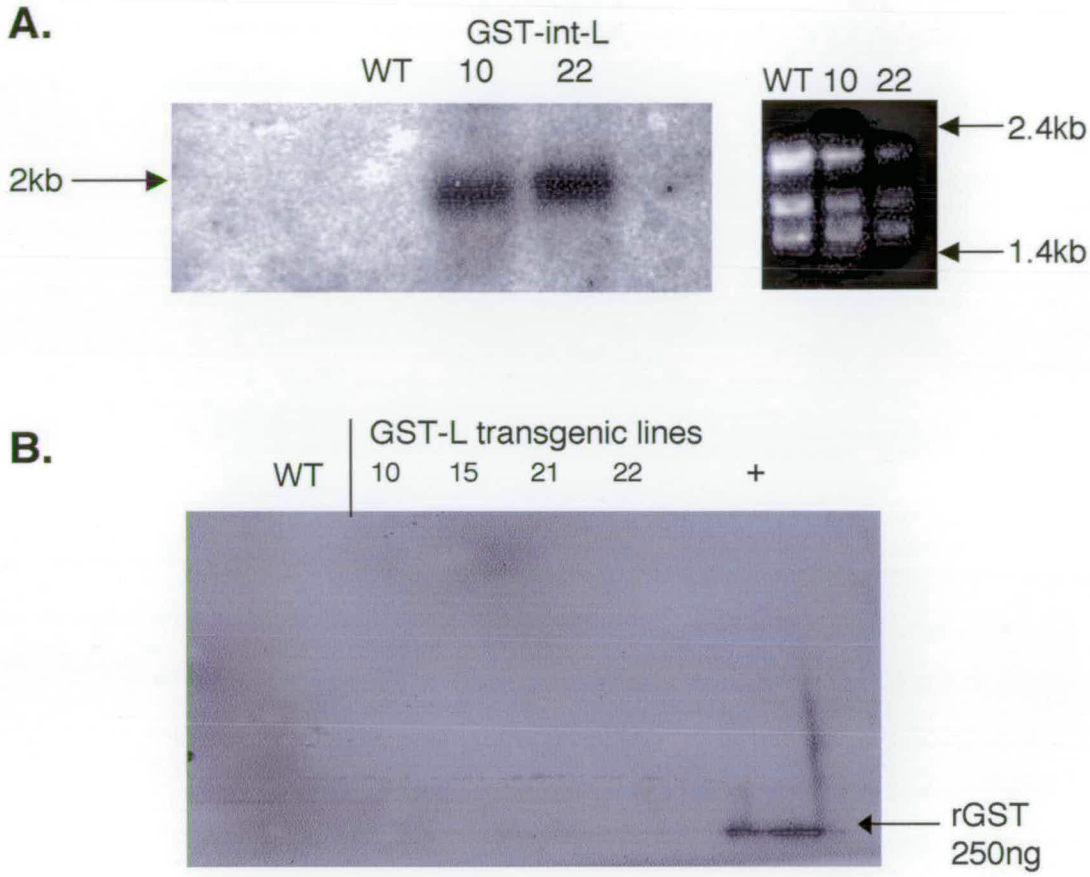


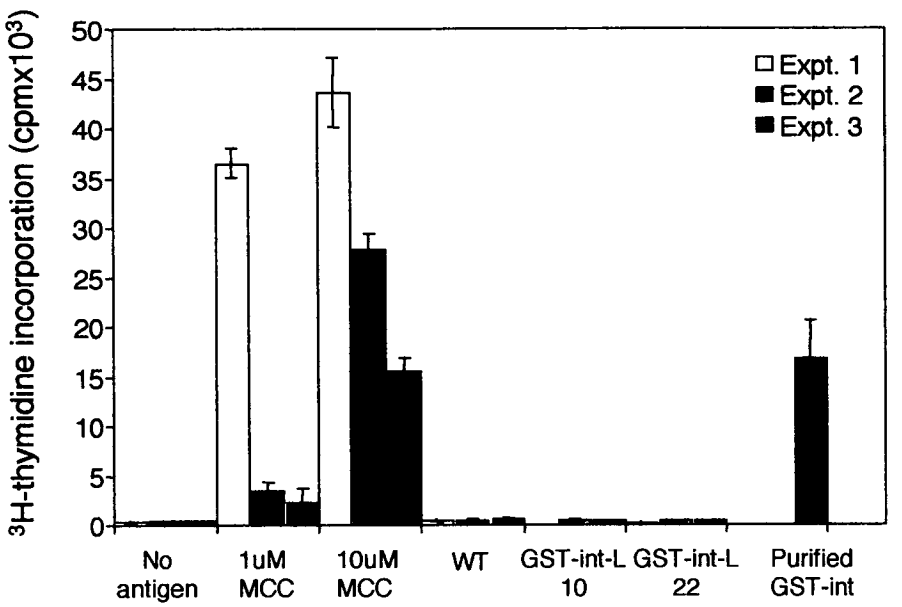
Figure 3.6: Expression of mRNA, but not Protein, by GST-int-L Parasites



A. 5μg of total RNA from *L. mexicana* wild type (WT) or GST-int-L parasites was run on a denaturing gel, blotted onto a nylon membrane, and probed with a labelled PCR fragment complementary to the GST sequence. A 2kb transcript was detected as predicted for the correctly expressed gene. The right hand panel shows ribosomal RNA as a loading control. *Leishmania* parasites express three rRNA bands between 1.4 and 2.4kb.

B. Transgenic GST-int-L amastigotes were cultured overnight at a concentration of 10^8/ml in 100μl of serum-free medium. Parasites were centrifuged the next day and supernatants were loaded onto a 12% SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose and the membrane incubated with a polyclonal antibody to GST (Amersham). No staining was seen in any parasite samples. This blot is representative of Westerns performed on promastigote supernatants, concentrated supernatants and promastigote and amastigote cell pellets.

Figure 3.7: DC Infected with GST-int-L Transgenic Parasites do not Stimulate a MCC-Specific T Cell Response



DC were cultured overnight with GST-int-L transgenic amastigotes, and then co-cultured with the T cell hybridoma 2B4. IL-2 production by 2B4 cells was measured using an IL-2-dependent indicator cell line. Values shown represent [^{3}H]-thymidine incorporation by IL-2-dependent indicator cell line C.C3.11.75.

DC were incubated overnight with 0.4mg/ml purified GST-int as a positive control for presentation of MCC to 2B4 cells from GST-int.

Each experiment was performed in triplicate.

(Figure 3.7). Since these results were as predicted by Western data GST-int was not pursued further as a potential epitope carrier, and an alternative carrier protein was sought.

3.4 Use of the *L. mexicana* Membrane-Bound Acid Phosphatase as a Carrier Molecule for MCC

3.4.1 Introduction

The *L. mexicana* membrane-bound acid phosphatase (MBAP) was originally identified using antibodies generated from mice immunised with Concanavalin A-binding membrane components of *L. mexicana* promastigotes (Menz *et al.* 1991). The *L. mexicana mbap* gene was subsequently cloned and characterised (Wiese *et al.* 1996); Figure 3.8), and shown, in promastigotes, to be localised to endosomal/lysosomal compartments between the nucleus and the flagellar pocket. Over-expression of MBAP, by episomal expression in pX, resulted in its re-localisation to the plasma membrane of promastigotes (Wiese *et al.* 1996), while deletion of the transmembrane domain (Figure 3.8) resulted in secretion of large amounts of active enzyme into the culture supernatant (Wolfram *et al.* 1996). MΦ infected by these transgenic parasites *in vitro*, but not with wild type parasites, were able to stimulate a MBAP-specific T cell line, indicating that localisation of MBAP to the parasite surface, or its secretion into the PV, was sufficient to facilitate the formation of MHC II-peptide complexes (Wolfram *et al.* 1996). *L. mexicana* MBAP therefore constituted an attractive carrier molecule in which to clone the MCC epitope. Furthermore, the 3D structure of MBAP, predicted based on the crystal structure of the related rat prostate gland acid phosphatase (rPAP) (M. Wiese unpublished results; (Schneider *et al.* 1992)) provided a means of designing fusion proteins anticipated to retain the structural integrity of native MBAP.

DNA sequence of the *L. mexicana* membrane-bound acid phosphatase (*mbap*) gene (accession number Z46971). Blue underlined codons are the translation start and stop codons; the peptide leader sequence is highlighted in green; the transmembrane domain is underlined with a dashed line. The sequence that was deleted to create Modlap, the secreted form of MBAP, is marked in bold.

Figure 3.8: Sequence of the *L. mexicana* Membrane-Bound Acid Phosphatase Gene

```
CCCCACACACACACATACATGCGAGCACCTAATACGACCTCTGTGCCATTGCCTACCGGA 60
AAGACGCAACCGCGCACCGTTTACATATGTTGCCTCTGTTACAGAAAGGTTGCCTTCCCC 120
ACTGTCTGCGTCCTTTGTTCTCTATTTTTTTTTTCTCTTCCCCACTCTCACGCCATTGTA 180
TGCACACGTTCATTGCCTGACCTTTTCCAACGCATGCACACGTGCACGGTCATCTTGTCG 240
TGCTGGCCATTTCCTTGTCTTTTGCAGGTATCCTCCCCGTCTTCTTCCCACTCCACTGCT 300
TGTGGGCTCTCACCCCCTTTTTTCGACCCATTTTACCGCCGGCGAACTCCTCTCATAGAA 360
GAGACTTCCCATCGCCCGCTTCCCTTCTCTTCCGCTATCATGCTCAGAGGCATCGGTTTC 420
CTCTACCGGTGCGTGGTGGTGGTGGTGGTGGCTGTGGTGGTCAGCGCAGCGGTGGTGTCG 480
                BsrGI
GCGGCACCGATGTACAAGGTGGAGCTGGTGCAGGTGGTGCACCGCCACGGAGCACGCTCC 540
CCGCTCGTCGATGACAACCA ACACTCATCTGCGGCACCGAGTTCCCGTGCGGGTTTCTC 600
AACTACGAGGGTCAGGCGATGCTGGTGAACCTCGGTAAGTATCTGCACCATCGCTACACG 660
GAGAACCCTTCGGTTGTGTCGAAGCCATACTTCCCGTACAGCTGGTACAACCTATCTATC 720
TCGTATACGCGCTCGACGGATGTGCTGCGCACGCTTCAGAGCGCCAACGGGCTTCTGCAG 780
GGCCTCTTCCCGAACATGTCGACTTTCTTTCCTGCAATCCACGCAGTGGGAAGAAAGGAG 840
GATGTGCTACTGCACAGCTACATGGTGCCGATGATTCGCGCTCGCTTCAATTACGCGAAG 900
GAGGAGCTGCGGGCCGTGTGCGACGAGGTGTTGGACAGACTAATGTCATTCGATCAGCTG 960
CAGGCGGTAGCGGCGGAGGTTCACTCGCAGAGGTTTTGCGCCAACTACACCCTGCGCTCG 1020
CGTTGTGCGAAGCGGCTGTGCGACATTGGGCGCGCGTACGAGCCCACTGGCCGCTTGGAA 1080
AGTCTCCCGCTGCTTAGTCGGCACCTGGACGACGTGTGCGCTGTGACGGCGATGAGCTCG 1140
TATTTCTATTTCGCTTACAACGCCAGCAATCCCGTCCATCAGAAGCAAGGCGCACCGTTC 1200
TACCACCTGGCGAAACTGCTGGTGAGCAACATGGTAGCGCACCAGCAGCGCGAGACGGCA 1260
CCGCCGTACAAGCTGTACGAGTACAGTGCACACGACACCACTATCTCGCCCCTGGCGGTT 1320
TCCTTCGGTGATAACTCGATGGAGGCGATGCTGCCGCCATTCGGCACAGCGTTTATCATA 1380
GAACTCCTGTCGCTGACGGACGCGCCTGCCGCGCCGTCGTCCTTCTACGTGCGGCTGCTG 1440
CGCGGTCACTCTGGTGTGAGGCCGGAAAGTAACTTCACCTTCGCTCTGAGTCACTTCGAC 1500
ATGCGCTGCCAGGACGCGACGGGCAACACGTACATTGCGACGGACAACATATGCCCCTTC 1560
GCCGACTTTGAGCGCTTTATAAACTCCACCGCGCCAACGAGCCCGATGGGCACGTGCTAC 1620
CTCGACCCTGGTCTTCTGTTCCGCATGGACTGTCCGATTGACGTCGTCAGCGACAACCGC 1680
AGCTTGTCGGAGGACTGCCTCTTCTACCGCCAGCACTGCAGCAACTACTCGTGCGGCACC 1740
GGCTACTACCTCGACGCGATCGACTACGGCTGCCACCGCATCCCGGCGAACAACTCAACG 1800
GCTGGATCGTCGCCCATGTCCAGCGGCGGGATTGCTGTCCTGAGCATCACACTCTTCATC 1860
GTCGGCGGAGTGGCGAGCGTCGGCGGTATGGAGGTGTGGAGACGCTACATGAAGTTCAAA 1920
AACAAGCAATCCGAGGCGATTATCGTCTGACAACCTATCGAACCCTGTAGCCATGCTTTT 1980
TTCCTTCGTAGCCTTTATTTTCCTTAAGCAAGAGCATAGCCTTACTTGCGGGCTTCCTCA 2040
ACAGTACACCCCCTATTCGACAGTGCTTCTGCGAGAAATCGTACATCTGCGCTCAGGTTG 2100
CAAATTCGCAAACGCCCAACACACGAACATTCTCTGATTTATTCTATCTGCCTCGGTACA 2160
TGCTTTTCTTCCTTCTGTGC GTGTGAAGCATATTTGCAGTGGATTATGCTTTCGCTTAG 2220
CCGTGTTTTTGTTTCTCCCACTACTACTGCTGCTACTGTTCTTTTCGTGTTCTAGCCCTT 2280
CGAGGGGCCCATCAGCTTCCCCGCCGTTGCGCTCACTCAGCGGTGGATCC           2330
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3.4.2 Generation of *L. mexicana* Parasites Expressing MBAP:MCC Fusion Proteins

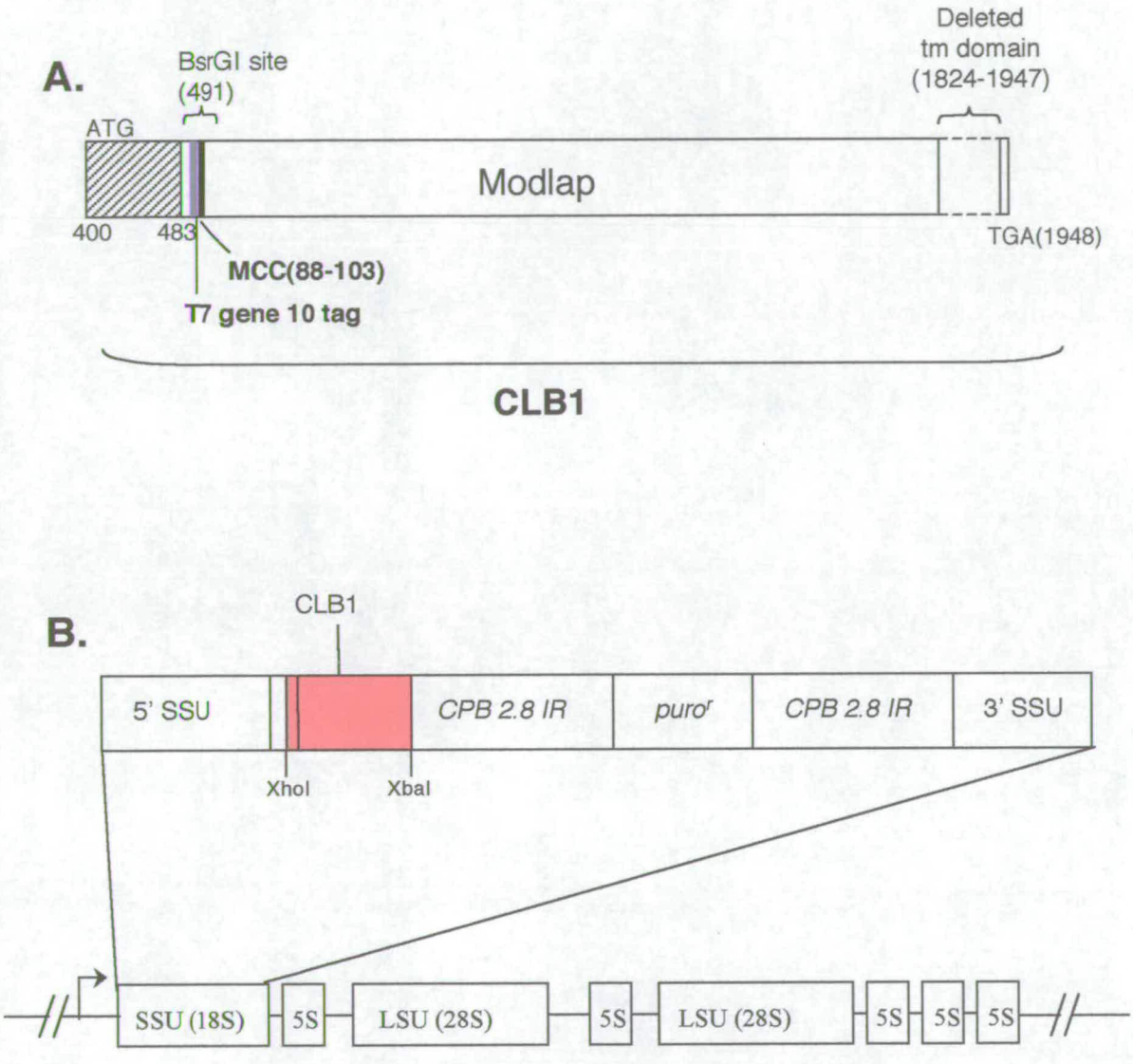
3.4.2.1 Integration of MCC into the N-terminus of MBAP

3.4.2.1.1 CLB1: Generation of Parasites and Integration into the rRNA SSU gene

A cloning strategy was designed in which MCC was cloned into the secreted form of MBAP, known as Modlap (kindly provided by Markus Wolfram, Tübingen; (Wolfram *et al.* 1996)). Modlap was chosen as secretion of high amounts of the acid phosphatase into the PV may facilitate processing of the protein and loading of epitopes onto intracellular MHC II molecules. $MCC_{(88\text{-}103)}$ was initially cloned adjacent to the N-terminus of MBAP. The extended MCC peptide was chosen because the sequence may include protease cleavage sites that would facilitate processing of the core 95-103 epitope. The sequence of the epitope was corrected for optimal codon usage by *L. mexicana* parasites (Langford *et al.* 1992). Modelling of the MBAP protein predicted that the N-terminus would not be contained within the globular structure of the enzyme, therefore, cloning into this site should not alter the correct folding and function of the protein. Expression of MCC at the exposed N-terminus of the protein should also ensure that it was easily accessible to host proteases. The T7 gene 10 epitope tag (Novagen) was included N-terminal to MCC in the fusion protein to facilitate analysis of transgene expression by immunohistochemistry and immunoprecipitation. The final construct was designated CLB1, the cloning strategy for which is described in section 2.1.8.3 (Figure 3.9 and Appendix A-b).

To generate lines of CLB1 transgenic parasites the linearised CLB1 construct was electroporated into *L. mexicana* promastigotes. Six Puromycin-resistant lines were selected and expanded. Genomic DNA from these lines was screened by PCR using primers specific to the rRNA promoter and to the splice leader of the integration cassette (Figure 3.10). A PCR product of the expected size (1.6kb) was amplified from all of the lines tested, indicating that they each contained the N-terminal MBAP:MCC construct

Figure 3.9: Schematic Diagram Showing Integration of CLB1 into the 18S SSU rRNA Gene

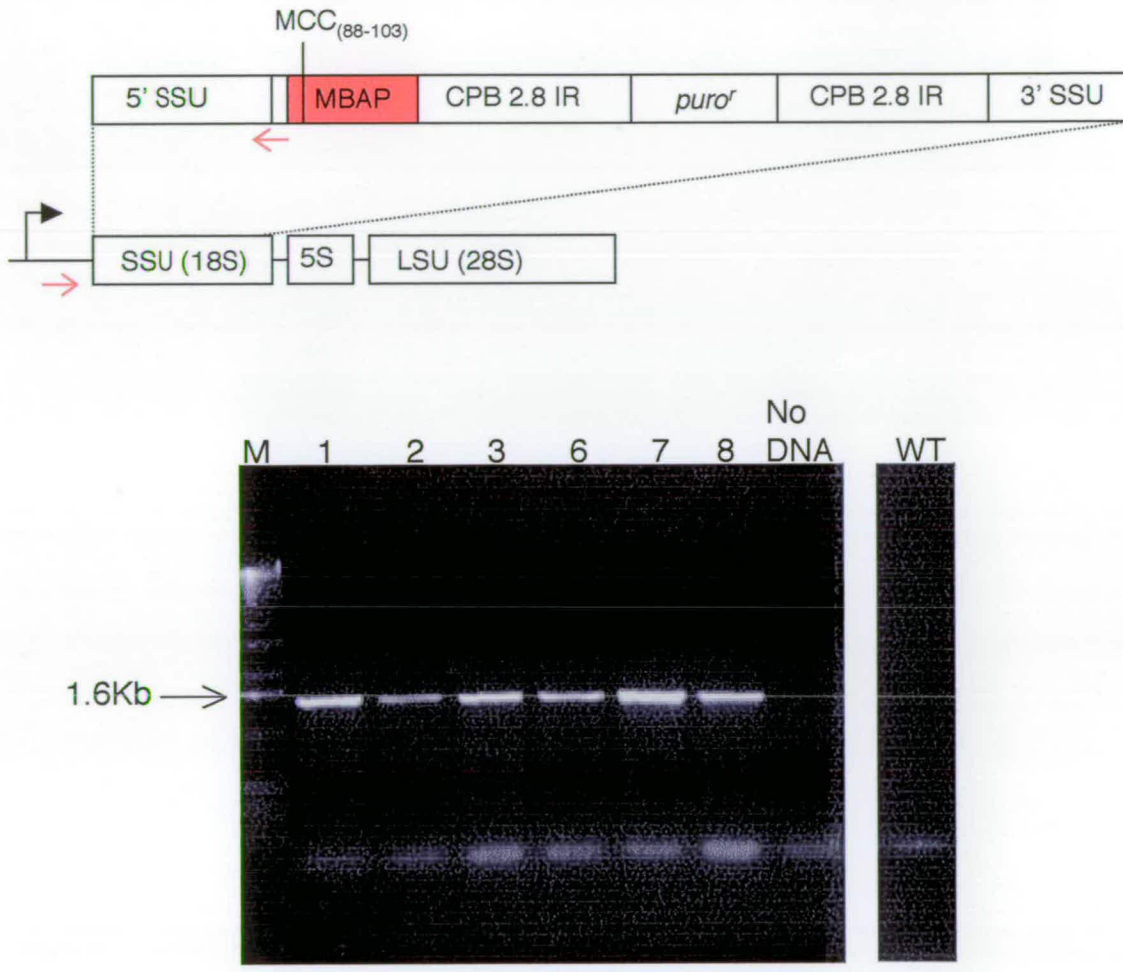


A. Schematic diagram of CLB1. The hatched box represents the *L. mexicana mbap* leader peptide. $MCC_{(88\text{-}103)}$ (black line) and the T7 gene 10 tag (grey line) were cloned into the BsrGI site downstream of the leader peptide. The diagram shows the secreted form of mbap, Modlap, in which the transmembrane (tm) domain was deleted.

Numbers refer to the DNA sequence shown in Figure 3.8.

B. Integration of CLB1 into the 18S small sub-unit rRNA gene.

Figure 3.10: PCR Analysis to Show Correct Integration of CLB-1 Lines into the Ribosomal Locus



Genomic DNA from CLB1 lines was analysed for integration into the ribosomal locus by PCR using a forward primer external to the 5' homology arm of the vector and specific to the promoter region, and a reverse primer specific to the splice leader sequence of the pSSU-int vector (red arrows). The expected size for the PCR product from these two primers if the transgene was correctly integrated into the ribosomal locus was approximately 1.6kb. A PCR product was not seen when these primers were used to amplify wild type (WT) genomic DNA.

Numbers represent wells containing Puromycin-resistant parasites from a 24 well plate.

M = 1kb ladder.

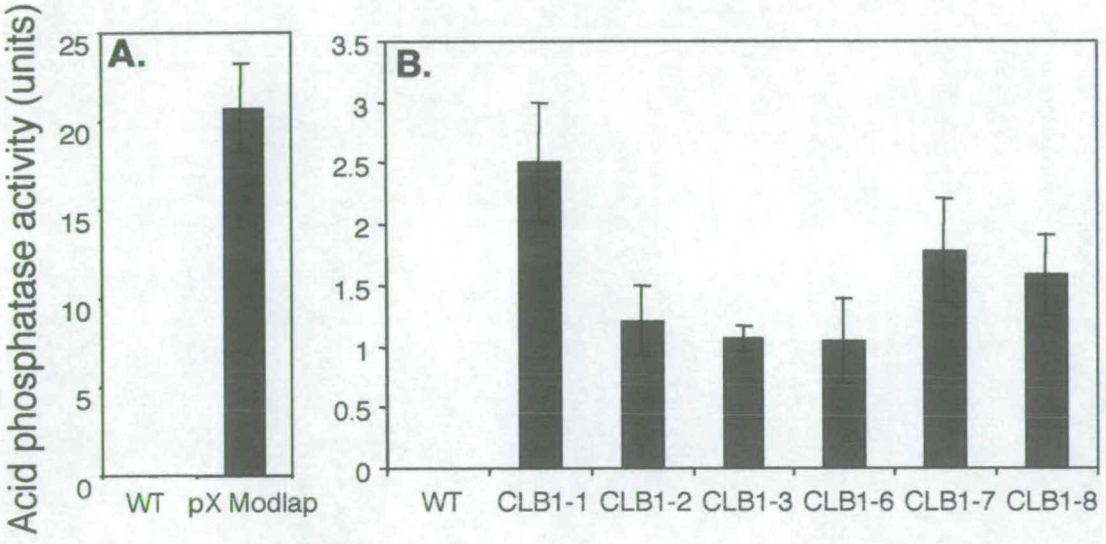
integrated into the 18S SSU rRNA gene (Figure 3.10). These lines were therefore tested for transgene expression, as described below.

3.4.2.1.2 CLB1: Up-regulation of Acid Phosphatase Activity in the Supernatant of CLB1 Parasites

Wild type *L. mexicana* amastigotes do not secrete acid phosphatases, therefore, Modlap expression levels could be assessed by assaying acid phosphatase activity in parasite culture supernatants. $1x10^7$ CLB1 axenic amastigotes from different transgenic lines were therefore cultured overnight in 1ml of serum-free medium. The supernatants were then collected and tested for acid phosphatase activity. The acid phosphatase activity was calculated in units, whereby 1 unit equals the amount of enzyme required to hydrolyse 1μM of substrate per minute (see section 2.2.2). Figure 3.11A shows that wild type *L. mexicana* amastigotes secrete no detectable acid phosphatase activity, whereas parasites in which Modlap had been cloned into pX (pX Modlap) secreted large amounts of the enzyme. CLB1 clones also secreted the functional enzyme, although at much lower levels than the pX Modlap parasites (Figure 3.11B). The lower expression levels in CLB1 clones are likely due to differences in copy number; only one copy of the CLB1 transgene will be expressed by each parasite whilst parasites containing pX Modlap, which is maintained episomally, will express multiple copies of the gene. These results demonstrated that CLB1 transgenic parasite lines over-expressed a functional secreted form of the membrane-bound acid phosphatase.

The existing anti-MBAP antibody, AP4, could not be used to detect CLB1 because it recognises an epitope that was disrupted by insertion of the MCC epitope. To determine whether the fusion protein could be detected using expression of the T7 gene 10 epitope tag, lysates derived from CLB1 parasites and culture supernatants were analysed by Western blotting. No proteins were identified using a mAb specific to the gene 10 epitope. Since repeated phosphatase assays prior to the Western blot analysis had demonstrated that the fusion protein was correctly expressed these data suggested that this peptide was not an appropriate epitope tag for use in this system.

Figure 3.11: Detection of Acid Phosphatase Activity in Supernatants from MBAP:MCC CLB1 Transgenic Amastigote Cultures



Acid phosphatase activity was measured in supernatants from $1x10^7$ wild type (WT), pX Modlap (A.) or CLB1 (B.) amastigotes cultured overnight in 1ml of serum-free medium. Activity was calculated from the change in absorbance of the substrate pNpp on incubation with culture supernatants and is expressed as units (1 unit = the amount of enzyme which hydrolyses 1μM substrate per min).

Values represent the average of 2 experiments. Error bars represent the s.e.m. from 2 experiments, each of which was performed in triplicate.

3.4.2.1.3 CLB1: MHC II-MCC Complexes can not be Detected on Infected DC

Since infected MΦ have repeatedly been shown to present *Leishmania*-derived antigens inefficiently to T cells, the utility of the CLB1 parasites for the experimental model described in section 3.2 was initially tested using infected DC. BM-DC were generated *in vitro* from CBA mice and incubated overnight with different transgenic lines of CLB1 axenic amastigotes at a ratio of 1:8 DC:amastigotes. The following day the cells were harvested, fixed, and incubated with D4 and an appropriate secondary antibody, both with and without permeabilisation, to detect the formation of surface and intracellular complexes. These cells were then analysed by flow cytometry. Figure 3.12 shows the results from infection of DC with CLB1-2 amastigotes, and reflects the results obtained by all the other lines tested. No D4 staining was detected in DC infected with transgenic promastigotes and amastigotes, amastigote supernatants or fixed amastigotes. To attempt to optimise these experiments DC were also infected for different time points, with promastigotes and amastigotes, however, D4 staining could still not be detected on or in infected cells (data not shown). Since presentation of Modlap epitopes was previously shown in MΦ, CLB1-2 amastigotes were also used to infect bone marrow-derived MΦ, according to the protocol described by (Wolfram *et al.* 1996). The cells were then harvested, fixed and stained as above with permeabilisation to detect the formation of intracellular MHC II-MCC complexes. D4 staining was not detectable in infected MΦ (data not shown). Therefore, although CLB1 parasites expressed the secreted acid phosphatase, the MCC epitope was not processed for presentation via MHC II molecules in infected DC or MΦ at levels detectable with D4 or 2B4 T cells.

Based on these results, it was decided that the lack of detectable presentation of MCC may have been due to incorrect processing of the epitope from MBAP. Alternative strategies were therefore devised, also using secreted MBAP as the carrier molecule.

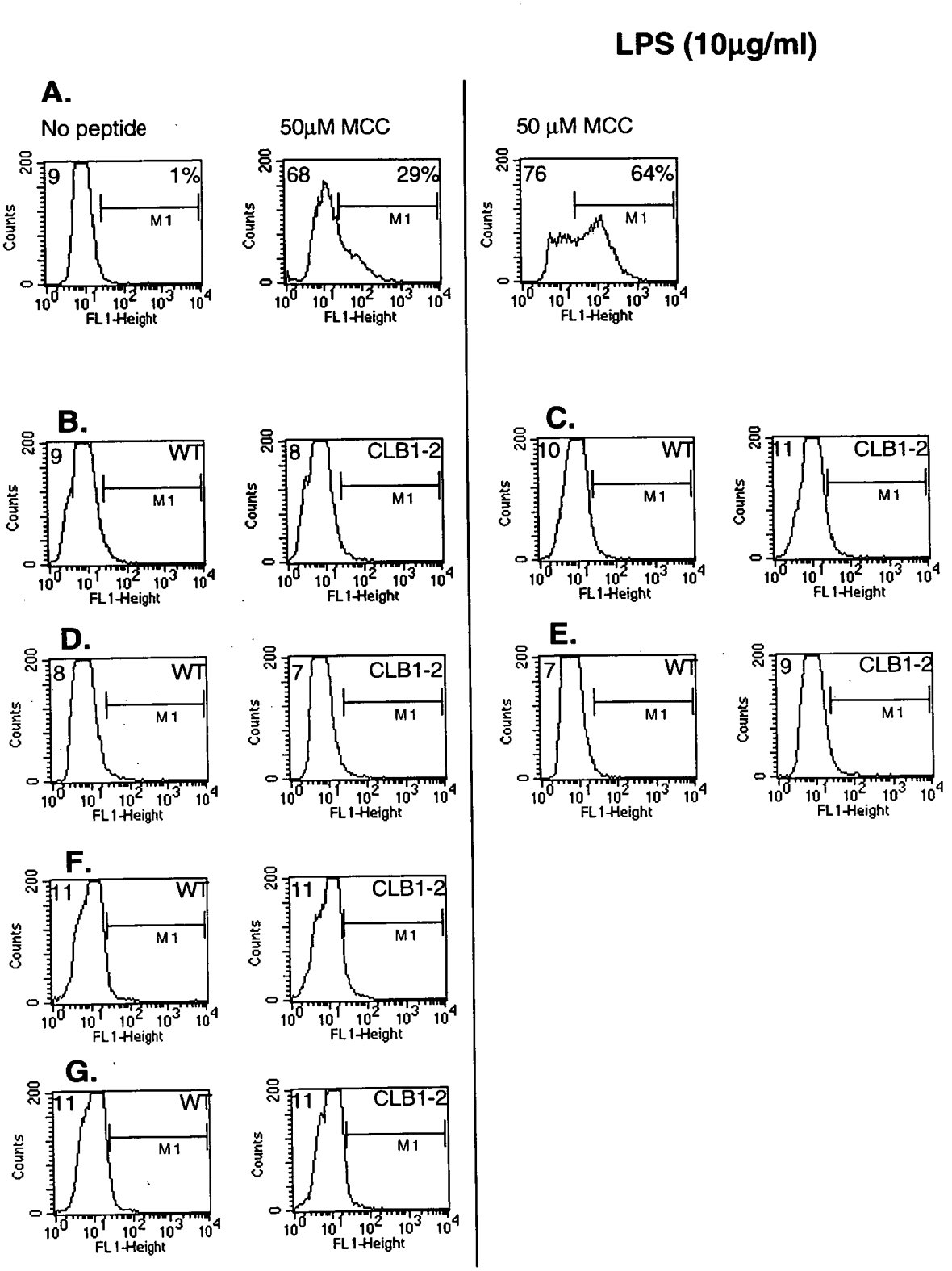
During experiments to attempt to optimise detection of MHCII-MCC complexes in infected cells, BM-DC were also infected with *L. mexicana* parasites and analysed by

Day 7 bone marrow-derived DC were incubated with MCC peptide, *L. mexicana* wild type (WT) amastigotes or CLB1-2 amastigotes for 18 hours at a ratio of 1:8. Cells were harvested and incubated with the complex-specific antibody, D4 followed by FITC-conjugated goat anti-mouse antibody.

A, surface stain on DC cultured without antigen or with 50μM MCC peptide, with or without 10μg/ml LPS; B, surface stain on amastigote-infected cells; C, as B but DC were activated with 10μg/ml LPS; D, intracellular stain on amastigote-infected cells; E, as D but DC were activated with 10μg/ml LPS; F, surface stain on cells incubated with fixed amastigotes; G, surface stain on cells incubated with supernatants from amastigote cultures.

M1 was set on DC incubated in the absence of antigen and stained with D4 and the appropriate secondary (first panel). Numbers in the top left hand corner of plots represent the mean of the population; percentages represent the percentage of cells in M1.

Figure 3.12: Detection of MHC II-MCC Complexes by D4



flow cytometry to ensure that the lack of presentation of MCC was not due to low level expression of MHC II molecules on the surface of infected cells. BM-DC were infected with EGFP-expressing amastigotes (see Chapter 4) overnight, harvested, fixed and stained with the anti-MHC II mAb 14.4.4. Figure 3.13 shows that, surprisingly, infected BM-DC did not up-regulate expression of surface MHC II. This effect is discussed further in Chapter 4. Based on this result, both immature BM-DC and BM-DC activated with LPS were used to assess presentation of MCC from fusion proteins.

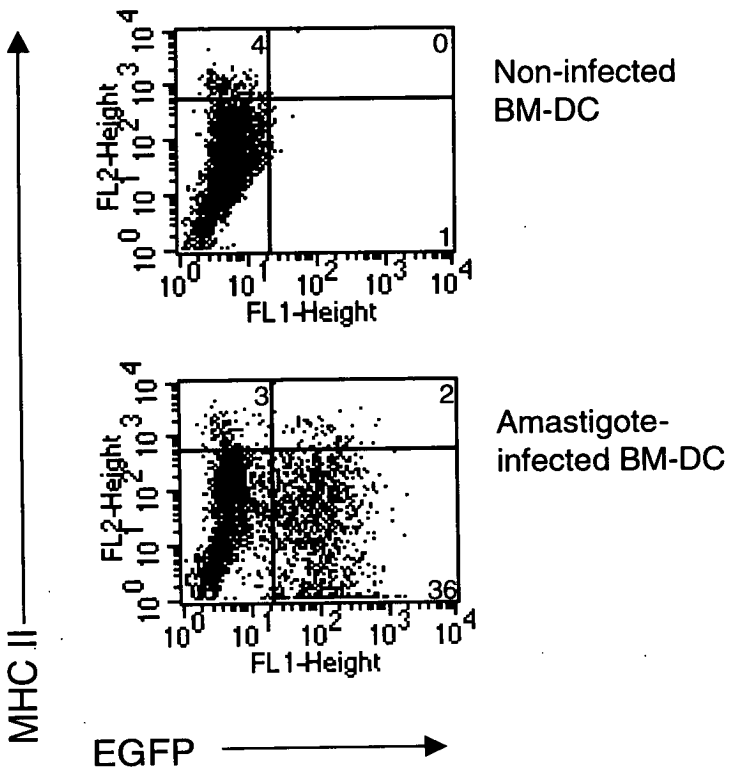
3.4.2.2 CLB2 and CLB3: Alternative MBAP Fusion Proteins

3.4.2.2.1 Integration of CLB2 and CLB3 Constructs into the rRNA SSU Gene

As neither GST-int-L nor CLB1 appeared suitable vehicles for developing a MCC fusion protein, useful for antigen processing and presentation studies, two further constructs were made. First, MBAP:MCC transgenic parasites were generated in which MCC was cloned into the N-terminal of Modlap, as in the CLB1 construct, but without the T7 gene 10 tag: this tag was omitted as it was not detectable by Western blot analysis in the previous fusion protein, and could have conceivably obstructed processing of MCC. Second, MCC was cloned near to the C-terminus of Modlap, immediately prior to the stop codon. For both of these constructs the core $MCC_{(88\text{-}103)}$ epitope was used. Again, the DNA sequence of the MCC epitope was corrected for optimal codon usage by *L. mexicana* parasites (Langford *et al.* 1992).

The new N-terminal MCC construct, CLB2, was generated as described in section 2.1.8.3. CLB2 was cloned into pSSU-int (Appendix A-b) and lines of transgenic parasites were generated and analysed for correct integration of the transgene. Six lines carrying the CLB2 transgene were identified by PCR on genomic DNA using a reverse primer specific to *mbap* and oligonucleotide corresponding to MCC as a forward primer to amplify a 900bp product. Of these lines, two contained the construct correctly integrated into the 18S SSU rRNA gene, as ascertained using a forward primer specific to the rRNA promoter and a reverse primer specific to the transgene (Figure 3.14).

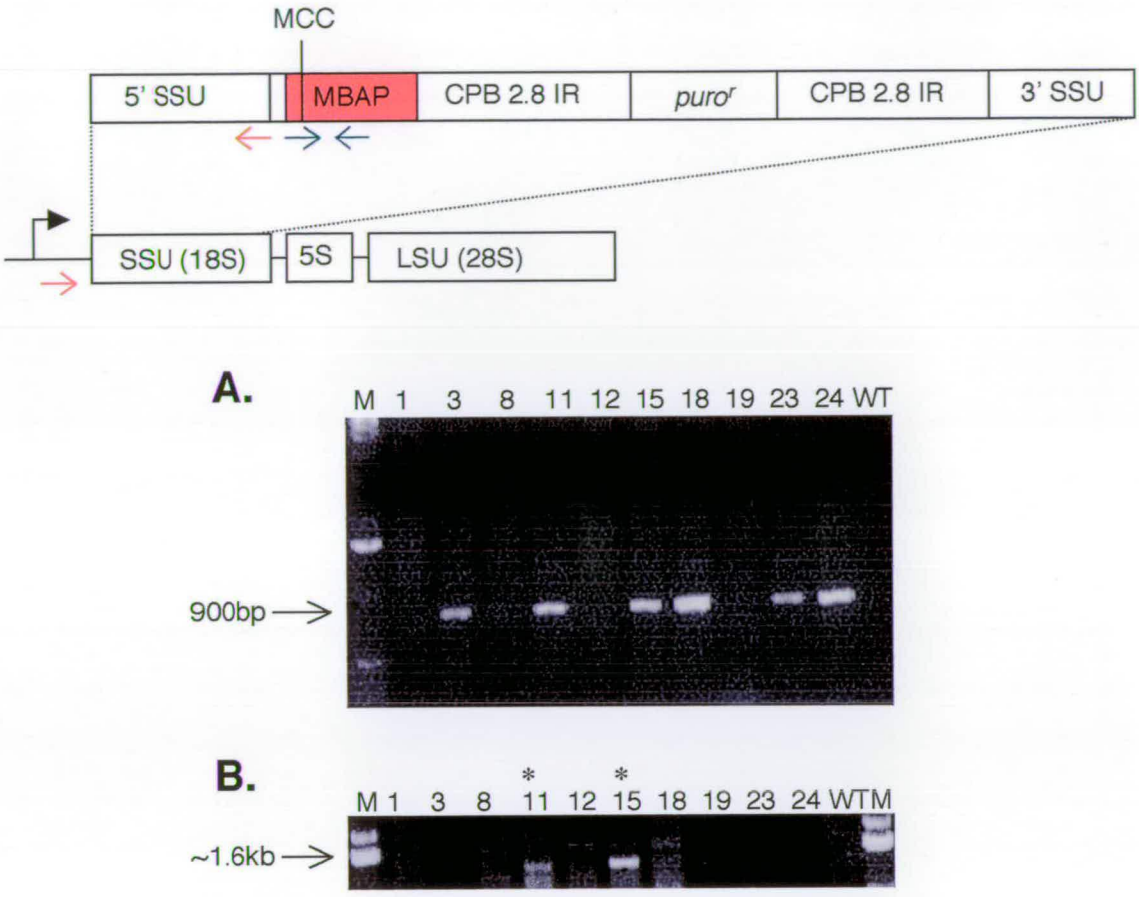
Figure 3.13: Infection of BM-DC with *L. mexicana* Amastigotes



Day 7 BM-DC were incubated with EGFP-expressing *L. mexicana* amastigotes overnight. Cells were then harvested, fixed, stained for MHC II expression and analysed by flow cytometry.

Numbers represent the percentage of cells in the respective quadrants.

Figure 3.14: PCR Analysis to Show Correct Integration of CLB2 Lines into the Ribosomal Locus



A. Genomic DNA from CLB2 clones was analysed by PCR for the presence of the mbap:MCC transgene. The forward primer was specific to the MCC oligonucleotide inserted into *mbap* and the reverse primer was specific to a sequence within *mbap* (blue arrows). The expected product size using these primers was 900bp.

B. Integration into the ribosomal locus was verified using a forward primer external to the 5' homology arm of the vector, and specific to the promoter region, and a reverse primer specific to the splice leader sequence of the pSSU-int vector (red arrows). The expected band size for the PCR product from these two primers if the transgene was correctly integrated into the ribosomal locus was approximately 1.6kb. A PCR product was not seen when these primers were used with wild type (WT) genomic DNA. * marks positive lines of parasites.

Numbers represent wells containing Puromycin resistant parasites in a 24 well plate.

M = 1kb ladder.

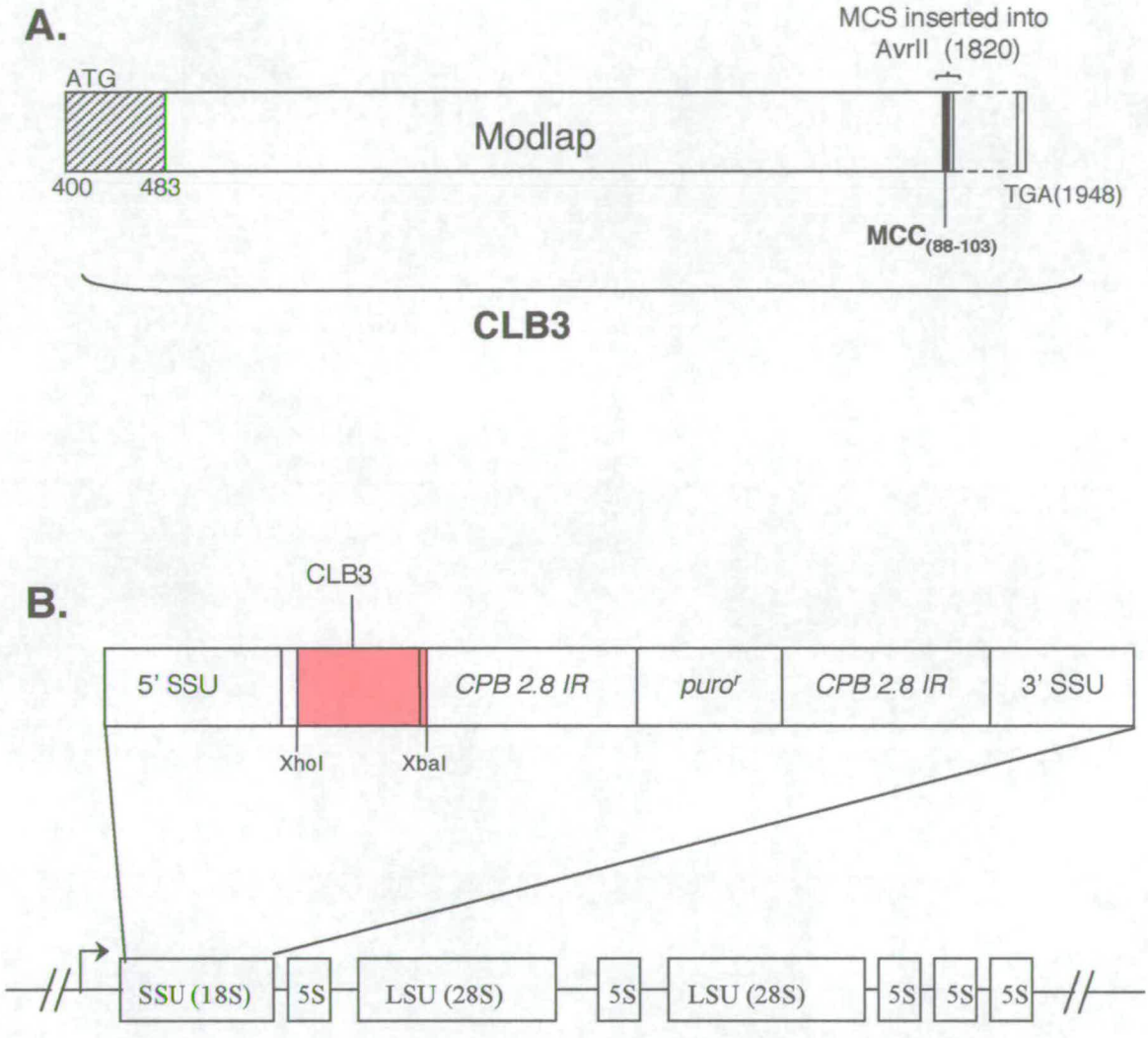
The cloning strategy for the C-terminal MCC fusion protein is outlined in section 2.1.8.3. In this construct, the MCC epitope was flanked by a multiple cloning site (MCS) which could be used, if necessary, to modulate the sequences surrounding the MCC epitope in order to facilitate processing by host cell proteases (Figure 3.15). The plasmid map for this construct, CLB3, is shown in Appendix A-b. Lines of transgenic parasites were generated and selected by culture with Puromycin. Lines in which the construct was integrated into the SSU locus were identified by amplification of PCR products from genomic DNA (Figure 3.16): internal primers, including an MCC oligonucleotide, confirmed the presence of the intact mbap:MCC transgene in five CLB3 clones; of these, PCR products were amplified from two clones using primers specific for integration of the construct into the rRNA SSU gene.

3.4.2.2.2 CLB2 and CLB3: Over-expression of Acid Phosphatase Activity in Culture Supernatants

Supernatants from CLB2 and CLB3 lines were assayed for acid phosphatase activity, as described in section 3.4.2.1.2. None of the CLB2 lines tested expressed the acid phosphatase (Figure 3.17). Since *L. mexicana* parasites express an endogenous copy of the *CPB 2.8* gene, and the vector used contains two copies of the intergenic region from this gene flanking the antibiotic resistance gene, the *puromycin*^{r} gene may have been integrated into the *CPB 2.8* gene by homologous recombination in resistant lines which were shown by PCR not to have the transgene inserted into the SSU locus. However, it is not clear why the parasites containing the correctly integrated transgene did not express high levels of acid phosphatase activity.

All of the CLB3 clones tested expressed the functional acid phosphatase. However there was large variation between transgenic lines (Figure 3.17). Surprisingly, expression of acid phosphatase was as high from non-SSU integrants as from parasites containing the correctly integrated transgene. These results indicated that the fusion proteins were produced in an enzymatically active form, suggesting correct folding of Modlap. Based on these results two clones were selected: CLB3-13 in which the transgene was correctly integrated into the rRNA 18S gene and which expressed high levels of secreted acid

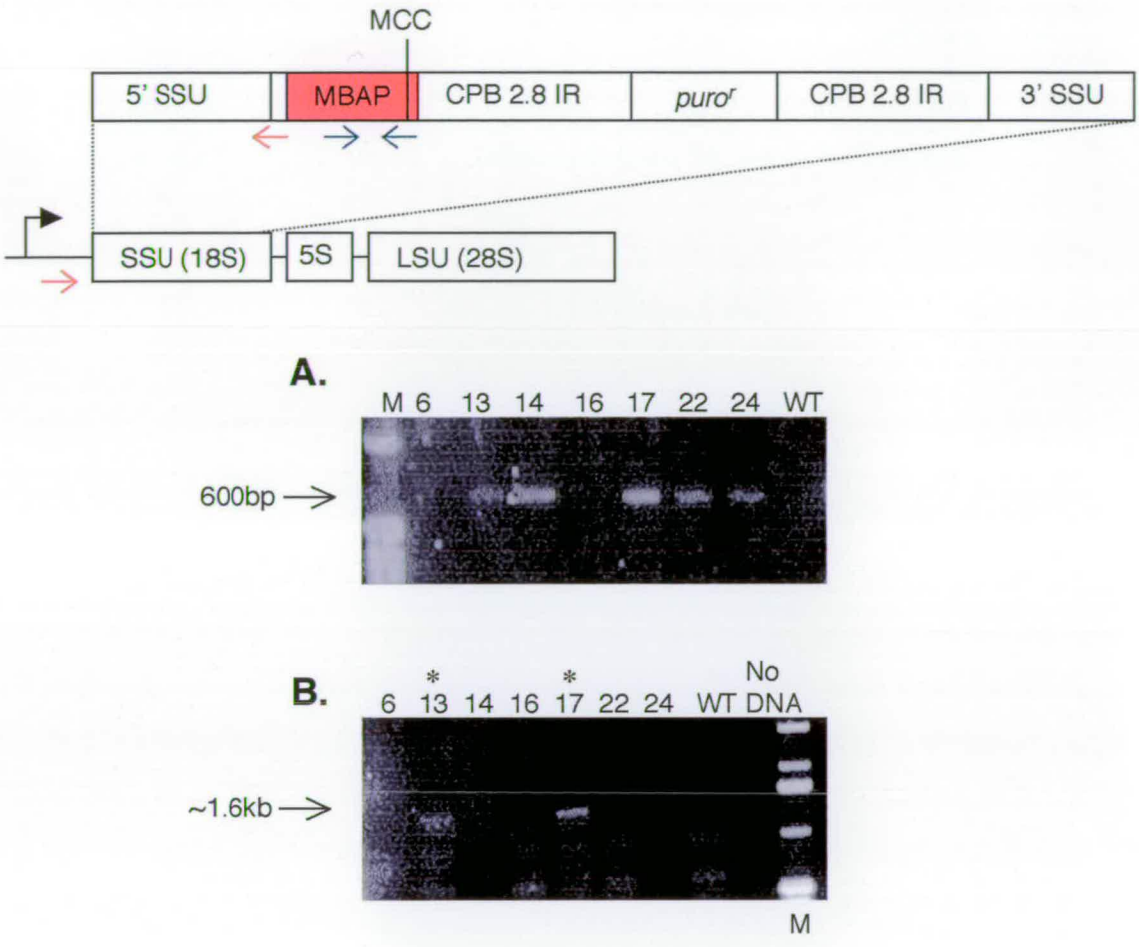
Figure 3.15: Schematic Diagram Showing Integration of CLB3 into the 18S SSU rRNA Gene



A. Schematic diagram of CLB3. The hatched box represents the *L. mexicana mbap* leader peptide. $MCC_{(88\text{-}103)}$ (black line) was cloned into a multiple cloning site that had been inserted into the AvrII(1820) site. Numbers refer to the DNA sequence shown in Figure 3.8.

B. Integration of CLB3 into the small sub-unit rRNA 18S gene.

Figure 3.16: PCR Analysis to Show Correct Integration of CLB3 Lines into the Ribosomal Locus



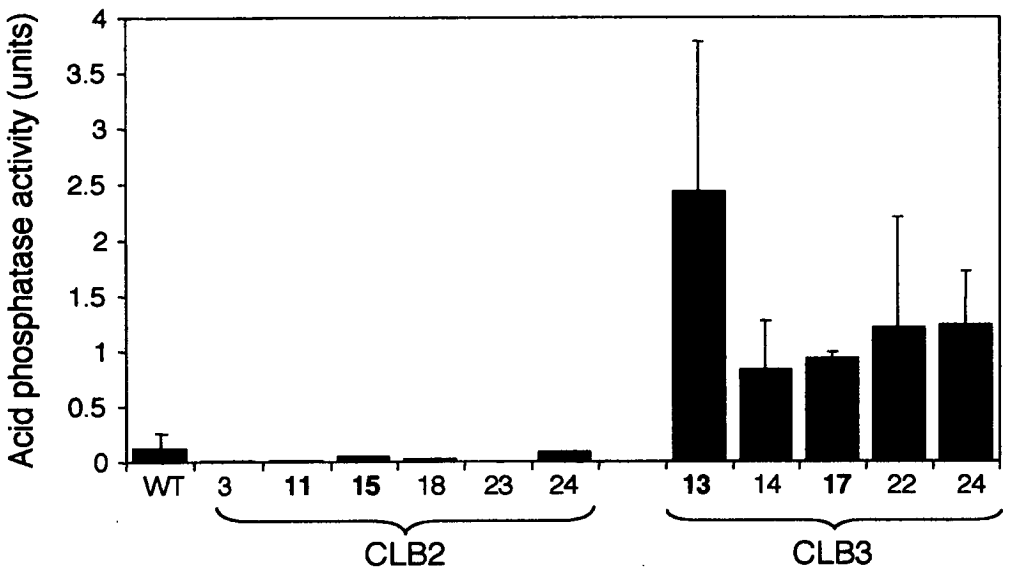
A. Genomic DNA from CLB3 lines was analysed by PCR for the presence of the mbap:MCC transgene. The forward primer was specific to a sequence within *mbap* and the reverse primer was specific to the MCC oligonucleotide inserted into *mbap* (blue arrows). The expected product size using these primers was 600bp.

B. Integration into the ribosomal locus was verified using a forward primer external to the 5' homology arm of the vector, and specific to the promoter region, and a reverse primer specific to the splice leader sequence of the pSSU-int vector (red arrows). The expected PCR product from these two primers if the transgene was correctly integrated into the ribosomal locus was approximately 1.6kb. A PCR product was not seen when these primers where used with wild type (WT) genomic DNA. * marks positive lines of parasites.

Numbers represent wells containing Puromycin-resistant parasites from a 24 well plate.

M = 1kb ladder.

Figure 3.17: Detection of Acid Phosphatase Activity in Supernatants from MBAP:MCC Transgenic CLB2 and CLB3 Amastigotes



Acid phosphatase activity was measured in supernatants from $1x10^7$ amastigotes cultured overnight in 1ml of serum-free medium. Activity was calculated from the change in absorbance of the substrate pNPP on incubation with culture supernatants and is expressed as units (1 unit = the amount of enzyme which hydrolyses 1μM substrate per minute).

Values represent the average of 2 experiments. Error bars represent the s.e.m. from 2 experiments, each of which was performed in triplicate.

Numbers in bold represent parasite lines in which the transgene was correctly integrated into the SSU locus.

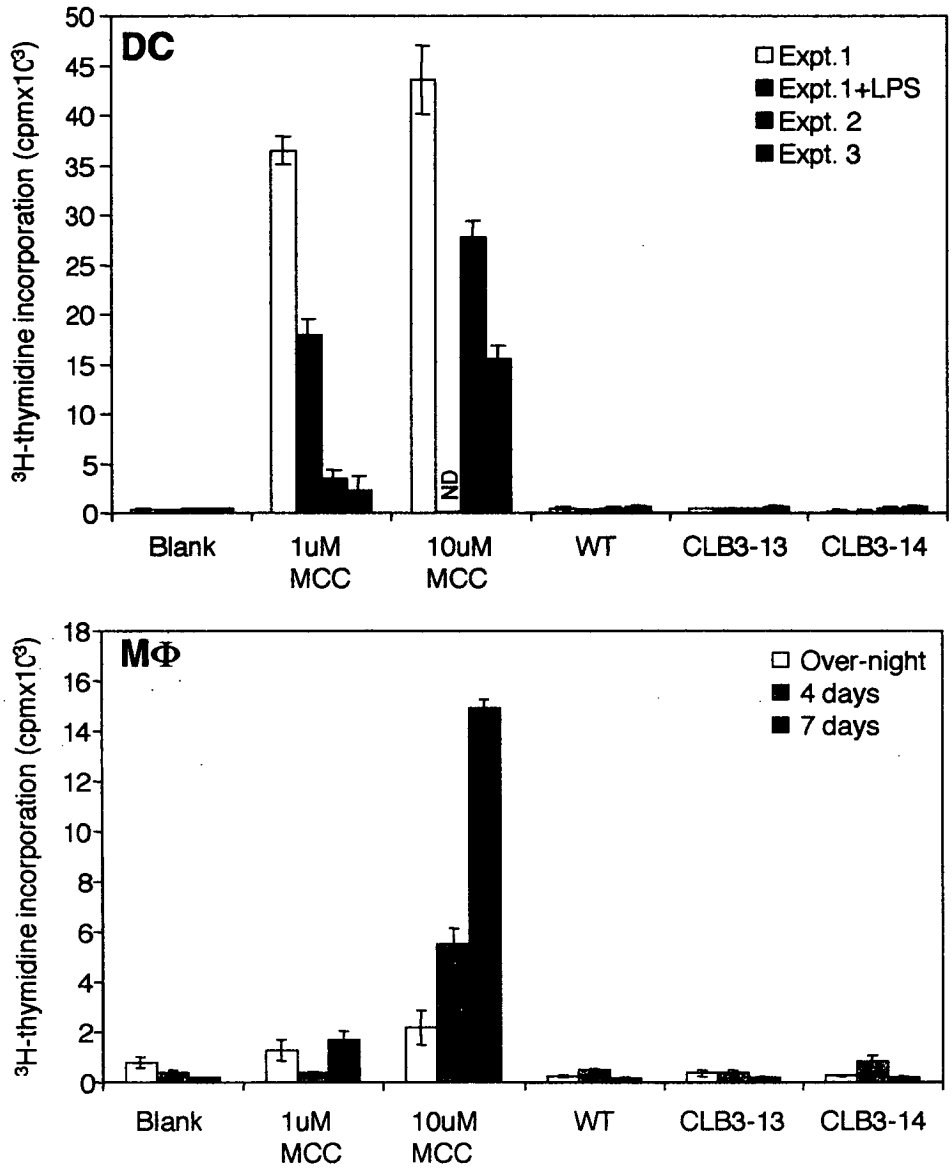
phosphatase; and CLB3-14 in which the transgene was integrated into the genome, although not downstream of the rRNA promoter, and which also expressed the acid phosphatase at high levels.

3.4.2.2.2 CLB3-infected DC and MΦ do not Stimulate the MCC-Specific T Cell Hybridoma 2B4

In order to analyse presentation of MCC from CLB3 transgenic parasites, CLB3-13 and CLB3-14 axenic amastigotes were used to infect CBA BM-DC, overnight, at a ratio of 8:1 amastigotes:DC. The following day the cells were washed thoroughly then incubated with MCC-specific T cells at a ratio of 1:10 for 18 hours. Stimulation of the T cells was followed by assaying levels of IL-2 in the culture supernatant using an IL-2-dependent cell line (Figure 3.18). As a positive control, 1μM or 10μM MCC peptide was incubated with non-infected DC for 4 hours prior to co-culture with T cells. Figure 3.18 shows the results from three independent experiments. These results demonstrate that DC infected with CLB3 transgenic parasites do not present the MCC epitope to T cells *in vitro*. Processing and presentation of *Leishmania*-derived MCC was also analysed in MΦ. It had previously been shown that MΦ infected for seven days with parasites that over-expressed Modlap were able to stimulate MBAP-specific T cells (Wolfram *et al.* 1996), possibly because high concentrations of the antigen could build up in the PV which form in these cells. Therefore, bone marrow-derived MΦ were generated from CBA mice and infected with CLB3-13 and CLB3-14 axenic amastigotes either overnight, for four or seven days. The cultures were given IFNγ either overnight or on days two, three and seven to up-regulate expression of MHC II molecules. Cells were then washed and incubated with T cells as above. Figure 3.18 shows that MΦ infected with CLB3 transgenic parasites did not stimulate MCC-specific T cells. Incubation of non-infected MΦ with MCC for four hours prior to adding the T cells resulted in IL-2 production, although levels were always much lower than with DC cultures.

These results were confirmed by flow cytometric analysis with D4. DC were incubated with CLB3-13 and CLB3-14 amastigotes over-night, harvested, fixed and stained with D4 and an appropriate secondary antibody. Figure 3.19 shows that no staining could be

Figure 3.18:Neither DC nor MΦ Infected with CLB3 Transgenic Parasites Stimulate a MCC-Specific T Cell Hybridoma



Bone marrow-derived DC or MΦ were infected with transgenic amastigotes either overnight (DC) or for different times (MΦ; see legend). Cells were then co-cultured with the MCC-specific T hybridoma 2B4 for 18 hours. IL-2 production by 2B4 cells was measured using an IL-2-dependent cell line. The values shown represent [^{3}H]-thymidine incorporation by the IL-2 dependent cells.

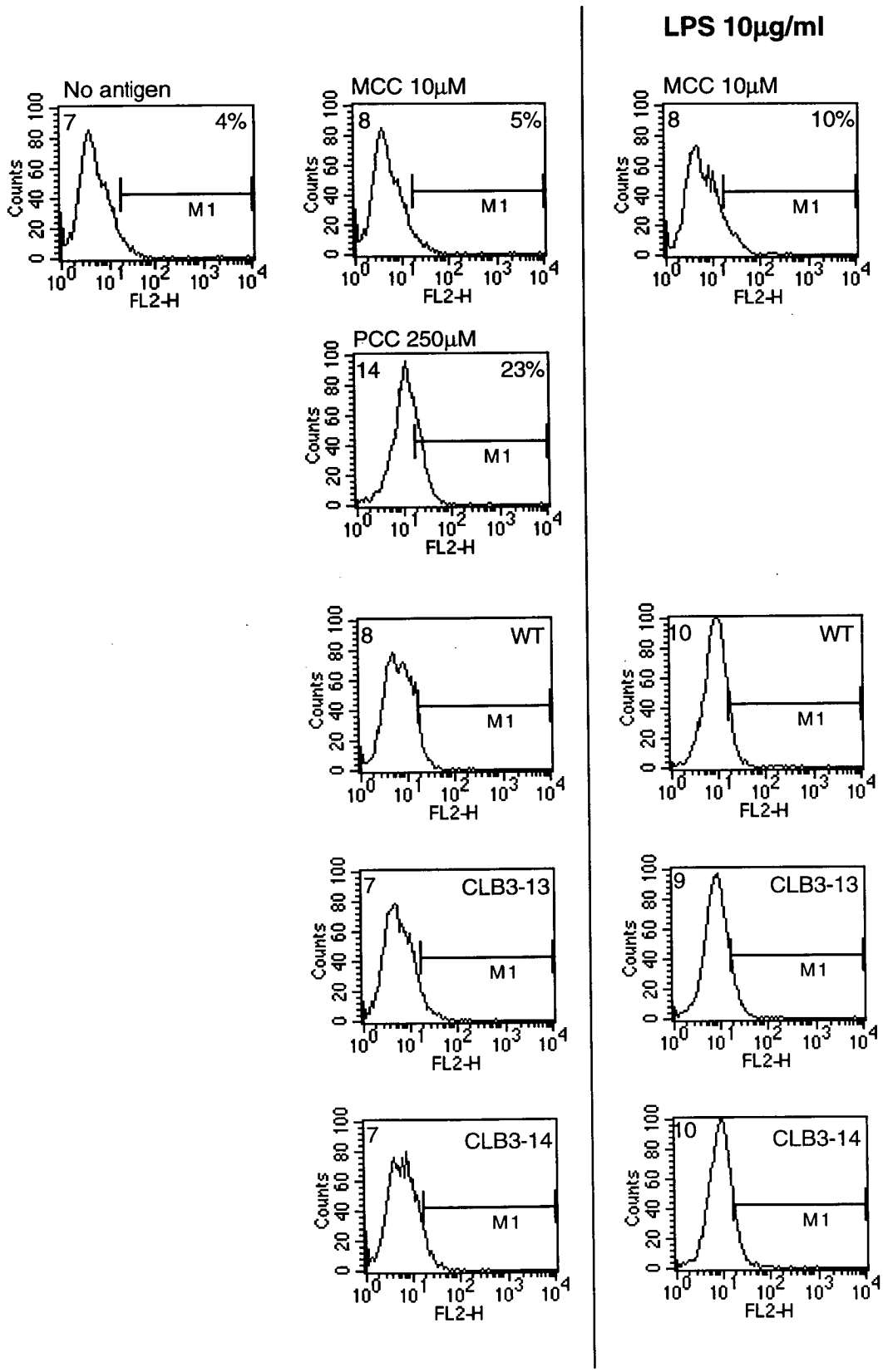
As a positive control MCC peptide was incubated with DC or MΦ for 4 hours prior to culture with 2B4 cells.

Each experiment was performed in triplicate. ND, not done.

Day 7 BM-DC were incubated with 10μM MCC peptide, 250μM PCC protein or *L. mexicana* wild type (WT) or CLB3 amastigotes for 18 hours at a ratio of 1:8, with or without 10μg/ml LPS. Cells were harvested and incubated with the complex-specific mAb, D4 followed by a FITC-conjugated goat anti-mouse secondary antibody.

M1 was set on DC incubated in the absence of antigen, and stained with D4 and the appropriate secondary (first panel). Numbers in the top left corner represent the mean of the peak; percentages show the percentage of cells within M1.

Figure 3.19: Detection of MHC II-MCC Complexes in CLB3-Infected DC by D4



detected with the D4 antibody, indicating that MHC II-MCC complexes did not form at detectable levels in these cells.

3.5 Discussion

This chapter describes work that aimed to establish an experimental model with which to follow the fate of parasite-derived antigens *in vitro*, and in particular, to determine whether DC could efficiently process and present parasite antigens to naïve $CD4^+$ T cells for the initiation of the anti-*Leishmania* immune response. The experimental model was based on transgenic expression of the T cell epitope, $MCC_{(95\text{-}103)}$, within a carrier molecule that was expressed at high levels by the parasite. MHC II-MCC complexes would then be detected using the complex-specific mAb, D4, and a MCC-specific T cell hybridoma. Both of these read-outs provide highly sensitive means of detecting the formation of $H2\text{-}E^k$-MCC complexes.

It had previously been shown that over-expression of *Leishmania*-derived antigens is sufficient to surmount the block in antigen presentation observed for *Leishmania*-derived proteins in infected MΦ (Wolfram *et al.* 1996). Therefore, in our experimental model, transgenes were integrated into the *L. mexicana* genome downstream of a promoter that drives high-level gene expression. Carrier proteins for the MCC epitope were selected that, once over-expressed, should not be detrimental to the intracellular survival of the parasite. Secreted forms of the fusion proteins were engineered for this strategy, since this should result in high concentrations of the antigen being accumulated in the PV of infected cells, and would possibly improve accessibility of fusion proteins for antigen processing and presentation: *L. mexicana* antigens have been shown to be efficiently presented to T cells when secreted into the PV (Wolfram *et al.* 1996), and secreted antigens primed a protective immune response against *Listeria monocytogenes* more efficiently than somatic antigens when delivered as part of a recombinant *Salmonella* vaccine (Hess *et al.* 1996).

Two different carrier molecules were selected in which the MCC epitope was inserted at different positions within the gene. Of these, only the MBAP:MCC fusion proteins were successfully secreted at high levels by the parasites. However, MBAP:MCC-derived MHC II-MCC complexes could not be detected within, or on the surface of, DC and MΦ infected with these parasites using either D4 or MCC-specific T cells.

3.5.1 Integration of GST-int into the *L. mexicana* rRNA SSU Gene

In initial experiments GST was selected as the carrier molecule for MCC. A construct, GST-int, in which MCC was integrated into a non-conserved loop of the protein, had previously been generated (Colledge *et al.* 2001), and infection of DC with bacteria expressing this fusion protein resulted in the formation of MHC II-MCC complexes that could be detected with D4 (L. Colledge unpublished results). Since GST is a well-characterised protein, and is routinely over-expressed for the production of GST fusion proteins, this construct was an attractive molecule from which to deliver MCC in our system. The peptide leader sequence from *L. mexicana mbap* was fused onto the N-terminus of GST-int to target the protein for secretion. Parasite lines were generated with the GST-int-L transgene integrated into the rRNA 18S gene, and transgene expression was demonstrated by Northern blot analysis. However, GST protein could not be detected in these parasites or in culture supernatants, and DC infected with the transgenic parasites did not present MCC to T cells. These results suggested that the GST-int-L protein was not translated by these parasites, or was rapidly degraded upon synthesis, perhaps due to incorrect folding of the fusion protein. Therefore it was not a suitable epitope carrier for these studies.

3.5.2 Generation of Parasites Expressing MBAP:MCC Fusion Proteins

The *L. mexicana* membrane-bound acid phosphatase (MBAP) was chosen as an alternative carrier molecule for MCC. This protein was selected because it had previously been shown that deletion of the transmembrane domain resulted in secretion of the active enzyme by the parasites (Wolfram *et al.* 1996). Importantly, MΦ infected with these parasites stimulated MBAP-specific T cells, indicating that the protein was processed from the PV (Wolfram *et al.* 1996).

A first round of MBAP constructs was made in which $MCC_{(88\text{-}103)}$ was cloned into a site at the N-terminus of Modlap (i.e. secreted MBAP), immediately 3' to the leader peptide. An epitope tag was included to follow expression of the transgene. The extended $MCC_{(88\text{-}103)}$ peptide was chosen for this construct to maximise the chances of including protease recognition sites involved in mediating processing of the core 95-103 epitope. The construct, CLB1, was integrated into the 18S SSU rRNA gene, and transgenic lines were selected which over-expressed the acid phosphatase in the culture supernatant. MHC II-MCC complexes could not, however, be detected in DC or MΦ infected with CLB1 parasites using D4.

Since Modlap epitopes were previously shown to be presented by MΦ (Wolfram *et al.* 1996), we considered whether expression levels could explain this finding. The amount of protein produced was calculated using the specific activity of the enzyme, (specific activity = units of enzyme activity/mg protein; specific activity of Modlap = 380U/mg; (Wolfram *et al.* 1996)), and was estimated as $3.2x10^{-8}$ mg of protein produced by five CLB1-2 amastigotes over 18 hours. Assuming that the PV occupies most of the cell, the PV volume was estimated as $20\mu m^3$. Therefore, the concentration of Modlap within the PV, assuming five amastigotes occupied the compartment, should be around 4mg/ml, assuming limited proteolysis of protein. This is approximately four fold more than the amount of PCC protein given to DC for T cell assays with the MCC-specific T cell

hybridoma, 2B4 (see section 4.8). These calculations indicate that MBAP:MCC was produced at high enough levels to permit detection H2-E^k-MCC complexes, if they were formed.

Since Modlap was over-expressed by CLB1 parasites, but MCC was not presented by DC or MΦ, two alternative lines of parasites were generated. $MCC_{(88\text{-}103)}$ was cloned into the N-terminus of Modlap, as for the CLB1 construct, but without the T7 gene 10 epitope tag. Alternatively, $MCC_{(88\text{-}103)}$ was cloned into a site at the C-terminus of Modlap. Lines of CLB2 (N-terminal) and CLB3 (C-terminal) parasites were generated which contained the transgene inserted into the rRNA 18S SSU gene. However, MBAP was not present in the culture supernatant of CLB2 lines tested. The reason for this is not clear since CLB1 parasites, which also had MCC integrated into the same site, over-expressed the acid phosphatase, and all CLB2 constructs had been extensively sequenced.

CLB3 transgenic parasites did over-express the acid phosphatase, although there was variability between lines of parasites. Of these lines, two were selected, one in which the transgene was inserted into the rRNA locus, and which secreted high amounts of MBAP (CLB3-13), and a second in which the mbap:MCC transgene had inserted into genomic DNA outside the rRNA locus, and which also over-expressed the protein (CLB3-14). CLB3-infected APC did not stimulate T cells; MHC II-MCC complexes could not be detected by incubating infected DC and MΦ with the specific T cell hybridoma, 2B4. The lack of complex formation was confirmed by flow cytometric analysis of DC infected with CLB3-13 and CLB3-14 amastigotes, using D4.

These data demonstrate that MCC was not processed and presented from the MBAP carrier molecule in infected DC and MΦ, at levels that could be detected with D4 or specific T cells. Since several different constructs had been generated in which MCC was integrated at different positions within the protein, this work suggested that MCC would not be presented from MBAP-based fusion proteins without modifications to the current experimental model.

3.5.3 Processing of a Parasite-Derived Antigen by Infected DC and MΦ

Three different hypotheses can be put forward to explain why MCC was not presented by DC or MΦ infected with transgenic *L. mexicana* parasites expressing MCC fusion proteins: first, the fusion proteins were degraded in the PV; second, functional proteins accumulated in the PV but MCC was not correctly processed by host proteases; third, the fusion proteins were not expressed at sufficiently high levels to overcome the inefficiency of antigen presentation in infected cells.

L. mexicana promastigotes and amastigotes produce high levels of cysteine proteases (Mottram *et al.* 1997) that have been implicated in the degradation of MHC II molecules within the PV (De Souza Leao *et al.* 1995). These proteases may also degrade parasite-derived antigens, which are secreted into the PV, before they can be bound by MHC II molecules. A role for parasite inhibition of presentation of parasite antigens is supported by data showing that intracellular *L. mexicana* antigens were presented to T cells only following drug-induced death of intracellular parasites (Wolfram *et al.* 1995).

The role of cysteine proteases in degrading MCC fusion proteins would be difficult to address: *L. mexicana* parasites in which cysteine protease genes had been deleted were less infective than wild type parasites (Mottram *et al.* 1996) and were rapidly killed once taken up by MΦ (Frame *et al.* 2000); inhibitors of cysteine proteases also reduced the infectivity of wild type promastigotes (Frame *et al.* 2000). These studies indicate that inhibitors of *L. mexicana* cysteine proteases could not be used to promote processing of intact MCC in this experimental model, since they also play a role in the survival of *Leishmania* parasites. Protease inhibitors would also inhibit Cathepsins S, which is essential for processing of T cell epitopes by the host cells. Therefore, while it is likely that the abundant cysteine proteases produced by amastigotes will not favour processing of the MCC epitope for presentation in the context of MHC II, this would be difficult to address directly within this model. An alternative approach would be to include sites in the constructs flanking MCC that stabilise the protein: the introduction of N-

glycosylation sites or Serine/Threonine-rich repeats, which have been shown to be sites for O-linked post-translational modification of *Leishmania* proteins (Wiese *et al.* 1995), proximal to MCC might protect the fusion protein from proteases in the PV. However inclusion of these sites could also occlude processing of the epitope by host proteases.

A second hypothesis to account for the lack of detectable MHC II-MCC complexes in cells infected with MBAP:MCC parasites is that the protein is not degraded but that MCC is not correctly processed for loading onto MHC II molecules. A core peptide and its flanking sequences provide the signals necessary for its processing, independent of the carrier molecule in which it sits (Lo-Man and Leclerc 1997). However, the tertiary structure of the protein will determine which parts are the most accessible to the host processing machinery. It has also been shown that flanking residues of long peptide fragments, which bind MHC II but remain outside the peptide-binding groove, can hinder the interaction between the loaded MHC II molecule and the TCR (Moudgil *et al.* 1998), thus if MCC was not correctly processed the epitope could actively inhibit presentation. MCC was cloned into N-or C-terminal sites of *mbap* that, according to the predicted 3-D structure, would not be contained within the folded enzyme. The epitope should therefore be accessible to intracellular proteases. Protease cleavage sites are not well defined, but certain residues have been shown to be important: characterisation of the epitopes cleaved from tetanus toxin by Cathepsins D and E revealed that hydrophobic residues often flank the cleaved site (position P1 and P1') and there is low tolerance for positively charged polar residues in the site adjacent to this (P2) (Hewitt *et al.* 1997). The sequence of the MCS surrounding the C-terminal MCC was designed to avoid residues that are known to inhibit processing, but this was limited by constraints imposed by sequences for restriction enzyme sites; the amino acid at P1' was arginine, which is a basic, non-hydrophobic amino acid.

MBAP-specific T cells have previously been shown to be stimulated by MΦ infected with *L. mexicana* amastigotes that over-express Modlap at very high levels (Wolfram *et al.* 1996), indicating that the inaccessibility of parasite-derived antigens can be overcome by increasing the concentration of antigen. It may be, therefore, that while

CLB1 and CLB3 parasites, in theory, produced sufficient protein for MHC II-MCC complexes to be detectable with D4, it was not sufficient to over-ride the inefficient processing of antigens that has been shown to occur in infected MΦ. In other studies in which MΦ infected with transgenic parasites stimulated T cells specific for parasite-derived antigens, the transgenes were expressed from the episomal vector, pX, which is present at high copy numbers within the cells (Kaye *et al.* 1993; Wolfram *et al.* 1996). Thus, it would be useful to express the MCC fusion proteins generated here from pX and compare these parasites to those in which the transgene was integrated into the 18S rRNA locus. Use of pX would, however, introduce other limitations into the system, as described in section 3.2.1. In the present, pSSU-int-based system, incubation of MBAP-specific T cells with DC and MΦ infected with CLB transgenic parasites would determine whether the concentration of Modlap in the PV was sufficient for MBAP epitopes to be processed and presented in these cells and if so, whether the defect in presentation of MCC was due to the amount of antigen secreted into the PV.

3.5.4 Replacement of a Defined T Cell Epitope from Gp63 with MCC

In the light of the results using the MBAP transgenic parasites an alternative carrier molecule was chosen from which to express the MCC epitope. Unfortunately, time constraints prevented generation of these transgenic parasites, and therefore only the cloning strategy is presented in this thesis.

Glycoprotein 63 (gp63; also known as Leishmanolysin) is a 63 KDa zinc proteinase that is abundantly expressed on the surface of *Leishmania* promastigotes (Russell and Wilhelm 1986). T cells from cured Leishmaniasis patients produced IFNγ in response to restimulation with gp63 protein or peptide epitopes from gp63, indicating that presentation of gp63 antigens may be important in the development of the healing Th1 response *in vivo* (Russo *et al.* 1991; Russo *et al.* 1993). A number of defined T cell epitopes have also been identified in murine gp63, of which PT3 was shown to protect

immunised mice against challenge with *L. major* promastigotes (Jardim *et al.* 1990), indicating that gp63 epitopes are highly immunogenic *in vivo*. This is supported by the fact that mice can be successfully immunised against Leishamaniasis by vaccination with Bacillus Calmette-Guerin (BCG) (Connell *et al.* 1993) or *Salmonella typhi* (Gonzalez *et al.* 1998) (Yang *et al.* 1990) vectors expressing gp63 epitopes. Gp63 was therefore considered a good candidate carrier molecule for MCC.

3.5.4.1 The Cloning Strategy

The gp63 DNA (Figure 3.20) and protein structure is highly conserved between different *Leishmania* species, with most of the differences being clustered at the N-terminal pro-peptide region or at the carboxy terminus (Medina-Acosta *et al.* 1989; Medina-Acosta *et al.* 1993). *Gp63* genes are present as multiple copies of a 3.1kb unit, which is tandemly repeated on a single chromosome. *L. mexicana* was shown to contain three classes of structurally distinct genes, C1, 2 and 3, containing five, four and one copy of the gene respectively. While comparable levels of gp63 RNA were found in both *L. mexicana* promastigotes and amastigotes, C1 transcripts were enriched in amastigotes, suggesting developmental regulation of different classes of *gp63* genes (Medina-Acosta *et al.* 1993). Abundant expression of *gp63* by promastigotes is thought to be due to polycistronic transcription of the *gp63* genes, which are subsequently trans-spliced into multiple mRNAs (Button *et al.* 1989).

A cloning strategy (see section 2.1.8.4) was therefore devised in which the amino acid sequence encoding a stretch of known gp63 T cell epitopes was replaced by the $MCC_{(95-103)}$ epitope (Figure 3.21). The protease sites that flank the endogenous gp63 T cell epitope should thus mediate the correct processing of MCC. The *gp63 C1* gene was amplified from *L. mexicana* wild type genomic DNA by PCR. Mutation of the GPI anchor addition site (Asn 577) has been shown to result in secretion of the intact gp63 protein (McGwire and Chang 1996). A truncated form of the *gp63 C1* gene was therefore cloned by insertional mutagenesis to create a TGA stop codon adjacent to Asp 560. It is from this point that the greatest divergence occurs between the amino acid sequences of different *Leishmania* species, indicating that this region is not essential for

DNA sequence of the *L. mexicana* gp63 C1 gene (accession number X64394). Blue underlined codons are the start and stop codons; the amino terminus of the mature protein is underlined and marked with an asterix (*); letters indicate the amino acids which make up the MΦ binding motif and the HEXXH motif of the active site; regions covering human and mouse T cell epitopes are underlined.

A stop codon was introduced immediately downstream of the aspartate residue at 1927 (green) to remove the GPI anchor addition site and produce a secreted protein. The maximum sequence divergence between species is within this region. The cytosine of the glutamine residue of the active site was mutated to a guanosine (red codon) to inactive the protease. The Hind III sites (AAGCTT) used to insert MCC are shown in red.

Differences between the published *L. mexicana* sequence and the *L. mexicana* strain used for these experiments are indicated with red stars.

Figure 3.20: Sequence of the *L. mexicana* gp63 C1 Gene

```
AATTCCCCATTTCCGCCATTCACAGACCCATCTCCCCGTGCCCCCTCCCTGTCCCCTCCC 60
TCCCCAGATCCACCAGCGCATCCCATCCCGCTATACCCTCTCCCCCGCCCGCACGCACGC 120
GCACACCGCCGTGCACAAGCCCTCGCCCTCGCCACCACACCCCACCGCCCGGCCCAGCGC 180
CCCCGCGCCCGCAGACCCATGCCCGTCGACAGCAGCAGCACGCACCGGCACCGCTGCGTC 240
GCCGCGCCCCTGGTGCGCCTCGCGGCTGCCGGCGCCGCAGTCACCGTCGCTGTCGGCACC 300
GCGGCCGCGTGGGCACACGCCGGTGCGCCCCAGCACCGCTGCATCCACGACGCGATGCAG 360
GCCCGCGTGCTGCAGTCGGTGGCGGCTCAGCGCATGGCCCCCAGCGCGGTGTCCGCGGTG 420
GGCCTGCCGTACGTGTCCGTGGTCCCCGTCGAGAACGCCAGCACCCTCGACTACTCGCTA 480
TCGGACAGCACGTCGCCCGGTGTTGTGCGCGCCGCGAACTGGGGCGCGCTGCGAGTCGCC 540
GTCTCCGCCGAAGACCTCACCGACCCCGCCTACCACTGCGCTCGTGTTGGGCAGCAGGTC 600
AACAACCACGCCGGCGACATCGTCACCTGCACCGCCGAGGACATCCTCACCGACGAGAAG 660
CGCGACACCCTCGTCAAGCACCTCGTCCCGCAGGCGCTGCAGCTGCACAGGGAGCGCCTG 720
AAGGTGCGGCAGGTGCAGGGCAAGTGGAAGGTGACGGGCATGGCGGACGTGATCTGTGGC 780
GACTTCAAGGTGCCGCCGGAGCACATCACGGAAGGCGTGACCAACACCGACTTCGTGCTG 840
TACGTCGCCTCCGTGCCGAGCGAGGAGAGTGTGCTGGCGTGGGCCACGACCTGCCAGGTG 900
TTCCCTGACGGCCACCCAGCCGTCGGCGTCATCAACATCCCCGCGGCGAACATTGCGTCG 960
CGGTACGACCAGCTCGTCACGCGTGTCGTCACGCACGAGATGGCGCACGCGGTGGGCTTC 1020
AGCGGCACATTCTTTGGGGCCGTCGGCATCGTGCAAGAGGTGCCGCACCTTCGGCGCAAG 1080
GACTTTAATGTGTCGGTGATCACCAGCAGCACGGTGGTGGCGAAGGCGCGTGAGCAGTAC 1140
GGCTGCAACAGCTTGGAGTATCTGGAGATTGAGGACCAGGGCGGTGCGGGCTCCGCCGGG 1200
TCGCATATCAAGATGCGCAACGCCAAGGACGAGCTCATGGCGCCTGCCGCATCTGCCGGG 1260
TACTACACCGCCCTGACCATGGCCGTCTTCCAGGACCTCGGCTTCTACCAGGCGGACTTC 1320
AGCAAGGCCGAGGAGATGCCGTGGGGCCGGAACGTCGGCTGCGCCTTCCTCAGCGAGAAG 1380
TGCATGGCGAAGAACGTCACGAAGTGGCCGGCGATGTTCTGCAATGAGAGTGCGGCCACC 1440
ATACGGTGCCCCACCGACCGTCTGAGAGTCGGAACTTGTGGTATAACAGCATACAATACT 1500
TCGTTGGCGACGTACTGGCAGTACTTCACCAATGCGTCCCTCGGGGGCTACTCGCCATTC 1560
CTGGACTACTGCCCGTTTGTTGTTGGCTACAGGAATGGCTCGTGCAATCAGGATGCGTCG 1620
ACGACACCGGACCTTCTCGCTGCGTTCAACGTCTTCTCCGAGGCCGCGCGGTGCATCGAT 1680
GGCGCCTTCACGCCGAAGAACAGAACCGCTGCGGATGGATACTACACCGCCCTGTGCGCC 1740
AACGTGAAGTGCGACACGGCCACGCGCACGTACAGCGTCCAGGTGCGCGGCACGAACGGC 1800
TACGCCAACTGCACGCCGGGCCTCAGAGTTAAGTTGAGCAGCGTGAGCGACGCCTTCGAG 1860
AAGGGCGGCTACGTCACGTGCCCGCCGTACGTGGAGGTGTGCCAGGGCAACGTCAAAGCT 1920
GCCAAGGACTTTGCAGGCGACACCGACAGCTCCAGCAGCGCCGATGACGCTGCCGACAAA 1980
GAGGCGATGCAGCGGTGGAGTGACAGGATGGCCGCCTTGGCTACTGCGACGACGCTGCTG 2040
CTAGGAATGGTGCTCTCTCTCATGGCACTCCTCGTGGTGCGGCTACTCCTTACCAGCTCC 2100
CCCTGGTGCTGCTGCAGACTGGGGGGGCTCCCGACGTGAGTTGCGGCGGCCCACTAGCTT 2160
GAAACGGCGTGAAGAGGCTGGGCATGG                                    2187
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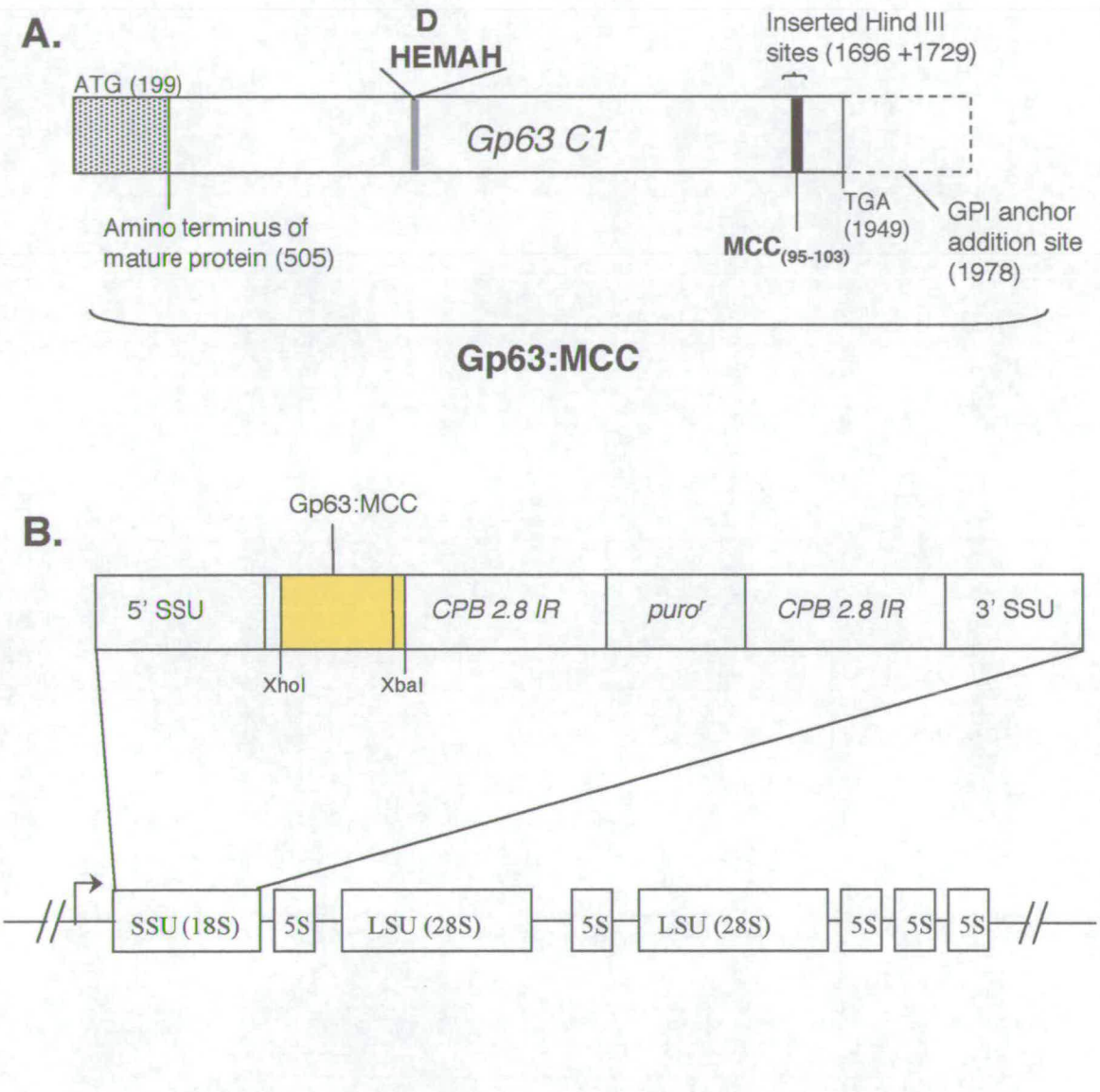
R Y D H E M A H S

AAGCTT AAGCTT

Figure 3.21: Schematic Diagram Showing Integration of Gp63:MCC into the 18S SSU rRNA Gene



A. Schematic diagram of gp63:MCC. The hatched box represents the pro-peptide which is cleaved to form the mature protein. $MCC_{(95\text{-}103)}$ (black line) was cloned into a Hind III site that had been inserted immediately upstream of the deleted C-terminus. The amino acid sequence of the active site is shown, demonstrating the site-directed mutation from glutamate (E) to aspartate (D). Numbers refer to the DNA sequence shown in Figure 3.20.

B. Integration ofgp 63:MCC into the 18S small sub-unit rRNA gene.

the correct folding and function of the protein. Sequencing of the cloned *gp63 C1* gene revealed a number of differences compared to the sequence published in Genbank (Figure 3.20). These differences were, however, consistent among all of the clones tested, indicating that they were due to differences between the strain of *L. mexicana* parasites used, and not mutations introduced by PCR. This was confirmed by direct sequencing of the *gp63* gene from wild type *L. mexicana* genomic DNA.

Gp63 is a metalloprotease and secretion of large amounts of this enzyme into the PV may not favour the generation of intact T cell epitopes. It had previously been shown that mutation of Glutamate 265, within the HEXXH motif of the active site, was sufficient to create a mutated protein that was expressed at the same level as the wild type protein, but the enzyme activity of which was greatly reduced (McGwire and Chang 1996). Therefore, this amino acid was mutated by site-directed mutagenesis in the cloned gp63 constructs by converting the GAG glutamate codon to a GAC aspartate codon (E265 to D265; see section 2.1.8.4).

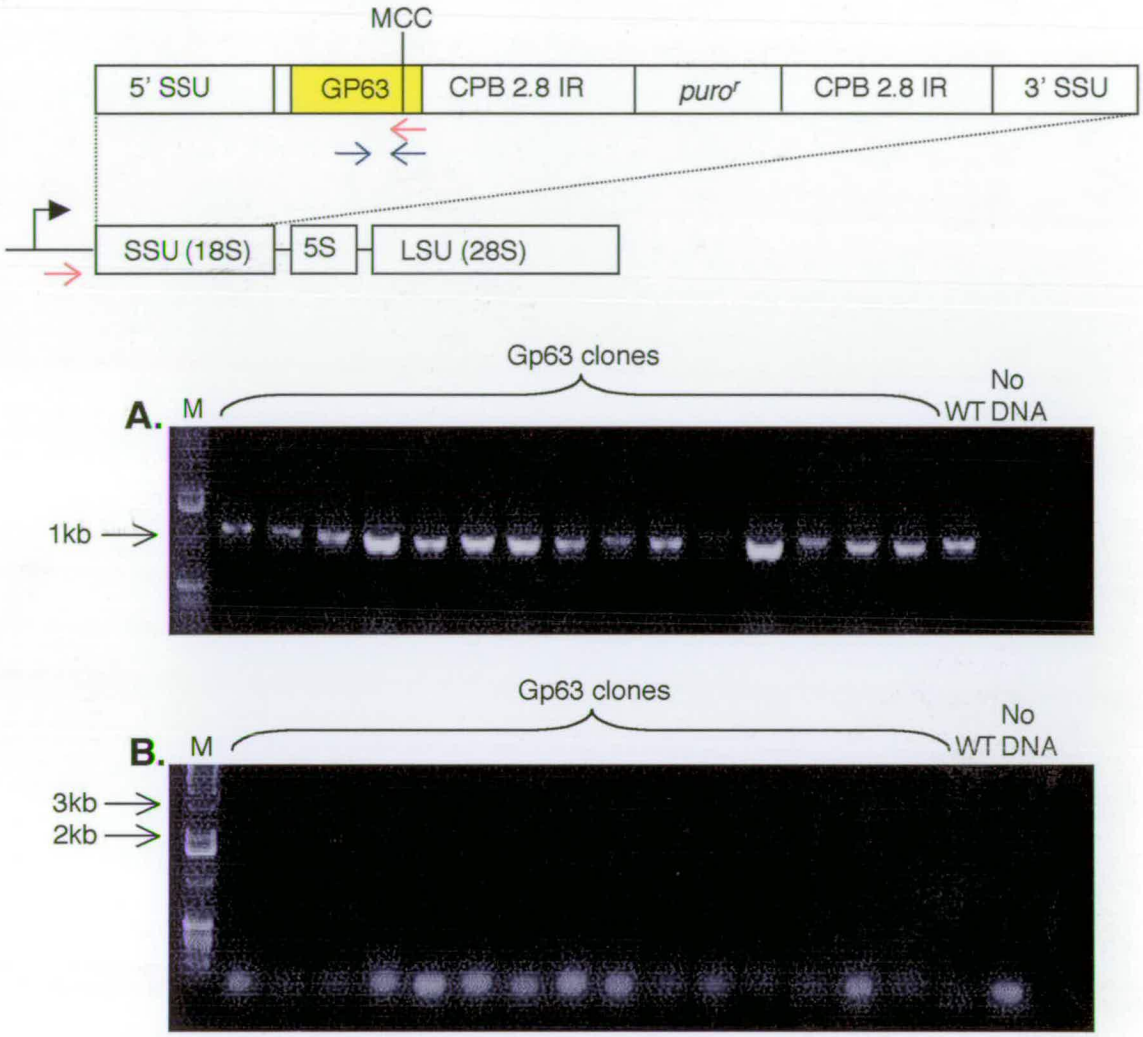
The dominant murine and human gp63 $CD4^+$ T cell epitopes have been well characterised (Jardim *et al.* 1990; Russo *et al.* 1993). Immunisation of BALB/c mice with the murine PT3 epitope, but not other putative epitopes, resulted in delayed lesion development, suggesting that T cells which recognise this peptide are important in controlling the response (Jardim *et al.* 1990). However, this epitope overlaps the active site of gp63, including the HEXXH motif and is highly conserved between species, indicating that disruption of the amino acid sequence would be detrimental to the folding and function of gp63. Therefore, the core $MCC_{(95\text{-}103)}$ epitope was cloned into an alternative region at the C-terminal of the gene that contained three overlapping T cell epitopes, PT6, PT7 and PT8 (Figure 3.20). GP63 has a highly globular tertiary structure, suggesting that domains in the middle of the protein would be relatively inaccessible to host proteases. Cloning of MCC into a site toward the exposed C-terminus of the protein may therefore have the added benefit of promoting cleavage by proteases. The amino acid sequence chosen was relatively similar to the MCC epitope, and avoided potential N-glycosylation sites that are important for the stability of the protein (McGwire and

Chang 1996). An epitope tag was not included in the construct at this point, but would be included in a second round of constructs if the transgene was correctly expressed by transfected parasites.

3.5.4.2 Integration of Gp63:MCC Fusion Proteins into the rRNA SSU Gene

The gp63:MCC construct was electroporated into *L. mexicana* promastigotes and individual lines were expanded, as described previously. Genomic DNA was extracted and analysed by PCR for correct integration of the transgene. Figure 3.22 shows that amplification of genomic DNA with primers specific for gp63 produced the expected 1kb band in all but one of the lanes, including the wild type. When the forward primer, which recognises a sequence in the promoter region of the rRNA gene, was used with a reverse primer specific for gp63, however, the predicted 3kb product was not amplified (Figure 3.22). Three rounds of transfectants were analysed (72 lines of Puromycin resistant parasites) but in none was the transgene integrated into the 18S rRNA small sub-unit gene. This strongly indicated that the transgene and antibiotic resistance gene were preferentially targeted to a locus other than the ribosomal locus. Gp63:MCC may have been integrated by homologous recombination into one of the clusters of *gp63* genes expressed by *L. mexicana* parasites, however, this does not account for the fact that the antibiotic resistance marker was also expressed by these parasites. These results indicate that there may be a strong selective pressure against parasites that express high levels of gp63, although the protease should be inactive in these parasites, and integration into the 18S rRNA small sub-unit gene does not always drive high levels of transgene expression. Furthermore, over-expression of gp63 has not been reported to be detrimental to the survival of the parasites (Liu and Chang 1992). Large numbers of parasites may, therefore, need to be screened before positive clones are identified. Unfortunately, time constraints meant that this was not possible within the time frame of this thesis.

Figure 3.22: PCR Analyses to Show Correct Integration of Gp63:MCC Lines into the Ribosomal Locus



A. Genomic DNA from gp63:MCC lines was analysed for the presence of the gp63 as a positive PCR control. All clones and wild type (WT) genomic DNA should be positive because these primers will recognise the endogenous copy of the gene. An approximately 1kb product was predicted from these primers (blue arrows).

B. Genomic DNA from gp63:MCC lines was analysed for integration into the ribosomal locus by PCR using a forward primer external to the 5' homology arm of the vector, and specific to the promoter region, and a reverse primer specific to a sequence within gp63 (red arrows). The predicted product would be approximately 3kb. No product was amplified by PCR from any of the lines indicating that the gp63:MCC transgene had not been targeted to the correct locus.

M = 1kb ladder.

3.6 Concluding Remarks

Leishmania-derived antigens are not efficiently processed and presented by infected MΦ. This chapter has described attempts to establish an experimental model system with which to explore the fate of parasite-derived antigens in infected DC and MΦ. Presentation of antigens from *Leishmania*-infected DC has not been well characterised, although it is assumed that MHC II-*Leishmania* antigen complexes on the surface of activated DC prime the initial anti-parasite immune response.

DC infected with parasites expressing the T cell epitope MCC did not stimulate antigen-specific T cells, and MHC II-MCC complexes could not be detected using the highly sensitive complex-specific mAb, D4. This indicates that the experimental model proposed at the beginning of this chapter requires further modification before MHC II-parasite-derived antigen complexes can be directly visualised *in vitro*. All of the hypotheses addressed in section 3.6 may contribute to the inefficient processing of *Leishmania*-derived antigens in infected DC and MΦ and it will be important to generate transgenic lines of parasites that express very high levels of the fusion protein to ensure that antigen concentration is not a limiting factor in these experiments. As exogenous antigens are efficiently processed and presented by *Leishmania* promastigote- or amastigote-infected DC (see Chapter 4), MHC II-parasite-derived MCC complexes should be detectable in infected DC once the carrier molecule and processing of the epitope have been optimised. The experiment presented in Figure 3.13 indicated that, unexpectedly, infection of BM-DC with *L. mexicana* amastigotes did not induce up-regulation of surface MHC II. This observation led to a more extensive analysis of the effect of up-take of *L. mexicana* parasites *in vitro*.

Chapter 4:

Characterisation of Infection of Bone Marrow-Derived DC with *L. mexicana* Parasites

(Bennett *et al* EJI (2001) see Appendix C)

4.1 Introduction

MΦ are the primary reservoir for *Leishmania* parasites. However, they are not activated upon infection but rather become refractory to additional stimuli (Weinheber *et al.* 1998), and are inefficient in the presentation of *Leishmania* antigens to T cells (Kima *et al.* 1996; Prina *et al.* 1996; Wolfram *et al.* 1996). DC are required for the initiation of primary T cell responses, and it is assumed that parasite-infected DC must prime the T cell response to *Leishmania*. Supporting this, it has been demonstrated that infected LC can migrate from the site of infection to the draining LN, and that DC from LN of infected mice can stimulate parasite-primed T cells *in vitro* (Moll *et al.* 1993). Parasites and parasite-derived material can also be detected in DC in the LN (Moll *et al.* 1993) and spleen of infected mice (Gorak *et al.* 1998). However, the specific interaction between DC and *Leishmania* parasites has not been examined in detail, and is of particular interest given the close lineage relationship between DC and MΦ.

Incubation of *L. mexicana* parasites expressing the MBAP:MCC fusion protein with BM-DC did not induce up-regulation of surface levels of MHC II (see Figure 3.13). This was contrary to expectations since *Leishmania* have been assumed to activate DC upon uptake similar to other pathogens (Reis e Sousa *et al.* 1999): several studies have documented the up-regulation of activation markers on the surface of different DC populations upon uptake of *Leishmania* amastigotes and/or promastigotes (von Stebut *et al.* 1998; Marovich *et al.* 2000; Qi *et al.* 2001), while others have reported infection-induced production of IL-12 (Gorak *et al.* 1998; Konecny *et al.* 1999). However, *ex*

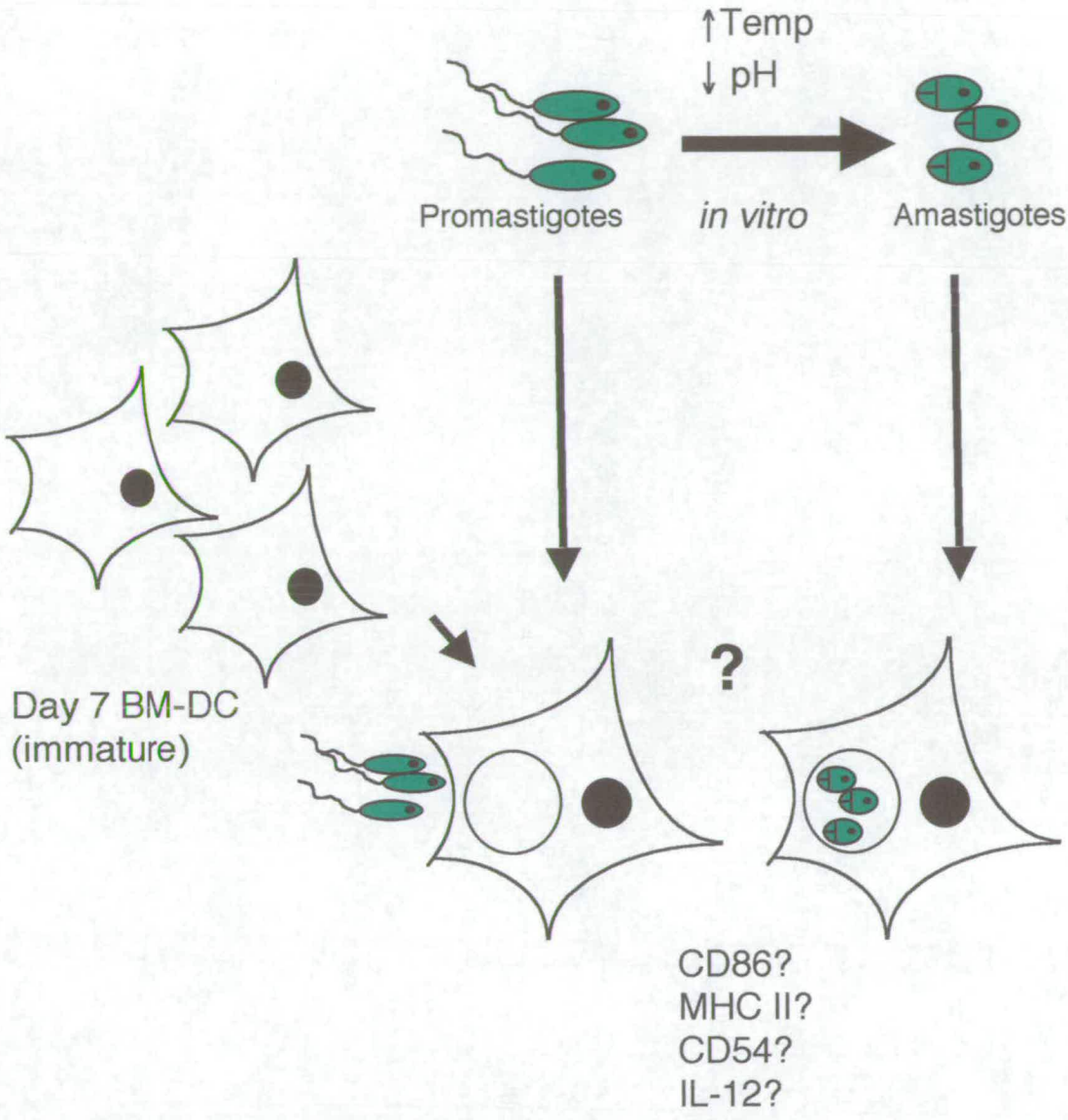
vivo-derived DC are sensitive to manipulation and constitutively mature upon culture *in vitro* (Gallucci *et al.* 1999). Furthermore, lesion-derived parasites will be opsonised by complement and Ig (Guy and Belosevic 1993; Peters *et al.* 1995) and exposed to other host proteins in the inflamed tissue (Winter *et al.* 1994). Thus, it is difficult to define in these systems the ability of *Leishmania* parasites *per se* to activate DC in the absence of other contaminating factors.

This chapter describes experiments that aimed to characterise the interaction between *L. mexicana* parasites and DC, through the use of a highly controlled *in vitro* model.

4.2 The Experimental Model (Figure 4.1)

DC can be generated in culture from bone marrow-derived precursors cultured with granulocyte/MΦ-colony stimulating factor (GM-CSF). Unlike *L. major* parasites, *L. mexicana* maintain their virulence after long-term culture *in vitro* and *L. mexicana* promastigotes can be transformed into axenic amastigotes without passage though a host (Bates *et al.* 1992; Bates 1994); see also section 2.3.1.2)). Axenic amastigotes of different *Leishmania* species have been well characterised and display a number of morphological and biochemical markers which distinguish them as *bone fide* amastigotes (Gupta *et al.* 2001): ultrastructural analyses revealed the presence of amastigote-specific organs such as the megasome and a non-emergent flagellum (Bates *et al.* 1992); axenic amastigotes have increased cysteine protease (Bates *et al.* 1992) and nuclease activity (Bates 1994); they show decreased total protein content, including down-regulation of the secreted acid phosphatase (Bates 1994); surface LPG is down-regulated (Saar *et al.* 1998); and axenic amastigotes are more infective than promastigotes *in vitro* and *in vivo* (Gupta *et al.* 2001). The combination of these tools provided an *in vitro* model in which BM-DC were incubated with *L. mexicana* promastigotes and amastigotes *in vitro*. In order to identify cells infected with live parasites, these experiments utilised transgenic parasites in which the enhanced green

Figure 4.1: The Model



Schematic representation of an *in vitro* system with which to study the effects of uptake of *Leishmania* parasites on the activation status of DC.

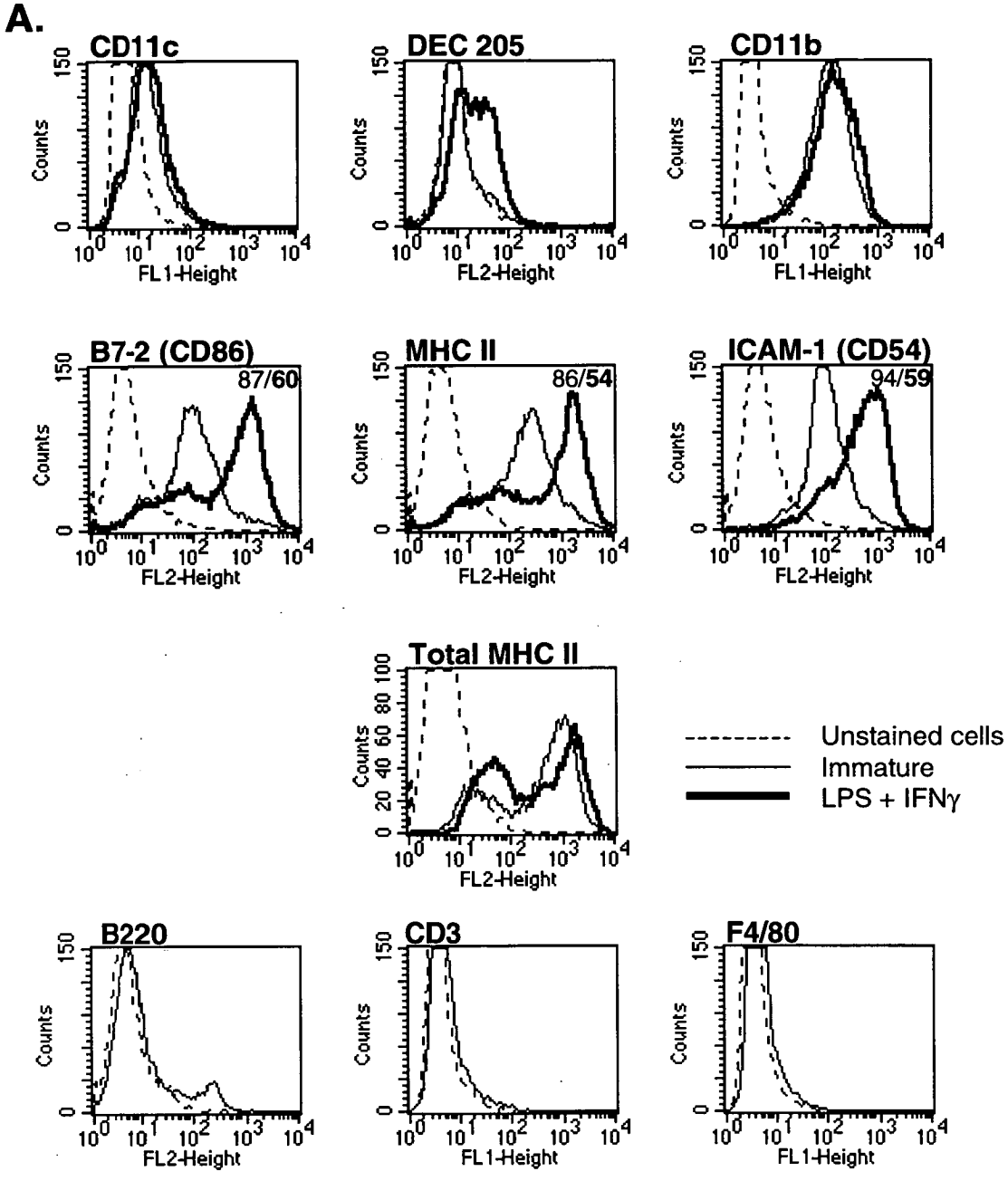
fluorescent (EGFP) gene was integrated into a small subunit ribosomal locus by homologous recombination (Misslitz *et al.* 2000): integration of the transgene into this locus ensures high expression levels (see Chapter 3). Characterisation of these transgenic parasites demonstrated that the virulence of EGFP-expressing parasites was unimpaired compared to wild type parasites *in vivo*, although lesion development was marginally delayed (Bennett *et al.* 2001). Thus, in the experiments described below, cultured EGFP-expressing *L. mexicana* parasites were used to infect *in vitro*-generated immature BM-DC. The activation status of DC was subsequently followed by measuring up-regulation of CD86, MHC II, CD54 and production of IL-12.

4.3 Phenotypic Characterisation of BM-DC

BM-DC were generated by culture of bone marrow precursors in medium containing GM-CSF, as described in section 2.4.3. DC, which are semi-adherent, were harvested into new plates on day 7, by vigorous washing, leaving behind the firmly adherent MΦ. This protocol was carefully optimised to minimise the presence of contaminating MΦ since *Leishmania* parasites may show a preference for this cell type over DC. Once harvested, DC were cultured for 18 hours with or without LPS and IFNγ as maturation stimuli. The purity of these DC preparations was assessed by phenotypic characterisation with a panel of surface markers (Figure 4.2A).

Immature BM-DC derived by this protocol were routinely $CD11c^+$, $CD11b^+$ and $DEC\text{-}205^{lo}$, consistent with the immunophenotype of myeloid lineage DC (Kamath *et al.* 2000). More than 80% of the cells were routinely intermediate for surface CD86, CD54 and MHC II, and expressed high intracellular levels of MHC II (Figure 4.2A). Overnight incubation of immature DC with 1μg/ml LPS and 10^3 U/ml IFNγ resulted in the up-regulation of CD86, CD54 and MHC II in at least 50% of the population (Figure 4.2A). Double staining with MHC II and CD11c indicated that 70% of the population were DC (Gallucci *et al.* 1999) (Figure 4.2B). No contamination with T cells or MΦ was apparent

Figure 4.2: Phenotype of Bone Marrow-derived DC

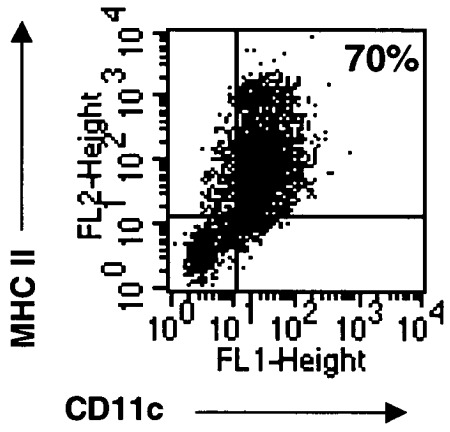


A. Day 7 BM-DC were incubated for 18 hours with or without 1μg/ml LPS and 10^3U/ml IFNγ as maturation stimuli. Cells were harvested and stained for surface antigens. For total MHC II cells were fixed and permeabilised before staining. Dotted lines represent isotype controls except CD11c = unstained cells and DEC205 = secondary antibody only.

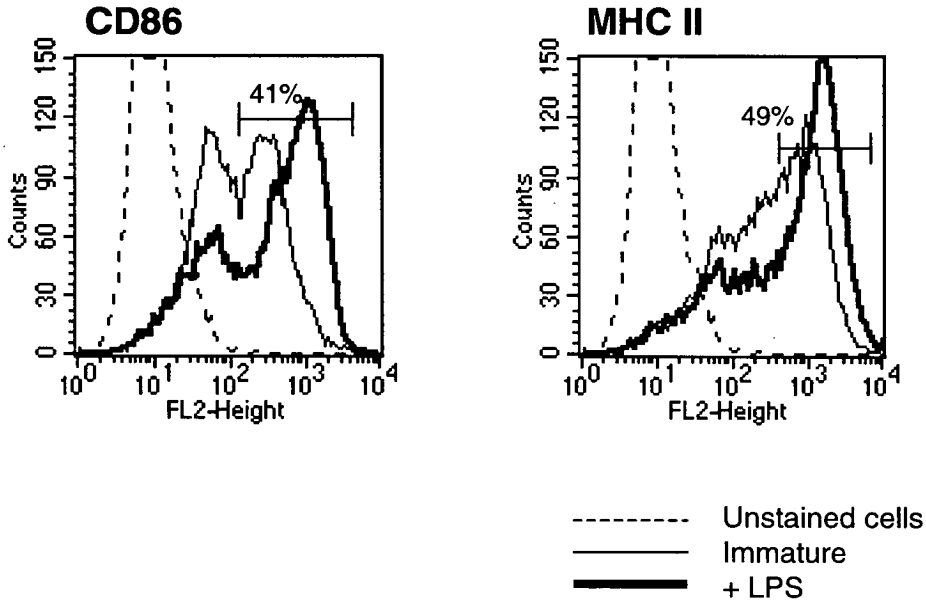
Numbers represent the percentage of positive cells for that marker / the percentage of cells which up-regulated that marker on addition of LPS and IFNγ.

Figure 4.2: Phentoype of Bone Marrow-derived DC

B.



C.



B. B M-DC were co-stained for CD11c and MHC II. 70% represents the number of cells gated in the upper right quadrant.

C. Day 7 BM-DC were harvested and enriched for CD11c positive cells by magnetic sorting. $CD11c^+$ DC were then plated out for 18 hours with or without 1μg/ml LPS, harvested and stained for surface activation markers.

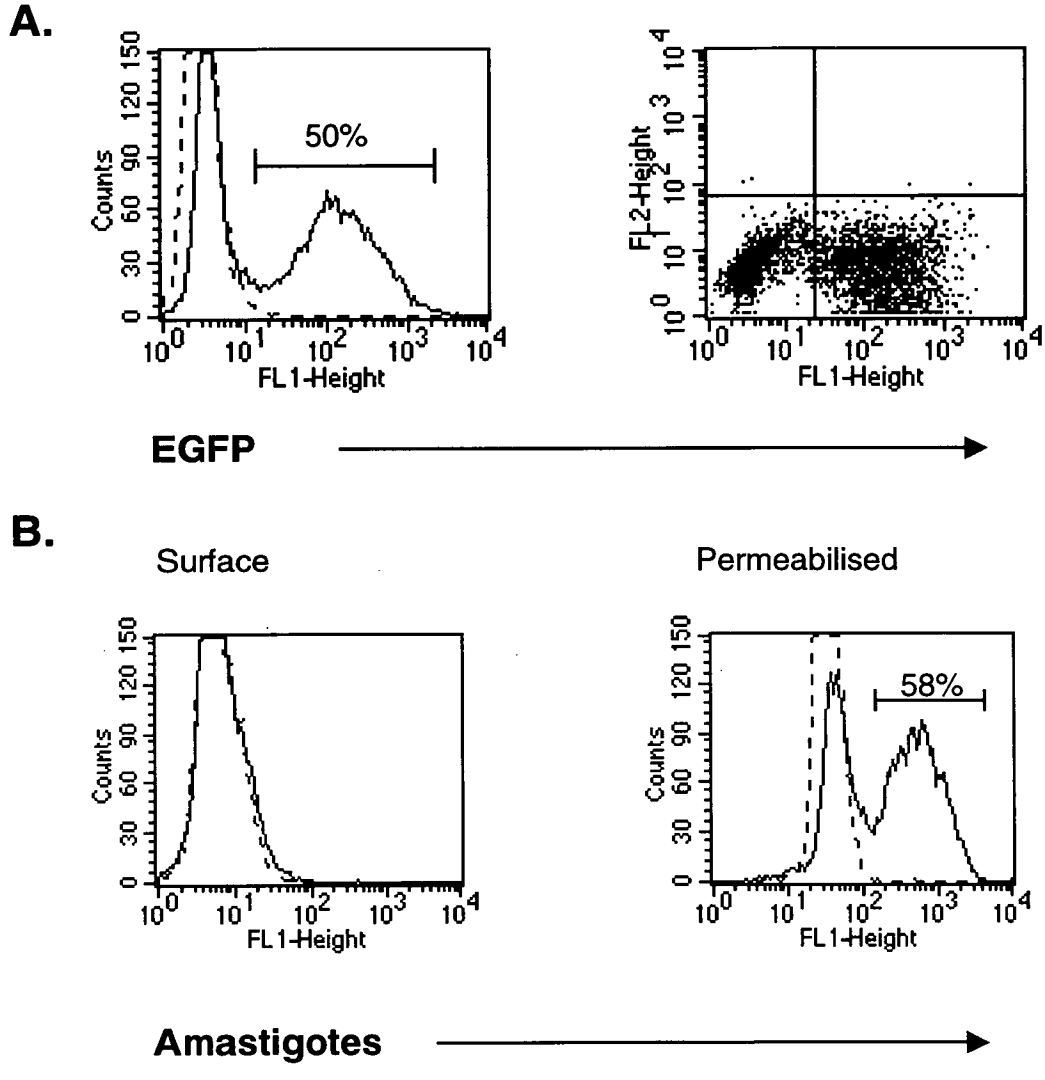
Numbers indicated the percentage of activate cells within the immature population (thin lines).

in these preparations, as determined by staining with anti-CD3 and F4/80 respectively (Figure 4.2A). Staining with anti-B220 revealed a small population of B cells. These were generally smaller cells and were therefore excluded from further analysis by gating on forward and side scatter. Although further purification of day 7 BM-DC by enrichment for $CD11c^+$ cells by magnetic activated cell sorting (MACS) resulted in a DC population which was more than 85% pure (based on CD86 and MHC II levels in immature cells), phenotypic analysis indicated that these DC became activated upon replating and therefore, this procedure was not used for this study (Figure 4.2C).

4.4 *Leishmania* Parasites are Internalised by BM-DC

In order to verify that *L. mexicana* parasites were taken up by DC, day 7 BM-DC were incubated with stationary phase EGFP-parasites at a ratio of 1:8 for 18 hours at 37°C. This ratio was selected because it resulted in optimal uptake of parasites, with few extracellular parasites remaining in the culture after 18 hours. Preliminary experiments had demonstrated that the effect of uptake of *L. mexicana* parasites was the same at 4, 12, 18 and 24 hours (data not shown). Thus, an 18 hour incubation was routinely used for these experiments. In contrast to *in vitro* infection of MΦ, parasite uptake by DC could not be monitored by light microscopy, as parasites were not clearly visible inside DC. However, microscopic analysis indicated that they had disappeared from the culture medium. Infection was therefore monitored by flow cytometry. $EGFP^+$ DC were clearly visible by FACS (Figure 4.3A), suggesting that parasites had been taken up by DC in culture. However, this analysis could not determine whether parasites had been internalised or were adhered to the cell surface. Therefore, to ascertain whether *Leishmania* were truly internalised by BM-DC, cells were infected with wild type parasites, fixed, and stained with an anti-parasite serum. Figure 4.3B shows that anti-parasite staining could be seen only when the cells were permeabilised, indicating that the parasites were intracellular and not on the surface. Incubation of BM-DC with *Leishmania* amastigotes resulted in infection of 37.0±3.9% (mean ±s.e.m.) of the cells

Figure 4.3: Internalisation of *Leishmania* Parasites by BM-DC



A. D ay 7 BM-DC were incubated with EGFP-expressing *L. mexicana* amastigotes for 18 hours. Cells were harvested, fixed and analysed by flow cytometry.

B. B M-DC were incubated with wild-type *L. mexicana* amastigotes for 18 hours. Cells were harvested, fixed and stained with an anti-parasite serum with or without permeabilisation.

Numbers represent the percentage of cells within the marker

based on percentages of $EGFP^+$ cells in the population. Incubation with promastigotes lead to infection of 22.7±4.1% of the cells, although green fluorescence was always decreased in promastigote-infected cultures probably due to higher levels of degradation of promastigotes than amastigotes, suggesting this value may be an underestimate. Table 4.1 presents the complete FACS data set for internalization of *Leishmania* parasites in six different experiments.

4.5 Incubation of BM-DC with *L. mexicana* Promastigotes or Promastigote Lysate Results in Activation of a Small Percentage of BM-DC

To determine whether uptake of *L. mexicana* promastigotes *in vitro* could activate BM-DC, DC were incubated with EGFP-promastigotes, harvested, fixed, and stained for surface activation markers. Flow cytometric analysis revealed that approximately 90% of the population remained immature, as indicated by surface levels of CD86, MHC II and CD54. This immature population contained both infected and non-infected cells (Figure 4.4A).

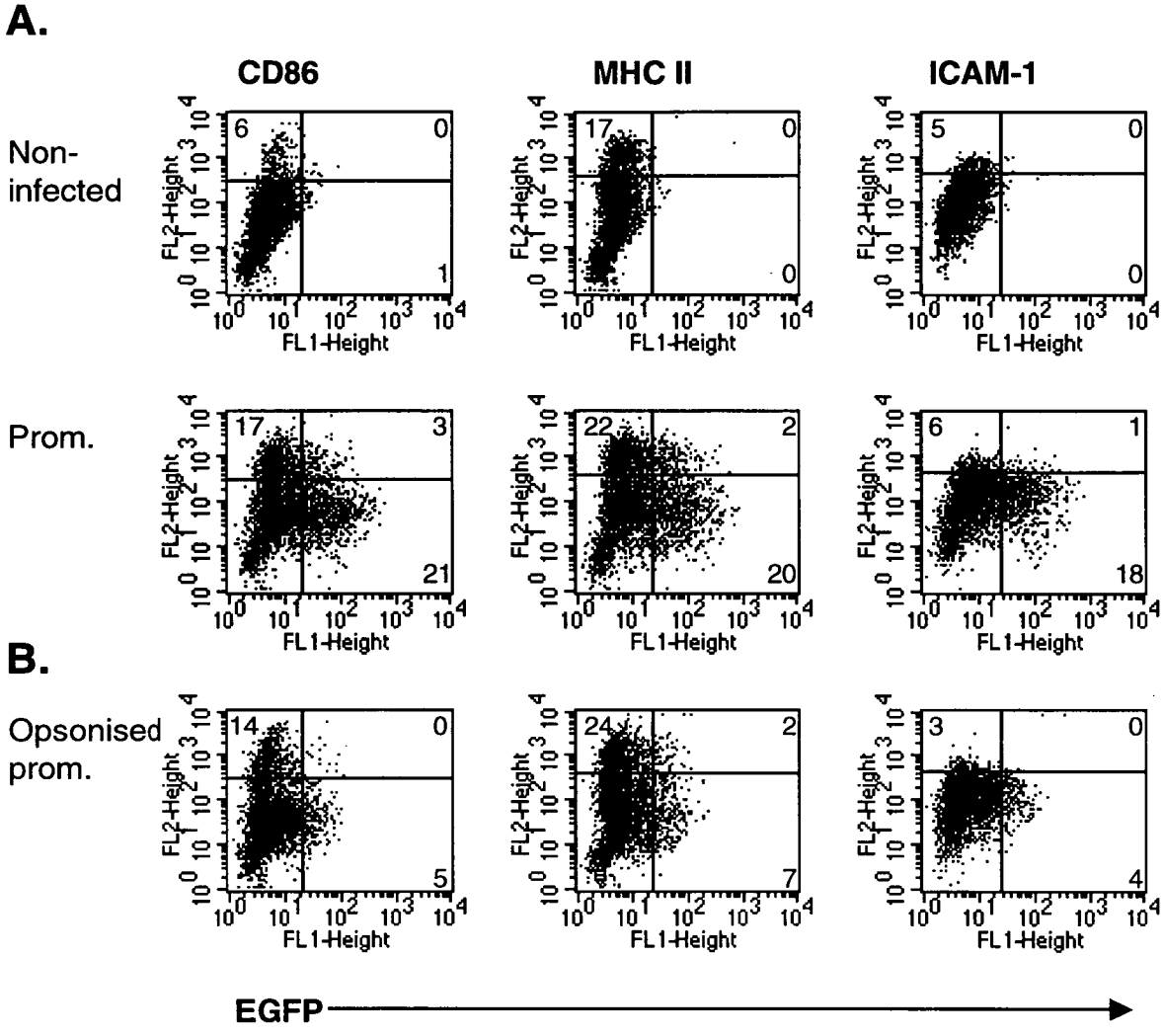
A small, but statistically significant percentage of cells (14.7±2.2%) showed increased levels of CD86 expression compared to immature non-infected DC, indicating activation (immature DC versus promastigote-infected cultures, $p<0.01$; statistical analyses are based on the numbers of $CD86^+$ cells in the upper quadrants of dot plots, and are calculated from the average of five different experiments using Student's T test; statistical analyses based on MHC II expression are shown in Table 4.2; CD54 levels were not included in these calculations because the difference in CD54 expression between immature and activated DC was much smaller than for CD86 and MHC II, and therefore is not a sensitive marker for the effect of uptake of parasites on the cells).

Table 4.1:
Complete FACS Data Showing Infection Levels of BM-DC Incubated with *L. mexicana* Promastigotes or Amastigotes.

	Percentage of $EGFP^+$ cells	
	Promastigotes	Amastigotes
Expt. 1	8.8	32.1
Expt. 2	28.2	22.2
Expt. 3	17.9	35.6
Expt. 4	29.2	39.3
Expt. 5	29.1	49.7
Expt. 6		43.3
Mean	22.7	37.0
s.e.m.	4.1	3.9

Numbers indicate the percentage of EGFP positive cells after incubation of day 7 BM-DC with EGFP-expressing *L. mexicana* promastigotes or amastigotes for 18 hours.

Figure 4.4: Incubation of BM-DC with *L. mexicana* Promastigotes



A. D ay 7 BM-DC were incubated with stationary phase EGFP-expressing *L. mexicana* promastigotes for 18 hours. Cells were harvested, fixed, stained for surface activation markers and analysed by flow cytometry.

B. P romastigotes were opsonised by incubation with 5% normal mouse serum and washed thoroughly before incubation with day 7 BM-DC. After 18 hours incubation, cells were harvested, fixed, stained for surface activation markers and analysed by flow cytometry.

Numbers represent the percentage of cells in the respective quadrants. The plots shown are representative of 5 separate experiments.

Numbers indicate the percentage of cells in the upper left (UL) and upper right (UR) quadrants for FACS plots of immature BM-DC, or BM-DC incubated with *L. mexicana* amastigotes, promastigotes, opsonised promastigotes or 3.0μm latex beads, for 18 hours and stained with anti-CD86 or anti-MHC II antibodies. For amastigote-, promastigote- and opsonised promastigote-infected populations the values in the upper left quadrant represent the activated EGFP negative cells and the values in the upper right quadrant represent the activated EGFP positive cells.

Values are not included for CD54 data because CD54 levels were not as indicative of activation as CD86 or MHC II.

CD86: Immature BM-DC versus promastigotes $p<0.01$; immature versus amastigotes, $p=0.24$; promastigotes versus opsonised promastigotes $p=0.76$; immature versus beads $p=0.43$; amastigotes versus beads $p=0.89$; promastigotes $EGFP^-$ versus $EGFP^+$ $p<0.01$; amastigotes $EGFP^-$ versus $EGFP^+$ $p<0.01$.

MHC II: Immature BM-DC versus promastigotes $p=0.76$; immature versus amastigotes, $p=0.67$; promastigotes versus opsonised promastigotes $p=0.63$; immature versus beads $p=0.80$; amastigotes versus beads $p=0.50$; promastigotes $EGFP^-$ versus $EGFP^+$ $p=0.15$; amastigotes $EGFP^-$ versus $EGFP^+$ $p=0.48$.

Table 4.2:
Complete FACS Data Showing Infection of BM-DC with *L. mexicana* Parasites or Latex Beads.

CD86										
	Immature		Amastigotes		Promastigotes		Ops. prom.		Beads	
	UL	UR	UL	UR	UL	UR	UL	UR	UL	UR
Expt.1	4.7	0.1	4.4	1.5	7.8	0.9	15.6	1.4	2.1	1.3
Expt.2	4.2	0.1	4.5	1.0	13.0	6.6	13.8	0.2	5.1	0.0
Expt.3	4.6	0.1	2.9	1.3	9.3	2.0	10.1	7.0	8.1	0.1
Expt.4	5.8	0.3	6.2	3.5	16.8	3.1	13.5	0.4	4.1	0.1
Expt.5	5.4	0.1	3.8	2.5	10.6	3.2			9.6	0.0
Mean	4.9	0.1	4.4	2.0	11.5	3.1	13.3	2.3	5.8	0.3
s.e.m.	0.3	0.0	0.5	0.5	1.6	1.0	1.2	1.6	1.4	0.3

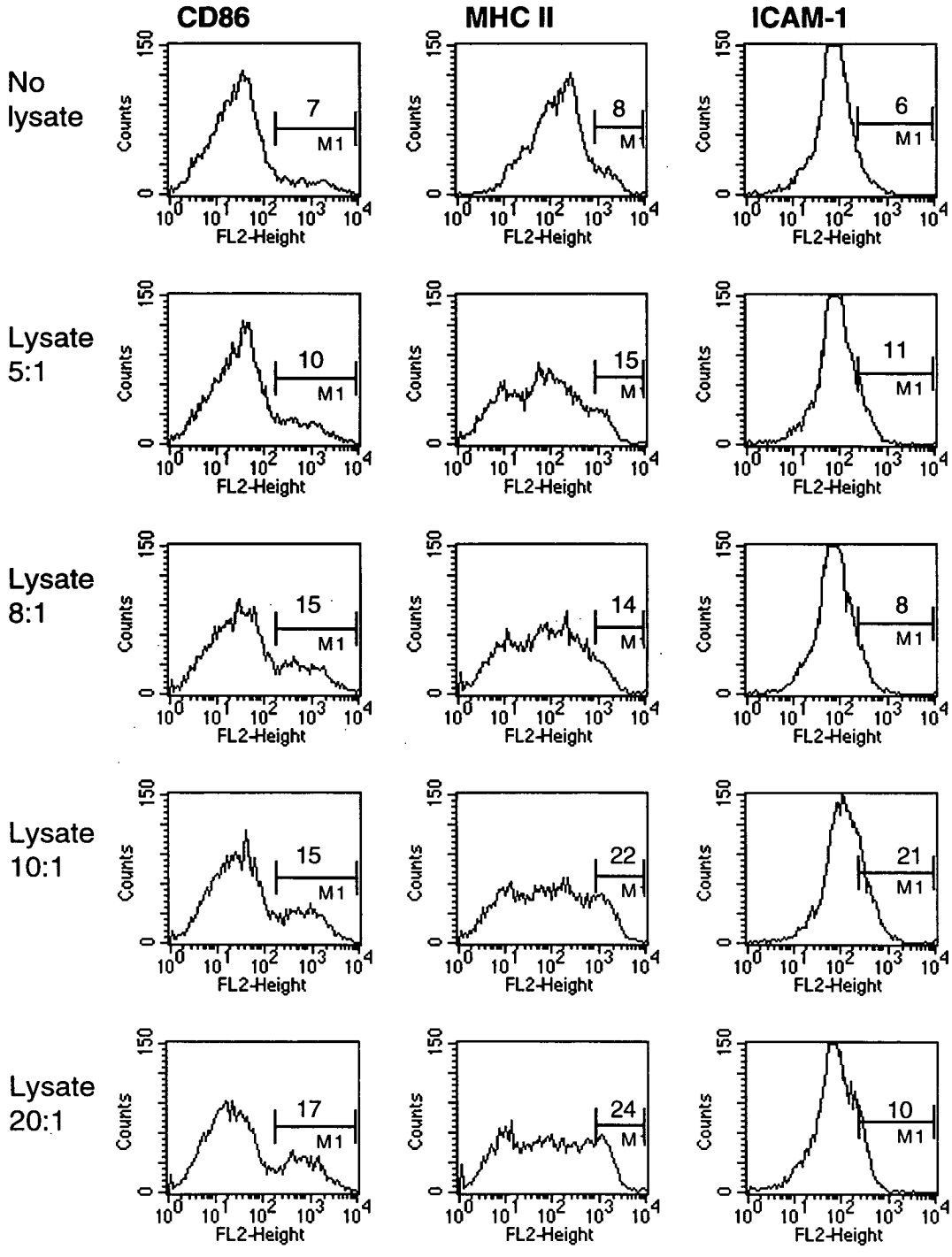
MHC II										
	Immature		Amastigotes		Promastigotes		Ops. prom.		Beads	
	UL	UR	UL	UR	UL	UR	UL	UR	UL	UR
Expt.1	5.8	0.0	3.1	0.8	3.6	0.3	7.7	0.2	2.5	2.2
Expt.2	5.6	0.0	4.4	0.7	2.4	2.3	6.0	0.1	5.0	0.0
Expt.3	4.2	0.1	2.5	1.1	2.2	0.3	7.0	0.6	4.8	0.0
Expt.4	17.0	0.1	9.3	4.0	22.2	1.5	23.5	1.5	4.1	0.1
Expt.5	3.6	0.1	7.9	11.5	6.3	2.2			13.5	0.2
Mean	7.2	0.1	5.4	3.6	7.3	1.3	11.1	0.6	6.0	0.5
s.e.m.	2.5	0.0	1.3	2.1	3.8	0.4	4.2	0.3	1.9	0.4

However activated BM-DC tended to be negative for EGFP, suggesting that these cells did not contain live parasites. The data presented in Figure 4.4 indicate that there is a larger increase in CD86 expression on incubation with *L. mexicana* promastigotes than MHC II, indicating differential regulation of these surface markers. The mean percentage of cells expressing high levels of CD86 on incubation with promastigotes is 14.7±2.2 compared to 5.1±0.3% of immature cells, whereas 8.7±3.9% and 7.2±2.5% of the cells, respectively, up-regulated MHC II (Table 4.2). These data indicate that CD86 expression is more sensitive to promastigote-derived activating stimuli than MHC II.

In vivo, uptake of *Leishmania* promastigotes is likely to be mediated by opsonisation of parasites with Ig. In order to determine whether opsonisation of promastigotes enhanced uptake and activation of DC, promastigotes were incubated with 5% inactivated normal mouse serum for 5 minutes and washed thoroughly before their addition to the BM-DC cultures (Figure 4.4B). Opsonisation of promastigotes did not enhance activation of DC. 15.5±0.9% of the cells up-regulated expression of CD86. This percentage was not significantly different from the percentage of cells activated upon incubation with non-opsonised promastigotes ($p=0.76$, numbers represent the mean ±s.e.m of four different experiments, see Table 4.2). Infection levels, as measured by green fluorescence appeared lower in these cells, probably due to enhanced degradation of these opsonised parasites.

Since incubation of BM-DC with *L. mexicana* promastigotes induced activation of a small percentage of cells that did not appear to contain green parasites, it was interesting to assess whether parasite debris alone could also activate DC. BM-DC were incubated with promastigote freeze/thaw lysates for 18 hours and then analysed for surface levels of activation markers. Incubation of BM-DC with lysates from promastigote numbers which were equivalent to giving intact promastigotes at a ratio of 1:8 led to activation of 13.6±1.8% of the population, based on levels of CD86 expression (Figure 4.5 and Table 4.3). This was equivalent to the percentage of cells activated on incubation with intact promastigotes (percentage of cells activated upon incubation with promastigotes versus percentage of cells activated upon incubation with an equivalent amount of promastigote

Figure 4.5: Incubation of BM-DC with Different Amounts of *L. mexicana* Promastigote Lysate



Day 7 BM-DC were incubated with different amounts of promastigote freeze/thaw lysate for 18 hours. Cells were then harvested and stained for surface activation markers. Amounts of lysate were calculated based on the numbers of intact promastigotes lysed. Ratios represent number of parasites lysed:DC.

M1 marks the percentage of activated cells compared to the immature population. Histograms are representative of 3 separate experiments.

Table 4.3:
Complete FACS Data for BM-DC Incubated with Promastigote Lysate.

CD86							
	Immature		Promastigote Lysate 8:1		Promastigote lysates		
					5:1	10:1	20:1
	UL		UL				
Expt.1	4.1		13.7		17.3	23.7	31.9
Expt.2	3.3		8.7		10.3	15.0	16.7
Expt.3	4.5		11.2		16.4	15.4	20.0
Expt.4	6.9		15.2				
Expt.5	3.4		19.3				
Mean	4.4		13.6		14.7	18.0	22.9
s.e.m.	0.7		1.8		2.2	2.8	4.6

MHC II							
	Immature		Promastigote Lysate 8:1		Promastigote lysates		
					5:1	10:1	20:1
	UL		UL				
Expt.1	25.4		20.7		39.6	42.4	40.7
Expt.2	12.8		14.1		14.5	21.9	24.2
Expt.3	8.1		12.5		13.8	19.5	21.6
Expt.4	8.1		14.2				
Expt.5	11.2		13.3				
Mean	13.1		15.0		22.6	27.9	28.8
s.e.m.	3.2		1.5		8.5	7.3	6.0

Numbers on the left hand side of the tables indicate the percentage of cells in the upper left (UL) quadrants of FACS plots of non-infected BM-DC or BM-DC incubated with *L. mexicana* promastigote freeze / thaw lysates at an equivalent of 8 parasites : 1 DC. Cells were harvested after 18 hours incubation with lysate, stained with anti-CD86 or anti-MHC II antibodies and analysed by flow cytometry.

Numbers on the right hand side of the tables are from 3 different experiments in which BM-DC were incubated with different amounts of promastigote lysate.

CD86: Immature BM-DC versus promastigote lysate 8:1, $p<0.01$; promastigotes versus lysate 8:1 $p=0.73$.

MHC II: Immature BM-DC versus promastigote lysate 8:1, $p=0.62$; promastigotes versus lysate 8:1 $p=0.23$.

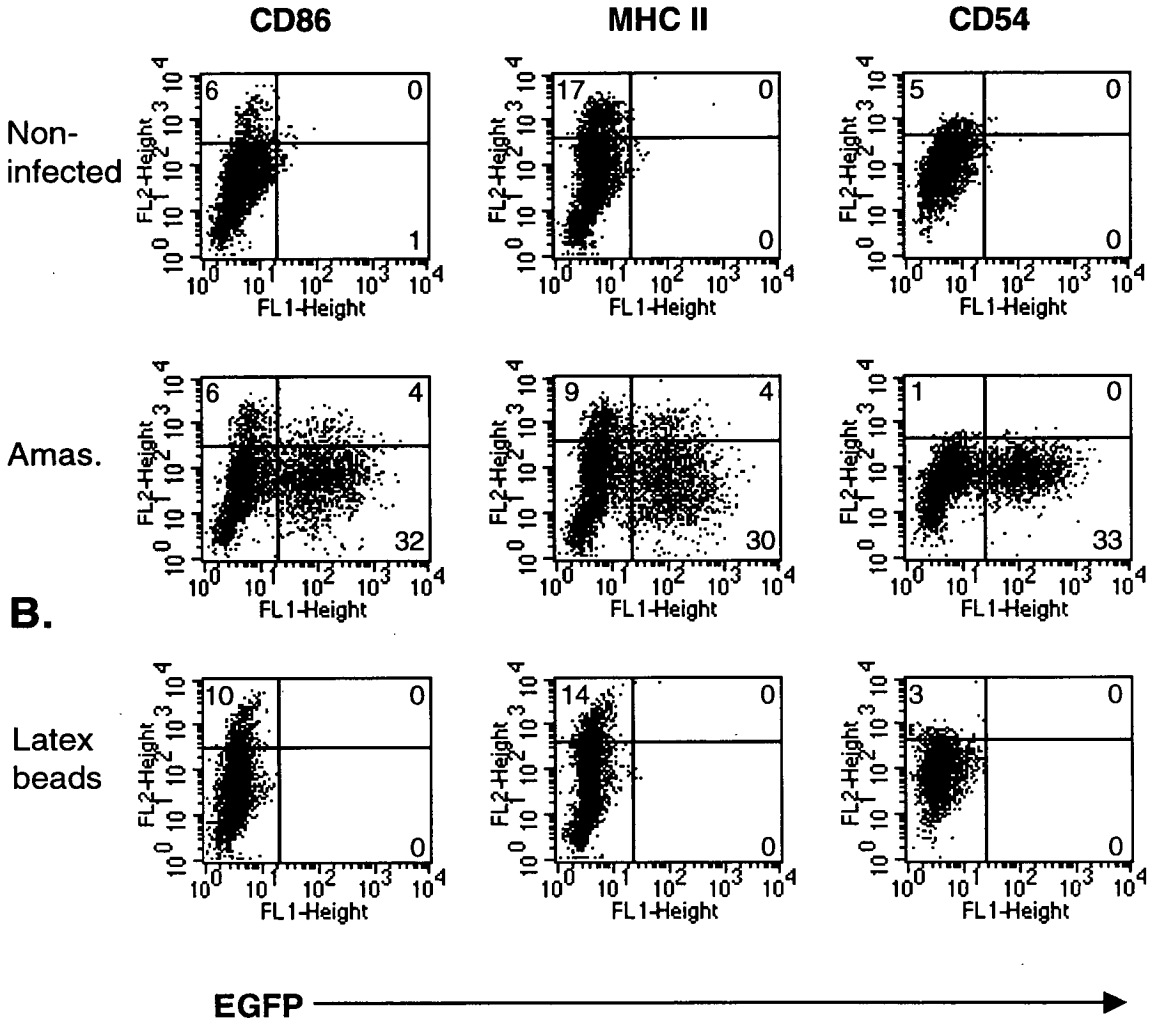
lysate p=0.73; value calculated from four different experiments) (see Figure 4.4). However, this percentage could not be increased on addition of more lysate, possibly due either to the large amount of proteases released on lysis of *Leishmania* parasites, or due to the limited number of cells in the culture that were susceptible to this activating stimulus. The data presented in Figure 4.5 suggest that, as for incubation with live promastigotes, CD86 is more sensitive to the promastigote-encoded activating stimulus than MHC II or CD54. This difference was not, however, reflected in the other experiments included in this data set (see Table 4.3).

Therefore, these data indicate that uptake of intact *L. mexicana* promastigotes *in vitro* does not activate BM-DC, but that a promastigote product is able to activate a percentage of BM-DC.

4.6 Infection with *L. mexicana* Amastigotes does not Induce Activation of BM-DC

BM-DC were also incubated with *L. mexicana* axenic amastigotes. Figure 4.6A illustrates that infection with amastigotes does not induce activation of BM-DC. Both the $EGFP^+$ and $EGFP^-$ populations remained immature, as judged by levels of surface expression of CD86, MHC II and CD54. There was no significant difference between the percentage of $CD86^+$ cells in the upper quadrants of flow cytometric plots of immature DC versus those incubated with amastigotes (p=0.24, values based on percentages from five different experiments, Table 4.2). There was no significant difference between MHC II levels on $EGFP^+$ and $EGFP^-$ DC (upper left versus upper right quadrants) derived from the same culture (p=0.48), however, $EGFP^+$ cells within the amastigote-infected cultures did appear to be retarded in expression of CD86 compared to the $EGFP^-$ cells (upper left versus upper right $p<0.01$). This observation supports the idea of differential regulation of CD86 and MHC II expression on the surface of BM-DC. The findings from these experiments are in contrast to those of von

Figure 4.6: Infection of BM-DC by *L. mexicana* Amastigotes does not Up-regulate Surface CD86, MHC II or CD54



A. D ay 7 BM-DC were incubated with EGFP-expressing *L. mexicana* axenic amastigotes for 18 hours. Cells were harvested, fixed, stained for surface activation markers and analysed by flow cytometry.

B. D ay 7 BM-DC were incubated with 3.0µm latex beads for 18 hours, harvested, fixed, stained and analysed by flow cyometry.

Numbers represent the percentage of cells in the respective quadrants. The plots shown are representative of 5 different experiments.

Stebut and colleagues (von Stebut *et al.* 1998), who demonstrated activation of foetal skin-derived DC by lesion-purified *L. major* amastigotes. These data indicate that uptake of *L. mexicana* amastigotes, in the absence of exogenous factors, does not activate DC. This could be due to *Leishmania* amastigotes presenting as inert particles, so that uptake by the DC is effectively silent. Alternatively, uptake may suppress activation of DC, as has been reported with *Leishmania*-infected MΦ (Kaye *et al.* 1994; Weinheber *et al.* 1998).

The following experiments attempted to differentiate between these two hypotheses. First, day 7 BM-DC were incubated for 18 hours with 3.0μm latex beads, which are similar in size to *L. mexicana* amastigotes, at a ratio of 1:8. Phagocytosis of latex beads did not induce activation of BM-DC, and there was no statistical difference between the effect of beads versus the effect of amastigotes on the percentage of activated cells in the cultures ($p=0.89$, based on the percentages of $CD86^+$ cells in the upper quadrants of dot plots from five different experiments, Table 4.2) (Figure 4.6B).

To further investigate whether *L. mexicana* amastigotes could suppress activation of DC, BM-DC were simultaneously incubated for 18 hours with *L. mexicana* amastigotes and activated with 0.5μg/ml LPS and 500U/ml IFNγ. Flow cytometric analysis showed that infected BM-DC were clearly activated by these stimuli, and there was no difference between the number of activated cells in infected and non-infected cultures ($p=0.91$, based on the number of activated $CD86^+$ cells from five different experiments) (Figure 4.7A). When the complete data set for CD86 and MHC II expression from the five different experiments is examined, there appears to be a slight retardation in the activation of infected ($EGFP^+$) cells in the population (Table 4.4). This retardation is not, however statistically significant: $CD86^+$ $EGFP^-$ versus $EGFP^+$ cells $p=0.09$; MHC II^+ $EGFP^-$ versus $EGFP^+$ cells $p=0.20$. Infected DC could also be activated to equivalent levels as uninfected DC with lower doses of stimuli (0.1μg/ml LPS and 500 U/ml IFNγ) (Figure 4.7B). Therefore, these data suggest that lack of activation by *L. mexicana* amastigotes is not due to strong suppression of DC function by the parasites.

A. Day 7 BM-DC were simultaneously infected with *L. mexicana* amastigotes and activated with 0.5μg/ml LPS and 500U/ml IFNγ for 18 hours. Cells were then harvested, fixed, stained for surface activation markers and analysed by flow cytometry.

B. Activation with 0.1μg/ml LPS and 500U/ml IFNγ is not suppressed by simultaneous infection with amastigotes. Day 7 BM-DC were simultaneously infected with *L. mexicana* amastigotes and activated with 0.1μg/ml LPS and 500U/ml IFNγ for 18 hours. Cells were then harvested, fixed, stained for CD86 and MHC II and analysed by flow cytometry.

Numbers represent percentages of cells in the respective quadrants. Dot plots are representative of 5 (A) and 1 (B) different experiments.

Figure 4.7: Infection by *L. mexicana* Amastigotes does not Suppress Activation of BM-DC

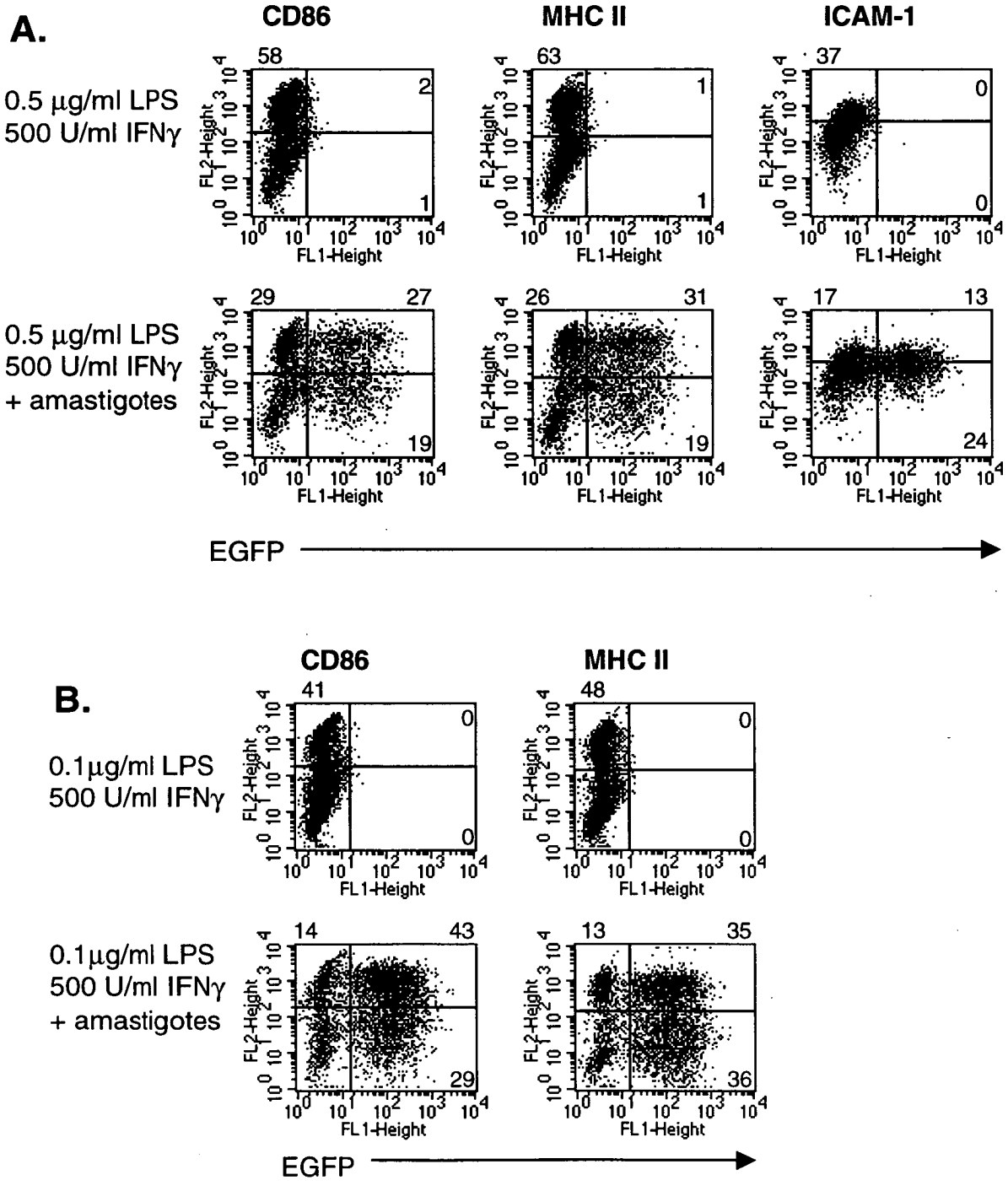


Table 4.4:
Complete FACS data for BM-DC Activated with LPS and IFNγ with or without Concomitant Infection with *L. mexicana* Amastigotes.

CD86					
	LPS/IFNγ		Amas. + LPS/IFNγ		
	UL	UR	UL	UR	Total
Expt.1	56.3	0.4	29.9	16.2	46.1
Expt.2	24.7	0.2	20.1	7.8	27.9
Expt.3	35.9	0.1	25.5	14.6	40.1
Expt.4	58.0	1.6	29.3	26.8	56.1
Expt.5	30.5	0.3	15.3	14.8	30.1
Mean	42.8	0.5	24.0	16.0	40.1
s.e.m.	4.4	0.3	2.8	3.1	5.2

MHC II					
	LPS/IFNγ		Amas. + LPS/IFNγ		
	UL	UR	UL	UR	Total
Expt.1	51.9	0.3	20.9	12.4	33.3
Expt.2	35.8	0.2	22.9	8.1	31.0
Expt.3	25.7	0.0	18.1	12.5	30.6
Expt.4	62.8	1.4	26.2	31.4	57.6
Expt.5	46.3	0.3	21.8	15.8	37.6
Mean	44.5	0.4	22.0	16.0	38.0
s.e.m.	6.4	0.3	1.3	4.0	5.1

Numbers indicate the percentage of cells in the upper left (UL) and upper right (UR) quadrants for FACS plots of BM-DC activated with LPS and IFNγ and incubated with or without *L. mexicana* amastigotes. Cells were activated with either 0.5μgml LPS and 500U/ml IFNγ or with 10μg/ml LPS alone (expt. 2 and 3), harvested after 18 hours, stained with anti-CD86 or anti-MHC II antibodies and analysed by flow cytometry. For the amastigote-infected cultures the values in the upper left quadrant represent the activated EGFP negative cells and the values in the upper right quadrant represent the activated EGFP positive cells.

CD86: Activated BM-DC versus amastigotes p=0.91; amastigotes $EGFP^-$versus $EGFP^+$ p=0.09.

MHC II: Activated BM-DC versus amastigotes p=0.45; amastigotes $EGFP^-$versus $EGFP^+$ p=0.20.

4.7 The effect of *L. mexicana* Infection on IL-12 Production by BM-DC

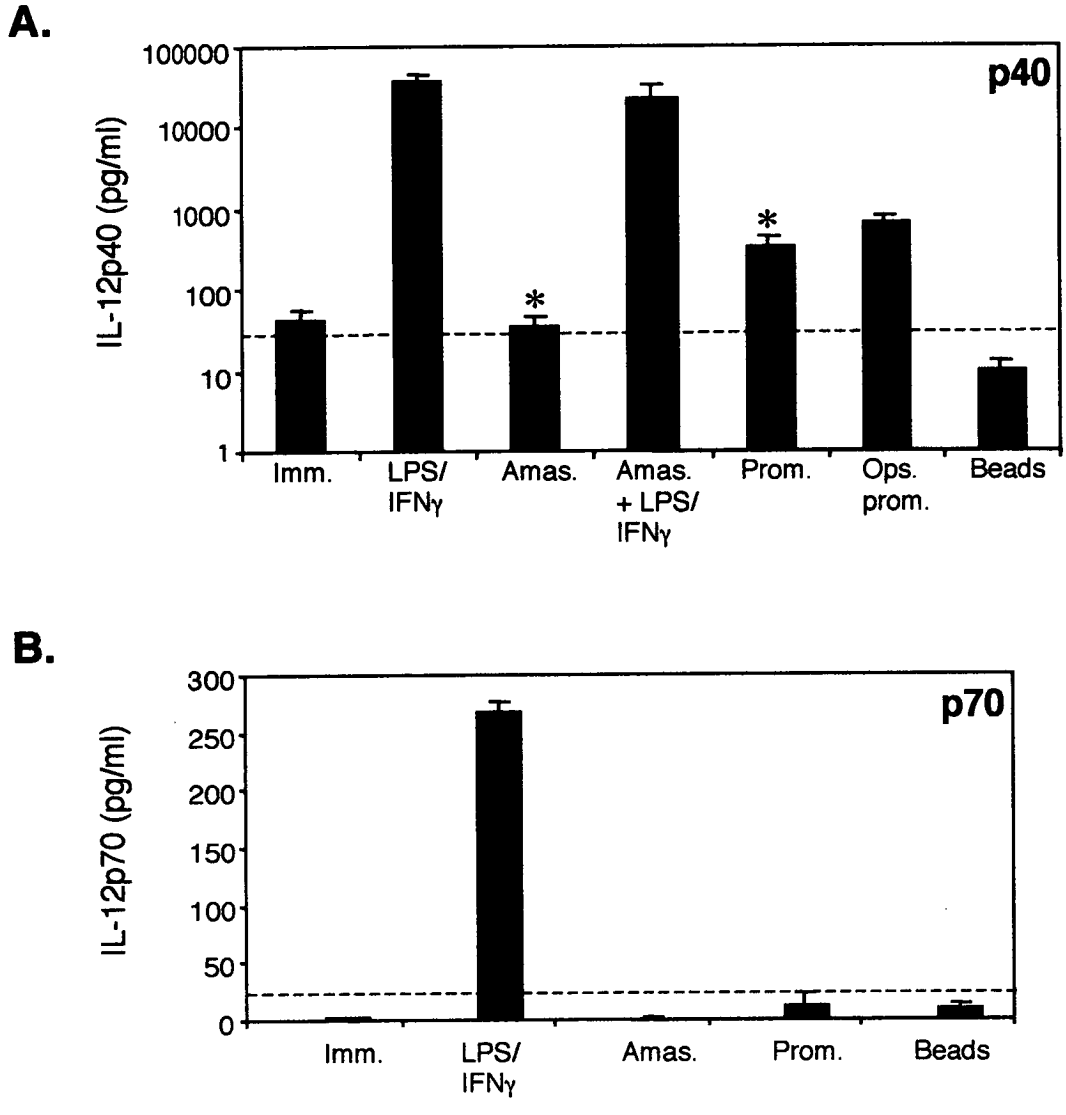
Activation of DC is associated with the production of pro-inflammatory cytokines including TNFα, IL-6 and IL-12. In order to determine whether incubation with promastigotes or amastigotes could induce secretion of IL-12, the concentration of the IL-12 p40 subunit was measured in culture supernatants (Figure 4.8A). Immature DC did not secrete IL-12 p40, but stimulation with LPS and IFNγ induced production of high levels of this protein. BM-DC incubated with *L. mexicana* amastigotes or latex beads were not stimulated to produce IL-12. Incubation with promastigotes induced levels of IL-12 p40 that are significantly higher than in non-infected or amastigote-infected cells, consistent with the activation of a small percentage of the population. Thus, production of the IL-12p40 subunit reflects the results obtained by flow cytometry.

Secretion of the IL-12 p40 subunit is indicative of the production of bioactive IL-12p70 in DC, but does not reflect levels of functional IL-12 since p40 can homodimerise and inhibit binding of IL-12p70 to the IL-12 receptor (Gillessen *et al.* 1995). Therefore, levels of IL-12 p70 were measured in the supernatant of infected cells (Figure 4.8B). As expected, immature DC did not produce IL-12p70 and p70 could only be detected on addition of LPS and IFNγ. No bioactive IL-12 was detected in cultures from promastigote- or amastigote-infected cells.

4.8 Presentation of Exogenous Antigen to T cells by *L. mexicana*-Infected BM-DC

L. mexicana-infected MΦ are impaired in their ability to present exogenous antigen to T cells, even after only a two hour incubation with parasites (Fruth *et al.* 1993). DC from infected mice have been reported to efficiently stimulate an anti-parasite T cell response

Figure 4.8: Production of IL-12 by *L. mexicana*-infected BM-DC



Day 7 BM-DC were incubated for 18 hours with *L. mexicana* amastigotes, promastigotes or latex beads, with or without 1μg/ml LPS and 10^3U/ml IFNγ. The presence of (A) the p40 subunit (n=4) or, (B) the p70 heterodimer (n=3) were measured in 18 hour supernatants by ELISA. The lower sensitivity limit of the assay was 30 pg/ml (dashed line). Values represent concentrations of cytokine in supernatants from ~$2x10^5$ cells (means ± s.e.m.).

* p40: amastigotes vs promastigotes: p=0.035; immature DC vs amastigotes: p=0.711; amastigotes vs beads p=0.067.

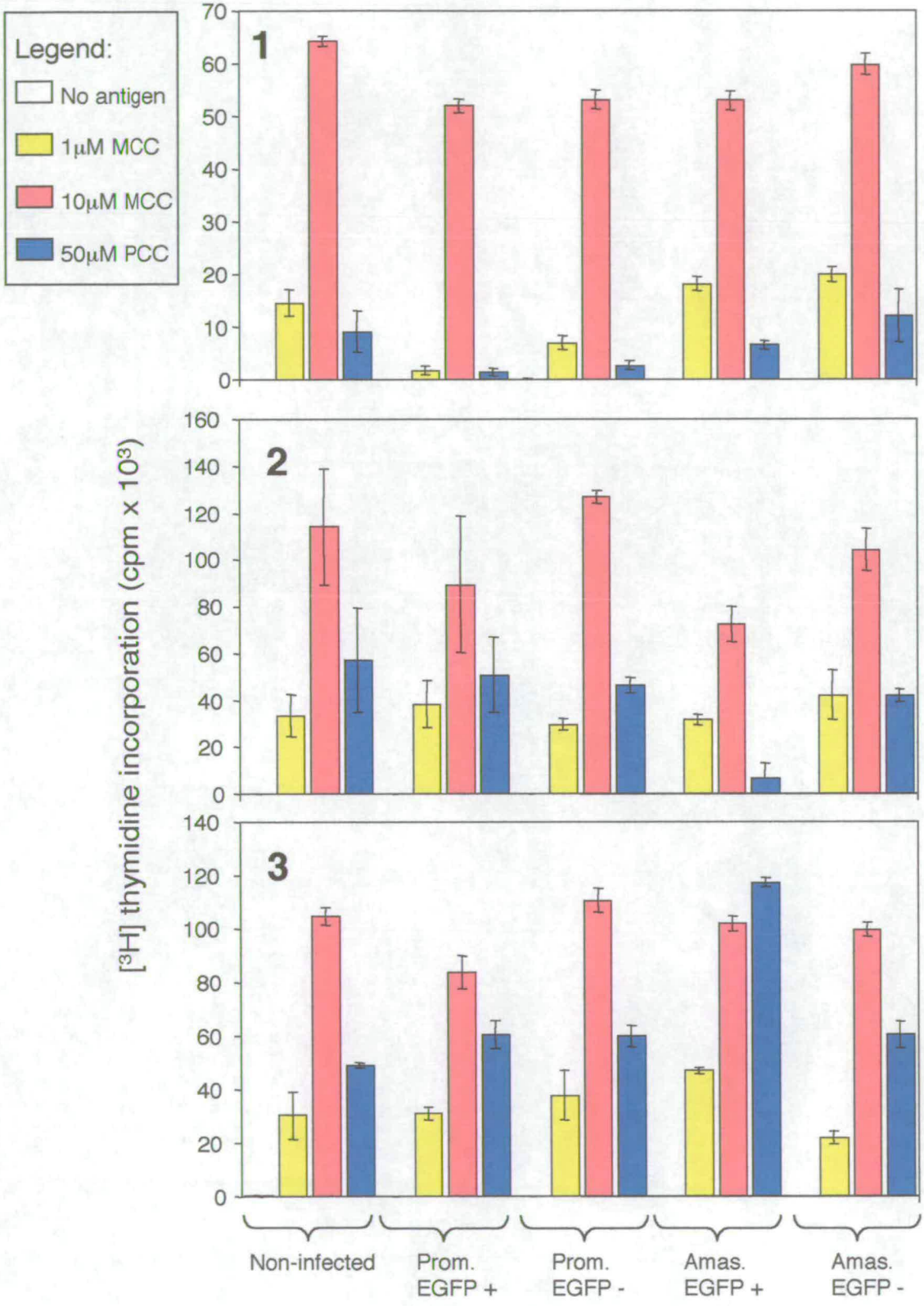
(Moll *et al.* 1993), however it has not been shown whether these cells contained intact parasites. Therefore, BM-DC incubated with EGFP-expressing parasites were used to investigate whether *Leishmania*-infected DC are competent to stimulate an efficient T cell response *in vitro*.

BM-DC were infected for 18 hours with EGFP-expressing parasites, as described above. The cells were then harvested, washed and sorted flow cytometrically into EGFP positive and EGFP negative populations. Immature DC were also harvested and sorted as a non-infected, sorted control. The cells were then replated in 96-well plates and pulsed with 1 or 10μM MCC peptide or 50μM PCC protein for 4 hours. They were then co-cultured with the PCC-specific T cell line 2B4 (Reay *et al.* 2000) at a ratio of 1 DC:10 T cells and supernatants collected after 18 hours. Production of IL-2 by activated T cells was monitored using an IL-2-dependent cell line (Swain *et al.* 1981; Lawrence *et al.* 1994). Figure 4.9 shows the results from three separate experiments. These data show good concordance between experiments 2 and 3 but variation between these experiments and experiment 1, probably due to variation in responses by the T cell hybridoma, and also due to variable effects which sorting and replating of the DC may have on DC maturation status. When comparing non-infected controls with promastigote $EGFP^+$ or $EGFP^-$ samples or amastigote $EGFP^+$ or $EGFP^-$ samples within each experiment there was no significant difference between any of the variables ($p>0.05$) for all except two of the samples. The exceptions were in experiment 1, non-infected cells versus promastigote $EGFP^+$ cells pulsed with 1μM MCC, $p<0.01$, and in experiment 3, non-infected cells versus amastigote $EGFP^+$ cells pulsed with 50μM PCC, $p<0.01$. However, when the results for the three experiments were pooled there was no significant difference between any of the samples ($p>0.3$). Therefore, these results indicate that infection of DC with *L. mexicana* promastigotes or amastigotes does not alter the efficiency with which DC present exogenous antigen to T cells.

Day 7 BM-DC were incubated with *L. mexicana* EGFP-expressing promastigotes or amastigotes or left uninfected for 18 hours. Cells were harvested and sorted into EGFP positive (infected) and negative (non-infected) populations. Non-parasite exposed DC were sorted as a control. Sorted populations were replated, pulsed with peptide or protein for 4 hours and incubated with the T cell hybridoma 2B4 for 18 hours. IL-2 levels in the T cell supernatant were measured by assessing [^{3}H] thymidine incorporation by an IL-2-dependent cell line.

The data shown here are from 3 independent experiments, with each experimental group performed in triplicate (mean ± s.e.m.).

Figure 4.9: *L. mexicana*-infected DC can Stimulate an Efficient T cell Response *in vitro*



4.9 Discussion

4.9.1 Infection by *L. mexicana* Amastigotes does not Activate BM-DC

The data presented in this chapter demonstrate that internalisation of *L. mexicana* amastigotes *per se* does not classically activate BM-DC. Rather these parasites appear to be recognised by DC as inert particles, similar to latex beads, at least in terms of the expression of surface activation markers and production of IL-12. This was surprising since phagocytosis has been reported to be an activation stimulus in some models (Randolph *et al.* 1998). Weinheber and colleagues (Weinheber *et al.* 1998) also showed that *L. mexicana* amastigotes present as inert particles on infection of MΦ, since infection-induced suppression of IL-12 could be mimicked by phagocytosis of latex beads. However, in contrast, to the situation in MΦ, the data presented here indicate that infection does not suppress the ability of DC to respond to other activating stimuli. Therefore infection of BM-DC by *L. mexicana* amastigotes may be regarded as silent based on the parameters tested.

In addition to these findings, the data presented in this chapter suggest that uptake of intact *L. mexicana* promastigotes may not induce activation of BM-DC. A small percentage of cells up-regulated expression of the surface markers tested upon incubation with promastigotes, however, these cells were not positive for EGFP, indicating that metabolically active parasites are not required for activation of BM-DC. This is supported by the fact that promastigote lysate induced similar levels of activation when added to the culture. The observation that addition of increasing amounts of lysates only marginally increased the number of cells activated indicates that only limited numbers of DC can respond to the promastigote-encoded signal, however, loss of parasite integrity will also release proteases that will have negative effects on the host cells and may restrict the proportion of BM-DC that are able to respond to this signal.

Interestingly, the data presented on infection of BM-DC with *L. mexicana* promastigotes suggests that CD86 and MHC II expression are differentially regulated, such that CD86 levels are more sensitive to low levels of an activating stimulus than MHC II. This observation was not, however, evident in other experiments, indicating that this difference is only apparent in the presence of very low levels of activating stimuli.

The discovery that receptors on the surface of DC recognise conserved pathogen-derived molecules has led to the paradigm that uptake of pathogens activates DC. This scenario is apparently supported by published studies which have examined the effect of *Leishmania* infection on *ex vivo* DC; von Stebut *et al* (von Stebut *et al.* 1998) reported activation of foetal skin-derived DC by lesion-derived *L. major* amastigotes but not promastigotes, whereas Qui *et al* (Qi *et al.* 2001) reported activation of BM-DC by *L. amazonensis* promastigotes and amastigotes. In contrast, the data presented in this chapter show that *L. mexicana* amastigotes *per se* do not express PAMP.

The discrepancies between our findings and those of others may be a function of the respective experimental systems, and in particular due to the use of lesion-derived amastigotes (von Stebut *et al.* 1998; Qi *et al.* 2001). Parasites derived *ex-vivo* are coated with Ig (Peters *et al.* 1995) and other proteins (Winter *et al.* 1994). Ligation of parasite-bound Ig by DC surface receptors may have two non-exclusive consequences: the interaction might directly activate DC; receptor-mediated endocytosis via FcR may transport the parasites to distinct intracellular compartments in which they may be more efficiently degraded, rendering intracellular *Leishmania* PAMP more accessible to internal DC pattern receptors. As ligation of FcγR by immune complexes induces maturation of DC (Regnault *et al.* 1999), the presence of Ig on lesion-derived (von Stebut *et al.* 1998; Qi *et al.* 2001), but not *in vitro* cultured, amastigotes is likely to explain the apparent differential ability of these parasite populations to activate DC. To address this hypothesis, the effect of uptake of Ig-opsonised axenic amastigotes on DC should be examined. In this experimental model, however, incubation of promastigotes with normal mouse serum did not increase activation of DC, although this would lead to coating of parasites with Complement rather than Ig. The observation that opsonisation

of promastigotes with 5% normal mouse serum led to increased degradation of the parasites supports the hypothesis that uptake of opsonised parasites may result in their internalization in distinct compartments. Although opsonisation of promastigotes with normal mouse serum did not apparently lead to increased uptake by DC, it may be that promastigotes were more efficiently internalised but that this was balanced by the increased rates of degradation.

An additional reason for the differential effects on uptake of *Leishmania* parasites by DC is that *ex vivo* DC may have been primed by endogenous factors *in vivo* before isolation and may therefore be more sensitive to activation upon culture *in vitro*. Konecny *et al* (Konecny *et al.* 1999) reported IL-12 production from $CD11c^+$ splenic DC infected with *L. major* promastigotes. As shown herein, selection with CD11c results in activation of DC upon replating overnight in the absence of additional stimuli, suggesting that CD11c-purified DC may be pre-disposed to maturation upon phagocytosis of the parasites.

The experimental model described herein has the unique advantage that it allows investigation of the DC:*Leishmania* interaction in a highly controlled system. Infection of BM-DC by EGFP-expressing parasites that have been cultured *in vitro* allowed the effects of this interaction to be addressed in the absence of contaminating host-derived factors, which may prime the activation of DC independently of the effect of the parasites. The results presented in this chapter indicate that, *in vitro*, intact promastigotes and axenic amastigotes bind DC receptors on uptake that do not result in activation of the DC. It is not clear, however, which receptors the parasites are binding in this experimental model: promastigotes utilise the mannose receptor for entry into host cells, however as yet, no evidence suggests that binding of this receptor activates DC (Reis e Sousa *et al.* 1999); promastigotes also bind CR1 and CR3. BM-DC express CR3 (CD11b, see Figure 1.2), however, ligation of CR3 has been shown to suppress activation of MΦ, and there does not appear to be any suppression in these experiments (Marth and Kelsall 1997; Sutterwala *et al.* 1997). The receptors involved in uptake of amastigotes in the absence of Ig therefore remain to be elucidated.

4.9.2 *L. mexicana*-Infected BM-DC can Efficiently Process and Present Exogenous Antigen to T cells.

Leishmania-infected MΦ do not efficiently process and present exogenous antigens. Therefore, in view of the close lineage relationship between DC and MΦ it was of interest to investigate the ability of infected DC to stimulate T cells *in vitro*. DC were incubated with EGFP-expressing promastigotes and amastigotes for 18 hours and sorted into EGFP positive and negative populations. These cells were pulsed with MCC peptide or PCC protein for four hours and then incubated with a MCC-specific T cell hybridoma. Use of EGFP-expressing parasites permitted direct investigation of the ability of infected DC to stimulate T cells. These data indicated that infected DC present exogenous antigen to T cells as efficiently as parasite-exposed non-infected DC, and non-infected control DC. Despite significant variation between the different experiments, all of the DC populations induced similar levels of proliferation of the T cell hybridoma within each experiment. It is important to note that T cell hybridomas are often co-stimulation-independent so these data only reflect the ability of infected DC to process and present exogenous antigens in association with the MHC II molecule H2-E^k. DC and MΦ may therefore respond differently to infection with *L. mexicana*. While others have shown that infection strongly suppresses activation of MΦ and alters the kinetics of antigen presentation, in our studies DC responses appear to be relatively unaffected by internalisation of *Leishmania* parasites. This difference may be related to the observation that *L. mexicana* parasites do not appear to be able to establish a chronic infection in DC as successfully as they do in MΦ (see section 5.4).

4.10 Concluding Remarks

The data presented here indicate, for the first time, that the receptors utilised by intact *L. mexicana* promastigotes or amastigotes for entry into DC do not trigger maturation of

this exquisitely sensitive cell type. The data presented in this chapter imply that activation of DC *in vivo*, and the consequent initiation of the anti-*Leishmania* T cell response, is not due to the uptake of intact parasites, but may be due to uptake of Ig-opsonised intact amastigotes and/or other exogenous activating stimuli. Injection of parasites into the epidermis will induce production of pro-inflammatory factors on wounding, and this innate immune response will also cause further tissue damage. This will result in the production of pro-inflammatory cytokines such as TNFα that activate maturation of immature DC. Sandfly saliva, which is injected into the host along with promastigotes, has also been shown to have an important immunomodulatory effect; inoculation of saliva from non-infected sandflies induces a delayed-type hypersensitivity response upon re-exposure to sandfly saliva, and can immunise against challenge with *L. major* promastigotes (Kamhawi *et al.* 2000). The data presented here also demonstrate that infection does not suppress activation and that infected DC are fully competent to process and present antigen to T cells. Thus, in the context of infection *in vivo*, infected DC should be able to initiate the anti-parasite T cell response.

Chapter 5:

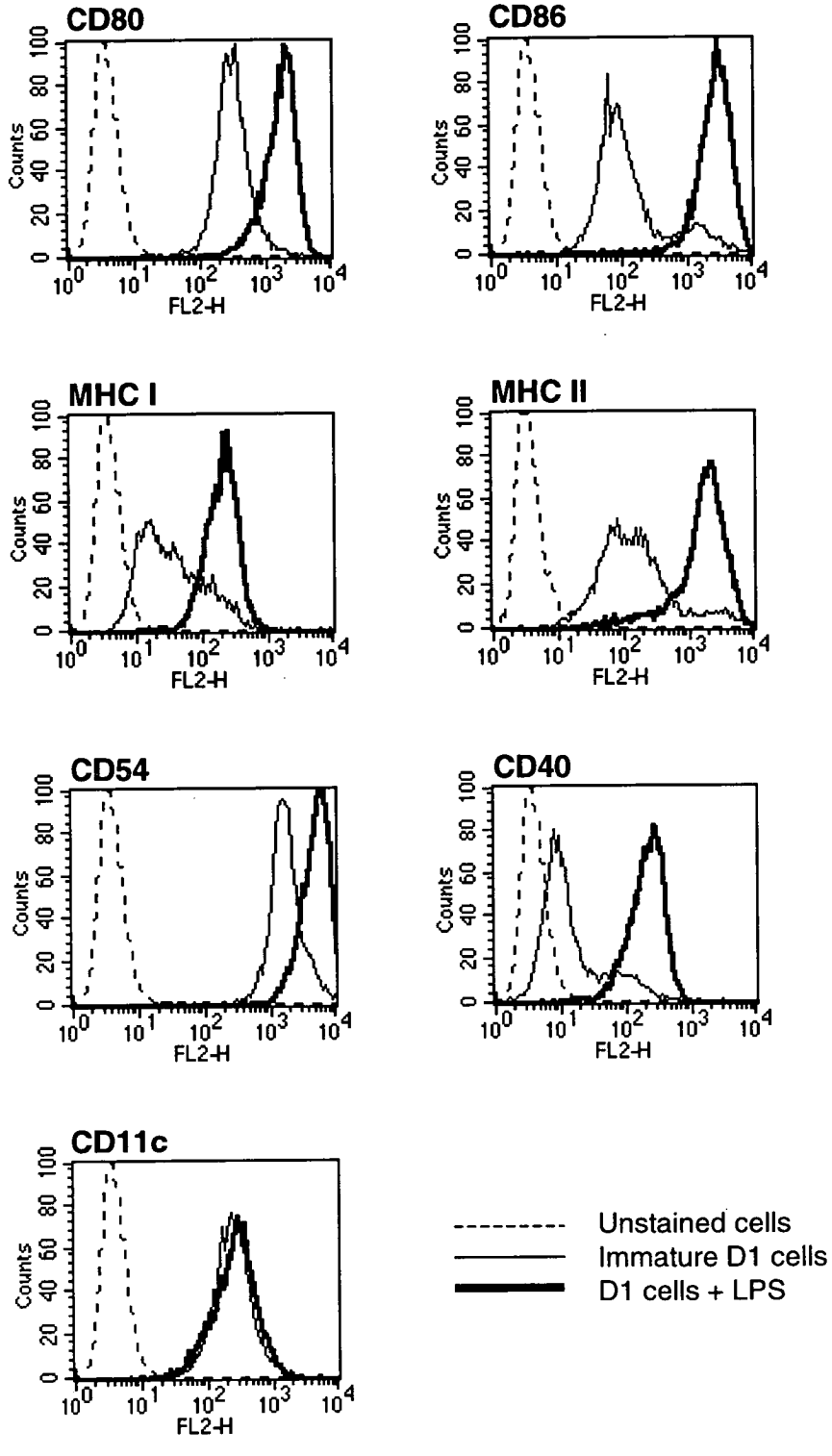
Characterisation of Infection of an Alternative DC Population with *L. mexicana*

5.1 Introduction to D1 Splenic DC

A continuous DC culture has been derived from C57BL/6 splenocytes that were expanded in the presence of conditioned medium from fibroblasts engineered to secrete recombinant GM-CSF. These factor-dependent, long-term, relatively homogeneous DC cultures have been termed D1 cells (Winzler *et al.* 1997). D1 cells have all the properties of an immature DC population: they have a surface immunophenotype characteristic of immature splenic DC and up-regulate molecules associated with interaction with T cells upon stimulation with LPS (Figure 5.1); they are highly efficient at taking up antigen, and this decreases upon activation; activation leads to increased motility; and D1 cells potently stimulate an allogeneic T cell response (Winzler *et al.* 1997). Phenotypically immature D1 cells are rounded, but extend long dendrites upon activation. Unlike other *ex-vivo*-derived DC, D1 cells can be cultured long-term in an immature form as a fairly homogeneous population of cells, which can be matured synchronously with LPS. This is a distinct advantage over BM-DC that are heterogeneous in terms of their differentiation from monocyte precursors into DC and their activation status, mature spontaneously over time, and generally die by day nine or ten of culture.

Data presented in the previous chapter demonstrated that infection with intact *L. mexicana* promastigotes or amastigotes *in vitro* does not induce activation of BM-DC. In order to test whether this was a general phenomenon, the effect of infection on an alternative *in vitro* DC culture was investigated. The experiments described in Chapter 5 aimed to characterise the effect of infection with *L. mexicana* on D1 splenic DC

Figure 5.1: Phenotype of D1 Cells



D1 cells were plated out for 18 hours with or without 10μg/ml LPS as a maturation stimulus. The cells were harvested, stained for surface DC markers and analysed by flow cytometry (see also Winzler *et al* 1997).

(Winzler *et al.* 1997). D1 cells were infected with *L. mexicana* promastigotes and amastigotes and the effect on surface activation markers and cytokine production was measured. These cells also provided an *in vitro* culture system with which to carry out a kinetic analysis of gene expression in *L. mexicana* promastigote- or amastigote-infected DC. Finally, culture of immature D1 cells facilitated investigation of long-term infection of DC with *L. mexicana* parasites, in particular, whether the formation of the large communal parasitophorous vacuole (PV) characteristic of infected MΦ is induced in DC.

5.2 Infection of D1 cells with *L. mexicana* Promastigotes and Amastigotes

5.2.1 Characterisation of Infection of D1 cells with *L. mexicana*

In order to compare the effect of infection of D1 cells with *L. mexicana* to the studies performed with BM-DC (Chapter 4), D1 cells were incubated for 18 hours with EGFP-expressing stationary phase *L. mexicana* promastigotes, axenic amastigotes, or 3.0μm latex beads at 37°C. The cells were then harvested, washed, fixed, stained for the surface markers CD86, MHC II and CD54 and analysed by flow cytometry.

D1 cells efficiently internalised the parasites after 18 hours in culture; 48.0±3.7% (mean±s.e.m) and 45.4±2.9% of the D1 cells internalised promastigotes and amastigotes respectively, (results are taken from five different experiments, see Table 5.1). Incubation with *L. mexicana* promastigotes resulted in more clearly defined $EGFP^+$ populations than were observed with the BM-DC cultures, perhaps due to the relative homogeneity of D1 cultures compared to BM-DC.

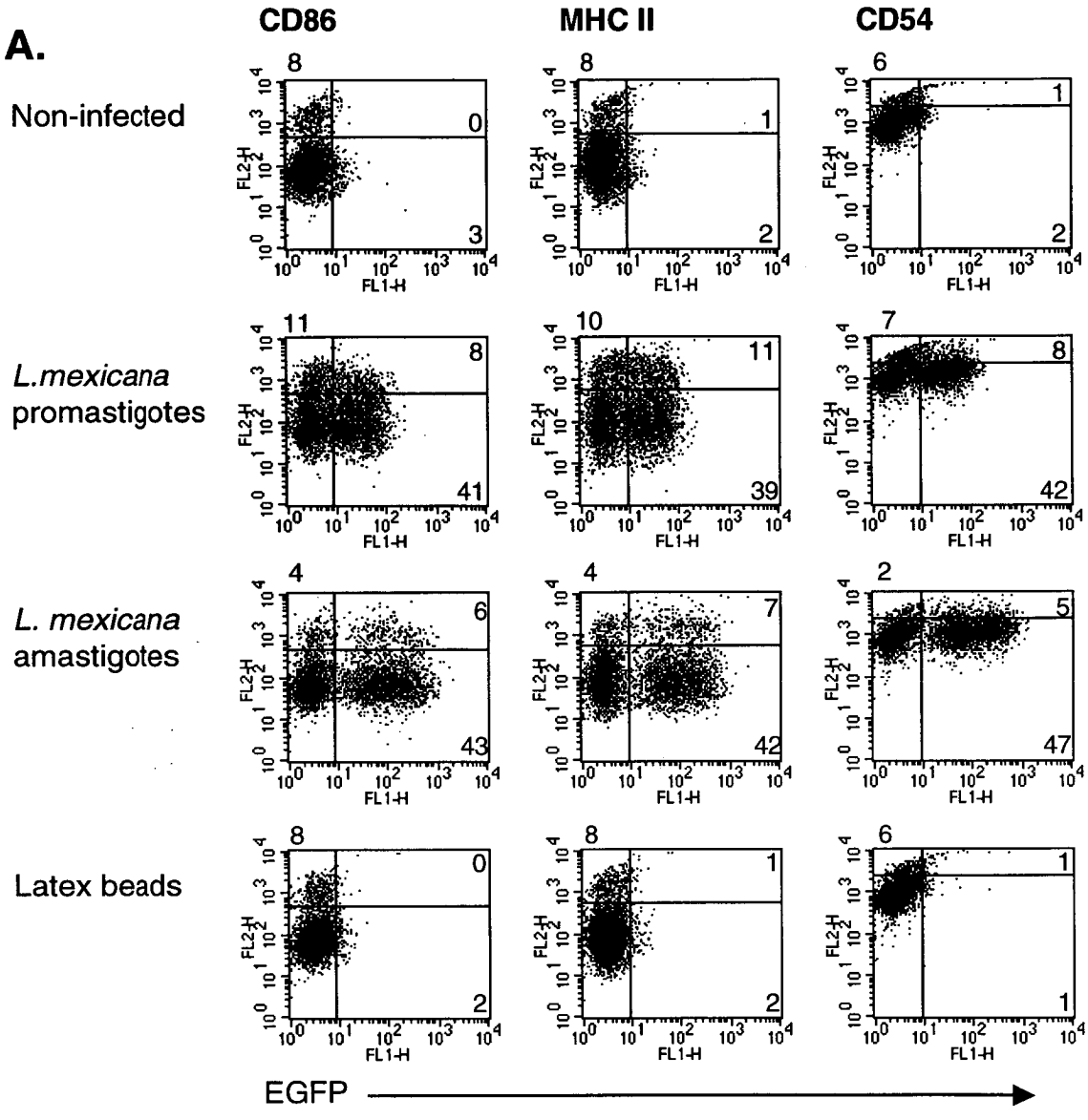
Incubation of D1 cells with stationary phase *L. mexicana* promastigotes induced activation of 33.1±5.4% of the cells (Figure 5.2), which was statistically different from the non-infected culture ($p<0.01$, based on percentages of activated $CD86^+$ cells from

Table 5.1:
Complete FACS Data Showing Infection Levels of D1 DC Incubated with *L. mexicana* Promastigotes or Amastigotes.

	Percentage of $EGFP^+$ cells	
	Promastigotes	Amastigotes
Expt. 1	49.1	43.1
Expt. 2	50.1	46.1
Expt. 3	60.0	47.9
Expt. 4	42.6	54.0
Expt. 5	38.0	36.1
Expt. 6	37.0	
Mean	48.0	45.4
s.e.m.	3.7	2.9

Numbers indicate the percentage of EGFP positive cells after incubation of D1 DC with EGFP-expressing *L. mexicana* promastigotes or amastigotes for 18 hours.

Figure 5.2: Infection of D1 Cells with *L. mexicana* EGFP- expressing Promastigotes and Amastigotes.



D1 cells were incubated for 18 hours with EGFP-expressing *L. mexicana* stationary phase promastigotes, axenic amastigotes or 3.0μm latex beads. Cells were harvested, fixed, stained for surface activation markers and analysed by flow cytometry.

Numbers represent the percentage of cells in the respective quadrants. FACS plots are representative of 5 different experiments.

five different experiments; statistical analyses on MHC II levels are shown in Table 5.2). Unlike BM-DC, the activated population of D1 cells comprised both infected and non-infected DC, and there was no difference between the $EGFP^+$ and $EGFP^-$ cells in the cultures ($CD86^+$ cells, upper left quadrant versus upper right $p=0.79$). Interestingly, the percentage of $CD86^+$ and MHC II^+ cells were very similar in these experiments (see Table 5.2), indicating that the differential regulation of these markers by low levels of activating stimuli did not occur in the D1 cells. Opsonisation of promastigotes with mouse complement enhanced neither uptake nor activation of DC (data not shown). The data from five independent experiments is presented in Table 5.2.

Figure 5.2 shows that, as with BM-DC, uptake of *L. mexicana* amastigotes did not induce activation of D1 cells, and that this resembled uptake of latex beads: there was no significant difference between the percentage of $CD86^{hi}$ cells in non-infected D1 cultures compared to cells which had been incubated with *L. mexicana* amastigotes ($p=0.21$), or latex beads ($p=0.92$); statistical analyses were performed on percentages of cells in the upper quadrants of plots from five different experiments (see Table 5.2). Moreover, as with the BM-DC cultures, there was no difference between $EGFP^+$ and $EGFP^-$ cells in amastigote-exposed cultures ($p=0.86$).

Analysis of cytokine production by infected D1 cells supported the flow cytometric data (Figure 5.3A): infection with *L. mexicana* promastigotes, but not amastigotes or latex beads, induced secretion of statistically significant levels of TNFα, but no IL-6 or IL-1β. The D1 cultures did not produce IL-12p70, which was expected since CD40 was not cross-linked in these experiments (Winzler *et al.* 1997). IL-10 has been shown to block up-regulation of co-stimulatory molecules on DC, and inhibit production of IL-12 (Steinbrink *et al.* 1997). Therefore, supernatants from parasite-infected cultures were also tested for IL-10 to investigate whether amastigote-induced IL-10 could be preventing the activation of infected DC. Both immature and LPS-matured D1 cultures produced IL-10 in these experiments (Figure 5.3B). No IL-10 was detected in promastigote-infected cultures, while low levels, below those seen in the immature non-infected cultures, were detected in amastigote-infected cultures, and in cultures

Numbers indicate the percentage of cells in the upper left (UL) and upper right (UR) quadrants for FACS plots of immature D1 DC, or D1 DC incubated with *L. mexicana* amastigotes, promastigotes, or 3.0μm latex beads, for 18 hours and stained with anti-CD86 or anti-MHC II antibodies. For amastigote- and promastigote-infected populations the values in the upper left quadrant represent the activated EGFP negative cells and the values in the upper right quadrant represent the activated EGFP positive cells.

CD54 data is not shown because expression of this marker was high in immature cells and CD86 and MHC II were thought to provide a more sensitive read-out of activation in this system.

CD86: Immature D1 cells versus promastigotes $p<0.01$; immature versus amastigotes $p=0.21$; immature versus beads $p=0.16$; amastigotes versus beads $p=0.92$; promastigotes $EGFP^-$ versus $EGFP^+$ $p=0.79$; amastigotes $EGFP^-$ versus $EGFP^+$ $p=0.86$.

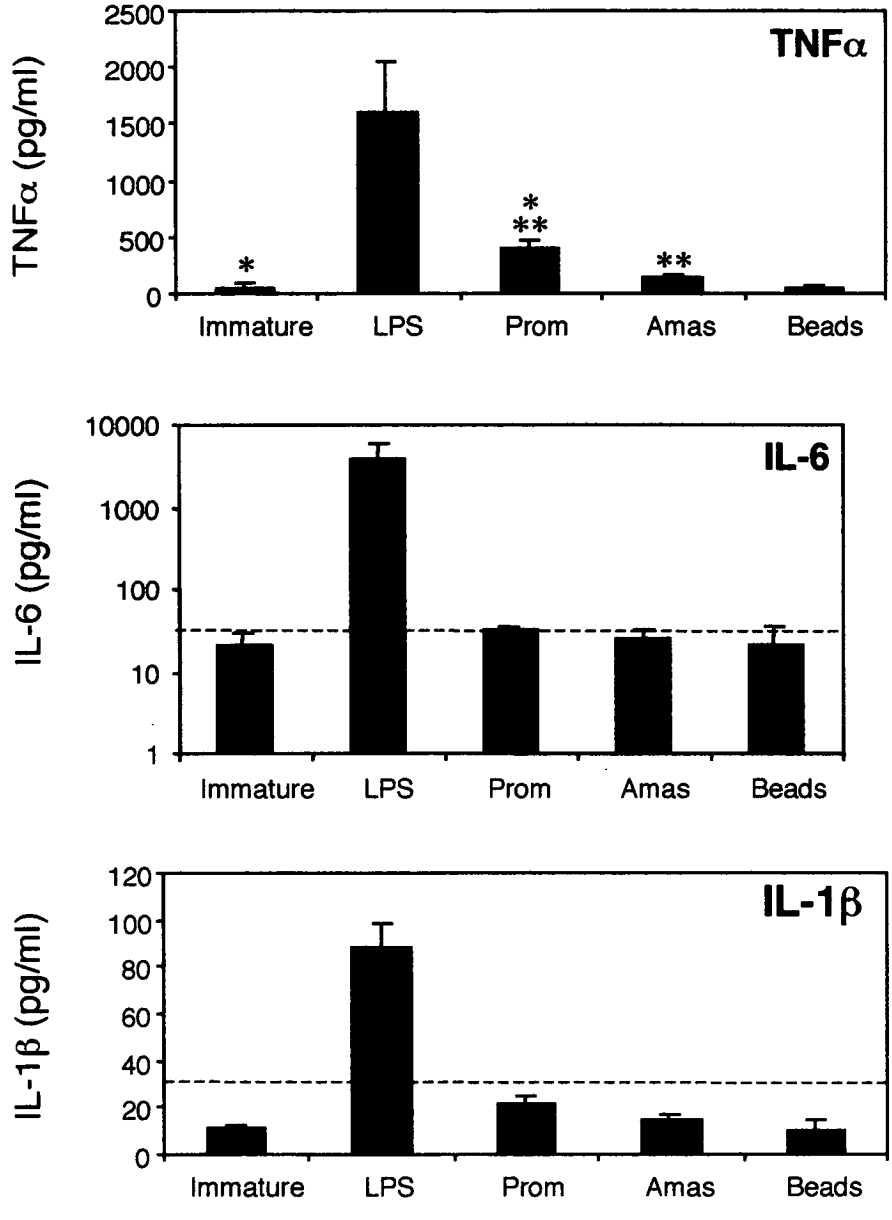
MHC II: Immature D1 cells versus promastigotes $p<0.01$; immature versus amastigotes $p=0.94$; immature versus beads $p=0.74$; amastigotes versus beads $p=0.70$; promastigotes $EGFP^-$ versus $EGFP^+$ $p=0.80$; amastigotes $EGFP^-$ versus $EGFP^+$ $p=0.64$

Table 5.2:
Complete FACS Data Showing Levels of Surface Activation Markers on D1 DC Incubated with *L. mexicana* Parasites or Latex Beads.

CD86							
	Immature		Amastigotes		Promastigotes		Beads
	UL	UR	UL	UR	UL	UR	UL
Expt.1	3.6	0.2	4.0	3.5	21.2	16.6	8.6
Expt.2	5.9	0.0	3.2	4.9	26.7	22.3	9.9
Expt.3	7.8	0.4	3.7	5.5	10.6	8.0	7.7
Expt.4	8.2	0.4	6.9	9.0	12.8	23.5	19.8
Expt.5	12.9	0.8	13.9	11	14.4	9.3	17.5
Mean	7.7	0.34	6.3	6.8	17.4	15.9	12.7
s.e.m.	1.5	0.1	2.0	1.4	3.0	3.2	2.5

MHC II							
	Immature		Amastigotes		Promastigotes		Beads
	UL	UR	UL	UR	UL	UR	UL
Expt.1	17.7	0.8	3.0	1.8	21.4	17.9	9.6
Expt.2	5.6	0.0	3.7	5.6	23.5	22.0	10.4
Expt.3	8.0	0.8	4.0	7.2	10.2	11.4	8.3
Expt.4	14.1	0.4	7.9	10.3	11.5	23.8	17.9
Expt.5	16.5	1.0	10.6	9.3	11.3	8.2	25.8
Mean	12.4	0.6	5.8	6.8	15.6	16.7	14.4
s.e.m.	2.4	0.2	1.5	1.5	2.8	3.0	3.3

Figure 5.3A: Production of Pro-inflammatory Cytokines by D1 Cells

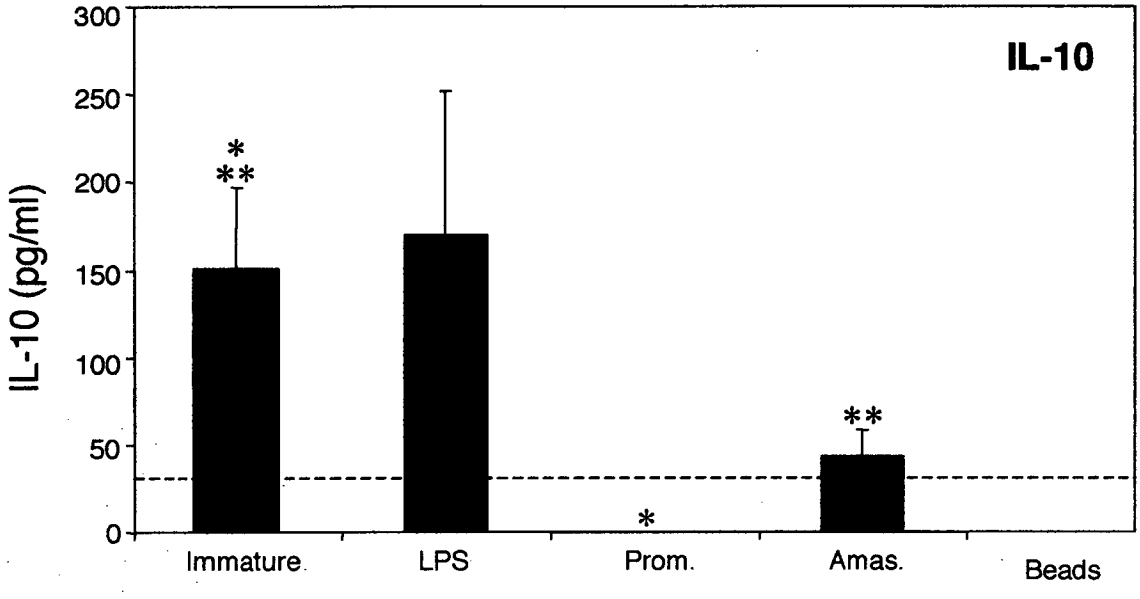


D1 cells were incubated for 18 hours with 10μg/ml LPS, promastigotes, amastigotes or latex beads. Supernatants were assayed by ELISA for the presence of TNFα, IL-6 and IL-1β (n=3). The sensitivity limit for these assays is 30 pg/ml (dashed line). Values represent concentrations of cytokine in supernatants from ~1X10^5 cells (mean ± s.e.m.).

TNFα * Immature cells vs. promastigotes: $p < 0.01$

** Promastigotes vs. amastigotes: $p = 0.02$

Figure 5.3B: IL-10 Secretion is not Induced by Infection of D1 Cells with *L. mexicana* Promastigotes or Amastigotes



D1 cells were incubated for 18 hours with 10μg/ml LPS, promastigotes,amastigotes or 3.0μm latex beads. Supernatants were assayed by ELISA for the presence of IL-10 (n=4). The sensitivity limit for these assays is 30 pg/ml (dashed line). Values represent concentrations of cytokine in supernatants from ~1X10^5 cells (mean ± s.e.m.).

* Immature cells vs promastigotes: p =0.03; ** immature cells vs amastigotes: p=0.07; *** amastigotes vs beads: p=0.12.

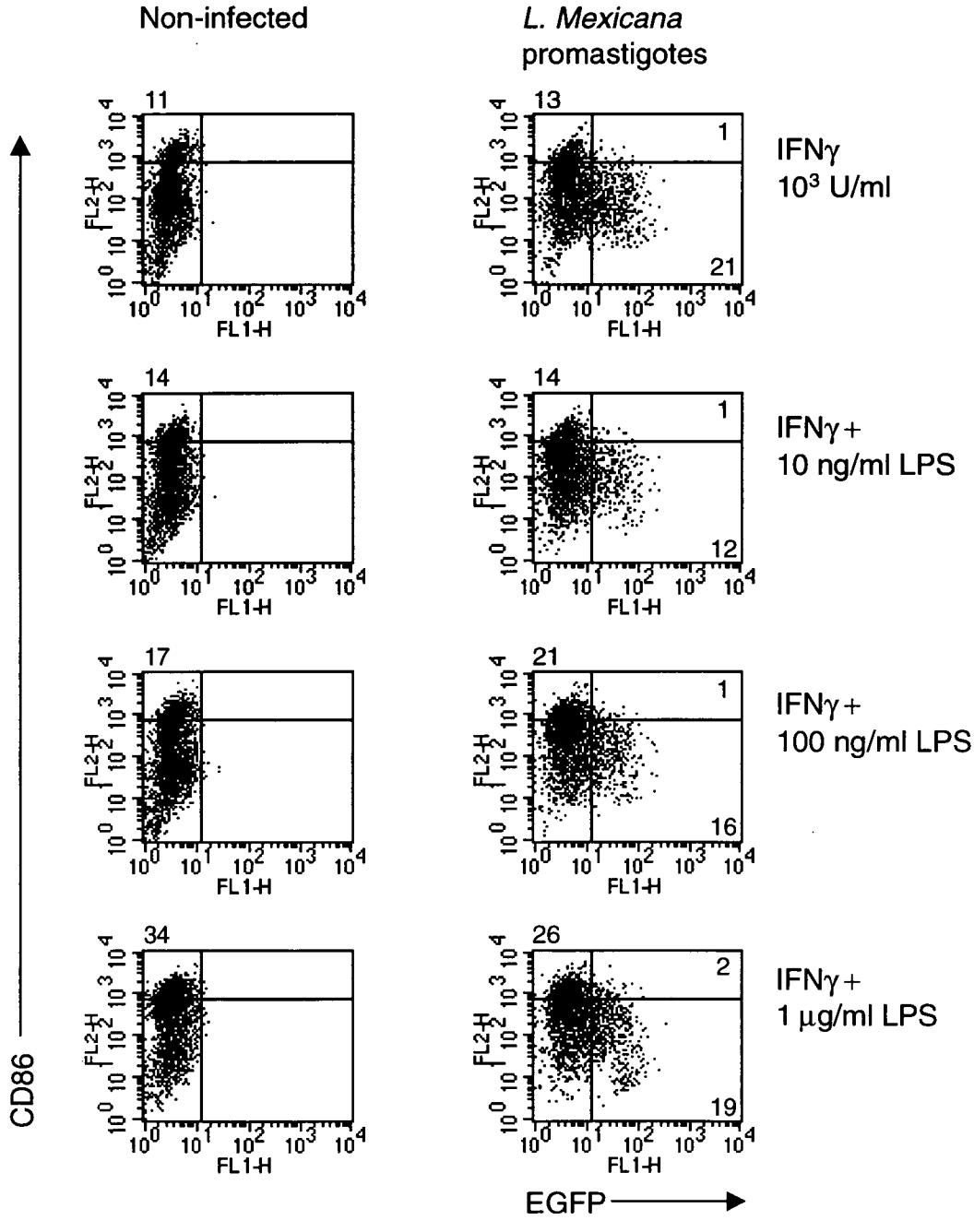
incubated with 3.0μm latex beads. Lack of IL-10 in the amastigotes-infected culture was not statistically significant compared to non-infected cultures ($p=0.07$) (see Figure 5.3B), due to the large amount of variability in the assay, however, cultures containing amastigotes did not produce significantly less IL-10 than cultures containing latex beads ($p=0.12$) indicating uptake of inert particles represses production of IL-10 by D1 cells. Therefore, these data suggest that the lack of activation of *L. mexicana* amastigote-infected D1 cells is not due to inhibition by IL-10.

5.5.2 Partially Activated BM-DC do not Become More Sensitive to Activation by Promastigotes

The enhanced activation of D1 cells compared to BM-DC on incubation with *L. mexicana* promastigotes might be explained if the D1 cells were partially activated in culture prior to infection with promastigotes as this might render them more sensitive to activating stimuli. A difference in basal activation state between D1 cells and BM-DC was suggested by the high levels of CD54 expressed by D1 cells versus BM-DC. Therefore, the effect of providing low levels of activating stimuli to BM-DC prior to infection was investigated in these experiments: BM-DC were "tickled" with sub-optimal levels of IFNγ and LPS for two hours before infection with *L. mexicana* promastigotes. Figures 5.4A and B demonstrate that infection of sub-optimally activated BM-DC did not result in increased expression of surface CD86 or MHC II compared to non-infected cells. These data therefore suggest that the difference between infection of D1 cells and BM-DC does not reflect prior conditioning of the D1 cells to a more responsive state.

The data presented in Figure 5.4A support the data from Chapter 4 (section 4.5) showing that activated BM-DC do not contain $EGFP^+$ parasites. These data suggest either that *L. mexicana* promastigotes suppress activation of DC, or that the parasites are rapidly degraded upon activation of the cells.

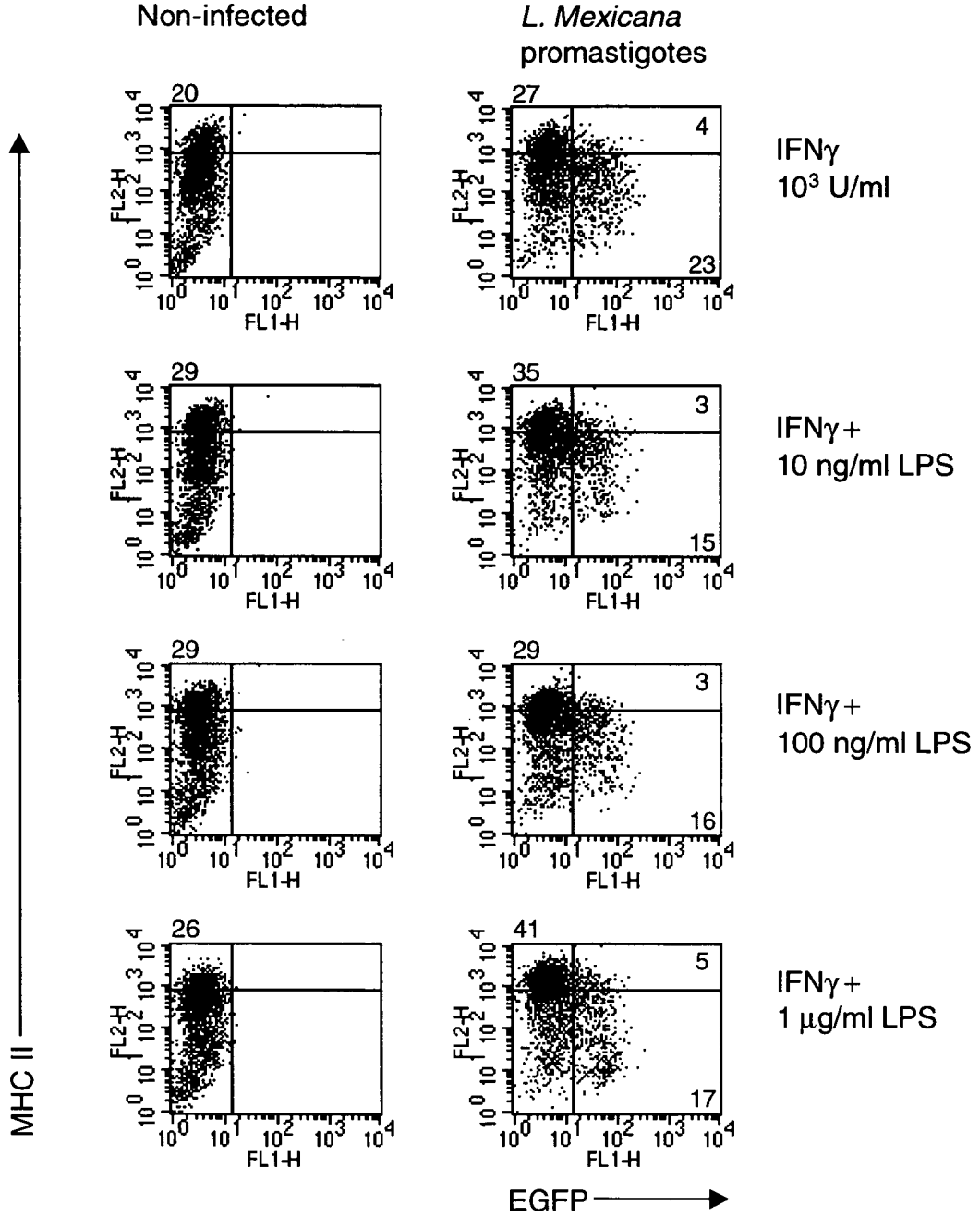
Figure 5.4A: Infection of Sub-Optimally Activated BM-DC with *L. mexicana* Promastigotes



Day 7 BM-DC were incubated for 2 hours with activating stimuli of different concentrations before infection with stationary phase *L. mexicana* promastigotes for 18 hours. The cells were then harvested, stained for surface expression of CD86 and analysed by flow cytometry.

Numbers indicate the percentage of activated cells in the labelled quadrants. Quadrants were set on immature, non-activated DC. The dot plots are representative of the results from two different experiments.

Figure 5.4B: Infection of Sub-Optimally Activated BM-DC with *L. mexicana* Promastigotes



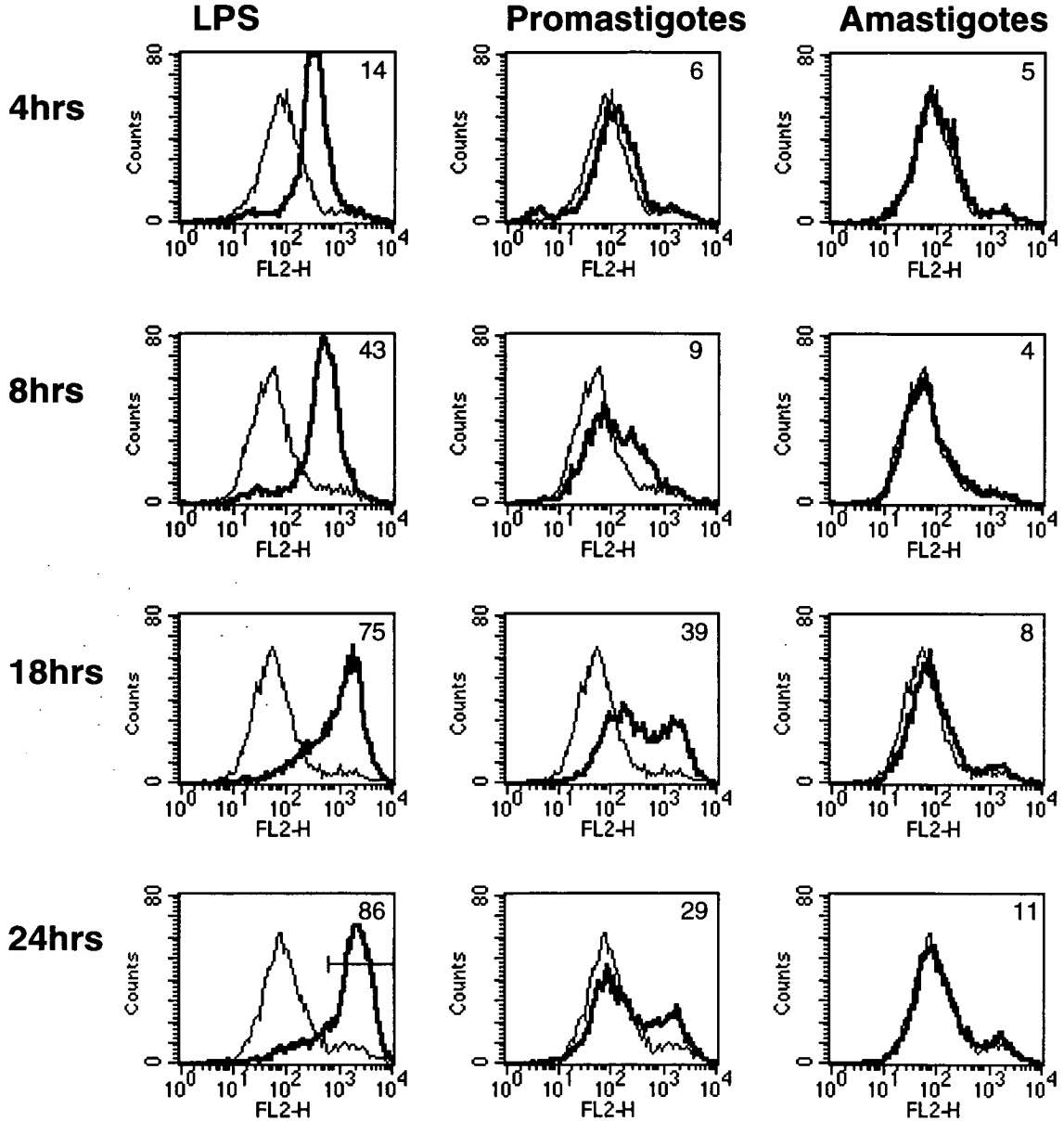
Day 7 BM-DC were incubated for 2 hours with activating stimuli of different concentrations before infection with stationary phase *L. mexicana* promastigotes for 18 hours. The cells were then harvested, stained for surface expression of MHC II and analysed by flow cytometry.

Numbers indicate the percentage of activated cells in the labelled quadrants. Quadrants were set on immature, non-activated cells. The dot plots are representative of the results from two different experiments.

5.3 Kinetic Characterisation of the Effect of *L. mexicana* Infection on D1 Cells.

In order to assess whether the data obtained from 18 hour incubation of D1 cells with *L. mexicana* parasites was representative of the effect of the parasites at earlier or later time points, as had been suggested by preliminary experiments on BM-DC prior to the work presented in Chapter 4, a kinetic analysis was performed to analyse the levels of surface activation markers on D1 cells incubated with *L. mexicana* promastigotes and amastigotes for different times. D1 cells were incubated with EGFP-expressing stationary phase *L. mexicana* promastigotes or amastigotes for 4, 8, 18 and 24 hours, then washed, fixed and stained. Figures 5.5A, B and C show surface levels of CD86, MHC II and CD54 expression respectively throughout the time course, (histograms are representative of three different experiments). CD86 and MHC II levels indicate that activated cells first become apparent 8 hours after infection with promastigotes, and a distinct population is seen by 18 hours. This is less obvious with the cells stained for CD54, as D1 cells consistently express very high levels of CD54. Maximal activation by promastigotes is seen by 18 hours post-infection, but this never reaches levels induced by LPS, and is not sufficient to activate the whole population. In contrast, *L. mexicana* amastigote-infected cultures always resemble the non-infected controls. At 18 hours post-infection MHC II levels on the amastigote-infected culture appeared below levels on the non-infected cells in the experiment shown, however this trend was not repeated in later experiments (see Appendix B). Therefore, these data confirmed that *L. mexicana* amastigotes did not activate D1 cells, and that maximal activation on incubation with promastigotes appears to be reached by 18 hours. Based on this time course, D1 cells were infected, harvested at different times and the RNA extracted for a kinetic analysis of the effect of infection with *L. mexicana* parasites on global gene transcription in D1 cells (see Appendix B).

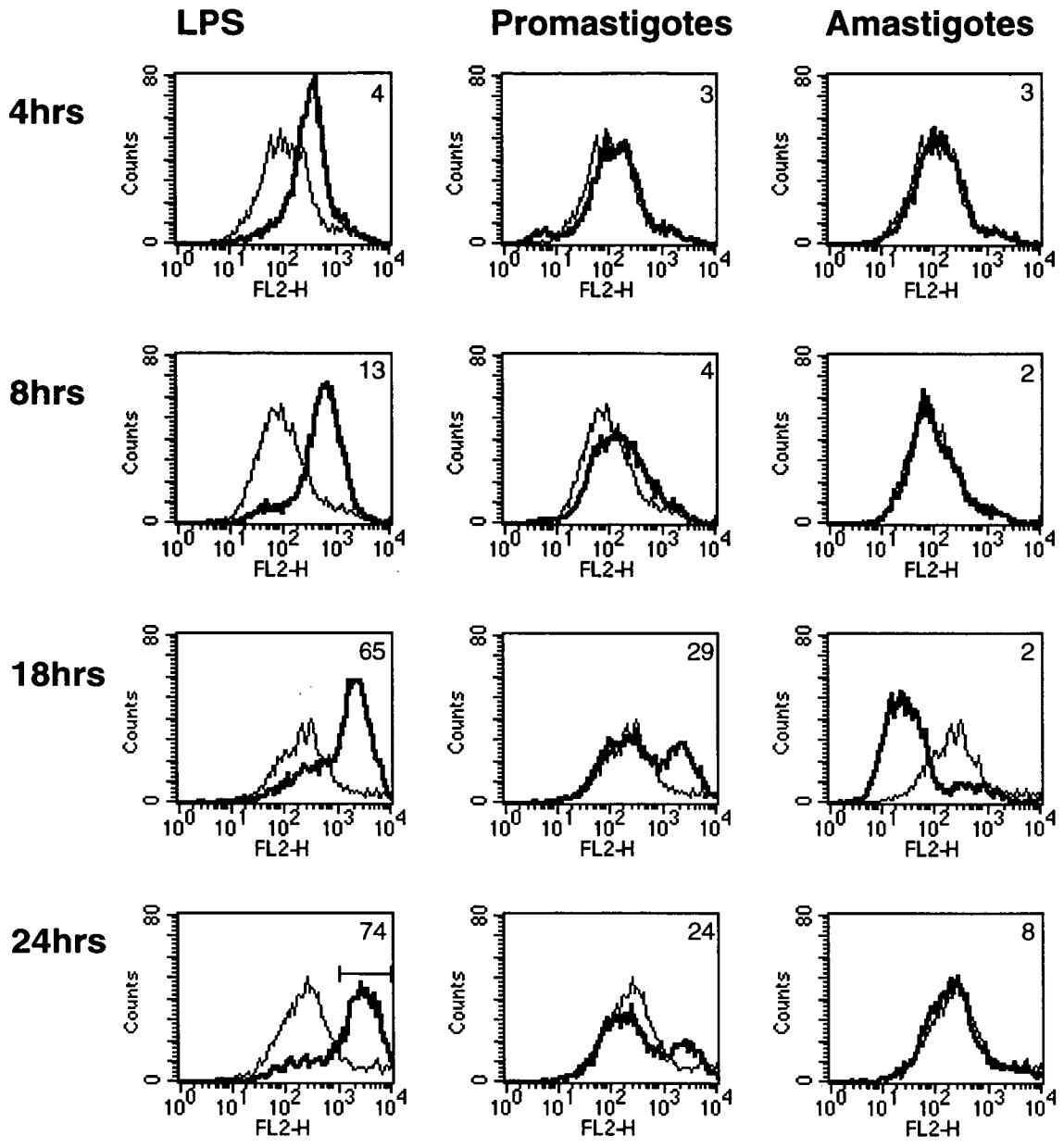
Figure 5.5A: Kinetic analysis of CD86 Expression on *L. mexicana* Promastigote and Amastigote-infected D1 Cells



D1 cells were infected with *L. mexicana* promastigotes, amastigotes or activated with 10μg/ml LPS, for the times indicated. Cells were then harvested, fixed and stained for the surface activation marker CD86. Histograms show non-infected D1 cells (thin lines) and cells incubated with LPS or *Leishmania* parasites (thick lines).

These results are representative of 2 separate experiments. Numbers represent the percentage of activated cells based on the marker set on LPS-activated cells at 24 hours.

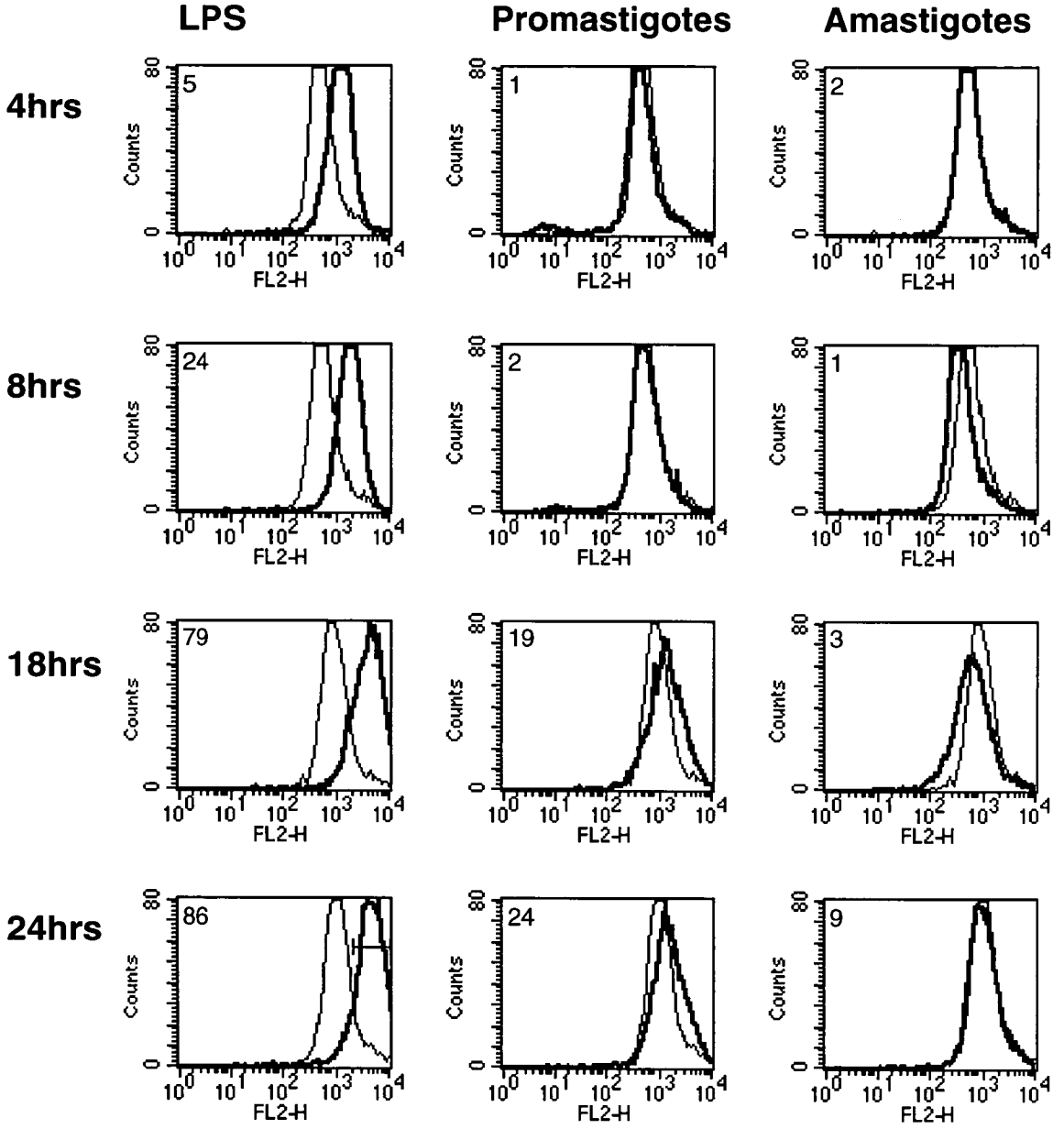
Figure 5.5B: Kinetic Analysis of MHC II Expression on *L. mexicana* Promastigote and Amastigote-infected D1 Cells



D1 cells were infected with *L. mexicana* promastigotes, amastigotes or activated with 10μg/ml LPS, for the times indicated. Cells were then harvested, fixed and stained for surface MHC II. Histograms show non-infected D1 cells (thin lines) and cells incubated with LPS or *Leishmania* parasites (thick lines).

These results are representative of 2 separate experiments. Numbers represent the percentage of activated cells based on the marker set on LPS-activated cells at 24 hours.

Figure 5.5C: Kinetic analysis of CD54 expression on *L. mexicana* Promastigote and Amastigote-infected D1 Cells



D1 cells were infected with *L. mexicana* promastigotes and amastigotes, or activated with 10μg/ml LPS, for the times indicated. Cells were then harvested, fixed, stained for the surface activation marker CD54 and analysed by flow cytometry.

Histograms show non-infected D1 cells (thin lines) and cells incubated with LPS or *Leishmania* parasites (thick lines).

These results are representative of 2 separate experiments. Numbers represent the percentage of activated cells based on the marker set on LPS-activated cells at 24 hours.

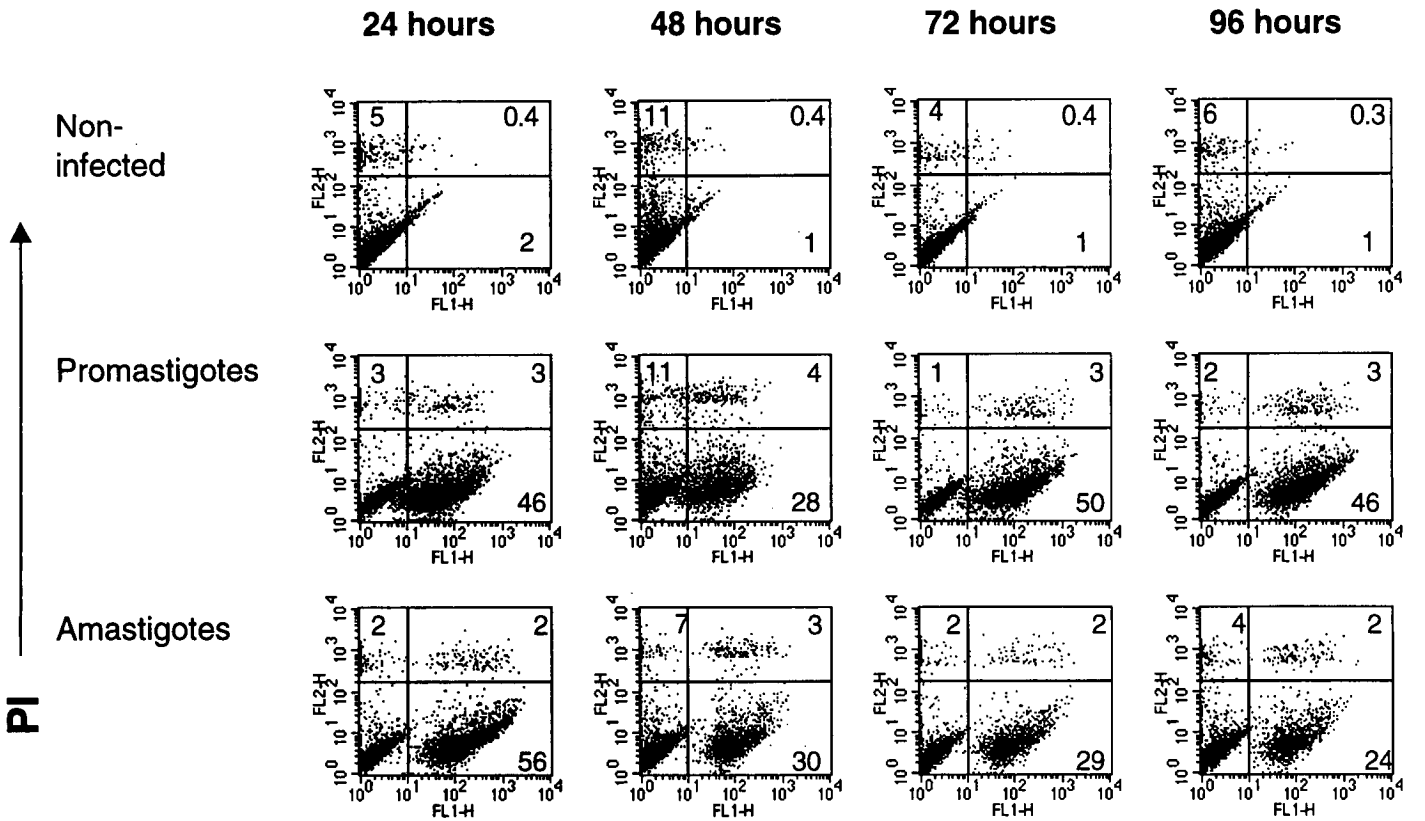
5.4 Characterisation of long-term infection of D1 cells with *L. mexicana*

Infection of MΦ by *L. mexicana* is characterised by the formation of a large phagolysosome known as the PV, which is clearly visible 48 hours after infection. Fusion of *Leishmania*-containing vesicles with other MΦ endocytic compartments begins to occur within the first 12 hours of infection, while the parasites are still at the promastigote stage (Courret *et al.* 2001). Upon transformation, *L. mexicana* amastigotes secrete large amounts of a proteophosphoglycan (aPPG), which induces expansion of the compartment to a large communal PV (Peters *et al.* 1997). Long-term culture of D1 cells was used to investigate whether DC could also support chronic infection with *Leishmania* parasites, characterised by formation of a communal PV.

5.4.1 Effect of Long-term Infection on the Viability of D1 Cells

D1 cells were infected with EGFP-expressing *L. mexicana* promastigotes or amastigotes and cultured at 34°C for up to 96 hours. Cells were harvested at the time points indicated and stained with propidium iodide (PI) to assess viability. Figure 5.6 shows that throughout the 96 hour period of the experiment, there were few PI positive cells in either the infected or non-infected populations, and that the number of $EGFP^+$ PI^+ cells never exceeded 4% of the population. The percentage of promastigote-infected cells stayed fairly constant throughout the experiment, whereas the percentage of amastigote-infected cells decreased to approximately half the value at 24 hours. This observation may be due to killing of intracellular parasites by D1 cells, or may be the result of inter-sample variation in the experiment. The presence of an EGFP positive population at 96 hours post-infection with promastigotes or amastigotes supports that fact that *Leishmania* remain viable within DC, since EGFP is rapidly lost upon parasite death (T. Aebischer personal communication). Therefore these data show that DC are permissive for long-term infection with *L. mexicana* parasites, and that both the DC and the parasites remain viable.

Figure 5.6: D1 Cells Support Long-term Infection with *L. mexicana* Parasites



D1 cells were incubated with EGFP-expressing *L. mexicana* promastigotes or amastigotes at 34ºC for the times indicated. Cells were then harvested and stained with propidium iodide (PI) to identify dead cells by flow cytometry.

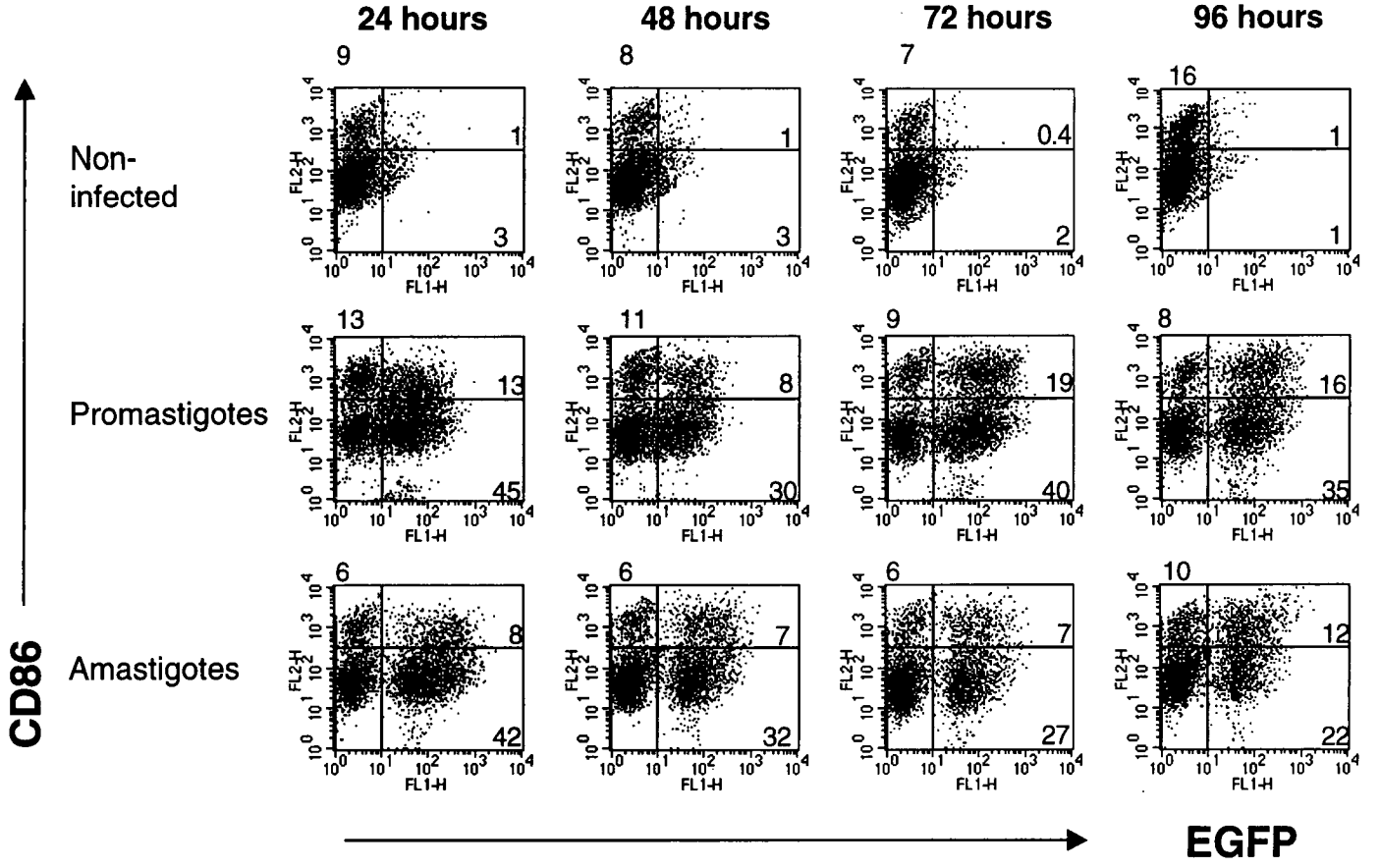
Numbers represent the percentage of cells in the respective quadrants. These results are from a single experiment.

Infected cultures were also stained for surface CD86 to follow the activation status of infected DC over the time course. Figure 5.7 shows that there is some spontaneous maturation in all three samples by 96 hours, but amastigote-infected cells always have surface levels of CD86 that are more similar to the non-infected cells than the promastigote-infected cells. Maximal activation by promastigotes occurs within the first 24 hours and numerically, based on percentages in the upper left and right quadrants, does not increase beyond this. It is interesting to note, however, that at 24 and 48 hours, the cells expressing the highest levels of CD86 are $EGFP^-$ but that as the experiment progresses the $EGFP^+$ cells express increasingly higher levels of CD86. This implies a slight retardation of activation of cells containing intact promastigotes. This experiment must be repeated to ascertain whether this observation is significant, but the trend was consistent in cells stained for MHC II during the same experiment (data not shown).

5.4.2 Characterisation of PV Formation in Infected D1 Cells

In order to investigate whether communal PV formed in infected DC, the D1 cells were infected for 72 hours at 34°C, harvested and fixed onto poly-L-lysine-coated coverslips for examination by confocal microscopy (coverslips were coated with 0.5mg/ml poly-L-lysine (Sigma), washed with H_20 and air dried). Data were kindly collected by A. Misslitz and T. Aebischer, Berlin. At 72 hours infection is well established in MΦ and large PV containing multiple parasites are clearly visible (Alexander and Russell 1992; Antoine *et al.* 1998) (see also Figure 5.8). Figure 5.9 shows D1 cells infected for 24 or 72 hours with EGFP-expressing promastigotes. Infected DC were not easily identified by phase contrast microscopy alone, however fluorescence microscopy revealed that $EGFP^+$ parasites were contained within the cells. Communal PV were virtually never seen in promastigote-infected DC: 77 out of 186 cells examined contained promastigotes; in only 10 of these the parasites appeared to be contained within individual vacuoles while parasites in the remaining infected cells were not clearly bound by a membrane; in one cell three parasites appeared to be contained within a single vacuole (see Figure 5.9, arrow). Figure 5.10 illustrates that amastigote-infected

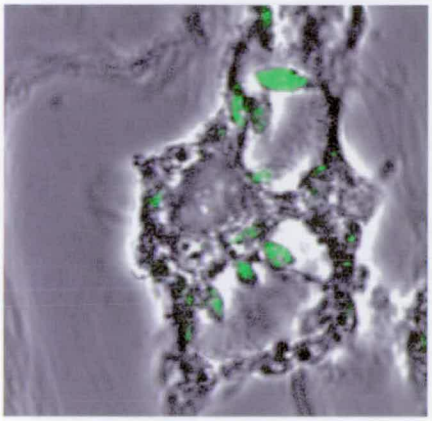
Figure 5.7: Long-term Infection with *L. mexicana* does not Increase Activation of D1 Cells



D1 cells were incubated with EGFP-expressing *L. mexicana* promastigotes or amastigotes at 34ºC for the times indicated. Cells were then harvested, stained with the activation marker CD86 and analysed by flow cytometry.

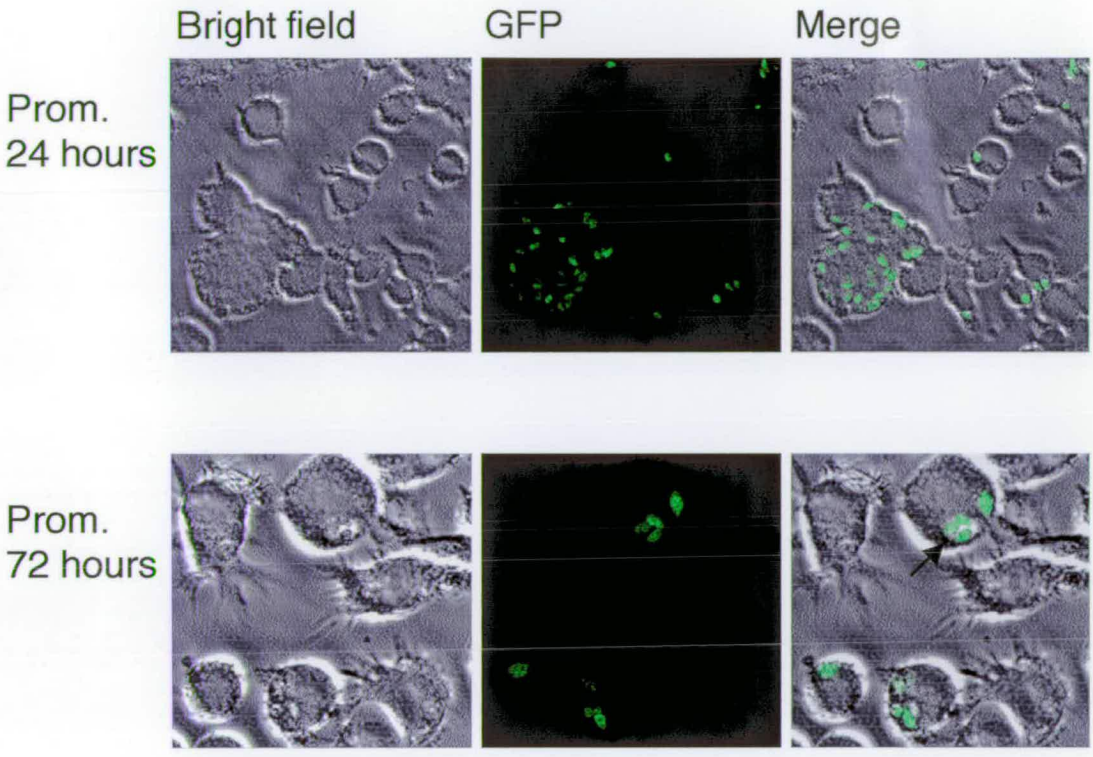
Numbers represent the percentage of cells in the respective quadrants.

Figure 5.8: PV Formation in *L. mexicana*-Infected MΦ



Confocal image showing PV development in a bone marrow-derived MΦ that has been infected with EGFP-expressing *L. mexicana* promastigotes for 72 hours *in vitro*. Kindly provided by A. Misslitz and T. Aebischer, Berlin.

Figure 5.9: Long-term Infection of D1 Cells by *L. mexicana* Promastigotes does not Induce Formation of Large PV.

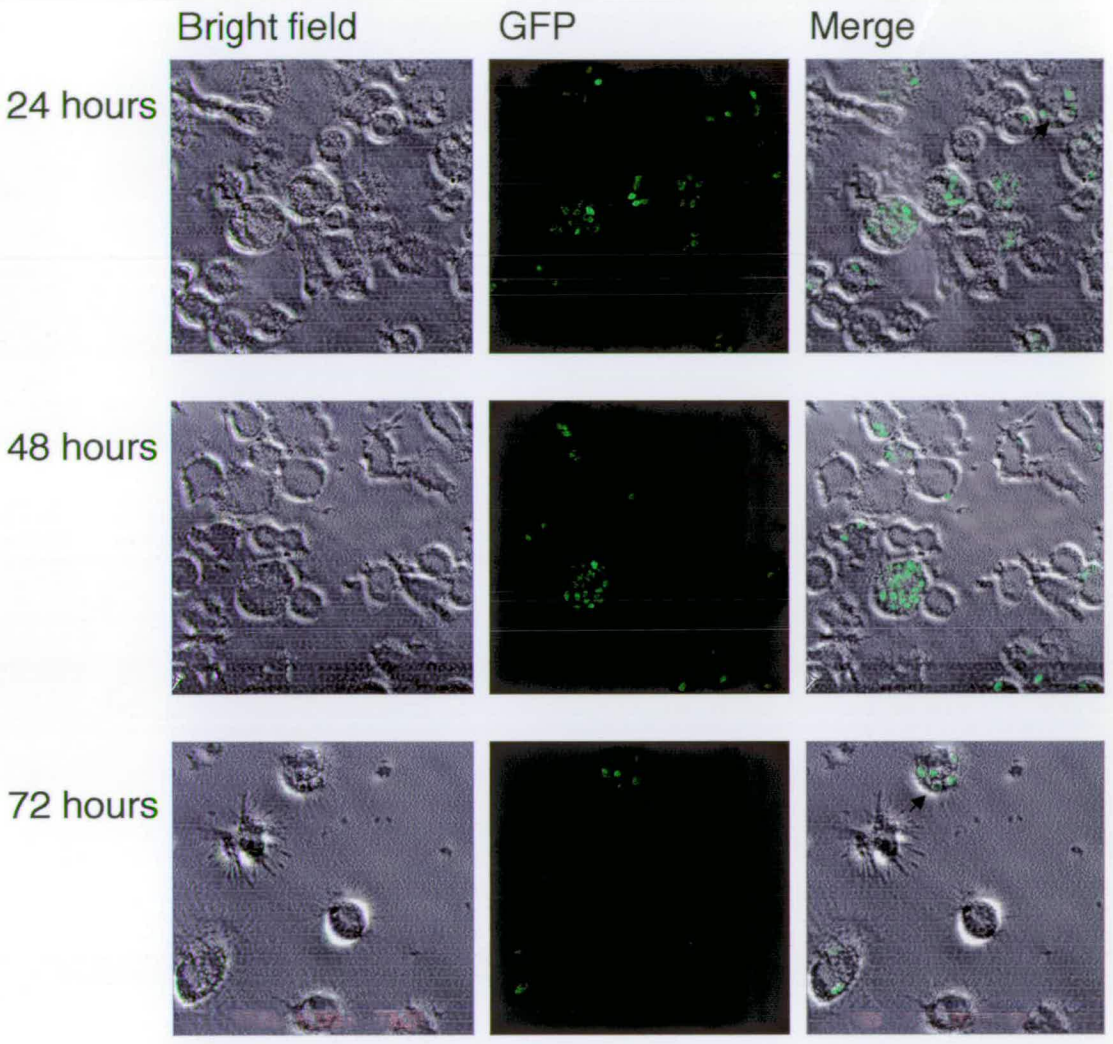


Cells examined	No. of cells containing prom.	Promastigote-infected cells		
		No apparent vacuole	Single vacuole	Communal vacuole
186	77	66	10	1

D1 cells were incubated with EGFP-expressing *L. mexicana* promastigotes at 34°C for different times. Cells were then fixed onto coverslips and analysed by confocal microscopy. Images are individual sections through cells and are representative of all images captured. Images were captured using a X63 objective.

The arrow points to a potential vacuole containing three parasites.

Figure 5.10: Long-term Infection of D1 Cells by *L. mexicana* Amastigotes does not Induce the Formation of Communal PV *in vitro*



Cells examined	No. of cells containing amas.	Amastigote-infected cells		
		No apparent vacuole	Single vacuole	Communal vacuole
157	59	53	6	0

D1 cells were incubated with EGFP-expressing *L. mexicana* amastigotes at 34°C for different times. Cells were fixed onto coverslips and analysed by confocal microscopy. Images are individual sections through cells and are representative of all images captured. Images were captured using a X63 objective.

Arrows mark parasites contained in vacuoles.

D1 cells also did not develop the large PV seen in MΦ: of the 157 cells screened from amastigote-infected cultures, 79 were infected and of these only 6 of the cells contained parasites that appeared to be within small individual vacuoles. Vacuoles were not seen in cells that had been incubated with amastigotes for less than 72 hours. Therefore, these data indicate that although promastigotes and amastigotes are viable within D1 cells, infection of D1 DC by *L. mexicana* parasites *in vitro* does not induce formation of the large PV characteristic of infected MΦ.

5.5 Discussion

5.5.1 *L. mexicana* Promastigotes but not Amastigotes Activate a Percentage of D1 Cells upon Infection

The data presented in this chapter indicate that uptake of *L. mexicana* amastigotes does not trigger activation of D1 cells. This supports the idea developed in Chapter 4 that amastigotes use receptors for entry into DC that do not trigger classical activation upon ligation. Incubation with amastigotes did not induce production of the anti-inflammatory cytokine, IL-10, and in fact appeared to repress it, indicating that *L. mexicana* amastigotes do not inhibit activation of DC via production of IL-10.

Uptake of intact *L. mexicana* promastigotes, as with BM-DC, only activated a proportion of the D1 cell population. These results, however, differed from the BM-DC results presented in Chapter 4 in two ways; approximately two fold more D1 cells were activated, which represented a discrete population of cells, and activated D1 cells included both $EGFP^+$ and $EGFP^-$ cells.

The apparent increased sensitivity of D1 cells to promastigote-derived activating stimuli may reflect genetic differences in the origins of D1 cells (C57BL/6) compared to the BM-DC (CBA) used in Chapter 4. However, infection of BM-DC from C57BL/6 mice with promastigotes did not result in an increase in the percentage of activated cells, indicating that this difference was not responsible for this observation (data not shown). It was hypothesised that D1 DC were partially activated in culture and that this would synergise with promastigote-derived signals. "Tickling" of BM-DC with low levels of IFNγ and LPS prior to infection with promastigotes did not, however, increase activation of these cells indicating that the differences between D1 cells and BM-DC are more fundamental than this, indeed, the observation that CD86 and MHC II surface expression levels are not differentially regulated in D1 cells, as they appeared to be under some circumstances in BM-DC, supports the notion that there may be inherent differences between these two DC cultures.

Activation of D1 cells by stimuli such as LPS or TNFα induces a synchronous shift of surface marker expression in the population (Winzler *et al.* 1997). Activated DC are induced to secrete TNFα, which will activate other cells in the culture by a positive feedback loop. Therefore, it was surprising that incubation with promastigotes only activated a percentage of D1 cells. These data indicate that *L. mexicana* promastigotes induce only low levels of TNFα secretion, suggesting that they encode only a weak activating stimulus, which does not induce sufficient levels of TNFα to activate other cells in the culture. In future work, this hypothesis could be tested by addition of equivalent amounts of TNFα to D1 cultures to that produced by promastigote-infected cultures (411±61.3pg/ml). It is likely, however, that this would induce a small, but synchronous shift in the culture and would not be the reason why promastigotes only activate a proportion of D1 cells. Therefore, activation of only a percentage of D1 cells by promastigotes is most probably due to heterogeneity in the D1 cell culture such that only a fraction of the cells are susceptible to promastigote-derived activation signals.

The observation that the fraction of D1 cells activated by *L. mexicana* promastigotes contained both EGFP positive and negative cells indicates that metabolically active *L.*

mexicana promastigotes do activate D1 cells. These data suggest that BM-DC or D1 cells are activated upon uptake of intact parasites, but while they become rapidly degraded in BM-DC, D1 cells are less able to kill intracellular parasites. This is supported by the observation that $EGFP^+$ populations were always more defined in promastigote-infected D1 cells compared to BM-DC, and by the data presented in Figure 5.4A which demonstrate that activated cells in BM-DC cultures incubated with TNFα or IFNγ are $EGFP^-$.

Kinetic characterisation of the effect of infection on D1 cells confirmed that these cells are not activated upon infection with *L. mexicana* amastigotes, since they remain immature throughout the experiment. Activation induced by *L. mexicana* promastigotes was first seen by 8 hours post-infection and this increased throughout the time course, reaching a maximum at 24 hours. This kinetic analysis of infection was extended by investigating the effects of *Leishmania* infection at the level of transcription (see Appendix B). The data from this experiment, however, awaits in depth analysis, and is only summarised in this thesis.

5.5.2 Characterisation of Long-term Infection of D1 cells with *L. mexicana*

MΦ infected with *L. mexicana* can be cultured *in vitro* for over a week, during which time a chronic infection is established. Large communal PV, in which amastigotes persist, are clearly seen by 48 hours post-infection (Courret *et al.* 2001). The data presented here demonstrate that D1 cells internalise *L. mexicana*, and that the parasites persist within the cells. However, large communal PV do not develop. Instead, D1 DC appear to contain intracellular promastigotes or amastigotes, which are retained within small individual vacuoles that do not expand during infection. This supports the work by Konecny *et al* (Konecny *et al.* 1999) who also did not see formation of classic PV in splenic DC incubated with *L. major* promastigotes for 24 hours.

An *in vivo* analysis has shown that communal PV do form in infected DC in the LN of mice infected with *L. mexicana* for one year, but that these are consistently smaller than in infected MΦ (T. Aebischer personal communication). Confocal analysis of optical sections confirmed that the discrepancy between the results obtained *in vitro* and *in vivo* was not due to the fact that MΦ are firmly adherent to tissue culture plates whereas DC are only semi-adherent and tend to be more rounded. Thus, by light microscopy the PV would appear to take up more room in the flattened MΦ than in cultured DC *in vitro*. The limited expansion of PV could be envisaged to be due to inhibition of fusion of the phagosome with host endocytic compartments or slower kinetics of PV formation in DC compared to MΦ. If the latter hypothesis was correct larger PV might be expected to form if the infected DC were cultured longer. This might therefore account for the fact that communal vacuoles were not seen after 72 hour infection *in vitro*, but were seen in mice which had been infected for one year. However, it is not possible distinguish between long-term infected DC and those which have recently taken up parasites released from neighbouring cells *in vivo*. Therefore, in order to test this hypothesis D1 cultures need to be infected for extended periods *in vitro* to ascertain whether communal PV can eventually establish in these cells. If these experiments demonstrate inhibition of PV development by DC, important consequences for the survival/growth of the parasite and also for the trafficking of parasite antigens through host MHC II processing and presentation pathways could be inferred. The experiments reported in Chapter 4 (section 4.8) however, demonstrate that infected DC are fully functional in their ability to process and present exogenous antigen to T cells. This strongly suggests that the formation of a PV in MΦ but not DC may play a critical role in the differential ability of these antigen presenting cells to efficiently process and present parasite antigens to T cells.

5.6 Concluding remarks

D1 cells provide a unique and versatile DC culture with which to dissect the interaction between DC and *Leishmania* parasites. The data presented in this chapter reinforce the observation that uptake of *L. mexicana* amastigotes *per se* does not classically activate DC. However, unlike BM-DC, some activated D1 cells containing intact *L. mexicana* promastigotes were observed. This indicates that promastigotes do encode a weak activating stimulus but that the D1 cell population is heterogeneous in its ability to respond to this signal, and that this stimulus is not strong enough to induce a synchronous activation of the entire D1 population as detected by flow cytometry. Differences between the sensitivity of D1 cells and BM-DC to this activating stimulus cannot be wholly ascribed to the activation status of the cells, or to genetic differences, indicating that more fundamental differences exist between these *in vitro* DC cultures.

Finally, characterisation of long-term infection of D1 cells supported the hypothesis that *L. mexciana* parasites do not establish a chronic infection in DC since large communal PV characteristic of infected MΦ did not form in these cells *in vitro*. This observation has important implications in the role of DC as hosts for *Leishmania* parasites, and also in the efficiency with which infected DC can process and present parasite-derived antigens for the activation of an appropriate anti-*Leishmania* $CD4^+$ T cell response.

Chapter 6:

The role of *L. mexicana* LPG as a Protozoan PAMP

6.1 Introduction

The experiments described in Chapters 4 and 5 demonstrate that around 15% or 33% of BM-DC or D1 cells respectively are activated upon incubation with *L. mexicana* promastigotes, but that *L. mexicana* amastigotes do not activate these cells. These results suggest that *L. mexicana* promastigotes encode a PAMP that induces activation of those DC bearing the relevant pattern receptor. The experiments described in this chapter aimed to identify this promastigote-derived PAMP via a "candidate molecule" approach.

6.2 Incubation of DC with *L. major* Promastigotes

Initial experiments were performed in which both BM-DC and D1 cultures were infected with *L. major* promastigotes, since the results from these experiments would indicate whether the activating molecule was conserved between different *Leishmania* species. EGFP-expressing *L. major* promastigotes (kindly provided by T. Aebischer; (Misslitz *et al.* 2000)) were used for these experiments, to facilitate visualisation of infected DC. *L. major* promastigotes were transformed from amastigotes freshly isolated from mouse lymph nodes, and passaged once *in vitro* before use to remove contaminants associated with purification. Stationary phase metacyclic promastigotes were then incubated with BM-DC and D1 cells, as described in Chapters 4 and 5.

L. major promastigotes were efficiently taken up by both BM-DC and D1 cells; 40.1±10.1% of BM-DC, and 47.6±4.2% of D1 cells were $EGFP^+$ after 18 hours of

culture with *L. major* promastigotes (percentages are averages from four different experiments (mean ±s.e.m.), see Table 6.1). Incubation of BM-DC with *L. major* promastigotes resulted in the presence of a distinct $EGFP^+$ population, thus differing from infection with *L. mexicana* promastigotes where the parasite-infected DC did not form such a clear population (Figure 6.1A compared to Figure 4.4). This observation may reflect a differential ability of *L. major* rather than *L. mexicana* promastigotes to survive in BM-DC in culture, or might be due to the fact that *L. major* promastigotes survive for longer at 37°C than *L. mexicana* promastigotes (T. Aebischer personal communication).

Uptake of *L. major* promastigotes did not activate BM-DC; expression of the surface markers CD86, MHC II and CD54 remained intermediate in both the $EGFP^-$ and $EGFP^+$ populations of BM-DC (Figure 6.1A). The activation status of *L. major*-infected BM-DC cultures reflected that of non-infected cultures or *L. mexicana* amastigote-infected cultures (see Figure 4.6), and the small percentage of activated cells characteristic of BM-DC infected with *L. mexicana* promastigotes was not seen (non-infected culture versus *L. major*-infected cultures $p=0.90$, percentages calculated from activated $CD86^+$ cells from four different experiments; for statistical analyses of MHC II levels see Table 6.2). These experiments also reinforced the observation made in Chapter 4 that surface levels of CD86 and MHC II are differentially regulated upon infection of BM-DC with *Leishmania* parasites; $EGFP^-$ cells expressed significantly higher levels of CD86 than $EGFP^+$ cells within the same sample ($p=0.04$), whereas $EGFP^-$ and $EGFP^+$ cells did not express significantly different levels of MHC II ($p=0.11$, see Table 6.2).

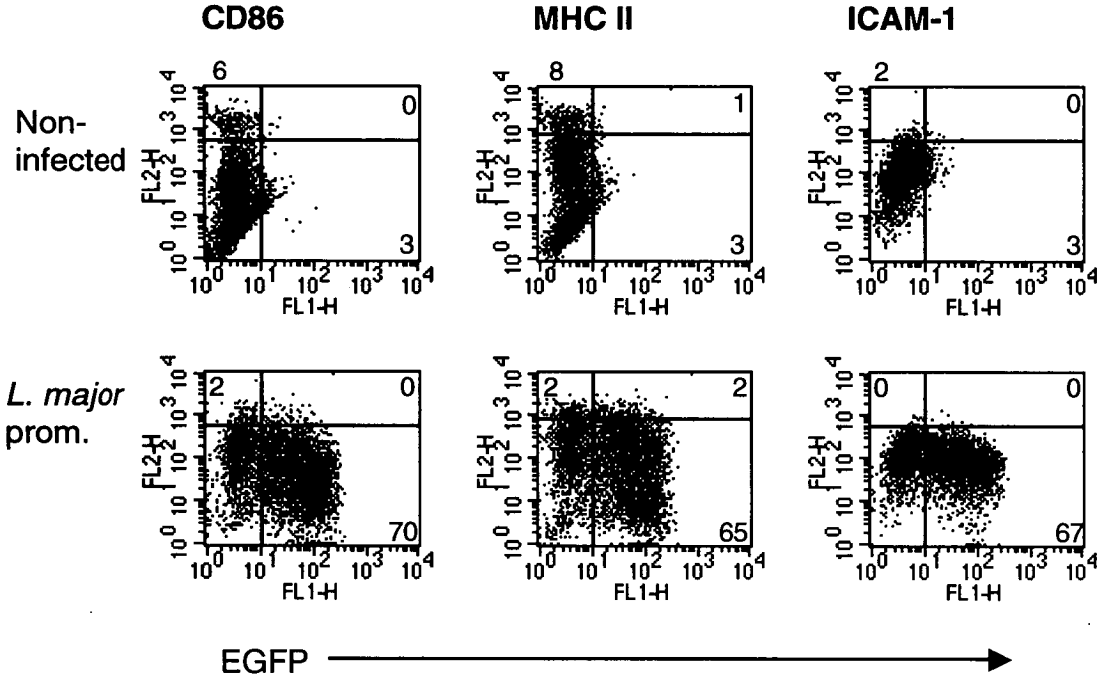
During an initial experiment up-take of *L. major* promastigotes also did not activate D1 cells (Figure 6.1B). However, although this experiment was repeated, spontaneous activation of D1 cells in control cultures made the data unusable. Despite this, D1 cells incubated with *L. major* promastigotes were not induced to secrete the cytokines TNFα, IL-6, or IL-1β (Figure 6.2), even in cultures in which the 'immature' D1 cells had spontaneously activated; supernatants from *L. major*-infected cultures contained

Table 6.1:
Complete FACS Data for Infection Levels of DC Incubated with *L. major* Promastigotes.

	Percentage of $EGFP^+$ cells	
	BM-DC	D1 cells
Expt. 1	22.2	43.2
Expt. 2	37.3	57.6
Expt. 3	69.0	51.1
Expt. 4	32.0	38.5
Mean	40.1	47.6
s.e.m.	10.1	4.2

Numbers indicate the percentage of EGFP-positive cells after incubation of day 7 BM-DC or D1 cells with EGFP-expressing *L. major* promastigotes for 18 hours.

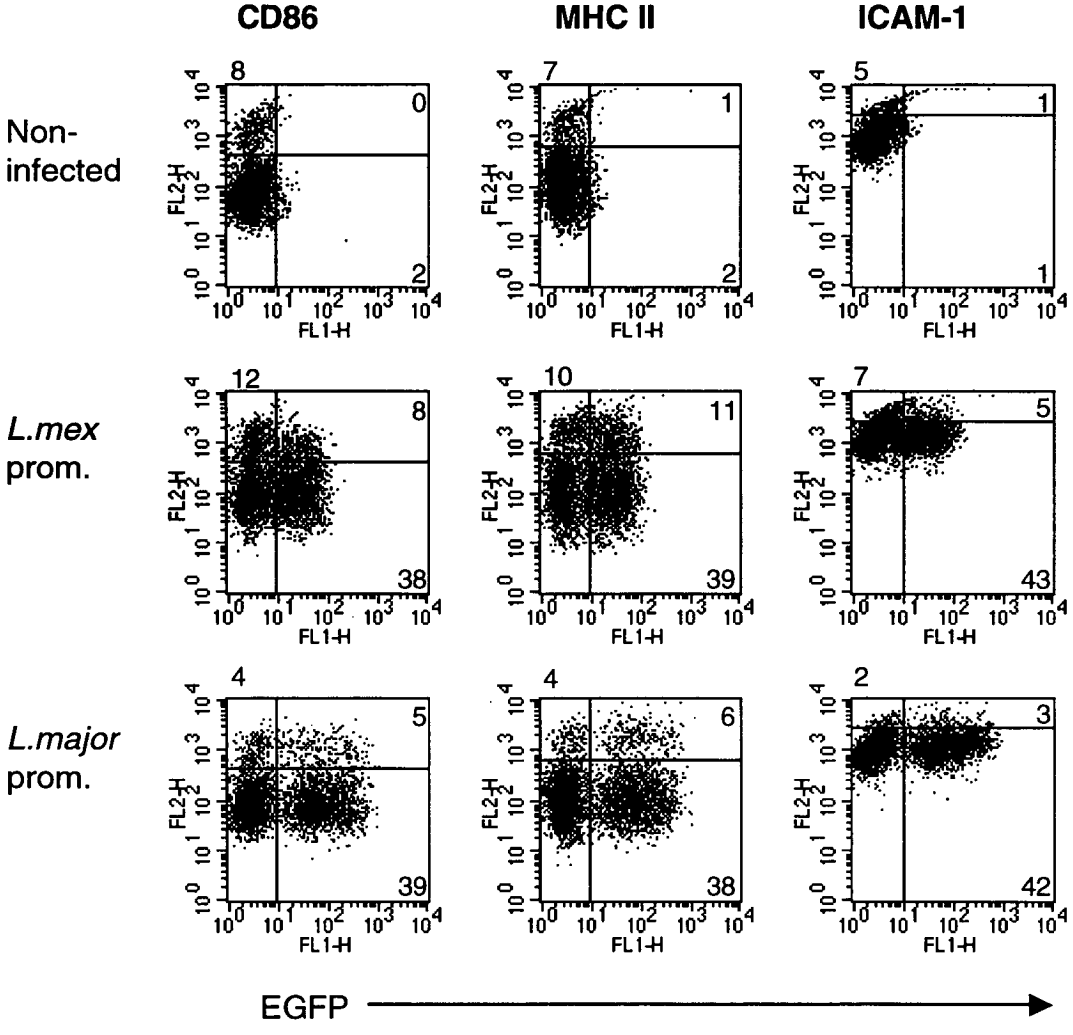
Figure 6.1A: Incubation with *L. major* Promastigotes does not Induce Activation of BM-DC



BM-DC were incubated for 18 hours with EGFP-expressing stationary phase *L. major* promastigotes. Cells were harvested, fixed, stained for surface activation markers and analysed by flow cytometry.

Numbers represent the percentage of cells in the respective quadrants. FACS plots are representative of 4 separate experiments.

Figure 6.1B: Incubation with *L. major* Promastigotes does not Activate D1 Cells



D1 cells were incubated for 18 hours with EGFP-expressing stationary phase *L. major* promastigotes. Cells were harvested, fixed, stained for surface activation markers and analysed by flow cytometry.

Numbers represent the percentage of cells in the respective quadrants. These data are representative of a single experiment.

Table 6.2:
Complete FACS Data for Infection of BM-DC with *L. major* Promastigotes.

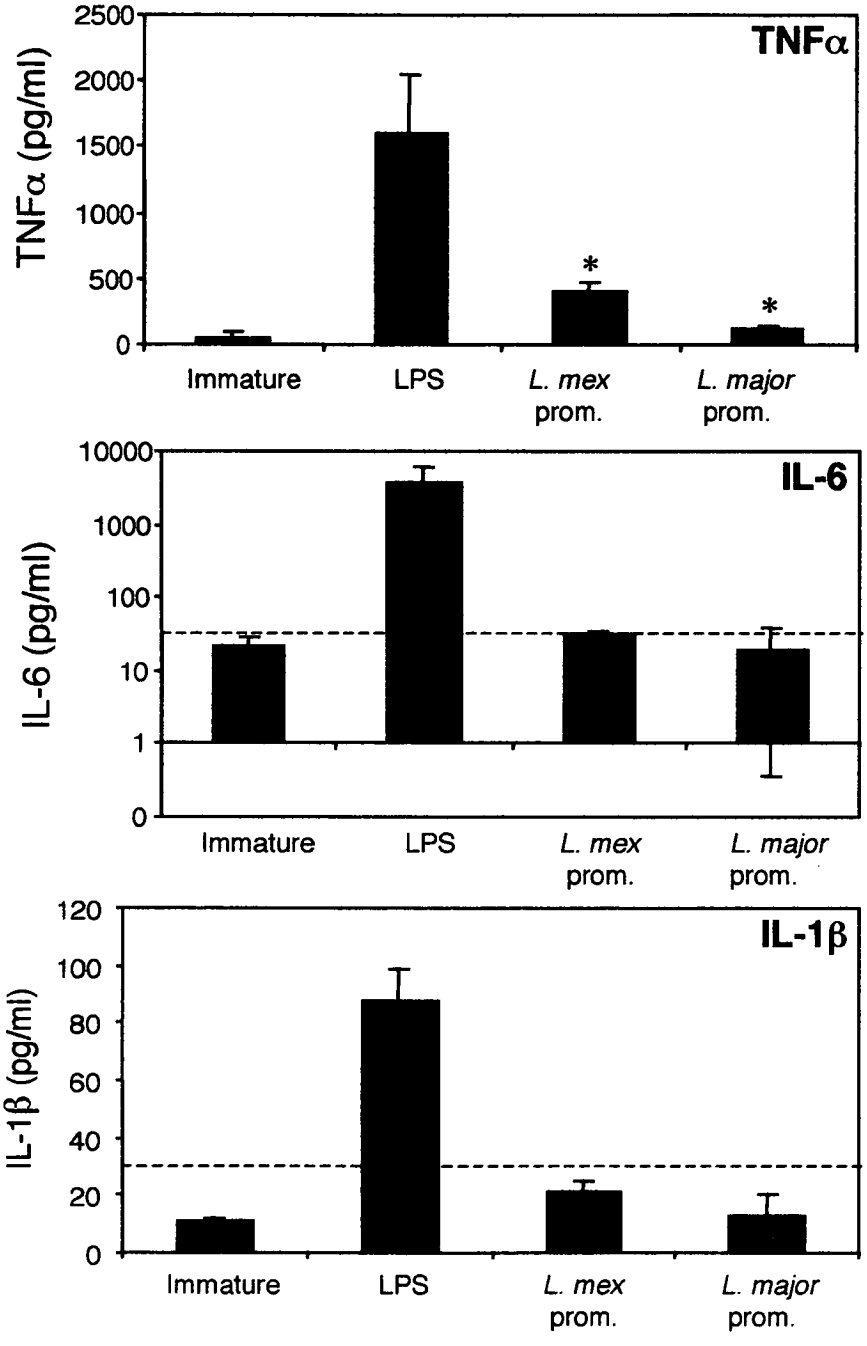
CD86						**MHC II**				
	Immature		Promastigotes				Immature		Promastigotes	
	UL	UR	UL	UR			UL	UR	UL	UR
Expt.1	4.3	0.2	3.1	0.4			8.6	0.8	6.7	1.5
Expt.2	4.8	0.0	6.7	1.9			11.1	0.0	7.6	3.6
Expt.3	5.8	0.4	2.1	0.2			8.0	0.8	2.1	1.5
Expt.4	3.0	0.4	3.5	1.8			3.0	0.4	2.5	0.9
Mean	4.5	0.3	3.9	1.1			7.7	0.5	4.7	1.5
s.e.m.	0.6	0.1	1.0	0.5			1.7	0.2	1.4	0.6

Numbers indicate the percentage of cells in the upper left (UL) and upper right (UR) quadrants for FACS plots of BM-DC incubated with *L. major* promastigotes for 18 hours. Cells were harvested, fixed, stained with anti-CD86 or anti-MHC II antibodies and analysed by flow cytometry. For promastigote-infected populations the values in the upper left quadrant represent the activated EGFP negative cells and the values in the upper right quadrant represent the activated EGFP positive cells.

CD86: Immature BM-DC versus *L. major* promastigotes p=0.90; *L. major* promastigotes $EGFP^-$ versus $EGFP^+$ p=0.04.

MHC II: Immature BM-DC versus *L. major* promastigotes p=0.55; *L. major* promastigotes $EGFP^-$ versus $EGFP^+$ p=0.11.

Figure 6.2: Infection of D1 Cells with *L. major* Promastigotes does not Induce Production of Pro-inflammatory Cytokines



D1 cells were incubated for 18 hours with 10μg/ml LPS, *L. mexicana* or *L. major* stationary phase promastigotes. Supernatants (n=3) were assayed by ELISA for the presence of TNFα, IL-6 and IL-1β. The sensitivity limit of these assays was 30 pg/ml (dashed line). Values represent the concentrations from the supernatant of ~ $1X10^5$ cells (mean ± s.e.m.).

*TNFα: *L. mexicana* promastigotes versus *L. major* promastigotes: p = 0.01

significantly less TNFα than those from D1 cells incubated with *L. mexicana* promastigotes (p=0.01, see Figure 6.2).

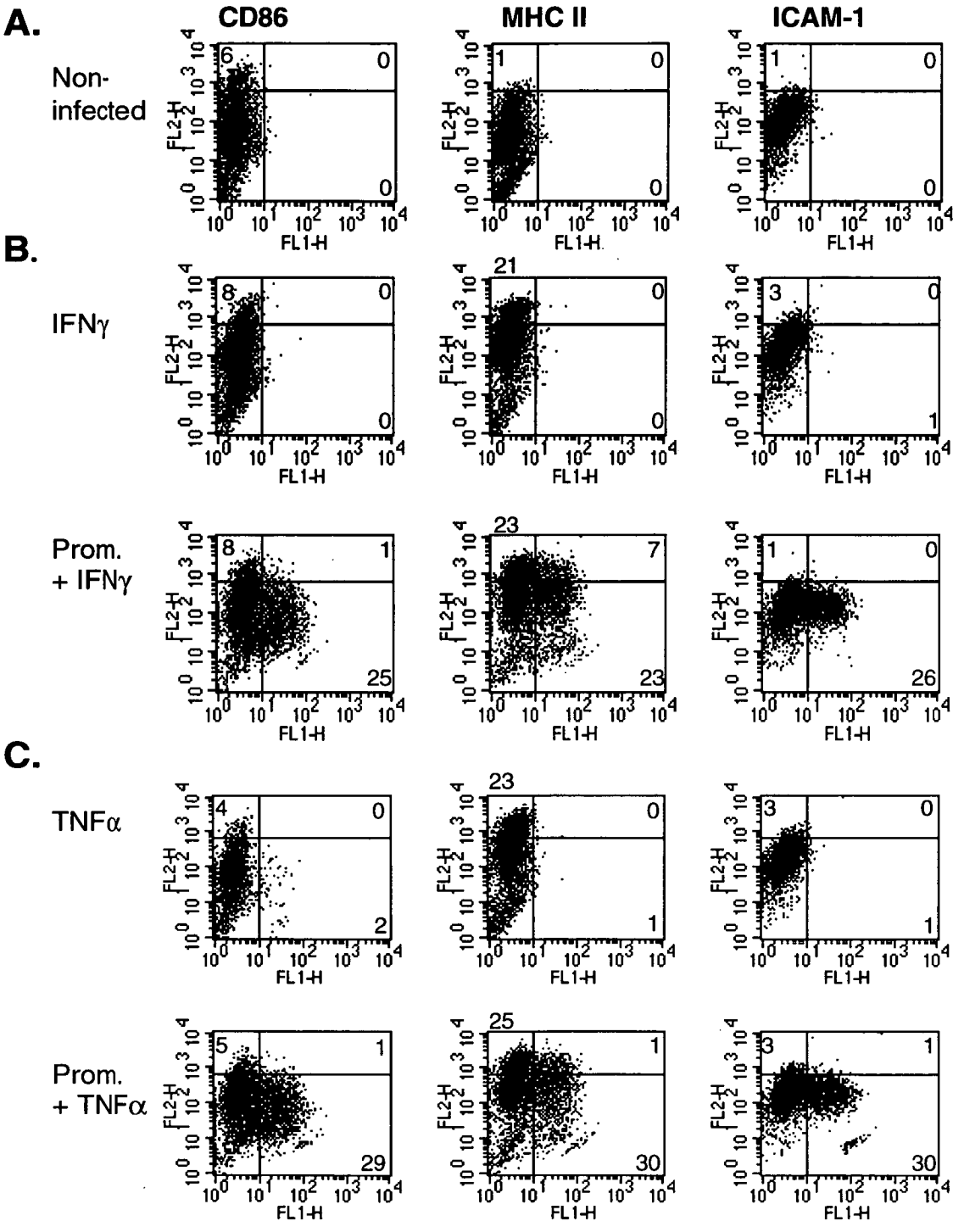
The FACS plots presented in Figure 6.1A indicate that $EGFP^+$ cells expressed lower levels of CD86 and CD54 than the non-infected $EGFP^-$ cells. In order to investigate whether *L. major* promastigotes were suppressing activation of DC, BM-DC were infected for 18 hours with stationary phase promastigotes and then activated for 3 hours with low levels of TNFα (10 ng/ml) or IFNγ (10^3 U/ml). The FACS plots presented in Figure 6.3 are representative of two different experiments. Numerically, based on percentages of cells in the upper two quadrants, infection does not appear to suppress activation with IFNγ or TNFα. However, $EGFP^+$ cells consistently appear to express lower levels of CD86 and CD54 than the $EGFP^-$ cells. These data therefore suggest that infection with *L. major* promastigotes does retard activation of BM-DC with low levels of activating stimuli. Alternatively, however, these data may support the idea proposed for infection of BM-DC with *L. mexicana* promastigotes, that the parasites are more rapidly degraded in infected cells, so that the activated cells are consistently negative for EGFP. This idea is discussed further in Chapter 7.

6.3 Identification of *L. mexicana* LPG as a *Leishmania* PAMP

6.3.1 Introduction to LPG

The surface of *Leishmania* promastigotes is dominated by the glycoconjugate, lipophosphoglycan (LPG), which forms a thick glycocalx around the entire surface of the parasite, including the flagellum (Turco and Descoteaux 1992). The LPG of all *Leishmania* species is comprised of four domains (Figure 6.4): a phosphatidylinositol lipid anchor attached to the plasma membrane; a hexasaccharide glycan core; a phosphoglycan domain and a neutral cap consisting of galactose and mannose residues.

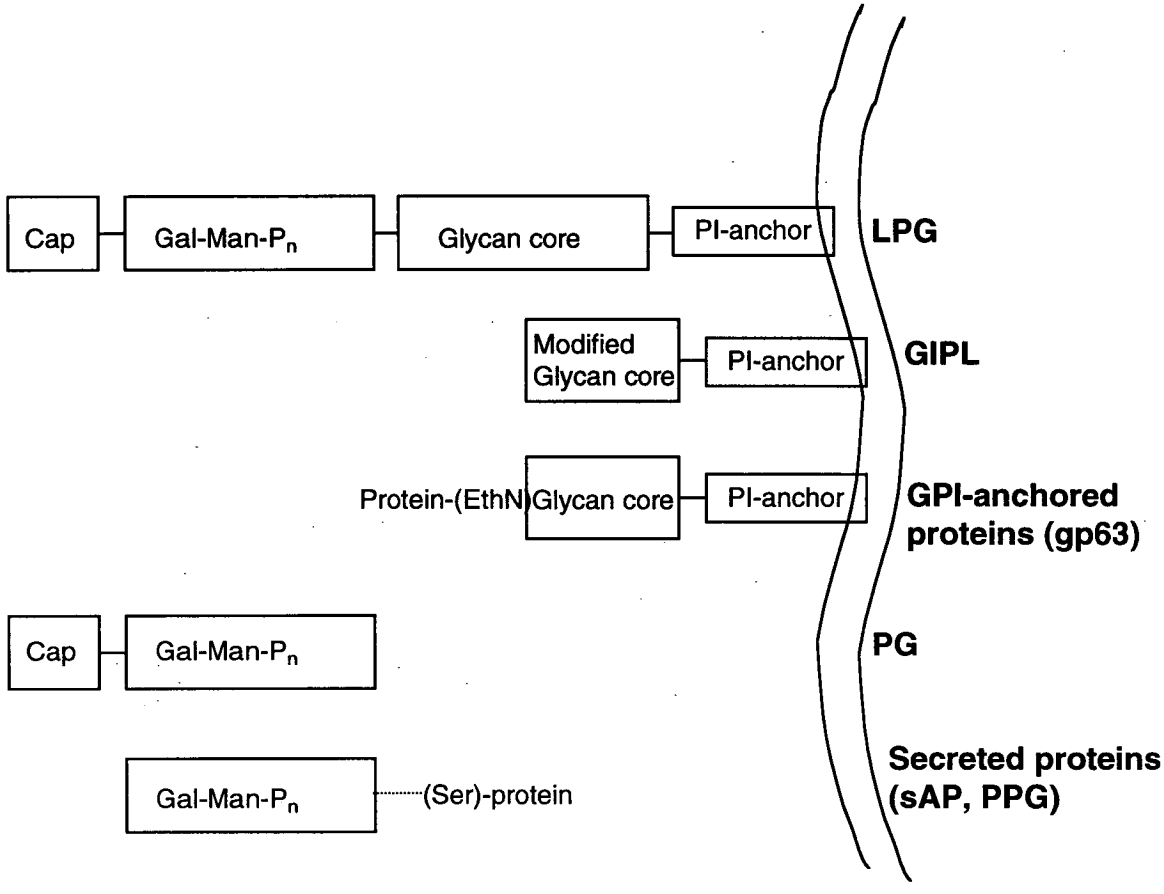
Figure 6.3: Suppression of Activation of BM-DC by *L. major* Promastigotes?



BM-DC were incubated with *L. major* promastigotes for 18 hours and then activated with 10^3U/ml IFNγ or 10ng/ml TNFα for 3 hours. Cells were then harvested, fixed, stained for surface activation markers and analysed by flow cytometry.

Numbers represent the percentage of cells in the respective quadrants. FACS plots are representative of 2 different experiments.

Figure 6.4: Schematic Representation of *Leishmania* Phosphoglycan-containing Glycoconjugates



Diagramatic representation of *Leishmania* phosphoglycan-containing glycoconjugates: LPG, lipophosphoglycan; GIPL, glycoinositol phospholipid; GPI, glycophosphatidylinositol; PG, phosphoglycan; sAP, secreted acid phosphatase; PPG, proteophosphoglycan (Sacks *et al* 2000).

Differences between species arise in substitutions made to the phosphoglycan core; in *L. mexicana* promastigotes approximately 25% of the galactose molecules are substituted on the third position with glucose molecules (Ilg *et al.* 1992), whereas in *L. major* 87% of the galactose molecules are substituted with different side chains. These substitutions include galactose molecules, linear saccharide chains, or, in metacyclic promastigotes, arabinose molecules (McConville *et al.* 1990; Sacks *et al.* 1990).These side chains are exposed in the folded LPG molecule and would be accessible for interactions with different host cell receptors (McConville and Ferguson 1993). Transformation from non-infective promastigotes in the sandfly gut to infective metacyclics is associated with a doubling in size of the LPG molecule due to an increase in the number of phosphoglycan repeat units, resulting in a thickening of the surface glycocalyx (Sacks *et al.* 1990). *L. major* promastigotes bind to the insect midgut epithelial cells via β-galactose residues terminating the LPG side chains. Metacyclogensis results in the production of LPG molecules in which these terminal residues are replaced by α-arabinose, leading to release of the parasites and migration of the now virulent forms to the mouthparts of the sandfly (Pimenta *et al.* 1992). The importance of LPG for midgut attachment was demonstrated definitively using LPG knock-out promastigotes, which were lost from the sandfly with excretion of the blood meal (Sacks *et al.* 2000).

Within the host the thickened LPG glyocalyx of metacyclic promastigotes probably protects them from complement-mediated lysis (Puentes *et al.* 1988) and masks other surface components from recognition by host opsonising antibodies (Karp *et al.* 1991). It may also mediate direct binding of parasites to MΦ (Handman and Goding 1985; Talamas-Rohana *et al.* 1990). Once in the cell LPG plays a number of roles in protecting the parasites until they have transformed into amastigotes, which are better adapted for intracellular survival: LPG protects *Leishmania* from oxidative attack (Chan *et al.* 1989); fusion of the phagosome with host endsomal compartments containing proteolytic enzymes is inhibited by LPG (Dermine *et al.* 2000); and expression of iNOS, IL-12 and IL-1β can be down-regulated in a LPG-dependent manner (Hatzigeorgiou *et al.* 1996; Proudfoot *et al.* 1996; Piedrafita *et al.* 1999). The latter may be partly due to inhibition of protein kinase C by LPG (Descoteaux *et al.* 1992; Giorgione *et al.* 1996)

and the consequent disruption of a number of signal transduction pathways. LPG may also have an anti-inflammatory effect upon infection since it has been shown to inhibit monocyte migration and responses to chemotactic factors (Lo *et al.* 1998).

The multifunctional role of LPG in promoting promastigote survival lead to the assumption that it was a key *Leishmania* virulence factor. This was supported by naturally occurring LPG mutants (Handman *et al.* 1986) or mutants generated by mutagenesis screens (Elhay *et al.* 1990) which were no longer able to survive in MΦ. However, *Leishmania* promastigotes express a number of related phosphoglycans on their surface (McConville and Ferguson 1993) which share common epitopes with LPG (Ilg *et al.* 1991) and which may also be mutated in these screens. The recent generation of LPG-specific knock-outs has, however, not clarified the issue: LPG mutants were independently generated by knocking out the β-galactofuranosyl transferase (*lpg1*) gene which is specifically involved in modifying the LPG glycan core, thus leaving intact other proteoglycan synthesis pathways (Ilg 2000; Spath *et al.* 2000). Ilg (Ilg 2000) demonstrated that *L. mexicana* promastigotes lacking LPG were unimpaired in their ability to infect, and survive in, MΦ. These parasites were as virulent, if not more so, as wild type promastigotes *in vivo*. However, while Spath *et al* (Spath *et al.* 2000) agreed that $LPG^{-/-}$ *L. major* promastigotes were efficiently taken up by MΦ in the presence of Complement, they reported decreased intracellular survival of $LPG^{-/-}$ mutants, which were approximately one hundred times less virulent than wild-type promastigotes *in vivo*. The differences between the results published by these two groups may reflect differences in the relative importance of LPG for amastigotes of different *Leishmania* species; *L. major* but not *L. mexicana* amastigotes express low levels of LPG (Turco and Sacks 1991; Bahr *et al.* 1993) that may play a role in their intracellular survival. Therefore it follows that loss of *L. major* amastigote-expression of LPG may explain the decreased survival of *L. major* parasites *in vivo*.

The data presented in section 6.2 indicated that activation of a percentage of DC *in vitro* was due to an interaction with a *L. mexicana* promastigote molecule that was not structurally conserved between *L. major* promastigotes. Since activated D1 cells were

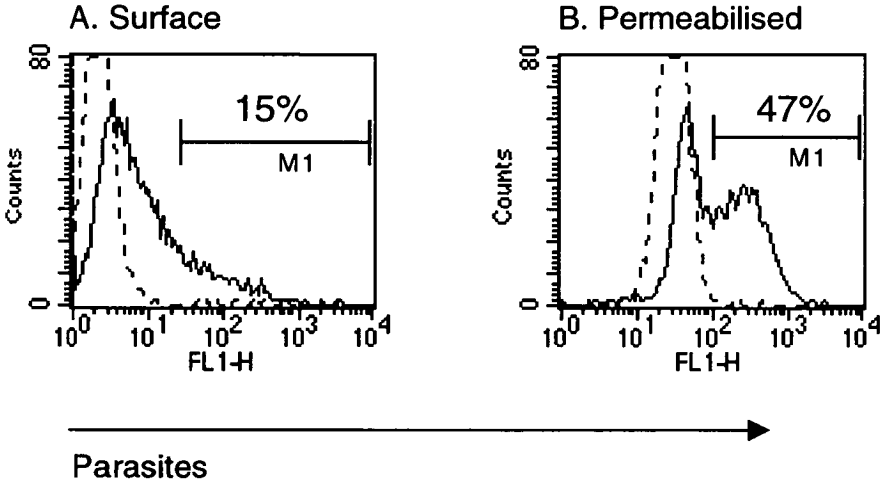
shown to contain intact EGFP$^+$ parasites (Chapter 5), it was likely that this molecule was expressed on the surface or was secreted by the parasites. LPG was therefore an attractive candidate for the *L. mexicana* promastigote PAMP. The hypothesis was proposed that pattern receptors expressed by a fraction of DC in BM-DC or D1 cell cultures recognise components of the metacyclic *L. mexicana* LPG molecule, but that the more complicated substitutions to metacyclic *L. major* LPG render these components inaccessible to DC receptors. In order to address this hypothesis, BM-DC were incubated with *L. mexicana* LPG$^{-/-}$ promastigotes *in vitro*.

6.3.2 Internalisation of LPG$^{-/-}$ Promastigotes by BM-DC

Two independent clones of LPG mutants which had been generated by targeted deletion of the *lpg1* gene (Ilg 2000), and were kindly provided by T. Ilg, Tübingen, were used to infect BM-DC. These parasites lack LPG, but display abundant non-LPG phosphoglycans on their surface (Ilg 2000). The results from both clones were identical, and data from only one of the clones, I/8D, is presented in this chapter.

LPG has been shown in a number of studies to mediate Complement-independent uptake of promastigotes by MΦ (Handman and Goding 1985; Talamas-Rohana *et al.* 1990). Studies using *L. mexicana* LPG$^{-/-}$ parasites reported no impairment in the ability of these parasites to be taken up by MΦ (Ilg 2000), however it was important to confirm that DC efficiently internalised LPG$^{-/-}$ promastigotes. Day 7 BM-DC were incubated with *L. mexicana* LPG$^{-/-}$ or wild type promastigotes for 18 hours. Cells were then harvested, fixed, permeablised, stained with an anti-parasite serum and analysed by flow cytometry. Some non-permeabilised cells were positive with the anti-parasite serum (16.8±2.1%) indicating the attachment of some parasites to the surface of the cells, however permeabilisation of cells prior to incubation with the anti-parasite serum showed that the majority of the parasites were intracellular (Figure 6.5), and that

Figure 6.5: LPG$^{-/-}$ Promastigotes are Internalised by BM-DC



BM-DC were incubated with LPG$^{-/-}$ promastigotes for 18 hours. Cells were harvested, fixed and stained with an anti-parasite antibody with or without permeabilisation. Dotted lines represent A. non-stained cells and B. cells incubated with normal mouse serum and the secondary antibody. Solid lines represent cells from parasite-infected cultures.

This data is representative of four separate experiments

41.6±2.5% of the cells were infected (average from four different experiments, see Table 6.3).

6.3.3 Incubation of DC with *L. mexicana* LPG$^{-/-}$ Promastigotes

To investigate whether uptake of LPG$^{-/-}$ promastigotes activated BM-DC *in vitro*, BM-DC were incubated with *L. mexicana* LPG$^{-/-}$ promastigotes for 18 hours, harvested, fixed, and stained for surface activation markers before being permeabilised and stained with the anti-parasite serum to identify infected cells by flow cytometry. Infection of BM-DC with LPG$^{-/-}$ promastigotes did not induce up-regulation of the surface markers CD86, MHC II or CD54 (Figure 6.6). Co-staining of BM-DC with the anti-parasite serum and antibodies against CD86, MHC II or CD54 demonstrated that both the infected and non-infected populations retained low levels of the surface activation markers tested. Interestingly, as with the *L. major* data in section 6.2, the lack of up-regulation of surface markers was more apparent with CD86 expression than MHC II. (Figure 6.6; Table 6.4). These data indicate that *L. mexicana* promastigotes lacking LPG no longer activate BM-DC (percentage of CD86^{hi} cells in BM-DC cultures versus LPG$^{-/-}$ cultures p=0.50; *L. mexicana* wild type versus LPG$^{-/-}$ promastigotes p=0.06, see Table 6.4).

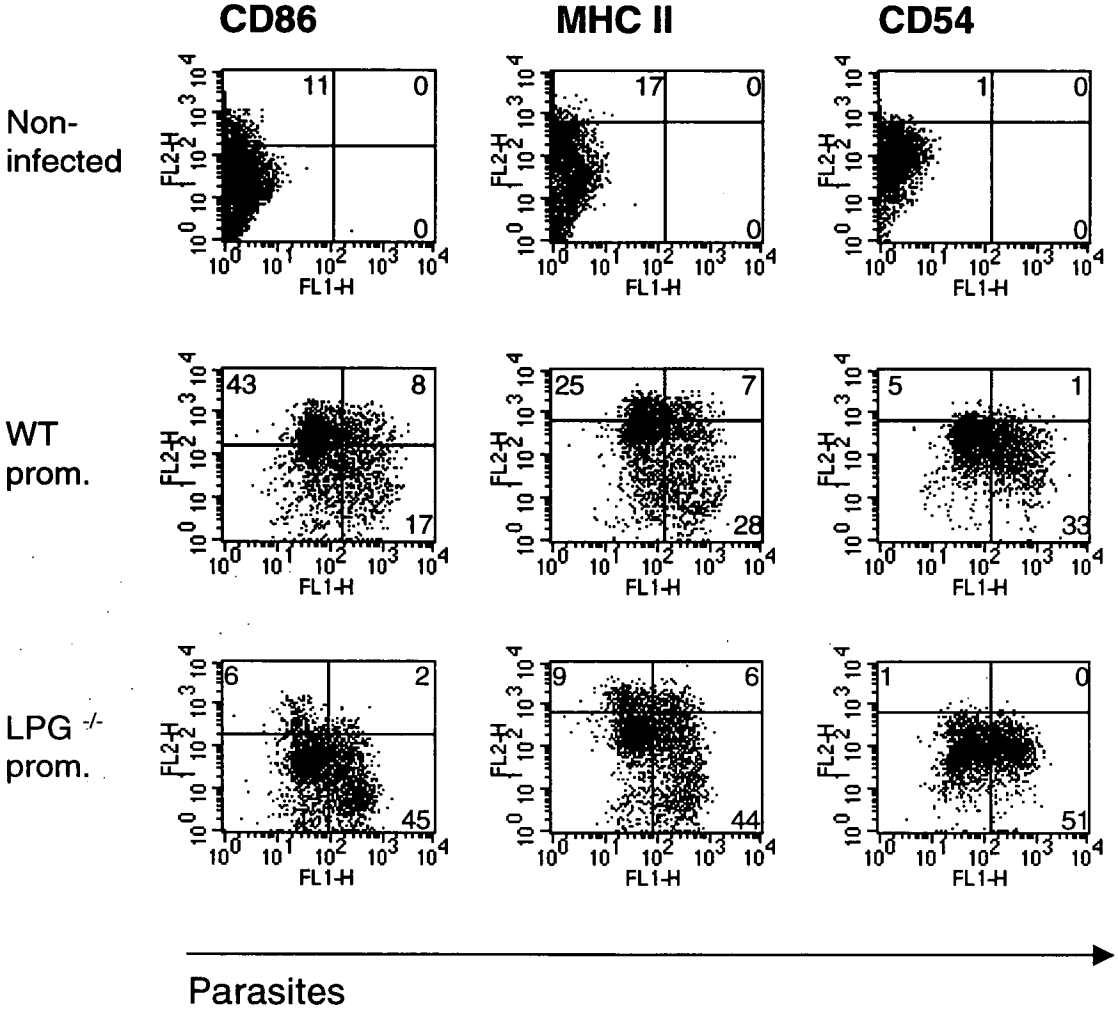
The effect of uptake of LPG$^{-/-}$ promastigotes was also examined in D1 cells. Figure 6.7A shows that incubation of D1 cells with LPG$^{-/-}$ promastigotes did not result in the up-regulation of the surface markers CD86, MHC II and CD54 seen on approximately 33% of the cells upon incubation with wild-type EGFP-expressing promastigotes, confirming the results from the BM-DC experiments (wild type EGFP-expressing promastigote-infected culture versus LPG$^{-/-}$ promastigotes p=0.04; value based on percentage of activated CD86^{+} cells from five different experiments, see Table 6.4). It should be noted that infected and non-infected D1 cells were not distinguished in experiments using LPG$^{-/-}$ promastigotes.

Table 6.3:
Complete FACS Data for Infection Levels of BM-DC Incubated with *L. mexicana* LPG$^{-/-}$ Promastigotes.

	Percentage of cells stained with the anti-parasite serum	
	Permeabilised	Surface
Expt. 1	35.0	18.8
Expt. 2	46.7	14.7
Expt. 3	43.6	
Expt. 4	41.1	
Mean	41.6	16.8
s.e.m.	2.5	

Numbers indicate the percentage of cells stained with the anti-parasite serum in BM-DC cultures incubated with *L. mexicana* LPG$^{-/-}$ promastigotes for 18 hours. Cells were harvested, fixed, stained with or without permeabilisation to distinguish between surface-bound and intracellular parasites and analysed by flow cytometry.

Figure 6.6: *L. mexicana* LPG$^{-/-}$ promastigotes do not activate BM-DC upon infection



BM-DC were incubated with stationary phase wild-type or LPG$^{-/-}$ *L. mexicana* promastigotes for 18 hours. Cells were harvested, fixed, stained for surface activation markers then permeablised and stained with an anti-parasite serum. Cells were then analysed by flow cytometry.

Numbers represent the percentage of cells in the respective quadrants. FACS plots are representative of plots from 4 different experiments.

Numbers indicate the percentage of cells in the upper left (UL) and upper right (UR) quadrants for FACS plots of immature BM-DC and D1 DC, or BM-DC and D1 DC incubated with *L. mexicana* $LPG^{-/-}$ promastigotes for 18 hours. Cells were harvested, fixed, stained with anti-CD86 or anti-MHC II antibodies, then permeabilised and incubated with anti-parasite serum followed by the appropriate FITC-conjugated secondary. Cells were then analysed by flow cytometry. For promastigote-infected populations the values in the upper left quadrant represent the activated serum negative cells and the values in the upper right quadrant represent the activated serum positive cells.

A. **CD86:** Immature BM-DC versus $LPG^{-/-}$ p=0.50; wild type promastigotes versus $LPG^{-/-}$ p=0.06.
MHC II: Immature BM-DC versus $LPG^{-/-}$ p=0.54; wild type promastigotes versus $LPG^{-/-}$ p=0.03.

B. **CD86:** Immature D1 cells versus $LPG^{-/-}$ p=0.13; wild type promastigotes versus $LPG^{-/-}$ p=0.04
MHC II: Immature BM-DC versus $LPG^{-/-}$ p=0.09; wild type promastigotes versus $LPG^{-/-}$ p=0.07.

Table 6.4: Complete FACS data for infection of DC with *L. mexicana* LPG$^{-/-}$ promastigotes.

A. BM-DC

CD86						
	Immature		WT prom.		LPG$^{-/-}$ prom.	
	UL	UR	UL	UR	UL	UR
Expt.1	6.2	0.0	12.4	2.4	9.0	3.0
Expt.2	11.0	0.0	43.2	8.1	6.4	2.1
Expt.3	9.3	0.0	35.5	2.5	6.2	1.1
Expt.4	6.0	0.0	12.5	3.0	7.5	1.7
Mean	8.1	0.0	25.9	4.0	7.3	2.0
s.e.m.	1.2	0.0	7.9	1.4	0.7	0.4

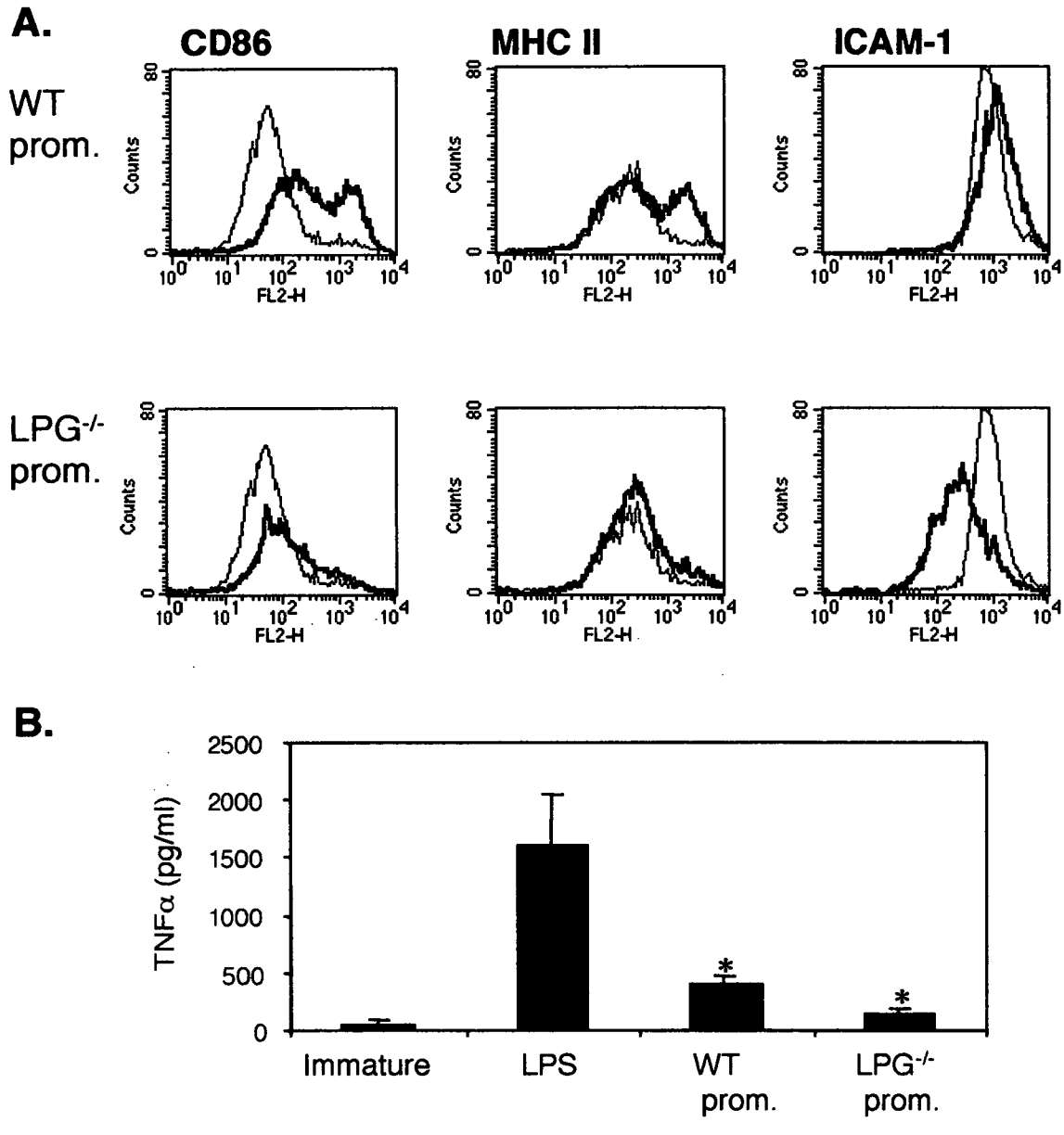
MHC II						
	Immature		WT prom.		LPG$^{-/-}$ prom.	
	UL	UR	UL	UR	UL	UR
	10.1	0.0	17.0	6.4	9.8	3.3
	16.8	0.0	24.8	6.5	9.0	6.4
	13.9	0.0	32.5	7.2	15.3	2.7
	9.6	0.0	14.2	4.0	5.6	4.5
	12.6	0.0	22.1	4.8	9.9	4.2
	1.7	0.0	4.1	0.7	2.0	0.8

B. D1 cells

CD86						
	Immature		EGFP prom.		LPG$^{-/-}$ prom.	
	UL	UR	UL	UR	UL	UR
Expt.1	3.6	0.2	21.2	16.6	13.0	0.3
Expt.2	5.9	0.0	26.7	22.3	11.4	0.0
Expt.3	12.9	0.8	14.4	9.3	22.6	0.6
Expt.4	16.9	0.2	40.3	18.6	28.6	0.0
Mean	9.8	0.3	25.7	16.7	18.9	0.2
s.e.m.	3.1	0.2	5.5	2.7	4.1	0.1

MHC II						
	Immature		EGFP prom.		LPG$^{-/-}$ prom.	
	UL	UR	UL	UR	UL	UR
	17.7	0.8	21.4	17.9	25.3	0.2
	5.6	0.0	23.5	22.0	14.5	0.1
	16.5	1.0	11.3	8.2	22.8	0.8
	16.0	0.3	31.7	14.8	27.2	0.0
	14.0	0.5	22.0	15.7	22.5	0.2
	2.8	0.2	4.2	2.9	2.8	0.3

Figure 6.7: Incubation with *L. mexicana* LPG$^{-/-}$ Promastigotes does not Activate D1 Cells



A. D1 cells were incubated with stationary phase *L. mexicana* wild type (WT) or LPG$^{-/-}$ promastigotes for 18 hours. Cells were harvested and stained for surface activation markers. Thin lines represent non-infected cells and thick lines represent cells incubated with *Leishmania* parasites. FACS plots are representative of 4 different experiments.

B. Supernatants from LPG$^{-/-}$ infected cultures were assayed for TNFα (n=3) by ELISA. The lower sensitivity limit of this assay was 30 pg/ml. Values represent concentrations in supernatants from approximately $1X10^5$ cells (mean ± s.e.m.).
* WT promastigotes versus LPG$^{-/-}$ promastigotes: p=0.03

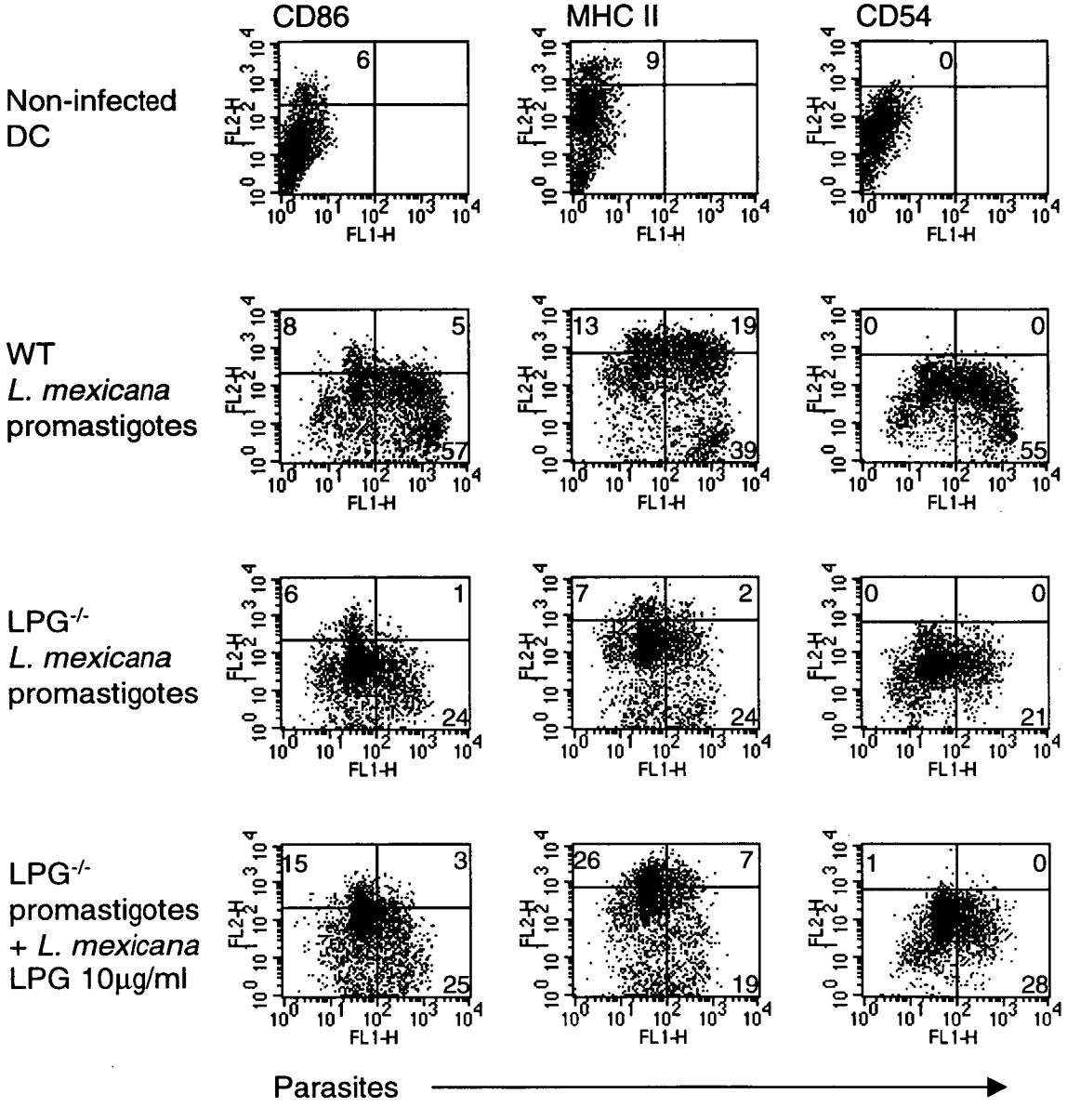
The lack of activation of DC on incubation with $LPG^{-/-}$ promastigotes was confirmed by analysing TNFα production in infected D1 cell cultures, since this cytokine had been induced on incubation with wild type promastigotes (Figure 5.3); incubation with $LPG^{-/-}$ promastigotes did not induce TNFα production by D1 cells (Figure 6.7B); wild type promastigotes versus $LPG^{-/-}$ promastigotes p=0.03.

6.3.4 Incubation of BM-DC with Purified LPG

In order to determine whether LPG itself could activate DC *in vitro*, BM-DC were incubated with purified *L. mexicana* or *L. major* LPG (kindly provided by T. Ilg, Tübingen), for 18 hours, harvested, stained for surface activation markers and analysed by flow cytometry. $LPG^{-/-}$ parasites were also incubated with purified *L. mexicana* LPG to test whether this was sufficient to restore the activation of 15% of the cells seen on incubation with wild type promastigotes.

Simultaneous incubation of $LPG^{-/-}$ promastigotes and 10μg/ml *L. mexicana*–derived LPG with BM-DC restored activation of BM-DC to levels seen on infection with wild type *L. mexicana* promastigotes (Figure 6.8; wild type promastigotes versus $LPG^{-/-}$ promastigotes with 10μg/ml LPG p=0.57, based on CD86 expression from three separate experiments, see Table 6.5). 10μg/ml LPG was chosen for these experiments based on a titration of the effect of LPG on infected MΦ (T. Aebischer personal communication). Incubation of BM-DC with 10μg/ml purified *L. mexicana* LPG activated 23.0±2.3% of the cells (based on CD86 expression from five separate experiments; Table 6.6, see also Figure 6.9). Incubation with 50μg/ml *L. mexicana* LPG did not significantly increase the percentage of activated $CD86^{+}$ or MHC II^{+} cells in the population, confirming the idea that BM-DC are heterogeneous in their ability to respond to the promastigote-derived activating signal.

Figure 6.8: Incubation of LPG$^{-/-}$ Promastigotes and Purified *L. mexicana* LPG with BM-DC Restores Activation



Day 7 BM-DC were incubated with *L. mexicana* wild type (WT) or LPG$^{-/-}$ promastigotes, or LPG$^{-/-}$ promastigotes with 10µg/ml purified *L. mexicana* LPG for 18 hours. Cells were harvested, fixed and stained for surface activation markers, then permeabilised and stained with an anti-parasite serum and FITC-conjugated goat anti-rabbit secondary antibody. Cells were then analysed by flow cytometry.

Quadrants were set on non-infected DC. Numbers represent the percentage of cells in the respective quadrants. Dot plots are representative of 3 different experiments.

Numbers indicate the percentage of cells in the upper left (UL) and upper right (UR) quadrants for FACS plots of immature BM-DC, or BM-DC incubated with *L. mexicana* wild type (WT) or $LPG^{-/-}$ promastigotes, or $LPG^{-/-}$ promastigotes and 10mg/ml purified *L. mexicana* LPG, for 18 hours. Cells were harvested, fixed, stained with anti-CD86 or anti-MHC II antibodies, then permeabilised and stained with an anti-parasite serum and a FITC-conjugated secondary antibody. Cells were then analysed by flow cytometry.

CD86: Wild type promastigotes versus $LPG^{-/-}$ promastigotes + 10μg/ml LPG p=0.57; $LPG^{-/-}$ versus $LPG^{-/-}$ +10μg/ml LPG p=0.09; wild type versus 10μg/ml *L. mexicana* LPG p=0.93.

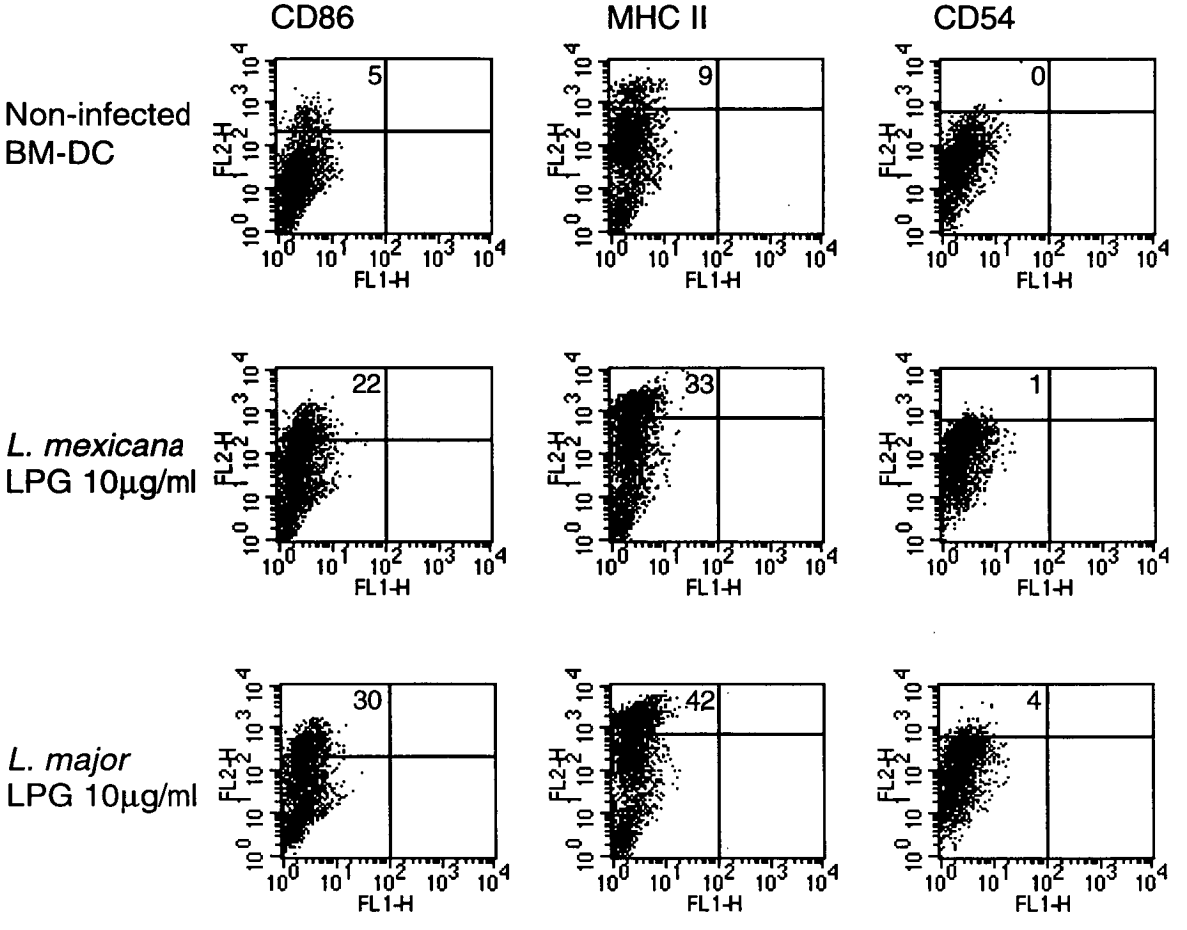
MHC II: Wild type promastigotes versus $LPG^{-/-}$ promastigotes + 10μg/ml LPG p=0.63; $LPG^{-/-}$ versus $LPG^{-/-}$ +10μg/ml LPG p=0.07; wild type versus 10μg/ml *L. mexicana* LPG p=0.64.

Table 6.5:
Complete FACS Data for Infection of BM-DC with *L. mexicana* Wild Type or LPG$^{-/-}$ Promastigotes, or LPG$^{-/-}$ Promastigotes and Purified *L. mexicana* LPG

CD86											
	Immature		WT promastigotes			LPG$^{-/-}$ promastigotes			LPG$^{-/-}$ prom. + 10μg/ml LPG		
	UL	UR	UL	UR	Total	UL	UR	Total	UL	UR	Total
Expt.1	8.9	0.0	9.9	3.4	13.3	7.2	1.0	8.2			
Expt.2	6.6	0.0	6.8	3.1	9.9	5.8	0.5	6.3			
Expt.3	5.5	0.0	8.1	4.7	12.8	6.6	0.9	7.5	15.4	3.4	18.8
Expt.4	6.2	0.0	27.4	25.0	52.4	6.5	0.7	7.2	11.7	4.2	15.9
Expt.5	9.0	0.0							39.5	16.6	56.1
Mean	7.2	0.0	13.1	9.1	22.1	6.5	0.8	7.3	22.2	8.1	30.3
s.e.m.	0.7	0.0	4.8	5.3	10.1	0.3	0.1	0.4	8.7	4.3	12.9

MHC II											
	Immature		WT promastigotes			LPG$^{-/-}$ promastigotes			LPG$^{-/-}$ prom. + 10μg/ml LPG		
	UL	UR	UL	UR	Total	UL	UR	Total	UL	UR	Total
Expt.1	16.6	0.0	11.4	7.7	19.1	4.9	5.2	10.1			
Expt.2	27.0	0.0	8.4	8.4	16.8	7.5	2.9	10.4			
Expt.3	8.6	0.0	12.6	19.0	31.6	7.4	2.0	9.4	26.4	6.7	33.1
Expt.4	21.6	0.0	ND	ND	69.3	11.2	3.9	15.1	17.2	13.2	30.4
Expt.5	12.8	0.0							4.1	7.1	11.2
Mean	17.3	0.0	10.8	11.7	34.2	7.8	3.5	11.3	15.9	9.0	24.9
s.e.m.	3.2	0.0	1.3	3.7	12.1	1.3	0.7	1.3	6.5	2.1	6.9

Figure 6.9: Activation of BM-DC by Purified *L. mexicana* or *L. major* LPG



Day 7 BM-DC were incubated with 10µg/ml purified *L. mexicana* or *L. major* LPG for 18 hours. Cells were then harvested, fixed, stained for surface activation markers and analysed by flow cytometry.

Quadrants were set on dot plots from non-infected BM-DC. Numbers represent the percentage of cells in the upper left quadrant. Dot plots are representative of plots from 4 different experiments.

Table 6.6:
Complete FACS Data for Incubation of BM-DC with Purified *L. mexicana* and *L. major* LPG.

A. *L. mexicana* LPG

CD86					MHC II				
	LPG (μg/ml)					LPG (μg/ml)			
	1	**5**	**10**	**50**		**1**	**5**	**10**	**50**
Expt.1			20.9	44.5				41.3	53.4
Expt.2			16.0	36.4				41.3	41.8
Expt.3			21.9	38.2				33.4	43.8
Expt.4			26.9	17.9				53.5	8.8
Expt.5	12.3	11.8	29.2			19.5	19.7	30.1	
Expt.6	10.6	35.6				18.0	27.7		
Mean	11.5	23.7	23.0	34.3		18.8	23.7	39.9	37.0
s.e.m.	0.9	11.9	2.3	5.7		0.8	4.0	4.1	9.7

B. *L. major* LPG

CD86				MHC II			
	LPG (μg/ml)				LPG (μg/ml)		
	1	**5**	**10**		**1**	**5**	**10**
Expt.1			35.3				34.8
Expt.2			29.5				41.9
Expt.3			22.2				55.8
Expt.4			24.3				36.0
Expt.5	10.9	31.5	43.7		18.2	30.6	43.8
Expt.6	36.8	30.4			20.7	37.9	
Mean	23.9	31.0	31.0		19.5	34.3	42.5
s.e.m.	13.0	0.6	3.9		1.3	3.7	3.8

Numbers indicate the percentage of cells in the upper left (UL) and upper right (UR) quadrants for FACS plots of BM-DC or D1 cells incubated with *L. major* promastigotes for 18 hours. Cells were harvested, fixed, stained with anti-CD86 or anti-MHC II antibodies and analysed by flow cytometry.

Unexpectedly incubation with 10μg/ml *L. major*-derived LPG potently activated BM-DC (Figure 6.9); 31±3.9% of the cells up-regulated surface expression of CD86 on incubation with *L. major* LPG (statistical analysis based on five separate experiments, see Table 6.6). *L. major* LPG also appeared to activate BM-DC more potently than *L. mexicana* LPG at lower concentrations (Table 6.6). In order to demonstrate that this activation was not due to contamination of LPG samples with endotoxin, BM-DC were incubated with 10μg/ml LPG that had been pre-incubated with 50μg/ml Polymyxin B (Sigma), which binds to the lipid A component of LPS thus inhibiting its activity. Table 6.7 shows that *L. mexicana* and *L. major* LPG activate BM-DC in the presence of Polymyxin B, indicating that the results described above are not due to endotoxin contamination of the samples. Thus, these data demonstrate that binding of *Leishmania* LPG activates a proportion of BM-DC.

6.4 Discussion

To investigate the nature of the *L. mexicana* promastigote-derived signal responsible for activating 15% or 33% of BM-DC or D1 cells respectively, experiments were performed in which metacyclic *L. major* promastigotes were incubated with DC *in vitro*. The data presented in this chapter indicate that uptake of stationary phase *L. major* promastigotes does not activate BM-DC and therefore, that infection with these parasites resembles infection with *L. mexicana* amastigotes in its overt effect on DC maturation. LPG is an abundant *Leishmania* surface glycoconjugate, the structure of which differs between metacyclic *L. mexicana* and *L. major* promastigotes, and LPG expression is down-regulated on transformation of *L. mexicana* promastigotes into amastigotes. Thus, *L. mexicana* LPG appeared an attractive candidate PAMP.

To address this possibility, BM-DC and D1 cells were incubated with *L. mexicana* LPG$^{-/-}$ mutants. These parasites were efficiently taken up by both cell types, but did not induce activation of DC as assessed by expression of surface activation markers and

Table 6.7:
Incubation of BM-DC with Purified LPG and Polymyxin B

CD86					
			Polymyxin B		
	Immature	LPS 100ng/ml	*L. mexicana* LPG 10µg/ml	*L. major* LPG 10µg/ml	100ng/ml LPS
Expt.1	9.1	33.0	20.9	72.9	14.8
Expt.2	7.7	ND	23.6	59.6	11.8

MHC II					
			Polymyxin B		
	Immature	LPS 100ng/ml	*L. mex* LPG 10µg/ml	*L. major* LPG 10µg/ml	100ng/ml LPS
Expt.1	13.1	30.3	23.9	34.3	7.9
Expt.2	7.2	ND	12.1	43.8	3.7

Day 7 BM-DC were incubated with LPS alone or purified *L. mexicana* LPG, *L. major* LPG and LPS with 50µg/ml Polymyxin B for 18 hours. Cells were then harvested, fixed, stained for the surface activation markers CD86 and MHC II and analysed by flow cytometry.

Numbers represent percentages of cells in the upper left quandrants of dot plots. ND, not done.

cytokine production. Rather, infection appeared to resemble incubation of the cells with *L. mexicana* amastigotes, or *L. major* promastigotes. Therefore, these data support the hypothesis that binding of *L. mexicana* LPG to receptors on DC induces activation of DC *in vitro*.

To further test this hypothesis, BM-DC were incubated with purified *L. mexicana* LPG. The results from this experiment would determine whether LPG itself is an activating stimulus, or whether uptake of parasites expressing LPG results in internalisation of parasites in compartments where they are accessible to other host pattern receptors. Incubation of BM-DC with purified *L. mexicana* LPG alone or LPG with $LPG^{-/-}$ promastigotes resulted in activation of a proportion of the BM-DC population. Therefore, these data indicate that LPG is the promastigote-encoded molecule that directly induces activation of a percentage of BM-DC when incubated with intact promastigotes or promastigote lysate.

LPG derived from both *L. mexicana* and *L. major* metacyclic promastigotes is composed of the same neutral cap strucure and phosphatidylinositol lipid anchor. However *L. mexicana* LPG contains a glycan core substituted with occasional glucose molecules, whereas in metacyclic *L. major* LPG the glucose residues are replaced by more complex saccharide units, including arabinose moelcules (McConville and Ferguson 1993). Since *L. mexicana*, but not *L. major*, promastigotes activated BM-DC, this observation indicates that DC receptors that recognise *L. mexicana*, but not *L. major*, promastigotes bind to determinants on the phosphoglycan core. Thus, the more complex substitutions to the *L. major* LPG glycan core may occlude access of these receptors to these determinants. Addition of purified *L. major* LPG to BM-DC culture resulted in potent activation of the cells. However, purification of LPG would result in loss of the ordered arrangement of the LPG molecules that occurs on the surface of the parasites, and DC receptors would therefore have access to activating determinants on the phosphoglycan core. This idea could be tested by reconstituting $LPG^{-/-}$ parasites with *L. mexicana* and *L. major* LPG, as described by (Handman *et al.* 1986).

6.5 Concluding Remarks

The results from experiments described in this chapter strongly implicate *L. mexicana*-derived LPG as a potential activation signal for DC, since in the absence of LPG *L. mexicana* promastigotes could not activate DC, and purified LPG activated BM-DC. Therefore, *L. mexicana* LPG is the first example described of a protozoan PAMP. Activation of DC is likely to be due to recognition of a part of the *L. mexicana* phosphoglycan core by DC sugar/lectin receptors since it is within this region that the main differences occur between *L. mexicana* and *L. major* LPG. Binding of LPG only triggers low levels of activation in DC cultures, however, indicating that the DC population is heterogeneous in terms of susceptibility to LPG activation. This observation implies that, *in vivo*, binding of LPG on uptake of metacyclic *L. mexicana* promastigotes may not be sufficient to activate DC at the site of infection. Thus activation by LPG would likely synergise with the effect of other exogenous factors, such as pro-inflammatory cytokines, to initiate the primary anti-*Leishmania* T cell response.

Chapter 7: Discussion

The work presented in this thesis had two aims: first to establish and test a model system with which to follow the fate of *Leishmania*-derived antigens in infected cells; second to investigate the specific interaction between *Leishmania* and DC using a defined *in vitro* model.

Do infected DC process and present *Leishmania*-derived antigens?

A model experimental system was designed with which to follow the fate of *Leishmania*-derived antigens in infected cells. This model was based on the transgenic expression of a defined MHC II-restricted T cell epitope, MCC, by *L. mexicana* parasites. H2-E^k-MCC complexes would be detected using the complex-specific mAb D4 and the H2-Ek-MCC-specific T cell hybridoma, 2B4. MCC was cloned into two different carrier proteins; GST and a secreted form of *L. mexicana* MBAP. MBAP:MCC fusion proteins were expressed by transgenic parasites, but MCC was not presented at detectable levels by infected DC and MΦ. Published data indicated that MBAP epitopes are present on the surface of infected MΦ in the context of MHC II molecules, and, therefore, that MCC should be presented in this system. A number of different factors are likely to contribute to the lack of presentation from transgenic parasites in this experimental system: the secretion of amastigote cysteine proteases into the PV may result in the destruction of T cell epitopes before they are bound by MHC II molecules. Incubation of infected cells with protease inhibitors would result in inactivation of the host processing machinery and could not be used to avoid this problem. However, expression of high levels of fusion protein by transgenic parasites may saturate proteases in the PV, resulting in the presentation of intact T cell epitopes. Second, sequences surrounding the MCC epitope may not favour optimal processing of this peptide by host cell proteases; replacing a known T cell epitope of gp63 with MCC should circumvent this problem. It is not known whether parasite antigens bind MHC II in the PV or

whether the antigens are transported out of the PV to join the host endosomal pathway in an alternative compartment. If complexes form in the PV then it may be that transport from the PV to the cell surface is less efficient that conventional processing and presentation pathways, and that high concentrations of parasite antigens are required to overcome this inefficiency. *Leishmania*-infected DC were fully competent to process exogenous antigen for presentation to T cells, therefore, once levels of expression of fusion proteins and processing of the epitope have been optimised, this experimental model should be appropriate for following the fate of parasite-derived antigens in infected cells.

Does uptake of *Leishmania* parasites *per se* activate DC?

Leishmania parasites, as pathogens, have been assumed to activate DC upon uptake. However the published data is conflicting regarding whether promastigotes or amastigotes, or both, can provide the activating stimulus. Following initial and unexpected observations that amastigote infection did not cause upregulation of MHC II on BM-DC, an experimental model was established to address the question of whether, in a highly controlled system, uptake of *Leishmania* parasites *per se* could activate DC. EGFP-expressing *L. mexicana* parasites, which can be maintained *in vitro*, were used to distinguish between infected and non-infected cells in the population. The results reported in this thesis demonstrate that uptake of *L. mexicana* amastigotes does not activate BM-DC or D1 cells either in terms of upregulation of surface activation markers or increased cytokine production. Thus, *L. mexicana* amastigotes do not express molecules that signal activation upon binding by DC receptors. This result apparently contradicts previous reports that lesion-derived *L. major* or *L. amazonensis* amastigotes activated DC upon up-take (von Stebut *et al.* 1998; Qi *et al.* 2001); a discrepancy likely to be due to the fact that lesion-derived amastigotes will be coated with Ig and other host proteins from the inflamed tissues that may trigger activation of DC. Differences in the species of parasite used may also account for the results obtained by different groups on infection of DC with *Leishmania* promastigotes; the data presented in this thesis demonstrate, in accordance with von Stebut and colleagues (1998) that uptake of *L.*

major promastigotes does not activate BM-DC; *L. mexicana* promastigotes however could activate at least some BM-DC and D1 cells.

While *L. mexicana* amastigotes do not induce upregulation of DC activation markers, it seems unlikely that there will be no interaction at all between the parasites and DC. This is supported by the clusteral analysis from the GeneChip® analysis of *L. mexicana*-infected D1 cells. Provisional data from this experiment indicates that transcription of 880 DC genes was altered by at least three fold on infection with *L. mexicana* amastigotes. Thus, these data, together with the flow cytometry data, indicate that amastigotes do not behave as inert particles on infection of D1 cells, but that this interaction does not result in classical activation of the cells.

Uptake of *L. mexicana* promastigotes activated a percentage of BM-DC, and this effect was more pronounced with D1 cells. Activated BM-DC were consistently $EGFP^-$ but it was subsequently shown that activated D1 cells were infected with intact, $EGFP^+$, parasites. Taken together these data suggest that the activating signal was not intracellular, as initially proposed in Chapter 4, but that BM-DC had been activated upon uptake of intact parasites that were subsequently degraded. These results suggest that *Leishmania* parasites are more rapidly degraded in BM-DC than D1 cells.

Experiments to address the nature of the *L. mexicana* promastigote activating signal demonstrated that *L. major* promastigotes did not activate BM-DC or D1 cells upon uptake, and resembled *L. mexicana* amastigotes in their interaction with this host cell type. The hypothesis was therefore proposed that metacyclic *L. mexicana* LPG might be the activating signal, since *L. mexicana* amastigotes do not express LPG and the composition of the sugar side chains on this molecule differs between *L. mexicana* and *L. major*. Experiments using $LPG^{-/-}$ promastigotes indicated that LPG was a *L. mexicana* PAMP and this was supported by incubation of BM-DC with purified LPG. It is proposed that the receptor for LPG recognises determinants on the *L. mexicana* LPG phosphoglycan core. The more complicated sugar substitutions on *L. major* LPG would block access to this core, preventing activation of DC via signalling through these

receptors. Only a limited number of cells in DC cultures responded to the promastigote-derived activating stimulus in these experiments, suggesting heterogeneity amongst both BM-DC and D1 cells in the expression of the LPG receptor, and that a higher percentage of D1 cells expressed this receptor than BM-DC.

The potential of LPG as a protozoan PAMP

LPG fulfills the requirements put forward by Medzhitov & Janeway (Medzhitov and Janeway 1997) that PAMP should be signature molecules for a range of pathogens, which are essential for their survival, and so will be relatively invariant. LPG is expressed by all species of *Leishmania* parasites and, while depending on the species, it may not be essential for survival in the host, it is critical for the passage of promastigotes through the sandfly (Sacks *et al.* 2000). LPG also shares structural similarities with LPS, a well-defined bacterial PAMP. Infact, LPG was originally characterised as "a *Leishmania* lipopolysaccharide molecule (L-LPS)"(Handman *et al.* 1986). Thus, LPG is proposed to be the first example identified of a protozoal PAMP. The similarities between LPG and LPS and the fact that LPG epitopes transiently appear on the surface of MΦ upon infection with *L. mexicana* promastigotes (Stierhof *et al.* 1991) suggest that LPG could mediate activation of DC by binding to the receptors utilised by LPS, including TLR4 in association with CD14. However, preliminary data indicates that LPG does not bind to TLR4, since BM-DC from B10 mice, which carry a natural mutation in the TLR4 gene, were still activated upon incubation with *L. mexicana* promastigotes (data not shown). Thus the receptor for *L. mexicana* LPG awaits identification. However, it is likely to be a sugar or lectin receptor if it recognises determinants on the phosphoglycan core.

Do *Leishmania* parasites suppress activation of DC?

DC are exquisitely sensitive to activating stimuli. Therefore, the lack of activation by *L. mexicana* amastigotes or *L. major* promastigotes implied that these parasites are able to suppress maturation of DC, as has been documented for infected MΦ. *L. mexicana* amastigotes, however, did not suppress simultaneous activation of BM-DC with LPS

and IFNγ and also did not induce IL-10 production in D1 cultures. It was more difficult, however, to define whether intracellular *L. major* promastigotes suppressed activation of DC; *L. major* promastigote-infected cells expressed consistently lower levels of activation markers than the non-infected cells in the culture. This was particularly evident when BM-DC were activated with low levels of activating stimuli (TNFα or IFNγ) prior to infection. This observation could imply either that activation of infected cells was suppressed, or that parasites were more rapidly degraded in activated cells. In order to distinguish between these two possibilities, one would ideally mark cells containing parasites in such a way that this label persisted once the parasites had been degraded. One way to approach this may be to infect DC with $EGFP^+$ parasites and sort them into a homogeneous infected population by flow cytometry. The $EGFP^+$ DC could then be cultured with low levels of activating stimuli and stained for surface activation markers. If parasites were suppressing activation then there should be very few activated cells in the culture. By comparison, if parasites were degraded in activated cells then an $EGFP^-$ population would emerge expressing high levels of surface activation markers.

D1 cells versus BM-DC

The experiments described in Chapters 4, 5 and 6 used two *in vitro*-cultured populations of DC as *in vitro* models of DC *in vivo*. BM-DC are derived from bone marrow precursors that have been cultured in the presence of GM-CSF. These cultures are fairly heterogeneous, containing DC precursors as well as immature, and spontaneously maturing DC, and are likely to reflect the myeloid DC that will be recruited to the site of infection from the blood *in vivo*. D1 cells are a long-term growth factor-dependent population of splenic DC, which are also cultured in the presence of GM-CSF. The D1 population is more homogeneous than BM-DC, and can be maintained as an immature population of DC in culture. Thus, they provide a useful system with which to investigate the interaction between *Leishmania* parasites and DC *in vitro*, and were an ideal population with which to perform the kinetic analysis described in Appendix B. The experiments described in this thesis, however, highlighted some differences between BM-DC and D1 cells: experiments performed using BM-DC indicated that CD86 was more sensitive to *Leishmania*-derived activating signals than MHC II,

suggesting that surface expression of these two markers was differentially regulated. This was not the case in the D1 cells. This observation may imply that long-term culture of D1 cells in conditioned medium has resulted in a homogeneity in the responses of these cells that does not reflect the situation *in vivo*. Furthermore, expression profiles of surface DC markers and the activation of only 33% of D1 cells on incubation with *L. mexicana* promastigotes indicates that these cells are not as homogeneous as they sometimes appeared. Finally, *L. mexicana* promastigotes were more rapidly degraded in activated BM-DC than activated D1 cells, indicating again that BM-DC are more responsive to infection than D1 cells. Thus, while the D1 cell culture provides an invaluable tool for the investigation DC interactions *in vitro*, these observations indicate the importance of interpreting the results from D1 experiments in the light of results from other DC cultures.

Relevance of the *in vitro* model to *Leishmania* infection *in vivo*

The results presented in this thesis use *in vitro* model experimental systems to explore the interaction between *Leishmania* and DC. In order to understand the complex interactions that occur *in vivo* during the initiation of a primary T cell response, it is important to be able to dissect the individual parts that contribute to this response. The experimental system described in Chapter 4 allowed the specific interaction between *Leishmania* parasites and DC to be examined in a highly controlled manner in the absence of other exogenous factors. Thus, the precise question of whether uptake of *Leishmania* parasites *per se* activated DC could be addressed. These results must, however, be considered within the context of the micorenvironment in which the interaction will occur *in vivo*: *L. mexicana* parasites will be injected into the epidermis of the host along with sandfly saliva, which has been shown to be immunomodulatory. In addition, wounding, caused by injection of the parasites, will initiate inflammatory responses in the host. Thus, promastigotes will be injected into an environment containing pro-inflammatory cytokines that may synergise with LPG to activate immature DC, that will then migrate to the draining LN and stimulate the primary anti-*Leishmania* T cell response. These DC may contain intact parasites, degraded parasites

or have picked up parasite debris at the site of infection. The apparent heterogeneity of expression of the putative LPG receptor in DC cultures may reflect the restricted expression of this receptor by distinct DC subsets *in vivo*, implying that activation of a response to *Leishmania* will depend on the subset of DC encountered by the parasites. It would therefore be interesting to investigate the interaction of *Leishmania* with LC in more detail, since these are the DC subset that first encounters the parasites on injection into the epidermis, notably whether these cells are more sensitive to activation by LPG than BM-DC.

An interesting extension of this work would be to investigate whether the was any difference between the ability of promastigote- or amastigote-infected DC, or DC incubated with LPG, to stimulate Th1 versus Th2 responses *in vitro* and on adoptive transfer *in vivo*. In particular it would be interesting to explore the consequence of the apparent 'silent' infection of DC by amastigotes on polarisation of developing Th cells. It might be expected that these DC would induce expansion of Th2, cells since previous studies have demonstrated that incubation of DC with a pathogen-derived product which does not induce up-regulation of DC surface activation markers, resulted in the development of DC which induced polarisation of Th2 cells *in vivo* (MacDonald *et al.* 2001). However, CD40 signalling has recently been shown to be important for the induction of a Th2 response by DC (MacDonald *et al.* 2002). CD40 expression was not measured in the experiments described in this thesis, but, based on the lack of activation of other surface markers, CD40 would be anticipated to be upregulated on the surface of promastigote-infected, but not amastigote-infected, DC. This would suggest that promastigote-infected DC may polarise a Th2 response but amastigote-infected DC would polarise a Th1 response. Finally, it would also be interesting to consider whether activation of DC upon uptake of *L. mexicana*, but not *L. major*, LPG could be partly responsible for the differential ability of C57BL/6 mice to control infection with these parasites, since these mice mount a polarised Th1 response to infection with *L. major*, but a non-polarised Th response to infection with *L. mexicana*.

DC will initially take up *L. mexicana* promastigotes at the site of infection, thus the primary immune response will be primed to promastigote antigens. Intracellular promastigotes will then transform into amastigotes, which express different antigens, so how is the immune response to *Leishmania* maintained? Bystander activation of amastigote-infected MΦ may occur by anti-promastigote effector cells which have been recruited to the site of infection and which will interact with MΦ that have taken up promastigote debris and degraded intracellular promastigotes. This would lead to the killing of intracellular amastigotes and the release of amastigote debris, thus increasing the pool of available amastigote antigens, which will be taken up and presented by DC activated at the site of infection.

The lack of classical activation of DC by amastigotes may, however, be important for the persistence of parasites in a 'cured' host. In the absence of pro-inflammatory cytokines, when the anti-*Leishmania* response has died down, amastigotes, which are released upon lysis or death of host cells, will be taken up by DC at the site of the lesion, or in the LN, without triggering activation. This may contribute to the persistence of parasites in the immune host.

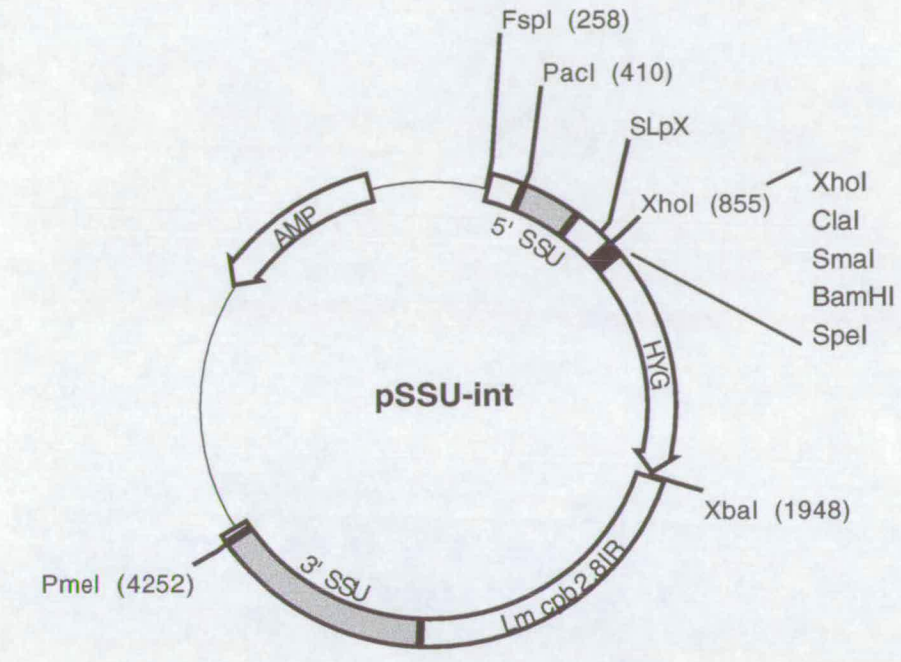
DC are not the natural reservoirs for *Leishmania* parasites

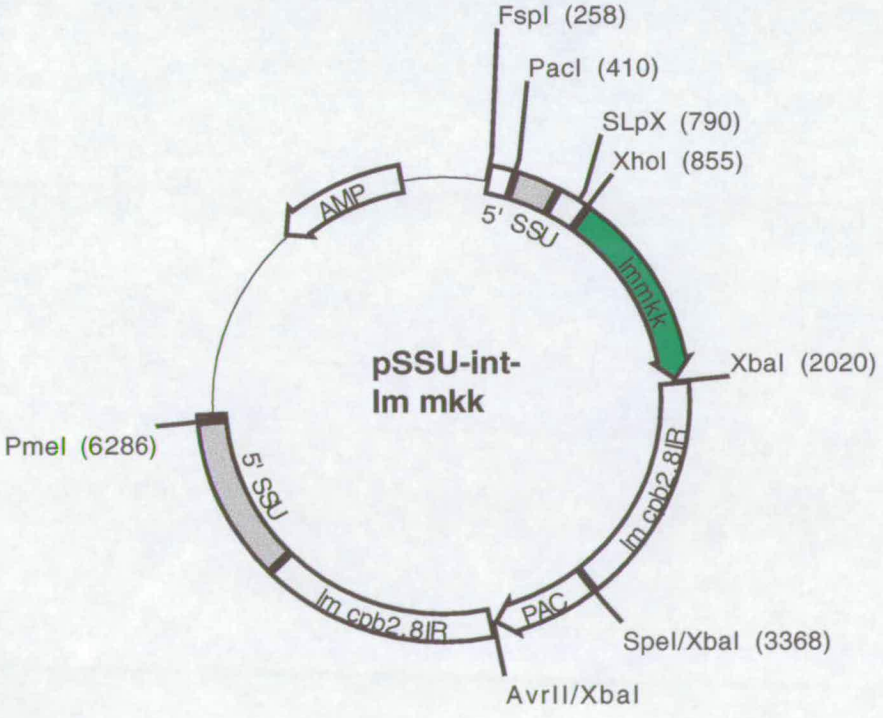
Infection of D1 cells facilitated investigation of the effect of long-term infection of DC with *L. mexicana* parasites. Infected MΦ develop large communal PV within 48 hours of infection that contain multiple parasites. It was proposed that *Leishmania* parasites would not be able to establish infection in DC in the way that they do MΦ, and that this was related to the observation that infected DC can efficiently process and present exogenous antigen *in vitro*. The results presented in Chapter 5 demonstrate that live *L. mexicana* promastigotes and amastigotes persist for up to 96 hours in D1 cells. This observation should be confirmed in an alternative DC population because, as discussed above, D1 cells do not degrade intracellular parasites as readily as BM-DC. Unfortunately, BM-DC die after 48 hours infection and so the experiment cannot be repeated in these cells. Confocal analysis of infected D1 cells showed that large

communal PV did not form in D1 cells infected with *L. mexicana* promastigotes or amastigotes within the 96 hour time frame of the experiment. This work was supported by the observation that PV in infected DC *in vivo* were significantly smaller than those formed in infected MΦ (T. Aebischer personal communication). This suggests that *L. mexicana* parasites cannot establish a chronic infection in DC as efficiently as in MΦ and that, *in vivo*, DC will not constitute a significant parasite reservoir. The hypothesis is proposed that the differential ability of *L. mexicana* parasites to form large PV in MΦ but not DC determines the efficiency with which *Leishmania* antigens are presented by these cell types. If so, then inhibition of PV formation in MΦ should alter the kinetics of antigen presentation in these cells. Care would have to be taken, however, not to inhibit transport of MHC II complexes in these cells. Results from the GeneChip® experiment may give clues about differential expression of DC genes related to vacuole formation and transport along the endosomal pathway, for example, transcriptional regulation of *rab* genes.

In conclusion, this thesis demonstrates that *Leishmania*-infected DC are capable of initiating the primary T cell response because they efficiently present antigen to T cells. However, uptake of *Leishmania* parasites *per se* is probably insufficient to initiate the primary anti-parasite immune response. Thus, the anti-*Leishmania* T cell response is likely to be primed via activation of DC at the site of infection by factors produced in response to injection of parasites by the insect vector, such as pro-inflammatory cytokines.

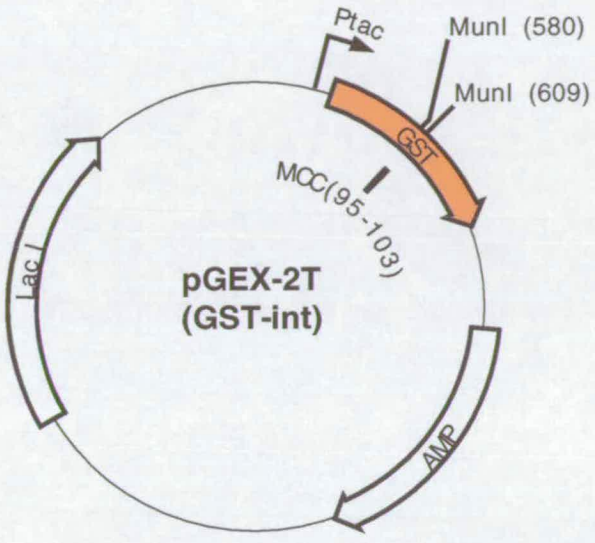
Appendix A:
a) Plasmids used for Cloning Strategies



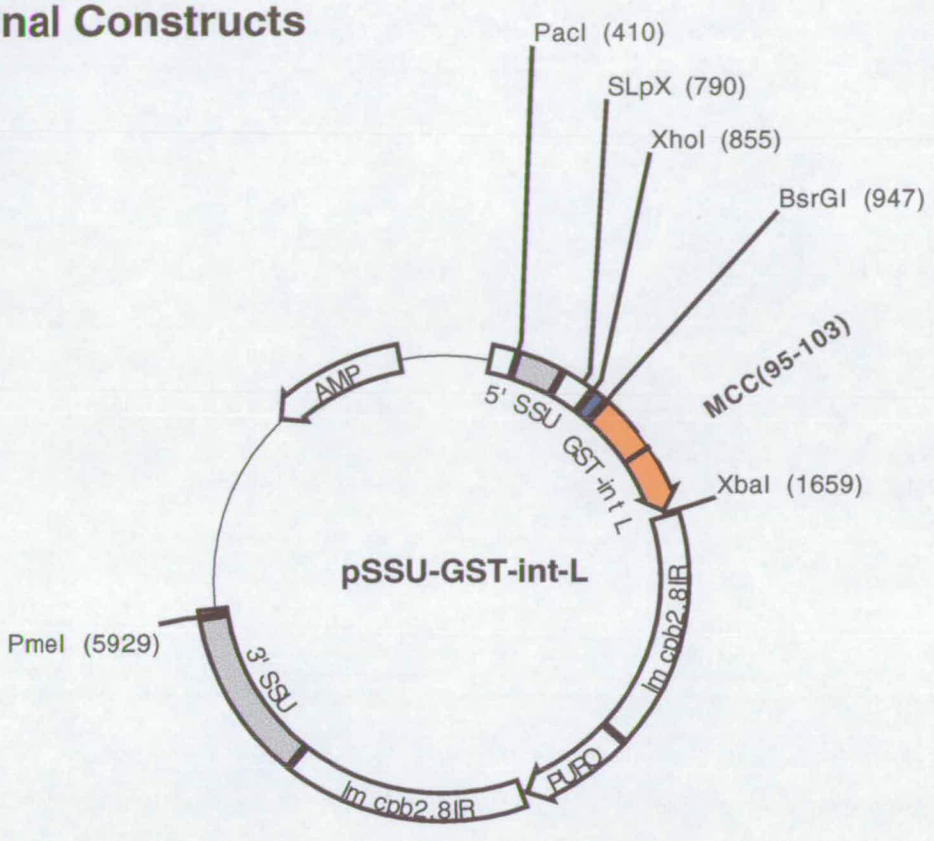


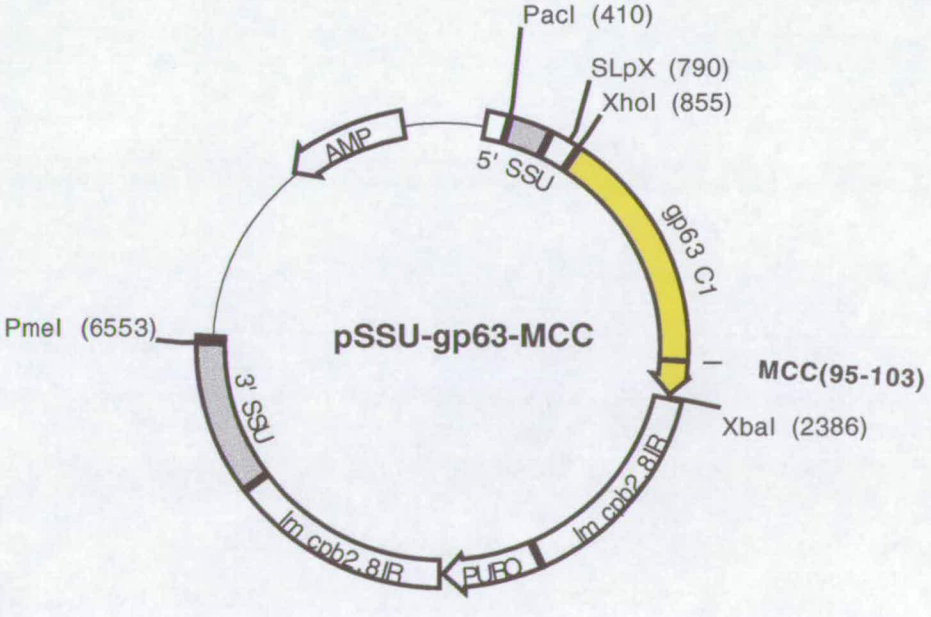
See also Figure 3.2

Appendix A:
a) Plasmids used for Cloning Stategies



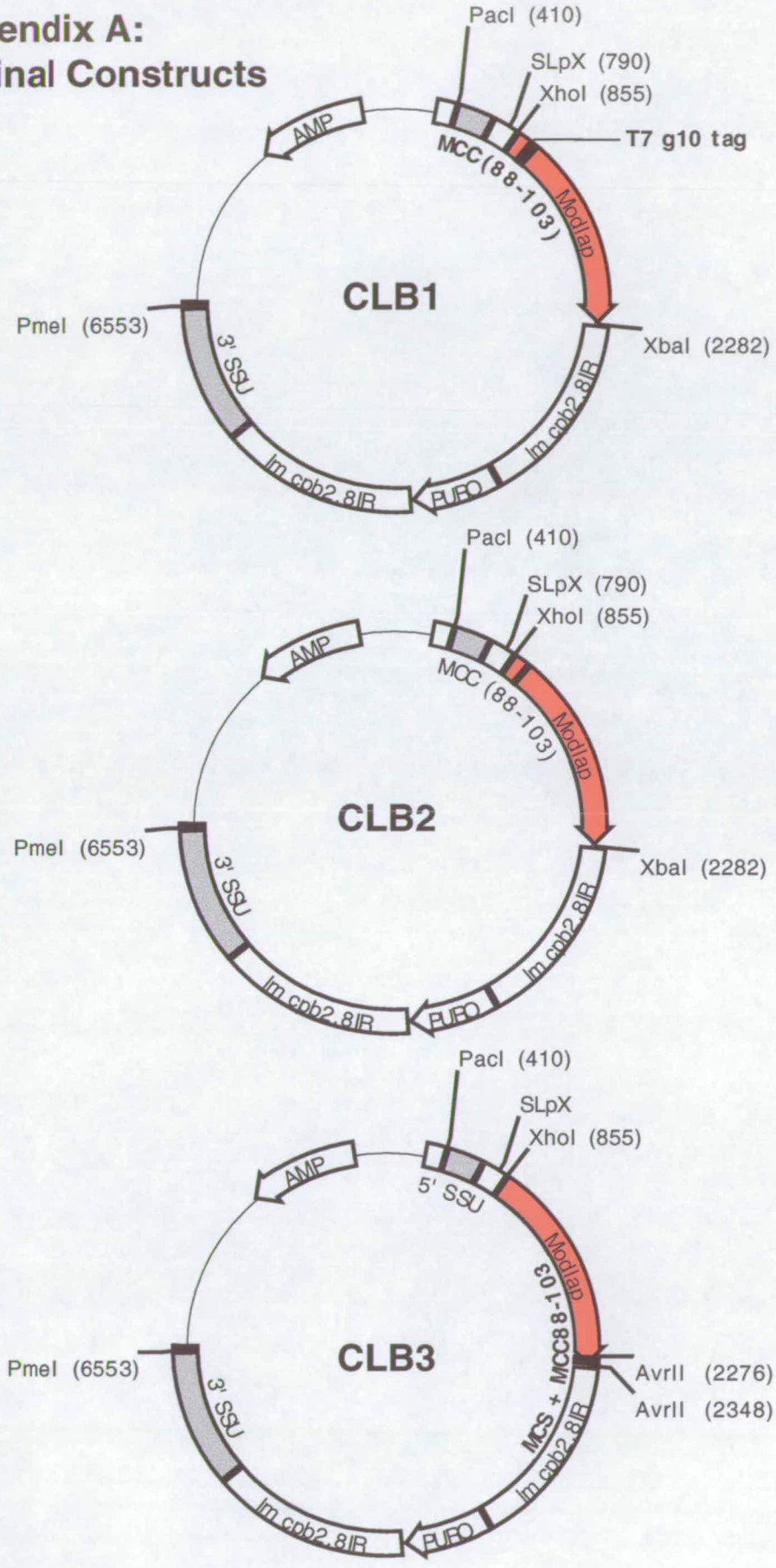
Appendix A:
b) Final Constructs





See also Figures 3.4 and 3.21

Appendix A:
b) Final Constructs



See also Figures 3.9 and 3.14

Appendix B:

Transcriptional Analysis of the Effect of Incubation of D1 Cell Cultures with *L. mexicana* Wild Type or LPG$^{-/-}$ Parasites.

Introduction

Oligonucleotide microarrays have recently been used to analyse the response of DC to infection with different organisms at the level of transcription (Granucci *et al.* 2001; Huang *et al.* 2001). Experiments comparing the effect of infection with different well characterised pathogens, or key molecular components from these pathogens, demonstrated that infection initiates a core response involving the up-regulation of over one hundred genes, as well as activating transcription of other genes more specific to the response to that class of pathogen. Interestingly, incubation of DC with purified molecular components from these pathogens (LPS, mannan or double stranded RNA) mimicked the effect of infection with the whole organism, in particular when comparing LPS and the gram negative bacteria, *E coli* (Huang *et al.* 2001). Thus, a kinetic analysis was performed to investigate the effect of uptake of *L. mexicana* promastigotes and amastigotes on the transcription of D1 DC genes. LPG$^{-/-}$ *L. mexicana* promastigotes were also included to investigate the LPG-specific up-regulation of gene expression in infected cells, since LPG is a major determinant on the surface of *L. mexicana* promastigotes but is lacking from the surface of amastigotes.

Preliminary Results and Discussion

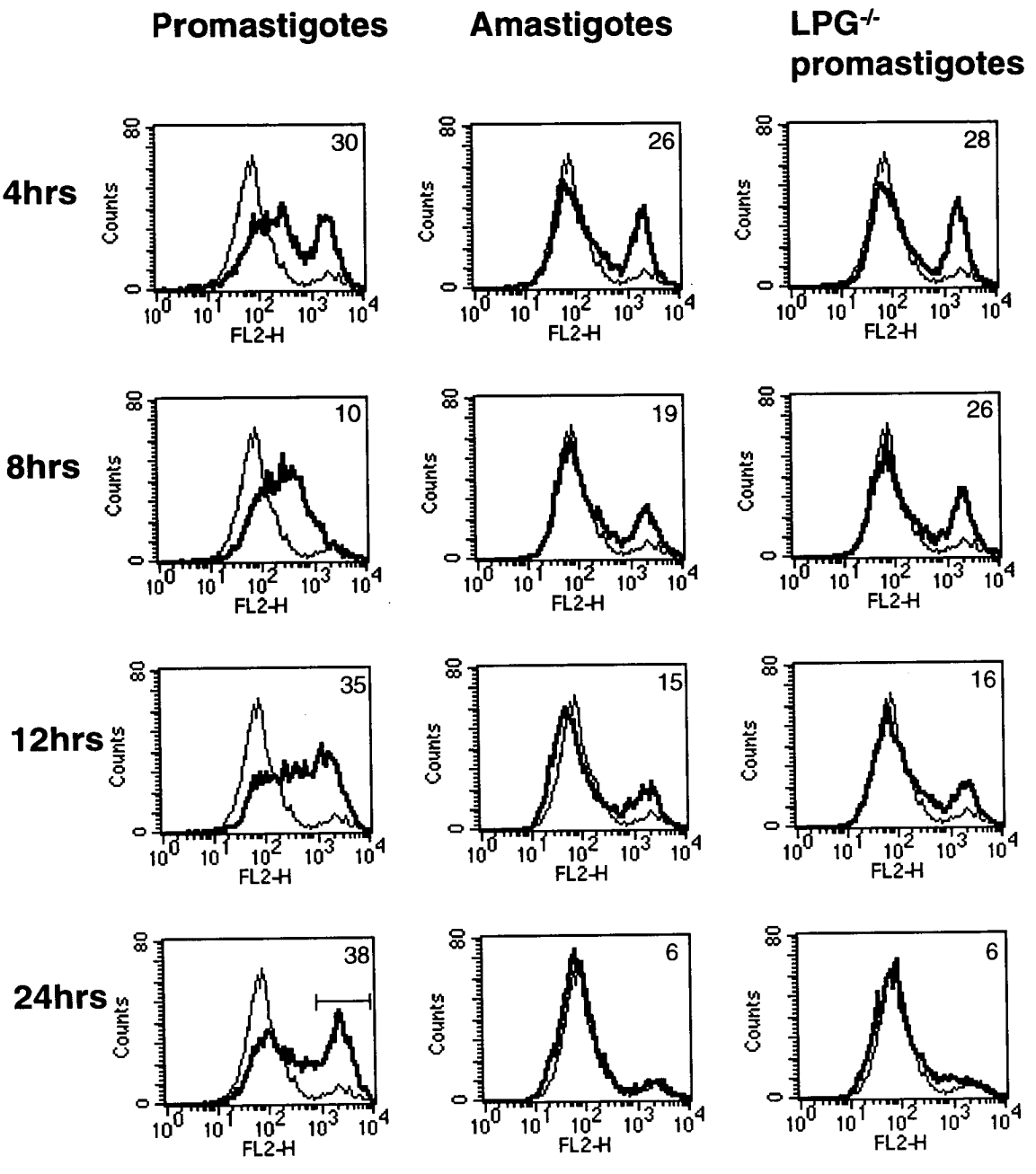
Based on the results from the time course performed on *L. mexicana* promastigote- and amastigote-infected cells (see Chapter 5, Figure 5.5), time points of 4, 8, 12 and 24 hours were chosen for the transcriptional analysis. A 12 hour time point was included because the maximum change of surface activation marker expression on incubation

with promastigotes occurred between 8 and 18 hours (Figure 5.5). $2x10^6$ D1 cells were incubated for 4, 8, 12 or 24 hours with stationary phase wild type *L. mexicana* promastigotes or amastigotes, or stationary phase *L. mexicana* $LPG^{-/-}$ promastigotes at a ratio of 1:5. This ratio of DC:parasites was chosen to maximise parasite uptake but minimise the number of excess parasites remaining in the culture medium, thus reducing the risk of contamination of samples with *Leishmania* RNA from extracellular parasites. Cells were then harvested, washed thoroughly and cell pellets frozen at –80°C. Aliquots of cells were tested by flow cytometry at each time point (Figure B.1). This analysis confirmed that uptake of *L. mexicana* amastigotes or $LPG^{-/-}$ promastigotes did not activate D1 cells, but that promastigotes activated approximately 37% of the cells after 24 hours. An early peak of activated cells that decreased through-out the time course was detected by flow cytometry for the amastigote- and $LPG^{-/-}$ promastigote-infected cultures, while this peak merged into the activated population of the promastigote-infected cultures. However, since this early peak appeared to be non-specific, and since it was present at the onset of the time course, it was likely to be due to the surface display of pre-existing molecules and should not be reflected at the level of transcription.

RNA was extracted from frozen pellets and used to generate biotin-labelled cRNA for hybridisation onto two high density oligonucleotide arrays (Affymetrix Mulik GeneChip® array) which collectively displayed 11 000 genes and ESTs (Expressed Sequence Tags). RNA extraction and chip hybridisation was performed by Caterina Vizzardelli (Milan). Statistical and filtering analyses were performed by Mattia Pellizola (Milan).

At present, only preliminary analyses have been performed on these data. Figure B.2 depicts the correlation between the genes that are up- or down-regulated by more than three fold upon incubation of D1 cells with *L. mexicana* promastigotes, amastigotes or $LPG^{-/-}$ promastigotes. This diagram illustrates that large numbers of genes are differentially regulated in the three groups. Notably, although infection with *L. mexicana* amastigotes did not induce up-regulation of surface activation markers or production of

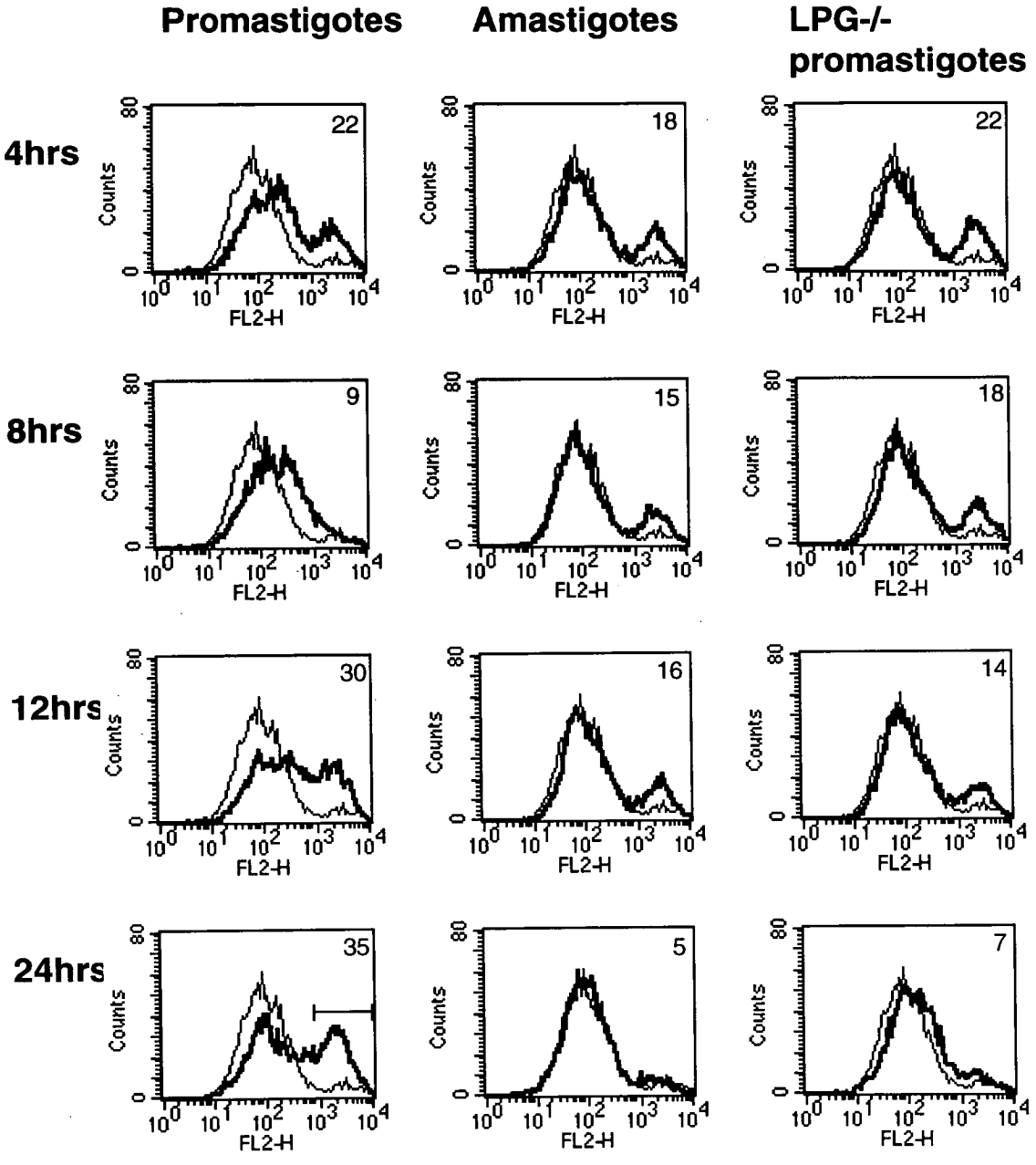
Figure B.1A: CD86 Expression on *L. mexicana*-Infected D1 Cells Used for the GeneChip® Analysis



D1 cells were infected with *L. mexicana* promastigotes, amastigotes, or LPG$^{-/-}$ promastigotes, for the times indicated. Cells were then harvested, fixed and stained for the surface activation marker CD86. Histograms show non-infected D1 cells at 4 hours (thin lines) and cells incubated with *Leishmania* parasites for 4, 8, 12 or 24 hours (thick lines).

Numbers represent the percentage of activated cells based on a marker set on activated cells at 24 hours.

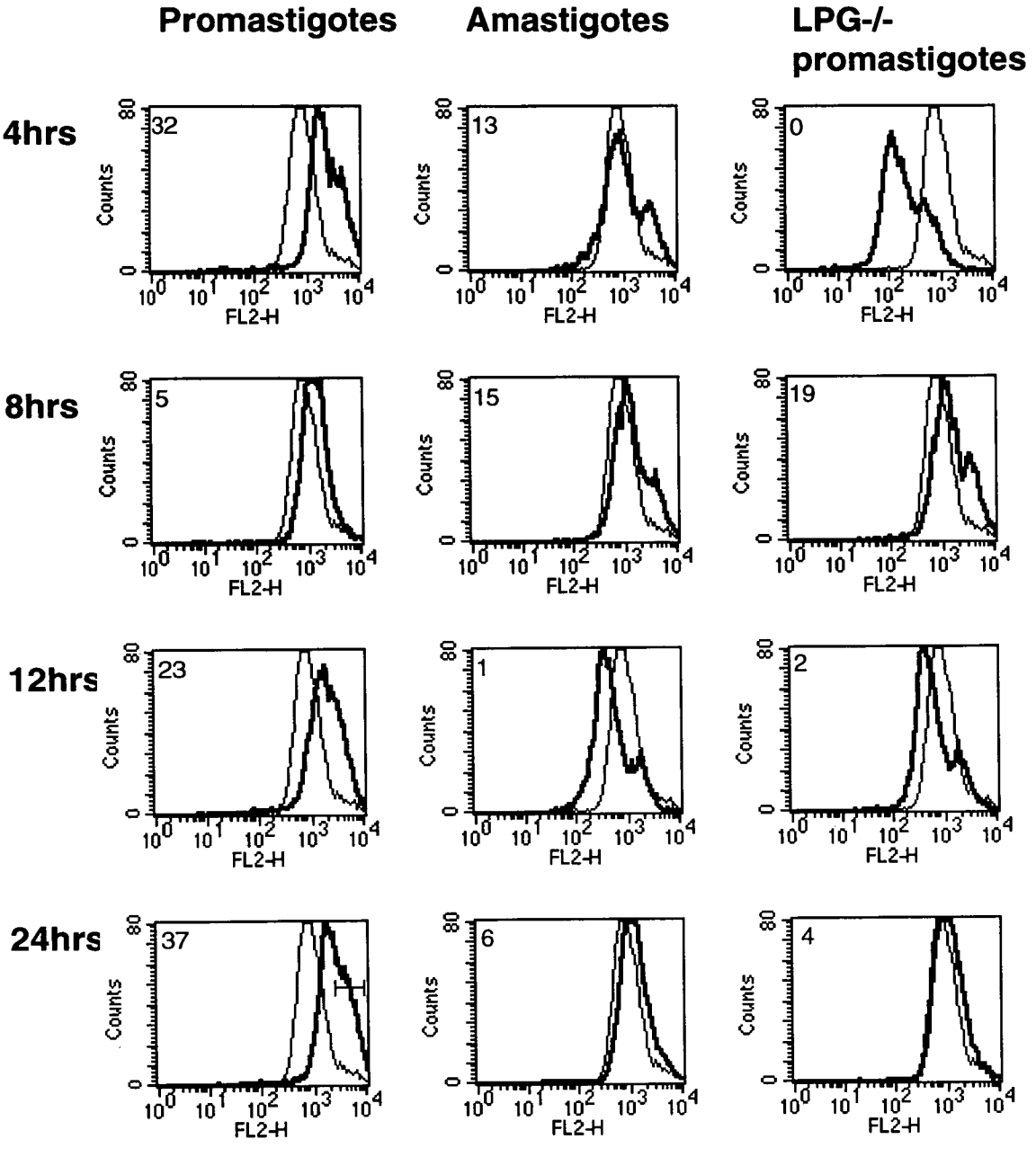
Figure B.1B: MHC II Expression on *L. mexicana*-Infected D1Cells Used for the GeneChip® Analysis



D1 cells were infected with *L. mexicana* promastigotes, amastigotes, or LPG$^{-/-}$ promastigotes for the times indicated. Cells were then harvested, fixed and stained for surface MHC II. Histograms show non-infected D1 cells at 4 hours (thin lines) and cells incubated with *Leishmania* parasites for 4, 8, 12 or 24 hours (thick lines).

Numbers represent the percentage of activated cells based on a marker set on activated cells at 24 hours.

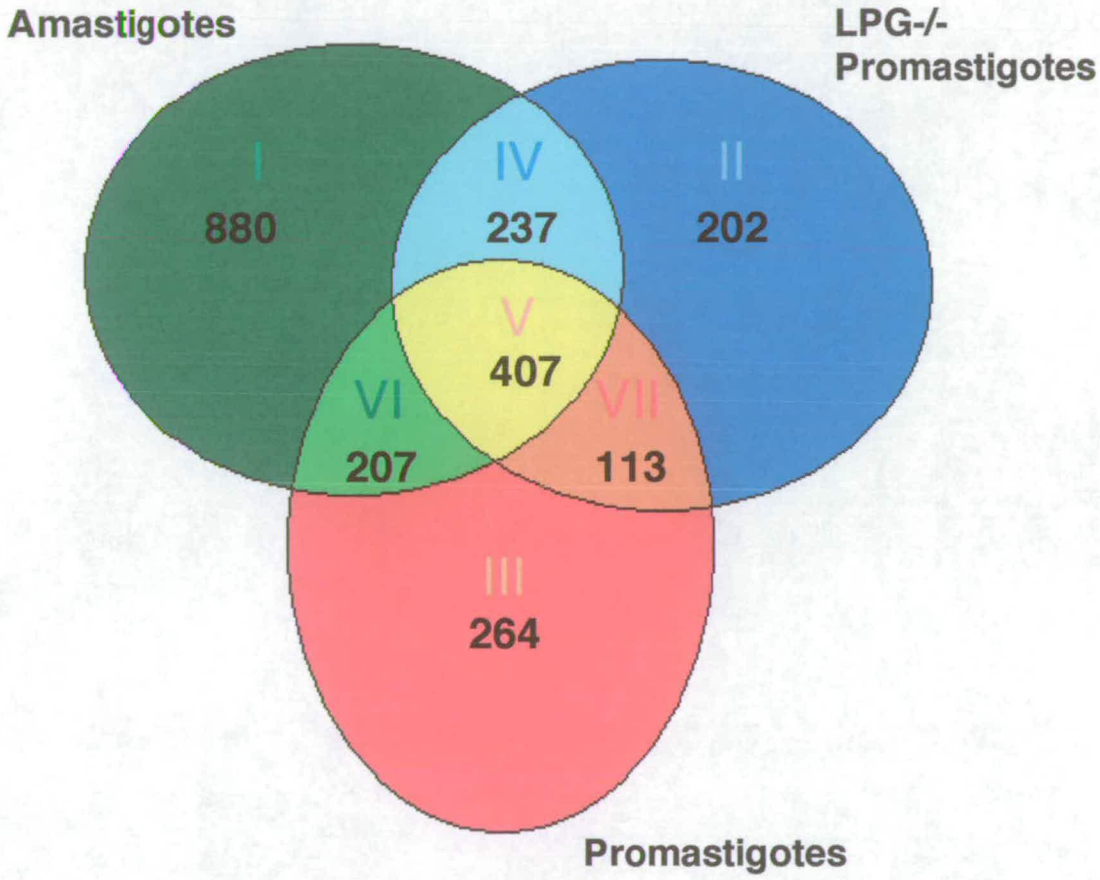
Figure B.1C: CD54 Expression on *L. mexicana*-Infected D1 Cells Used for the GeneChip® Analysis



D1 cells were infected with *L. mexicana* promastigotes, amastigotes or LPG$^{-/-}$ promastigotes, for the times indicated. Cells were then harvested, fixed and stained for the surface activation marker CD54. Histograms show non-infected D1 cells at 4 hours (thin lines) and cells incubated *Leishmania* parasites for 4, 8, 12 or 24 hours (thick lines).

Numbers represent the percentage of activated cells based on a marker set on activated cells at 24 hours.

Figure B.2: Diagram Showing Correlations Between Genes Expressed by Wild Type *L. mexicana* Promastigote-, Amastigote- or LPG$^{-/-}$ Promastigote-infected D1 Cells.



Venn diagram showing the correlation between gene expression of D1 cells incubated for 4, 8, 12 or 24 hours with wild type *L. mexicana* promastigotes, amastigotes or LPG$^{-/-}$ promastigotes.

Numbers represent the number of pooled genes in each subset, expression of which is regulated by three fold or more upon incubation with promastigotes, amastigotes or LPG$^{-/-}$ promastigotes.

cytokines, 880 D1 genes were regulated in an amastigote-specific manner. This is much higher than the number of differentially regulated genes in the promastigote-specific groups. It is also significant that incubation of D1 cells with $LPG^{-/-}$ parasites results in the differential expression of 202 genes. This implies that interactions between D1 cell molecules and *L. mexicana* LPG have an important modulatory effect on the cell.

Figure B.3 shows the results from hierarchal clustering of genes regulated on incubation with *Leishmania* parasites. This analysis was performed as described by (Granucci *et al.* 2001). These diagrams illustrate the differential expression of groups of genes in the three infection groups, demonstrating the extent to which D1 cells respond to infection by *L. mexicana* promastigotes, amastigotes or $LPG^{-/-}$ promastigotes at the level of gene transcription.

The results discussed in this appendix are preliminary and the data awaits further analysis. However, initial analyses indicate that the microarray data supports the results presented in Chapters 4, 5 and 6; surface activation markers are not transcriptionally up-regulated upon incubation of D1 cells with *L. mexicana* amastigotes or $LPG^{-/-}$ promastigotes, and infection with wild type *L. mexicana* promastigotes specifically up-regulates expression of the IL-12 p40 gene. These data also indicate that, while infection with amastigotes or $LPG^{-/-}$ promastigotes appeared to be silent according to the parameters tested, hundreds of genes are regulated in response to these interactions, and it is evident that the parasites are not merely behaving as inert particles. However, it remains to be seen whether any of these responses are related to the activation of DC, and the efficacy with which they can initiate the anti-*Leishmania* T cell response.

Hierarchal clusters showing the different types of gene expression profiles obtained. For each group the kinetic points (0, 4, 8, 12, and 24) are represented from left to right. The label above the upper left corner of each box identifies the cluster and the number above the upper right corner indicates the number of genes in each cluster. Genes were clustered as described by Granucci *et al.* (2001). A filter was used to examine only those genes which showed a difference in expression of at least 3 fold between the maximum and minimum kinetic expression values.

Colour changes from black to red as expression increases.

Figure B.3: Hierarchal Clusters Showing Gene Expression Profiles Obtained from Infection of D1 Cells with Wild Type *L. mexicana* Amastigotes, Promastigotes or LPG$^{-/-}$ Promastigotes

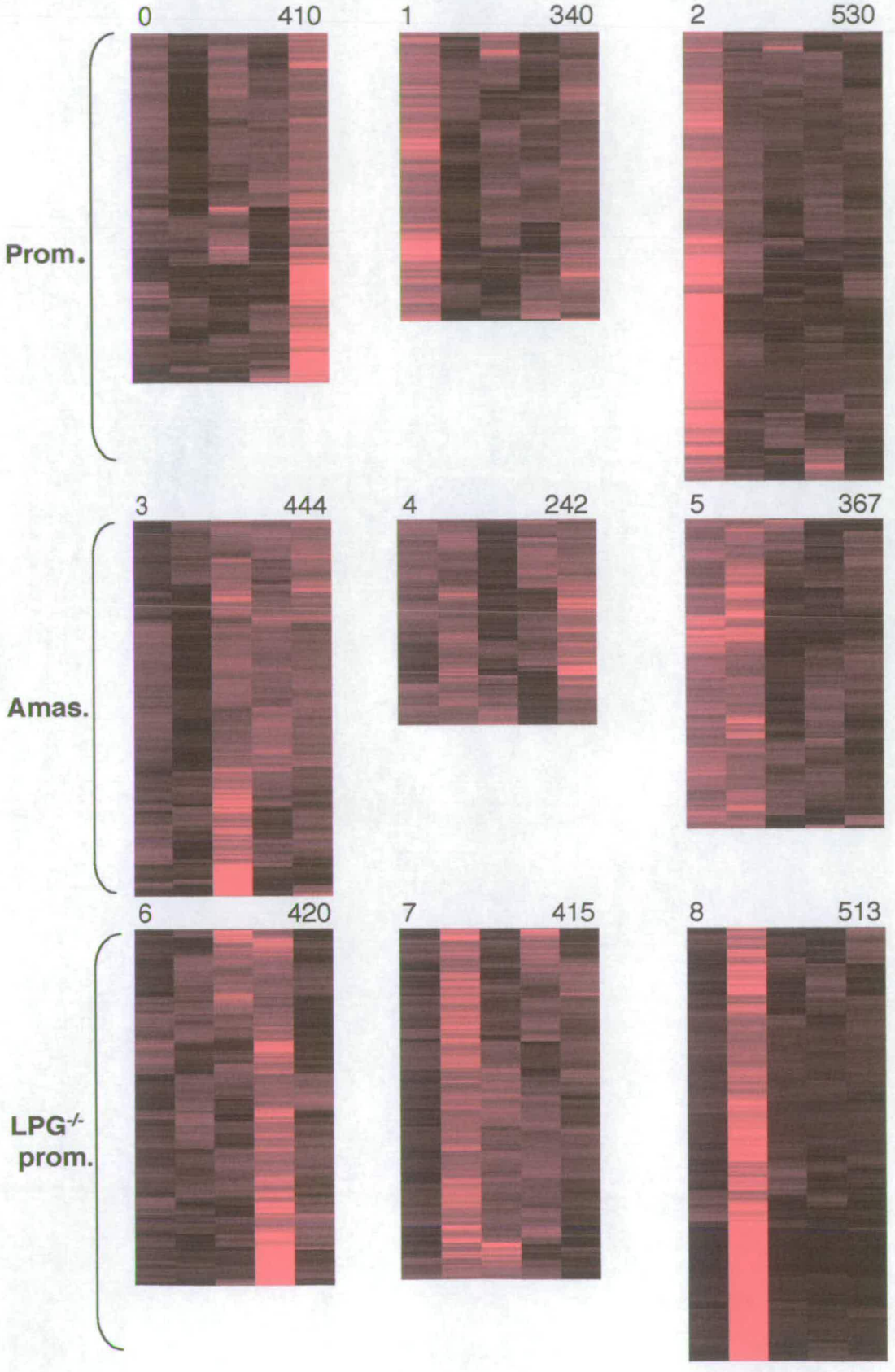


Table 1. Infection of BM-DC by *L. mexicana* amastigotes does not induce secretion of IL-12 (p40)[a]

		IL-12 (p40) pg/ml
BM-DC alone		43.4 ± 15
+	LPS + IFN-γ	$3.6 \times 10^4 \pm 1 \times 10^4$
+	Amastigotes[b]	36.1 ±11
+	Amastigotes + LPS + IFN-γ	$2.7 \times 10^4 \pm 9 \times 10^3$
+	Promastigotes[b]	341.0 ± 112
+	Opsonized promastigotes	632.7 ± 184
+	Latex beads	ND

a) Day 7 BM-DC (n = 4) were incubated for 18 h with *L. mexicana* amastigotes, promastigotes, or latex beads, with or without 1 μg/ml LPS and 10^3 U/ml IFN-γ. The p40 subunit of IL-12 was measured in 18 h supernatants by ELISA. The sensitivity of the assay was 30 pg/ml. Values represent concentrations of cytokine in supernatants from ~ 2×10^5 cells (mean ± SEM).
b) Amastigotes *vs.* promastigotes: $p = 0.035$.

DC activation. Our data indicate that axenic *L. mexicana* amastigotes do not activate BM-DC, but present to DC as inert particles similar to latex beads during infection *in vitro*. Incubation with promastigotes results in the activation of a fraction of BM-DC, which is not increased by parasite opsonization with 5 % mouse serum. However, the majority of promastigote-infected BM-DC retain an immature phenotype, suggesting that the promastigote surface is also devoid of BM-DC-activating signals. In contrast to the situation documented in MΦ, where infection suppresses subsequent activation [4, 6, 18, 20], BM-DC incubated with either promastigotes or amastigotes could be fully matured upon addition of LPS and IFN-γ, indicating that *L. mexicana* do not suppress the ability of DC to respond to activating stimuli.

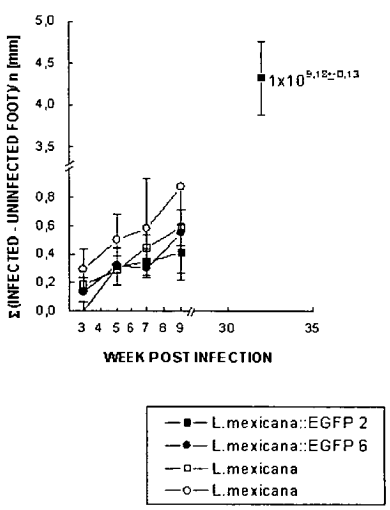


Fig. 5. Infection of EGFP-expressing parasites *in vivo*. C57BL/6 mice were infected with two clones of *L. mexicana:* EGFP (filled symbols) or two wild-type clones (open symbols) in the left hind footpads (10^7 promastigotes). Thereafter lesion development in this mouse strain (left part) was monitored at biweekly intervals, commencing at week three. Lesion size is also shown for BALB/c mice (right part) 32 weeks post-infection, by which time all animals had developed large, non-healing lesions. Data points refer to mean lesion size ± SD; numbers refer to the geometric mean of the parasite burden in the lesions at 32 weeks ± SD.

Activation of immature DC is believed to be triggered by "danger signals". These include conserved structural motifs on pathogenic organisms which are recognized by specific pattern recognition receptors [11–13], and host-derived factors. The list of pattern receptors currently includes members of the Toll-like receptor family involved in recognition of bacterial lipopolysaccharide or lipoteichoic acids [21–23], the multi-lectin family including DEC-205 [24] and mannose receptors [25, 26], and the presumptive receptor for unmethylated CpG islands [27]. In addition, pathogens can be opsonized by soluble factors such as complement and immunoglobulins, and sampled by DC that express complement (CD11b and c/CD18) and Fc receptors [28–30]. Since the surface of different *Leishmania sp.* is understood in some detail, our results can be considered in the context of the molecular composition of these surfaces, and their known and potential interactions with different classes of pattern receptors.

The surface of promastigotes is dominated by phosphoglycans, in particular lipophosphoglycan, and the glycoprotein GP63. These molecules activate complement via the alternative or the MBlectin pathway (for review see [31, 32]). Binding of cultured promastigotes to host cells via these surface molecules is mediated by CD11b and c/CD18 (directly to the lectin domain or indirectly, if opsonized, through the iC3b binding site) and mannose receptors [33–35]. Binding of these receptors appears not to change the expression of DC surface activation markers (this study; [14, 36]) implying that engagement of the mannose receptors, and of CD11b and c/CD18 (after reacting with 5 % serum; see [36]), by *Leishmania sp.* promastigotes is sufficient for triggering phagocytosis but does not cause DC maturation and activation.

Konecny and colleagues have reported that incubation of $CD11c^+$ splenic DC with *L. major* promastigotes increased IL-12 production [14], while von Stebut et al. infected Langerhans cell-like foetal skin-derived DC (FSDDC) with *L. major* promastigotes, but did not note IL-12 expression [36]. In our hands selection with CD11c

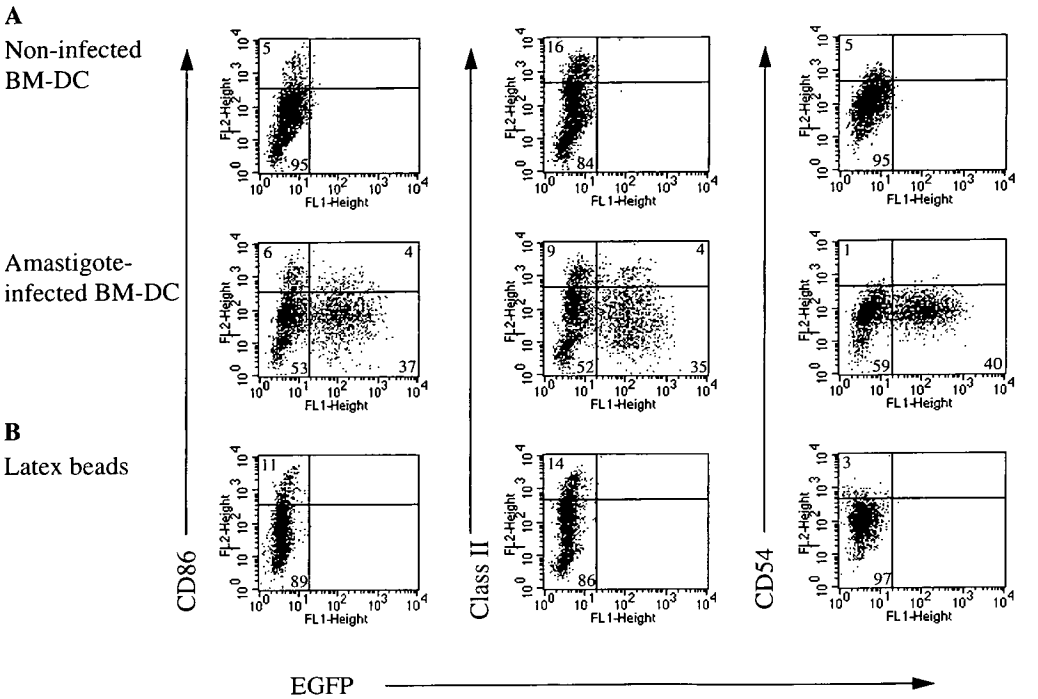


Fig. 3. Infection by *L. mexicana* amastigotes does not activate BM-DC. (A) Replated day 7 BM-DC were incubated with EGFP-expressing amastigotes for 18 h, fixed and stained with antibodies against DC activation markers. (B) "Infection" of BM-DC with 3.0-μm latex beads. The results are representative of at least five different experiments.

induced low levels of IL-12 production, which were significantly higher than those produced by amastigote-infected cells, consistent with the activation of a small percentage of the population (Fig. 2). These data support those presented in Sect. 2.4.

3 Discussion

In this study, we have investigated the interaction between *L. mexicana* parasites cultured *in vitro* and BM-DC, choosing to use this *in vitro* model in order to define the extent to which parasite-derived factors contribute to

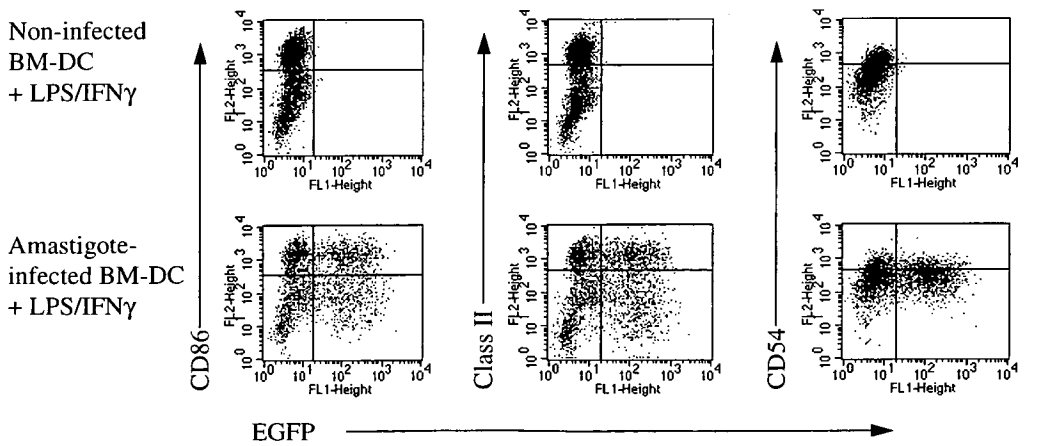


Fig. 4. L. mexicana amastigotes do not suppress activation of BM-DC. Day 7 BM-DC were incubated for 18 h with 1 μg/ml LPS and 10^3 U/ml IFN-γ, with or without EGFP amastigotes, then fixed and stained for DC activation markers. These results are representative of at least five different experiments.

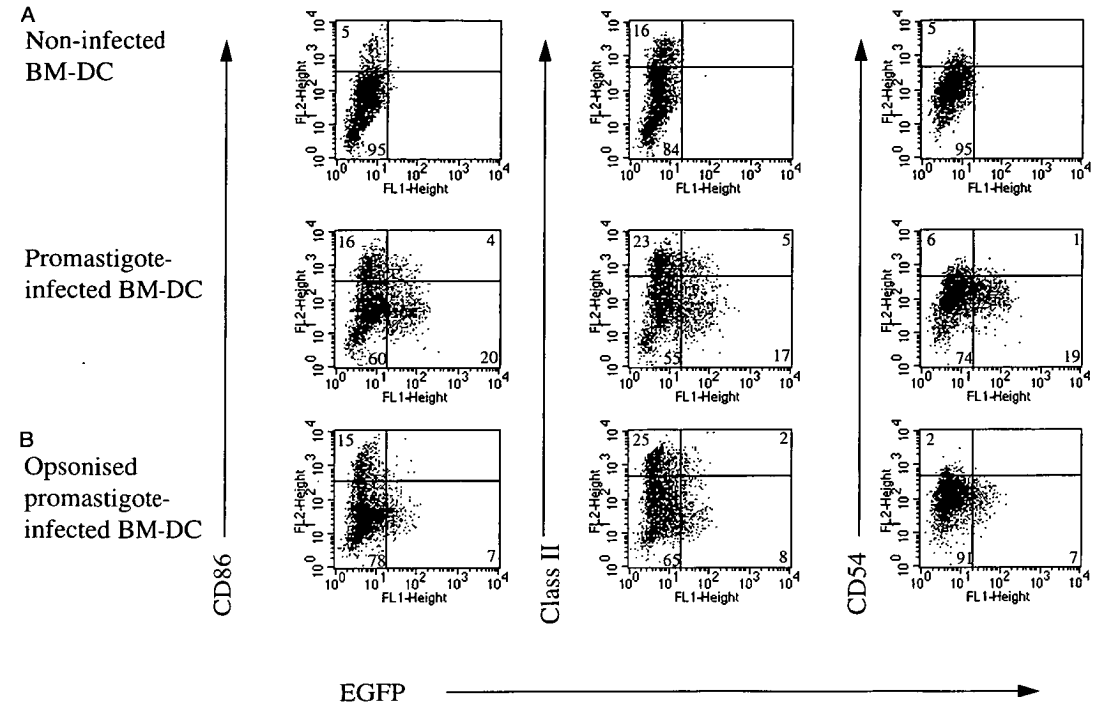


Fig. 2. Infection with EGFP-expressing *L. mexicana* promastigotes or opsonized promastigotes activates a small percentage of BM-DC. Replated day 7 BM-DC were incubated with promastigotes or opsonized promastigotes for 18 h, then fixed and stained with antibodies against DC activation markers. These results are representative of at least five different experiments.

2.3 Infection with *L. mexicana* amastigotes

The effect of *L. mexicana* amastigotes on BM-DC was investigated by harvesting day 7 BM-DC and replating with axenic amastigotes. BM-DC infected with *L. mexicana* amastigotes retained an immature phenotype and did not up-regulate expression of the DC activation markers CD86, CD54 or MHC class II (Fig. 3 A). In this respect, the *L. mexicana* amastigotes behaved similarly to latex beads, phagocytosis of which also did not activate BM-DC (uptake of latex beads was confirmed by microscopy; Fig. 3 B). Infected BM-DC were stained with an anti-amastigote serum to confirm that the parasites were truly intracellular and not attached to the surface of the cell; fluorescence was seen only in permeabilized cells (data not shown).

2.4 *L. mexicana* infection does not suppress activation of immature BM-DC

Infection of MΦ by *Leishmania sp.* was previously shown to suppress up-regulation of co-stimulatory molecules [18] and production of IL-12 [6]. Immature DC were therefore given LPS/IFN-γ at the time of infection, or at various times thereafter, to establish whether *L. mexicana* promastigote or amastigote-infected BM-DC were similarly refractory to activation. In all cases, this induced activation of infected BM-DC to levels equivalent to those seen in LPS/IFN-γ-activated, non-infected BM-DC (Fig. 4 and data not shown). Infected BM-DC could also be activated by lower concentrations of these stimuli (data not shown). Thus, *L. mexicana* did not actively suppress activation of BM-DC.

2.5 Effect of *L. mexicana* infection of IL-12 secretion by BM-DC

We also investigated the effect of these parasites on IL-12 production by BM-DC. Day 7 BM-DC were incubated with parasites for 18 h with or without LPS and IFN-γ, after which the supernatants were collected and assayed by ELISA for production of IL-12. Table 1 shows that LPS/IFN-γ-activated, but not immature, BM-DC secreted high levels of IL-12, as expected [19]. Immature BM-DC infected by *L. mexicana* amastigotes did not secrete IL-12, although addition of LPS/IFN-γ resulted in production of IL-12 by infected cells. Incubation of BM-DC with promastigotes or opsonized promastigotes

to dissect the parasite:DC interaction through use of the highly controlled *in vitro* system afforded by *L. mexicana* parasites and bone marrow-derived DC (BM-DC), which permits discrimination between true parasite-mediated effects and effects mediated by host-derived factors associated with infection. Specifically, we have investigated whether *L. mexicana* promastigotes or amastigotes provide activation signals for immune BM-DC.

2 Results

2.1 Phenotypic characterization of BM-DC

Immature DC, derived from bone marrow precursors by *in vitro* culture with GM-CSF, express markers consistent with the immunophenotype of myeloid lineage DC [15]. *Leishmania* exhibit a strong tissue tropism for MΦ, and care was therefore taken to ensure high levels of purity in harvested BM-DC (>70% BM-DC). Fig. 1 shows the phenotype of BM-DC harvested after 7 days of culture, and replated for a further 18 h with or without LPS and IFN-γ. Staining with T cell (CD3) and MΦ (F4/80) markers showed no contamination with these cell types. Staining with B220 revealed a small population of B cells, which were excluded from subsequent analyses by gating. Further purification of the BM-DC, *e.g.* by enrichment for $CD11c^+$ cells, resulted in maturation of the BM-DC population upon replating (data not shown), and therefore was not used in this study.

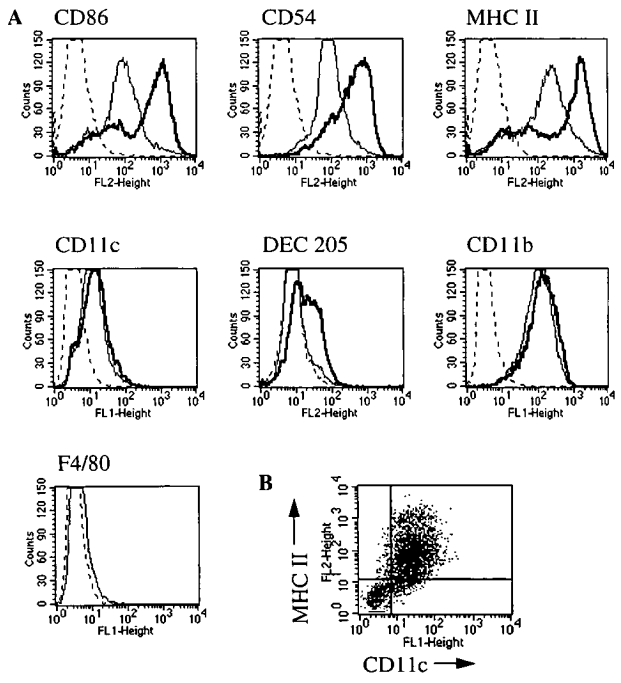


Fig. 1. Immunophenotype of day 7 BM-DC. BM-DC were harvested on day 7, replated and incubated for a further 18 h with or without activation stimuli. (A) Thin lines represent immature BM-DC and thick lines show BM-DC activated for 18 h with 1 μg/ml LPS and 10^3 U/ml IFN-γ. Dotted lines are negative controls. Small and dead cells were excluded from the plots by gating. (B) Day 7 BM-DC are positive for both CD11c and MHC class II.

2.2 Infection of BM-DC by *L. mexicana* promastigotes

Promastigotes are injected by the sandfly into the host epidermis, where they become opsonized, enhancing uptake by the phagocyte [16]. We therefore investigated whether infection with promastigotes or opsonized promastigotes could provide a maturation signal for immature BM-DC. To this end, green-fluorescent parasites were generated by integrating the enhanced green fluorescent protein (EGFP) gene into a ribosomal RNA locus of *L. mexicana* by homologous recombination [17]. Briefly, a DNA cassette containing the EGFP reporter gene fused to the *L. mexicana* cysteine protease B 2.8 intergenic gene region, and followed in tandem by the hygromycin-resistance gene fused to a copy of the same intergenic region, was integrated into a genomic small subunit rRNA locus. Drug-resistant clones expressing high levels of EGFP were selected. The recombinant parasites expressed high levels of EGFP as both promastigotes and amastigotes, and expression levels between axenic amastigotes and lesion-derived amastigotes remained similar for at least 10 months post-infection.

Day 7 BM-DC were incubated for 18 h with stationary-phase EGFP-promastigotes. Flow cytometric analysis of this population (Fig. 2 A) revealed that the majority of cells (85–90%) remained immature, and that the immature population contained both infected and non-infected BM-DC. A small but consistent percentage (10–15%), consisting mainly of uninfected cells, up-regulated the surface activation markers CD86 and MHC class II. Opsonization of the promastigotes with 5% mouse serum did not significantly increase the proportion of activated cells in the population (Fig. 2 B). These data indicate that infection with *L. mexicana* promastigotes did not stimulate BM-DC to mature within the 18-h period investigated. However, promastigotes, or promastigote-derived material, could occasionally activate immature BM-DC. The lower levels of GFP fluorescence observed in experiments using opsonized compared to non-opsonized promastigotes was probably due to enhanced degradation of opsonized parasites.

Silent infection of bone marrow-derived dendritic cells by *Leishmania mexicana* amastigotes

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Resolution of infection by *Leishmania sp.* is critically dependent on activation of $CD4^+$ T helper cells. Naive $CD4^+$ T helper cells are primed by dendritic cells which have responded to an activation signal in the periphery. However, the role of *Leishmania*-infected dendritic cells in the activation of an anti-*Leishmania* immune response has not been comprehensively addressed. Using the highly controlled model system of bone marrow-derived dendritic cell infection by *Leishmania mexicana* cultured *in vitro*, we show that uptake of *L. mexicana* parasites does not result in activation of immature dendritic cells or secretion of IL-12. Incubation with *L. mexicana* promastigotes results in the activation of a small percentage of dendritic cells which do not appear to contain whole parasites. Activation of dendritic cells is not suppressed by infection, since infected cells can be fully activated on addition of activating stimuli. Therefore, uptake of intact *Leishmania mexicana* parasites is not sufficient to activate dendritic cells *in vitro*. We propose that these data provide a basis for interpreting the interactions between dendritic cells and all *Leishmania sp.*

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1 Introduction

The protozoan parasites *Leishmania sp.* are the causative agents of a spectrum of diseases in humans. They undergo a digenetic lifecycle: the promastigote form resides in and is transmitted by its insect vector, the sandfly, while the amastigote form is an obligate intracellular resident of host monocytic phagocytes [1]. MΦ are the predominant host for *Leishmania sp.* in infected mammals and are also the principal effector cells in the clearance of intracellular parasites, through the activation of nitric oxide synthase [2, 3]. However, *Leishmania* infection itself does not appropriately activate MΦ [4–6] and resolution of the infection is entirely dependent on the presence of MΦ-activating type 1 phenotype parasite-specific $CD4^+$ T helper cells [7].

The *Leishmania*-specific T cell response is thought to be initiated by dendritic cells (DC), since parasites and parasite-derived material can be detected by immunohistochemistry in DC in the LN [8] and spleen [9] of infected mice. Some of these DC populations stimulate specific T cells *in vitro* ([8]; T.A., unpublished data). DC must mature to strongly stimulate T cells. Tissue DC are mostly immature, but are actively phagocytosing and processing antigens for loading onto largely intracellular MHC class II molecules. Activation signals stimulate DC to undergo dramatic phenotypic changes, which result in the redistribution of peptide-loaded MHC II molecules to the cell surface, up-regulation of co-stimulatory molecules, expression of cytokines such as IL-12, and down-regulation of phagocytosis. The maturing DC migrate to T cell areas of the draining LN where they can activate specific naive $CD4^+$ T helper cells [10].

It has recently been suggested that DC provide a link between the innate and adaptive immune responses, and that recognition of pathogen-derived "danger" signals activates DC and consequently enables them to stimulate appropriate T cell responses [11–13]. However, this emerging paradigm has not been rigorously investigated for complex pathogens such as protozoa and macroparasites. Several studies have reported effects of *Leishmania* infection on DC maturation [14]; however, these do not yet comprise an exhaustive survey of DC infection by different *Leishmania sp.* and moreover, are open to alternative interpretations. Here, we have sought

[I 20820]

The last two authors contributed equally to the work.

Abbreviations: DC: Dendritic cell **BM-DC:** Bone marrow-derived dendritic cell **EGFP:** Enhanced green fluorescent protein

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antibodies is by itself an activation signal, possibly because of extensive cross-linking, and phagocytosis of the parasites may provide some synergy at the level of IL-12 expression, providing a possible explanation for the discrepancy between these results. In our study, a small percentage of BM-DC incubated with *L. mexicana* promastigotes was activated. These cells did not exhibit green fluorescence, indicating that they harbored parasite debris rather than live parasites. This is supported by the fact that incubation with promastigote lysates activates a small fraction of BM-DC, equivalent to that seen on incubation with whole parasites (CLB, CCB unpublished data). We therefore suggest that intracellular proteins released as a result of lost parasite integrity may be responsible for the observed BM-DC activation by promastigotes. Precedent for this is suggested by a previous report that LelF, a leishmanial homologoue of eukaryotic initiation factor 4A, could induce monocyte-derived DC activation [37]. Liberated parasite DNA could also contain sequences with CpG activity, though we have thus far been unable to demonstrate this for *L. mexicana* genomic DNA (T. A., unpublished data).

The surface of axenic *L. mexicana* amastigotes [38] has previously been characterized, and is covered with a small GPI-anchored glycolipid, EpiM3. EpiM3 contains mannoses and an ethanolamine-phosphate group, but no equatorial OH groups to engage mannose receptors [39]. Thus, cultured amastigotes do not bind mannose receptors, CR3 or FcRγII [40]. However, although the receptors involved in their uptake remain elusive, cultured *L. mexicana* amastigotes are clearly phagocytosed by BM-DC: our data indicate that they do not encode surface-associated "danger" signals capable of activating BM-DC. Infection with lesion-derived *L. major* amastigotes has, in contrast, been shown to induce CD40, CD54, CD80 and CD86 as well as IL-12 secretion in FSDDC [36]. Lesion-derived parasites are opsonized by complement and Ig [40–42] and are co-purified with other host proteins present in the inflamed tissue [38]. Therefore, their interaction with DC is mediated at least by complement receptors and Fc receptors, ligation of the latter of which has been shown to induce DC maturation [28].

In murine models of Leishmaniasis the place of DC-parasite encounter is known to bias the development of DC-induced effector T cell populations: in experimental visceral leishmaniasis, intravenous injection of *L. donovani* leads to IL-12 expression in splenic DC harboring parasite material [9], and apparently, a type 1, though not protective, T cell response [43]. We propose that intact *Leishmania sp.* promastigotes and amastigotes lack adjuvant activity. An important consequence of our findings is that, in the absence of such activity, DC maturation and activation during experimental *Leishmania* infection *in vivo* will be mediated by host-derived factors, such as cytokines released due to the trauma of injection or biting by the sand fly vector, and the array of receptors involved in the interaction. These are likely to depend on the microenvironment in which DC interaction with the parasites occurs, for instance, whether the parasite inoculum has been fully exposed to serum components, or whether the locale is conditioned by components present in the insects' saliva [44–46].

4 Materials and methods

4.1 Mice

Six- to eight-week-old female CBA mice were obtained from a colony maintained at the Centre for Genome Research animal facility.

4.2 Parasite cultures

L. mexicana (strain MNYC/BZ/62/M379) promastigotes were cultured *in vitro* in semi-defined medium/10 % heat inactivated FCS at 26 °C. Amastigotes were cultured axenically at 34 °C in Schneider's *Drosophila* medium (Gibco BLR) supplemented with 20 % heat-inactivated FCS and 3.9 g/l 2-(N-morpholino)ethanesulphonic acid (Sigma, GB). EGFP-expressing parasites are virulent upon infection *in vivo* (Fig. 5) with a tendency to slightly delayed lesion development compared to wild-type in C57BL/6 mice, which show intermediate resistance when infected with *L. mexicana*. At 32 weeks post-infection the parasite burden in lesions in fully susceptible BALB/c mice is identical to that reported for wild-type infections [47], indicating no gross difference in virulence between wild-type and transgenic parasites [17].

4.3 Isolation and propagation of BM-DC

BM-DC were cultured *in vitro* according to a method adapted from Inaba et al. [48]. Briefly, BM-derived white cells were plated at a density of 3.75×10^5/ml in RPMI (Sigma) supplemented with 10 % FCS, 2 mM glutamine/pyruvate and 10 ng/ml murine GM-CSF (Peprotech), in 24-well plates, at 1 ml/well, and incubated at 37 °C. The cultures were washed on days 3 and 6 to remove nonadherent granulocytes and lymphocytes. On day 7 loosely adherent BM-DC were removed by more vigorous washing and replated, to exclude firmly adherent MΦ from subsequent culture. Where necessary, DC were stimulated for 18 hours with 1 μg/ml *E. coli*-derived LPS (Sigma) and 10^3 U/ml IFN-γ (R&D systems).

4.4 Infection of BM-DC with *L. mexicana* parasites and phagocytosis of latex beads

Day 7 BM-DC were incubated with well-washed *L. mexicana* amastigotes, stationary-phase promastigotes, or latex beads, at a ratio of 1:8, for 18 h at 37 °C. We used 3.0-μm beads (Sigma) since this is approximately the size of an amastigote. Stationary-phase promastigotes were opsonized by incubation with 5 % normal mouse serum at 37 °C for 15 min, followed by thorough washing. BM-DC were then harvested and fixed (Sect. 4.5) before staining, and the supernatant was stored frozen at −70 °C for analysis for IL-12 content.

4.5 Flow cytometry

BM-DC were harvested into 5-ml polypropylene tubes (Becton Dickinson), washed with PBS/10 % FCS, then stained using standard protocols. For intracellular staining cells were fixed and permeabilized using a Fix & Perm kit (TCS Biological Ltd). The following mAb were used: GL1-PE, specific for CD86; 14-4-4S-PE, specific for I-E^k; HL3-FITC, specific for CD11c; anti-B220-FITC; anti-CD3-PE (all from Pharmingen); KAT-1-PE, specific for ICAM1 (Cambridge Bioscience) and anti-F4/80-FITC (Caltag). Appropriate isotype controls were also used. An anti-DEC-205 antiserum (Serotec) was used with an anti-rat-PE secondary Ab (Pharmingen). Intracellular infection by amastigotes was confirmed with an anti-*L. mexicana* rabbit serum, generated by immunization of rabbits with *L. mexicana* promastigotes, and visualized using an anti-rabbit-FITC secondary Ab (Sigma). DC were fixed and blocked before staining using 2.4G2 (Fc Block™, Pharmingen) and 10 % normal mouse serum. Flow cytometric analysis was performed using a FACScan (Becton Dickinson) and analyzed using CellQuest 3.1 software.

4.6 Cytokine detection

The p40 subunit of IL-12 in culture supernatants was measured using a commercially available ELISA kit (Pharmingen). Statistical analyses were performed using the Student's *t*-test.

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AEP	Asparaginyl endopeptidase
APC	Antigen presenting cell
bp	Base pairs
BM-DC	Bone marrow-derived dendritic cells
CASM	Concanavalin A-stimulated medium
CCR	C-C chemokine receptor
CD40L	CD40 ligand
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxyfluoroscein diacetate succinimidyl ester
CLIP	Class II associated invariant chain peptide
CIIV	Class II-containing vesicle
CPB	Cysteine protease B
CR	Complement receptor
DC	Dendritic cells
DEPC	Diethylpyrocarbonate
DMSO	Dimethlysulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
dsRNA	Double stranded RNA
EGFP	Enhance green fluorescent protein
ELISA	Enzyme-linked immunosorbant assay
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FcR	Fc receptor
FITC	Fluoroscein isothiocyanate
GFP	Green fluorescent protein
GM-CSF	Granulocye/macrophage-colony stimulating factor
GPI	Glycosyl-phosphatidylinositol
GST	Glutathione S-transferase
HA	Haemagglutinin
HRP	Hydrogen peroxide
ICAM-1	Intercellular adhesion molecule-1
Ig	Immunoglobulin
Ii	Invariant chain
IL	Interleukin
IL-12R	IL-12 receptor
IFN	Interferon
IRAK	IL-1 receptor activated kinase
KLH	Keyhole limpet haemocyanin
LACK	*Leishmania* homologue of receptor of activated C kinase
LC	Langerhans cells
LN	Lymph node

LPB	Lipopolysaccharide-binding protein
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
LTA	Lipoteichoic acids
mAb	Monoclonal antibody
MΦ	Macrophage
MAC	Membrane attack complex
MACS	Magnet activated cell sorting
MBAP	Membrane-bound acid phosphatase
MCC	Moth cytochrome C
MCS	Multiple cloning site
MHC	Major histocompatibility complex
MIIC	MHC II-containing compartment
MIP	Macrophage inflammatory protein
MLR	Mixed lymphocyte reaction
MMTV	Mouse mammary tumour virus
MyD88	Myeloid differentiation factor 88
kb	Kilobase pairs
Kda	Kilodalton
NFκB	Nuclear factor κB
NK	Natural killer cells
NO	Nitric oxide
INOS	Inducible nitric oxide synthase
OVA	Ovalbumin-derived peptide
PAMP	Pathogen-associated molecular pattern
PBL	Peripheral blood lymphocytes
PCC	Pigeon cytochrome C
PCR	Polymerase chain reaction
PDBu	Phorbol-12,13-dibutyrate
PDG	Peptidoglycan
PE	R-phycoerythrin
PI	Propidium iodide
PNA	Peanut agglutinin
PNPP	p-Nitrophenol phosphate
PRR	Pattern recognition receptor
PS	Phosphatidyl serine
PV	Parasitophorous vacuole
RANTES	Regulated on activation, normal T cell expressed and secreted
RIL-12	Recombinant IL-12
RNA	Ribonucleic acid
RRNA	Ribosomal RNA
SAP	Shrimp alkaline phosphatase
SCID	Severe combined immunodeficiency
SDM	Semi-defined medium
SDM	Site-directed mutagenesis
SDS-PAGE	SDS-polyacrylamide gel elecrophoresis

SEA	Schistosome egg antigen
SLA	Soluble *Leishmania* antigen
SLC	Secondary lymphoid tissue chemokine
SSU	Small subunit
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
Th	T helper cell
Th1	T helper type 1
Th2	T helper type 2
TGF	Transforming growth factor
TIR	Toll/IL-1 receptor domain
TIRAP	(TIR)-domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF	TNF receptor associated factor
TRANCE	TNF-related activation-induced cytokine

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