NOTTINGHAM TRENT UNIVERSITY

Studies on Immunology of *Leishmania mexicana*

Hossein Rezvan

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Communications

Conferences:

Rezvan H, Ali SA and Rees RC

Leishmania Parasite Subunit Vaccine in HLA-A2 transgenic mouse model

13th Multi-disciplinary Iranian Researchers, 2nd and 3rd of July 2005, Leeds University, England

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CTL Activity Induced by Immunisation with Leishmania mexicana gp63 cDNA. DNA Vaccines Conference, May2007, Malaga, Spain.

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Development of a peptide-based sub-unit vaccine by selecting peptides from the essential surface glycoproteins of Leishmania parasites (gp63) using transgenic mouse models.

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Publications:

CTL activity in BALB/c immunised with *L. mexicana* gp63 cDNA using gene gun
Ali S Rezvan H, McArdle S, Rees R
School of Biomedical and Natural Science, Nottingham Trent University, Clifton Lane, Clifton, Nottingham, NG11 8NS
Email: selman.ali@ntu.ac.uk
Manuscript in preparation

Compression study of immunity induced by *L. mexicana* gp63 cDNA and Soluble Leishmania Antigen (SLA) in *L. mexicana* infection
Rezvan, McArdle, Rees R, Khodadadi A, Ali S.A,
School of Biomedical and Natural Science, Nottingham Trent University, Clifton Lane, Clifton, Nottingham, NG11 8NS
Email: selman.ali@ntu.ac.uk
Manuscript in preparation

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Abstract

Leishmaniasis is a worldwide disease prevalent in many tropical and sub tropical countries. Treatment of Leishmaniasis by chemotherapy is not wholly effective and is usually accompanied by unpleasant side effects. The development of an effective and inexpensive vaccine represents a practical way to control the disease, however at present no safe and effective vaccine is available.

In the first part of the present study, the immunity induced by four different *L. mexicana* potential vaccines, including killed leishmania vaccine, Soluble *L. mexicana* Antigen (SLA), *L. mexicana* gp63 cDNA and CT26 tumour cells transfected with *L. mexicana* gp63, were compared.

It was shown that DNA immunisation using *L. mexicana* gp63 generated the highest immunity to the parasite among the four tested vaccines where the killed leishmania vaccine and *L. mexicana* gp63 transfected CT26 tumour cells did not generate significant immunity.

The efficacy of DNA immunisation by intramuscular injection or using gene gun, in generating immunity to leishmania was compared. Gene gun immunisation induced more immunity to the parasite and high levels of Th1 immune response, which were detected, one week after immunisation through determination of the IgG2a levels in blood serum. Gene gun immunisation also induced long-lasting CTL activity, which was detectable before and during the course of infection for up to 6 months.

Immunogenicity of MHC class I restricted peptides derived from *L. mexicana* gp63 have been investigated. Using "SYFPEITHI" software, four peptides with high affinity to human HLA-A2 and four peptides with high affinity to mouse H2-L^d were predicted, synthesized and tested in HHD II and BALB/c mice respectively. Only three of the peptides predicted with high affinity to HLA-A2 were immunogenic but only two of them were likely to be naturally processed, however, none were protective in HHD II mice against leishmania infection.

Purification and application of OX40L, a ligand for T-cell co-stimulatory receptor, was investigated in *L. mexicana* BALB/c model. In addition to purification by protein A sepharose, the murine OX40L-IgG fusion protein produced by B9B8E2 cells (cells transfected with OX40L and IgG) was successfully purified by two novel resins, MBI & MEP. The biological activity of the OX40L-IgG purified by MBI resin was significantly higher than that of MEP or protein A sepharose resins. Application of OX40L-IgG resulted

in healing of leishmania lesions or delaying in development of the lesions in leishmania-infected mice.

Abbreviation

ALM	Autoclaved *L. major*
alum	Aluminium hydroxide
APC	Antigen Presenting Cells
B8	HLA-A2 restricted peptide with sequence of LLVAALLAV
BCA	Bicinchoninic Acid Kit for Protein Determination
BCG	Bacillus Calmette-Guerin
BSA	Bovine Serum Albumin
BT1	*L. donovoni* bioprotein transporter
C	constant region of T-cell receptors or immunoglobolins
C1	HLA-A2 restricted peptide with sequence of RLSLGACGV
C2	HLA-A2 restricted peptide with sequence of RLAAAGAAV
CCIEP	Counter Current Immunoelectrophoresis
CFA	Complete Freud's Adjutant
CL	Cutaneous Leishmaniasis
CLIP	Class II-Associated Invariant-chain Peptide
CM4	HLA-A2 restricted peptide with sequence of AAAGAAVTV
CP	*L. mexicana* Cystein Proteinase
CTL	Cytotoxic T Cells
DAT	Direct Agglutination Ttest
DC	Dendritic Cells
ds	double-stranded RNA
ELISA	Enzyme Linked Immunosorbent Assay
FAST	Fast Agglutination-Screening Test
FC	constant domain of antibodies
FCS	Fetal Calf Serum
G418	Geneticin
GBP	Gene B Protein
GCP	Good Clinical Practice
GLP	Good Laboratory Practice
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GMP	Good Manufactory Practice
gp63	Leishmania Zinc Metalloprotease

H_2O_2	Hydrogen Peroxide
HASPB1	Recombinant Acylated Surface Protein B1
HBr^-	Hypobromite
HCIC	Hydrophobic Charge Induction Chromatography
HHD II	HLA-A2 Transgenic Mice
HLA	Human Leukocyte Antigen
HPLC	High Performance Liquid Chromatography
I.D.	Intradermal
I.M.	Intramuscular
I.V.	Intravenous
IFA	Immunofluorescent Antibody
IFA	Incomplete Freud's Adjuvant
IFAT	Indirect Flourcent Antibody Test
IFN-γ	Interferon-γ
Ig	Immunoglobulins
IgG	Immunoglobulin G
IHA	Indirect Haemagglutination test
Ii	MHC class II-associated invariant chain
LPG	Lipophosphoglican
LPS	Lipopolysacaride
LST	Leishmanin Skin Test
MBLectin	Mannos-Binding Lectin
MCL	Mucocutaneous leishmaniasis
MHC	Major Histocompatibility Complex
MM1	mOX40-mIgG1
NK	Natural Killer cells
NKT	Natural Killer T cells
NO	Nitric Oxide
O_2^-	Superoxide anion
OCl^-	Hypochlorite
OH^*	Hydroxil radical
OX40L	OX40 ligand
PAMP	Pathogen-Associated Molecular Pattern

PCR	Polymerase Chain Reaction
PKDL	Post-Kala azar Dermal Leishmaniasis
PMNS	Polymorphonuclear Neutrophils
PSA-2	Surface Antigen complex2
PSI	Pounds Square Inch
PVP	polyvinylpyrollidone
S.C.	Subcutaneous
SLA	Soluble Leishmania Antigen
TAP1	Transports Associated with antigen Processing1
TAP2	Transports Associated with antigen Processing 2
TBS-T	TBS + 0.05% Tween 20
TCR	T-cell Receptor
Th1	T helper1
Th2	T helper2
TLRS	Toll-Like Receptors
TNF	Tumour Necrosis Factor
TNFR	Tumour Necrosis Factor Receptor
V	Variable region of T-cell receptors or antibodies
VL	Visceral Leishmaniasis

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Chapter 1 Introduction

1.1 Pathogens

Robert Koch in late 19th century was first described micro-organisms as the cause of infectious diseases [Sonnichsen, 1982]. Now it is known that the majority of micro-organisms are harmless and many of them are even beneficial and used in food industries and biotechnology. Only a small group of micro-organisms have properties to cause disease in mammalians, ”pathogens” [Somova & Pechurkin, 2005].

The term of pathogen is derived from a Greek word "pathos", which means “birth of pain or suffering”. A simple definition for pathogens is given as organisms that can dominate the host’s defence mechanisms and induce deleterious changes in the host [Basset et al., 2003] causing disease or illness to its host. Today pathogens are classified into four main categories; viruses, bacteria, fungi, and parasites. Viruses are obligate intracellular parasites, which can only replicate inside the living cells using the host cell’s metabolic machinery. Bacteria are single-cell micro organisms. Some bacteria are obligate pathogens that their lives are totally dependent on the host nutrients. Other pathogenic bacteria might have a free life in the environment; however, when they arrive into the host’s body they induce pathogenic effects in the host. Fungi are eukaryotic organisms which may be unicellular (yeast), multicellular or exist in both forms. Most pathogenic fungi are opportunistic pathogens. They can live freely in the environment and their host but only when the host’s immune system is weakened they can over grow and cause disease. Parasites are including two main groups, protozoan, and helmets.

Helmets are multicellular and usually macroscopic, which may have a size between 3 millimetres to 25 meters long. Many helmets can infect humans and animals causing serious disease or provoke allergic reactions by their persistent presence in host tissues.

Protozoan parasites are unicellular eukaryotic organisms. Some of these parasites can live freely in the environment as well as inside the host but some are obligate parasitic organisms, which need to live for a part or whole their life cycle inside a mammalian host. These parasites often have several life cycle stages; having sexual and asexual reproduction in different stages. The completion of some protozoan parasites' life cycle depends upon the insect or arthropod vectors to transmit them from one host to another.

1.2 Immune system and Immunity to pathogens

Our body is constantly exposed to pathogens in the environment and during infection. Pathogens are often sophisticated to cause disease in their hosts, however, majority of micro-organisms that daily encountered are detected and destroyed by the immune system within minutes or hours [Valiante et al., 2003]. The immune system, to defend against pathogens, has developed two main strategies, called innate and adaptive immune response.

1.2.1 Innate immune system

The innate immune system is the first line of immune defence, which does not rely on clonal expansion of antigen-specific effector cells and does not require prolonged induction phase. Although the innate immune system does not generate immunological memory, it can efficiently be activated immediately after a pathogenic invasion encountering and removing majority of pathogens and activating inflammatory mechanisms prior to the establishment of the infection. Therefore, only few among all pathogens, which enter the body, can cause disease [Valiante et al., 2003]. The innate immune system has many inhibitory properties against pathogens. The first line is the epithelia that acts as a physical barrier and comprise the skin and the epithelia surface of the internal organs, which called mucosal epithelia [Basset et al., 2003].

The cells engaged in the innate immune defence include epithelial cells, mast cells, phagocytic cells, such as macrophages and Polymorphonuclears, natural killer cells and dendritic cells [Basset et al., 2003].

1.2.1.1 Mononuclear and poly morphonuclear phagocytes

Macrophages, which are usually considered as the first cells encountering the pathogens, are differentiated from monocytes. Monocytes circulate in the blood stream and when they migrate into the tissues, they differentiate to macrophages. Most of pathogenic micro-organisms are immediately encountered by mononuclear phagocytes or “macrophages” that reside in tissues [Hume, 2006].

Polymorphonuclears including neutrophils are the second major family of phagocytes. They reinforce macrophages soon in the site of infection. Macrophages and neutrophils can recognize pathogens by means of their cell surface receptors. Ligation of these receptors leads to phagocytosis. Phagocytosis is an active process by which the phagocytes first recognize the microoganism and engulf it in a membrane-bound vesicle called phagosome.

In the next step the phagosome will be fused with one or more lysosomes creating a phagolysosome where the pathogen is attacked by lysosomal enzymes. In addition to lysosomal enzymes, macrophages and neutrophils also produce other toxic products, which help degradation of the engulfed pathogen. The main toxic product of macrophages and neutrophils is nitric oxide (NO). Superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). Other products are also involved including the hydroxyl radical (OH^*), the hypochlorite (OCl^-) and hypobromite (HBr^-) ions [Kobayashi et al., 2005; Mayer-Scholl et al., 2004; Raines et al., 2006].

1.2.1.2 Natural Killer Cells

Natural killer cells (NK cells) are normally accounted as a part of innate immune system. They develop from the CD34(+) haematopoietic progenitor cells and then migrate into the blood stream [Freud et al., 2006]. NK cells are often larger than lymphocytes and are characterized by the expression of NK receptors such as NKp46, NKp30, NKp44 and NKG2D as well as the CD56 surface antigen and the lack of CD3 [Moretta & Moretta, 2004; Smyth et al., 2001]. A subset of NK cells called natural killer T (NKT) cells constituting a subpopulation of lymphocytes expressing the NK receptors, CD56, CD3 and T-cell receptor (TCR) [Capone et al., 2003; Papamichail et al., 2004; Wajchman et al., 2004]. The presence of NK/NKT cells is crucial in the host's defence particularly against tumours and viral infection as they mainly act in early phases of immune response, before B cells and T cells generate an antigen-specific immunity [Papamichail et al., 2004]. The antigen recognition of NK cells is based on recognition of up regulation or down regulation of self- proteins such as MHC molecules in infected cells[Raulet, 2004]. Down regulation of MHC class I molecules in infected cells is shown to be an indicator by which NK cells recognize the infected cells. The interaction of dedicated receptors on NK cells and MHC molecules on target cells regulates the NK cells activity [Andrews et al., 2005].
The mechanisms of cytotoxicity applied by NK cells are similar to those of CD8+ T cells as they release cytotoxic granules such as perforin, which makes pores onto the target cell membrane or granzymes (trypsin, chymotrypsin, granulysin), which induce a programmed cell death via the surface of the target cells. NK cells also produce a set of Th1 and Th2 cytokines including IFN-γ, TNF-α, TNF-β, IL-10 and GM-CSF. It has been shown that cytokines produced by other immune cells like macrophages or DCs can also activate NK cells mainly via IL-18, IFN-αβ, IL-15, IL-2 and IL-12 [Ferlazzo & Munz, 2004; Papamichail et al., 2004].

1.2.1.3 DC cells

Dendritic cells (DC) were first described by Paul Langerhans in 1968. These cells are accounted as a part of innate immune system and can be derived from either or both of myeloid and lymphoid progenitors. They generate different sets of receptors (see1.2.1.4) for recognizing different sets of antigens [Burgdorf et al., 2006; Kadowaki et al., 2001]. DCs are most professional antigen-presenting cells (see 1.2.2.1.1) acting as a bridge between the innate and adaptive immune system [McCormick et al., 2006]. The main known function for DCs is to present the antigen to T cells. Therefore, they take up and process the pathogen and carry it away to the local lymph node where they present and activate naïve T cells. The local lymph nodes are the last destination for DCs where they eventually die. The antigen uptake and presentation ability of DCs are being developed during a process called maturation. Maturation of DCs cause up-regulation of MHC class I, class II molecules and co-stimulatory molecules such as CD40, CD83, CD80, and CD86 [Hoebe et al., 2004; Saalmuller, 2006; Villadangos et al., 2005].

The role of dendritic cells is particularly crucial in stimulation of T cell responses to viruses because not all viruses can induce co-stimulatory activities in other types of antigen presenting cells. Viruses bind to several molecules on the surface of dendritic cells and/or become engulfed but not destroyed by them. The viruses synthesize their particle using the DCs machinery and then the antigenic peptides of those viral proteins is presented to CD8+ T cells through MHC class I molecules [Yan et al., 2005].

1.2.1.4 Antigen recognition by cells of the innate immune system

The recognition of antigens by the innate immune system is based upon the detection of limited conserved patterned molecule on pathogens called “pathogen-associated molecular pattern (PAMP)” by pattern recognition receptors [Janeway & Medzhitov, 2002]. Microoganisms normally bear repeating patterns of molecular structures on their surface membrane or their DNA, for example some bacteria express lipopolysacaride (LPS), lipoproteins, peptidoglycan, lipoarabinomannan and oligosaccharides on their cell membrane or may contain repeats of dinucleotide CpG in their DNA. Viruses, on the other hand, almost invariably bear double stranded RNA as a part of their life cycles [Akira, 2006]. The innate immune cells including epithelial cells, macrophage-monocytes, granulocytes, mast cells and dendritic cells bear a series of receptor to recognize and bind to these PAMP; these receptors are sometimes called “pattern-recognition receptors”. Toll-

like receptors, scavenger receptors, manose-binding lectin, which activate complement; macrophage mannose receptor on the surface of macrophages are examples of pattern recognition receptors [Basset et al., 2003; Hoebe et al., 2004; Hornung et al., 2002; Lund et al., 2004].

1.2.1.4.1 Toll-like receptors

Toll-like receptors (TLRs) are a series of pattern recognition receptors expressed on the surface of monocytes/macrophages, dendritic cells, NK cells, B cells, neutrophils and at very low level on T cells that are used to recognize pathogens [Hayashi et al., 2003; Hornung et al., 2002]. Although the diversity of known TLRs in mammals is limited to 10, they still recognize a broad range of pathogens. The activation of these receptors leads to activation of both innate and adaptive immune responses through the induction of phagocytosis, and production of cytokines and chemokines. They also induce the up-regulation of MHC molecules and co-stimulatory molecules such as B7.1 (CD80) and B7.2 (CD86) [Iwasaki & Medzhitov, 2004; Takeda et al., 2003; Underhill & Ozinsky, 2002].
Each TLR is dedicated to recognize a certain set of molecular proteins, for instance in mammals, TLR-4 on macrophages in association with CD14 acts as a receptor for LPS [da Silva Correia et al., 2001]. The TLR9 is a sensor for the unmethylated DNA. The TLR3, although evolutionarily distant from TLRs 7, 8, and 9, is a sensor for double-stranded (ds) RNA [Crozat & Beutler, 2004]. Recent experiments have shown that all TLRs may act as a unique concert with multiple binding properties to acquire maximum sensitivity and specificity. The location at which different TLRs are expressed also influences which molecules they are likely to encounter [Crozat & Beutler, 2004; Lund et al., 2004].

1.2.1.5 Complement cascade

Complement is a system of plasma proteins that interacts with pathogens to either destroy them or mark them for phagocytosis. Complement was first discovered by Jules Bordes as an effector arm of the antibody response, however, it is now accounted as a part of innate immune system and can be activated even in the absence of antibodies. The complement system is made up of more than 30 distinct plasma proteins that react one to another in a cascade to opsonise pathogens inducing a series of inflammatory responses at the site of infection [Endo et al., 2006].

The complement system is activated through a triggered-enzyme cascade. In such a cascade, an active component enzyme generates by cleavage of its zymogene precursor. The active component in turn cleaves another zymogene precursor to its active form in the complement pathway. By this way, because of the amplification of each enzymatic reaction by another one, activation of a small number of complement proteins at the start of the complement pathway results in a rapid generation of a massive complement response.

Complement can be activated on the surface of pathogens through three distinct ways; the classical pathway, mannose-binding lectin pathway (MBLectin) and the alternative pathway [Gal & Ambrus, 2001; Seelen et al., 2005]. The initiation of each pathway depends upon the type of antigen that complement is activated by but they converge to generate the same set of effector molecules.

There are three effector mechanisms for complement action: First, complement system generates a huge amount of some of the activated complement proteins. These proteins can covalently bind to the surface of pathogens opsonising them to enhance engulfing by phagocytes, carrying receptors for complement proteins. Second, the small fragments of complement particles, because of their chemotactic properties, can recruit more of other phagocytes to the site of infections. Third, the terminal component of complement can create pores in the wall of certain bacteria to disrupt their membrane and damage them [Rus et al., 2005].

1.2.2 Adaptive immune system

Due to the vast variation in the pathogen that individuals encounter in their life, the innate immune system needs to recognize or eradicate all of them, therefore, a more complicated system is needed to defend against each pathogen individually. This is called “adaptive immune system” and the pathogens that bypass the innate immune system are encountered and destroyed. The innate and adaptive immune systems are complementary to each other as the innate immune system has a crucial role in priming adaptive immune response; if the innate immune system fails to control the infection, it initiates an acquired immune response (Figure 1-1). The cells involved in the adaptive immune system consist mainly of T and B-lymphocytes, which have different properties in antigen recognition and effector function. However, there is a degree of overlapping between them as dendritic cells which are accounted as a part of the innate immune system (see 1.2.2.1.1), have a very important and crucial role in the initiation of adaptive immune response as antigen presenting cells

[Howard et al., 2004]. Besides, macrophages, under the control of lymphocytes, become more activated in destroying the pathogens (see 1.4.2).

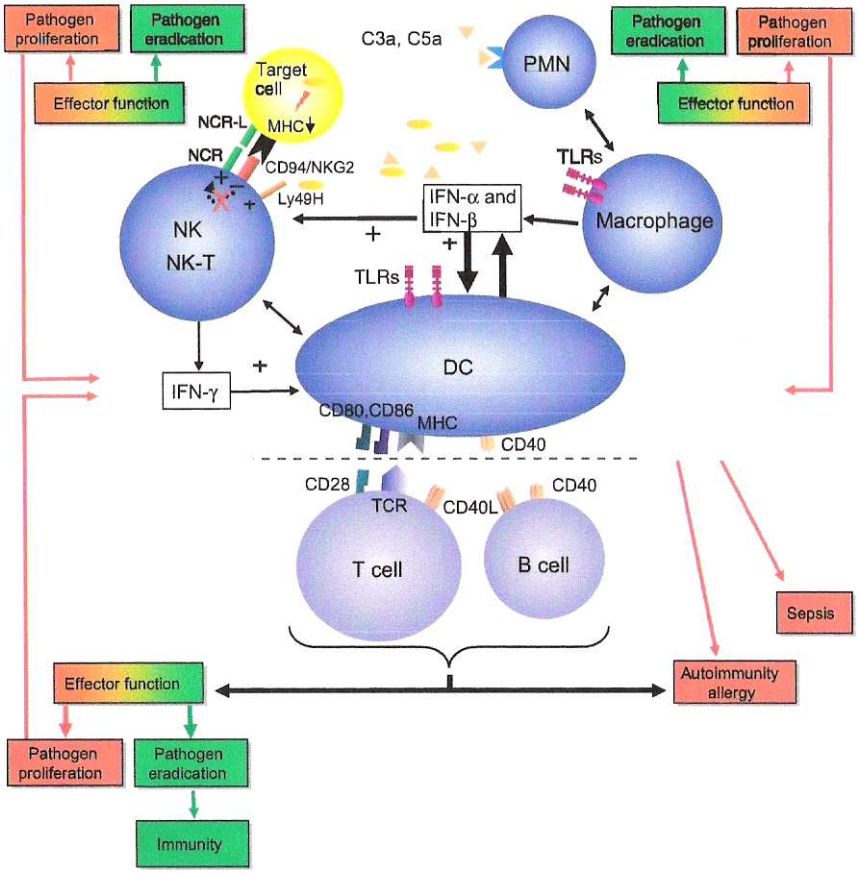


Figure 1-1 The interface between the innate and adaptive immune systems copied from [Hoebe et al., 2004] with permission.
The immune system has two arms the innate and adaptive, which are complementary. Antigens by passing the innate immune system, are presenting to the adaptive immune system by DCs to generate a specific immunity to the pathogen**.**

1.2.2.1 Antigen recognition in adaptive immune system

1.2.2.1.1 Antigen presenting cells

In order to generate specific immunity against pathogens, T and B lymphocyte must recognize immunogenic antigens of pathogens and become activated. B cells can recognize Ags directly with their antibody receptors, which will be discussed later but T cells can only detect the pathogenic product where they are displayed along with a complex molecule called major histocompatibility complex (MHC) class I or class II molecule on the surface of the cell. However, a second stimulation is also needed for the activation of T

lymphocytes and it is provided through co-stimulatory molecules such as CD80, CD86 and CD40 on Antigen Presenting Cells (APC). Interaction of CD40 and its ligand on T cells up regulate the expression of CD80/CD86 as well as the priming capacity of APCs [Haase et al., 2004; Probst & van den Broek, 2005].

Dendritic cells, macrophages and B lymphocytes were shown to efficiently take up the antigen and present them to T lymphocytes after folding up them with MHC molecules. These cells, which are called antigen-presenting cells (APC), can also express co-stimulatory molecules, which are necessary for activation of T cells [Bryant & Ploegh, 2004].

1.2.2.1.2 Major Histo compatibility Complex (MHC) molecules

Major histo compatibility genes were first discovered when their role in the rejection of transplanted tissues became clear and the peptide-binding glycoproteins encoded by these genes are still known as the MHC molecules. The MHC genes in mouse are called H-2 genes and located on chromosome 17. In human they are called leukocyte antigen (HLA) and located on a chromosome 6 [Chaplin, 2006; Goldmann et al., 2005].

The MHC molecules have been classified into two groups -MHC class I and class II- which are recognized by CD8+ and CD4+ T lymphocytes respectively [Kosor et al., 2003]. MHC class I and class II are completely different in structure, synthesis and expression pattern on cells. It has been shown that except red blood cells, central nervous system, fetotrophoblast, testis, and the anterior eye chamber all cells express the MHC class I [Ambagala et al., 2005; Ruckert et al., 1998] but the MHC class II molecules are only expressed on T cells, B cells, macrophages, dendritic cells, eosinophils and also thymic epithelial cell [Baecher-Allan et al., 2006; Jabrane-Ferrat et al., 2002; Padigel et al., 2006]. However, some other cells such as fibroblasts and epithelial cells but not trophoblasts, in the presence of IFN-γ, express MHC class II. B-lymphocytes loose the expression of these molecules on transformation to plasma cells [Buttice et al., 2006; Denning et al., 2000; Manz et al., 1998; Murphy et al., 2004].

Two different polypeptide take part in the structural formation of the MHC class I molecule. The first part is a polymorphic polypeptide chain consisting of three parts - α_1, α_2 and α_3 - and the second is a smaller polypeptide chain called β-microglobulin (Figure 1-2), which is not encoded by the MHC locus and its gene is located on chromosome 15. In

human three different genes are encoding for the HLA class I -α chain that make three subclasses of MHC class I called HLA –A , HLA-B, HLA-C [Chaplin, 2003].

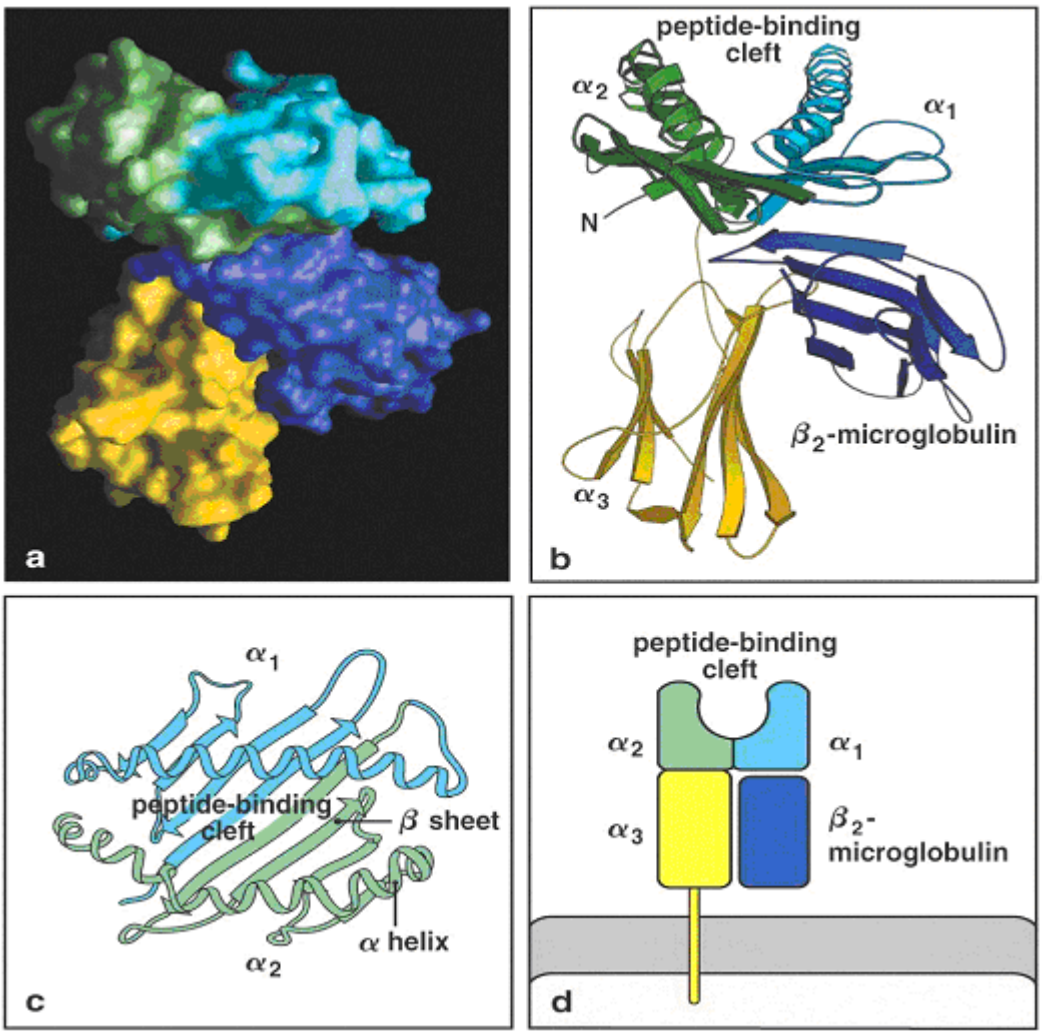


Figure 1-2: MHC class I molecule as described by x-ray crystallography taken from [Janeway & Travers, 2005].

Because of the extreme polymorphism in MHC molecules and the co-dominant expression of MHC gene products, there are variety of alleles in each subclass like HLA-A1, HLA-A2.

The MHC class II molecule consists of two chains, α and β. Both α and β chains span the cell membrane and are encoded by MHC genes. Each chain has two noncovalent domains (α1, α2 and β1, β2) (Figure 1-3). There are three types of MHC class II genes in human, HLA-DR, HLA-DP, HLA-DQ, and because of the polymorphism, each type has a variety of subtypes like HLA-DR1, HLA-DR2. In some cases there are two genes encoding the β-chain in HLA-DR cluster that means the three types of HLA class II molecule can give rise to four [Chaplin, 2006].

Because of the polygeny in MHC genes, each individual express three different types of MHC class I molecules and three or four types of MHC class II molecules and the polymorphicity of MHC also creates multiple variants of each gene in the population as a whole. Therefore, a wide range of peptides will be presented to T cells and the pathogens will have a lower chance to evade the immune system.

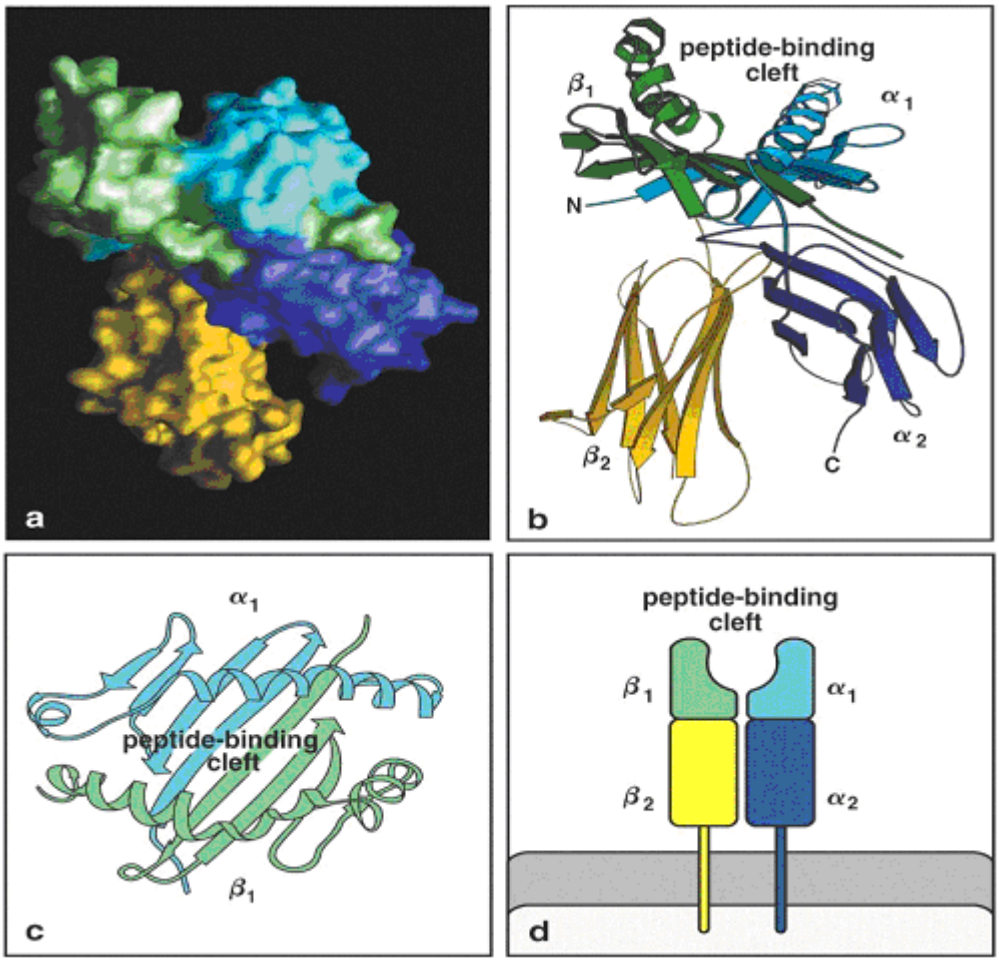


Figure 1-3: MHC class II molecule as described by x-ray crystallography taken from [Janeway & Travers, 2005].

1.2.2.1.2.1 Antigen processing of MHC class I antigens

It has been shown that the expression of empty MHC molecules (without bound peptide fragment) on the cell membrane, is unstable and binding to a peptide is stabilizing it. The peptide-binding cleft in MHC class II is far wider than class I. So that the peptides bound to MHC class II are longer- at least 13 amino acids- than those bound to class I, which are between 8-10 amino acids [Murugan & Dai, 2005; Schoenhals et al., 1999].

Among intracellular pathogenic agents, viruses and certain bacteria use the cell machinery to reproduce in the cytosol or in the contiguous nuclear compartments whereas bacteria

and some intracellular parasites that are picked up by phagocytes live in the phagosome. All antigen fragments of proteins made up inside the cell bind to MHC class I molecules. It has been well demonstrated that the proteosome, a large multicatalytic protease complex, in cytoplasm is responsible for the degradation of most cytosolic proteins and the production of peptide fragments for MHC class I [Bouvier, 2003]. Because the MHC class I molecules are synthesised inside the endoplasmic reticulum, the peptide fraction are loaded on MHC class I molecule and transported to the cell surface. The peptide fragments are transported to the endoplasmic reticulum by transports associated with antigen processing-1 and 2 (TAP1 and TAP2). In the endoplasmic reticulum some accessory proteins -calexin, tapsin, ERP57, and TAP- with chaperon-like function, help in folding and assembly of MHC class I and the loading of a suitable peptide fragment. The completed MHC molecule and the bound peptide can now be transported to the cell surface (Figure 1-4). The bound peptide helps in stabilizing and maintenance of the MHC molecule on the cell surface [Bouvier, 2003; Diedrich et al., 2001].

1.2.2.1.2.2 Antigen processing of MHC class II antigens

Due to the pathogenic process, some other pathogenic agents like most of bacteria and intracellular parasites replicate in phagosomal cell compartments in phagocytes. Therefore, the proteins of these pathogens are surrounded by a vesicle membrane and are not accessible to the proteosome. Thus, the peptides of these proteins bind to MHC class II to be recognized by CD4+ T lymphocytes. All extra cellular proteins and the proteins recognized by B cells are also processed through this pathway [Silacci et al., 1994]. Endosomes containing the proteins or pathogens as they progress into the interior of the cell become increasingly acidic until they eventually fuse with lysosome. The proteins inside these vesicles undergo unfolding and disulphide reduction and are degraded into peptides by lysosomal protease enzymes, which have optimal activity at low pH of the phagosome [Robinson & Delvig, 2002].

The main function of the MHC class II molecules is to bind to the peptide fractions generated in intracellular vesicles carrying them onto the cell surface and present them to T lymphocytes. The biosynthesis of MHC class II molecules are carried out in the endoplasmic reticulum [Robinson & Delvig, 2002].

To prevent binding to an undesired peptide, and during the assembly of the MHC molecule, a protein called the MHC class II-associated invariant chain (Ii) binds to the

binding site of the newly constructed MHC class II molecules. This is followed by the dissociation and transportation of the completed assembly from the endoplasmic reticulum to an endosome and degredation of the invariant and membrane-associated fragments of Ii, which leaves a small fraction of Ii called class II-associated invariant-chain peptide (CLIP) on the binding site of the MHC class II. (Figure 1-4). Finally, the endosome containing the new constructed MHC class II is fused to an incoming endosomes containing degraded antigenic proteins. An MHC class II-like molecule called HLA-DM in human and H-2M in mouse is responsible for catalizing the release of CLIP and the binding of a new peptide fragment. Thus, the antigenic peptide fragments bind to the MHC class II molecules and are transported to cell surface [Lee et al., 2006; Robinson & Delvig, 2002].

The empty MHC class II molecules like class I are unstable and they are rapidly degraded. In uninfected cells the MHC class II molecules are loaded by peptide fragments derived from self-proteins. It has been shown that some of peptide fragments derived from extracellular pathogens are presented by MHC class I. This process is called cross-priming or cross-presentation of antigens. However, the mechanism by which the peptides are loaded on the MHC class I molecules, is not very clear [Stoitzner et al., 2006; Tewari et al., 2005].

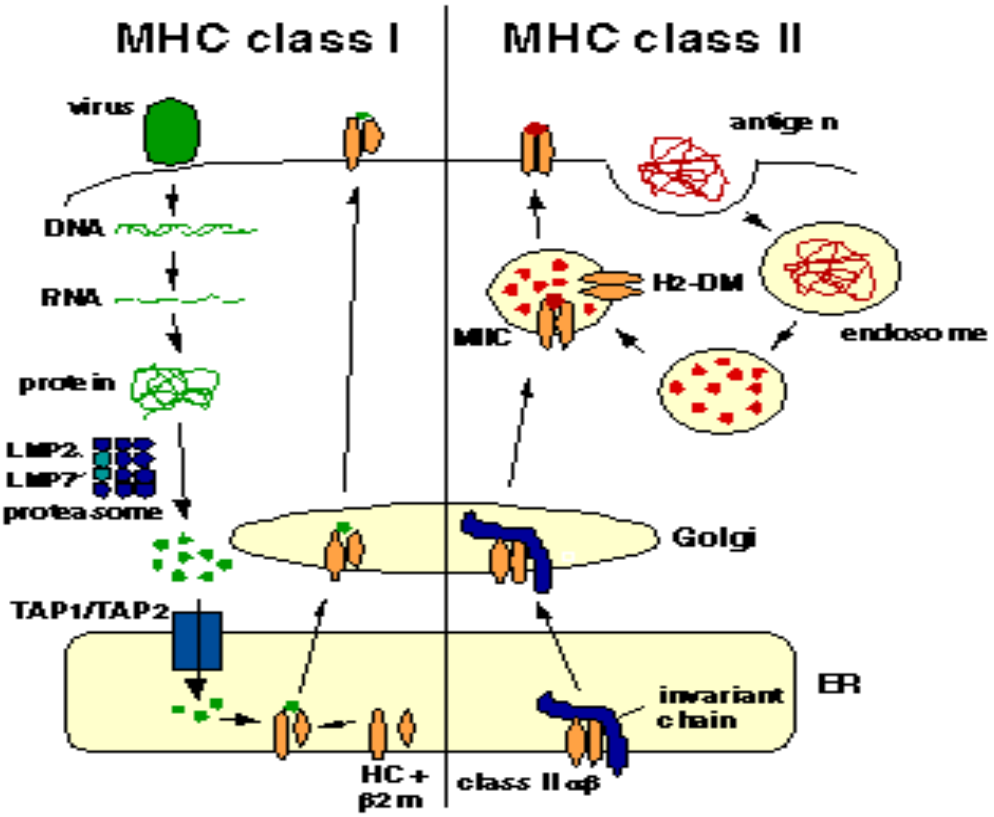


Figure 1-4: Antigen presenting procedure by MHC class I and class II.
Intracellular antigens are expressed through the MHC class I and extra cellular antigens are expressed through the MHC class II molecules. Intracellular antigens are chopped by proteosome inside the cytoplasm and their peptides are transported to the reticulum endoplasmic through TAP1 and TAP2 molecules. In the reticulum endoplasmic the peptides are loaded on the MHC class I molecules and transported to the cell surface. For extra cellular antigens, the MHC class II molecules are assembled in the reticulum endoplasmic and move to the cytoplasm through Golgi particles. The Golgi particles containing the MHC classII are fused to the phagolysosomes containing degraded extra cellular proteins. Each MHC class II then binds to a 15 to 22 mer peptide and move to the cell surface.

1.2.2.1.3 T cell receptors

T cells recognize the antigens displayed on the surface of other cells via the receptors they bear on their surface. Each T cell receptor, similar to that of B cells, consists of two polymers chains, α and β, which are linked together with a disulphide bond [Housset et al., 1997]. In a minority of T cells, a different pair of polypeptides, γ and δ, make the receptor. Although the function of γ-δ T-cell receptor has not been entirely clarified, it seems that they have different antigenic recognition properties from the α-β type [Born et al., 2006; Mincheva-Nilsson, 2003].

Each T cell receptor has two parts: the first part is the variable region (V), which make the contact with the MHC-antigen complex and has a homology to the V part of immunogloubulims . The second is a constant region (C), which is attached to the cell membrane, with a homology to the constant domain of immunoglobulins. The V and C domains are linked together by a short hinge region containing a cystein residue that forms the interface disulphide bond. (Figure 1-5) [Housset et al., 1997].

In contrast to B-cell receptors (see 1.2.2.1.4), which interact directly to intact antigens, T-cell receptors can only respond to processed antigens (peptides), which are bound and presented by major histocompatibility complex (MHC) molecules. In another word, T-cell receptors recognize peptide fragments only when they are sandwiched within a MHC molecule [Hennecke & Wiley, 2002; Wang et al., 1998].

The interaction of T-cell receptors and MHC class I or II molecules does not stimulate T-cells unless some other molecules so-called co-receptors or co-stimulatory molecules are engaged. In fact, co-stimulatory molecules interact with their ligand producing a complementary signal in T cells. The signals posed by co-stimulatory molecules complete the signals of T-cell receptors and lead to activating T lymphocytes. Thus, only APCs, which have co-stimulatory molecules on their surface, have the potential to activate T cells. The most known co-receptor molecules are CD4 on a subset of T cells, which

specifically binds to MHC class II molecules, and CD8+ molecules on the other subtype of T cells, which binds to MHC class I molecules [Julius et al., 1993]. The best characterised co-stimulatory molecules are CD80 (B7.1) and CD86 (B7.2) on APCs that bind to CD28 on T cells and CD40 on APCs that binds to CD40 ligand (CD154) on T cells. CD3 is also a functional receptor that is necessary for signalling of T-cell receptors. It has been shown that T-cell receptors bind to the MHC molecules having expressed the antigen but without CD3, cannot signal the presence of the antigen they have recognised [Julius et al., 1993].

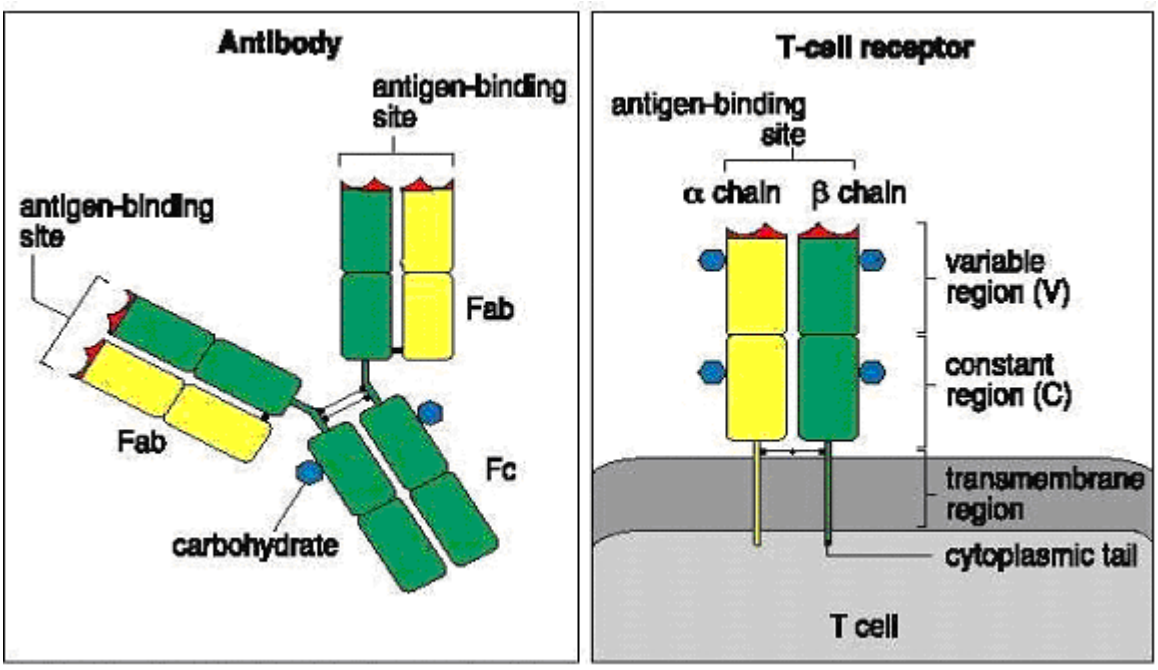


Figure 1-5: A schematic diagram of B-cell and T-cell receptors taken from [Janeway & Travers, 2005]. B-cell receptors are similar to antibodies unless they are fixed on B-cell surface. B-cell receptors or antibodies are composed of two heavy, green, and two lights, yellow, chains. Each antibody consists of two domains. The constant domain (FC) is bound to the cell membrane variable domains, which have antigen-binding sites capable of binding to the specified antigen. T-cell receptors are similar to antibodies but two chains with same size and one site for antigen binding. T-cell receptors can only recognise the antigen when it is expressed through MHC molecules.

1.2.2.1.4 B-cell receptors/Immunoglobulins

B cells are leukocytes defined by their production of the immunoglobulin (antigen-binding proteins) and represent approximately 15% of peripheral blood cells [Chaplin, 2006]. Immunoglobulins (Ig) or antibodies are proteins produced by B lymphocytes against antigens. These molecules are produced in vast specifities as almost each B cell produces the antibody with a single specifity. Antibodies are normally produced by terminally differentiated B cells called plasma cells. However, membrane-bound immunoglobulins on B cells act as the B-cell receptor. There are five classes, isotypes, of antibodies called IgD,

IgM, IgE, IgA and IgG, which are different in shape. Each Ig class is divided into a number subclasses; IgG into four subclasses, IgG1, IgG2, IgG3 and IgG4, and IgA into two subclasses, IgA1 and IgA2. The subclasses are named in order of their abundance in serum [Matousovic et al., 2006; Putnam, 1995; Toptygina et al., 2005]. The production of antibody can switch from one isotype to another one and CD40 on B cells as well as CD40L on T cells have a crucial role in isotype switching [He et al., 2003]. IgG is a large molecule of about 150 KD. Two heavy chains of 50KD each and two light chains of 25 KD each contribute in the antibody structure. The antibody consists of two main parts: The variable region (V), which specifically binds to a part of the antigenic molecules and the constant domain (C), which binds to FC receptors on the immune cells [Edelman, 1994; Faber et al., 1998] (Figure 1-5).

The secretion of antibodies is the result of activation of the humoral immune response. The main functions of antibodies are to protect the body from the extracellular pathogens and their products. Many of pathogenic agents multiply in extracellular spaces of the body and most of intracellular agents also use the extracellular spaces to move from cell to cell and spread in the body.

Antibodies exert their effects against pathogens in three different ways: 1) via neutralization when they bind to bacteria, viruses or toxins to deny them access to infect and to induce damages to the susceptible cells [Hangartner et al., 2003]. 2) Via opsonisation when antibodies bind to the antigen coating their external surfaces. The opsonised pathogens are easily recognized by phagocytes through the FC receptors expressed on their surface, which bind to the FC part of the antibodies. 3) Via their distinctive role in activation of the complement [Boruchov et al., 2005], which results in the attraction and activation of complement proteins. The type of complement activation mechanism induced by the antibody depends upon the isotype and class of the antibody engaged. Complement proteins are also recognized by their receptors on the phagocytic cells. Complement components can recruit other immune cells like phagocytes to the site of infection (see 1.2.1.5). They also lyse certain types of microorganisms by forming pore on their cell membrane.

1.2.2.2 CD4+ and CD8+ T cells in adaptive immune response

T lymphocyles fall into two main groups with different effector function, which are distinguished by distinctive protein molecules, CD4 or CD8, expressed on their cell

membrane. CD8+ T cells interact with MHC class I and CD4+ T cells interact with MHC class II molecules and the presece of CD8 and CD4 is crucial for inter cellular interaction and activation. CD4+ T cells fall into two functional sub groups called T helper1 (Th1) and T helper2 (Th2). It has been shown that IL-12, IL-2 and IFN-γ have an essential role in Th1 pathway but IL-4, IL-5, IL-13 and IL-10 are involved in the Th2-type immune response [de Jong et al., 2005; Mackay, 2000].

Th1 and Th2 T cells are very different in function. The main function of Th2 cells is the activation of B cells to proliferate and differentiate to an effector plasma cells that produce antibodies whereas Th1 cells enhance the potency of macrophages to phagocytose and degrade the pathogen [Mack et al., 2005; Munder et al., 1999; Yun et al., 2003] (see 1.2.1.1).

The mechanisms that determine the type of immune pathway, are not yet fully understood. However, it has been shown that toll-like receptors can have a role in deciding the immune response type; Th1 or Th2 pathways. Activation of a set of these receptors including TLR9, activated by interaction with CpG DNA, lead to Th1 pathway. In contrast, activation of other TLRs including TLR2 can lead to Th2 pathway [Chaplin, 2006; Redecke et al., 2004]. Furthermore, secretion of IL-4 in early phases of immune response lead to Th2 response but the lack of IL-4 can help estabilishing a Th1 immune response [Sacks & Noben-Trauth, 2002; Yun et al., 2003]. Nevertheless, T cells, during their activation, need to receive two different signals. The first signal is provided by T-cell receptors, which bind to MHC-peptide molecules and the second one comes through engagement of co-stimulatory molecules. Accumulating evidence supports the notion that co-stimulatory molecules play important roles in T cell activation, differentiation, survival and effector function. Activation of T cells without co-stimulation may lead to T cell anergy, T cell deletion or the development of immune tolerance. One of the best characterized co-stimulatory molecules expressed by T cells is CD28, which interacts with CD80 (B7.1) and CD86 (B7.2) on the membrane of APC [Freeman et al., 1993; Harding et al., 1992; Lenschow et al., 1996]. Other co-stimulatory molecules such as CD40 and OX40 also play an important role in interaction of T lymphocytes with other immune cells. CD40, which mainly binds to CD154 on T cells, has a role in activation of T cells and B cells [Banchereau et al., 1994]. Interaction of OX40 and OX40L on APCs promotes activation of naive T cells with some IL-2 secretion and has synergy with B7-1. APCs co-expressing OX40L with B7-1 induce large quantities of IL-2 and promoted proliferation compared to B7-1 alone. OX40/OX40L interactions act to prolong clonal expansion and

enhance effector cytokine secretion, and may be involved in promoting long-lived primary CD4 responses [Gramaglia et al., 1998]. In addition, there are accumulating evidences to suggest that the interference with co-stimulatory signals can modulate Th1/Th2 cytokine expression levels and immune deviation [Jankovic et al., 2004]. For instance, it has been shown that the interaction of CD28 or OX40 with their receptor/ligand , dependent on the dose of antigen, can promote either Th1 or Th2 immune response [Rogers & Croft, 2000].

CD8+ T lymphocytes, which are called cytotoxic T cells (CTL) can recognize infected or abnormal cells that display the antigenic peptides on their surface through MHC class I. These cells will be killed by cytotoxic T cells through releasing lytic granules, which lyses the cells or inducing programmed cell death, apoptosis. Moreover, cytotoxic T cells release a large amount of IFN-γ, TNF-α and TNF-β, which contribute in host defence [Ambagala et al., 2005]. IFN-γ directly inhibits viral replication and enhances the MHC class I expression and other mechanisms involved in peptide loading of the newly synthesized MHC class I proteins in infected cells [Zhang et al., 2002]. IFN-γ also enforces Th1 pathway by activating macrophages to kill the engulfed microorganism. TNF-α and TNF-β act in synergy with IFN-γ [Olleros et al., 2005; Romagnani, 2000; Saito & Nakano, 1996].

The potent activation of CD8+ cytotoxic T lymphocytes in killing the infected cells requires co-stimulation through interaction of B7 and other co-stimulatory molecules similar to those in CD4+ T cells and the presence of CD4+ T cells, which can recognize a related antigen on the surface of the same antigen-presenting cells [Serre et al., 2006].

1.3 Leishmaniasis

Leishmaniasis is a worldwide human and animal disease caused by a malaria-like parasite called Leishmania. First species of these parasites, named *Leishmania donovoni*, was described by Leishmon and Donovoni in 1905 [Herwaldt, 1999]. So far, approximately 30 species of these parasites are known from which 20 species are pathogenic for human and canine [Ashford, 2000].

1.3.1 Classification of leishmania species:

As it appears from the name of some species of leishmania, the classification of leishmania parasites was first based upon the clinical, biological, geographical and epidemiological criteria. Now different methods are being used for the classification of these parasites including phenotypic, immunological and molecular methods. Isoenzyme analysis is one of the most sophisticated taxonomic techniques, which still remains as a standard technique for leishmania taxonomy [Cupolillo et al., 1994; Lainson & Shaw, 1989; Miles et al., 1980; Thomaz-Soccol et al., 2000].

Application of monoclonal antibodies is another technique, which is used to identify leishmania species, however, the specificity of the technique is not very high, which has made it less reliable [Falqueto et al., 2003; Grimaldi et al., 1987; Ilg et al., 1993].

Molecular biological methods including chromosomal rearrangements [Britto et al., 1998] and DNA-based methods have also been successfully applied for the characterization of leishmania isolates at a genus, species and even strain level. DNA-based methods are mainly applied by performing PCR and using leishmania specific genes such as beta-tubulin that is present in all strains tested belonging to the Leishmania (Viannia) subgenus [Eisenberger & Jaffe, 1999; Luis et al., 2001; Noyes et al., 1996; Uliana et al., 1991].

Different classifications have been suggested for leishmania parasites. In one of the latest classifications proposed by Lainson & Shaw, the genus *Leishmania* has been classified as a member of the kingdom Protista; Sub-kingdom Protozoa; Phylum Sarcomastigophora; Sub-phylum Mastigophora; Class Zoomastigophora; Order Kinetoplastida; Sub-order Trypanosomatina; Family Tripanosomatidae.

Based on the site of growth of the parasite in the midgut of the sandfly vector the genus *Leishmania* has been divided in two subgenera; *Leishmania* and *Viannia* [Lainson & Shaw, 1987]. In subgenus *Leishmania*, promastigotes develop in the midgut and foregut, Suprapylaria, of the insect, which is called “suprapylarian development”, whereas their growth in the subgenus *Viannia* is restricted to the hindgut, “Peripylarian development”

[Correa et al., 2005]. Species of the subgenus *Leishmania* are devided into 3 clusters or complexes: Leishmania donovoni complex (*L. donovoni, L. infantum, L. chagasi [*American *L. infantum], L. archibaldi*); Leishmania tropica complex (*L. tropica, L. aethopica, L. major*) and Leishmania mexicana complex (*L. mexicana, L. amazonensis, L. pifanoi, L. garhami, L. venezuelensis*). However, species of the subgenus Viannia are grouped in one Leishmania brasiliensis complex (*L. guyanensis, L. naiffi, L. peruviana, L. panamensis and L. shawi*) [Shaw, 1994].

1.3.2 Leishmania life cycle

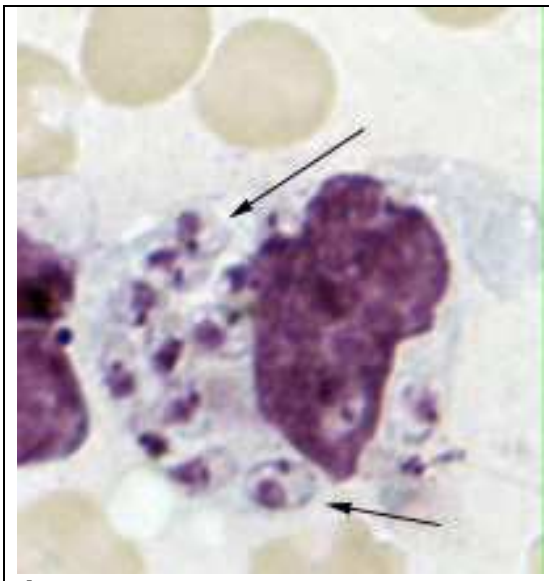
Leishmania parasites life cycle has been reviewed by Hommel [Hommel, 1999]. In brief, leishmania parasites need to pass through two different hosts to complete their life cycle; mammalian host and the sandfly vector.

Mammalian hosts of leishmania are mainly the human and dog, although some other mammalian species occasionally are infected with the parasite. Basically, humans are the most sensitive host for leishmania parasites and others including wolves, rodents, foxes, jackals and dogs, gerbils and also humans serve as reservoirs [Britto et al., 1998; el-Hassan et al., 1995; Hommel, 1999; Lainson & Rangel, 2005]. The role of dogs in harbouring and transmitting the parasite to the vector and in turn to humans is much more important than other hosts due to its close relation and association with humans [Reithinger & Davies, 1999].

The second host for leishmania parasites is the female blood sucking species of Phlebotomine sandfly (Figure 1-6 C), which carry, propagate and complete the life cycle of the parasite. The male sandflies feed on plants so that they cannot carry the parasite. Over 40 species of genus Phlebotomus (sandflies) act as a vector for leishmania in the old world (Asia, Africa, Europe), while a further 30 species belong to genus Lutzemia sandflies take role in the epidemiology of the parasite in the new world (Americas) [Dedet, 2005; Maroli & Khoury, 2004; Murray et al., 2005]. The feeding habit of the sandflies of each area mainly determines whether their main reservoir is humans or animals as some species are used to feeding on animals and some not [Hommel, 1999].

In the vector, the flagellar form, promastigotes, of the parasite lives extra cellularly (Figure 1-6 A). Virulent promastigotes express surface glycoprotein (gp63), lipophosphoglycan (LPG) and mannose receptors on their surface, which are crucial in their uptake by macrophages [Chakrabarty et al., 1996; Chakraborty et al., 2001; Chakraborty et al., 1998; Chaves et al., 2003].

The sand fly becomes infected during feeding on the blood of the infected mammalian host or reservoir. Macrophages containing the parasite, are ingested by the fly and amastigotes transform to elongated motile promastigotes (10-12µM), which have a flagella on one pole. While inside the gut of the sandfly, promastigotes multiply by binary fission and then migrate in the alimentary tract of the sandfly passing through several stages of procyclic, nectomonad, haptomonad and mammal-infecting metacyclic. The whole process takes few days to complete [Awasthi et al., 2004; Bates, 1994b; Bates & Rogers, 2004; Killick-Kendrick, 1990]. Metacyclic promastigotes move toward the oesophagus and salivary glands ready to transfer to the mammalian host. The vector saliva plays an important role in transmission of the parasite to the mamalian host by preventing the blood from clotting [Norsworthy et al., 2004]. During a blood meal bite of the vector, promastigotes are transferred to the mammalian host tissue to be easily picked up by macrophages, which act as the first line of the host's immune defence. While inside the macrophages, promastigotes loose their flagella and become spherical in shape to be called amastigote (Figure 1-6 B). Amastigotes are normally between 2.5 to 5µM long and are contained within the parasitophagous of macrophages. Transformation into the amastigote form makes the parasite more resistant against the antimicrobial activity of the macrophage so that it can survive and multiply in the macrophage eventually destroying it. The released amastigotes are taken up by new macrophages and, ultimately, all the organs containing macrophages such as spleen, liver and bone marrow become infected. A new sand fly vector will become infected when it has bitten an infected mamalian host taking up the infected macrophages and, thus, the life cycle of the parasite continues (Figure 1-7) [Awasthi et al., 2004; Davies et al., 2003; Hommel, 1999].



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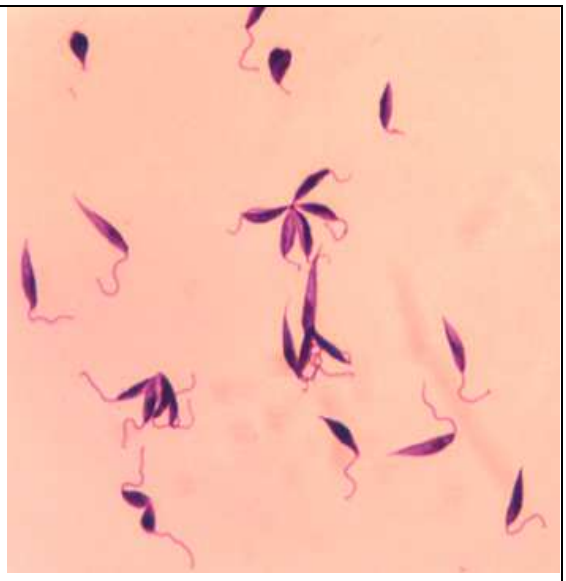

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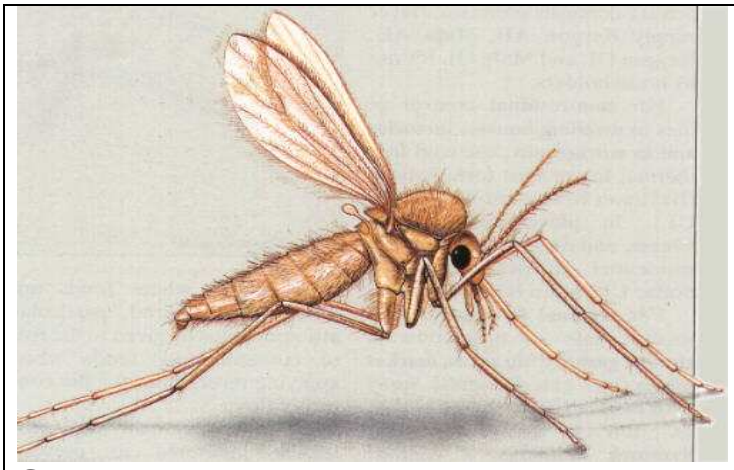


C:

Figure 1-6: A: Amastigotes of leishmania in mammalian host's macrophages B: Flagellar promastigotes of leishmania parasite C: leishmania vectors. Taken from [WHO, 2004].

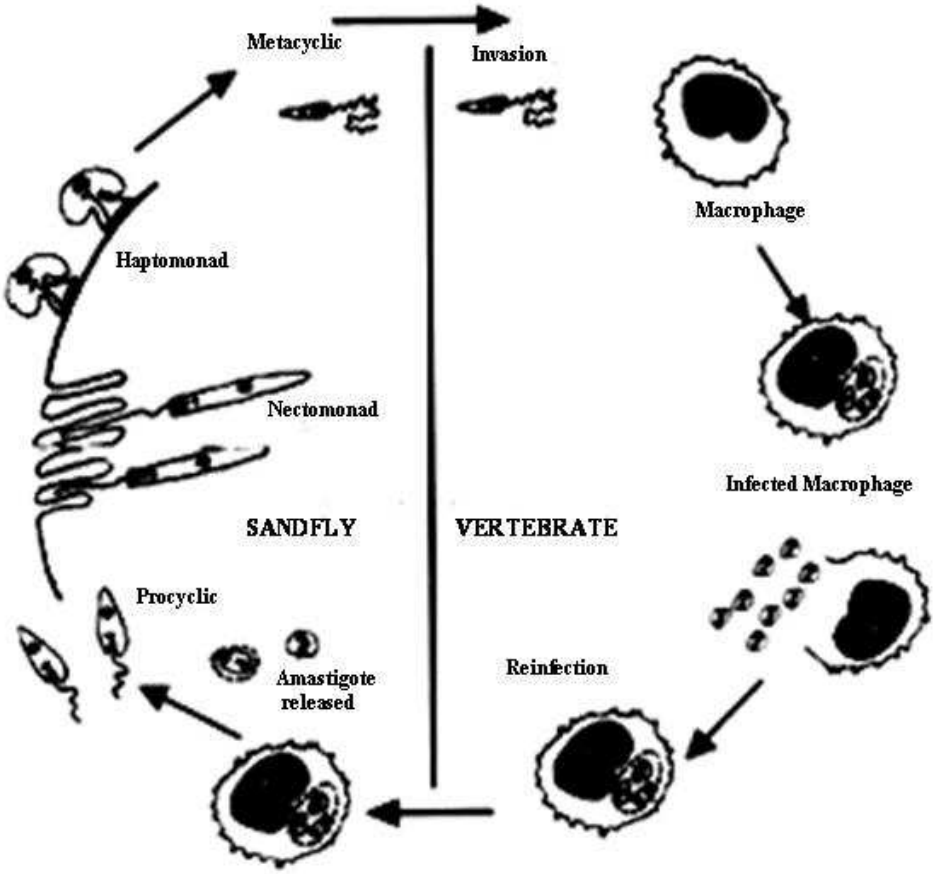


Figure 1-7: Life cycle of leishmania in its vertebrate and invertebrate hosts.
Leishmania parasites need to pass through two different hosts, the mammalian host and the sandfly vector, to complete their life cycle. The sandfly become infected when it feeds from the blood of an infected mammalian host, which are either human or some species of animals. The promastigotes grow in the sandfly vector and go through a few stages until they become infective for the mammalian host (explained in the text). The infective promastigotes called metacyclics are transferred to the non-infected mammalian host during the next blood meal of the sandfly. Adapted from [Hommel, 1999].

1.3.3 Leishmaniasis: clinical manifestations

Both sub-genera of genus Leishmania are infective and accounted as the causative agent of leishmaniasis [Rotureau, 2006]. According to the clinical spectrum of the disease, the human leishmaniasis has been classified into four main forms:

Dermal cutaneous leishmaniasis: This form of leishmaniasis is mainly caused by *L. major, L. mexicana* and *L. tropica* (Table 1-1) producing skin lesions in any part of the body mainly in the face, arms and legs [Dowlati, 1996; Murray et al., 2005]. After the initial infection, in some cases the infection may remain subclinical. The incubation period varies between 1 to 12 months and after that it produces progressive papules, which usually ulcerate and secondary bacterial infection may be also involved. The typical ulcer is usually painless with a raised, indurated margin and necrotic centre. However, some

lesions may not ulcerate. The lesion size is varied from 0.5 to 3 cm diameter (Figure 1-8). There are no systemic symptoms such as fever, anaemia, spleen, and/or liver enlargement and most of lesions usually heal in a 3 to 12 month period without taking treatment but they leave scar [Calvopina et al., 2004; Hepburn, 2003; Weina et al., 2004].

Figure 1-8: Leishmania lesions in American solders serving in Iraq.
Ranged from papular eruptions *(left)* to more classic erosive craters *(center)* and were sometimes surrounded by concentric desquamation *(right).* Adopted from [Weina et al., 2004]

Diffuse cutaneous leishmaniasis: This form of the disease is a progressed form of Cutaneous leishmaniasis and mainly caused by the same species of the parasite. This form of the disease is difficult to treat due to disseminated lesions that resemble leprosy and do not heal spontaneously [Silveira et al., 2004; Weina et al., 2004].

Mucocutaneous leishmaniasis (MCL): This form of leishmaniasis, which is called 'Espundia' in South America, generates lesions in mucous membranes particularly in areas where mucous attach to the skin. It may affect the nasal mucosa, septum and turbinate, upper lip, pharynx, larynx and face causing dyspnoea, producing deformities and malfiguration in these areas (Figure 1-9) [Herwaldt, 1999; Murray et al., 2005]. MCL can be the result of the dissemination of cutaneous leishmaniasis to mucousal tissues caused by certain species such as *L. braziliensis* (Table 1-1) [Calvopina et al., 2004; Silveira et al., 2004].

Visceral leishmaniasis [Dumonteil et al.]: Visceral leishmaniasis also called 'Kala Azar' is always accompanied by systemic symptoms such as irregular fever, cough, weight loss, cachexia, hepatosplenomegaly, splenomegaly and anaemia. It is the most sever form of the disease and patients often die if they are not given health care. Visceral leishmaniasis is associated with *L. infantum* and *L. donovani* in the Old World and *L. amasonensis* in the New World (Table 1-1). Unlike the cutaneous forms, visceral leishmaniasis involves only the internal organs and does not develop lesions on the skin during the course of disease, however, after the recovery, patients may develop a chronic form of cutaneous leishmaniasis called post-kala azar dermal leishmaniasis (PKDL) that requires long medical treatment [Awasthi et al., 2004; Herwaldt, 1999; Weina et al., 2004].

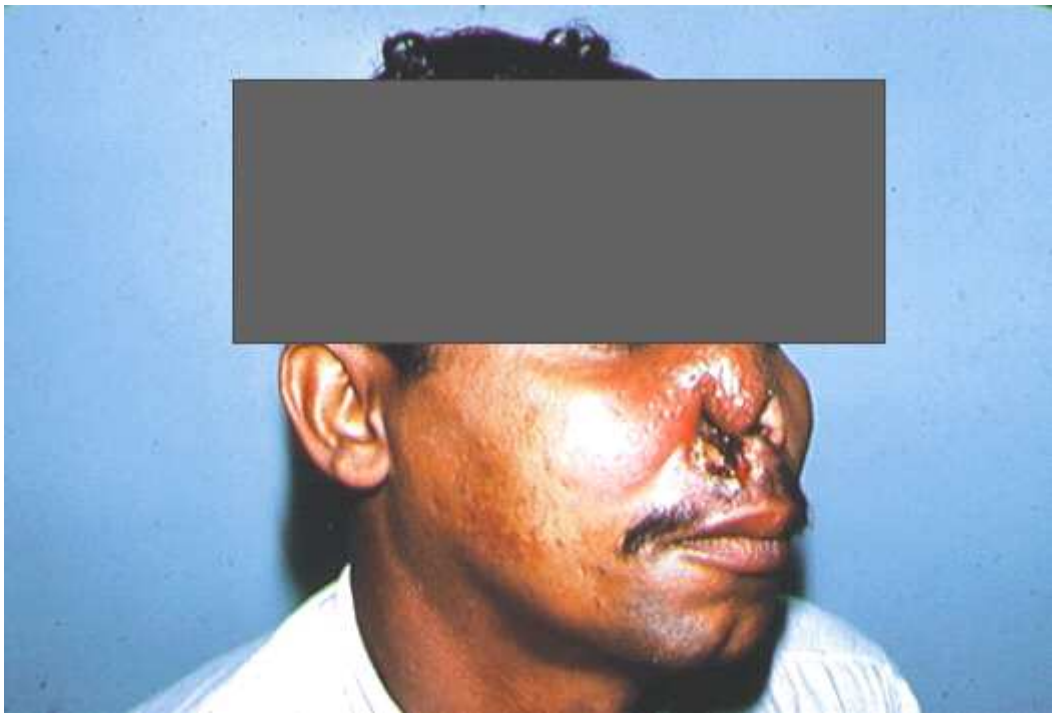


Figure 1-9: Malformation caused by Mucocutaneous leishmaniasis in face

Species	Geographic distribution	Reservoir	Clinical syndrome
L.chagasi (New world)	Mexico, Surinam, Brazil,Paraguay, Argentina, Venezuela, Brazil, Bolvia	Canine	VL, CL, PKDL
L. amazonensis (New World)	Brazil, Costa Rica, Texas, Guyana, Peru, Bolvia, Venezuela	Rodents, Marsupial	CL, VL, PKDL, MCL, DCL
L. major-like Isolates (New world)	Colombia, Panama, Venezuela	Canines	CL, DCL
L. mexicana (New world)	Mexico, Guatemala, Texas, Costarica, Panama	Forest Rodent	CL, DCL
L. major (Old world)	Middle East, Indian Subcontinent, northwestern China, Africa	Humans, rodents, mustelids, hedgehogs,rabbits	CL
L. tropica (Old world)	Middle East, India, Mediterranean littoral, western Asiatic areas	Canids and perhaps some rodent	Dry cutaneous lesions
L. donovani (Old world)	Africa, India, East Asia	primates, equids, rodents	VL, PKDL
VL, Visceral leishmaniasis; CL, cutaneous leishmaniasis; PKDL, post kala azar dermal leishmaniasis; MCL, mucocutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis			

Table 1-1: Old and new world leishmania species and geographical distribution adopted from [Awasthi et al., 2004]

1.3.4 Epidemiology of leishmaniasis

Today, leishmaniasis is considered to be endemic in many countries (16 developed countries and 72 developing countries) on four continents. An estimated 12 million cases of leishmaniasis exist worldwide and a further 367 million are at the risk of acquiring the

disease. Moreover, an estimated number of 1.5 - 2 million new cases are occurring annually; 1 - 1.5 million cases of cutaneous leishmaniasis and 0.5 million cases of visceral leishmaniasis resulting in 75,000 deaths per year [Desjeux, 2004; Griekspoor et al., 1999; Kar, 1995; WHO, 2002a]. The geographical distribution of leishmaniasis is restricted to tropical and temperate regions and the living area of the sandfly in Asia, India, Africa and the Mediterranean (Old World) and Americas (New World) [Royer & Crowe, 2002]. Ninety percent of the cases with cutaneous forms of leishmaniasis occur in Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria, while ninety per cent of the visceral leishmaniasis cases are found in Bangladesh, Brazil, India, Nepal and Sudan (Figure 1-10) [Hepburn, 2003; WHO, 2002a].

There is convincing evidence that the number of cases of leishmaniasis has been increasing in several areas of the world e.g. CL in Brazil (1998: 21800 cases; 1999: 30550 cases; 2000: 35000 cases), CL in Kabul, Afghanistan (1994: 14200 cases, 1999: 200,000 cases), and CL in Aleppo, Syria (1998: 3900 cases; 1999: 4700 cases; 2000: 5900 cases) [WHO, 2002a]. Visceral leishmaniasis has also caused large-scale epidemics with high case fatality. For instance, Western Upper Nile State in South Sudan experienced a major outbreak of visceral leishmaniasis between 1984 and 1994, which claimed 100,000 lives in a population of around 300,000 [Herwaldt, 1999]. This is thought to be due to the increase of risk factors such as man-made environmental changes which increase human exposure to the sand fly vector or the movement of susceptible populations into endemic areas, including large-scale migration of populations for economic reasons. Moreover since the parasite may survive for decades in asymptomatic infected people, who are of great importance for the transmission, the asymptomatic infected people can act as a reservoir which is the third risk factor for the disease [WHO, 2002b].

It has been shown that AIDS patients are more sensitive to leishmania infection than healthy individuals as many of such new cases have been reported from 30 countries around the world (Figure 1-10). Epidemiological data reveal that the human leishmaniasis and HIV virus co-infection, especially among adults, has also been progressively increasing. Now, it is thought that 50% of all adult cases of visceral leishmaniasis are HIV-positive. In south–western Europe, 1.5–9% of patients with AIDS are suffering from newly acquired or re-activated VL and the recorded number of cases has increased from about 700 cases in 1995 to more than 1500 cases in 2001 [Puig & Pradinaud, 2003; WHO, 2002a]. It has recently been shown that the conventional transmission of leishmania parasites among leishmania/HIV co-infected patients has changed where the parasite can

easily be transmitted between intravenous-drug users by needle sharing [Molina et al., 2003]. A congenital transmission of visceral leishmaniasis has also been reported from an asymptomatic mother to her child [Meinecke et al., 1999].

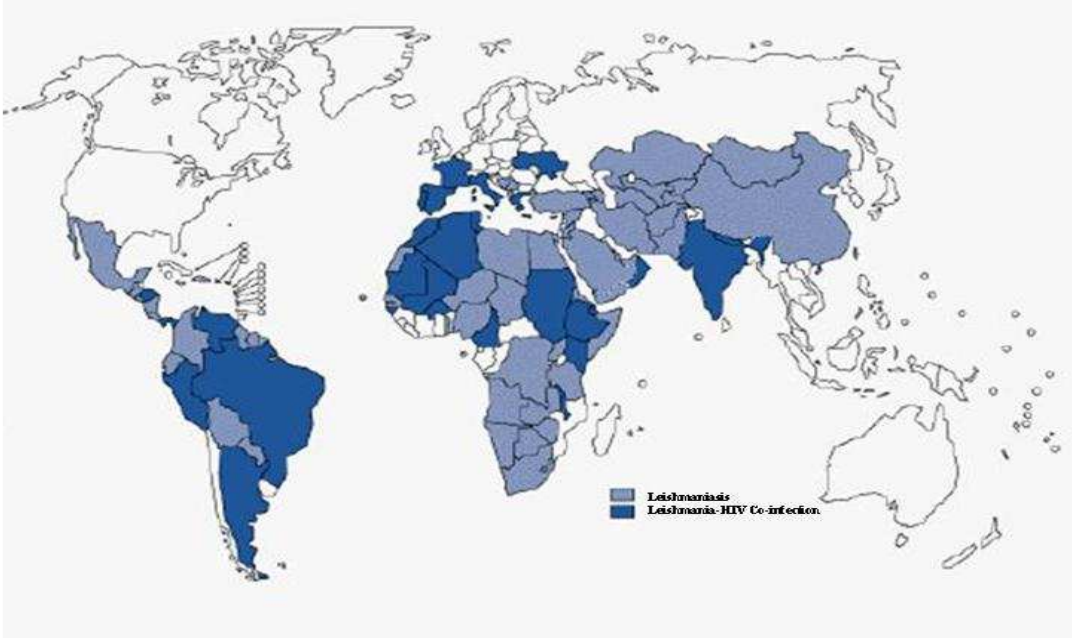


Figure 1-10: Distribution of leishmaniasis and leishmania-HIV co-infection in the world [WHO, 2000].

1.3.5 Control of leishmaniasis

Depending upon the epidemiological studies in leishmania parasites, a number of strategies have been considered to control leishmaniasis. The current control strategies of leishmaniasis are based on early diagnosis, treatment and the control of vectors and reservoirs, however, early diagnosis and treatment strategies are considered are the most effective [WHO, 2004].

1.3.5.1 Control of vectors and reservoirs

The best known strategy to control the vector-born diseases is to reduce or disrupt the man-vector contact. There are attempts to control the sand fly reproduction by spreading insecticides and damaging their living places. Control by chemical reagents in some areas like India, Bangladesh or Nepal is the only choice and for that DDT is vastly being used [Alexander & Maroli, 2003; Kishore et al., 2006; Maroli & Khoury, 2004]. However, this is limited by cost and development of the poison resistant flies. Moreover, this strategy because of ecological effects of the chemicals, is not practical in some areas [Alexander &

Maroli, 2003; WHO, 2002a]. In areas, where dogs play as the main host or reservoir, killing the wild dogs in the area might be considered as an essential method [Reithinger & Davies, 1999; Reithinger & Davies, 2002].

1.3.5.2 Chemotherapy in leishmaniasis

Although chemotherapy is considered as the only available effective method for the treatment of leishmaniasis, there are various shortcomings for each drug currently in use. For instance, the anti-leishmania drugs are often toxic and usually accompanied by unpleasant side effects [Velez et al., 1997]. In addition, the drug-based treatment of leishmaniasis is expensive , which makes it unavailable for most of people especially in poor countries, and the parasites usually become resistant to chemotherapy following few times of administration [Griekspoor et al., 1999; Rosenthal & Marty, 2003; Sundar & Chatterjee, 2006; WHO, 2004].

A limited number of drugs are available for treatment of leishmaniasis. Pentavalent antimony is an old known drug, which is still accounted as the first line of anti-leishmania drugs and the cornerstone of chemotherapy in leishmaniasis. The course of treatment by antimony is often long and lasted for a period of 3-4 months. A high level of drug resistance has been reported from endemic areas such as India [Hadighi et al., 2006; Murray, 2004; Singh, 2006; Sundar & Chatterjee, 2006]. Now, different products of antimony are available but the mechanisms by which antimonals act against leishmania is still unclear. However, it is thought that they target important biological activity of the parasite and affect a number of factors such as cytokines and T-cell subsets that have a significant anti-leishmania role [Ouellette et al., 2004]. In spite of massive work that has been carried out in the past few years to reveal the mechanisms used by leishmania parasites to resist antimonals, they are still unclear [Singh, 2006]. Failed treatment may be accountable for making the parasite resist to the drug [Rojas et al., 2006].

Other available anti-leishmania drugs including Pentamidine, Amphotericin B and Miltefosine, which are accounted as the second line of chemotherapy [Singh et al., 2006; Sundar, 2001]. The efficacy of Pentamidine has been shown declining in India suggesting the possibility of the parasite resistance against the drug [Ouellette et al., 2004]. Amphotricin B is basically an anti fugal drug, which has also potency to leishmania. The highly effective dose of Amphotricine B is usually accompanied by sever side effects and that it remains as the drug of choice only in the areas, where the parasite is resistant to other drugs [Ouellette et al., 2004; Sundar, 2001]. The high cost of amphotricin B also

makes it unavailable for many patients [Sundar, 2001; Sundar & Chatterjee, 2006]. Miltefosine has shown a high affectivity against leishmania in a phase III trial in adults and it can be considered as a break through in leishmania treatment [Jha et al., 1999; Sundar et al., 2002]. However, other aspect of this drug still needs to be investigated. Other drugs including metronidazole, ketoconazole, fluconazole, itraconazole and terbinafine were shown to have different range of effectiveness to leishmania in animal models and human clinical trials so that their efficacy still needs to be investigated [Alrajhi et al., 2002; Gangneux et al., 1999].

1.3.6 Diagnosis of leishmaniasis:

Diagnosis of leishmaniasis is dependent on a combination of clinical symptoms, parasitological detection, immunological tests and molecular techniques

1.3.6.1 Clinical symptom

A series of clinical manifestations can be seen in visceral leishmaniasis including long-term unexplained fever, cachexia and hepatosplenomegaly. In cutaneous leishmaniasis changes on the skin are the most important symptom, which can lead to the diagnosis of the disease (see 1.3.3), however, all forms of the disease need to be confirmed by other diagnostic methods.

1.3.6.2 Parasitological diagnosis

In visceral leishmaniasis, the amastigote form can be easily detected by the microscopic examination of stained smear of aspirates derived from lymph nodes, bone marrow, liver, or spleen [Bhattacharya et al., 2006; Markle & Makhoul, 2004]. There is always a risk of haemorrhage and complication for splenic and liver aspiration, which is also painful and unpleasant for the patients [Osman et al., 1997]. In cutaneous leishmaniasis, the detection of amastigotes by microscopic methods is based on obtaining the smear from the skin lesion biopsy or other methods like biochemical or immunohistochemical tests. The aspirate can also be cultured for recovering the parasite [Markle & Makhoul, 2004]. The culture method is simple, cheap and relatively sensitive but suffers from its vulnerability to contamination. In addition, the culture method of the parasite is usually time consuming, which makes it not an ideal method for field use.

In occult and sub-clinical infections, both direct microscopy and cultured-based methods have a low sensitivity and cannot distinguish between the amastigotes of different species,

so that, no species identification can be applied by these methods [Osman et al., 1997; Singh & Sivakumar, 2003]. In visceral leishmaniasis the sensitivity of the methods for the splenic aspirates are quite high (98%) but it is lower for other organs indicating a very high level of infection in splenic macrophages (Table 1-2) [Singh & Sivakumar, 2003].

1.3.6.3 Immunological tests

Immunological tests are based upon the detection of anti-leishmanial antibodies and leishmanial antigens and are useful in both individual diagnosis and epidemiological surveys. Serodiagnosis of the disease is sometimes accompanied by shortcomings due to antibody prevalence in endemic areas specially in post-infected cases, absence of antibody during the incubation period, or cross-reactivity with other pathogenes such as malaria, trypanosoma, schistosoma or leprosy [Kar, 1995]. A number of methods have been described for immunological test of leishmaniasis including Leishmanin Skin Test (LST), Indirect Flourcent Antibody Test (IFAT), Enzyme Linked Immunosorbent Assay (ELISA), Direct Agglutination Test (DAT), Indirect Haemagglutination test (IHA), Immunodiffusion test, Counter current immunoelectrophoresis (CCIEP), immunoblotting, and antigen detection .

Investigation	Sensitivity	Specifity
Splenic aspirate smear	80 –98%	100%
Splenic aspirate culture	70-98%	100%
Bone marrow smear	60-85%	100%
Bone marrow culture*	40-50%	100%
Liver aspirate smear	50-75%	98%
Lymphnode smear	40-50%	95%
Buffy coate culture	0-30%	100%
Complement fixation test	70-80%	60-73%
Immunodiffusion test	60-75%	90-95%
CCIEP test	80-90%	50-70%
IHA test	73-75%	80-95%
IFA test	55-96%	70-98%
DAT	90-100%	80-95%
ELISAs **	36-100%	85-100%
* Hampered by high contamination rate of the cultures.		
** Depending on the antigen used		

Table 1-2: Sensitivity and specificity of various laboratory tests used for visceral leishmaniasis adapted from [Singh & Sivakumar, 2003].

1.3.6.4 Molecular Techniques

Recently, several molecular biological techniques have been developed for a more sensitive detection and identification of leishmania parasites [Bastrenta et al., 2002; Mary et al., 2004; Smyth et al., 1992]. The main approaches of nucleicacid-based detection is based on amplification techniques such as the polymerase chain reaction (PCR) for the detection of DNA, which allows sensitive, specific and fast detection of minute amounts of pathogen DNA. PCR is based on the amplification of a known, specific sequence of DNA using oligonucleotide primers (typically 20-mers), which specifically bind to the DNA flanking the region of interest. Then the target sequence is amplified using a heat-stable DNA polymerase. Recent studies have shown that kinetoplast minicircle is an ideal target DNA in leishmania parasite as there are 10,000 copies of the DNA per cell and its sequence is known for most of species [Aransay et al., 2000]. In visceral leishmaniasis, PCR has opened a new window for diagnosis of leishmaniasis using blood samples with high sensitivity, which is very simple to obtain compare to spleen and bonemarrow aspirates. The sensitivity of the test using blood samples is reported as 70-96% [Osman et al., 1997; Salotra et al., 2001]. In PKLD, PCR with either lymph node or skin aspirates is more sensitive than microscopy for the diagnosis [Osman et al., 1998]. The sensitivity of the test in PKLD patients is between 93.8-96%. The specifity of the test is 100%, which is even higher than ELISA [Faber et al., 2003; Salotra et al., 2003; Salotra et al., 2001]. In cutaneous and mucocutaneous leishmaniasis the test has also shown better sensitivity compared to other tests; up to 100% for CL and 86.4% for MCL [Disch et al., 2005; Faber et al., 2003].

1.4 Immunity to leishmania

1.4.1 Early mechanisms in immunity to leishmania

1.4.1.1 Opsonisation and Immune adherence

Opsonisation is accounted as the earliest phenomenon after the arrival of metacyclic promastigotes into the blood stream. Opsonisation is basically results from the interaction of leishmania and serum proteins leading to the covarage of the parasite by the serum proteins. The leishmania opsonisation takes place through different ways. Interaction of leishmania with natural antibodies is the main and most usual way for opsonisation of the parasite. It has been long known that sera of non-infected vertebrate have anti-leishmania antibodies [Nunes & Ramalho-Pinto, 1996; Puentes et al., 1988], which is independent to the parasite species [Rezai et al., 1975] that means non-infected vertebrates normally have a level of anti-trypanosomatid antibodies in their blood. The natural antibodies constitutes of natural IgM antibodies, which have cross reactivity with leishmania antigen. There are also evidence to suggest that some other serum proteins such as manan-binding lectin, C-reactive protein and C3, the third protein in complement cascade, can take part in the opsonisation of the parasite [Culley et al., 1996; Green et al., 1994; Mosser & Brittingham, 1997]. The time course of opsonisation is species independent and is approximatly 3 minutes long and the most out come of opsonisation is the triggering of the complement cascade, which means the rapid opsonisation of the parasite (3 minutes) [Dominguez et al., 2003].

In addition, it has been shown that human C3-opsonised promastigotes and human serum opsonised amastigotes bind to blood erythrocytes. So that, it is believed the immune adherence has a role in dissemination of the parasite in the host [Dominguez & Torano, 1999]. Natural antibodies-coated parasites deposit C3 on their surface and C3 coated promastigotes adhere to erythrocytes. The adherence is the result of co-operation between CR1 receptors of erythrocytes and the very few C3b ligand on promastigotes [Chevalier & Kazatchkine, 1989; Dominguez & Torano, 1999]. The kinetic of the binding is extremely rapid, which takes 40 sec to complete and leads to transferring leishmania to receiver leukocytes for endocytosis to ensue. Polymorphonuclears have been shown to take role as principal receiver cells in the initial leishmania-host contact. Therefore they can help in dissociation of the promastigote-erythrocyte in the blood [Dominguez et al., 2003].

Leishmania-platelets immune adhesion is reported in non-primate vertebrate and dogs [Dominguez et al., 2003]. Thus, the immune adherence is a phenomenon, which occurs in early stages of leishmania infection in primate and non-primate mammals [Dominguez & Torano, 2001]. In primate, erythrocytes act as an inert shuttle to carry C3-opsonised promastigotes to phagocytes [Lindorfer et al., 2001] and there are studies indicating an anti-microbial role for platelets, however , the main role of platelets is remained unknown [Umekita et al., 1998]. Therefore, the main important function of immune adherence is to promote phagocytosis of opsonised immune complexes and microbes by professenal phagocytes. Hence, the immune adherence may facilitate the uptake of leishmania promastigotes by phagocytes.

1.4.1.2 Complement

The potency of cytotoxic effect of human serum against leishmania parasites has been previously identified [Dominguez et al., 2002] and the role of complement as the main non-cellular immune mechanism in leishmaniasis was thoroughly investigated. However, it has only recently been proven that complement has a complex function against the parasite by posing a strong defence line to the parasite's survival before it enters a permissive phagocyte. The kinetic of complement activation in leishmania infections is not yet fully understood however, it has been shown that the process starts approximately 1 minutes after the parasite arrives into the blood stream and completes in 2.5 minutes [Mosser & Edelson, 1984]. These phenomena strongly supported by real time kinetoplast analysis, as 90% of promastigotes were killed after 2.5 minutes of serum contact. For *L. donovoni* the lytic process induced by complement starts in 60 sec from serum contact and reaches 50% in 80 sec and most of the parasites are killed in 2.5 minutes [Dominguez et al., 2002].

Though, most of the studies at first was focused on the alternative complement pathway, it has now been shown that both classic and alternative pathways can be activated by leishmania parasites [Mosser et al., 1986] and the classic pathway accounts for 85% of complement activity. Thus, the classic pathway is relevant pathway in a kinetic quantitative term and this is confirmed using the sera of patients congenitally deficient in C1 or C2 complement factors [Dominguez et al., 2002].

In leishmania infection, the classic pathway is normally triggered by natural antibodies in which 85% is triggered by IgM and 15-20% by IgG during the first course [Navin et al., 1989]. However, it has also been shown that complement can be activated by antibody-independent mechanism [Mosser et al., 1986]. The classic pathway rises up in 2-3 minutes

but the alternative pathway is activated there after and because most of studies measure the activation of complement in 15-30 minutes of the infection the activity of the classic pathway was first missed out [Puentes et al., 1989; Puentes et al., 1988]. Finally, deposition of C3b on the parasite, which can serve as C5 convertase, can lead to the activation of lytic complement cascade that kills the parasite. Besides, it has been shown that the survival of the parasite after the inoculation is limited. Sandflies normally take 100-300 ηl blood meal and the number of parasites, which can be transmitted is roughly 25-75. As the viability of promastigotes in contact to non-immune serum after 2.5 minutes is >25% and the number of macrophages/monocytes are roughly 7% of human blood leukocyte [Dominguez et al., 2003]. The success rate of inoculation is not known but most of inoculations might be aborted by innate immune system.

1.4.2 Cellular mechanisms in immunity to leishmania

1.4.2.1 Macrophages and their effector function in leishmania infection

Macrophages are the main phagocytes, which take up leishmania parasites. These cells eradicate the parasite efficiently when they are activated by CD4+ T cells. Non-activated macrophages can not kill the parasite effectively and are used as the host cells by leishmania. In mammals, leishmania lives exclusively in macrophages, although the parasite lacks special properties to penetrate its host cells and therefore, its survival is totally dependent on phagocytosis by the macrophage. It has been shown that complement receptors 1 and 3 (CR1 &CR3) and C3b bind to leishmania promastigotes and help them be internalised by macrophages. CR1, with the help of the mannose/fucose receptor and surface glycoproteins such as gp63, has a major role in ligation of promastigotes to macrophages. For internalisation of amastigotes FCIg and CR3 might take a significant role [Chakrabarty et al., 1996; da Silva et al., 1988; Da Silva et al., 1989; Guy & Belosevic, 1993; Wilson & Pearson, 1986].

The activation of macrophage by T cells is the main mechanism leading to destruction of the engulfed parasite (see 1.2.2.2). IFN-γ is accounted as an essential cytokine produced by T cells for activation of macrophages to eliminate leishmania and IFN-γ knocked out mice can not resist *L. major* resulting in fatal infection [Swihart et al., 1995]. IL-12 produced by macrophages and DCs also plays an important role in immunity to leishmania through the interaction of CD40-CD40L [Campbell et al., 1996; Kato et al., 1996; Yamane et al.,

1999]. This was clearly demonstrated in IL-12 knocked out mice where they failed to prevent leishmania infection [Taylor & Murray, 1997]. Activated macrophages also produce other cytokines like TNF-α, IL-6, IL-18 and IFN-γ [Awasthi et al., 2004]. Some cytokines like TNF-α synergises with IFN-γ in induction of NO production *in vitro* [Taylor & Murray, 1997]. Administration of TNF-α to susceptible infected mice was shown help to terminate the course of disease. Blocking TNF in vivo by passively administering anti-TNF antibodies exacerbated the course of *L. major* infection [Liew et al., 1990; Titus et al., 1989]. Administration of other cytokines after infection like IL-2, IL-7, IL-4 and IL-18 induced synergy to IFN-γ in activating the macrophages [Belosevic et al., 1990; Bogdan et al., 1991; Gessner et al., 1993; Tapia et al., 2003]. Instead administration of IL-4 before infection, abrogates the activation of macrophages [Heinzel et al., 1989; Leal et al., 1993].
The macrophage-destroying mechanisms, presumably mediated by toxic mediators, are to some extent effective against on leishmania. The main mediators acting against the parasite in macrophages are toxic mediators of oxygen including superoxide anion (O^-_2), hydrogen peroxide (H_2O_2) and nitric oxide (NO). Production of nitric oxide (NO) is the main sophisticated pathway to make macrophages resistant to the parasite. It has been shown that inhibition of production of NO from iNO render macrophages unable to resist to leishmania. Administration of NO inhibitors abrogates the ability of macrophages to resist leishmania [Assreuy et al., 1994; Awasthi et al., 2004; Evans et al., 1993; Green et al., 1990].

1.4.2.2 Role of neutrophils in leishmania infection

Neutrophils (PMNS) in leishmania infection normally function as the primary effector or phagocytic cells. They are the first cells migrating to the site of infection to phagocyte leishmania parasites and perhaps demonstrate a lethal effect on them. Although the anti-microbial activity of PMNSs is some time effective against the parasite [Chang, 1981; Pearson & Steigbigel, 1981], the parasite usually survive in PMNSs, which help the parasite escaping the complement function [Laufs et al., 2002]. There are studies suggesting a role for PMNSs in generating Th2 immune response [Tacchini-Cottier et al., 2000]. PMNS-secrete chemokine-like IL-8 and leishmania chemokine factor to bring more neutrophils to the site of infection . It has been reported that infection with *L. major* induces production of MIP-2 and KC (two functional murine homologues of IL-8) in the skin at the site of infection [Awasthi et al., 2004].

Because of the short life-span of PMNSs (6-10 h in circulation), which is usually ended by apoptosis [Squier et al., 1995] and phagocytosis by macrophages, neutrophils are actually helping leishmania to enter macrophages. However, leishmania can increase neutrophils' life span by delaying apoptosis for 2-3 days [Aga et al., 2002]. apoptotic PMNSs are recognised by phosphatidylserine expressed on their surface [Fadok et al., 1992]. Ingestion of apoptotic leishmania infected PMNSs, does not activate the antimicrobial mechanisms of the macrophage against leishmania. Therefore, infection of PMNSs makes a smooth way for leishmania to enter macrophages.

PMNSs seem to present a contradiction function. It has been shown that 47% of peripheral human neutrophils express CD28, which on ligation induce IFN-γ and T-cell chemotactic factors indicating the role of neutrophils in initiating of adaptive immune response. In addition, it has been shown that IFN-γ promotes the macrophage activity for processing and presentation of antigens to T cells. Thus, neutrophils indirectly can induce the initiation of adaptive immunity against leishmania [Awasthi et al., 2004].

1.4.2.3 Role of CD4+ T lymphocytes in immunity to leishmania

T cells are the main cells responsible for generating specific immune responses against pathogens (see 1.2.2.2) including leishmania parasites. It has been shown that resistance to leishmania can be transferred from one mouse to another by transferring specific CD4+ T cells [Holaday et al., 1991; Moll et al., 1988]. There is convincing evidence, in mammalian hosts, to suggest that the immunity against leishmania parasites relies on generating a cellular immune (Th1) response. This is clearly demonstrated by the genetic predisposition of susceptibility to *L. major* infection in mice, which correlates with the domination of IL-4-driven Th2 response and resistance is linked to an IL-12-driven, interferon-γ (IFN-γ)-dominated Th1 response that promotes healing and parasite clearance respectively [Sacks & Noben-Trauth, 2002]. This is fully described in non-healing BALB/c and self-healing C57 mice where they express transcripts for IL-4 and IFN-γ respectively [Locksley et al., 1987]. IL-12 has a major role in the development of Th1 immune response. This has been shown using transgenic mice where T-cell receptors were specifically characterised for peptides derived from ovalbumin. In this model it was shown that dendritic cells were capable of induction and clonal expansion of T cells but they were unable to induce differentiation of T cells toward Th1 or Th2 without the addition of IL-4 or IL-12 respectively [Macatonia et al., 1993]. In other experiments, IL-12- deficient genetically resistant mice and susceptible BALB/c mice both developed a strong Th2 response with

high levels of IL-4 mRNA and low levels of IFN-gamma mRNA in CD4+ T cells [Mattner et al., 1996]. In addition, *in vivo* administration of neutralisation anti IL-12 during a leishmania infection, made resistant mice more susceptible to leishmania [Hondowicz et al., 1997]. Endogenous IL-12 plays a decisive and crutial role in leishmania infection [Murray, 1997]. It has been reported that unresponsiveness of T cells to IL-12 in BALB/c mice was the main cause of lack of Th1 immune response and IL-12 receptors and responsiveness are suppressed by IL-4 [Himmelrich et al., 1998; Launois et al., 1997; Macatonia et al., 1993]. In BALB/c mouse model, it has also been revealed that during early stages of *L. major* infection (detected in 4 days) there is a mixture of Th1/Th2 immune response. IFN-γ is variable in different strains but strikingly IL-4 was produced in all of the examined mice that fully generated Th2 immune response. Administration of anti-CD4 and anti-IL-4 antibodies resulted in healing of the lesions indicating the crucial role of IL-4 and T cell, which produce IL-4 [Locksley et al., 1993; Uzonna & Bretscher, 2001].

Further observations suggested that the Th1 suppression role of IL-4 was under the control of IL-2 suggesting IL-2 as the leishmania susceptibility factor [Heinzel et al., 1993; Louis et al., 1998]. Moreover, other studies showed a dual role for IL-4 in *L. major* infection, which has been shown to be dependent upon the phase of response and the antigen-presenting cells [Biedermann et al., 2001]. IL-10 is another cytokine that may affect susceptibility to leishmania infection [Kane & Mosser, 2001]. It has also been reported that blocking of IL-10 using anti-IL-10 in *L. major* infection further reduced the succeptibility of IL-4 receptor α gene deficient mice [Noben-Trauth et al., 2003]. Administration of anti-IL-10 cure the leishmania infection [Belkaid et al., 2001]. The role of T-regulatory (CD4+ CD25+) cell or T reg cells in leishmania infection has recently been investigated. T reg cells effectively suppress the effector activity of other T lymphocytes against self-antigens as well as foreign antigens. It has been reported that in leishmania infection T reg cells might play a regulatory role in generation of immunity to the parasite by producing IL-10 [Campos-Neto, 2005]. There are also studies suggesting immunosuppressive roles for IL-13 and TGF-β in immunity to leishmania, which correlates with a suppression of IL-12 and IFN-γ expression [Li et al., 1999; Matthews et al., 2000].

It has been shown that administration of IFN-γ as single dose or sustained delivery cannot shift Th2 immune response to Th1 to stop BALB/c mice developing progressive lesion or alter the course of infection. This suggest that the role of IFN-γ in maintaining Th1

immune response is independent and early production of IL-4 and IL-10 is not due to lack of IFN-γ [Awasthi et al., 2004]. The role of other types of CD4+ T cells such as Th-17 cells in immunity to leishmania is not clear yet and needs to be investigated, howevere, they may play a positive role by their contribution in the production of IL-12.

1.4.2.4 The role of CD8+ T cells in immunity to leishmania

The role of CD8+ T cells in immunity against intracellular parasites was first reported in malaria by two independent groups in 1987 and 1988 [Schofield et al., 1987; Weiss et al., 1988]. In subsequent years, the participation of CD8+ T cells was also described in immunity to other intracellular pathogens such as *Toxoplasma gondii, Trypanosoma cruzi and Mycobacterium tuberculosis* [Rodrigues et al., 2003; Serbina & Flynn, 2001].

In leishmania, the function of CD8+ T cells in generation of immunity has been undefined for many years and it still remains as a dark area in immunity to leishmania. Initial studies failed to indicate a role for CD8+ T lymphocytes [Erb et al., 1996; Huber et al., 1998]. However, later studies showed that the role of CD8+ T cells in pathogenesis and immunity to leishmania can be demonstrated under certain conditions. In studies reported by Belkaid [Belkaid et al., 2002], C57BL/6 mice with CD8+ T cell deficiencies, including mice without CD8 ($CD8^{-/-}$)or treated with anti-CD8 mAb, failed to control the *L. major* infection following the inoculation of 100 metacyclic promastigotes into the ear dermis. Also, these animals demonstrated a severe and delayed dermal pathology when compared to wild-type animals. In this model of infection, reconstitution of resistance was achieved when both CD4+ and CD8+ T cells were adoptively transferred [Belkaid et al., 2002]. These results were in agreement with some previous studies where immune BALB/c mice rechallenged with *L. major* showed production of IFN-γ from CD8+ T cells [Muller et al., 1993], which was associated with the production of nitric oxide by macrophages [Stefani et al., 1994]. In addition, β2-microglobulin and perforin deficient mice primed with leishmania antigen failed to control the infection after a challenge with leishmania suggesting a role of CD8+ T cells [Colmenares et al., 2003].These results are supported by a number of studies demonstrating an effective role for CD8+ T cells in activating macrophages by secreting IFN-γ in leishmania infections [Bottrel et al., 2001; Colmenares et al., 2003; De Luca et al., 1999; Pompeu et al., 2001]. The cytolytic activity of CD+ 8 T cells in leishmania infection has also been investigated. It has been shown that CD8+ T lymphocytes are highly cytolytic *in vitro* against leishmania-infected macrophages [Brodskyn et al., 1997].

In conclusion, it is believed that CD8+ T cells take a significant part in immunity against leishmania [De Luca et al., 1999; Rodrigues et al., 2003]. It is likely that the presence of CD4+ T cells and their contribution is necessary for the activation of CD8+ T cells [Colmenares et al., 2003; Erb et al., 1996] and CD8+ T cells function as effectors by posing a direct cytotoxic activity to infected macrophages or by releasing IFN-γ to activate them against the parasite. CD8+ T cells may also contribute in the long-last immunity to leishmania [Awasthi et al., 2004]. Nevertheless, the importance of CD8+ T cells in immunity to leishmania is far from being clear and remains to be further elucidated.

1.4.2.5 The role of other lymphocytes in immunity to leishmania

The role of other NK cells and γ/δ T cells in immunity to leishmania is not fully understood. It was reported that the course of leishmaniasis caused by *L. tropica* in Beige mice lacking NK activity with the background of C57 mice, was similar to that of normal C57 mice but they failed to control *L. donovoni* [Kirkpatrick & Farrell, 1982]. In another study, it was shown that NK cells induced early IFN-γ dependent protective response in resistent C3H/HeN mice against *L. major* compared to diminished activity of NK cells in susceptible BALB/c mice [Scharton & Scott, 1993], suggesting a role for these cells in resistance to leishmania. Also, in C57BL/6 resistant mice, depletion of NK cells before the infection induced marked exacerbation in local tissue swelling and increased the number of parasites in the lesions which was accompanied by less IFN-γ production in the first two weeks of infection [Laskay et al., 1993]. Neutralization of IL-12 by anti IL-12 antibodies has also led to abrogating the protective role of NK cells in C57 mice [Scharton-Kersten et al., 1995] indicating the role of IL-12 in early production of IFN-γ by NKs, which mediates Th1 immune response. It has been shown that BALB/c mice lack early NK cell response after *L. major* infection due to simultaneous production of IL-12 inhibitor factor like IL-4, IL-10 and TGF-β. This indicates that early NK immune response is lacking in the susceptible BALB/c mice, which may be due to presence of inhibitory factors like IL-10 and IL-4 [Scharton-Kersten et al., 1995].

An *in vitro* studies on γ/δ T cells have demonstrated a marked increase in contact of these celld with leishmania parasites [Saha et al., 1999], however, expansion of γ/δ T cells *in vivo* was shown to be mediated through Th2 cytokines and activation of Th2 lymphocytes results in the expansion of γ/δ T cells [Rosat et al., 1995]

1.4.3 Immune evasion by leishmania

After entering the macrophage, the parasite evades the proteolytic action of the macrophage and use the macrophage as a site to live and propagate. Two surface molecules of leishmania parasites, the gp63 surface protease and a lipophosphoglycan (LPG), have been implicated in the attachment and uptake of promastigotes by host cells. Interestingly, these proteins complement the effect of each other in the immune evasion mechanism. During the initial stages of the infection of the macrophage with *L. donovoni*, LPG promotes the intracellular survival of promastigotes by inhibiting the fusion of the parasite –containing phagosome with the lysosomes [Desjardins & Descoteaux, 1997]. LPG also blocks the oxidative burst of the macrophage via inhibition of protein kinase C [Giorgione et al., 1996]. In the phagolysosome form, gp63 acts to protect the parasite by inhibiting chemotaxis and the degradative phagolysosmal enzymes [Sorensen et al., 1994]. In addition, the parasite transformation inside the macrophage from the promastigote to amastigote makes it more resistance against the low pH of the phagolysosome because amastigotes are metabolically more active in an acid than the neutral environment [Zambrano-Villa et al., 2002]. The parasite also promotes its survival inside the macrophage by preventing apoptosis, antigen presentation procedure and responsiveness to cytokines [Moore & Matlashewski, 1994]. For instance, leishmania inhibits the expression of the MHC class II molecules so in turn it decreases peptide presentation by macrophage [Reiner et al., 1987]. Similarly, gp63 from *L. major* and *L. donovoni* cleaves CD4 molecules on T cells interfering with the stabilisation of the interaction between antigen-presenting cells and T helper cells. Besides, amastigotes internalize and degrade class II MHC molecules as well as down regulate the expression of co-stimulatory molecules such as B7-1 [De Souza Leao et al., 1995; Kaye et al., 1994]. Leishmania also induces the release of PGE2 and TGF-β, which inhibit macrophage. The other evading system of leishmania parasites is to control the response of infected macrophages through its LPG by down regulating the expression of the TNF-α receptor and inhibiting the chemotaxis of neatrophiles and monocytes [Zambrano-Villa et al., 2002]. The mechanism developed by the parasite to evade complement could be among the most sophisticated ones that used by parasites to evade immunity. Promastigotes of leishmania, probably due to high expression of LPG molecule in their surface can resist the complement complex C5-C9. gp63 can also process to protect the parasite from complement through the proteolytic conversion of C3b to C3bi on the parasite surface and leishmania protein kinases can phosphorylate some complement components such as C3, C5 and C9 thus blocking both pathways of activation

[Brittingham et al., 1995; Puentes et al., 1990; Zambrano-Villa et al., 2002]. Another strategy for leishmania to evade the immune system is suppressing IL-12 transcription gene. Because IL-12 is a major promoter of IFN-γ production, its suppression can, in turn, inhibit the IFN-γ production, which provide a survival advantage to the parasite [Zambrano-Villa et al., 2002].

1.5 Leishmania vaccines

The complexity of host-leishmania parasite interaction demands a high level of basic knowledge coupled with clinical research to pave the way for designing and production of an effective vaccine against the parasite. For production of each vaccine utmost attention must be paid to assure safety, reproducibility and efficacy. Each vaccine has to meet several criteria to be counted as a safe and effective vaccine. The requirements are including Good Laboratory Practice (GLP), Good Manufactory Practice (GMP) and Good Clinical Practice (GCP) [Khamesipour et al., 2006]. To assure safety each vaccine, during its development, needs to pass through 5 stages, discovery, pre-clinical development, clinical development, registration and post-marketing evaluation [Khamesipour et al., 2006].

Different strategies have been considered to develop an effective vaccine for leishmaniasis but according to WHO's report, there is not a wholly effective vaccine available for leishmania parasites yet although different preventive or even therapeutic vaccines are currently is under investigation [Coler & Reed, 2005; Machado-Pinto et al., 2002].

1.5.1 Killed leishmania Vaccine

Several studies have been carried out to prove the immunogenicity of killed leishmania parasite. The earliest attempt to construct a killed leishmania vaccine for leishmania took place in 1940s in Brazil. After that in 1970s, a killed vaccine composed of 5 isolates of 4 different species was developed by Mayrink. Later, this was simplified to contain only killed *L. amazonensis* and used for vaccination in Colombia and Equador and was also used as an adjuvant for chemotherapy in Brazil [Khamesipour et al., 2006]. Later a vaccine made up of autoclaved *L. mexicana* together with BCG was introduced for immunotherapy and immunochemotherapy [Convit et al., 1987]. It has also been confirmed that using killed parasite can act as an adjuvant and helps in reducing the dose of anti-leishmanial drugs used for treatment of leishmania patients [Machado-Pinto et al.,

2002]. In Venezuela, autoclaved *L. mexicana* is now used to treat leishmaniasis [Convit et al., 2003]. In Equador, two injections of a killed vaccine composed of *L. mexicana* and *L. amazonensis* together with BCG resulted in 73% protection in Ecuadorian children [Armijos et al., 1998] however further studies did not confirm the result [Armijos et al., 2004]. In Colombia testing this vaccine did not show much difference between vaccinated and non-vaccinated individuals in a double-blind placebo control efficacy study against natural infection [Velez et al., 2005]. In another attempt, it was tried to use autoclaved *L. major* (ALM) mixed with one tenth dose of BCG used for tuberculosis vaccine in non-endemic area. Although, this vaccine was tested in Phase III field efficacy trials against cutaneous and visceral leishmaniasis, in a randomized double-blind trial in Sudan against VL and in some prospective studies in Iran against CL no significant protection was observed following the injection of the vaccine (ALM + BCG) against either VL or CL compared to BCG alone [Khalil et al., 2000; Sharifi et al., 1998]. The results indicated that the mixture was safe and induced leishmania Skin Test (LST) conversion with weak but measurable IFN-γ production. In the field only 16.5% of LST conversion was seen in anthroponotic areas of Bam, Iran. With two doses of the vaccine, 43% of LST converted volunteers showed immunity to VL in Sudan. There was no difference between one and three doses injection in prevention of CL [Khalil et al., 2000; Sharifi et al., 1998]. Using aluminium hydroxide (alum) as adjuvant in order to enhance the immunogenicity of the vaccine, better results were observed in monkeys and dogs. Thus, combination of alum precipitated ALM and BCG seems to be more optimistic in leishmania vaccine development [Khamesipour et al., 2006].

Other studies showed protection to *L. major* in mice immunised with killed promastigotes or recombinant proteins plus IL-12 as an adjuvant but the mice lost protective immunity after 12 weeks [Sacks & Noben-Trauth, 2002]. However, in another study, ALM induced a higher level of protection in monkeys when combined with rIL-12 and alum as adjuvants. Further studies to test this combination are being carried out in Sudan and Iran [Dumonteil et al., 2001]. Oral vaccination with whole leishmania antigen has been tried as another strategy in mouse model; two oral doses with 100mg killed *L. amasonensis* whole antigens rendered BALB/C and C57BL/6 mice more resistant against subsequent infection with *L. amasonensis*. Orally vaccinated BALB/c mice with the killed leishmania vaccine were also protected against *L. major* infection [Pinto et al., 2003].

1.5.2 Live leishmania Vaccines

Using live leishmania parasite as a vaccine to prevent the possible future infection is called Leishmanization [Khamesipour et al., 2006]. Developing a potent vaccine based on inoculation of attenuated live leishmania parasites or Leishmanization has been considered as another strategy in the prevention of leishmaniasis. Since long time ago, it was clear that patients recovered from cutaneous leishmaniasis are resistant to re-infection. Thereafter some attempts were made to use live parasite derived from patients exudates to create self-healing lesion in some parts of the body preventing future infection that may cause lesion on the face and other exposed parts of the body similar to the approach used in cow-pox vaccine [Sacks & Noben-Trauth, 2002]. In mouse models vaccination with radiation attenuated or virulent promastigotes has also resulted in better protection than that achieved by inoculation of immunogenic leishmania proteins such as gp63 [Rivier et al., 1999]. There have been attempts in Iran and Israel for developing a live leishmania prophylactic vaccine for cutaneous leishmaniasis. No significant reduction is reported in a large prognosis study carried out in 1980s [Khamesipour et al., 2006].

Basically, the live vaccine is of low cost and highly immunogenic but the main problem associated with this vaccine is the lack of standardization and quality control as the parasite looses infectivity in long *in vitro* culture. In addition, the vaccine produces lesions on the site of inoculation that may last between 3-13 months if left untreated by anti-leishmania drugs. Some live vaccines may cause chronic lesions that do not easily respond to chemotherapy [Khamesipour et al., 2006]. In mice, it has been shown that the parasite persists in the infected tissues for a long time after healing. Therefore, the application of the live parasite in HIV and other immonosuppressed patients and even in the populations at risk of HIV due to the possible recurrence of the infection is restricted [Aebischer et al., 1993; Montalban et al., 1989].

1.5.2.1 Use of genetically modified parasite as a vaccine

Genetically modification of the parasite is considered as a new strategy for application of live leishmania vaccination. Genetically modified parasites do not normally cause clinical symptoms but are able to induce immunity to the wild type parasite. Developing genetically modified leishmania is carried out either by mutagenesis and selections [Daneshvar et al., 2003] or gene targeting methods in which either a foreign gene is introduced into the parasite genome or by knocking out virulency genes [Joshi et al., 1998]. In a different strategy it has been tried to introduce genes encoding proteins, which

are harmful for the parasite itself but not for the host upon the exposure of harmless products [Muyombwe et al., 1998; ten Asbroek et al., 1990]. For instance, introducing foreign genes to the parasite to make it more sensitive to particular drugs has recently been studied [Davoudi et al., 2005]. The results have revealed that the elimination of the parasite even as early as day 7 after infection can stimulate a high level of IFN-γ production leading to protection in mouse model, however, none of these products have yet reached clinical development studies. Some studies tried to knock out several genes in *L. major* and *L. donovoni* models. Dihydrofolate reductase knocked out *L. major* showed good protection in mice but not in monkeys where the results were disappointing [Khamesipour et al., 2006; Titus et al., 1995; Veras et al., 1999]. In *L. donovoni* bioprotein transporter (BT1) and in *L. mexicana* cystein proteinase (CP) showed similar results in BALB/c mice [Frame et al., 2000; Papadopoulou et al., 2002]. In leishmania parasites, Centrin function as a calcium-binding cytoskeletal protein essential for centrosome duplication or segregation. It has been reported that certain knocked out mutants parasites show irregularity in their growth [Selvapandiyan et al., 2004; Selvapandiyan et al., 2001]. So, the Centrin-knocked out mutants of leishmania are considered as potential live vaccines and the immunogenicity of them is now under investigation in our lab [personal communication with Dr Nakhasi].

1.5.3 Recombinant protein Vaccination

Recently, recombinant proteins of leishmania species have been used to produce the immunity against different species of leishmania. Different proteins have been tested to be used as a vaccine including gp63, HASP-B1 and PSA-2, which are immunogenic and can develop varriable levels of immunity in different animal models. Gp63 in *L. major* is an extremely potent immunogenic protein compared to a standard protein (ovalbumin) and its injection even in salin induced significant protection. Injection of gp63 in saline, Complete Freud's Adjutant (CFA), BCG and *Corynebacterium parvum* induced significant protection in BCA mice. Combination of gp63 and adjuvant resulted in different levels of protection depending upon the site of vaccination relative to that of the challenge infection. The vaccination in the tail close to the site of infection led to a stronger induction of immunity in mice [Rivier et al., 1999]. In addition, recombinant acylated surface protein B1 (HASPB1) of *L. donovoni* is able to confer protection against the experimental challenge. unlike soluble leishmania Ag + IL-12, rHASPB1 did not require adjuvant and was sophisticated to control the parasite burden in the spleen [Stager et al., 2000]. Surface

Antigen complex2 (PSA-2) showed potent immunogenicity but the level of immunity developed after vaccination was not good enough to protect the animals against challenge with the parasite [Handman et al., 1995a; Handman et al., 1995b].

1.5.4 Peptide vaccination

It is known that proteins taken by antigen presenting cells (APC) are cut into peptides and presented to T cells through MHC class I and II molecules. Therefore, finding a strong immunogenic peptide with high affinity to MHC class l or ll, which can be presented by APCs would be a feasible strategy for developing a novel vaccine against pathogeneses such as leishmania parasites. Many studies have been taken place to find an immunogen peptide, which can be used as a vaccine in leishmaniasis. Some of those peptides have been discussed in chapter 5.

1.5.5 DNA vaccination in leishmania

Genetic immunization is a newly developed approach in prevention and treatment of infectious diseases. In this method, DNA Plasmids encoding one or more proteins of the pathogen are directly introduced into host cells and decoded into the protein. So, specific cell mediated and /or humoral immune responses are elicited against the encoded protein. Effectiveness of DNA vaccination has been shown in different studies against tumour and intracellular parasites [Westermann et al., 2007; Zapata-Estrella et al., 2006]. In leishmania parasites, it has been demonstrated that immunization with plasmid DNA encoding single or multiple leishmania antigens is a particularly effective approach to generate strong and long-lasting protection against *L. major*. Using this approach for the leishmania vaccination to induce cell mediated immunity to *L. major*, DNA plasmid constructed with *L. major* gp63 gene (gp63-pcDNA3) were injected to BALB/c mice intradermally resulted in the protection of 30% of mice from leishmania infection. CD4+T cells from gp63-pcDNA3-immunized mice proliferated and produced IFN-γ (but not IL-4) upon stimulation with freeze –thawed parasites indicating a Th1 response [Walker et al., 1998; Xu & Liew, 1995]. In another study, the *L. major* gp63 gene was cloned into an eukaryotic expression plasmid pcDNAI with CMV or RSV promoters and administrated in BALB/c mice. Intramuscular injection of mice with 100 µg of the plasmid DNA induced a significant level of immunity in immunised mice compared to controls [Xu & Liew, 1994]. Similar results were achieved by cDNAs encoding *L. mexicana* gp63 in BALB/c mice when the mice were immunized with two i.m. injections of 100µg of plasmid DNA and challenged

by *L. m. mexicana* parasites in the footpad. The size of lesion indicated that the immunized mice were partially protected against the infection [Dumonteil et al., 2003]. In a comparative study, the immunogenicity of DNA plasmids encoding *L. major* LACK, PSA2, Gp63, LeIF and two newly identified p20 and Ribosomal like proteins, in addition to other truncated portions of the LACK antigen were compared. Neither of the DNA vaccine candidates was able to mount a full protection in BALB/c mice challenged with a highly virulent *L. major* strain. However, the most promising gene was LACK and it was more protective when it was used as a p24 truncated form [Ahmed et al., 2004]. In another study, *L. m. mexicana* gp63 and CPb, *L. m. amazonensis* gp46, and *L. major* LACK were compared. The results indicated that BALB/c mice immunised with plasmids VR012-GP46, VR012-GP63 and VR1012-CPb were partially protected against *L. mexicana* infection, whereas VR1012-LACK had no effect [Dumonteil et al., 2000]. A DNA vaccine composed of leishmania Parasite Surface Antigen Complex 2, PSA-2 is also under investigation by Noormohammadi. The advantage of this antigen is being present in several leishmania species; it provides an opportunity to protect individuals against several forms of the disease. It has been shown that PSA-2 DNA is immunogenic but surprisingly co-administration of IL-12 with PSA-2 DNA abrogates the immunogenicity [Noormohammadi et al., 2001].A further study about the potency and quality of the vaccine is being continued.

DNA vaccines are inexpensive, simple to use and easy to produce. It is also possible and managable to put different genes in one plasmid construct [Almeida et al., 2002]. These vaccines can target both MHC class I and II molecules and the immunogenic protein can, therefore, be presented through both of them that enhances the efficacy of the vaccine, which is important for leishmania vaccination as; both CD4+ and CD8+ T cells take role in immunity to leishmania [Gurunathan et al., 2000a]. Moreover, these vaccines produce a long-term production of immunogenic protein, which is similar to the natural infection leading to long memory [Scott et al., 2004].

1.6 Animal models in leishmania studies

Due to the ethical issues related to studies performed on human, leishmania studies directly in humans are cumbersome. Therefore, there is a crucial need for the development of animal models for leishmania studies. Animal models are expected to mimic the pathological features and immunological responses observed in humans when exposed to a

variety of leishmania species. with different pathogenic characteristics. Many experimental models have been developed, each with specific features, but none accurately reproduces what happens in humans [Garg & Dube, 2006].

In cutaneous leishmaniasis, *L. enriettii* infection of guinea pigs was the first model to be well characterized. Guinea pigs develop T-cell responses to parasite antigens within 2 weeks of infection, and the lesions heal within about 10 weeks. A major attraction of this animal model is the fact that the host-parasite combination is a natural one and that the disease pattern is similar to that observed in human cutaneous leishmaniasis caused by *L. major* [Handman, 2001]. Due to difficulties associated with the guinea pig animal models, this model is now replaced with inbred mouse animal models. However, the spectrum of disease manifestations observed in human leishmaniasis is not perfectly mimicked in the laboratory by infection of different inbred strains of mice. BALB/c mice are highly susceptible; upon infection with *L. major*, they develop skin ulcers, which expand and metastasize, leading to death. C57BL/6 and CBA/N mice are more resistant, develop small lesions which cure in 10 to 12 weeks, and are resistant to reinfection. Most other strains of mice are intermediate in susceptibility[Handman, 2001].

For visceral leishmaniasis, several attempts were made in the past to use small rodents for *L. donovani* infection. These include hamster (European, Chinese and Syrian), mouse (BALB/c, NMRI, DBA/1, C57BL/6), rat, mastomys, squirrel, gerbil *etc*. Of the various animals tried, BALB/c mice and Syrian golden hamsters are the commonest and currently used animal models for drug and vaccine testing against VL. [Hommel et al., 1995]. The golden hamster was used in one of the early animal models for the study of visceral leishmaniasis. Infection with *L. donovani* causes visceral leishmaniasis, which might lead to death in human. Anaemia, hyperglobulinemia, and cachexia are aspects of the human disease mimicked in the hamster, making it a useful tool for the characterization of molecules and mechanisms involved in the pathogenesis [Hommel et al., 1995]. However, in recent years, the interest in the hamster animal model has waned and this model is now used preferably as a source of *L. donovani* amastigotes. The passage through hamsters seems to be a required life cycle stage for infection of mice, which are currently accounted as the preferred animal models for visceral leishmaniasis (Table 1-3).

The mouse model reproduces many aspects of the human disease, including a range of susceptibility states depending on the strain of mouse used. Although the mouse model is useful in many ways, it must be remembered that the mouse model for leishmaniasis is just a model and that the mechanisms of pathogenesis and immunity may be a little different in

humans and extrapolation from mouse to human requires much care [Kelso, 1995; Kelso, 1998] due to the different circumstances in human leishmaniasis. For instance, under natural conditions, the infected sandfly deposits a few hundred metacyclic promastigotes into the dermis of the host, whereas experimental infections are usually induced by subcutaneous (s.c.) or intravenous (i.v.) injection of millions of promastigotes grown under *in vitro* conditions or amastigotes recovered from cutaneous lesions or infected spleens [Garg & Dube, 2006]. In addition, the immune responses following infection of inbred mouse strains with viscerotropic leishmania species, such as *L. donovani, L. chagasi* or *L. infantum*, are similar to those observed in the *L. major* mouse model. However, BALB/c mice do not appear to exhibit a similarly high susceptibility to these parasites, since intravenous injection of visceral leishmania species results in a self-healing of chronic infection. Furthermore, cytokine phenotypes elicited by viscerotropic leishmania in this mouse model are not typical of a Th2-type response [Lehmann et al., 2000]. Therefore, it is believed that BALB/c mouse model for VL is considered to be a good model since the infection progresses for the first few weeks and then controlled by the host immune response [Murray et al., 1987]. The other difficulty with the mouse as a model for human visceral leishmaniasis is the need to inject amastigotes intravenously in order to induce a reproducible pattern of colonization of the liver and spleen. This route of administration does not mimic the natural infection by the sandfly. In addition, there is no evidence of wasting, as in the human disease, hence the infection is chronic but not fatal [Ahmed et al., 2003]. Dog has also been reported as suitable animal model for visceral leishmaniasis in which relevant immunological studies and vaccine development could be performed. With the recent cloning of several dog genes encoding cytokines and immunologically important cell markers, as well as the development of monoclonal antibodies to these molecules, there is hope for a more sustained exploitation of this excellent animal model.[Garg & Dube, 2006; Handman, 2001].

In visceral leishmaniasis, the mouse model is mainly comparable to self-controlled oligosymptomatic cases and therefore it is useful for the study of the protective immune response. On the other hand, a more suitable model to study the progressive disease is the hamster, which infected with *L. donovani* or *L. chagasi* that develop a disease similar to human progressive visceral leishmaniasis with hepatosplenomegaly, hypoalbuminaemia, hypergammaglobulinaemia, and pancytopenia. Therefore, this model is mainly used to study the mechanisms of immunosuppression [Rodrigues Junior et al., 1992]. Due to the close phylogenic relation of primates to humans, leishmania infected monkey model using

Aotus trivirgatus (owl monkeys) and *Saimir sciureus* (Squirrel monkeys) has also been used for tertiary preclinical testing of vaccines for visceral leishmaniasis [Chapman & Hanson, 1981; Chapman et al., 1983].

Animal/strains		Parasite	Route of inoculation	Characteristics
Mouse:	BALB/b	*L. chagasi*	i.v	-Self curing to non healing type
	BALB/c	*L. donovani*	i.d	-Th1/Th2 response
	C57BL/6	*L. infantum*	s.c	-All immunological reagents are available
				-Good model for dissecting protective immune response
Hamster:	Golden	*L. chagasi*	s.c	-Progressive fatal infection
	Chinese	*L. donovani*	i.p	-Severe immunosuppression (Th2 response)
		L. infantum	i.c	-Reagents for T-cell response not available
				-Good experimental model for initial vaccine trial
Dog:	Stray	*L. infantum*	i.v	-Natural reservoir (not in India)
	Beagle	*L. chagasi*	i.d	-Subclinical/asymptomatic to progressive fatal infection
	Mongrel			-Immunosuppression (Th2 response)-Reagents for cytokine response not available-Good secondary model for pre clinical vaccine trial
Monkey:	Owl	*L. donovani*	i.v.	-Sub clinical to fulminating progressive fatal infection
	Squirrel		i.d	-Severe immunosuppression (Th2 response)
	Vervet			-All immunological reagents are available
	Langurs			-Good secondary model for pre clinical vaccine trial but difficult to use due to cost, handling and immunological black boxes

i.v., intravenous; i.d., intradermal; s.c., subcutaneous; i.p., intraperitonial; i.c., intracardial

Table 1-3: Experimental models used for vaccine trials against visceral leishmaniasis (VL) [Garg & Dube, 2006]

1.7 Aim of the study

The role of CD8+ T cells in immunity to leishmania has not yet fully understood and most of previous studies have assessed CD8+ T cells involvement by measuring proliferation or the secretion of IFN-γ by these cells. The main objective of this study is to develop a mouse model to assess CTL responses to leishmania vaccine candidates by developing a cytotoxicity assay using tumour cells transfected with leishmania antigen as a surrogate target cells. This model will be also used to evaluate the contribution of CD4 T cells and APC in immunity against leshmania infection. Two mouse models will be used in this study including the conventional BALB/c, and transgenic HHDII models.

DNA immunization is a newly developed approach in leishmania vaccination. In this method DNA encoding an immunogenic protein is directly introduced to the host cells to generate immunity to the parasite. DNA vaccines are effective and simple to use, and it has been shown that alteration of the method of inoculation can alter the immunity induced by the vaccine. In this study, the efficacy of DNA vaccine in BALB/c mouse model will be evaluated by two DNA immunisation methods (administration by intramuscular or gene gun) using leishmania gp63 DNA plasmid construct.

Dendritic cells are the most professional antigen presenting cells, which may have important roles in immunity to leishmania. Using DCs as adjuvant to enhance immunity to leishmania is a novel approach in leishmania vaccine investigation. This study is proposed to investigate the role of DCs in immunity against leishmania infection. The immunogenicity of DCs alone or loaded with lesihmanai antigen(s) will be tested in BALB/c mouse model against infection with lesihmanai parasite. The potency of DCs in induction of Th1/Th2 or CTL immune response will also be determined.

Peptide-based vaccine is a promising approach in vaccine development. In this study the web-based software “SYFPEITHI” will be used to select potential immunogenic peptides from leishmania gp63 antigen to be tested in BALB/C and HHDII mice. Immunogenicity will be determined by immunisation and in vitro immunological assays.

OX40 is an important co-stimulatory receptor expressed on T cells. Interaction of OX40 and OX40L on APC induces T-cell activity, which results in a higher immune response. In this study the potency of recombinant OX40L molecule in up- or down-regulation of immunity against leishmania in BALB/c mouse model will be investigated. Co-administration of OX40L and the leishmania vaccine candidates will also be tested. In order to produce the OX40L, protocols for purification of OX40L from B9B8E2 cell

culture supernatant will be established and optimized using different buffers, gels and methods of purification.

Chapter 2 Materials and methods

2.1 Cells & Animals

2.1.1 Cells

Various cell lines used in this study are described below

Name	Description	Media	Source
CT-26	N-methylurethane-induced BALB/c murine colon carcinoma	DMEM+10% FCS	Prof Ian Hart (St Thomas Hospital)
A20	Murine B cell lymphoma	RPMI 1640+2 mM L-glutamine	ATCC
RMA/S-A2	Transgenic lymphoblastoid	RPMI 1640+2 mM L-glutamine+10% FCS+G418	Dr. F Lemonnier (Institut Pasteur, Paris)
T2	Human Lymphoblastoid	RPMI 1640+2 mM L-glutamine +G41810% FCS	Dr. F Lemonnier (Institut Pasteur, Paris)
B9B8E2	Chinese Hamster Ovary cell line transfected with the murine OX40L and IgG plasmids	DMEM medium supplemented with 15 mg/ml MSX, 1% glutamine synthetase (GS) and 10% Bio-FCS (FCS without bovine IgG).	Xenova plc, (Cambridge, UK)

Table 2-1: Cell Lines and their descriptions

2.1.2 Generation of DCs

BM-DC were generated as described by Inaba and coworkers with some modifications [Inaba et al., 1992]. Briefly, hind limbs of naïve BALB/c mice were collected and all muscle was removed using scalpel and tweezers. After cutting the ends of the bone, bone-marrow cells were flushed out media and harvested. The bone marrow cells were then centrifuged and resuspended in 1ml BM-DC media, counted and plated at 1×10^6 cells per well/ml with 100ng/ml of mGM-CSF (X63 supernatant). The cells were then incubated overnight at 37° C, 5% CO_2. On day 2 and day 4, non-adherent cells were washed out by gently replacing 700µl of media with fresh DC media containing GM-CSF. On day 6, BM-DC were replated and split into two groups. The first group (test) were pulsed with 10µg/ml SLA and the second group was used as control. Control and test groups were pulsed 4-6 hours later by 1µg/ml LPS to induce maturation. The following day, BM-DCs were washed in serum free RPMI 1640 media, counted and injected intradermally at 2×10^6 per mouse or used as target cells in standard 4-hour cytotoxicity assay.

2.1.3 Leishmania parasites

L. mexicana promastigotes strain Hd18 were kindly gifted by Dr. Varley, the London School of Hygiene and Tropical Medicine (LSHTM), and cultured in Schneider media (Sigma) supplemented with 10% FCS at 25 ºC as described by [Bates, 1994a].

2.1.4 Animals

HLA-A2 transgenic (HHDII) mice, a generous gift from Dr. F Lemonnier (Institute Pasteur, Paris) were housed and bred at the Nottingham Trent University.

BALB/c mice were purchased from the Harlan Olac (Oxon, UK) housed and bred at the Nottingham Trent University. All animals were handled in accordance with the Home Office Codes of Practice for the housing and care of animals.

2.2 Preparation of Reagents

2.2.1 Peptides

A list of the peptides used in this study are shown in Table 2-2. The peptides were predicted by SYFPETHI web-based software and synthesised by Alta Bioscience.

Gene	Sequence	Abbreviation	Class I	Score
L. major gp63	LLVAALLAV	B8	HLA-A2	28
L. mexicana/ major gp63	RLAAAGAAV	C2	HLA-A2	25
L. major gp63	RLSLGACGV	C1	HLA-A2	23
L. mexicana/major gp63	AAAGAAVTV	CM4	HLA-A2	24
L. mexicana/major gp63	YYTALTMAI	A3	H2-Kd	21
L. mexicana/major gp63	DYTNCTPGL	A4	H2-Kd	20
L. mexicana/major gp63	VPNVRGKNF	A5	H2-Ld	22
L. mexicana/major gp63	ASLLPFNVF	A6	H2-Ld	21

Table 2-2: Predicted peptides from Leishmania gp63 proteins by Web-based software "SYFPEITI".

2.2.2 Preparation of SLA (Soluble Leishmania Antigen)

The *L. mexicana* SLA was prepared according to procedure previously described by Dumonteil [Dumonteil et al., 2003]. Briefly, late log phase *L. mexicana* promastigotes were washed 4 times in PBS and resuspended in 100mM Tris buffer, pH 7.3 containing 1mM EDTA, 0.5mM PMSF (Sigma) and 2.5g/ml Leupeptin (Sigma). The parasites were lysed by sonication and the lysate was centrifuged at 13000rpm for 20 minutes. The

supernatant was centrifuged again for 4 h at 39,000rpm, and then was dialysed against 5 litters of cold PBS overnight with continuous agitation and several changes of the PBS. The lysate was sterilised by passing through 22µm filters (Sartorius).

2.2.3 Tumour Cell Lysate Preparation

Cells were harvested and washed twice in ice cold PBS by centrifugation at 400 rpm for 3 minutes at 4°C. The cell pellet was resuspended in 500µl of lysing buffer (150mM NaCl, 50mM Tris-Base pH 8.0, 5mM EDTA, 1% v/v IGEPAL CA-630, 0.5% w/v sodium deoxycholate, 0.1% w/v SDS, 1mM benzamidine, 0.1mM PMSF, 1mM sodium ortho-vanadate, 1mM sodium azide) and the tubes were agitated for 30 minutes at 4°C. The tubes were then allowed to stand on ice for one hour followed by centrifugation at 14000 rpm at 4°C for 30 minutes. Supernatants were transferred to fresh eppendorfs and stored at –20°C until analysed by protein assay and SDS-PAGE.

2.2.4 Coating of gold particles by DNA

DNA was coated onto 1.0 Micron gold particles (Biorad, Hemel Hempstead, Hertfordshire, UK) using manufacturers' instruction and administered by Helios Gene Gun (Biorad). Breifly, 200 µl of spermidine was added to 16.6 µg of gold in a 1.5ml epindorf. 36µg of DNA was added followed by sonication. 200 µl of 1M calcium chloride was added to the DNA-Spermidine solution followed by incubation at room temperature for 10 minutes. Tubes were centrifuged at 13,000 rpm for 1 minute and gold particles resuspended in dry ethanol (Sigma). After repeating the above step 2 more times, particles were resuspended in 0.025mg/ml of polyvinylpyrollidone (PVP) in dry ethanol. During these steps, the plastic tubing was dried using nitrogen for 15-20 minutes using nitrogen gas. The resuspended gold particles were loaded into the dried tubing using a syringe and the tubing was placed on the roller/dryer (Biorad) followed by incubation for 15 minutes. The PVP-dry ethanol was gently removed using the syringe and the tube was rotated on the roller along with nitrogen gas being passed through it for 5 minutes. Bullets were then cut using guillotine and stored at 4ºC until used for immunisation.

2.3 FACS Analysis

2.3.1 BM-DC phenotyping

5×10^5 per tube DCs were harvested for FACS analysis. Cells were washed twice in PBS + 0.1%BSA + 0.02%NaN_3. Rat anti-mouse CD80, Macrophage/Monocyte marker (F4/80), DEC205, I-A (murine class II) and CD45, and hamster anti-mouse CD11c monoclonal antibodies were added. Appropriate isotype controls were used in each experiment. The cells were incubated on ice for 30 minutes with primary antibodies. Cells were then washed twice in PBS + 0.1%BSA + 0.02%NaN_3 and incubated for 30 minutes on ice with FITC coupled goat anti-rat IgG or goat anti-hamster IgG as secondary antibodies as appropriate. Finally the cells were washed in PBS + 0.1%BSA + 0.02%NaN_3 and resuspended in 500µl of sheath fluid, and analysed by FACS.

2.3.2 Detection of *L. mexicana* gp63 Protein in Transfected CT26 Cells

The method used was as explained in section 2.3.1 with the exception of using rabbit anti *L. mexicana* gp63 (gifted by Dr Brad McGwire, The Ohio State University) and FITC conjugated goat anti-rabbit antibodies (DAKO).

2.4 DNA Preparation

2.4.1 Preparation of *L. mexicana* cDNA

CMV promoter VR1012 *L. mexicana* gp63 DNA was bulked up by transformation of *E. coli* followed by purification using Quia-gen EndoFree plasmid purification Maxi Prep Kits and all the products were evaluated by UV spectrophotometer at 260 and 280nm. The ratio OD260/OD280 was always more than 1.7. The construct was also sequenced by MWG-Biotech using the primers shown in Table 2-3 and checked for mismatches against the sequence obtained from the gene bank.

2.4.2 PCR Amplification

PCR was performed on a DNA Thermal cycler (Thermo Hybaid, USA). Primers were supplied by Sigma Genosys (UK). All the primers used for screening mGAPDH and *L. mexicana* gp63 genes are shown in Table 2-3. For amplification by PCR, 1µl of cDNA was

mixed with 5 µl of 10x PCR buffer, 0.8 µl each of 10mM dNTP, 20 pM each of primer solutions, 1.25 unit of thermostable Taq polymerase (Bioline), 1.5 mM $MgCl_2$ (Bioline), and water to a final volume of 50 µl. PCR was initiated by a melting step at 95˚ C lasting for 5 minutes, followed by 33 cycles of denaturation at 95˚ C for 1 minute, annealing at 58˚ C for and extension at 72˚ C for 45 sec. It was followed by a final extension step at 72˚ C for 5 minutes. PCR products were visualized using a 1.5% (wt/vol) agarose gel containing 1 µg/ml of ethidium bromide (BDH Laboratories, UK).

Application	Name	Sequence
Used for screening GAPDH	mGAPDH Forward	5'-ACTCCACTCACGGCAAATTC-3'
	mGAPDH Reverse	5'-CCTTCCACAATGCCAAAGTT-3'
Used for screening or sequencing *L. mexicana* gp63	*L. mexicana* Forward	5'-ACATCCTCACCGACGAGAAG-3'
	L. mexicana Reverse	5'-CTTGAAGTCGCCACAGATCA-3'
Used only for sequencing *L. mexicana* gp63	*L. mexicana* Forward	5'-GCTGCAACAGCTTGGAGTATC-3'
	L. mexicana Forward	5'-GATACTACACCGCCCTGTGC-3'

Table 2-3: Primers used for PCR, screening or sequencing of mouse GAPDH and *L. mexicana* gp63

2.4.3 Detection of *L. mexicana* gp63 gene by RT-PCR

The presence of the *L. mexicana* gp63 gene in the stable transfected CT26 tumour cells was screened by RT-PCR.

Total RNA was isolated from the cells using RNA STAT-60 (AMS Biotechnology, UK) following manufacturer's instructions. Briefly, CT26 *L. mexicana* gp63 were cultured in T25 tissue culture flasks. 1ml of RNA-STAT60 was added to the cell culture after discarding the media and incubated at room temperature for 5 minutes. The solution was transferred to a 1.5ml epindorf and 0.2ml of Chloroform was added and the homogenate was shaken vigorously for 60 seconds and then left at room temperature for 3 minutes. Samples were then centrifuged at 14,000 rpm for 10 minutes. The aqueous phase was transferred to a fresh eppendorf and 0.5 ml of isopropanol was added. Samples were incubated at room temperature for 8 minutes followed by centrifugation at 14,000 rpm for 15 minutes. Supernatant was discarded and RNA pellet was washed with 75% ethanol. RNA pellet was then dried and resuspended in molecular grade water and the concentration and purity of the RNA was measured on UV spectrophotometer. RNA was then reverse transcribed into cDNA as follow. 2µg of RNA was taken in an eppendorf along with 0.5µg of oligo (dT_{15}) primer. Tubes were heated at 70˚C for 5 minutes and then placed on ice. The following mixture was then added to the tube.

5 µl of 5x Reaction Buffer

1 µl of dNTPs (12.5 mM)

25 units rRNasin Ribonuclease Inhibitor

200 units of M-MLV Reverse Transcriptase.

Nuclease free water was then added to make the final volume to 25 µl. Contents of the tube were gently mixed and heated at 39.2˚C for 80 minutes followed by cooling on ice and heating at 95˚C for 5 minutes and then storing them at -20˚C. The reverse transcribed RNA was used as template for PCR amplification to screen the presence of *L. mexicana* gp63 gene.

2.4.4 Preparation of VR1012 empty vector

VR1012 *L. mexicana* gp63 vector was digested by EcoR I restriction enzyme and run onto the gel agarose. The heavier band corresponding to the molecular weight of the empty vector was then cut and the DNA was extracted by DNA extraction kit (GeneFlow) with accordance to the manufacturer's protocol. Two sides of the cut vector were ligated together by ligation enzyme (promega). The ligation was set up by adding 0.5µl ligation enzyme, 1µl buffer and 6.5µl water to 2µl DNA (adjusted at 10µl). The ligated DNA was incubated at 4 ºC overnight. The empty vector was reproduced by transforming *E coli* and extracting the DNA from the bacteria.

2.4.5 Gene sub-cloning

VR1012 *L. mexicana* gp63 and pcDNA vector were digested by EcoRI restriction enzyme and the digested products were run onto the gel agarose. *L. mexicana* gp63 and pcDNA3 bands were cut and the DNAs were extracted from the gel. Two ends of the EcoRI cut pcDNA3 vector were dephosphorylated using alkaline phosphatase (Promega). The dephosphorylated pcDNA3 vector and the *L. mexicana* gp63 DNA were ligated together as explained in section 2.4.4 with the exception of using 6.5µl *L. mexicana* gp63 instead of water. The direction of the gene in the vector was checked by cutting the new construct (pcDNA3 *L. mexicana* gp63) by Not I restriction enzyme.

2.5 Transfection of CT26 tumour cells with L. mexicana *gp63 "CTLX"*

2.5.1 Antibiotic sensitivity assay

1×10^6/well CT26 tumour cells were cultured in 24-well plates in presence of a serial concentration of Geneticin (G418) from 50 to 900µg/ml; wells for each concentration of the antibiotic were put in duplicate. The cells were incubated at 37 ºC with 0.5 CO_2 for 10 days. The lower concentration of the antibiotic in which all the CT26 tumour cell died in 7-10 day was chosen (500µg/ml).

2.5.2 Transfection of CT26

CT26 tumour cells were transfected with pcDNA3 *L. mexicana* gp63 using lipofectamine 2000 (Invitrogen) according to the manufacture's instruction for adherent cells with slight modifications. Briefly, CT26 tumour cells were cultured at 1×10^6 per well in 24-well plates; to produce 90% confluence on the day of transfection. Lipofectamine 2000 and pcDNA3 *L. mexicana* gp63 were diluted in serum free DMEM media at 2µl/50µl and 0.8µg/50µl respectively and incubated at room temperature for 5 minutes. The diluted lipofectamine 2000 and DNA were mixed together and incubated again for 20-30 minutes at room temperature. The CT26 cell culture supernatant was gently removed and the DNA-lipofectamine mixture was gently added followed by 4-6 hours incubation at 37 ºC with 0.5% CO_2. 1ml/well DMEM media complemented with 10% FCS was added. The media was replaced 16-24 hours later with a fresh media containing 500µg/ml G418.

2.6 Western Blotting

2.6.1 Protein Assay

An approximate total protein was measured using Sigma Bicinchoninic Acid Protein Assay Kit (BCA) according to the manufacturer protocol. Briefly, 25µl per well of the sample (SLA) and the standard proteins were mixed in duplicate in 96-well plates (Biorad). A serial dilution of 1mg/ml BSA in lysate buffer was used as standard. Reagents A and B were mixed in the ratio of 50:1 and 200µl of the mixture was added per well. The plate was wrapped in the foil and incubated at 37 ºC for 30 minutes to develop the reaction and then the plate was read at 570nm on a Spectrophotometer (Tecan).

2.6.2 SDS-PAGE and Membrane transfer

The gel tank was assembled according to the instruction and resolving gel 10% (1165µl Acrylamide/bis, 875 µl Tris 1.5 M HCl pH 8.8, 1460 µl H_2O option 4, 35µl Ammonium Presulphate 10%, 3.5 µl TEMED) was prepared and poured into the cassette. The gel was left until solidified. The 4% stacking gel (15% (v/v) acrylamide /bis, 25% 0.5M Tris HCL pH6.8, 60% dH_20 plus 0.1% (v/v) TEMED and 1% (v/v) 10% ammonium persulfate) was added on top of the resolving gel and the comb was inserted in it. To prepare the samples, 33µl of 1x reducing sample buffer was added to 100µl of each sample and heated to 95C for 5 minutes. 10-30µg of samples was run at 90V through the stacking gel and 120V through the resolving gel. Standard protein (Protomarker) was used to asses the molecular weight of the sample proteins. After running the samples throughout the gels, the resolving gel was incubated in transfer buffer (48mM tris, 39mM glycine, 200ml methanol, 800ml water) for 5 minutes and then proteins were transferred onto the Bio-trace membrane (nitrocellulose membrane) at 13V for 30 minutes through a semi-dry transfer system using trans-blot machine (Biorad) according to manufacturer instructions.

2.6.3 Western Blotting

Detection of leishmania gp63: Membranes were stained with Ponceau S, and the standard protein lane was cut from the rest of the membrane. The membrane was blocked overnight in TBS + 0.05% Tween 20 (TBS-T) + 5% Marvel milk powder at 4°C under constant agitation. The primary antibody (rabbit anti *L. mexicana* gp63, a gift from Dr Brad McGwire The Ohio State University) was then added at 1:1000 dilution in TBS + 0.05% Tween 20 + 5% Marvel milk powder and incubated for 1 hr at room temperature with vigorous shaking. After washing the membrane 3 times for 15 minutes in TBS-T at room temperature, the secondary antibody (HRP conjugated goat anti rabbit IgG (Biorad)) was added to the membrane at a 1: 2000 dilution in 5% milk-TBS-T and incubated for 1 hour at room temperature with vigorous shaking.

Detection of OX40L: Similar approach was used to detect OX40L by western blotting. Rat anti mouse OX40L and HRP coupled rabbit anti rat IgG antibodies were used.

The membrane was then washed 4 times for 15 minutes at room temperature in TBS-T, and revealed using ECL chemioluminescence kit (Amersham). Hyperfilm ECL (Amersham) films were used to detect the luminescence.

2.7 Immunisation/Infection protocols

2.7.1 Animal infection with leishmania parasite

Groups of 6 BALB/c and HHD II mice were injected with 2×10^6 and 1×10^7 log phase *L. mexicana in vitro* culture in a volume of 50µl/mouse, respectively. Mice were injected intradermally at the back about 1cm from the tail base. The progression of the lesions was monitored regularly twice a week. The lesion diameter was measured by a calliper and surface area was calculated by the equation of πr^2. Mice were sacrificed at 10-12 weeks or even earlier if the lesion size was excessive.

2.7.2 Immunisation of mice with killed leishmania parasites

In vitro cultures of *L. mexicana* promastigotes log phase were autoclaved at 121ºC under the pressure of 15 PSI for 20 minutes. Groups of 6 female BALB/c mice were immunised S.C. with 2×10^6 autoclaved *L. mexicana* admixed with the same volume of IFA. The immunisation was carried out twice at two weeks interval. The control group was injected with PBS. Two weeks later, the mice were challenged I.D. with 2×10^6 *L. mexicana* promastigote. The mice were monitored regularly twice a week.

2.7.3 Immunisation of mice with SLA

Two groups of 6 BALB/c mice were immunised S.C. with 100µg SLA in 50µl mixed with 50µl IFA (total volume of 100µl per mouse) or 100µlPBS twice at 2 weeks interval. Two weeks later, all mice were challenged with 2×10^6 *L. mexicana* promastigotes. The mice were monitored regularly twice a week.

2.7.4 Immunisation with DCs pulsed with SLA

Two groups of 6 female BALB/c mice were either immunised with 2×10^6 SLA-pulsed or control DCs. A third group of 3 mice was injected with PBS and used as additional control. All mice were injected intradermally twice at two weeks interval. Two weeks later all mice were challenged with 2×10^6 late log phase *L. mexicana* promastigotes and monitored regularly twice a week.

2.7.5 I.M. Immunisation with *L. mexicana* gp63 cDNA

Two groups of 6 female BALB/c mice were selected. The first group was injected I.M. with 100µg/mouse *L. mexicana* gp63 plasmid DNA (VR1012 vector). The second group

was injected with the empty vector or, in some experiments, with PBS. The mice were injected twice on day 0 and 14 intramuscularly in hind leg muscles. On day 28, mice were challenged with 2×10^6 log-phase *L. mexicana* promastigotes and then were monitored regularly twice a week for at least two months.

2.7.6 Gene gun immunisation with *L. mexicana* gp63 cDNA

1µg per mouse of *L. mexicana* gp63 plasmid DNA (VR1012) coated on gold particles was administered to a shaved area of the abdominal skin of BALB/c mice by gene gun (Biorad) on day 0 and 14. The control group was given 1µg of the empty vector coated on gold particles or injected with PBS. All mice were challenged with 2×10^6 log-phase *L. mexicana* promastigote on day 28, and were monitored regularly.

2.7.7 Immunisation with CT26 *L. mexicana* gp63

Three groups of BALB/c mice were injected S.C. with 5×10^5 irradiated (15000 rads) CT26 *L. mexicana* cells, non-transfected CT26 tumour cells or PBS on days 0, 14 and 28. Mice were challenged I.D. with 2×10^6 log phase *L. mexicana* promastigotes and then were monitored twice a week.

2.7.8 Immunisation to test CTL activity of immunogenic peptides

HHD II mice: 100µg of the peptide, 140µg of HAP-B as helper peptide and 50µl IFA were transferred to an epindrof. PBS was added in a total volume of 100µl per mouse. The injection was given at the base of the tail. Mice were sacrificed one week after the immunisation and their splenocytes were used in standard 4-hour cytotoxicity assay.

BALB/c mice: Peptides were prepared similar to those of HHDII mice with the exception of using a 15 mer peptide derived from bovine albumin with the sequence of ISQAVHAAHAEINEAGR as helper peptide. Mice were injected twice two weeks apart at the base of the tail and one week after the second immunisation they were sacrificed and their splenocytes were tested for CTL activity.

2.7.9 Immunisation to test for endogenous processing of immunogenic peptides

Three rounds of immunisations at one week interval were undertaken for immunisation of mice with gold particles coated with *L. mexicana* gp63 cDNA using the gene gun (Biorad).

In certain experiments, mice were injected with 100 µg *L. mexicana* gp63 cDNA two times at two weeks interval.

2.7.10 Immunisation to test CTL activity induced by *L. mexicana* gp63 cDNA or DCs pulsed with SLA

BALB/c mice were immunised twice at two weeks interval with *L. mexicana* gp63 cDNA by gene gun. Mice were sacrificed two weeks following the 2nd immunisation and spleens were collected and splenocytes were tested for CTL activity. For immunisation of mice with DCs pulsed with SLA, DC cells generated as outlined in part 2.1.2. One dose of 2×10^6 SLA loaded matured DC was administered per mouse and mice were sacrificed two weeks later.

2.8 CTL Assay

2.8.1 LPS Blast

Between 2-3 days prior to the removal of spleens from immunised mice, naïve splenocytes were cultured at 1.5×10^6 cells/ml in 40ml T cell media containing 25µg/ml LPS and 7µg/ml dextran sulphate in a T75 culture flask and incubated at 37^0C, 5%CO_2. On the day of isolation of immunised mice splenocytes, LPS treated naïve splenocytes were irradiated at 3000rads for 4 minutes. Cells were washed and pulsed with 100µg/ml of relevant or irrelevant peptides separately for at least 1 hour. Cells were then washed, counted and added to culture plates containing splenocytes from immunised mice at 5×10^5 /well.

2.8.2 *In vitro* generation of CTLs

One week after the last immunisation, spleens were harvested from the immunized mice and single cell suspensions were prepared in sterile conditions Cells were flushed out from the spleens by serum-free RPMI 1640 media using a 25-G needle and 10 ml syringe. The remaining splenic sac was cut and digested with 500µl of enzyme cocktail (1.6mg/ml collagenase and 0.1%DNAase in serum free medium (Sigma-Aldrich, Dorset, UK) at 37°C in 5%CO_2 for 1 hour (parenchymal fraction). The spleen tissue was disrupted by pipetting and the cells were also collected. All cells were centrifuged at 1500rpm for 3 minutes and resuspended in CTL media (RPMI 1640 supplemented with 1% L-glutamine, 10%FCS,

20mM HEPES buffer, 50µM 2-Mercapto Ethanol, 50U/ml penicillin, 50µg streptomycin and 0.25µg/ml fungizone). The cells were counted using white cell counting fluid (0.6% acetic acid in distilled water) and 0.1%Trypan Blue**,** and plated in a 24 well plate at 2.5×10^6 cells/500µl/well. 5×10^5 /500µl irradiated and peptide pulsed LPS blasts were added to the splenocytes to make a final volume of 1ml in each well of 24 well plate. Supernatants were collected usually on day 3 and 5 for cytokine testing. In certain experiments SLA was used instead of peptide to stimulate CTL activity in vitro.

2.8.3 Chromium Release Standard 4-hour Cytotoxicity Assay

On day 5 of *in vitro* stimulation, splenocytes were harvested, washed twice in serum free medium, counted and resuspended in CTL media and used as the effecter cells. Target cells (RMA/S-A2 or T2 for HHD II mice & CT-26 or A20 for BALB/c mice) were also harvested, washed and labelled with chromium-51 (Amersham,UK) followed by 1h incubation at 37°C. The labelled cells were then pulsed with relevant and irrelevant peptides separately and incubated for 1 hour at 37°C. In certain experiments *L. mexicana* gp63 transfected CT26 tumour cells or SLA pulsed DCs were used as targets for CTL assay. A standard 4 hour Cr release assay was performed and the specific cytotoxicity was determined using the following formulae.

$$\text{percentage cytotoxicity} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100$$

2.9 Antibody/Cytokine Response

2.9.1 Detection of anti-leishmania IgG2a and IgG1 isotype antibodies

After immunisation as described in part 2.7, mice were regularly bled for 7 times at a week interval starting a week after the last immunisation. The blood samples were harvested and span at 2000rpm for 10 minutes. The serum was collected and stored at -20 until tested for specific immunoglobulin IgG1 and IgG2a using ELISA. Serum samples from 9 naïve mice were used for control. *L. mexicana* Soluble Antigen (SLA) 1µg/well was coated on the flat bottom 96-well plates (Biorad) and incubated overnight at room temp. After 4 times wash with PBS, the plates were blocked with block buffer (1% BSA, 5% sucrose in PBS with 0.05 NaN_3) for 1hour. Plates were washed 4 times with PBS and 1:100 dilution of the serum samples in dilution reagent (1% BSA, 0.05% Tween 20 in 20mM Trizma base, 150mM NaCl, pH 7.2-7.4) was added in duplicate followed by 2h incubation at room temp

and 4 times washes with PBS. Rabbit anti-mouse IgG1 or IgG2a (Serotech) was added at 1:1000 followed by 1hour incubation at room temp and 4 times washes. HRP conjugated goat anti-rabbit antibody at 1:1000 dilution was added and the plates were stored at room temp for 1h followed by 4 times washes. 50µl of HRP substrate (DAKO) was added and kept at room temp for 20minutes for reaction development. 2.5M H2SO4 was added to stop the reaction and the OD was measured at 570 nm by spectrophotometer.

2.9.2 Cytokine Assays (IFN-γ & IL-4)

Splenocytes were prepared as outlined above in section 2.8.2. 1 ml of the supernatant was collected and stored at -20C until required. Cytokine analysis for IFN-γ and IL-4 using the ELISA kits (R&D Systems, Abingdon, UK) was performed according to the manufacturer's protocols.

2.9.3 Effect of *L. mexicana* infection on the expression of MHC class I

DCs were cultured as described in section 2.1.2. The cells were split into two groups. The first group was infected with ten times of the number of DCs *L. mexicana* promastigotes. Both groups were then pulsed with LPS after 4-6 hours. The expression of the MHC class I molecules in the infected and non-infected DC cells was analysed by FACS using mouse anti-mouse H2-L^d and FITC coupled rat anti-mouse (DAKO) antibodies.

2.10 OX40L: Purification/Application

2.10.1 Cells culture supernatant preparation

B9B8E2 cells were cultured in DMEM media supplemented with 1% glutamine synthetase (GS), 15 mg/ml L-methionine sulfoximine (MSX) and 10% Bio-FCS for 2-3 weeks. The cell culture supernatant was collected, passed through the 0/20µm filter and kept at -80°C until required. The OX40L fusion protein present in the supernatant was separated and purified using the antibody purification columns.

2.10.2 MBI column

MBI column (Biosepra) was used for OX40L-IgG fusion protein purification. The column was packed with 2mls of MBI resin To optimise the conditions of the loading buffer, sodium acetate 100µM and sodium acetate 100µM + NaCl 150µM at a set of pH from 4 to 6.5 were used. To set the pH of the column, before loading the OX40L sample, the column

was washed by the loading buffer. 2ml of the B9B8E2 cell culture supernatant was mixed up with the same quantity of loading buffer and the pH was adjusted to that of the loading buffer. After loading the sample the column was washed with the loading buffer. Finally the OX40L was eluted by tris buffer at pH 9. Similarly, the conditions of elution buffer were optimised. Sodium acetate at pH 4 was used as loading buffer and tris buffer at a set of pH from 7 to 10.5 were used as elution buffer.

2.10.3 MEP column

The column was packed with 2mls of MEP resin and the sample was loaded onto the column as described for MBI column. 50mM Tris buffer pH 8.0 was used for loading the samples and the elution was undertaken using 50mM sodium acetate pH 6.0, 5.8, 5.6, 5.4, 5.2, 5.0, 4.7, 4.5, 4.3, 4.0 and 3.0.

2.10.4 Protein A sepharose column:

Protein A sepharose column (Amersham Biosciences) was used to purify the OX40L-IgG fusion protein. First the column was washed by PBS at pH 7 and then samples were loaded. To remove all unbound proteins, the column was washed by PBS again. The column was then eluted by tris/glycin buffer at pH 3.

2.10.5 Application of OX40L

Purified samples were checked for OX40L by western blotting. Samples were then dialysed against 3-5 litres PBS at 4 ºC over night with vigorous agitation and a few changes in the PBS using the dialysing tubing. In certain cases the samples were concentrated by putting them in dialysing tubes, which were placed in propylene glycol. For sterilization, the samples were passed through 0.2µm filters.

500µg of the OX40L was injected I.P to groups of 10 female BALB/c mice 3 and 7 days following S.C. implantation of 2×10^4 CT26 tumour cells on their right flank. The mice were monitored regularly twice a week and the mice were killed when the tumour size exceeded 1 cubic centimetre. For application of OX40L against leishmania, similar protocol was used with exception of using 2×10^6 *L. mexicana*.

Chapter 3 Protection studies of vaccines against *L. mexicana*

3.1 Introduction

3.1.1 New strategies in leishmania vaccination

Vaccination is one of the most feasible and cost effective methods for the control of infectious diseases [Andre, 2003]. It is now believed that via vaccination some of disabling and lethal diseases like poliomyelitis and measles will be eradicated, with large number of children lives being saved by vaccination annually around the world [Andre, 2003].

Intracellular pathogens such as *Leishmania spp*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Toxoplasma gondii* and *Trypanosoma cruzi* cause diseases, which have a major impact on public health. Despite of the massive progress occurred in the basic knowledge of immunology, we still relatively little know of the mechanisms of the immune system that are involved in immunity to intracellular pathogens. The ability to survive and multiply within macrophages is a feature of intracellular pathogens that makes their pathogenesis even more complicated [Alexander et al., 1999].

Leishmania species are obligate intracellular parasites of the macrophage-dendritic cell lineage. Although the first species of leishmania parasite was known more than 100 years ago [Herwaldt, 1999], construction of an effective vaccine against the parasite has not yet been achieved [Selvapandiyan et al., 2006]. As Leishmania parasite lives intracellularly, in macrophages, the humoral immune system cannot be of great help in immunity and therefore the vaccine-developing strategies must involve the cellular immunity and direct the immune response towards the Th1 immune pathway. Due to the complexity of the mechanisms involved in the immunity to Leishmania, different vaccine strategies have been proposed. The first generation of leishmania vaccines was based upon using live parasites, “Leishmanization” and autoclaved-killed leishmania alone or with the addition of adjuvants such as *Bacillus Calmette-Guerin* (BCG) [Khalil et al., 2000; Khamesipour et al., 2006; Momeni et al., 1999].

In the second generation of leishmania vaccines, because of the new advances in cell biology, most of efforts were shifted to purified or recombinant parasite antigens, DNA vaccines [Selvapandiyan et al., 2006] and recently DCs.

3.1.2 DNA Vaccination

DNA vaccination is the latest method of immunisation implicated in leishmania vaccination, shown to have potential to induce immunity to leishmania in mice [Sjolander et al., 1998]. In this method, DNA sequences that encode a *Leishmania* antigens are spliced into an expression vector, which is administered to the host cells to promote the production of leishmania protein [Gurunathan et al., 2000a; Gurunathan et al., 2000c]. The DNA can be administered by injection, in vivo electroporation or gene gun. It is thought that the method of immunisation affects the nature of the immune response induced by the encoded antigen, however, this requires further investigation. The gene gun is one of the most advanced methods for the application of recombinant DNA vaccines. The gene gun was first designed by a horticultural scientist "John C. Sandford" in late 1980s for transforming plant cells and has found recent application in animal models. This method involves "shooting" heavy metal particles coated with plasmid DNA encoding a particular gene to target host cells. The gene of interest is first cloned into a suitable plasmid "vector", then, the DNA is bound to the heavy metal particles. Different heavy metals like tungsten, silver or gold can be used; however, gold is preferred because it carries the coated DNA into the cells without being toxic for them. In this method, the heavy metal functions as a shuttle to carry the plasmid DNA into the cells. Shooting gold particles coated with DNA directly penetrates the cell membrane into the cytoplasm and even the nucleus and bypasses the endosomal pathway/compartment releasing the DNA, which encodes the desirable protein [Niidome & Huang, 2002].

Gene gun has successfully been applied in DNA vaccination to generate immunity to pathogens such as leishmania and trypanosome [Li et al., 2004; Sakai et al., 2000] and is also considered for human application.

3.1.3 DCs in immunisation against leishmania

Dendritic cells, as professional antigen presenting cells, play a crucial role in immunity to leishmania. DCs initiate the adaptive immune response by phagocyting the leishmania parasite and processing their antigens, and present processed MHC-associated peptides to the lymphocytes. There are studies suggesting that a number of different subsets of DCs exist: CD11c+ DCs have bias to Th1 immune response and are involved in cross presentation of the intracellular microbial antigens through MHC class I and activate cytotoxic T lymphocytes [Jung et al., 2002]. There is also a possible role for subsets of

DCs in directing the immune response towards either Th1 or Th2 following the encounter of an infectious agent, which may determine whether the host will resist or succumb to that infection [Ahuja et al., 1999].

In recent studies, DCs have been demonstrated as potent candidates for immunotherapy of Leishmaniasis suggesting a new approach of immunisation/therapy by using DCs primed with the antigens. In order to elicit Ag-specific protective immune responses, DCs are first primed with relevant antigens *in vitro* and then injected to the animals. This has successfully been used in studies against tumour. It has been shown that loading DCs with anti-tumour antigens protected mice from tumour growth [Tegerstedt et al., 2007]. Also, DCs pulsed with lysate derived from tumour cells infected with vaccinia virus encoding IL-2 gene (DC-IL-2VCO) produced safe and effective immune responses in a murine CC-36 colon adenocarcinoma model [Jack et al., 2007].

In leishmania vaccination the potency and effectiveness of DC-based vaccines has been shown in both immunotherapy and chemotherapy [Ahuja et al., 1999; Berberich et al., 2003; Ghosh et al., 2003]. The cytokine profile of mice after DC-based vaccination has demonstrated a shift toward a Th1-type response in which IL-12 has a critical role [Berberich et al., 2003] and because DCs exposed to *L. major* readily produce IL-12, it may further increase the feasibility of using the DC-based vaccines [Moll & Berberich, 2001]. In addition, there are reports showing that DCs might play a contradicting role depending on the antigen and when pulsed with certain peptides they can promote a Th1 or Th2 immune response, exacerbating the disease [Tsagozis et al., 2004]. It has also been reported that adjuvants such as CpG may help in generating Th1-type immunity by DCs but the initiation of a protective Th1 cell response *in vivo* may be dependent on the ability of DCs in producing IL-12 [Ramirez-Pineda et al., 2004]. Therefore, the potency of DC based vaccine for different leishmania antigens varies and requires further investigation.

3.2 Results

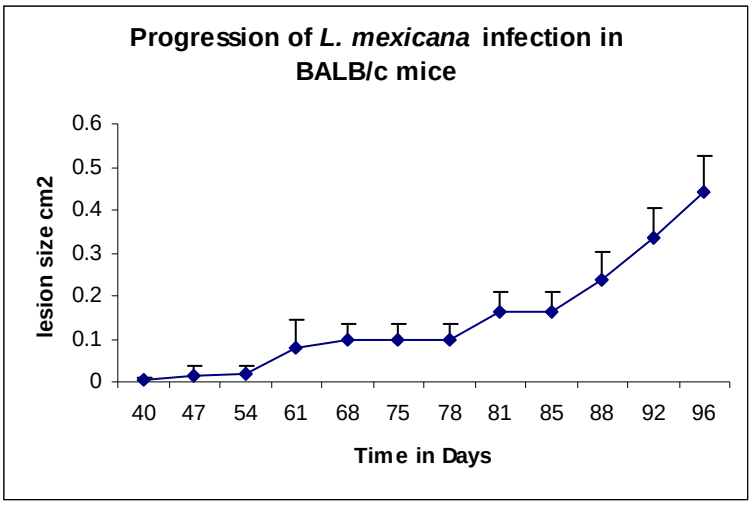
3.2.1 Animal Models to study *L. mexicana*

The first part of this study was devoted to establish a leishmania mouse model, which could be used in vaccine studies.

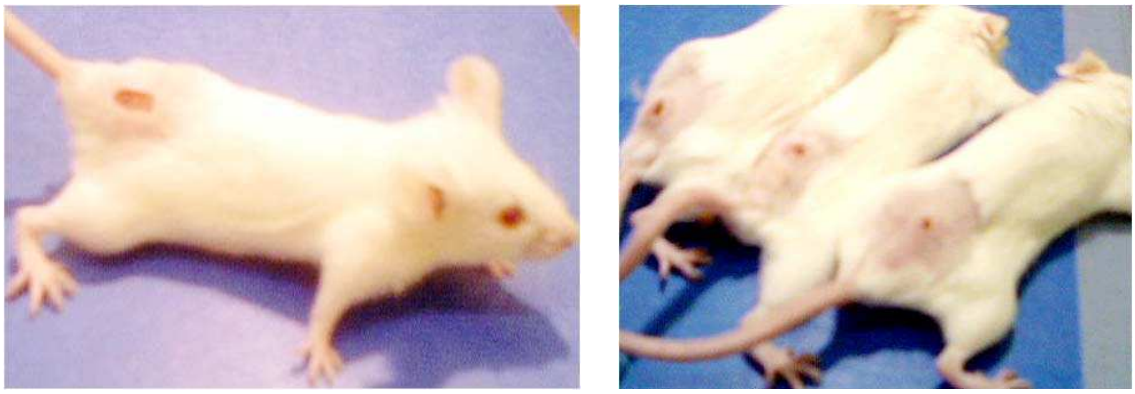
3.2.1.1 Conventional BALB/c mouse model

In order to study the manifestation spectrum of cutaneous leishmaniasis caused by *L. mexicana* in the inbred BALB/c mouse and establish the animal model, 2×10^6 *in vitro* cultured *L. mexicana* promastigotes in log growth phase were injected intradermally on the back of BALB/c mice about 1cm from the tail base. The inoculated mice were monitored regularly and the lesions produced by the parasite were compared with control mice injected with PBS, which remained free of lesions.

The incubation period was varied from 2 weeks to 2 months and almost all the inoculated mice developed lesion. The lesions in BALB/c mice were constantly progressive (Figure 3-1A); they were circular and usually raised from the skin base with tick edges (Figure 3-1B).



A:



B:

Figure 3-1: Progression of *L. mexicana* infection in BALB/c mice.
A group of 6 BALB/c mice were injected with 2×10^6 log phase *L. mexicana* promastigotes *in vitro* culture. The progression of lesions was monitored regularly twice a week. A: lesion progression curve in BALB/c mouse model. The graph represents 3 independent experiments. Bars represent the standard deviation n=3. B: Cutenaous leishmania in BALB/c mice

3.2.1.2 HLA-A2 transgenic (HHDII) mouse model

HLA-A2 transgenic (HHDII) mice have a C57 genetic background with the substitution of mouse H-2MHC class I with human HLA-A2 gene. Although the HHD II transgenic mouse model has been reported in Trypanosoma [Garcia et al., 2003], to the best of our knowledge, this is the first study,which used this mouse model in leishmania vaccine studies. To study the course of cutaneous leishmaniasis caused by *L. mexicana* in these mice, and whether the expression of human MHC class I molecule has any impact on the resistance of these mice to this parasite, naïve HHDII mice were injected intradermally at the back with 1×10^7 log phase *L. mexicana in vitro* culture. The progression and regression of the lesion were monitored regularly twice a week and compared with controls, which were injected with PBS.

The results indicated that the course of cutaneous leishmaniasis in HHDII mice is similar to that of their background C57B1/6; These mice are resistant to leishmania and their lesions slowly healed after 6 weeks [Beil et al., 1992]. Between 50 to 100% of mice showed lesions. The incubation period usually varied from 2 to 4 weeks. The size of the lesion was often smaller than that observed in BALB/c mice (Figure 3-2). Almost in all cases, after a period of progression, the lesions healed and finally disappeared leaving behind a small scar. However, when mice were injected with 2×10^7 parasites, the lesions grew quicker but the course of the disease was still similar.

The results show that the presence of human MHC class I (HLA-A2) in this mouse model (HHD II) did not affect the course of cutaneous leishmaniasis caused by *L. mexicana*.

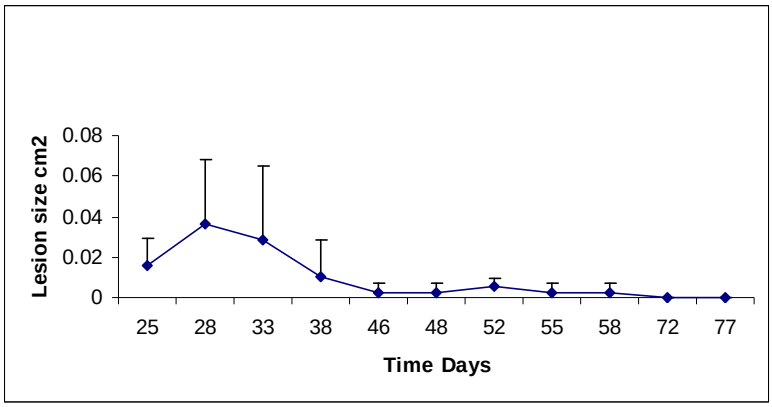


Figure 3-2: Progression of *L. mexicana* infection in HHD II transgenic mice.
1×10^7 log phase *L. mexicana* promastigote *in vitro* culture were injected I.D. at the back of HHD II transgenic mice. The mice were monitored regularly twice a week. The graph represents 4 independent experiments. Bars represent the standard deviation n=6.

3.2.2 Protection induced by killed leishmania parasite vaccine

To evaluate the efficacy of using killed leishmania parasites in generating immunity to challenge, 2×10^6 autoclaved *L. mexicana* mixed with the Incomplete Freund's Adjuvant (IFA) were injected S.C. at the base of the tail of BALB/c mice (see materials and methods). After two immunisations, the mice were challenged with 2×10^6 log growth phase *L. mexicana* promastigotes. No significant difference in the average of lesion sizes was observed between test (immunised) and control (injected with PBS) groups (Figure 3-3).

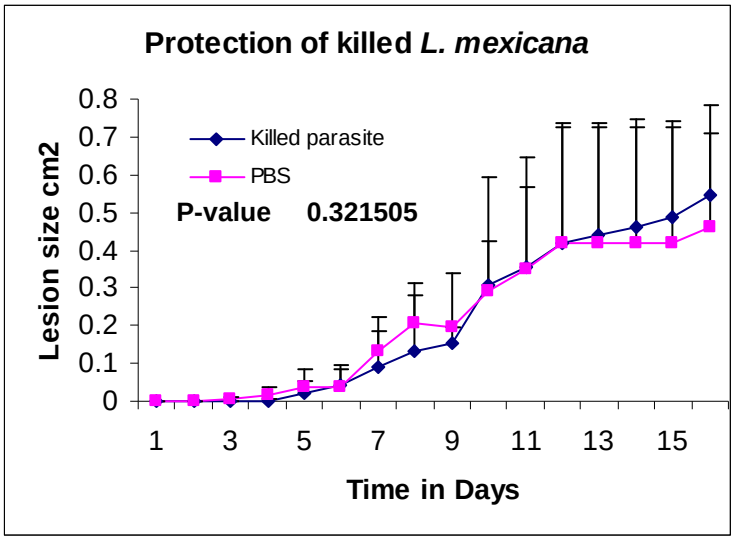


Figure 3-3: Protection induced by immunisation with autoclaved *L. mexicana*.
2×10^6 autoclaved *L. mexicana* mixed with IFA were injected subcutaneously into groups of 6 female BALB/c mice twice at two weeks interval. The control group were injected with PBS. The mice were challenged with 2×10^6 *L. mexicana* promastigote intradermally two weeks after the last immunisation. The challenged mice and control groups were monitored regularly. The graph represents 2 independent experiments. Bars represent the standard deviation n=6.

3.2.3 Protection studies of Soluble Leishmania Antigen (SLA)

3.2.3.1 Preparation of SLA

The SLA was also checked for the presence of *L. mexicana* gp63 protein by western blotting using anti-*L. mexicana* gp63 antibodies (Figure 3-4). A control cell preparation of CT26 tumour cells was used as a non-specific antigen/protein control.

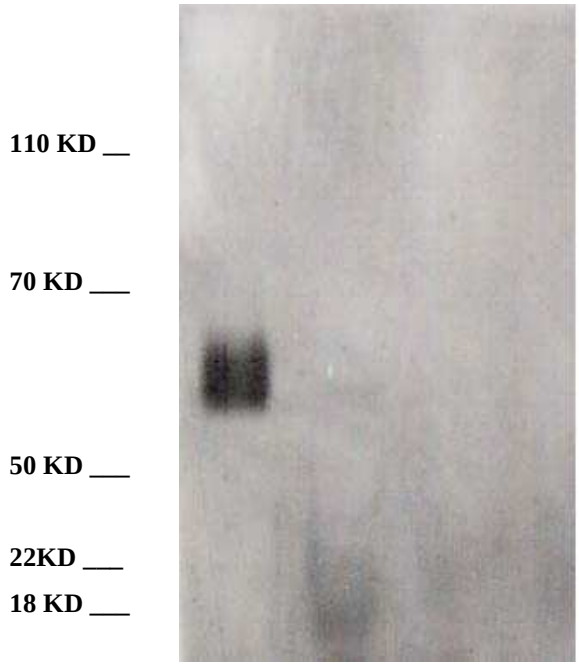


Figure 3-4: Detection of *L. mexicana* gp63 in SLA.
L. mexicana parasites were washed 4 times in PBS and then lysed in lysate buffer and sonicated. The SLA was analysed for *L. mexicana* gp63 by western blotting using anti *L. mexicana* gp63 antibodies.

3.2.3.2 Protection induced by Soluble Leishmania Antigen (SLA)

To investigate the ability of SLA to protect the animals from leishmania infection, a series of experiments were performed using test and control groups of leishmania sensitive BALB/c mice. The test group was injected with 100µg of *L. mexicana* SLA and IFA as adjuvant and the control group was injected with PBS. The results showed that two S.C. injections of SLA+IFA did not significantly decrease the size of the leishmania lesion compared to controls, althoght lesion progression was slower in the immunised group (Figure 3-5).

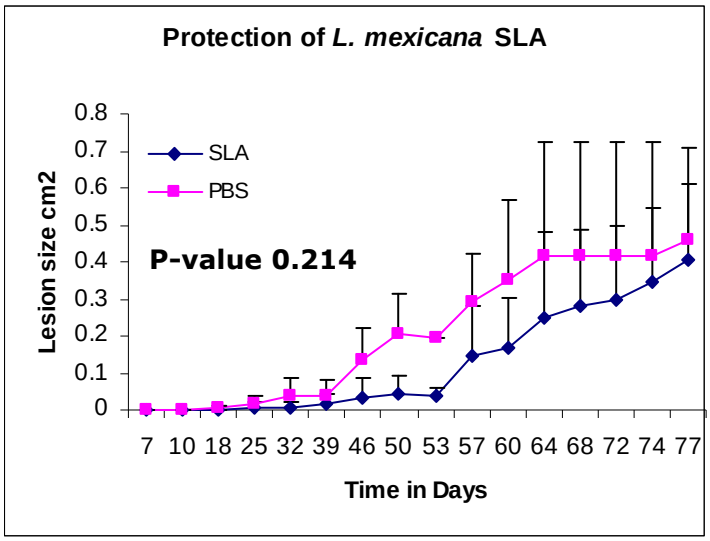
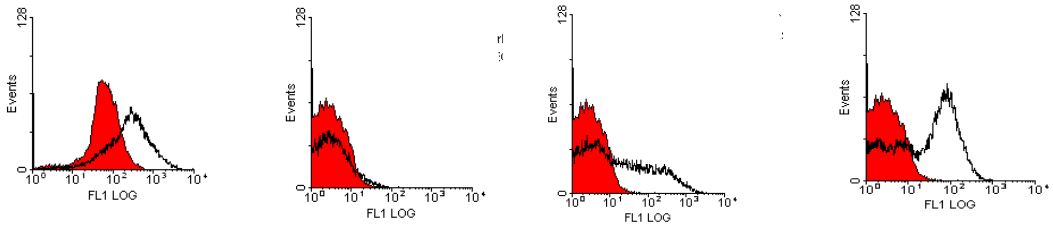


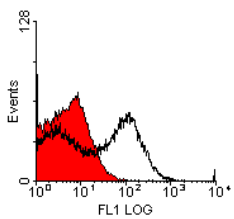
Figure 3-5: Protection induced by SLA admixed with IFA in BALB/c mice.
Tow groups of 6 BALB/c mice were immunised S.C. with 100µg +IFA or PBS twice at 2 weeks interval. Two weeks later mice were challenged with 2×10^6 *L. mexicana* promastigotes. The mice were monitored regularly and average of the surface of the lesions was measured. Student t test was used to statistically analyse the data. The graph represents 3 independent experiments. Bars represent the standard deviation n=6.

3.2.3.3 Protection induced by Dendritic Cells (DC) loaded with SLA

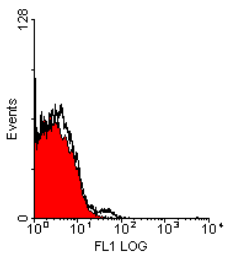
The effect of immunisation with dendritic cells loaded with SLA was investigated against infection with leishmania parasite. Bone-marrow cells were obtained from BALB/c mouse, and cultured with GM-CSF for 6 days with gentle washes every two days (see materials and methods). On day 6, DCs were replated at 1×10^6/ml and split into two groups. One group (test) was treated with the SLA at a concentration of 10µg/ml and after 4-6 hours they were also pulsed with 1µg/ml LPS to mature. The second DC group (control) was only pulsed with LPS. On day 7, 2×10^6 DCs per mouse were injected I.D. into groups of BALB/c mice. A third group of BALB/c mice were injected with PBS and used as an additional control. DCs phenotype was determined with a number of Abs and FACS analysis (Figure 3-6). Mice were immunised twice at two weeks interval and then challenged with 2×10^6 *L. mexicana* promastigotes. No protection but exacerbation of lesions was observed however the lesion exacerbation was not significant (Figure 3-7).



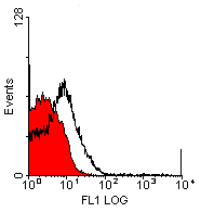
A: DEC205 B: F4/80 C: MHC Class II D: CD40



E: CD11c



F: CD45R



G: CD80

Figure 3-6 : DC phenotypic analysis.
Bone marrow cells obtained from BALB/c mice were cultured in presence of GM-CSF for 6 days with gentle wash every two days. On day 6 the DCs were treated with SLA (10-15µg/ml) and after 4-6h they were pulsed with LPS 1µg/ml to mature. On day 7 the cells were split into a number of groups stained with Abs and phenotyped by FACS analysis. Red line: control; Black line: test.

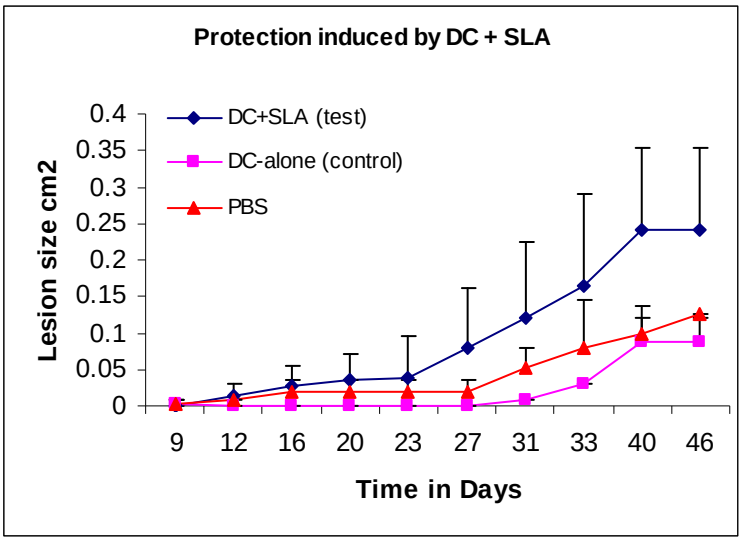


Figure 3-7: Protection induced by DCs loaded with SLA in BALB/c mice.
Bone-marrow cells derived from BALB/c mice were cultured with GM-CSF for 7 days. One day before immunisation, they were loaded with 10-15µg/ml SLA and pulsed with LPS 1µg/ml (see materials and methods). On day 7 DCs were phenotyped and then, 2×10^6 of each DC preparation was administered in a group of 6 BALB/c mice intradermally twice at two weeks interval. A control group of 3 mice were injected with PBS. Mice were challenged with 2×10^6 *L. mexicana* promastigotes two weeks after the last immunisation. The mice were monitored regularly. Student t-test was used to analyse the data. The graph represents 3 independent experiments. Bars represent the standard deviation n=6.

3.2.4 Protection induced by *L. mexicana* gp63 cDNA

Leishmania gp63 is a known immunogenic protein of Leishmania parasites but its role in CTL mediated immunity has not yet been determined. To assess the immunogenicity of *L. mexicana* gp63, the *L. mexicana* gp63 gene cloned into VR1012 plasmid vector, a gift from Dr Dumonteil Laboratorio de Parasitología Yucatan Mexico [Dumonteil et al., 2003], was used in this investigation (Figure 3-8).

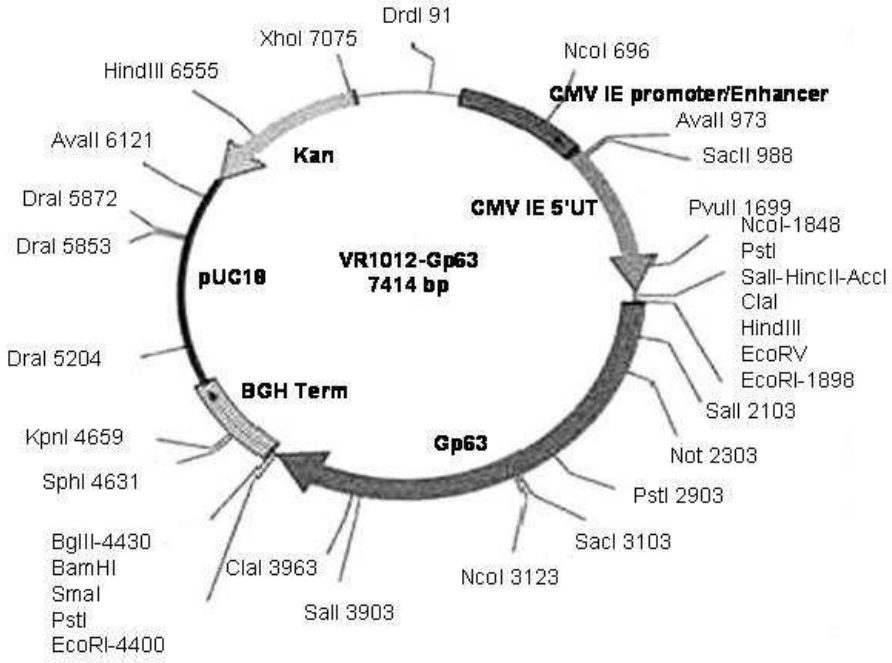


Figure 3-8: Map representing VR1012 plasmid vector containing *L. mexicana* gp63 gene (gift from Dumonteil [Dumonteil et al., 2003])

3.2.4.1 Confirmation and bulking of *L. mexicana* gp63 construct

The VR1012 plasmid containing *L. mexicana* gp63 DNA was bulked up using standard protocols (Materials & Methods) and PCR amplification was performed to confirm the presence of *L. mexicana* gp63 gene in the construct (Figure 3-9). Forward and reverse primers with the sequences of 5'-ACATCCTCACCGACGAGAAG-3' and 5'-CTTGAAGTCGCCACAGATCA-3' respectively were designed by a web-based software "Primer3" based on the sequence of the gene obtained from the gene bank and used in the PCR process.

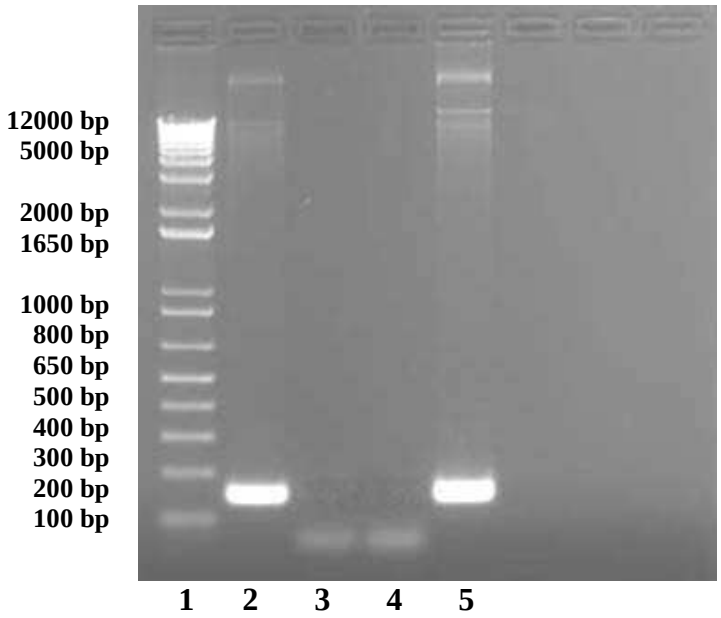


Figure 3-9: Confirmation of the existence of *L. mexicana* gp63 in VR1012 construct by PCR.
VR1012 *L. mexicana* gp63 was reproduced by transformation of *E. coli* followed by phenol chlorophorm precipitation using Quia-gen EndoFree plasmid purification Maxi Prep Kits and the presence of *L. mexicana* gp63 was confirmed by PCR amplification using 5'-ACATCCTCACCGACGAGAAG-3' forward and 5'-CTTGAAGTCGCCACAGATCA-3' reverse primers. The primers are expected to produce 180bp bands.

To determine the sequence of the *L. mexicana* gp63 gene, four primers including the primers used for the PCR amplification and two new designed primers with the sequence of 5'-GCTGCAACAGCTTGGAGTATC-3' and 5'-GATACTACACCGCCCTGTGC-3', were applied to complete the sequencing (Figure 3-10 and Figure 3-11).

atgcccgtcgacagcagcagcacgcaccggcaccgctgcgtcgccgcgcgcctggtgcgcctcgcggctgccggcgccgca
gtcaccgtcgctgtcggcaccgcggccgcgtgggcacacgccggtgcgccccagcaccgctgcatccacgacgcgatgcagg
cccgcgtgctgcagtcggtggcggctcagcgcatggctcccagcgcggtgtccgcggtgggcctgccgtacgtgtccgtggtcc
ccgtcgagaacgccagcaccctcgactactcgctatcggacagcacgtcgcccggtgttgtgcgcgccgcgaactggggcgcg
ctgcgcatcgccgtctccgccgaagacctcaccgaccccgcctaccactgcgctcgtgttgggcagcgcgtcaacaaccacgcc
ggcgacaccgtcacctgcaccgccgagg**acatcctcaccgacgagaag**cgcgacaccctcgtcaagcacctcgtcccgcag
gcctgcagctgcacagggagcgcctgaaggtgcggcaggtgcagggcaagtggaaggtgacgggcatggcggacg
tgatctgtggcgacttcaaggtgccgccggagcacatcacggaaggcgtgaccaacaccgacttcgtgctgta
cgtcgcctccgtgccgagcgaggagagtgtgctggcgtgggccacgacctgccaggtgttccctgacggccac
ccagccgtcggcgtcatcaacatccccgcggcgaacattgcgtcgcggtacgaccagctcgtcacgcgtgtcg
tcacgcacgagatggcgcacgcgctgggcttcagcggcacattctttggggccgtcggcatcgtgcaagaggt
gccgcacgttcgcggcaaggactttaatgtgtcggtgatcaccagcagcacggtggtggcgaaggcgcgtgag
cagtacg**gctgcaacagcttggagtatct**ggagattgaggaccagggcggtgcgggctccgccgggtcgcata
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ccgtttgttgttggctacaggaatggctcgtgcaatcaggatgcgtcgacgacaccggaccttctcgctgcgt
tcaacgtcttctccgaggccgcgcggtgcatcgatggcgccttcacgccgaagaacagaaccgctgcggatg**g**
atactacaccgccctgtgcgccaacgtgaagtgcgacacggccacgcgcacgtacagcgtccaggtgcgcggc
agcaacggctacgccaactgcacgccgggcctcagagttaagttgagcagcgtgagcgacgccttcgagaagg
gcggctacgtcacgtgcccgccgtacgtggaggtgtgccagggcaacgtcaaagctgccaaggactttgcagg
cgacaccgacagctccagcagcgccgatgacgctgccgacaaagaggcgatgcagcggtggagtgacaggatg
gccgccttggctactgcgacgacgctgctgctaggaatggtgctctctctcatggcactcctcgtggtgcggc
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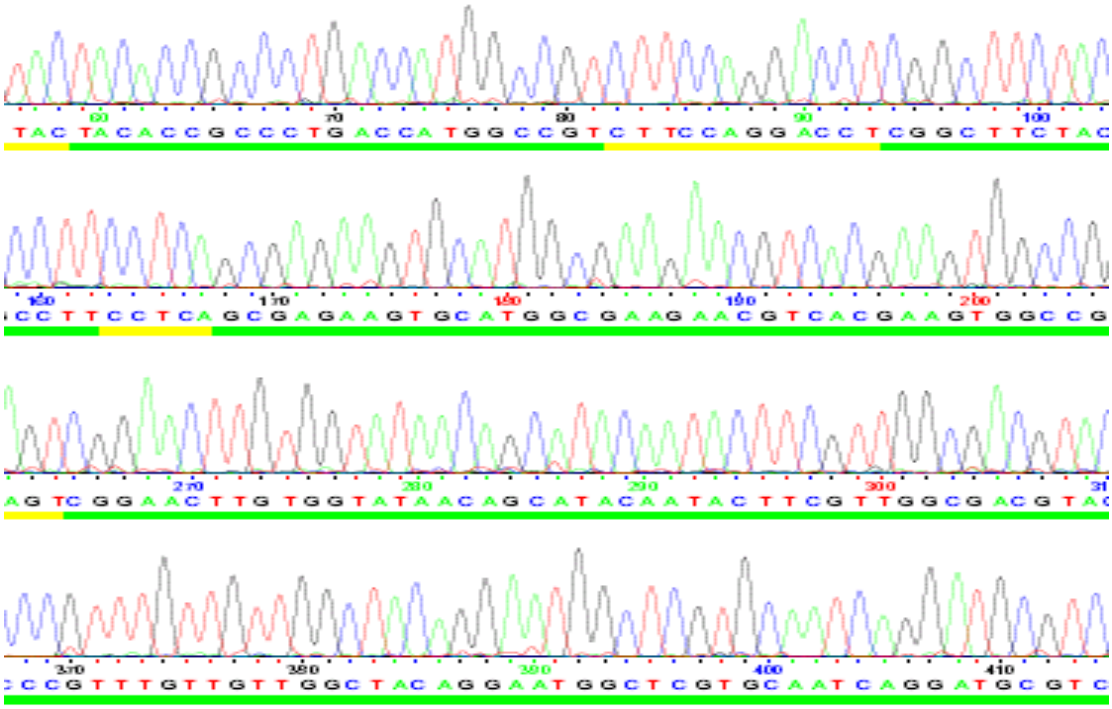


Figure 3-11: The chromatography of *L. mexicana* gp63 gene sequencing produced by MWG Biotech

3.2.4.2 Construction of VR1012 empty vector

In order to produce a VR1012 empty vector to be used as a negative control in the protection studies, the *L. mexicana* gp63 gene was cut and removed from this vector (Figure 3-8). The *L. mexicana* gp63 gene was cut out from the vector by digestion with EcoRI restriction enzyme and the product was run into the agarose gel (Figure 3-12 A). The band related to the vector was extracted from the gel. Both free ends of the vector that resulted from digestion with EcoR I were then ligated to each other by ligase enzyme (Figure 3-12 B). The absence of the gp63 gene in the empty vector was confirmed by sequencing the empty vector using primers specific for the gp63 gene or the vector. The sequencing confirmed the lack of *L. mexicana* gp63 in the VR1012 vector.

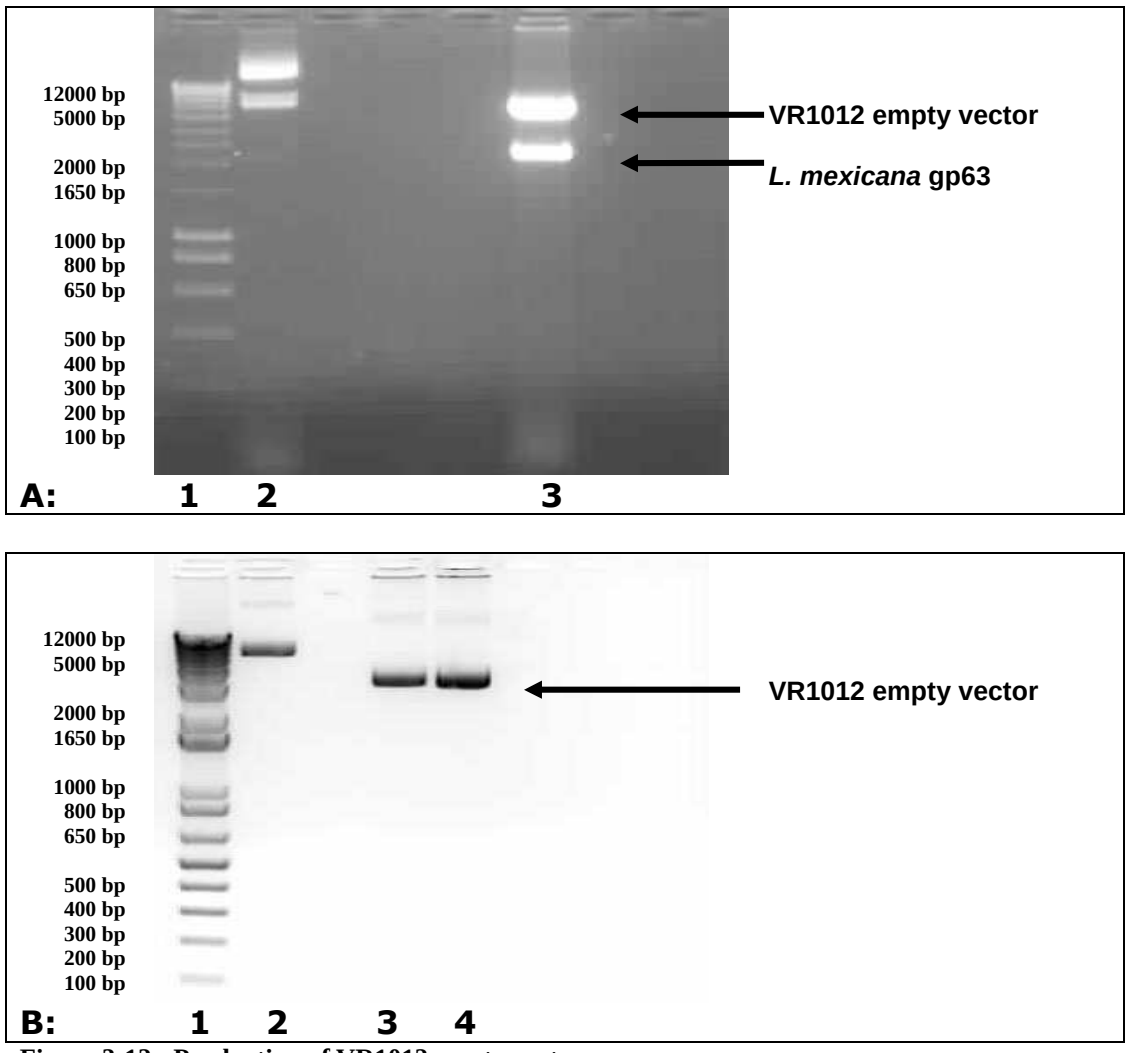
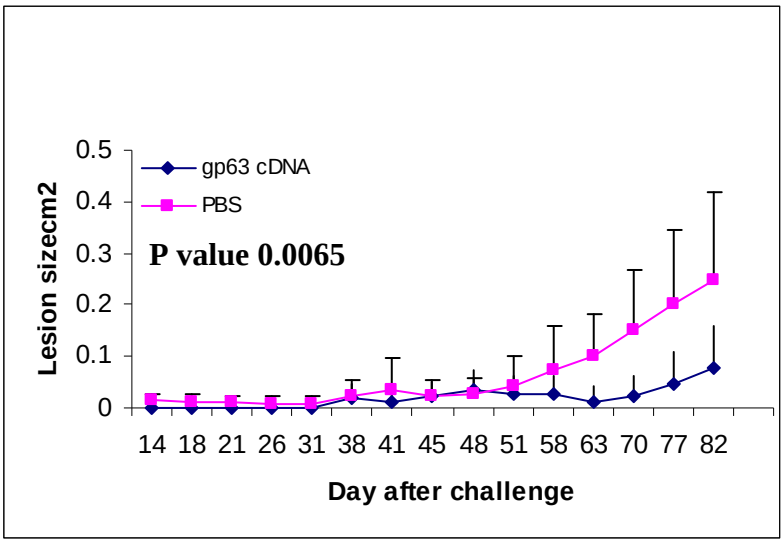


Figure 3-12 : Production of VR1012 empty vector.
A: Cutting VR1012 *L. mexicana* gp63 by EcoRI restriction enzyme 1- standard DNA ladder; 2- VR1012 *L. mexicana* gp63 with the size of 7414bp (control) the above band is a linear form of DNA; 3- VR1012 *L. mexicana* gp63 cut by EcoR I (4900bp) and *L. mexicana* gp63 (2500bp) **B:** Producing VR1012 empty vector; 1- standard DNA ladder; 2- VR1012 *L. mexicana* gp63 (control) 7414bp; 3, 4- VR1012 empty vector 2500bp

3.2.4.3 Immunisation with *L. mexicana* gp63 cDNA via I.M. injection

To assess the immunogenicity of *L. mexicana* gp63 cDNA, 100µg of *L. mexicana* gp63 cDNA (VR1012 vector) was administered to BALB/c mice by I.M. injection into the leg triceps muscle. The immunisation was carried out twice two weeks apart, and two weeks after the last immunisation, the mice were challenged with 2×10^6 *L. mexicana* promastigotes *in vitro* culture.

The results revealed that intramuscular injection of *L. mexicana* gp63 cDNA induced partial but significant protection since 2 out of 6 (33%) of the immunised mice remained lesion free (Figure 3-13). Immunisation with VR1012 empty vector did not protect the mice against challenge with live promastigotes (data not shown).



Lesion free mice: 2/6 (33%)

Figure 3-13 : Protection induced by intramuscular injection of *L. mexicana* gp63 cDNA vector (VR1012).
Two groups of 6 female BALB/c mice were used. The first group was injected with 100µg of *L. mexicana* gp63 plasmid DNA (VR1012 vector). The second group was injected with PBS. The mice were injected I.M. twice on day 0 and 14 in the triceps muscle of the leg and on day 28 were challenged with 2×10^6 log-phase *L. mexicana* promastigotes. Mice were monitored regularly. The graph represents 3 independent experiments. Bars represent the standard deviation n=6.

3.2.4.4 Immunisation with *L. mexicana* gp63 cDNA via gene gun

The gene gun was used in this study to immunise BALB/c mice (6mice per group) with *L. mexicana* gp63 cDNA. 1µg of *L. mexicana* gp63 cDNA (VR1012) coated on gold particles (see Materials & Methods) was administered by gene gun into a shaved area of the abdomen. A control group of 6 mice was administered with gold particles coated with empty plasmid by gene gun. The mice were immunised twice two weeks apart and the immunised mice were monitored regularly following the challenge with the parasite. The results showed that a significant protection was induced by immunisation with 1µg *L. mexicana* gp63 cDNA using the gene gun; 66% (4 out of 6) of the immunised mice remained free of lesion (Figure 3-14).

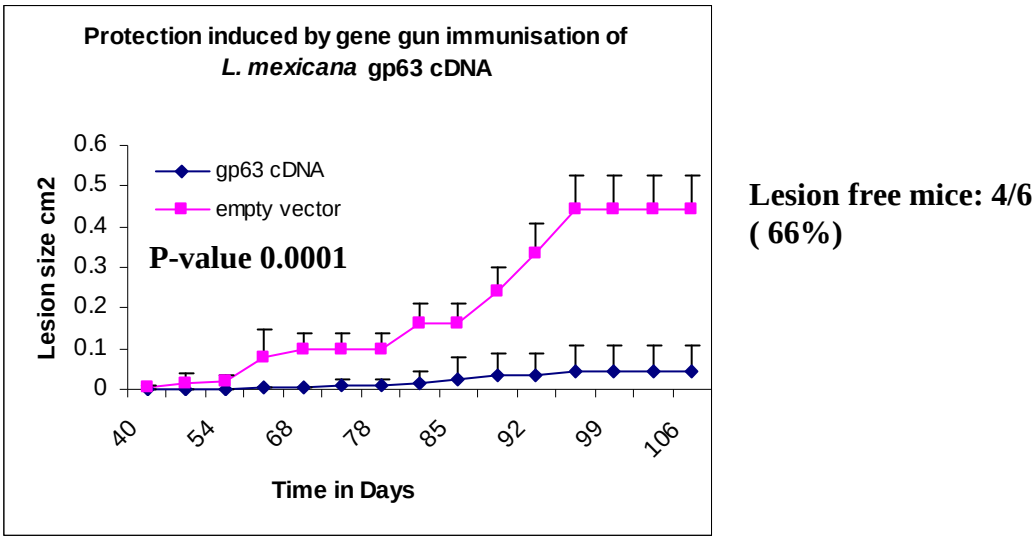


Figure 3-14 : Gene gun immunisation with *L. mexicana* gp63 cDNA.
1µg per mouse of *L. mexicana* gp63 plasmid DNA (VR1012) coated on gold particles was introduced to a shaved area of abdomenal skin of BALB/c mice by gene gun on day 0 and 14. The control group was given 1µg empty vector coated on gold particles. The mice were challenged with 2×10^6 log-phase *L. mexicana* promastigote on day 28, and were monitored regularly. The graph represents 3 independent experiments. Bars represent the standard deviation n=6.

3.2.5 Immunisation with CT26 tumour cells transfected with *L. mexicana* gp63 cDNA

To evaluate the efficacy of the leishmania gp63 recombinant protein in generating immunity to Leishmania when it is being produced and expressed by host cells, CT26 tumour cells were transfected with *L. mexicana* gp63 (see chapter 4). A group of 6 BALB/c mice were immunised subcutaneously with 5×10^5 irradiated CT26 *L. mexicana* gp63 tumour cells. Another 2 control groups of 6 mice were injected either with irradiated CT26 tumour cells or PBS. Two immunisations were given on day 0 and 14, and on day 28 all mice were challenged with 2×10^6 log-phase *L. mexicana* promastigotes. The results clearly demonstrated that no protection against *L. mexicana* was observed following immunisation with CT26 *L. mexicana* gp63 when compared with control group given PBS (Figure 3-15). However, immunisation with irradiated non-transfected CT26 tumour cells (control group) exacerbated leishmania lesions.

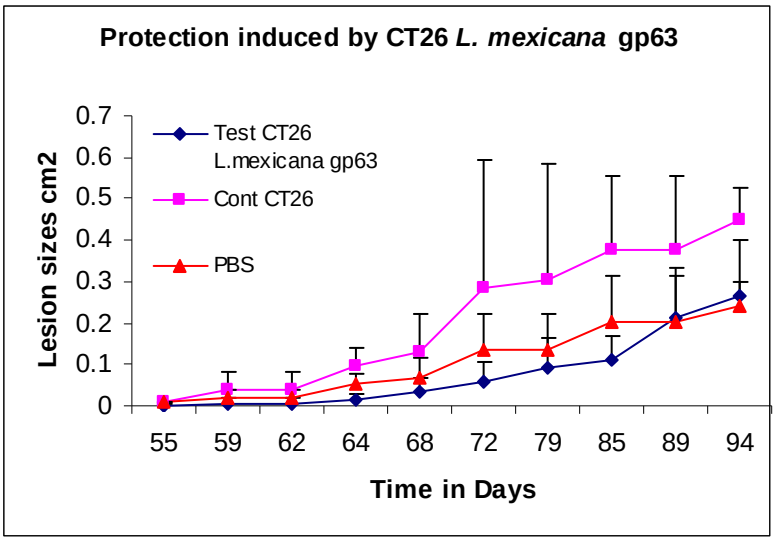


Figure 3-15: The immunogenicity of *L. mexicana* gp63 recombinant protein expressed by CT26 tumour cells in BALB/c mice.
Three groups of 6 BALB/c mice were used. The first group was subcutaneously immunised with 5×10^5 irradiated CT26 *L. mexicana* gp63 tumour cells. The second group was injected with irradiated CT26 tumour cells and the third group was injected with PBS. Two immunisations were given at two weeks interval and challenged two weeks later with 2×10^6 log-phase *L. mexicana* promastigotes. The mice were monitored twice a week. The graph represents 3 independent experiments. Bars represent the standard deviation n=6. P-value for groups of mice immunised with *L. mexicana* gp63 transfected CT26 and PBS was 0.215.

3.3 Discussion

3.3.1 Protection using autoclaved *L. mexicana*

Intracellular parasites are now accounted as a major health problem in the world. Developing vaccines for intracellular parasites has always been a goal for immunologists as relatively a certain amount is known about the interaction of these parasites with their host. Leishmania as an intracellular parasite presents a distinct interaction of intracellular parasites with the host. It is well known that leishmania patients develop a long-lasting immunity after recovery from the disease [Khamesipour et al., 2006]. This clearly rationalizes the attempts towards developing an effective vaccine to Leishmania parasites and different strategies have been implicated to develop potent leishmania vaccines. The use of autoclaved killed parasites to generate immunity to leishmania was one of the earliest strategies investigated for leishmania vaccination, which has been used for the prevention and treatment of leishmania patients [Convit et al., 2004; Khamesipour et al., 2006]. Using this approach of vaccination has always been produced contradictory results, since no protection was reported by some researchers using autoclaved leishmania parasites [De Luca et al., 1999; Velez et al., 2000; Velez et al., 2005]. Administration of adjuvants such as BCG, Aluminum Hydroxide (Alum) or both has been shown to be effective in enhancing the immunogenicity of killed leishmania parasites [Alimohammadian et al., 2002; Dube et al., 1998]. Other adjuvants such as IL-12 has also been tested in mouse models, which resulted in better protection by using Autoclaved *L. major* (ALM) + IL-12 followed by ALM + BCG [Michel et al., 2006]. In contrast, other studies reported no protection from immunisation with autoclaved parasites and BCG [Armijos et al., 2004; Khalil et al., 2000; Momeni et al., 1999; Sharifi et al., 1998]. This could be due to the differences in the method of immunisation, or the population tested. Recent studies have suggested for better outcome using a mixture of BCG and Alum as adjuvant [Khalil et al., 2006; Misra et al., 2001]. Nevertheless, in our studies, IFA was used as adjuvant to enhance the immunogenicity of the autoclaved parasite. IFA is a known adjuvant and has already been used in leishmania vaccination in combination with leishmania soluble antigen [Gabaglia et al., 2004; Sharma et al., 2006].

Our results clearly show that little protection was achieved by administration of autoclaved *L. mexicana* promastigotes plus IFA in BALB/c mice. Therefore, the application of IFA did not enhance the immunogenicity of autoclaved Leishmania in this mouse model.

3.3.2 Application using SLA in protection studies

The immunity induced by Soluble Leishmania Antigen has generated interest among leishmania researchers. In a study by Sharma [Sharma et al., 2006] *L. donovani* promastigote soluble antigens were encapsulated in non-phosphatidylcholine liposomes derived from *E. coli* lipids elicited a protective immune response against experimental visceral leishmaniasis. In another study, Immunization with soluble leishmania antigen in IFA plus Ad5IL-12 vector induced protection in BALB/c mice against *L. major* infection [Gabaglia et al., 2004].

In the present study, we examined the immunogenicity of *L. mexicana* soluble antigen in two modes of immunisation in protection investigation in BALB/c mouse model. In the first approach, two injections of BALB/c mice with 100µg/mouse of SLA mixed with 100µg/mouse of IFA did not significantly prevented *L. mexicana* infection. However, administration of SLA induced detectable levels of Th1 and Th2 immune responses (see chapter 4) indicating the existence of immunogenic proteins in this preparation, which provokes immunity to Leishmania. Therefore, the identification of these immunogenic proteins and using them in potential vaccines as well as developing new methods for vaccine administration to enhance immunogenicity are future areas for leishmania vaccine investigation.

The second approach tested was the application of DCs loaded with SLA. DCs have been shown to be a potent adjuvant in leishmania vaccination [Moll & Berberich, 2001] and their potency in the generation of immunity to intracellular pathogens is dependent on the production of IL-12, which results in shifting the immune response toward Th1-type. It has been shown that the protective potential of DCs pulsed with a given Leishmania Ag correlated with the level of their IL-12 expression [Berberich et al., 2003]. In a similar study, animals receiving DCs pulsed with *L. donovoni* soluble antigen either before or following infection had 1-3 log lower parasite burdens as well as enhancement of the parasite-specific IFN-γ response. The number of live parasites in the liver of mice was further reduced by vaccination with DCs transfected with IL-12 gene and loaded with SLA and the parasitological response was associated with a nearly normal liver histology [Ahuja et al., 1999].

Our results showed that immunisation with DCs pulsed with SLA obtained from *L. mexicana* did not protect BALB/c mice from leishmania infection. This was in contrast with the results obtained by Moll in which DCs pulsed by SLA protected BALB/c mice from *L. major* infection [Moll & Berberich, 2001]. The discrepancy could be due to the

difference between the species of leishmania used. However, other results (see chapter 4) showed that DCs pulsed with SLA were potent in generating CTL activity and inducing a mixed Th1/Th2 immune response. Further studies are required to clarify the role of DCs in these immune responses in protection against the infection.

3.3.3 DNA immunisation

DNA immunisation is a method that has recently been used in leishmania vaccination. Different studies using different genes have shown the potency of this method in generating immunity to Leishmania [Kedzierski et al., 2006; Tewary et al., 2006]. Gp63 is an immunogenic protein in Leishmania pariasites. It has been shown that administration of DNA encoding leishmania gp63 protein can generate immunity and partially protect BALB/c mice from the infection [Dumonteil et al., 2003; Walker et al., 1998].

It has been shown that the modification of the method of DNA administration, such as the application of heterologous prime-boost protocol enhances the efficacy of DNA vaccine [Stober et al., 2007]. In a study by Rafati, the potential protection of an immunogenic gene called SPase from *L. major* was evaluated using three different vaccination strategies (DNA/DNA, Protein/Protein and DNA/Protein) against *L. major* infection. The results indicated that the DNA/DNA strategy gave more effective protection than the other two approaches [Rafati et al., 2006]. Application of gene gun has recently been implicated in leishmania DNA immunisation [Sakai et al., 2000]. Here we compared two different methods of DNA immunisation based on the DNA/DNA strategy in BALB/c mice. In the first method of immunisation, mice were immunised with 100µg of *L. mexicana* gp63 cDNA construct intramuscularly and in the second one, 1µg of the same construct coated on gold particles was administered I.D. by gene gun.

Both methods of immunisation induced significant protection in immunised mice, confirming the results obtained by Dumonteil [Dumonteil et al., 2003]. Although for gene gun immunisation, the amount of the DNA applied was far less than that used for intramuscularly injection, the protection obtained by gene gun immunisation was much better, where 66% of immunised mice were free of lesions compared with 33% given intramuscular immunisation. The results, may for the first time, confirm the capability of gene gun immunisation in enhancing the immunogenicity of DNA opening a new window of opportunity in leishmania vaccine research.

3.3.4 Protection using gp63 recombinant protein expressed by CT26 tumour cells

Leishmania gp63 protein is shown to be immunogenic in BALB/c but cannot fully protect mice from Leishmania infection. Different strategies have been implicated to enhance the immunogenicity of this protein by applying different adjuvant or using different methods of immunisation [Berberich et al., 2003; Jaafari et al., 2006; Papadopoulou et al., 1998].

In order to investigate the possibility of enhancement of the immunogenicity of this protein, BALB/c mice were immunised with CT26 tumour cells, which were *in vitro* transfected with pcDNA3 *L. mexicana* gp63 plasmid DNA (expression of *L. mexicana* gp63 protein is given in chapter 4). The results indicated that little protection was achieved using this method of immunisation, where 0.5×10^6 CT26 *L. mexicana* gp63 expressing tumour cells were implanted subcutaneously into immunised mice; as these cells were irradiated, they had little chance to reproduce gp63 protein *in vivo* and perhaps the amount of gp63 protein expressed by the cells was not sufficient to protect the mice from the infection. The size of lesions in mice immunised with CT26 *L. mexicana* gp63 was similar to that of mice injected with PBS. Surprisingly the other group of control mice injected with normal CT26 tumour cells showed larger lesions (P-value 0.006). one possibility is that in these mice, the application of tumour cell antigen diverts the immune system toward non-leishmania antigens that could reduce the immune response to leishmania parasites.

Chapter 4 CTL Activity and Antibody responses in *L. mexicana* infection

4.1 Introduction

4.1.1 Immune response to intracellular pathogens vaccination

In infections caused by intracellular pathogens, due to the complexity associated with these pathogens, both innate and adaptive systems normally become involved. In adaptive immunity, although both humoral and cellular mechanisms are involved in immunity to these pathogens, the domination of one response over the other depends upon the life cycle and pathogenicity of the infectious agent. As most of intracellular pathogens spend one stage of their life cycle in the macrophage host cells [Alexander et al., 1999], it is believed that a cellular immunity based on releasing IL-12 and IFN-γ cytokines has an essential role in generating immunity to these pathogens. For instance, in Trypanosoma parasites, immune mice produce high levels of IFN-γ and low levels of IL-4 compatible with Th1 immune responses while non immune mice do not [Guinazu et al., 2004]. The transfer of Ag-specific Th1 cells but not Th2 cells protects non-immune mice from a lethal infection with *T. cruzi* [Kumar & Tarleton, 2001]. In acute stages of malaria the production of Th1 cytokines are dominant whereas, in the chronic stages the level of Th2 cytokines is higher [Su & Stevenson, 2002]. Therefore, it is believed that for generating a sufficient immunity to malaria, a vaccine should target the pre-erythrocytic stages of the parasites when the parasite is hidden inside the hepatocytes and induce a cell immune response, which is potent to irradicate the parasite [Todryk & Walther, 2005].

In leishmania infection, the presence of antibodies might facilitate the entry of parasite to host cells. This normally functions in favour of the parasite than the host and helps the parasite survive in the mammalian host. In addition, when the parasites lodge inside the macrophage, antibodies are ineffective unless the parasite is released from the macrophage. Instead, the role of Th1 immune response in immunity to Leishmania appears crucial and the lack of IL-4 and IL-10 in the initial steps of the immune response plays important roles in diverting the immune system to the Th1 immune response [Sacks & Noben-Trauth, 2002]. However, recent studies indicated a diversity in immunity to different species of Leishmania suggesting an important role for Th2 immune response in visceral leishmaniasis [Selvapandiyan et al., 2006], which requires further investigation.

To assess the Th1/Th2-type immune response induced by a given antigen, a model system is required by which pure Th1 or Th2 immune responses can be evaluated. No lymphocyte surface marker is yet known to exclusively differentiate T-cell sub-types secreting different types of cytokines [Sjolander et al., 1998]. The method that is currently being used to evaluate Th1/Th2 immune responses is to measure the production of subclass antibodies stimulated by either of immune pathways. In mouse, Th1-like immune responses are associated with a strong antibody production of IgG2a, IgG2b and IgG3 subclasses. The Th2-like immune responses characterized by the production of large amounts of IgG1 and IgE [Germann et al., 1995; Su & Stevenson, 2002].

4.1.2 Leishmania gp63 proteins

A Leishmania zinc-metaloproteinase called gp63 or leishmanolysin is a characterised protein of leishmania species. The natural substrate for this proteinase is not yet known. The optimum pH for activation of gp63 appears to be dependent on the nature of the substrate used for the *in vitro* assay [Seay et al., 1996]. The molecular weight of this enzyme is 63KDa and there are some variation in the protein sequences in different species [Seay et al., 1996]. Although gp63 is normally found in high density on the surface of promastigotes [Corradin et al., 2002], there is evidence to suggest that leishmania promastigotes produce more than one isoform of gp63 [McGwire et al., 2002]. Promastigotes release proteolyticaly active forms of gp63 by cleaving the gp63 from the cell surface or releasing a soluble form of gp63 directly from inside the cell [Jaffe & Dwyer, 2003]. Amastigotes also release the soluble intracellular isoform of gp63. The soluble isoform produced by the amastigotes and promastigotes of some *Leishmania* species are at lower levels than the promastigote surface enzyme [Corradin et al., 2002]. During the transformation of promastigotes to amastigotes in macrophages, changes may occur in the expression pattern of gp63. In a study on *L. chagasi*, it has been revealed that gp63 proteins are encoded by three different classes of genes. Using a human macrophage cell line “U937”, providing an *in vitro* model of phagocytosis, it was shown that there were three gp63 isoforms active in amastigotes [Streit et al., 1996]. In *L. mexicana* promastigotes the surface protease gp63 is amphiphilic and comprises approximately 1% of the cellular proteins. In contrast, in amastigotes the gp63-related proteins are predominantly hydrophilic and constitutes 0.1% of the cellular protein, mainly located in the lumen of the extended lysosomes (megasomes) [Bahr et al., 1993].

Despite extensive investigation, the role of gp63 in physiology and infectivity of the parasite [Corradin et al., 2002] and its function in insect vectors is still not clear [Sadlova et al., 2006]. It has been revealed that gp63 might have an important role in the initial stages of leishmania infection by inhibiting the chemotaxis of both neutrophils and monocytes [Sorensen et al., 1994]. Using genetically modified *L. amasonensis,* which were expressing gp63 proteins at lower levels, it has been shown that the down regulation of gp63 increases the extra cellular lysis of the parasite by complement *in vivo* and reduces the infection of macrophages resulting in a Th1-type immune response [Thiakaki et al., 2006]. gp63 also has an important role in the parasite's evading system (see chapter1). Moreover, gp63 plays a crucial role in protecting parasites from the killing and degradative activities of macrophages by preferentially accessing macrophages via CR3 and CR1; the signals produced by these receptors inhibits the macrophage respiratory burst. gp63 has also been associated with suppression of the oxidative burst and its protease activity protects the parasite from lysosomal cytolysis and degradation [Alexander et al., 1999; Seay et al., 1996]. There are studies suggesting that gp63 may function as a receptor for macrophages and is implicated in the attachment and uptake of promastigotes by the host cells [Handman et al., 1990]. There is also evidence that gp63 accelerates the phagocytic process by increasing the cell membrane motility and macrophage ruffling activity [Coelho Neto et al., 2005]. In a previous study the expression of gp63 was down regulated by 20-50 fold in attenuated parasites and was associated with failure in survival of the parasite in the macrophage phagosomes [Seay et al., 1996].

The immunogenicity of leishmania gp63 has been shown in different studies by several research groups [Lopez et al., 1991; Russell & Alexander, 1988]. To improve the immunogenicity of leishmania gp63, different adjuvants and different methods of immunisation including the use of recombinant gp63 protein, DNA immunisation and peptide immunisation have been implicated [Awasthi et al., 2004]. The protective immunity generated by gp63 in vaccinated mice was indicated by reduced inflammation and suppressed lesions after experimental challenge [Thiakaki et al., 2006]. In the first part of this chapter, the CTL activity induced by *L. mexicana* gp63 cDNA and SLA is explained. In the next parts, the role of leishmania infection in down regulation of MHC class I, and also antibody responses induced by different leishmania vaccines will be discussed.

4.2 Results

4.2.1 CTL activity induced by immunisation with gp63 cDNA construct

Standard (4hr ^{51}Cr release) cytotoxicity assay was used in this study to measure the CTL activity in mice immunised with *L. mexicana* gp63 cDNA (VR1012 plasmid) construct (map shown in chapter 3).

4.2.1.1 Preparation of CTL targets expressing *L. mexicana* gp63 protein

To prepare *L. mexicana* gp63 specific cell target to use in standard 4-hour cytotoxicity assay, the *L. mexicana* gp63 gene was cloned into pcDNA3 plasmid vector and transfected into CT26 tumour cells (see section 4.2.1.2).

4.2.1.1.1 Gene cloning of L. mexicana gp63 into pcDNA3

Prior to transfection of CT26 tumour cells with *L. mexicana* gp63, it was essential to clone the gp63 gene into pcDNA3 vector, which contained a mammalian selection antibiotic gene (Figure 4-1). *L. mexicana* gp63 was first cut from both sides by EcoRI restriction enzyme off the VR1012 vector (Figure 4-2). pcDNA3 vector was also cut using the same restriction enzyme. Then, the *L. mexicana* gp63 gene and the digested vector were ligated using a DNA ligase enzyme.

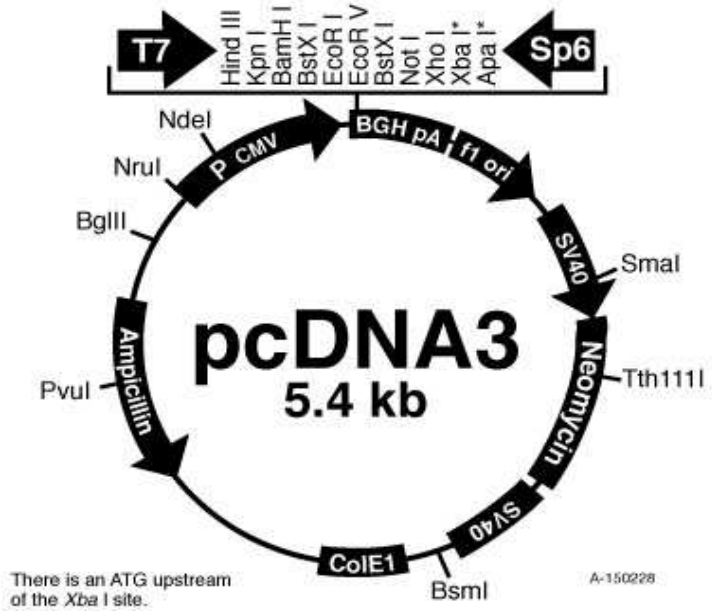


Figure 4-1 : Map of pcDNA3 vector used to transfect CT26 tumour cells with *L. mexicana* gp63 [Invitrogen web site].

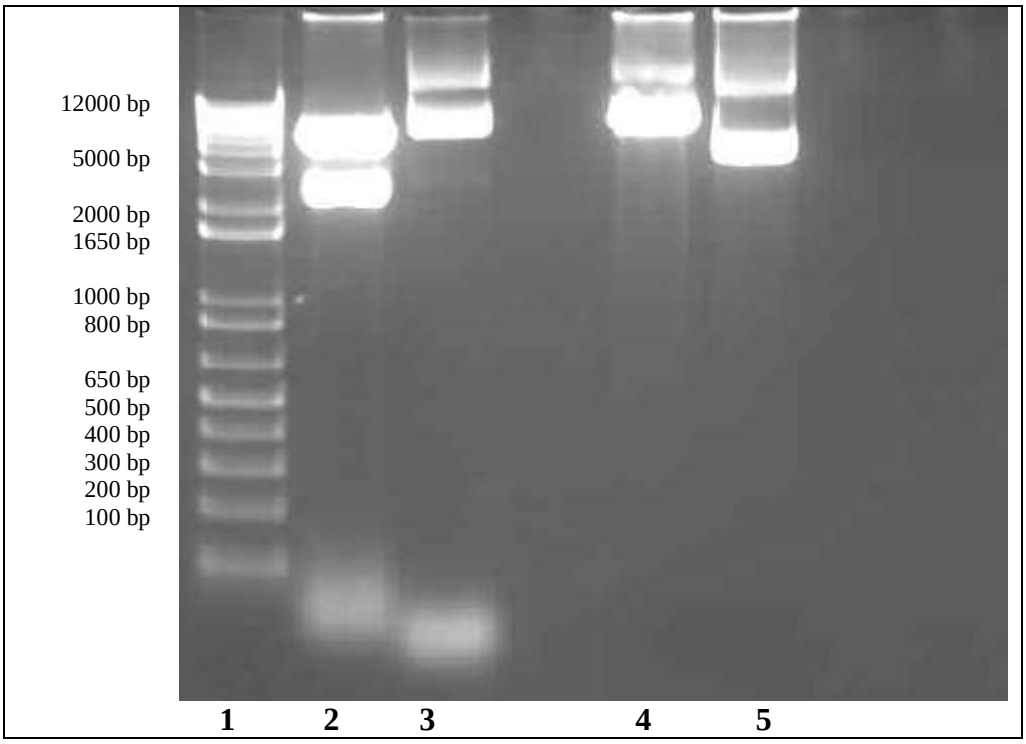


Figure 4-2: Digestion of VR1012 *L. mexicana* gp63 and pcDNA3 vector with EcoRI restriction enzyme. Lanes 1-standard DNA, 2-VR1012 *L. mexicana* gp63 cut by EcoRI restriction enzyme (VR1012 vector 4800bp and *L. mexicana* gp63 25bp), 3-VR1012 *L. mexicana* gp63 (uncut, 7414bp), 4-pcDNA3 vector cut by EcoRI restriction enzyme (54000bp), 5-pcDNA3 vector (uncut, 54000bp).

The presence of the *L. mexicana* gp63 gene in pcDNA3 vector was first determined by restriction enzyme digestion (Figure 4-3) and then by PCR amplification using 5'-ACATCCTCACCGACGAGAAG-3' forward and 5'-CTTGAAGTCGCCACAGATCA-3' reverse primers (Figure 4-4). Moreover, to ensure the sub-cloned gene is completed and no mismatches happened during the cloning procedure the whole gene (1900bp) was sequenced.

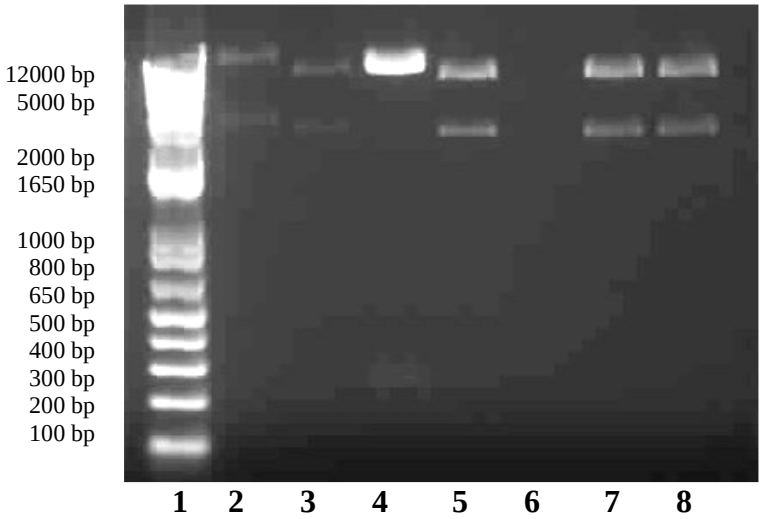


Figure 4-3: Detection of *L. mexicana* gp63 in pcDNA3 expression vector.
After ligation of *L. mexicana* gp63 gene and pcDNA3 vector, XLblu *Ecoli* were transformed by pcDNA3 *L. mexicana* gp63, plated and cultured in presence of Ampicilin. A number of single colonies were cultured separately and pcDNA3 *L. mexicana* gp63 was extracted from each colony separately. The presence of the gene was confirmed by cutting the gene using EcoRI restriction enzyme. **1**:standard DNA **2**:VR1012 *L. mexicana* gp63 **3, 5, 7 and 8**:pcDNA3 *L. mexicana* gp63 **4**: empty pcDNA3 vector

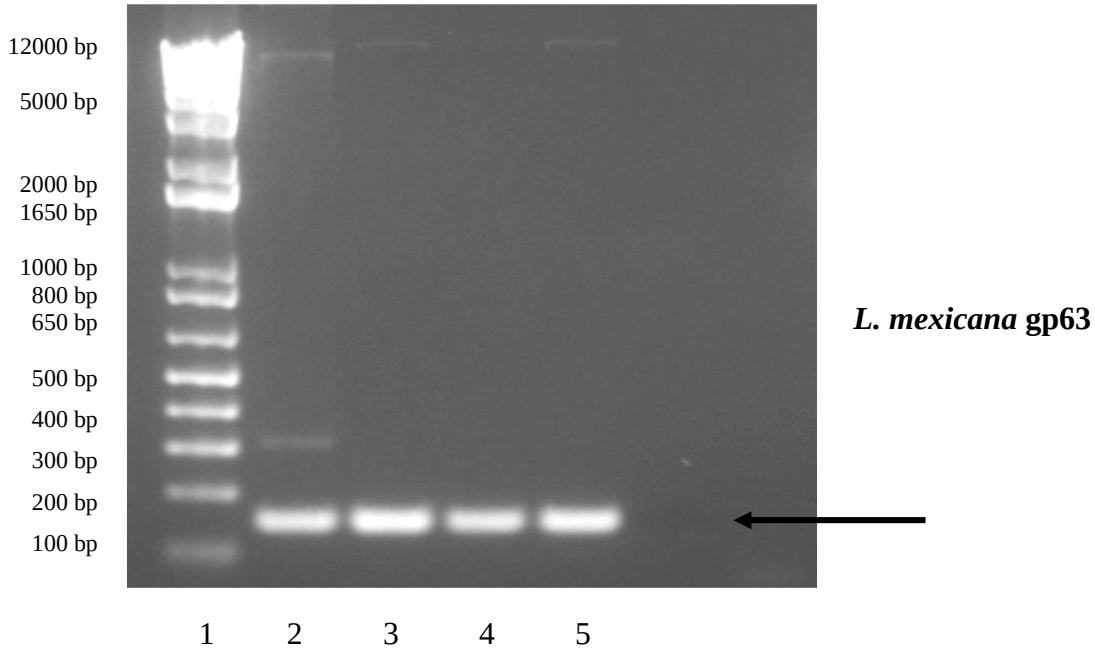


Figure 4-4: Detection of *L. mexicana* gp63 in pcDNA3 vector by PCR amplification.
The presence of *L. mexicana* gp63 in the new construct was confirmed by PCR amplification using 5'-ACATCCTCACCGACGAGAAG-3' forward and 5'-CTTGAAGTCGCCACAGATCA-3' revese primers. **1**:Standard DNA **2**:VR1012 *L. mexicana* gp63 **3-5**:pcDNA3 *L. mexicana* gp63 (the primers are expected to produce 180bp bands)

4.2.1.2 Transformation of CT26 tumour cells by *L. mexicana* gp63

4.2.1.2.1 Antibiotic sensitivity Assay

Prior to the transfection, the sensitivity of CT26 tumour cells to Geneticin (G418) was tested. Different doses rang from 200µg/ml to 850µg/mlof the antibiotic were applied in which the dose of 500µg/ml was effective enough to kill all the cells within 10 days. So, Geneticin at 500 µg/ml was used for selection and culture of transfected cells.

4.2.1.2.2 Transfection of CT26 cells

CT26 tumour cells were transfected with pcDNA3 *L. mexicana* gp63 plasmid construct using lipofectamine 2000 according to the manufacture's instruction. The presence of the *L. mexicana* gp63 gene was first determined in the stable transfected cells by RT-PCR. For unexplained reasons, non-transfected CT26 cells always showed a faint band when it was tested with the primers (Figure 4-5). The expression of gp63 protein was also determind by FACS analysis and western-blotting using anti *L. mexicana* gp63 antibodies (Figure 4-6, Figure 4-7).

A: GAPDH **B: gp63**

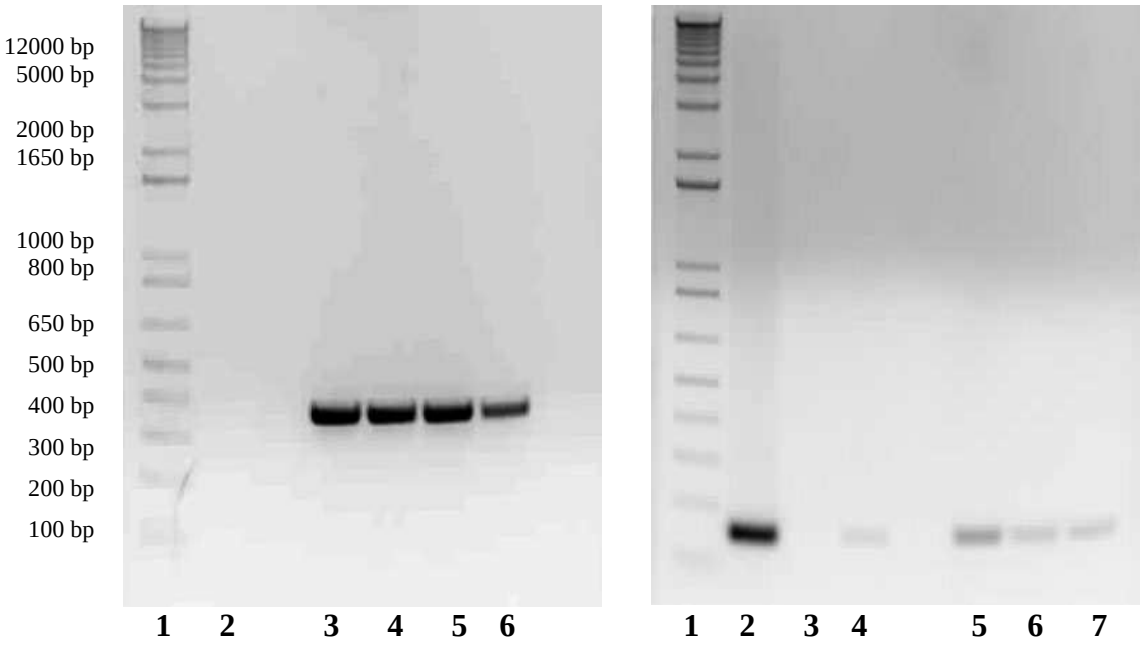


Figure 4-5: Expression of *L. mexicana* gp63 gene in transfected CT26 tumour cells detected by RT-PCR.
A: Expression of mouse GAPDH (primers are expected to produce 400bp) 1: standard DNA 2: PCR negative control 3-5: transfected CT26 tumour cells 6:non-transfected CT26 cells (control) **B:** expression of *L. mexicana* gp63 in CT26 tumour cells (primers are expecte to produce 180bp) 1: standard DNA 2: VR1012 *L. mexicana* gp63 (control) 3: PCR negative control 4: non-transfected CT26 (control) 5-7: transfected CT26 tumour cells

A: Non-transfected CT26 cells **B: Transfected CT26 cells**

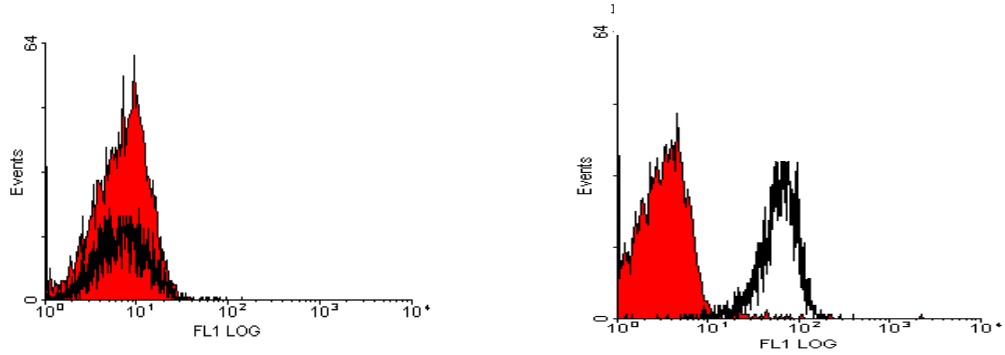


Figure 4-6: Expression of *L. mexicana* gp63 protein detected by FACS analysis.
The expression of *L. mexicana* gp63 protein was determined in CT26 transfected cells. CT26 *L. mexicana* gp63 (A) and non-transfected CT26 (B) were split equally into two tubes (test & control). The tube (black curves) was stained with rabbit anti *L. mexicana* gp63 and FITC conjugated anti rabbit antiboddies. The control tube was only stained with the FITC conjugated anti rabbit antibody. Results (histogram B) clearly show the high level of the protein expression on the transfected cells.

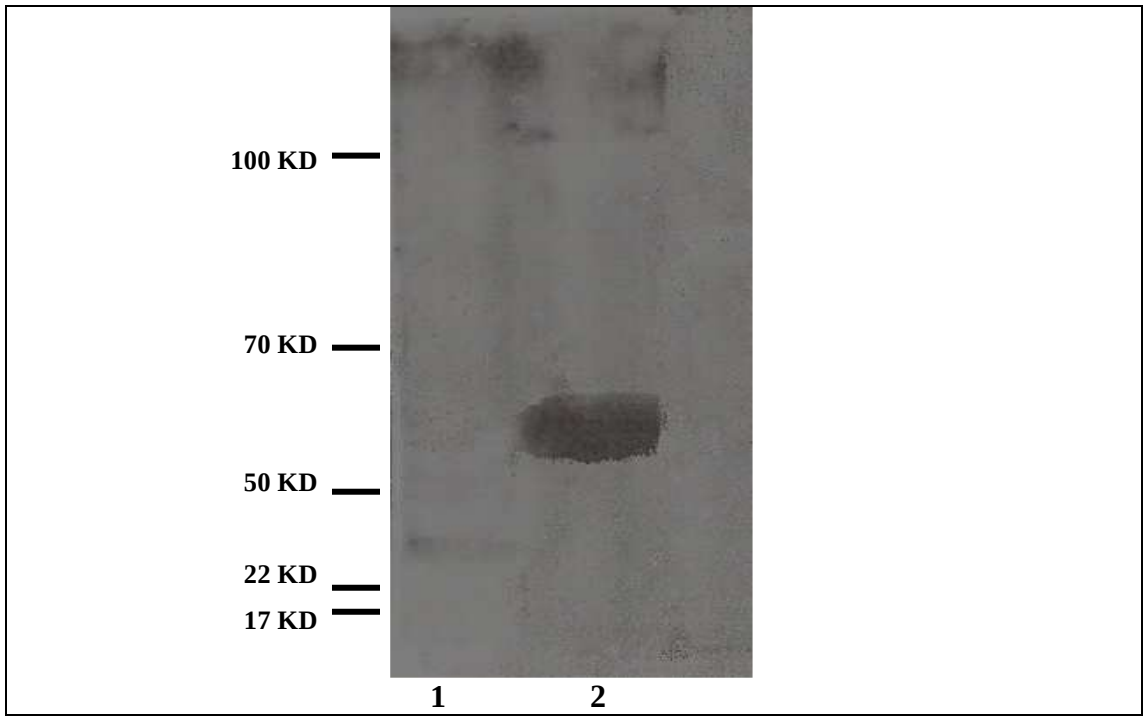


Figure 4-7: Expression of *L. mexicana* gp63 protein in CT26 tumour cells transfected with *L. mexicana* gp63 construct.

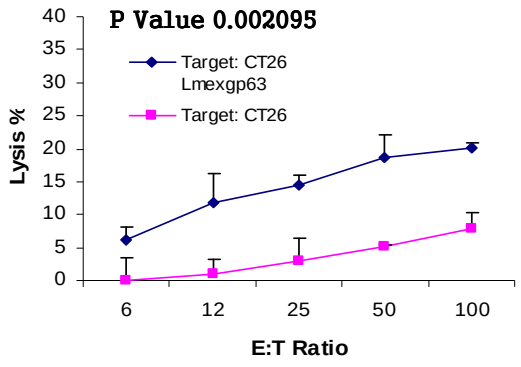
Transfected CT26 (*L. mexicana* gp63) and non-transfected CT26 cells (control) were lysed using lysate buffer. The cell lysates were run into the gel electrophoresis and the presence of *L. mexicana* gp63 protein was determined by western blotting analysis using rabbit anti-*L. mexicana* gp63 and HRP coupled goat anti-rabbit antibodies (see materials and methods) **1:** non-transfeted CT26 cell lysate **2:** CT26 *L. mexicana* gp63 cell lysate

4.2.2 Induction of CTL activity by immunisation with *L. mexicana* gp63 cDNA construct

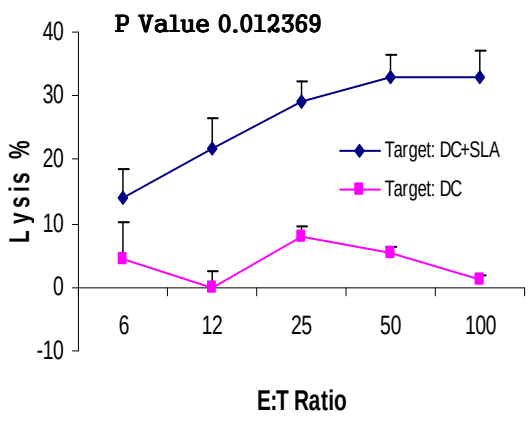
To evaluate the role of cytotoxic T cells in immunity to Leishmania, standard 4-hour cytotoxicity assay was used to assess the ability of *L. mexicana* gp63 cDNA to generate specific cytotoxic T lymphocytes. BALB/c mice were immunised twice at two weeks interval with *L. mexicana* gp63 cDNA by gene gun. Mice were sacrificed two weeks following the 2nd immunisation and spleens were collected. Splenocytes were harvested and cultured *in vitro* for 5 days together with blasts cells pulsed with LPS and SLA (SLA was shown to contain gp63 protein). On day 5, the splenocytes cells were used as effectors in standard 4-hour cytotoxicity assay against CT26 tumour cells transfected with *L. mexicana* gp63 (see materials and methods section CTL activity).

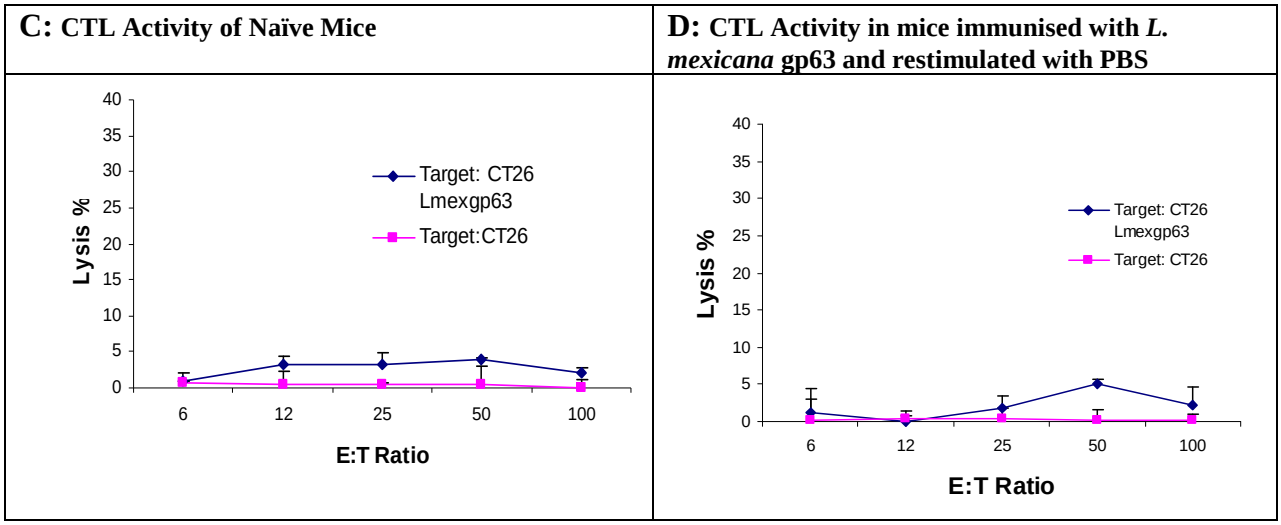
The results clearly revealed that immunisation of mice with *L. mexicana* gp63 cDNA induces specific CTL activity against CT26 tumour cells expressing *L. mexicana* gp63 (Figure 4-8A) and DCs loaded with SLA as targets; SLA was shown to contain gp63 (see chapter 3). The CTL activity demonstrated against DC targets was greater than that of CT26 *L. mexicana* gp63 (Figure 4-8B). The *in vitro* restimulation of CTLs by SLA loaded blast cells was crucial. It was shown that removing the *in vitro* restimulation of the splenocytes highly prevented the generation of CTL activity in immunised mice (Figure 4-8C) and levels was comparable with that of naïve mouse splenocytes restimulated *in vitro* by blast cells loaded with SLA (Figure 4-8D). *In vitro* depletion of CD8+ T cells by anti CD8 Ab and complement on day 5 significantly removed the CTL activity (Figure 4-8E) suggesting an effector role of CD8+ T cells in the CTL activity.

A: CTL Activity of *L. mexicana* gp63



B: CTL Activity of *L. mexicana* gp63





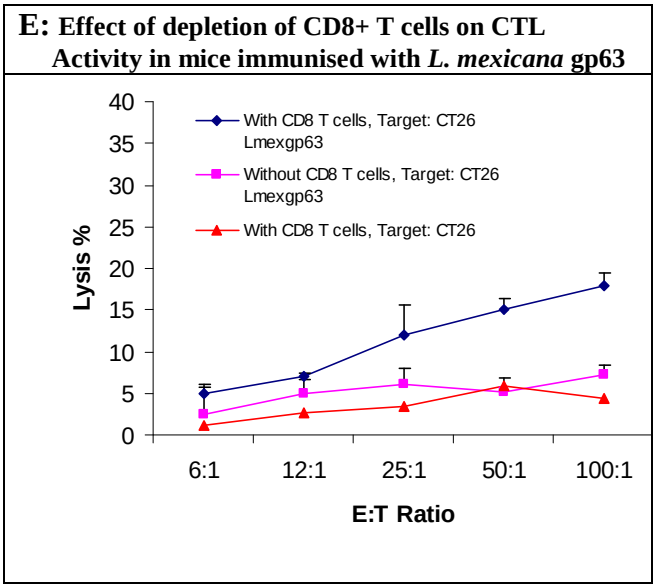


Figure 4-8: CTL activity induced by gene gun immunisation using *L. mexicana* gp63 cDNA.
BALB/c mice were immunised twice with *L. mexicana* gp63 using the gene gun twice; on day 0 and 14. On day 28 mice were sacrificed and the splenocytes were cultured *in vitro* with blast cells pulsed with LPS and SLA for 5 days. On day 5 the cells were used as effector in standard 4-hour cytotoxicity assay. A: DNA immunised mice restimulated with blast cells+SLA tested against CT26 *L. mexicana* gp63 cells. B: DNA immunised mice restimulated with blast cells+SLA tested against DCs pulsed with SLA **C:** naive mice restimulated with blast cells+SLA tested against CT26 *L. mexicana* gp63 cells. **D:** DNA immunised mice restimulated with blast cells+PBS tested against CT26 *L. mexicana* gp63 cells. **E:** DNA immunised mice restimulated with blast cells+SLA CD8+ T cells depleted and tested against CT26 *L. mexicana* gp63 cells. The results represent 8 mice in 4 independent experiments.

The persistence of CTL activity during the course of infection was also assessed by immunisation of BALB/c mice with *L. mexicana* cDNA using the gene gun followed by

challenging with 2×10^6 *L. mexicana* promastigotes. After 4 months of immunisation, splenocytes from the immunised mice still showed a significant level of CTL activity compared with control mice injected with PBS and then challenged with 2×10^6 of the parasite (Figure 4-9). This indicates that only immunisation but not infection induced CTL activity.

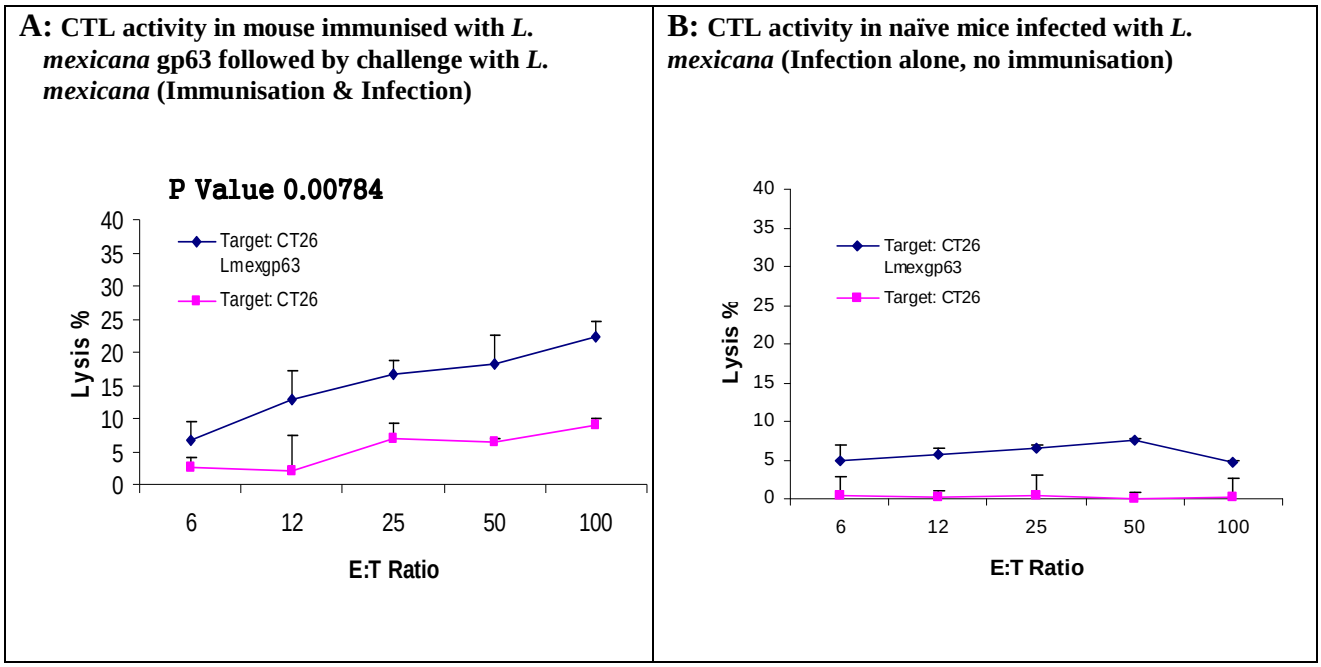


Figure 4-9: CTL activity in mice immunised with *L. mexicana* gp63 cDNA after being challenged with *L. mexicana.*

BALB/c mice were immunised with *L. mexicana* gp63 cDNA using gene gun. A group of 3 naïve mice were used for control. The mice were challenged by 2×10^6 *L. mexicana* promastigotes. After 4 month of infection the mice were sacrificed and splenocytes were restimulated with blast cells + SLA and cultured for 5 days, and then were tested against CT26 *L. mexicana* gp63 in a standard 4-hour cytotoxicity assay. **A:** DNA immunised mouse **B:** naïve mice. The results represents 6 mice in 3 independent experiments.

4.2.2.1 CTL activity in mice immunised with *L. mexicana* Soluble Antigen (SLA)

To assess the potency of SLA in inducing CTL activity, DCs were loaded with *L. mexicana* Soluble Antigen and injected into BALB/c mice intradermally at a dose of 2×10^6 cells per mouse. One injection of 2×10^6 SLA loaded matured DC induced high level of CTL activity, when tested against DCs loaded with SLA in standard 4-hour cytotoxicity assay (Figure 4-10).

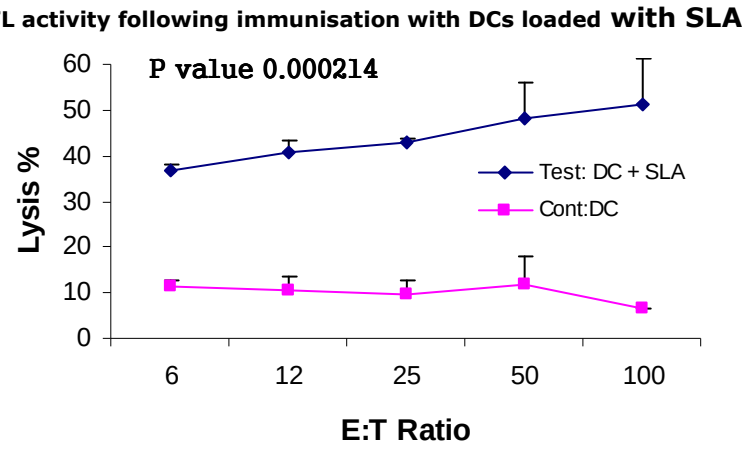


Figure 4-10: CTL activity in mice immunised with DCs pulsed with SLA.
BALB/c mice were immunised I.D. with 2×10^6 DCs loaded with SLA per mouse. After two weeks the mice were sacrificed and their splenocytes were cultured cultured *in vitro* for 5 days together with blast cells pulsed with LPS and SLA. On day 5 they were used as effector cells in a standard 4-hour cytotoxicity assay against DCs pulsed with SLA.

4.2.3 Effect of leishmania infection on the expression of MHC class I

The effect of leishmania infection on the expression of MHC class I at the cellular level was evaluated on bone marrow derived DCs infected with *L. mexicana*. In order to generate DCs, bone-marrow cells were cultured with GM-CSF for 6 days. On day 6 the cells were split into two groups. The first group of DCs were infected with *L. mexicana* at the ratio of 10 parasites to1 DC (Figure 4-11). The second group were treated with PBS. Both groups were also treated with LPS to induce maturation. Most parasites were shown to be taken up by DCs in the first few hours of the infection. On day 7 both groups of DCs were checked for the expression of MHC class I molecules on their surface using anti-mouse H2-L^d antibody.

Data obtained showed a down regulation of MHC class I molecules in leishmania infected DCs compared to controls (Figure 4-12A). Treatment of DCs with killed parasites or SLA failed to down regulate the expression of the MHC molecules (Figure 4-12B and C). In a time course study it was shown that the down regulation of the MHC molecules starts after 3 hours of the infection and is complete in 24 hours (data not shown). The effect of leishmania infection on the expression of MHC class I at gene level in DCs is now under further investigation.

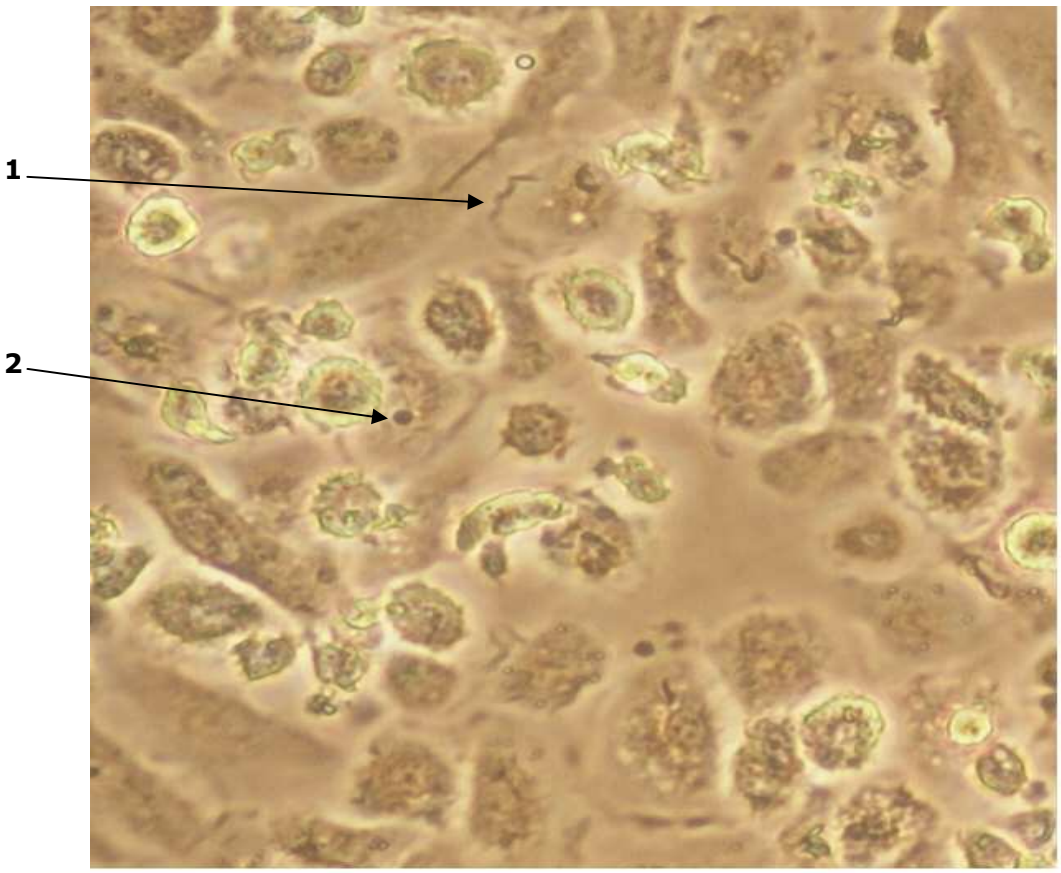
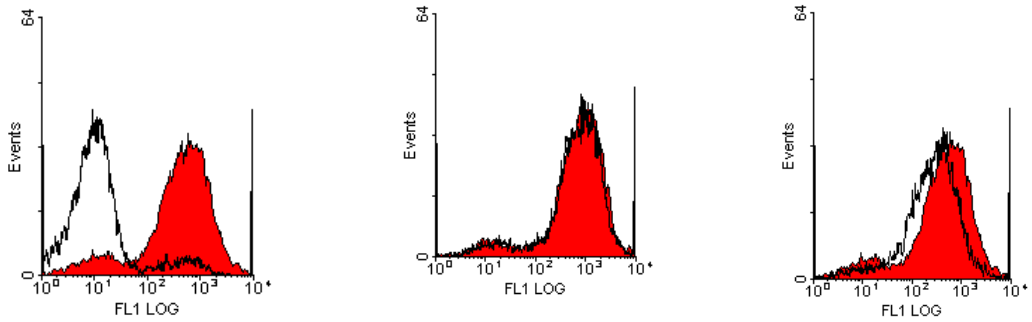


Figure 4-11: Infection of DCs with *L. mexicana.* 1- Leishmania promastigotes outside DCs 2- Leishmania amastigotes inside DCs



A: **B:** **C:**

Figure 4-12: Expression of MHC class I molecules in leishmania infected DCs.
Bone-marrow cells were cultured in presence of GM-CSF for 6 days with wash every 2 days. DCs were harvested and split into two groups. The first group were infected with 10 times number of leishmania to DCs for 24 hours. No parasite was added to the second group. Both groups were treated with 1µg/ml LPS. On day 7 both groups were checked for the expression of MHC class I by FACS analysis using FITC conjugated examined MHC class I antibody. **A:** non-infected DCs (red graph) show high expression of MHC class I where the expression of these molecules in Leishmania infected DCs (black graph) is highly down regulated. **B:** there is no difference between the expression of MHC I molecules on normal DC (red graph) and DCs infected with autoclaved parasite (black graph) **C:** the expression of the MHC I in normal DCs (red graph) is similar to that of DCs treated with SLA (black graph).

4.2.4 Antibody responses to leishmania vaccines

The potency of different immunisation strategies in inducing Th1/Th2-type immune response was assessed by measuring the level and the type of antibodies in leishmania sensitive BALB/c mice. In this study, the mice were immunised on day 0 and day 14 either with killed leishmania parasites, SLA, DCs pulsed with SLA, *L. mexicana* gp63 cDNA or CT26 *L. mexicana* gp63 cells, and bled weekly to determine the level of anti-leishmania isotype antibody associated with Th1 (IgG2α) or Th2 (IgG1) immune response.

4.2.4.1 Immunisation with DNA or gp63 transfected CT26 cells

To study the effect of the method of immunisation on "direction" of immune response, in a set of experiments, six groups of six female BALB/c mice were used. The first group was immunised with *L. mexicana* gp63 construct (VR1012) by injecting 100µg of the DNA intramuscularly. The second group was immunised with 1µg of the same construct using gene gun. The third group was immunised with empty plasmid vector (VR1012) by gene gun. The fourth group was immunised S.C. with 5×10^5 irradiated *L. mexicana* gp63 transfected CT26 tumour cells. The fifth group was injected S.C. with 5×10^5 parental CT26 tumour cells (irradiated-nontransfected). The sixth group was injected with PBS. The immunisation was carried out on day 0 and 14, and one week after the second

immunisation, the mice were bled once a week regularly. Serum was separated and stored at -20 for antibody typing by ELISA to determine the level of anti-leishmania IgG2a and IgG1 isotype antibodies.

The results clearly demonstrated a sharp increase of IgG2a in the mice immunised with *L. mexicana* gp63 construct by gene gun as early as 7 days after the immunisation, which slightly decreased afterward and remained at that level during the course of experiment. On the other hand, immunisation with the same construct by I.M. injection slightly increased the serum level of IgG2a on day 14 and peaked 6-7 weeks following immunisation. A very low level of IgG2a was observed throughout the experiment in mice immunised with CT26 cells transfected with gp63 construct or empty vector (Figure 4-13). This clearly indicates a strong Th1-type immune response to gene gun immunisation compare to other methods of immunisation. The results also showed that the gene gun immunisation induced an increase in the level of IgG1 after day 14 similar to that obtained by immunisation with the empty vector whereas intramuscular injection of the DNA and immunisation with *L. mexicana* gp63 transfected CT26 cells induced increases of IgG1 after four and five weeks of immunisation respectively (Figure 4-13). The IgG2a/IgG1 antibody responses demonstrates a complex Th1/Th2 immune response in all methods of immunisation including the gene gun immunisation, however, VR1012 vector may play a role in the increase of the IgG1.

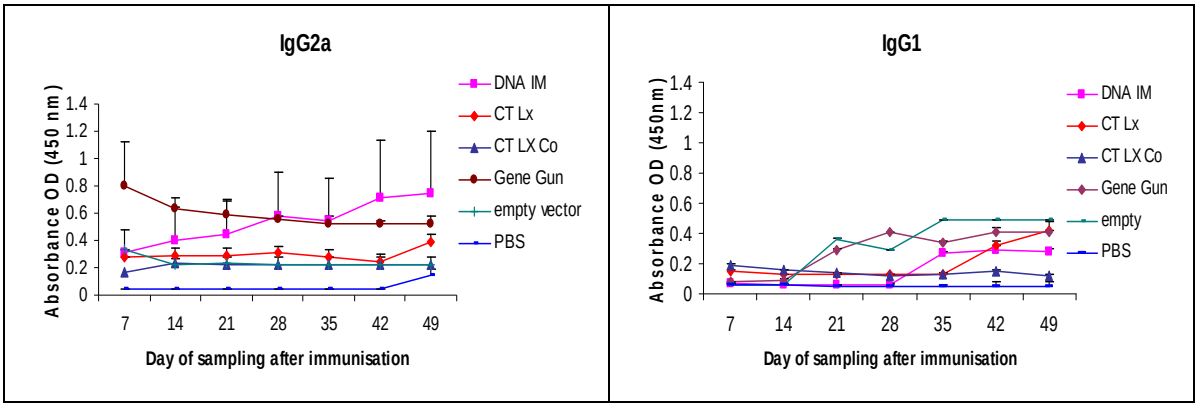


Figure 4-13: Th1/Th2 direction of immune response in mice immunised with DNA and transfected tumour cells.

Leishmania sensitive BALB/c mice were immunised with *L. mexicana* gp63 cDNA using the gene gun or intramuscularly injection of the DNA. A control group was also immunised with the empty vector. Mice were also immunised with 0.5×10^6 CT26 *L. mexicana* gp63 tumour cells (CTLX) alone or together with the same number of non-transfected CT26 (CTLX Co). Control mice were also injected with PBS. All groups of mice were immunised twice at two week interval. After one week of the second immunisation, mice were

bled regularly every 7 days and blood samples were collected. ELISA was implicated to determine IgG2a and IgG1 isotype antibodies against SLA.

4.2.4.2 Immunisation with SLA or autoclaved Leishmania parasites

In similar experiments to those described in 4.2.4.1, groups of BALB/c mice were immunised S.C. with 2×10^6/mouse autoclaved *L. mexicana* or 100µg/mouse SLA (see materials and methods) mixed with the same volume of IFA. The level of anti-leishmania IgG1 and IgG2a isotype antibodies in the serum of the immunised and control mice were determined by ELISA. Immunisation with *L. mexicana* soluble antigen (SLA) resulted in high levels of IgG1, in comparison to that induced by autoclaved parasites. The level of IgG2a induced by immunisation with SLA was lower than that of IgG1 during the course of study (Figure 4-14). Immunisation with autoclaved Leishmania parasites resulted in lower levels of IgG1 and IgG2a compared with that induced by SLA. The IgG1 was only detected 5 weeks after immunisation with autoclaved parasite (Figure 4-14).

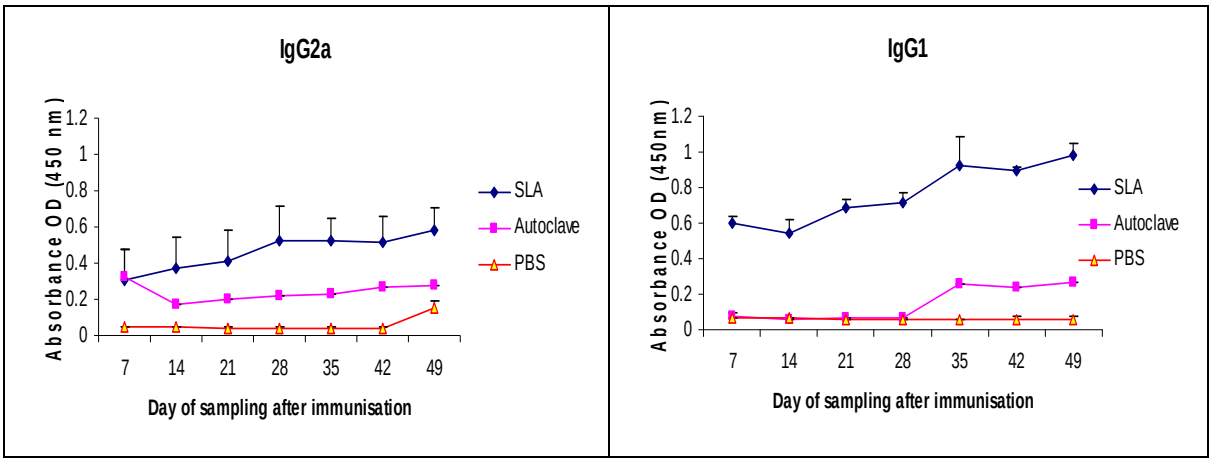


Figure 4-14: Th1/Th2 immune response in mice immunised with autoclaved Leishmania or SLA. BALB/c mice were immunised with either 2×10^6/mouse autoclaved *L. mexicana* or 100µg/mouse SLA mixed with 100µg/mouse IFA on day 0 and day 14. A group of control mice were injected with PBS. Serum samples were collected every 7 days after the second immunisation. The level of IgG1 and IgG2a was determined by ELISA and SLA as the antigen.

4.2.4.3 Antibody responses in mice immunised with DCs pulsed with SLA

To determine the type "direction" of the immune response following immunisation with of DCs pulsed with SLA, groups of 6 BALB/c mice were either immunised I.D. with 2×10^6 DCs loaded with SLA, or control DCs or PBS. DCs were prepared from bone marrow cells and loaded with10µg/ml SLA. 1µg/ml LPS was also added to induce DC maturation (see materials and methods). Two weeks after the second immunisation, all mice were bled to determine the level of total IgG, IgG1 and IgG2a isotypes. The results clearly showed significant increase in levels of total IgG, IgG1 and IgG2a in test groups compared with controls (Figure 4-15) indicating a rise of both Th1 and Th2-type antibody response in the immunised mice.

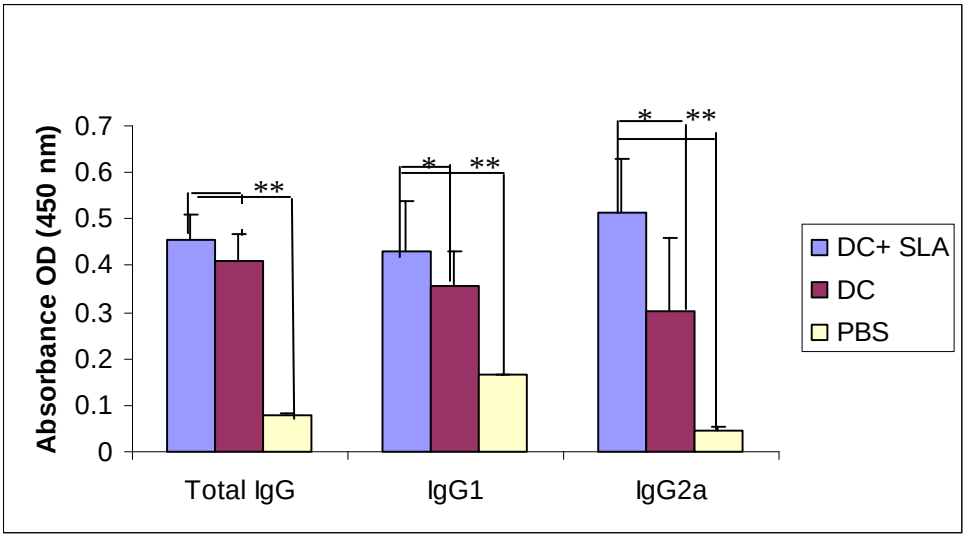


Figure 4-15: Ab responses in mice immunised with DCs loaded with SLA.
Groups of 6 BALB/c mice were immunised S.C. either with 2×10^6/mouse DCs alone or pulsed with SLA (see materials and meethods) on day 0 and day 14. On day 28 serum samples were collected and analysed by ELISA to determine the level of IgG, IgG1 and IgG2a. Data were analysed by student t-test. * p> 0.05, ** p>0.01, *** p>0.001.

4.3 Discussion

4.3.1 CTL activity induced by *L. mexicana* gp63 and SLA

The role of CD8+ T cells in immunity to Leishmania parasites is not yet fully established. There are studies demonstrating that Leishmania patients show high proportions of leishmania-reactive CD8+ T cells [Da-Cruz et al., 1994] and it is thought that that CD8+ T cells help raise immunity to Leishmania in two different ways. These cells release a large amount of IFN-γ that in turn promotes a Th1 immune response to activate macrophages against the parasite. Also, activated CD8+T cells can kill macrophages, which are invaded by the parasite (see chapter 1) and it has been shown that DNA and DC-based vaccines elicit CD8 immune response [Gruber et al., 2007; Kamath et al., 1999].

In the present study, the role of CTL T cells in the immunity induced by *L. mexicana* gp63 and SLA was investigated. The results revealed that immunisation of mice with *L. mexicana* gp63 elicited significant CTL activity in BALB/c mice as demonstrated against CT26 tumour cell, which were already transfected with the relevant gene and tested for the expression of *L. mexicana* gp63 protein. Similar results were also obtained when DCs were transfected with *L. mexicana* gp63 cDNA and used as target cells (data not shown). When CTLs were tested against DCs pulsed with SLA (containing gp63 protein), they exhibited strong activity against targets demonstrating that DCs successfully processed and expressed the gp63 protein. *In vitro* re-stimulation with SLA had a crucial role in inducing CTL activity as splenocytes from mice immunised with *L. mexicana* cDNA or DC's transfected with *L. mexicana* gp63 cDNA without *in vitro* re-stimulation did not generate CTL activity, indicating the importance of boosting vaccination in leishmania immunisation. In this animal model, T cells derived from non-immunised, but leishmania infected mice did not have significant CTL activity confirming that the CTL activity was induced by the DNA immunisation not the infection, contrasting with results obtained in human studies [Da-Cruz et al., 2002; Da-Cruz et al., 1994]. Lack of CTL activity in leishmania infected BALB/c mice might be a reason for the susceptibility of these mice to the parasites, which requires further investigation. The CTL activity induced by the DNA immunisation was detectable after four months of immunisation and was similar to what has been reported in human patients with mucosal and cutaneous leishmaniasis before and after cure [Da-Cruz et al., 2002].

CTL activity was also detected in mice immunised with DCs loaded with SLA. The mice showed a high level of CTL activity against DCs loaded with SLA. The CTL activity

induced by SLA was much higher than that of mice immunised with *L. mexicana* gp63 cDNA indicating the presence of other immunogenic proteins in the SLA.

4.3.2 Antibody responses to leishmania vaccines

It has been shown that the immunity to Leishmania is mainly based upon the induction of a Th1-type immune response. Therefore, the type of the immune response induced by a given vaccine has a direct impact on the resistance to the parasite [Awasthi et al., 2004; Sacks & Noben-Trauth, 2002]. It is thought that different parameters including the nature of antigen, type of the adjuvant and the method of immunisation influence the direction of immune response toward either Th1 or Th2 [Liu et al., 2005; Saldarriaga et al., 2006].

In the present study we sought to define the type of the immune response induced by a single antigen "*L. mexicana* gp63" or a cocktail of antigens "*L. mexicana* soluble antigens" using different methods of immunisation. The type of immune response was determined by establishing the level of IgG2a and IgG1 antibody subtypes in the blood serum that represent the Th1 or Th2-type immune response respectively.

L. mexicana gp63 cDNA was administered by three different methods; intramuscular injection, by gene gun using gold particles or by the administration of CT26 tumour cells transfected with *L. mexicana* gp63.

It has been reported that the mode of administration of the DNA vaccine can influence the type of immune response induced by the vaccine. Intramuscular injection of DNA was one of the first method described for gene immunisation [Wolff et al., 1990], which has been reported to lead the immune response toward Th1 type while application of gene gun using gold particles bombardment recruits inflammatory cells and leads to Th2 immune response [Feltquate et al., 1997; Liu et al., 2005]. Application of adjuvants such as IL-12 or CpG motif as Th1 immune response enhancers in DNA vaccination has also been reported [Liu et al., 2005; Schirmbeck & Reimann, 2001], wich may shift the direction of the immune response induced by gene gun immunisation from Th2 towards Th1 [Zhou et al., 2003]. Some studies indicated that the Th2 induction of gene gun is not due to the decreased amount of DNA used in gene gun immunisation [Weiss et al., 2002] but due to the nature of the antigen, which strongly influences whether a Th1 or Th2 immune response is induced [Aberle et al., 1999].

To the best of our knowledge, this study, for the first time, investigated the benefit of using gene gun DNA immunisation in leishmania mouse model. In addition, the potency of I.M. injection of DNA versus gene gun immunisation in generating immunity against

leishmania was compared. Intramuscular injection of *L. mexicana* gp63 cDNA resulted in a Th1-type immune response and that was compatible with other studies, however, the results obtained from the gene gun immunisation was in contrast with the previous studies [Liu et al., 2005], where gene gun immunisation had led to Th2 immune responses. Mice injected intramuscularly with 100µg DNA induced high levels of IgG2a isotype antibody, which gradually increased during the course of the experiment and at 7 weeks it reached the level comparable with gene gun immunisation, which was obtained at week 1. The levels of IgG1 for the intramuscular injection of the DNA remained low during the course of experiments. In contrast, administration of 1µg of the DNA by gene gun in BALB/c mice induced a sharp rise of IgG2a, which was detected one week after immunisation. The level of IgG1 was quite low for two weeks and slightly increased afterward.

Immunisation of mice with CT26 tumour cells transfected with *L. mexicana* gp63 failed to produce high levels of IgG2a at any time point during the course of the experiments. IgG1 levels were low for the first 5 weeks and gradually rose and levelled with that of the gene gun immunisation at week 7. The reason for using a transfected cell line to generate immunity to Leishmania was its similarity to the leishmania infection. In leishmania infection, the macrophage takes up the parasite cell expressing the gp63 protein. In this model, macrophages also phagocytose irradiated tumour cells, which express the leishmania protein. However, this model still requires further investigation.

Administration of SLA with IFA induced a high level of IgG1 and less IgG2a, however, both antibodies increased during the course of the experiments. The kinetic responses of the antibody isotypes in the serum revealed mixed Th1/Th2 immune responses, which might be due to the presence of several immunogenic antigens in the SLA. The effect of IFA in directing the immune response towards Th1 or Th2 was not determined in the study. DC-based vaccine potency in producing antibodies has already been shown in HIV vaccine studies [Gruber et al., 2007]. Application of DCs loaded with SLA resulted in similar profile of IgG2a and IgG1 isotype antibodies to that induced by SLA. Levels of antibodies in control groups injected with DCs alone or PBS could be due to the cross reactivity of natural antibodies, which detected by the secondary antibody in ELISA; the presence of natural antibodies cross reacting with leishmania parasites was already reported in pigs, rats, mice, hamsters, gerbils and humans [Nunes & Ramalho-Pinto, 1996; Schmunis & Herman, 1970].

Chapter 5 Identification of Immunogenic MHC class I epitopes of leishmania gp63

5.1 Introduction

5.1.1 Peptides as a new vaccine approach

The ultimate objective of developing vaccines against pathogens is inducing potent, specific and protective immunity in the host. In order to generate an effective immunity, different strategies have been developed and investigated where some approaches generate more potent immunity to a particular pathogen than others. The "conventional" vaccines were usually based on using whole pathogens either live or killed. Although these vaccines are effective against some pathogens, they are in general ineffective against a considerable number of other pathogens. Therefore, emphasise is now focused on using a single antigenic protein of pathogens in order to induce specific immunity, which in turn protective against the pathogen.

Immunogenic proteins of pathogens taken up by APCs are cleaved up into peptides, which are presented to either CD8+ or CD4+ T lymphocytes through MHC class I or II respectively [Chaplin, 2006]. Thus finding a strong immunogenic T cell epitope to use as a vaccine is a feasible strategy for developing vaccines effective against the pathogen. There are two immunological-based methods to identify the immunogenic epitopes. First, direct identification of the epitopes presented by APCs by eluting the epitopes from the surface of the MHC molecules and sequencing the peptides using mass spectrometry [Bonner et al., 2002; Lemmel et al., 2004] (direct immunology). Second, the affinity of the peptide motifs to MHC molecules is calculated by a computer algorithm and then their immunogenicity is confirmed by *in vitro* and *in vivo* immunological methods (indirect method or reverse immunology). Moreover, it should be more emphesised on natural processing of the immunogenic peptides by APCs and whether the immunogenic peptide has the potential to protect the host from infection. Based on information obtained from the processed peptides and using weight-matrix and algorithm methods, several data bases and software such as the novel Gibbs sampling approach [Nielsen et al., 2004], SVMHC [Donnes & Elofsson, 2002] and SYFPEITHI [Rammensee et al., 1999] have been developed to predict the peptides binding with high affinity to MHC class I or II from the protein sequences in human and other species of animals. SYFPEITHI is one of the most popular web-based

data bases, which are designed by The University of Tubingen for prediction algorithms of peptide/MHC interaction (www.syfpeithi.de) [Pelte et al., 2004]. Although several studies have been conducted based on SYFPEITHI and several epitopes of different antigens have been identified so far, it is believed that the immunogenicity of the predicted peptides still needs to be determined by immunological methods [Pelte et al., 2004].

5.1.2 Peptide immunisation

The potency of peptide subunit vaccines have been shown by generating immunity to cancers or pathogens. In melanoma, this approach has been successfully used and led to the testing of some of the peptide vaccines in clinical trials. In studies on patients with localized prostate cancer, it was shown that the peptide vaccination was safe and well tolerated with no major adverse effects. Increased CTL response and the anti-peptide IgG titre were also observed post-vaccination [Noguchi et al., 2007; van der Bruggen et al., 1994]. In contrast, in another trial study, melanoma patients immunised with 3 peptides: MART-1(26-35) (ELAGIGILTV), tyrosinase(368-376) (YMDGTMSQV), and gp100(209-217) (IMQVPFSV), admixed with tetanus toxoid and GM-CSF did not show significant immunity to the tumour nor raised IFN-γ [Bins et al., 2007]. The discrepancy might be due to the nature of the antigens used and the nature of the disease. Loading DCs with immunogenic peptides can also generate immunity by expanding Ag-specific CD8+ T cells even in advanced stage IV melanoma patients [Schuler-Thurner et al., 2000].

Peptide vaccines have also been used to generate immunity against pathogens. A recombinant subunit vaccine based on the insertion of a 27-amino acid sequence from Omp31 to the N-terminus of Brucella enzyme lumazine synthase (BLS) induced protection against *Brocella ovis* similar to that of the Rev.1 vaccine, inducing a strong peptide and BLS-specific humoral, Th1 and cytotoxic T-cell responses [Cassataro et al., 2007]. Also, inoculation of a synthetic peptide derived from *Eimeria acervulina* and *Eimeria tenella* antigens homogenized in IFA induced a high level of antibody and cellular responses associated with partial cross-species protection against challenge with sporulated oocysts of the parasites [Talebi & Mulcahy, 2005]. In a malaria vaccine study, the use of a 42 kDa fragment and a 19 kDa subfragment of C-terminal *Plasmodium falciparum* merozoite surface protein induced specific antibodies, although a better protection was achieved by administration of the 42 kDa fragment [Hui & Hashimoto, 2007]. Similar results were also obtained by application of Plasmodium *falciparum merozoite* surface protein 1(MSP1) at the site of MSP1-42 and MSP1-19 [Yuen et al., 2007]. It was also shown that an anti-HIV

lipopeptide vaccine injected to HIV-uninfected and HIV-1 chronically infected patient volunteers was well tolerated and able to induce a specific CD4+ and CD8+ T cell responses [Gahery et al., 2006]. The use of peptide is also a new approach that has been investigated in leishmania vaccination. Many studies have been carried out to identify immunogenic peptides, which can be used as a vaccine in leishmaniasis. Some of the leishmania identified immunogenic peptides are listed in Table 5-1.

The main shortcoming towards peptide vaccination is their limited immunogenicity. Therefore, different adjuvants, including IFA and alum, have been used to enhance the immunogenicity of the vaccine [McAnally et al., 2001; Valmori et al., 2003]. In one study, mice immunised with an ovalbumin peptide and polyinosinic-polycytidylic as an adjuvant combined with anti-CD137 rendered a massive functional and IFN-γ producing CD8+ T cell memory pool in lymphoid and non-lymphoid tissues for more than a year in which the adjuvant played an essential role [Myers et al., 2006]. Recently, a ceramic core based nanodecoy system was used as delivery vehicles, resulting in higher immunity compare to the conventional adjuvant alum [Goyal et al., 2006].

In present study, we sought to identify the MHC class I epitopes derived from *L. mexicana* gp63 to be used as vaccine to generate immunity to *L. mexicana.*

Protein	Model	Sequence	Tests	References
Gp63	BALB/c	A single synthetic T cell epitope (PT3) (16 mer)	long-lasting protection	[Spitzer et al., 1999]
Gp63	in BALB/c, C57BL/6, and CBA	15mer peptides	Th1 response	[Soares et al., 1994]
Gp63	CBA	467-482 (15 mer)	Significant protection	[Frankenburg et al., 1996]
recombinant KMP-11 plus six 20-mer	Human (HLA DRB1* 04 volunteers)	DEEFNKKNQEQ NAKFFADKP (20 mer) And FKHKFAELLEQQ KAAQYPSK (20 mer)	T-cell proliferation and cytokine production	[Delgado et al., 2003]
GP63	CBA	161-167 and 158-167 (7 & 10 mer)	T-cell proliferation	[Yang et al., 1993]
PSA-2	C3H/He	recombinant PSA-2 polypeptide	N/A	[Handman et al., 1995b]

Table 5-1: Immunogene peptide of Leishmania candidates for vaccine

5.2 Results

5.2.1 Identification of MHC class I immunogenic peptides derived from leishmania gp63 protein in HHD II transgenic mice

It has already been shown that leishmania gp63 is an immunogenic protein capable of inducing cytotoxic T-cell activity (see chapter 4). In present study, we attempted to identify immunogenic MHC class I peptides derived from leishmania gp63 protein (Table 5-2 & Table 5-3). The web-based software "SYFPEITHI" (Table 5-5) was used to predict the immunogenic peptides with high affinity to human HLA-A2.1 or mouse MHC H2-L^d or H2-K^d molecules.

MPVDSSSTHRHRCVAARLVRLAAAGAAVTVAVGTAAAWAHAGAPQHRCIHDAMQARVLQ
VAAQRMAPSAVSAVGLPYVSVVPVENASTLDYSLSDSTSPGVVRAANWGALRIAVSAEDLT
DPAYHCARVGQRVNNHAGDTVTCTAEDILTDEKRDTLVKHLVPQALQLHRERLKVRQVQG
KWKVTGMADVICGDFKVPPEHITEGVTNTDFVLYVASVPSEESVLAWATTCQVFPDGHPAV
GVINIPAANIASRYDQLVTRVVTHEMAHALGFSGTFFGAVGIVQEVPHVRGKDFNVSVITSST
VVAKAREQYGCNSLEYLEIEDQGGAGSAGSHIKMRNAKDELMAPAASAGYYTALTMAVFQ
DLGFYQADFSKAEEMPWGRNVGCAFLSEKCMAKNVTKWPAMFCNESAATIRCPTDRLRVG
TCGITAYNTSLATYWQYFTNASLGGYSPFLDYCPFVVGYRNGSCNQDASTTPDLLAAFNVFS
EAARCIDGAFTPKNRTAADGYYTALCANVKCDTATRTYSVQVRGSNGYANCTPGLRVKLSS
VSDAFEKGGYVTCPPYVEVCQGNVKAAKDFAGDTDSSSSADDAADKEAMQRWSDRMAAL
ATATTLLLGMVLSLMALLVVRLLLTSSPWCCCRLGGLPT*X

Table 5-2: Sequences of *L. mexicana* gp63 protein (gene bank ref X64394)

MSVDSSSTHRRRCVAARLVRLAAAGAAVTVAVGTAAAWAHAGALQHRCVHDAMQARVR
QSVADHHKAPGAVSAVGLPYVTLDAAHTAAAADPRPGSARSVVRDVNWGALRIAVSTEDL
TDPAYHCARVGQHVKDHAGAIVTCTAEDILTNEKRDILVKHLIPQAVQLHTERLKVQQVQG
KWKVTDMVGDICGDFKVPQAHITEGFSNTDFVMYVASVPSEEGVLAWATTCQTFSDGHPA
VGVINIPAANIASRYDQLVTRVVTHEMAHALGFSGPFFEDARIVANVPNVRGKNFDVPVINSS
TAVAKAREQYGCDTLEYLEVEDQGGAGSAGSHIKMRNAQDELMAPAAAAGYYTALTMAIF
QDLGFYQADFSKAEVMPWGQNAGCAFLTNKCMEQSVTQWPAMFCNESEDAIRCPTSRLSL
GACGVTRHPGLPPYWQYFTDPSLAGVSAFMDYCPVVVPYSDGSCTQRASEAHASLLPFNVF
SDAARCIDGAFRPKATDGIVKSYAGLCANVQCDTATRTYSVQVHGSNDYTNCTPGLRVELS
TVSNAFEGGGYITCPPYVEVCQGNVQAAKDGGNTAAGRRGPRAAATALLVAALLAVAL

Table 5-3: Sequences of *L. major* gp63 protein (gene bank re Y00647)

```
sequence1       MPVDSSSTHRHRCVAARLVRLAAAGAAVTVAVGTAAAWAHAGAPQHRCIHDAMQARVLQ-
sequence2       MSVDSSSTHRRRCVAARLVRLAAAGAAVTVAVGTAAAWAHAGALQHRCVHDAMQARVRQS
                *.********:********************************* ****:******** *

sequence1       VAAQRMAPSAVSAVGLPYVSVVPVENASTLDYSLSDSTSPGVVRAANWGALRIAVSAEDL
sequence2       VADHHKAPGAVSAVGLPYVTLDAAHTAAAADPRPGSARS--VVRDVNWGALRIAVSTEDL
                ** :: **.**********:: ....*:: *   ..: *  *** .**********:***

sequence1       TDPAYHCARVGQRVNNHAGDTVTCTAEDILTDEKRDTLVKHLVPQALQLHRERLKVRQVQ
sequence2       TDPAYHCARVGQHVKDHAGAIVTCTAEDILTNEKRDILVKHLIPQAVQLHTERLKVQQVQ
                ************:*::***  **********:**** *****:***:*** *****:***

sequence1       GKWKVTGMADVICGDFKVPPEHITEGVTNTDFVLYVASVPSEESVLAWATTCQVFPDGHP
sequence2       GKWKVTDMVGDICGDFKVPQAHITEGFSNTDFVMYVASVPSEEGVLAWATTCQTFSDGHP
                ******.*.. ********  *****.:*****:*********.*********.*.****

sequence1       AVGVINIPAANIASRYDQLVTRVVTHEMAHALGFSGTFFGAVGIVQEVPHVRGKDFNVSV
sequence2       AVGVINIPAANIASRYDQLVTRVVTHEMAHALGFSGPFFEDARIVANVPNVRGKNFDVPV
                ************************************.**  . ** :**:****:*:*.*

sequence1       ITSSTVVAKAREQYGCNSLEYLEIEDQGGAGSAGSHIKMRNAKDELMAPAASAGYYTALT
sequence2       INSSTAVAKAREQYGCDTLEYLEVEDQGGAGSAGSHIKMRNAQDELMAPAAAAGYYTALT
                *.***.**********::*****:******************:********:********

sequence1       MAVFQDLGFYQADFSKAEEMPWGRNVGCAFLSEKCMAKNVTKWPAMFCNESAATIRCPTD
sequence2       MAIFQDLGFYQADFSKAEVMPWGQNAGCAFLTNKCMEQSVTQWPAMFCNESEDAIRCPTS
                **:*************** ****:*.*****::*** :.**:*********  :*****.

sequence1       RLRVGTCGITAYNTSLATYWQYFTNASLGGYSPFLDYCPFVVGYRNGSCNQDASTTPDLL
sequence2       RLSLGACGVTRH-PGLPPYWQYFTDPSLAGVSAFMDYCPVVVPYSDGSCTQRASEAHASL
                ** :*:**:* : ..*..******:.**.* *.*:****.** * :***.* ** :   *

sequence1       AAFNVFSEAARCIDGAFTPKNRTAADGYYTALCANVKCDTATRTYSVQVRGSNGYANCTP
sequence2       LPFNVFSDAARCIDGAFRPKATDGIVKSYAGLCANVQCDTATRTYSVQVHGSNDYTNCTP
                 .*****:********* **   .    *:.*****:************:***.*:****

sequence1       GLRVKLSSVSDAFEKGGYVTCPPYVEVCQGNVKAAKDFAGDTDSSSSADDAADKEAMQRW
sequence2       GLRVELSTVSNAFEGGGYITCPPYVEVCQGNVQAAKD-GGNTAAG---------------
                ****:**:**:*** ***:*************:**** .*:* :.

sequence1       SDRMAALATATTLLLGMVLSLMALLVVRLLLTSSPWCCCRLGGLPTX
sequence2       --RRGPRAAATALLV------AALLAVAL-----------------
                  * .. *:**:**:       ***.* *
```

Table 5-4: Sequence alignment of *L. mexicana* and *L. major* gp63 proteins. Sequence1: *L. mexicana* gp63; Sequence2: *L. major* gp63

Peptide Position	Sequence	score
592	L **L** V A A L L A **V**	28
159	H **L** I P Q A V Q **L**	26
20	R **L** A A A G A A **V**	25
22	A **A** A G A A V T **V**	24
412	A **I** R C P T S R **L**	23
419	R **L** S L G A C G **V**	23
13	C **V** A A R L V R **L**	22
66	K **A** P G A V S A **V**	22
110	R **I** A V S T E D **L**	22
73	A **V** G L P Y V T **L**	21
71	V **S** A V G L P Y **V**	20
96	S **A** R S V V R D **V**	20
148	L **T** N E K R D I **L**	20
24	A **G** A A V T V A **V**	19
147	I **L** T N E K R D **I**	19
36	A **A** W A H A G A **L**	18

Table 5-5: Predicted peptides with high affinity to HLA-A2 molecules.
The protein sequences of *L. major* gp63 were pasted onto to SYFPEITHI software and the peptides with high affinity to HLA-A2 molecules were predicted. Peptides with a higher score have more affinity to HLA-A2 molecules. Peptides used in immunogenicity studies are underlined.

5.2.2 Peptide vaccination in HLA-A2.1 transgenic mice

Peptides from gp63 proteins of *L. major* and *L. mexicana* were selected for HLA-A2.1 class I molecules by using the prediction web-based software "SYFPEITHI" and their immunogenicity was determined in HLA-A2.1 transgenic (HHDII) mice.

Prior to the peptide immunisation, the efficacy of the CTL experimental protocol was confirmed by immunisation with the PAP135 peptide as the positive control. PAP135 (sequence: ILLWQPIPV) is an immunogenic peptide derived from prostatic acid phospatase, a protein associated with prostate cancer and the immunogenicity of which has previously been shown in HHDII mice [Machlenkin et al., 2005]. The results clearly showed that one immunisation with 100 μg PAP135 plus IFA adjuvant and helper peptide (see Materials and Methods) resulted in a high specific killing of target cells by effecter cells generated from cultured splenocytes in presence of APCs pulsed with PAP135 peptide (Figure 5-1).

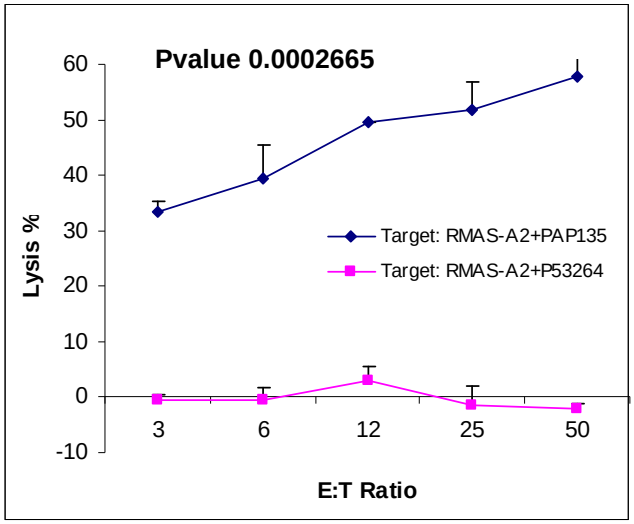


Figure 5-1: Immunogenicity of PAP135 tested in HHDII transgenic mice.
HHDII mice were immunized S.C. with 100µg of PAP135 peptide at the base of tail. One week later spleens were harvested, processed and splenocytes were cultured for 5 days with APCs pulsed with PAP135 peptide or non relevant P53 peptide separately. On day 5 the splenocytes were used as effector cells in standard 4-hour cytotoxicity assay against RMAS-A2 cell targets pulsed with relevant PAP135 and irrelevant P53 peptides.

The results were confirmed by assays to determine the levels of IFN-γ and IL-4. The amount of IFN-γ in samples collected from splenocytes of the immunised mice cultured with APC pulsed with relevant peptide (Test) was significantly higher than those pulsed with irrelevant peptide (control). No significant IL-4 (a key cytokine in activation of Th2 pathway) levels were detected (Figure 5-2).

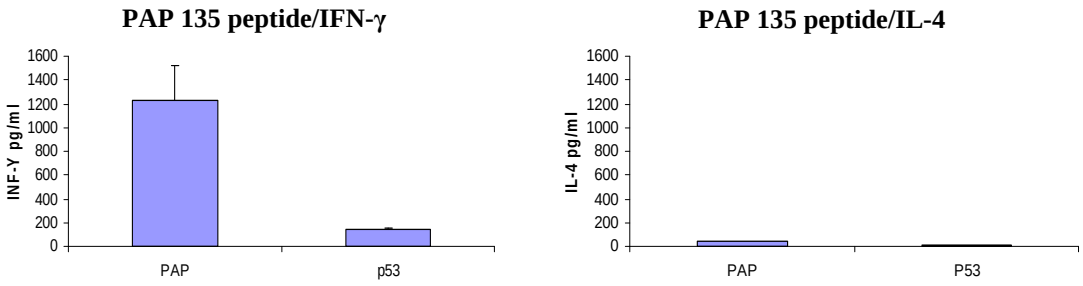


Figure 5-2: IFN-γ and IL-4 production by splenocytes from HHDII mice immunised with PAP135.
The supernatants from splenocytes cultured with APCs pulsed with PAP135 and irrelevant peptide P53 were collected on the day 2 and 5. The samples were stored at -20 until required. IFN-γ and IL-4 were measured using commercial kits according to manufacture's instruction (see materials and methods). The experiments repeated three times, obtaining similar results on each occasion. P-value for the level of IFN-γ between test and control was 0.012075

5.2.2.1 Immunogenicity of *L. major/L. mexicana* gp63 peptides predicted for HLA-A2.1 in HHDII mice

The immunogenicity of four peptides predicted for HLA-A2.1 selected from leishmania gp63 proteins of different species of leishmania parasites (Table 5-6), were tested for immunogenicity in HHDII transgenic mice; a summary of the results are shown in Table 5-7 and Figure 5-3. Mice were immunised once with 100µg of each peptide together with 100µg of IFA adjuvant and helper peptide, which were administered S.C. at the base of the tail (see materials and methods). Two peptides (C2 and B8) were highly immunogenic and the immunogenicity of the third one (CM4) was less but still significant (P=0.001) in comparison with an irrelevant peptide. The fourth peptide (C1) showed very weak immunogenicity. Boosting with a second immunization did not improve the immunogenicity of the C1 peptide. *In vitro* depletion of CD8+ T cells inhibited the cytotoxicity indicating a role of CD8+ T cells as mediators of cytotoxicity (data not shown).

	Leishmania species						
Peptide code	**major**	**mexicana**	**donovani**	**infantum**	**aethiopica**	**chagasi**	**tropica**
B8	+	-	+	+	-	+	-
C2	+	+	+	+	+	+	+
CM4	+	+	-	+	+	-	+

Table 5-6: Presence of immunogenic peptides in Leishmania species

NO	**Peptide**	**Sequence**	**Gene**	**Mouse**	**Score**	**Positive results**
1	C2	RLAAAGAAV	gp63	HHDII	25	4/5
2	CM4	AAAGAAVTV	gp63	HHDII	24	2/3
3	B8	LLVAALLAV	gp63	HHDII	28	5/5
4	C1	RLSLGACGV	gp63	HHDII	23	1/5

Table 5-7: Summary of the immunogenicity of gp63 HLA-A2 restricted peptides in HHDII mice

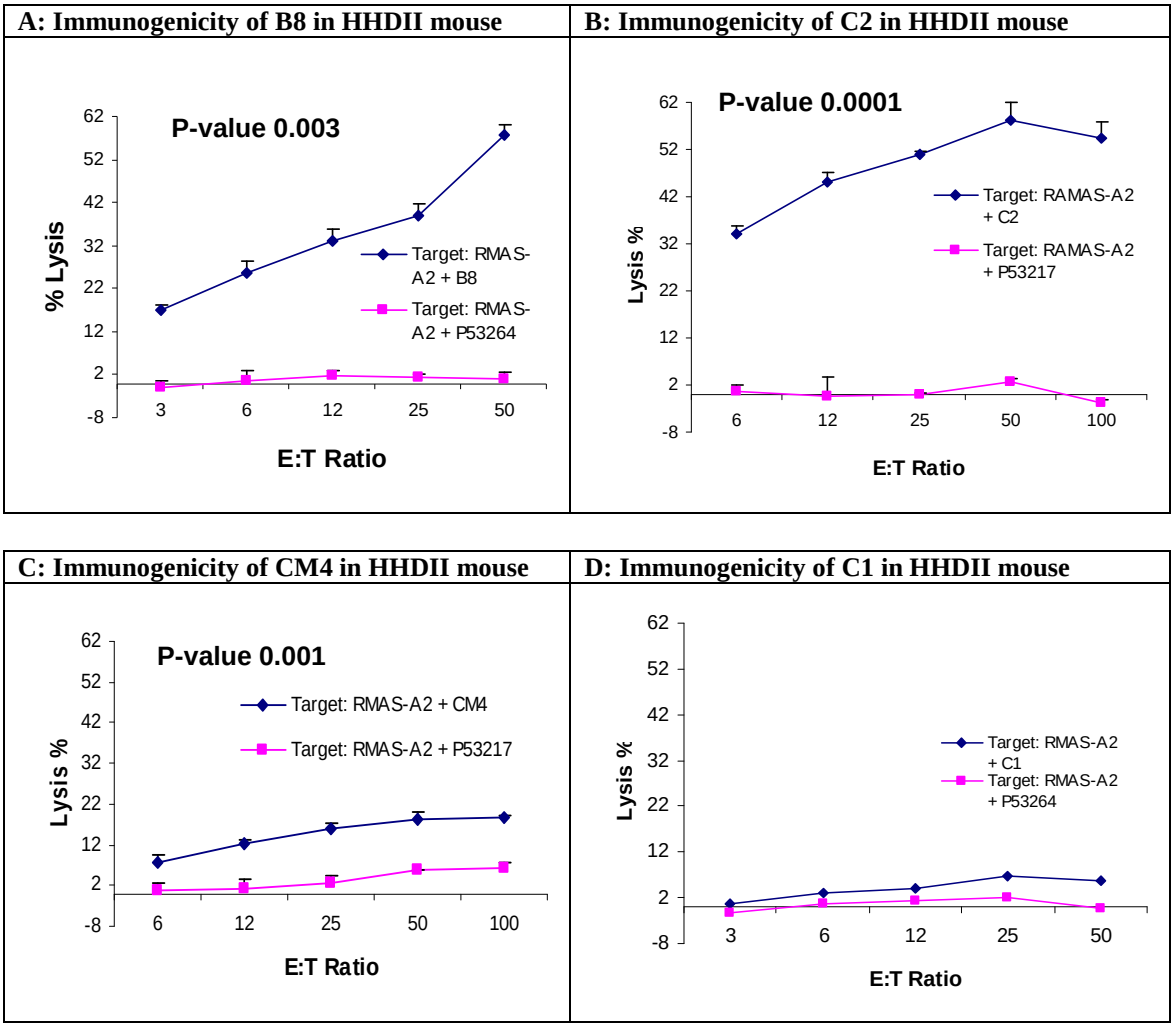


Figure 5-3: Immunogenicity of B8, C2 and C1 peptides in HHDII mice.
Four peptides of leishmania gp63 proteins were predicted for HLA-A2.1 using SYFPEITHI web-based software. 100µg of each peptide was injected S.C. at the base of tail of HHDII mice together with the helper peptide and IFA adjuvant. A week after the immunisation, spleens were harvested and splenocytes were cultured with spleen blast cells pulsed with relevant and irrelevant P53 peptides for 5 days. On day 5 the cells were used as effectors against target cells "RMAS-A2" pulsed with relevant and irrelevant P53 peptides using standard 4-hour cytotoxicity assay. Results of peptides B8, CM4 & C2 are representative of immunogenic peptides while peptide C1 represents a poor immunogenic peptide.

5.2.2.2 Cytokine Production of Splenocytes of HHDII mice Immunised with gp63 peptides

The cytokine assays to detect IFN-γ and IL-4, were conducted on supernatants collected from immunised mouse splenocytes cultured for 2 & 5 days, in order to confirm the immunogenicity of C2, CM4, B8 and C1 peptides. The results are shown in Figure 5-4.

For the highly immunogenic peptides (B8 & C2) the amount of IFN-γ detected in supernatants of splenocytes cultured with APCs pulsed with the relevant peptide was significantly higher than those cultured with blast cells (derived from mouse splenocytes) pulsed with the irrelevant peptide. For CM4 and C1, there was no significant difference in IFN-γ levels for splenocytes cultured with relevant compared to those cultured with irrelevant peptides. No significant IL-4 levels were detected for any of the peptides (Figure 5-4).

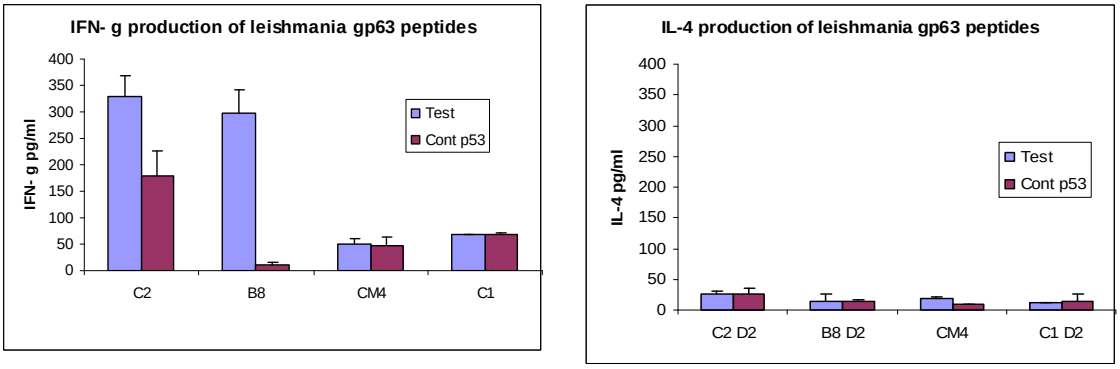


Figure 5-4: IFN-γ and IL-4 production by splenocytes cultured with relevant and irrelevant peptides. HHDII mice were immunized with the predicted peptides of gp63 and their splenocytes were cultured with splenocytes blast cells pulsed with the relevant peptides and an irrelevant peptide, P53"217" or PAP135, for 5 days. The supernatants were collected on day 2 and 5 and tested for IFN-γ and IL-4 using a commercial kit according to manufacture's instruction. Student t-test was used to statistically analyse the results and P-value for the level of IFN-γ between test and control for peptides C2 and B8 was 0.015 and 0.009 respectively.

5.2.3 Peptide vaccination in the BALB /c mouse model

To determine the efficacy of peptide vaccination in BALB/C mice, a 9 mere H2-L^d restricted peptide named TPH with the sequence of "TPHPARIGL" derived from β-galactosidase [Ali et al., 2004a; Saren et al., 2002], was used for immunisation (see Materials and Methods). Also, four peptides derived from *L. major* gp63 protein, "A3, A4, A5 and A6", predicted for MHC-class I H2-L^d and H2-K^d (Table 5-8) were assessed in Balb/c mice. Each mouse received two immunisations on days 0 and 7, and then the mice were sacrificed for standard 4-hour cytotoxicity assay on day 14. The results clearly show that immunisation with some peptides induced low but significant levels of cytotoxicity against targets pulsed with the corresponding peptide (Figure 5-5). The frequency of positive results for TPH was quite low (Table 5-8).

Administration of mouse CpG or altering the time intervals of immunisation failed to increase the immunogenicity of the predicted peptides and no significant increase of IFN-γ or IL-4 cytokines was observed when the immunised mice splenocytes were cultured with blast cells pulsed with the relevant peptides (data not shown).

NO	Peptide	Sequence	Gene	Mouse	Score	Results
1	TPH	TPHPARIGL	β-galactosidase	BALB/c	25	12/29
2	A3	YYTALTMAI	gp63	BALB/c	21	0/3
3	A4	DYTNCTPGL	gp63	BALB/c	20	0/4
4	A5	VPNVRGKNF	gp63	BALB/c	22	0/2
5	A6	ASLLPFNVF	gp63	BALB/c	21	0/4

Table 5-8: Immunogenicity of *L. major* gp63 peptides predicted for mouse MHC class I

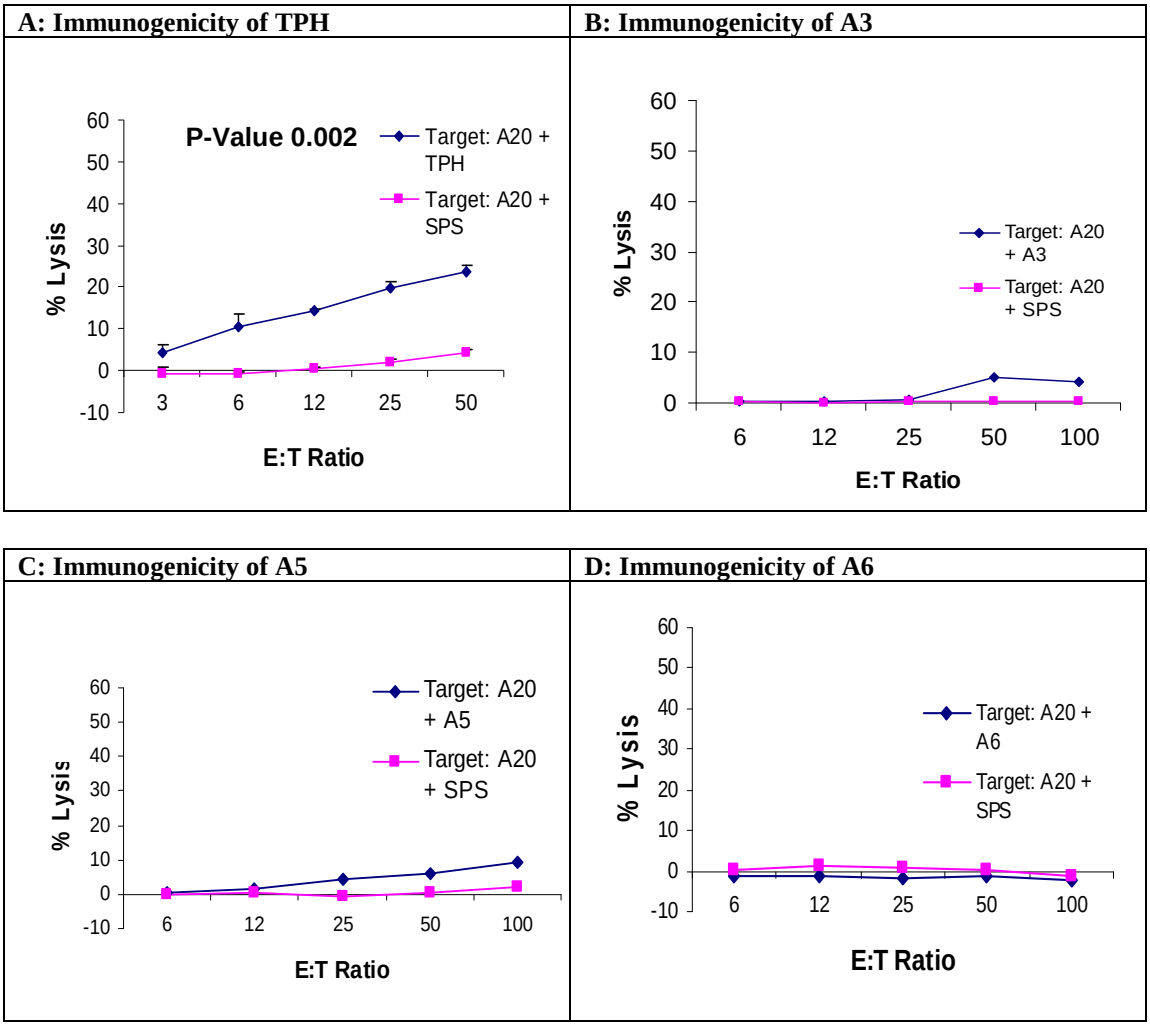


Figure 5-5: The immunogenicity of TPH and *L. major* gp63 peptides in BALB/c mice.
BALB/c mice were immunised twice at a week interval with 100µg of appropriate peptide together with the helper peptide and adjuvant (see materials and methods) S.C. at the base of tail. A week after the last immunization, spleens were harvested and splenocytes were cultured with APCs pulsed with relevant and irrelevant "SPSYVYHQF" peptides for 5 days. On day 5 splenocytes were used as effectors in standard 4-hour cytotoxicity assay against targets pulsed with relevant and irrelevant peptides.

5.2.4 Natural processing of the immunogenic class I peptides derived from leishmania gp63

An immunogenic peptide, to be used as a vaccine, needs to be naturally processed via the MHC class I in which the protein is first cleaved into peptides by the proteosome and then the peptide is expressed through MHC class I molecules.

DNA immunisation by I.M. injection and gene gun were performed to test the natural processing of the gp63 derived immunogenic peptides. Two plasmid constructs *L. mexicana* gp63 cDNA & *L. major* gp63 cDNA were used for immunisation by the gene gun (HHD II & BALB/c). After 3 immunisations at 1 week intervals, mice were killed and the splenocytes were flushed out and cultured with APCs pulsed with the relevant peptide for 5 days. On day 5 the splenocytes were used as effectors in cytotoxic assays using tumour target cells pulsed with the relevant peptides as targets (RMAS for C2, CM4, B8 & C1 and A20 for A3, A4, A5 & A6) (see materials and methods). The results indicated that none of the peptides showed immunogenicity when mice immunised by gene gun using chromium release assay (figure 5-6).

The supernatant collected from the splenocyte cell culture were analysed for IFN-γ and IL-4. No significant difference was observed between the level of IFN-γ or IL-4 in splenocyte cell culture supernatants cultured with APCs pulsed with relevant peptides (A3, A4, A5 & A6 for BALB/c and C2, CM4 & B8 for HHD II mice) and the irrelevant peptide (TPH for BALB/c & P53,264 for HHD II)(data not shown).

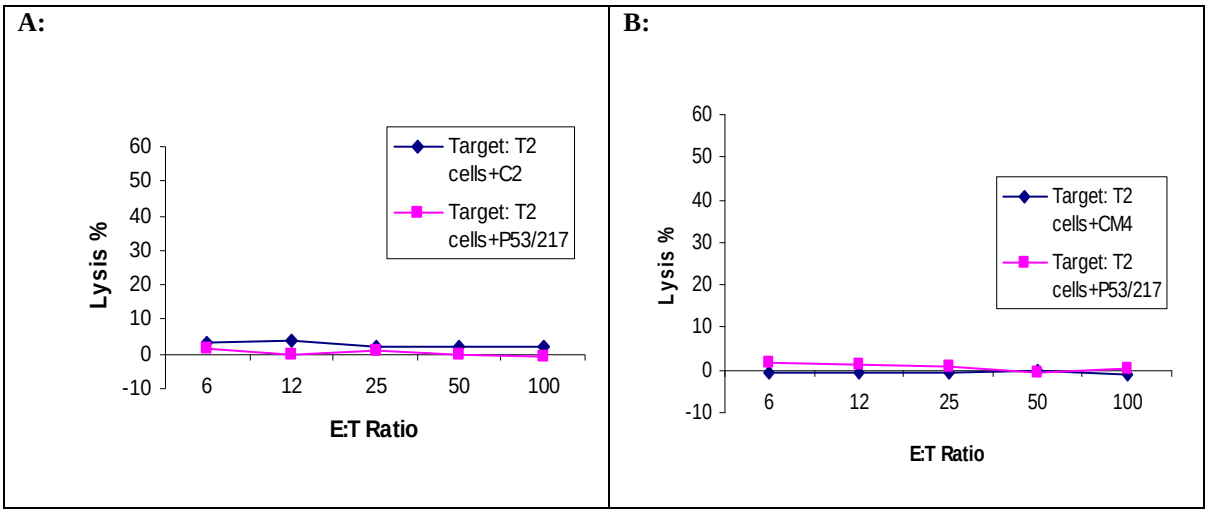


Figure 5-6: Assessment of natural processing of immunogenic peptides tested in HHD II mice.
HHD II mice were immunised by the gene gun with *L. major/L. mexicana* gp63 three times. After a week of the last immunisation mice were killed and the CTL activity was determined by standard 4-hour cytotoxicity

assay. Graphs represent 6 immunised mice for each peptide tested in three independent experiments. **A:** mouse immunised with C2 peptide **B:** mouse immunised with CM4 peptide

The natural processing of C2 and CM4 (HLA-A2 peptides/HHD II) was also assessed by intramuscular injection of DNA. Mice were injected I.M. with 100µg *L. mexicana* gp63 cDNA twice at two weeks interval. After two weeks of the last immunisation, mice were sacrificed and tested for the CTL activity as with that of gene gun immunisation. The results showed CTL activity in 1/6 immunised mice detected by standard 4-hour cytotoxicity assay for C2 (Figure 5-7).

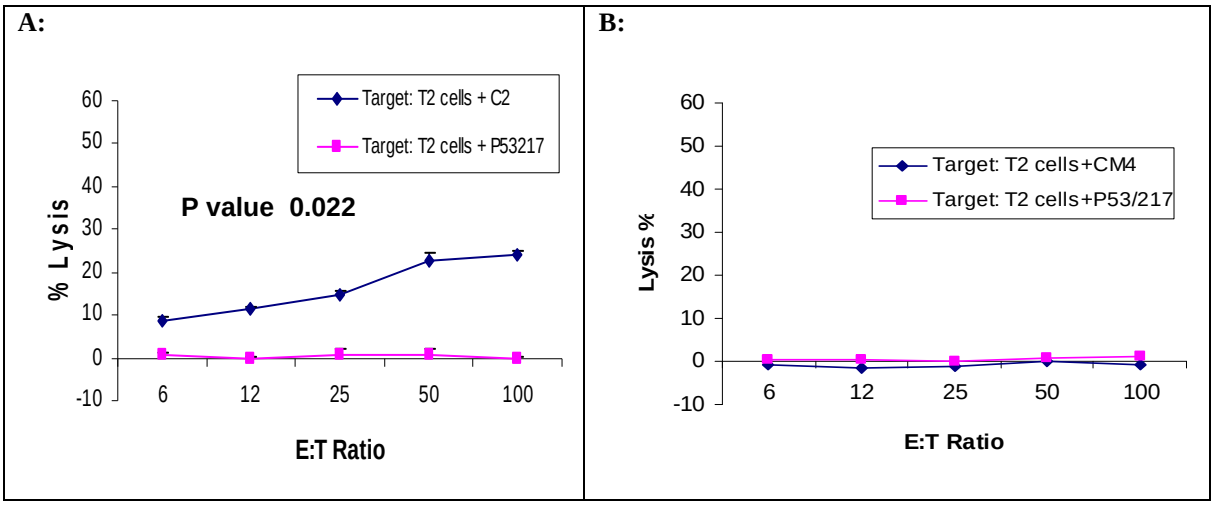


Figure 5-7: Immunogenicity of C2 and CM4 peptides by intramuscularly DNA immunisation.
BALB/c mice were intramuscularly injected with 100µg *L. mexicana* gp63 cDNA twice. Two weeks after the last immunisation they were killed and the splenocytes were cultured with APCs pulsed with C2 and CM4 peptides for 5 days. On day 5, the splenocytes were used as effectors in standard 4-hour cytotoxicity assay against tumour cells pulsed with the relevant peptides. Only 1 out of 6 mice showed immunogenicity against targets pulsed with C2 peptide. **A:** mouse immunised with C2 peptide **B:** mouse immunised with CM4 peptide

The supernatants collected from immunised mice cultured splenocytes were tested for the presence of IFN-γ and IL-4. In contrast to the results of the cytotoxicity assay, there was a significant increase in the level of IFN-γ but not IL-4 in the supernatants of splenocytes obtained from the immunised mice when they were cultured with LPS blast cells pulsed with both C2 and CM4 peptides (Figure 5-8).

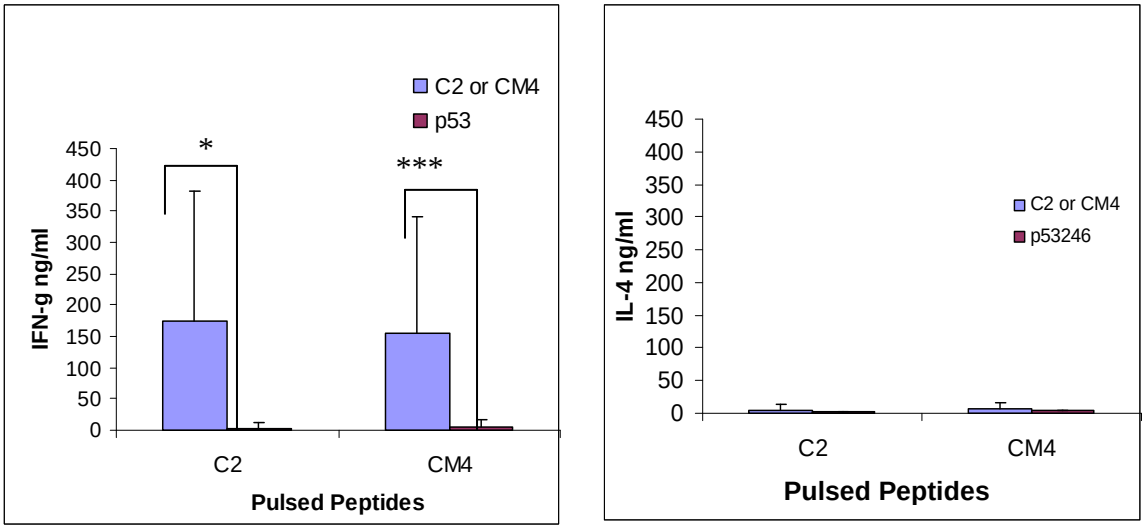


Figure 5-8: IFN-γ production of splenocytes from HHD II mice immunised with gp63 cDNA and stimulated with relevant and irrelevant peptides.
Supernatants collected from the culture of the splenocytes were tested for IFN-γ by ELISA using the commercial kit according to the manufacture's instructions. The graph represents three independent experiments and p value < 0.05, 0.01, 0.001 accounts for *, **, and *** respectively.

5.2.5 Protection induced by immunisation with C2 peptide in HHDII mice

HHDII mice were immunised twice at two weeks intervals with 100 µg of the C2 peptide (seq RLAAAGAAV). After two weeks of the last immunisation, mice were challenged with 1×10^7 log phase of *L. mexicana*. Mice were monitored for lesion development for at least 2 months. Two control groups of mice were used one injected with PBS and the other with an irrelevant peptide p53/246. The results showed no significant protection induced by immunisation with C2 peptide compared with controls (data not shown).

5.3 Discussion

5.3.1 Peptide immunization in HHDII mice

Peptide immunisation is a new vaccination approach that has not yet fully investigated in leishmania vaccination. Gp63, a leishmania antigen, has been postulated as a promising candidates for Leishmania peptide-subunit vaccine. In a study by Spitzer [Spitzer et al., 1999] a 16-mer synthetic peptide with the sequence of YDQLVTRVVTHEMAHA derived from *L. major* gp63, induced a detectable immunity in BALB/c mice. On the other hand, there are many studies, including our own, which have demonstrated immunogenicity and CTL stimulation of gp63 proteins in leishmania (see chapter 1) and that CD8+ T cells appear to have an important role in immunity to leishmania (see chapter 4). Therefore, it is appropriate to identify the MHC calss I restricted CTL epitopes that can be used as vaccine to leishmania either alone or in combination with other immunogenic or therapeutic agents. This study for the first time reports the identification of immunogenic MHC class I restricted epitopes from leishmania gp63 protein in both HLA-A2 transgenic (HHD II) and conventional BALB/c mouse models using reverse immunology. In order to identify immunogenic epitopes, which are presented through MHC class I molecules, the web-based software "SYFPEITHI" [Hundemer et al., 2006; Mishra & Sinha, 2006; Rammensee et al., 1999] was used to predict the immunogenic peptides for both models. The immunogenicity of the predicted peptides was determined by using a number of *in vivo* and *in vitro* immunological tests [Pelte et al., 2004]. Due to the ethical difficulties associated with studies on human subjects, HHD II mice were used to determine the immunogenicity of the peptides predicted for human HLA-A2.1 molecules. HLA-A2.1 transgenic (HHDII) mice have been described as a powerful model to study human immune responses *in vivo* [Firat et al., 1999; Hundemer et al., 2006; Ramage et al., 2004; van der Bruggen et al., 1994]. These mice model have already been used to study *Tripanozoma cruzi* in humans [Garcia et al., 2003] and the results obtained are inline with those of the present study. Using *L. major* gp63 sequences, four of nine mer peptides named C2, CM4, B8 and C1 (RLAAAGAAV, AAAGAAVTV, LLVAALLAV and RLSLGACGV) were predicted to have affinity to HLA-A2.1 molecules and were tested for immunogenicity in HHD II transgenic mice. Three peptides (C2, B8 and CM4) induced CTL activity in the immunised mice, however, the CTL activity induced by CM4 was weaker. The fourth peptide (C1) was non-immunogenic and produced weak CTL activity. Injection of C2 and B8 together (the two high immunogenic peptides) failed to induce strong CTL activity against targets

pulsed with either of the peptides in standard 4-hour cytotoxicity assay (data not shown) indicating the diversity of the immune response against two different immunogenic peptides. In order to obtain potent CTL activity during *in vitro* culture, the splenocytes were restimulated with APCs pulsed with the relevant peptide (see materials and methods). Either re-stimulation with no peptide or restimulation (with relevant peptides) without using APCs, failed to stimulate CTL activity indicating the importance of APCs in enhancing the CTL activity.

IFN-γ secreted by T cells has been shown to be essential for the development of Th1 responses and it has been used as a marker for the existence of the CTL activity, while IL-4 on the other hand indicates the bias immunity towards the Th2 pathway [Delespesse et al., 1998]. The level of IFN-γ produced by splenocytes from mice immunized with immunogenic leishmania gp63 peptides (B8, CM4 & C2) cultured with APCs pulsed with relevant peptides confirmed its role in the activation of Th1 pathway and/or CTL responses. Immunization with the C1 peptide failed to produce a significant level of IFN-γ. The lack of IL-4 secretion may indicate down regulation or the absence of Th2 responses in this model.

To determine the natural processing of the immunogenic peptides, DNA immunisation was performed using two methods; gene gun and intramuscular injection of the DNA, both were shown to induce protection against challenge with live parasites (see chapter 3). *L. mexicana* gp63 cDNA construct was used to immunise for peptides C2 & CM4 and *L. major* gp63 cDNA for B8 and C1. Immunisation of HHD II mice by the gene gun and restimulation the splenocytes with APCs pulsed with the immunogenic peptides failed to generate CTL activity, as measured by either standard 4-hour cytotoxicity assay or the production of IFN-γ in the splenocytes culture supernatants. In contrast, the splenocytes of mice immunised by I.M. injection of cDNA restimulated with splenocyte LPS blasts pulsed with C2 or CM4 produced high levels of IFN-γ compare to those restimulated with APCs pulsed with an irrelevant peptide. In addition, a low frequency of CTL activity was detected by standard 4-hour cytotoxicity assay only for C2 peptide (1 out of 6 mice). The results indicate that these peptides may be naturally processed but the cytotoxicity assay is not sensitive enough to detect the immune responses, which are detectable by IFN-γ ELISA.

The potency of I.M. injection of DNA in inducing CTL activity was inline with other studies, which reported CTL activity induced by S.C. injection of ß-gal plasmid DNA in BALB/c mouse model [Gurunathan et al., 1998].

5.3.2 Peptide immunization in BALB/c mice

To develop a peptide-based vaccination model in BALB/c mice, which are sensitive to Leishmania parasites [Soares et al., 1994], an immunogenic 9-mer peptide "TPHPARIGL" derived from β-galactosidase protein and four peptides derived from gp63 with the high affinity to H2-Ld and H2-Kd (identified by SYFPEITHI software) were tested in BALB/c mice. Although TPH has shown strong immunogenicity when BALB/c mice were immunized with a Disabled Infectious Single Cycle Herpes Simplex Virus (DISC-HSV) virus encoding β-galactosidase protein followed by in vitro restimulation with TPH [Ali et al., 2004a], immunisation with this peptide and adjuvant induced a low, but detectable, immune response (CTL activity) in 41% of immunised mice. Co-injection of DISC virus, CpG or Titer Max as adjuvants did not enhance the immunogenicity of the TPH peptide (data not shown). In addition, increasing the frequency of immunisation up to 3 times did not alter the immunogenicity of this peptide (data not shown). Increasing the frequency of in vitro restimulation (see Materials and Methods) did not positively alter the immunogenicity of peptides. When instead of the adjuvant IFA, Titer Max was used as an adjuvant and the splenocytes were pulsed by the relevant peptide without APCs according to the protocol used by Anne Saren [Saren et al., 2002], no killing of target cells pulsed with relevant peptide was observed compared to those pulsed with an irrelevant peptide (data not shown). Immunisation with peptides predicted for mouse MHC class I (A3, A4, A5 & A6 with the sequences of YYTALTMAI, DYTNCTPGL, VPNVRGKNF & ASLLPFNVF respectively) showed only low levels of immunogenicity. This indicates that the BALB/c system is a poor model for peptide vaccination possibly due to the bias of the immune system to a Th2 response rather than Th1 [Sacks & Noben-Trauth, 2002].

Perhaps, the results also suggest that the computer-based prediction is more accurate for human than mouse MHC class I epitopes, since none of the mouse MHC class I predicted peptides were immunogenic whereas the protein itself could induce CTL activity in BALB/c mouse model (see chapter 4).

Immunisation of HHDII mice with either C2 or CM4 peptides did not protect the mice from *L. mexicana* infection and the course of the disease in immunised mice was similar to that of the controls immunised with non-immunogenic peptide or PBS. This was in contrast with the results obtained by Spitzer in BALB/c mice using the 16-mer synthetic peptide with the sequence of YDQLVTRVVTHEMAHA derived from *L. major* gp63 [Spitzer et al., 1999] where the synthetic peptide protected the infected mice against the disease for 10 months. Our results may indicate that the peptide as administered is

insufficient to protect mice from the infection and/or induce CTL activity. The generation of a Th1 response may be a requirement.

Chapter 6 OX40L: Purification and Application in leishmania

6.1 Introduction

6.1.1 OX40 and OX40L

OX40 (CD134) is a membrane- associated glycoprotein, which is a member of the Tumour Necrosis Factor Receptor (TNFR) superfamily with molecular weight of 47000 – 51000. The OX40 ligand (OX40L), is a type Ⅱ transmembrane glycoprotein, which also belongs to the Tumour Necrosis Factor (TNF) super family [al-Shamkhani et al., 1996].

There are evidences to suggest different expression patterns for OX40 in different species. In rats, OX40 was only expressed on activated CD4+ T lymphocytes but in mouse, OX40 is expressed on both activated CD4+ and CD8+ T cells [al-Shamkhani et al., 1996]. In human, OX40 expression is mainly restricted to CD4+ T cells [Durkop et al., 1995], however, a naturally soluble OX40 has also been identified in human serum [Taylor & Schwarz, 2001]. OX40L is preferentially expressed on activated B cells [Satake et al., 2000], macrophages, DCs and endothelial cells at the site of inflammation. DCs express the OX40L constitutively (Figure 6-1) and the expression of this protein becomes further up regulated by LPS stimulation [Barrios et al., 2005; Brocker et al., 1999; Ohshima et al., 1997; Satake et al., 2000; Souza et al., 1999].

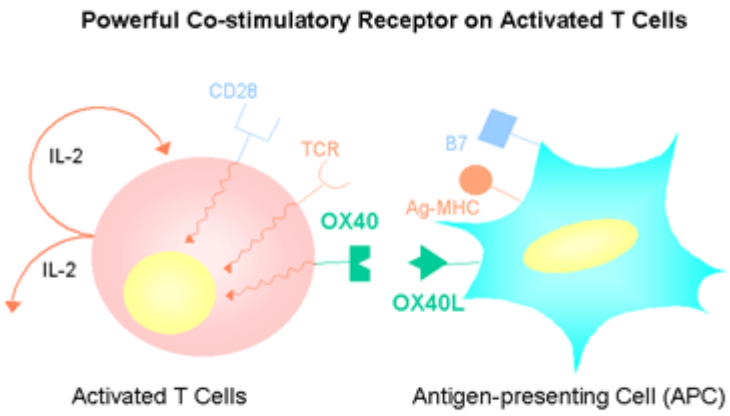


Figure 6-1: Interaction of OX40-OX40L on APC and activated T cells

6.1.2 Role of OX40 Ligand in immunity

It has been shown that OX40 - OX40L engagement is capable of signalling both the cells on which they are expressed and has a positive regulatory effect on division, survival, effecter function and the number of T cells at the peak of immune responses. This interaction induces a strong co-stimulatory signal, which promotes the activation and memory development of T cells [Gramaglia et al., 2000; Ohshima et al., 1997]. The interaction of OX40 - OX40L has a direct role in adhesion of vascular endothelial cells and T cells [Imura et al., 1996]. In early cognate interaction between B and T cells, OX40 - OX40L engagement also triggers an OX40L reverse signal that enhances IgG production of B cells and promotes the maturation of DCs as evidenced by increase expression of CD80, CD86, CD83 and CD115 [Ohshima et al., 1997; Wang et al., 2004]. It has also been shown that the engagement of OX40L on antigen presenting cells stimulate naïve T cells to differentiate into Th2 [Tanaka et al., 2000]. Furthermore, OX40 on activated naive human T cells increases their expression of IL-4, IL-5 and IL-13 [Delespesse et al., 1999; Ohshima et al., 1998]. On the other hand recent studies have demonstrated that OX40L promotes Th1 immune responses and down regulates the activity of CD4+ CD25+ T reg cells [Vu et al., 2007]. In studies carried out by Ito et al [Ito et al., 2006], it was reported that OX40L completely inhibited the generation of IL-10-producing Th1 cells from naive and memory CD4+ T cells. In other studies, ligation of OX40L on activated DCs enhanced their cytokine production (TNF-alpha, IL-12 p40, IL-1 beta, and IL-6) and increased CD80, CD86, CD54, and CD40 expression [Ohshima et al., 1997]. The role of OX40L in enhancement of CTL activity in a mouse tumour model has been shown by Ali et al [Ali et al., 2004b]. Dannull has also shown that the transfection of human DCs with OX40L mRNA effectively enhances the CTL activity and Th1 polarization of naive CD4+ T cells [Dannull et al., 2005]. Other studies suggest a possible function of OX40L / OX40, through T cell-T cell interaction inducing CTL activity and/or a reactivation of memory T cells in viral infections and cancers [Takasawa et al., 2001].

The potency of OX40L to enhance the immunogenicity of potential vaccines against leishmania is not yet fully investigated. Few studies have been carried out to determine the role of OX40-OX40L interaction in the immune response raised against parasitic infections. Some studies have stated that a down regulation of Th2-type immune response by blocking OX40-OX40L interaction using anti-OX40L mAb, which renders a therapeutic effect on the disease [Akiba et al., 2000], however, other studies are to show that administration of OX40L enhances the immunity against the parasite [Zubairi et al.,

2004]. Nevertheless, determination of the OX40L potential to be used in combination with vaccines or anti-leishmanial drugs is a new objective in leishmania research and needs more investigation.

The main objective of this study was to determine the effect of administration of OX40L in *L. mexicana* cutaneous infection and the potency of this protein in enhancing the immunogenicity of leishmania potential vaccines. OX40L-IgG fusion protein was produced and purified from transfected B9B8E2 cells. B9B8E2 cells are hamster kidney cells transfected with both mouse OX40L and mouse IgG1 capable to produce and release mouse OX40L-IgG fusion protein in the cell culture supernatant. For purification of OX40L-IgG from B9B8E2 culture supernatants, two novel resins named MBI and MEP were used. It is now clear that the main purpose of protein purification is the isolation of the given protein with maximum yield and highest purity while the protein holds its chemical and biological integrity. As proteins have different structures and different characteristics, the method of purification varies. Many studies have been carried out to develop methods by which proteins can be purified with a high yield and several methods have yet been developed. Most of protein purification methods are based on differences in the biochemical properties such as overall charge, size, and hydrophobicity between the protein of interest and the contaminants. Some of the common methods used for purification of proteins are, precipitation with ammonium sulfate $(NH_4)2SO_4$, ultracentrifugation and chromatographic methods. The basic procedure in chromatography relies on separating the protein passage through a column packed with different resins, which can interact with the protein of interest. These resins normally consist of a ligand, which binds to the protein of interest, the matrix, which is a solid phase to immobilize the ligand by covalent bonds and a spacer arm, which is normally included in-between the matrix and the ligand to encourage binding where the small size of the ligand prevents free access to proteins in the solvent [Weimer et al., 2000]. Based upon the interaction of the protein with the resins of the column, the chromatographic methods are divided into size exclusion chromatography (Gel filtration), affinity chromatography, ion exchange chromatography and Hydrophobic Charge Induction Chromatography (HCIC)**.** MBI and MEP resins are among resins that work with ion exchange while Protein A sepharose works based on affinity chromatography.

In this study, the purification conditions of B9B8E2 cell produced OX40L-IgG for both MBI and MEP resins were first optimised and then the biological activity of the OX40L purified by these resins was compared with that of protein A sepharose resin. Finally, the

therapeutic effects of MM1 (the OX40L produced by Xenova) and the MBI purified OX40L on *L. mexicana* cucaneous infection were investigated.

6.2 Results

6.2.1 Optimisation and purification of mouse OX40L-IgG by MBI resin

The structure of 2-mercapto-5-benzimidazole sulfonic acid is based upon the presence of a heterocyclic, a sulphur atom and an aromatic ring supporting a strong acidic group, which is negatively charged over the whole range of working pH (Figure 6-2). According to the information presented by the manufacturer, antibodies are adsorbed to the resin in physiological ionic strength whereas the elution occurs at bufferic pHs.

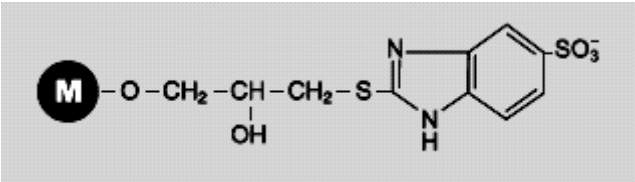


Figure 6-2: Structure of the MBI Ligand (Adapted from MBI Hypercel product note).
MBI ligand has a sulfonate group present on the aromatic ring.

Particle size	80-100µm
Dynamic binding capacity for hu IgG	20-40 mg/ml
Ligand	2-mercapto-5-benzimidazole sulfonic acid
Working pH	Adsorption: pH 5.0-5.5 Elution: pH 8.0-9.5
Cleaning pH	3-14
Pressure resistance	< 44 psi
Typical working pressure	< 14psi

Table 6-1: Key features of MBI Hypercel (Adapted from MBI Hypercel product note).

The OX40L-IgG producing cell line (B9B8E2) was cultured according to the protocol supplied by Xenova plc (see materials and methods). Briefly, the cell culture media was renewed every 5 to 7 days and the supernatants were collected and kept in –80 until required. MBI, a novel resin designed for purification of antibody, was used to purify OX40L-IgG from the B9B8E2 cell supernatant. To optimise the purification conditions, sodium acetate buffer and sodium acetate buffer plus NaCl at pH 4, 4.5, 5, 5.2, 5.5, 6 or 6.5 were used as loading buffers and tris buffer at pH 9 was used for elution. Fractions were collected and total protein was determined by measuring absorbance at 280nm using spectrophotometer (Figure 6-3) and also for selected fractions using BCA kit (see materials

and methods). The presence of OX40L was determined in all fractions by dot blotting and the concentration of OX40L was marked as + to +++ according to the size of spots (Table 6-2). The highest level of total protein was obtained in elution fractions when the samples were loaded at pH 4 (Figure 6-3). There was no significant difference between sodium acetate and sodium acetate plus NaCl at 280nm absorbance; however, the amount of protein in the eluting fractions was slightly higher in the presence of NaCl (Table 6-2).

Buffer: Sodium Acetate				**Buffer: Sodium Acetate+NaCl**			
Loading pH	4	Elution Fractions	10, 11	Loading pH	4	Elution Fractions	11, 12
Fraction	**Protein mg/ml**	**Fraction**	**Dot Blotting Results**	**Fraction**	**Protein mg/ml**	**Fraction**	**Dot Blotting Results**
1	0.120419	1	-	2	0.120013	3	-
2	0.119877	2	-	5	0.169938	4	-
5	0.159926	4	-	10	0.124884	5	-
7	0.153432	6	-	12	0.469833	8	-
9	0.19754	10	+++			11	+++
10	0.458671	11	++			12	+++
11	0.134625						

Table 6-2: Total protein and detection of OX40L in sample fractions purified by MBI resin

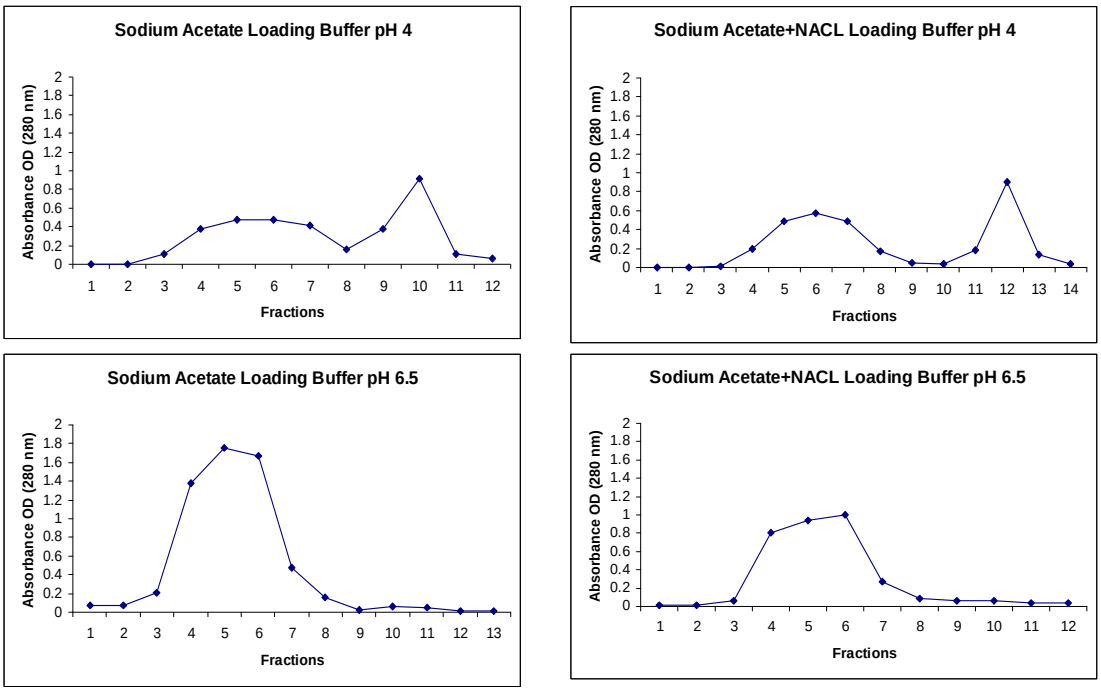
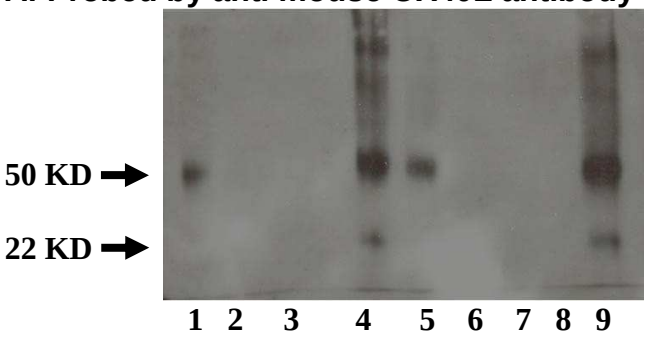


Figure 6-3: Fraction absorption at 280nm for samples loaded at pH4 using sodium acetate or sodium acetate+ NaCl for loading buffer.
2 ml of B9B8E2 cell culture supernatant was mixed up with same volume of 100mM sodium acetate or 100mM sodium acetate + 150mM NaCl at a set of pHs from 4 to 6.5. After loading the samples, the OX40L was eluted with Tris buffer at pH 9. All samples were collected in fractions of 6ml. the absorbance of fractions was measured at 280nm by spectrophotometer.

To confirm the presence of OX40L-IgG in the fractions and determine the purity of the samples, selected fractions were probed with anti-mouse OX40L, anti-mouse IgG and

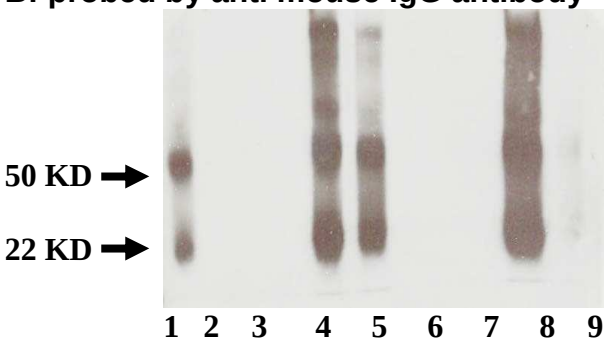
bovine IgG antibodies using western blotting. mOX40-mIgG1 (MM1) produced by Xenova plc was used as positive control (Figure 6-4).

A: Probed by anti mouse OX40L antibody



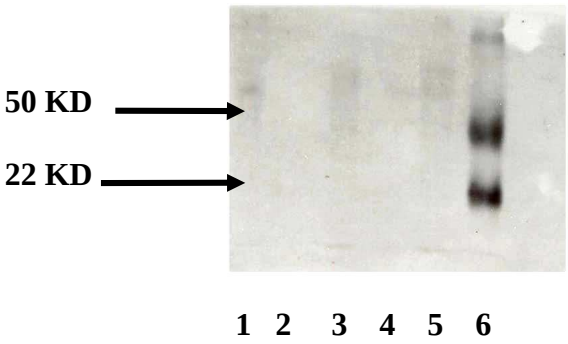
1- Unpurified sample
2, 3- Loading fractions (sodium acetate pH=4)
4, 5- Elution fractions (tris pH 9.5)
6, 7- Loading fractions (sodium acetate + NaCl pH=4)
8, 9- Elution fractions (tris pH 9.5)

B: probed by anti mouse IgG antibody



1- Unpurified sample
2, 3- Loading fractions (sodium acetate pH=4)
4, 5- Elution fractions (tris pH 9.5)
6, 7- Loading fractions (sodium acetate + NaCl pH=4)
8, 9- Elution fractions (tris pH 9.5)

C: probed by anti bovine IgG antibody



1- Unpurified sample
2- Loading fractions (sodium acetate pH=4)
3- Elution fractions (tris pH 9.5)
4- Loading fractions (sodium acetate + NaCl pH=4)
5- Elution fractions (tris pH 9.5)
6- MM1

Figure 6-4: Detection of OX40L purified by MBI resin using western blotting.
B0B8E2 cell culture supernatants were collected and purified by MBI resin. The presence of OX40L was detected in the sample fractions collected during loading and elution phases by gel electrophoresis and using anti-mouse OX40L antibodies. The presence of mouse IgG and bovine IgG were also determined by using anti-mouse or bovine IgG antibodies.

To optimise the pH for the elution buffer, Tris buffer at pH 7, 7.5, 8, 8.5, 9, 9.5 and 10 was used together with sodium acetate plus NaCl at pH 4 as loading buffer. The results clearly

showed that increasing the pH of the elution buffer from 7 to 10 increased the total protein in the eluted fractions. The highest protein measurement was for the fractions eluted at pH 9.5, which was finally selected as the optimum elution pH (Figure 6-5).

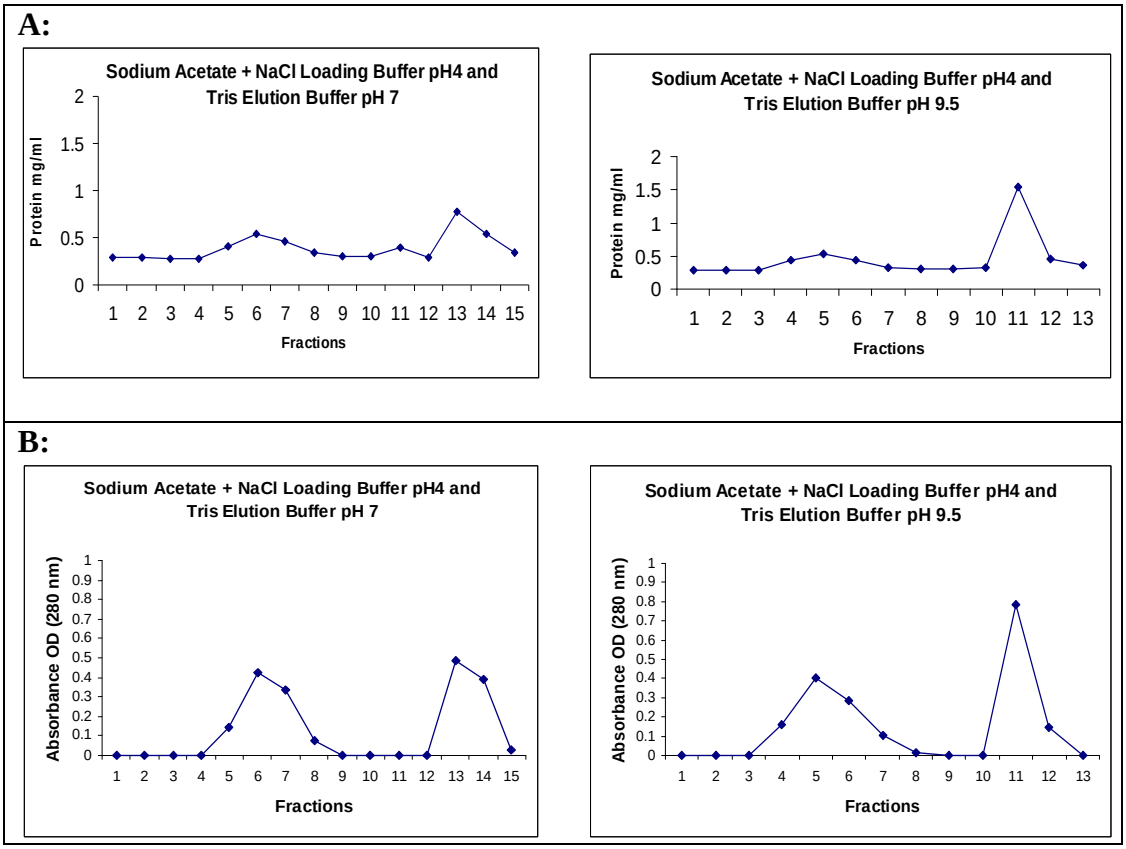
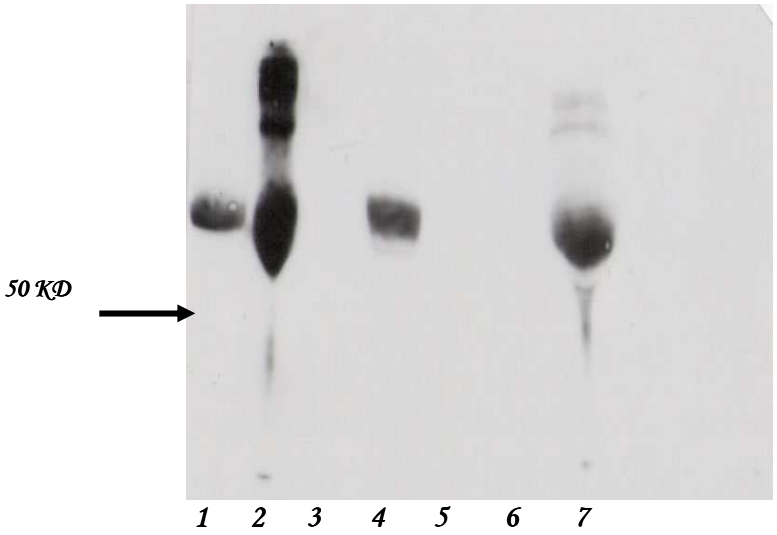


Figure 6-5: Total protein of sample fractions evaluated by protein assay or measuring the absorbance at 280 nm.
2 ml of B9B8E2 cell culture supernatant was mixed up with same volume of 100mM sodium acetate buffer containing 150mM NaCl and the pH was adjusted at 4. The OX40L was eluted with Tris buffer at a set of pHs from 7 to 10. All samples were collected in fractions of 6ml. **A**)The total protein was assessed by BCA kit according to the manufacturer's instructions. **B**) Absorbance was also measured at 280nm by spectrophotometer.

Due to the presence of FCS in the cell culture media of B9B8E2 cells, the sample fractions loaded at pH 4 and pH 5.2 were collected and checked for bovine serum albumin (BSA) by western blotting. The results clearly showed that the amount of BSA as detected by

western blotting in the elution fraction was much higher at pH 4 in comparison to pH 5.2 (Figure 6-6).



1. **Unpurified sample**
2. **BSA (positive control)**
3. **MM1**
4. **Loading fraction (loaded at pH 5.2)**
5. **Elution fraction (loaded at pH 5.2)**
6. **Loading fraction (loaded at pH 4)**
7. **Elution fraction (loaded at pH 4)**

Figure 6-6: Detection of BSA in OX40L samples purified by MBI resin at pH 4 and pH 5.2.
B9B8E2 cell culture supernatants were purified by MBI resin. The samples were loaded at a set of pH from 4 to 7 and eluted at pH 9.5. The purified samples were analysed for BSA by western blotting using anti-BSA antibodies.

6.2.2 Purification of mouse OX40L-IgG fusion protein by MEP Hypercel resin

The structure of 4-mecapto-ethyl-pyridine is based on the presence of a pyridine ring Figure 6-7. MEP is attached to a hydrophilic matrix through a hydrophobic spacer arm. The

hydrophobic spacer arm provides enhanced selectivity for the adsorption of antibodies. The presence of a pyridine ring is also shown to enhance antibody selectivity [Bak & Thomas, 2007; Mowry et al., 2004]. MEP has an isoelectric pH point of 4.8 and hence un-charged in neutral conditions. The resin becomes positively charged when the pH is below 5 due to repulsive forces between the resin and the positively charged antibody. Pyridine rings associated with sulphur atoms are relatively well documented as being able to separate immunoglobulins from complex mixtures such as serum proteins [Nopper et al., 1989]. The main properties of the MEP resin is shown in Table 6-3.

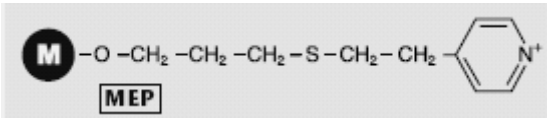


Figure 6-7: Structure of the MEP Ligand (Adapted from MEP Hypercel product note).

Particle size	80-100µm
Dynamic binding capacity for hu IgG	≥ 20mg/ml
Ligand	4-Mercapto-Ethyl-Pyridine
Ligand density	70-125µmol/ml
Working pH	3-12
Cleaning pH	3-14
Pressure resistance	< 44 psi
Typical working pressure	<14 psi

Table 6-3: Key features of MEP Hypercel (Adapted from MEP Hypercel product note).

To optimize the conditions of MEP resin for purification of the OX40L-IgG fusion protein, a constant pH 8 for loading and a set of pHs of 6.0, 5.8, 5.6, 5.4, 5.2, 5.0, 4.7, 4.5, 4.3, 4.0 and 3.0 for elution were applied (see materials and methods). Because in MEP resin different proteins are eluted at different pHs, elution buffers at different pHs were applied continuously in decreased order from high to low. The flow of each buffer pH was continued until the absorbance at 280nm was stabled at 0.01 (Figure 6-8). All fractions were collected and tested for OX40L by western blotting using anti mOX40L antibodies. The western blotting results clearly showed that the OX40L-IgG fusion protein bound to MEP resin starts eluting at pH below 5, which peaked at pH 4.5 (Figure 6-10).

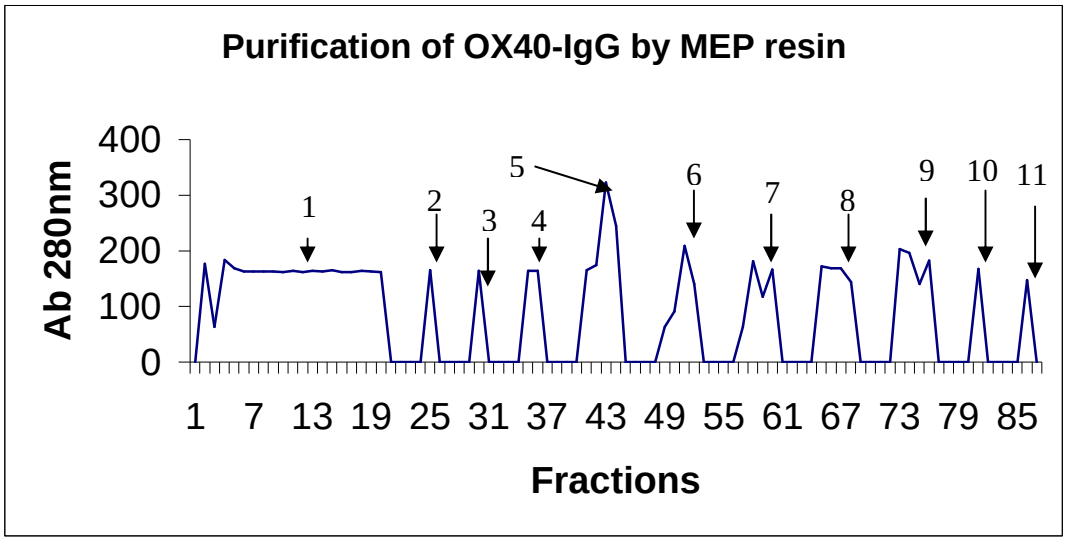


Figure 6-8: Optimisation of elution pH for purification of mouse OX40L-Ig fusion protein by MEP resin.
2 ml B9B8E2 cell culture supernatant was mixed with the same volume of 50mM tris buffer and adjusted at pH 8. After loading the sample, the loading buffer was run continuously until the absorbance at 280 was stabled at 0.01. Elution buffer (50mM sodium acetate) at a set of pH was applied for elution. 1: pH 8.0, 2 to 11: pH 6.0, 5.8, 5.6, 5.4, 5.2, 5.0, 4.7, 4.5, 4.3, 4.0 and 3.0 respectively.

In the second step to confirm the conditions of the purification, the samples were loaded at pH 8. Then, the resin was eluted, after washing with the loading buffer, with the elution buffers of pH 5, 4.5 and 3 continuously (Figure 6-9). Using this method the OX40L-IgG fusion protein was successfully purified at pH 4.5. The presence of OX40L in the elution

fractions was confirmed by western blotting using anti-OX40L antibodies (Figure 6-10A). To evaluate the purity of the OX40L, the MEP purified samples were also checked for the presence of mouse IgG, bovine IgG and bovine albumin (Figure 6-10B, C, D).

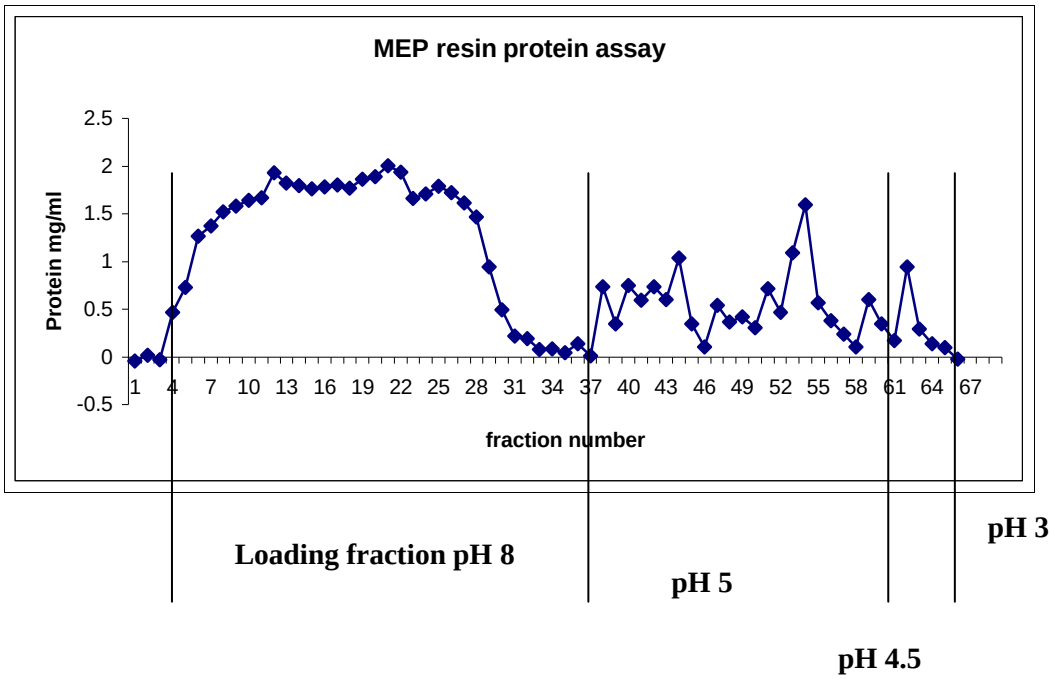
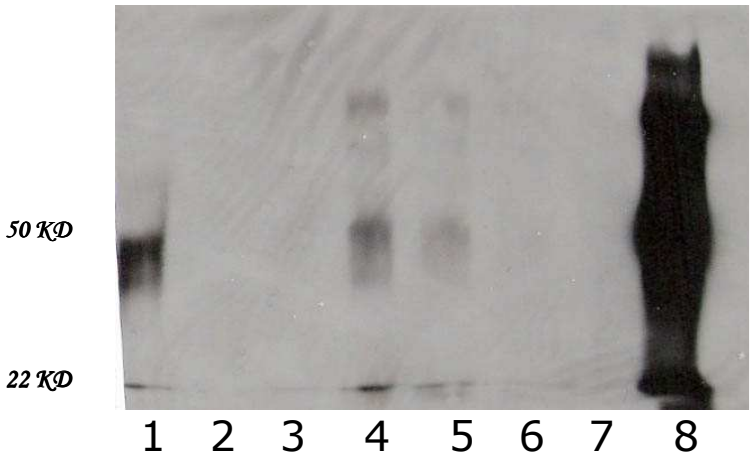


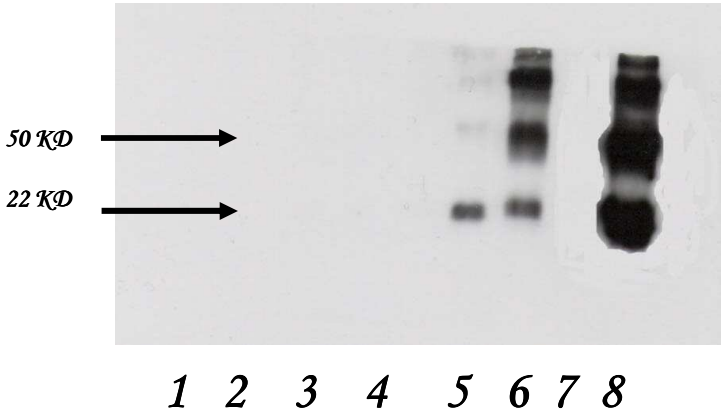
Figure 6-9: Improved method of purification of OX40L-IgG by MEP resin.
B9B8E2 cell culture supernatants were mixed up with same volume of the loading buffer (50mM tris buffer ph 8) and then loaded onto MEP resin after adjusting the pH to 8. The column was washed with the loading buffer until the absorbance at 280 was stabled at 0.01. The column was first eluted with the elution buffer (50mM sodium acetate pH) at pH 5 followed by pH 4.5 and pH 3.

A: OX40L purified by MEP resin probed by anti-OX40L antibody



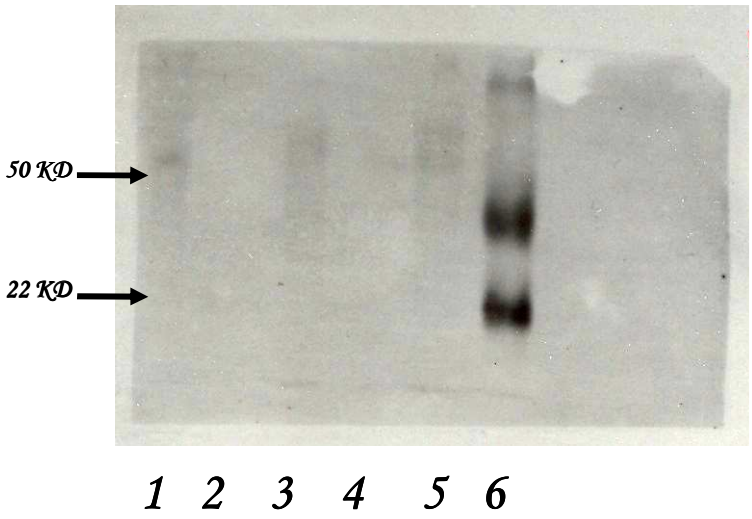
1. Unpurified sample
2. Loading fraction
3. Fractions eluted at pH 5
4. Fractions eluted at pH 4.5
5. Fractions eluted at pH 4.5
6. Fractions eluted at pH 3
7. Fractions eluted at pH 3
8. MM1

B: Mouse IgG



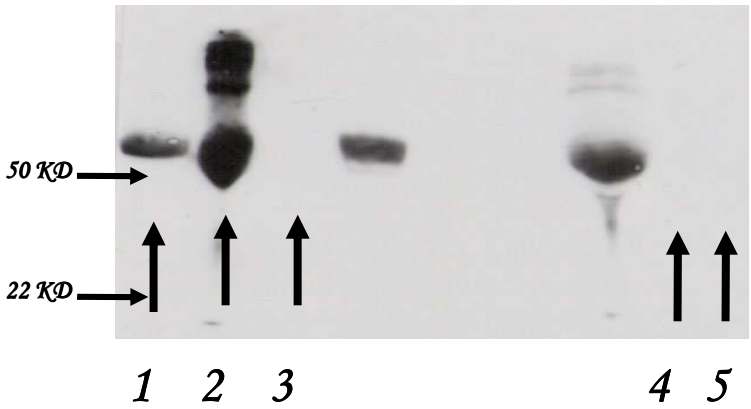
1. Loading fraction
2. Loading fraction
3. Fractions eluted at pH 5
4. Fractions eluted at pH 5
5. Fractions eluted at pH 4.5
6. Fractions eluted at pH 4.5
7. Fractions eluted at pH 3
8. MM1

C: Bovine IgG



1. **unpurified fraction**
2. **Loading fraction**
3. **Fractions eluted at pH 5**
4. **Fractions eluted at pH 4.5**
5. **Fractions eluted at pH 3**
6. **MM1**

D: BSA



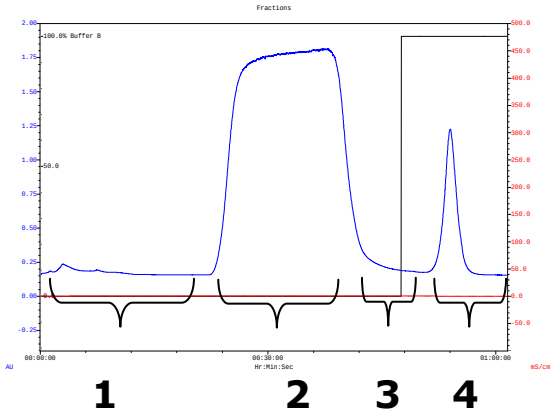
1. **Unpurified fraction**
2. **BSA (positive control)**
3. **MM1**
4. **Loading fraction**
5. **Fractions eluted at pH 4.5**

Figure 6-10: Detection of OX40L, mouse IgG, bovine IgG and BSA in samples purified by MEP resin. B9B8E2 cell culture supernatants were loaded at pH 8 (see Materials and Methods) and eluted at ph 5, 4.5 and 3 continuously. The presence of OX40L, mouse IgG, bovine IgG and BSA was checked in the fractions by western blotting using relevant antibodies.

6.2.3 Purification of OX40L-IgG fusion protein by protein A sepharose resin

As a standard method for purification of antibodies, Protein A Sepharose column was used to purify the OX40L-IgG fusion protein from the B9B8E2cell culture supernatant (Figure 6-11). B9B8E2 cell supernatant was loaded on the column at pH 7, washed with PBS and

eluted at pH 3 (see Materials and Methods). The fractions were analysed for the presence of OX40L by western blotting and compare with that of Xenova OX40L (Figure 6-12). The results clearly showed that purification was affected by the stringency and washing speed between the loading and elution (data not shown). Therefore, loading time, stringency of wash and elution were modified to optimise conditions for OX40L purification (data not shown).



1. **washing with PBS**
2. **Loading sample**
3. **PBS wash after loading**
4. **Eluting OX40L with Tris/ glycine pH3**

Figure 6-11: OX40L purification using Protein A Sepharose Column.
Protein A sepharose resin was washed with PBS and B9B8E2 cell culture supernatants were loaded onto the column. The column was given another PBS wash to remove all unbound proteins. The OX40L bound to the column was then eluted with tris/glycin at pH 3.

A: B:

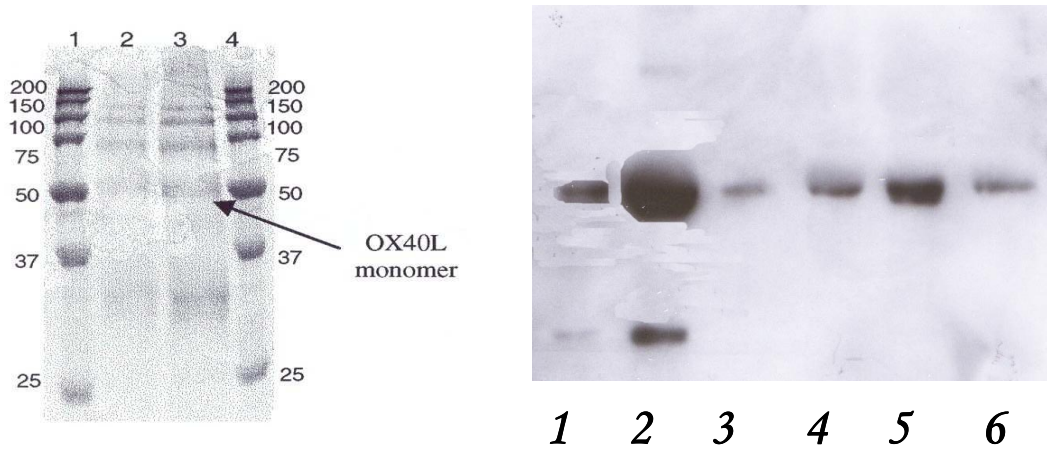
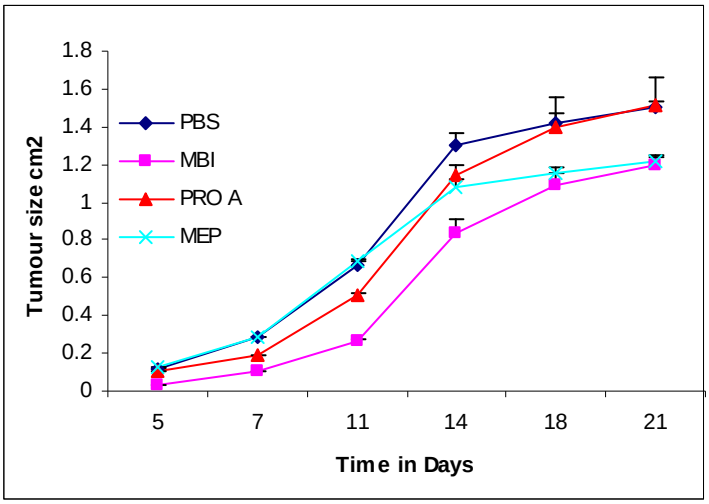


Figure 6-12: Purification of OX40L-IgG with protein A sepharose.
B9B8E2 cell supernatants were purified by protein A sepharose resin (see materials and methods). Alteration of the time or speed of the wash between loading and elution largely affected the concentration of the OX40L in the purified samples A: The OX40L-IgG (MM1) fusion protein purified by Xenova plc; B: in house experiment 1- Positive control, 2- MM1, 3-6 OX40L-IgG purified samples by protein A resin

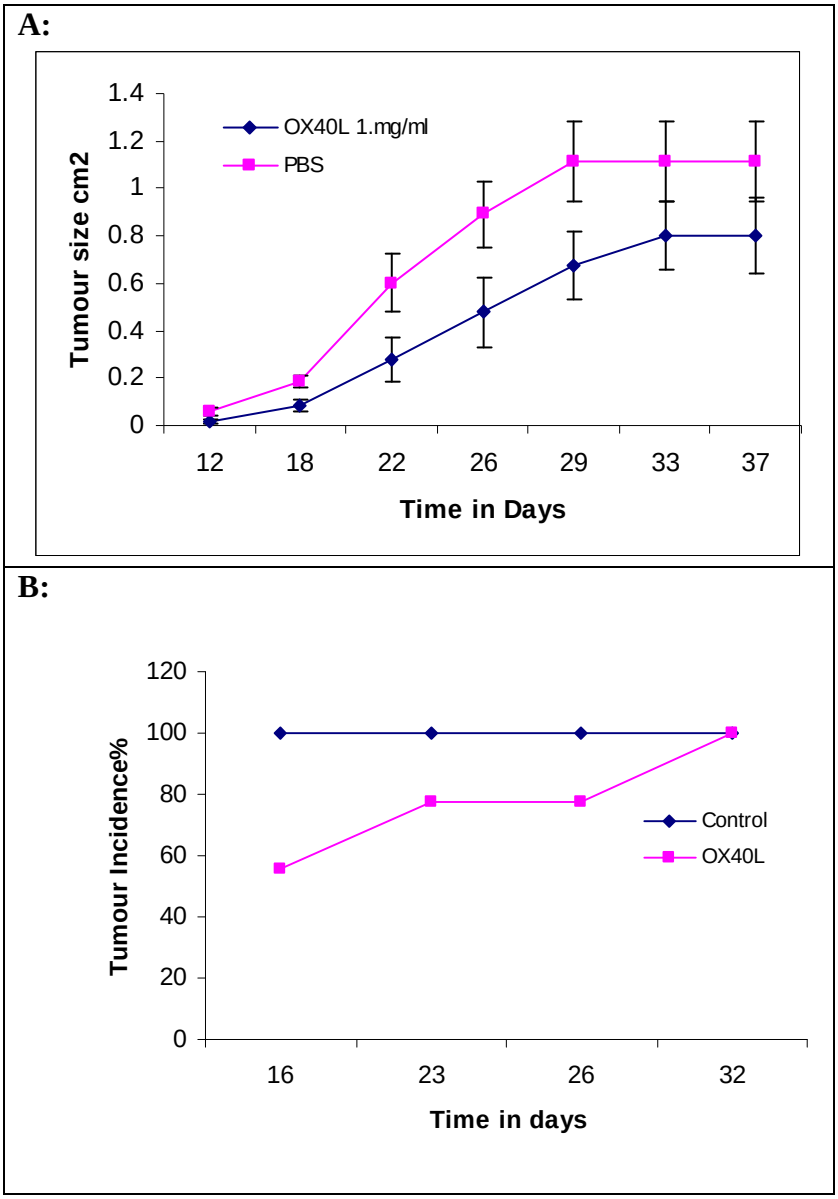
6.2.4 Biological activity of OX40L-IgG

The biological effect of the OX40L purified by MBI and MEP on the growth of CT26 tumour cells was assessed and compared with that purified by protein A sepharose resin. Four groups of 10 female BALB/c mice were implanted S.C. with 2×10^4 CT26 tumour cells on the right flank. On day 3 and 7, three groups were injected I.P. with 500µg/100µl/mouse OX40L-IgG purified by MBI, MEP and protein A sepharose accordingly. The fourth group was injected with 100 µl PBS and used as control. The results clearly showed a significant delay in tumour growth when mice were injected with the OX40L regardless of the resin used for purification. However, tumour progression in mice injected with the OX40L-IgG purified by MBI resin was significantly slower than those injected with the OX40L purified by the other resins. No significant difference was observed between biological activities of the OX40L purified by MEP and protein A sepharose resins (Figure 6-13). The MBI purified OX40L-IgG was also effective at a concentration of 1.3 mg and 40 µg/100 µl/mouse (Figure 6-14 A & B respectively).



P-value MBI, PBS	0.0020
P-value MEP, PBS	0.047
P-value Pro A, PBS	0.03
P-value MBI, Pro A	0.0026
P-value MBI, MEP	0.018
P-value MEP, Pro A	0.261

Figure 6-13: Efficacy of OX40L purified by MBI, MEP or proten A sepharose resin against the growth of CT26 tumour cells *in vivo*.
4 groups of 10 BALB/c mice were implanted S.C. with 2×10^4 CT26 tumour cells and injected I.P. with 500µg per mouse of OX40L-IgG purified by MBI, MEP and protein A sepharose resins. The fourth group was given 100µl per mouse PBS. The tumour growth was monitored regularly twice a week. Student t-test was used to analyse the data. The graph represents one experiment. Bars represent the standard deviation n=10.



P-value 0.0012
1/9 mice tumour free

P-value= 0.024

Figure 6-14: Effect of OX40L-IgG purified by MBI resin on CT26 tumour cell growth *in vivo*.
2 groups of 10 BALB/c mice were injected S.C. with 2 $\times 10^4$ CT26 cells. One group was treated intrapritonally with 1.3mg (graph A) and 40µg (graph B) OX40L-IgG fusion protein purified by MBI column. The OX40L-IgG protein was injected twice on day 3 and 7. Animals were monitored regularly for tumour growth. Student t-test was used to analyse the data. Each graph represents one experiment. Bars represent S.E. n=10.

6.2.5 Effect of OX40L-IgG (MM1) on leishmania infection

To assess the effect of OX40L-IgG on leishmania infection, two groups of 6 BALB/c mice were injected I.D. with 2×10^6 *L. mexicana* promastigotes. On days 3 and 7, the first group was injected I.P. with 100µg/mouse MM1 (OX40L-IgG fusion protein) and the 2nd group was given 100µl PBS. Mice were regularly monitored for two months. The results clearly showed significant reduction in size of cutaneous leishmania lesions in MM1 treated group compared to control (Figure 6-15A). The group injected I.P. with MM1 showed local acute inflammatory reactions at the site of lesions soon after the injection of the OX40L, which lasted for 3 weeks. This group also showed a formation of scar tissue at the site of infection (Figure 6-15B). After two months, 40% (2 out of 5) of mice injected with MM1 remained lesion free (Figure 6-15 C).

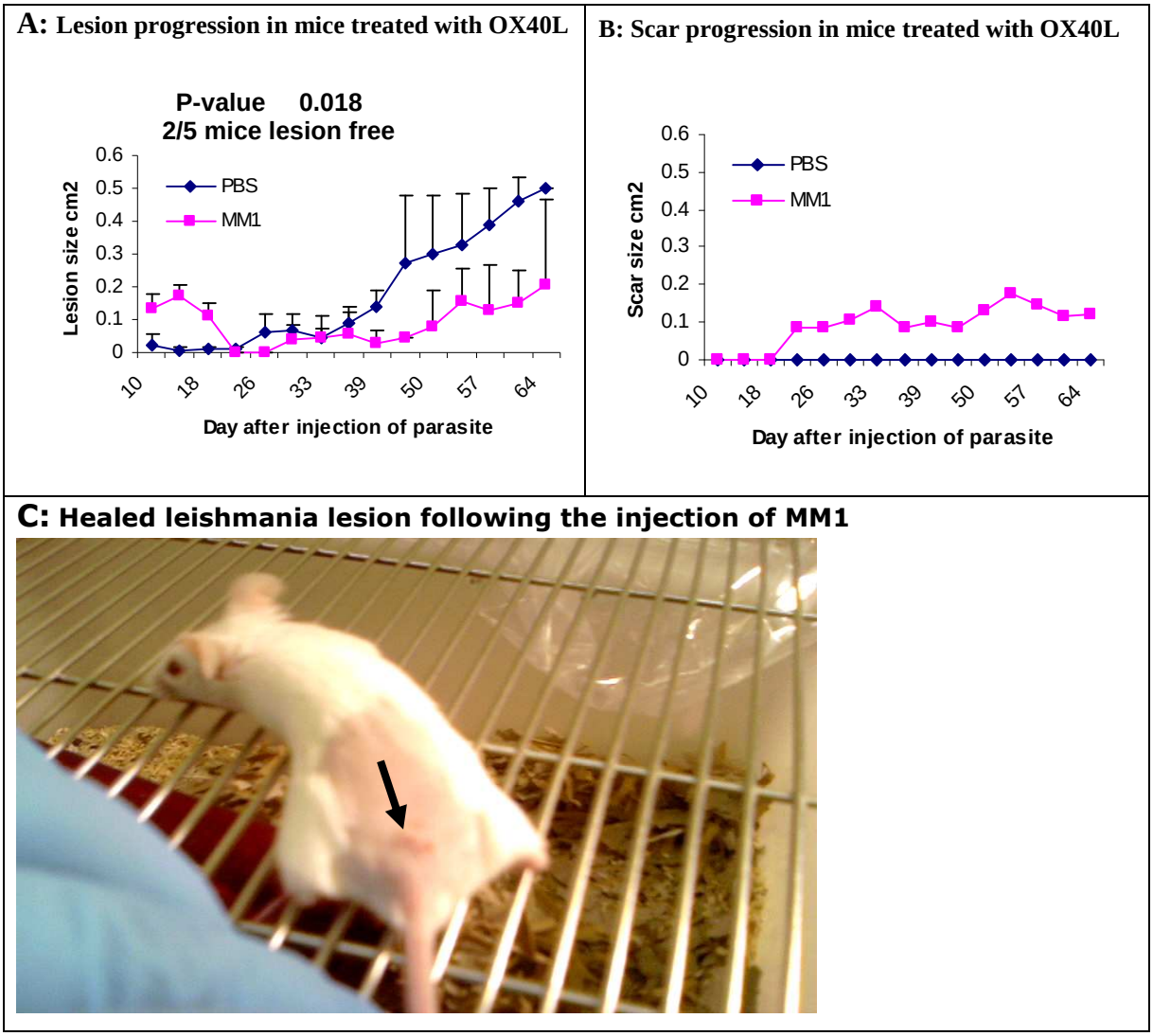


Figure 6-15: Effect of OX40L on *L. mexicana* infection.
Two groups of 6 BALB/c mice were inocluted with 2×10^6 *L. mexicana* promastigotes. The first group was injected with 100µg/100µl/mouse of MM1 and the second group with 100µl PBS. Mice were injected twice on day 3 and 7 after infection. Mice were monitored regularly two times a week. The graph represents 2 independent experiments. Bars represent the standard deviation n=6.

6.2.6 Effect of OX40L-IgG (MM1) on the immunogenicity of SLA

To assess the effect of MM1 on the enhancement of the immunogenicity of SLA, four groups of BALB/c mice were immunised with SLA, SLA in combination with MM1, MM1 and PBS. SLA was injected S.C. at 100µg per mouse mixed with the same volume of IFA at the base of the tail twice at two weeks interval and MM1 was injected I.P. at dose of 100µg per mouse on day 3 after SLA. Two weeks after the second immunisation, mice were challenged with 2×10^6 *L. mexicana*. No significant delay was observed in mice treated with SLA or MM1 alone compared to those receiving PBS. However, a small but not significant delay was observed in mice treated with SLA and MM1 compared to those given SLA alone or PBS (Figure 6-16). Also, no significant difference was observed between mice injected with MM1 and PBS. None of the mice were lesion free when the experiment was terminated. The results indicated a low effect for the OX40L when it was administered before challenging mice with the parasite compare to those injected after challenging with the parasite showed in 6.2.5.

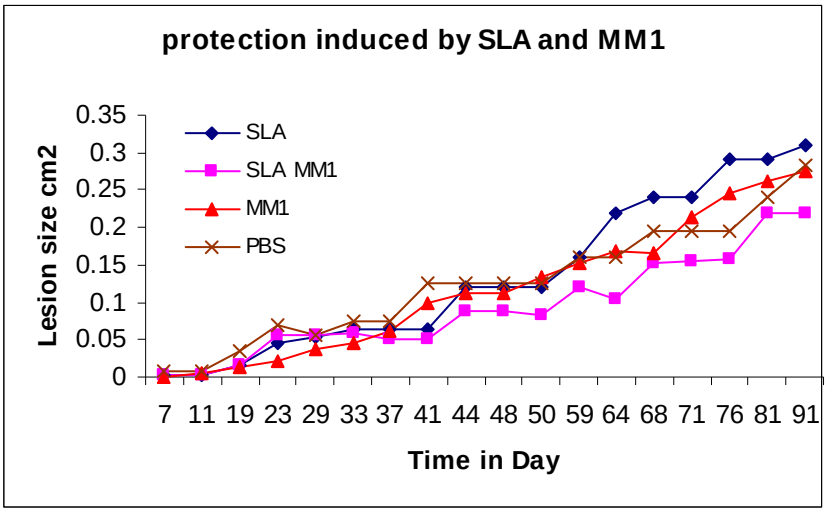


Figure 6-16: Protection induced with SLA and MM1against *L. mexicana* infection.
Four groups of 6 female BALB/c mice were selected. The first (-♦-) and second group (-■-) were immunised S.C. at the base of tail with 100µg SLA per mouse on days 0 & 14. Second (-■-) and third (-▲-) groups were given 100µg/mouse of MM1 I.P. on days 3 & 17 (3 days after immunisation with SLA). The group four (-×-) was injected with PBS. On day 28 all mice were challenged with 2×10^6 *L. mexicana* promastigotes. Mice were monitored regularly twice a week. Student t-test was applied to statistically analyse the data. The graph represents one experiment n=6.

6.2.7 Effect of MBI resin purified OX40L-IgG on leishmania infection

To assess the efficacy of the OX40L purified by MBI resin in leishmania infection, the progression of leishmania lesions in groups of BALB/ mice treated with MBI purified OX40L was investigated. In this study, two groups of mice were injected I.D. with 2×10^6 *L. mexicana* promastigotes and then one group was treated with 500µg of the OX40L purified by MBI resin given on days 3 and 7 after infection. The second group was injected with PBS. The results showed a significant delay in lesion progression in mice treated with the OX40L compared to those injected with PBS and 33% (2 out of 6) of mice receiving the MBI purified OX40L remained lesion free (Figure 6-17).

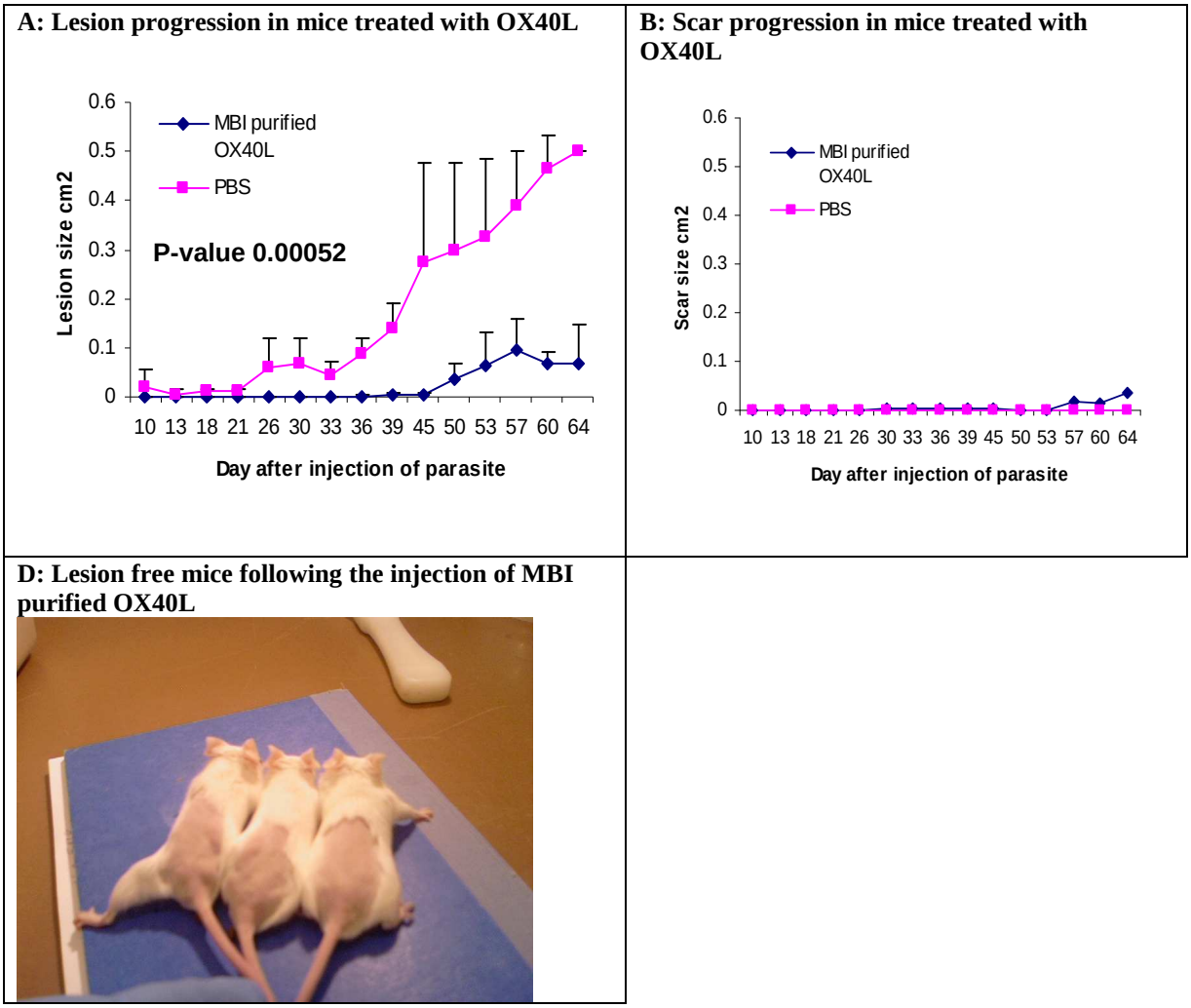


Figure 6-17: Effect of OX40L purified by MBI resin on *L. mexicana* infection.
Two groups of 6 female BALB/c mice were injected with 2×10^6 *L. mexicana* promastigotes. The first group was injected I.P. with two doses of 500µg MBI resin purified OX40L 3 and 7 days later. The second group was given PBS. Mice were monitored regularly twice a week and student t-test was used to analysed the data. The graph represents 2 independent experiments. Bars represent the standard deviation n=6.

6.3 Discussion

6.3.1 OX40L purification

Antibodies are essential in biomedical research and the optimal purification of antibodies has always been an objective for researchers. Finding a specific ligand, which can be used for purification of recombinant proteins, is sometimes difficult, therefore antibodies have been used to bind the protein of interest to make fusion protein to be used for purification. In this study, B9B8E2 cells, donated by Xenova plc, were transfected with the murine OX40L and IgG1 plasmids and were used to produce the mOX40-mIgG1 (MM1). The therapeutic efficacy of the MM1 fusion protein has previously been described in murine tumour models [Ali et al., 2004b]. In the present study, two novel resins (MBI & MEP) were used for the first time for the purification of OX40L-IgG fusion protein. To obtain optimum purification, the conditions of loading and elution were optimised and the purity was determined by western blotting using anti-murine OX40L antibodies. The biological activity of the product was also assessed against tumour growth and compared with that of the conventional methods (protein A sepharose), which were previously described by Xenova plc [Xenova plc MM1 product leaflet].

MBI Hypercel is a resin recently designed for specific chromatographic capture of antibodies [MBI Hypercel product note]. In a study carried out by Brenac, MBI resin was successfully used for purification of antibodies from a cell culture supernatant and it was shown that uptake increased when the pH decreased [Brenac et al., 2005]. Our results confirmed that lowering the pH increases the binding of proteins to the resin and as the pH increases, the binding of the OX40L-IgG to the resin reduces. The maximum binding of proteins including the OX40L-IgG fusion protein to the resin occurred at pH 4; decreased amount of unbound OX40L-IgG was detected during the loading of the sample at this pH 4. This is in contradiction with the results of Branac et al [Brenac et al., 2005] where the complete absorption of their antibody occurred at pH 5. The optimum pH for desorption of OX40L-IgG from the resin was pH 9.5 and this was similar to that of Branac et al [Brenac et al., 2005]. These results may indicate that for purification of each type of antibody, the conditions of purification by MBI resin need to be optimised.

The purity of the purified product was determined by testing representative samples for contamination with BSA and bovine IgG. When samples were loaded at pH 4, the BSA completely bound to the resin and there was no unbound BSA released during the loading phase (as determined by western blotting). When the loading pH was increased to 5.2, no

BSA bound to the resin. Therefore, loading the samples at pH 5.2 resulted in more pure OX40L-IgG fusion protein, although compared to pH 4, adsorption of OX40L-IgG fusion protein at pH 5.2 was slightly less. This specific pH adsorption (5.2) was in agreement with the results obtained by Brenac et al (5.0 to 6.0) and Girot et al (5.0 to 5.5) [Brenac et al., 2005; Girot et al., 2004]. No bovine IgG binding was detected under the same conditions. According to the datasheets produced by Xenova plc, MM1 has been produced through the purification of the cell supernatant by protein A sepharose resin. Thus, there may be a selectivity for binding of different antibodies to MBI resin where it is not for protein A sepharose resin, however this aspect requires further investigation.

MEP Hypercel resin is a hydrophobic charge interaction chromatography resin designed for purification of antibodies. It is shown that MEP Hypercel has a negative charge at pH 7 and above and a positive charge at lower pH; therefore, proteins bind to the resin at pH above 7 whereas they start eluting at pH below 7. This resin has successfully been used for purification of monoclonal antibodies [Dux et al., 2006; Mowry et al., 2004]. In the present study, MEP resin was used for the purification of OX40L-IgG fusion protein. To optimise the elution pH, a range of different pHs were tested, showing that the OX40L-IgG fusion protein is eluted only at pH 4.5. Testing the product for BSA and bovine IgG revealed that the OX40L purified by the resin was free of both BSA and bovine IgG, neither which was detected in the purified samples by western blotting indicating selectivity in the purification.

Additionally, OX40L-IgG was purified from B9B8E2 cell culture supernatant by protein A sepharose column (see materials and methods). Purification by protein A sepharose is based on the affinity of FC portion of the fusion protein for the protein A. It was shown that increasing the course or the speed of the wash after loading the sample caused the loss of majority of the OX40L in the samples. This clearly shows that protein A sepharose resin has potential to bind to the OX40L-IgG molecules but the binding is very weak. Therefore, the speed of sample flow through the resin or increasing the course of wash result in a reduction in the amount of purified OX40L-IgG molecules.

6.3.2 Biological activity

It has been shown that the OX40L can enhance T cells immunity [Ali et al., 2004b], however, the effect of the method of purification on the biological activity of the OX40L has not yet been fully investigated. To the best of my knowledge, this is the first study, which has investigated the effect of methods of OX40L purification and immunisation

relative to the biological activity of the OX40L-IgG fusion protein. The biological activity of the OX40L purified by MBI, MEP and protein A sepharose were compared in a tumour growth inhibition experiment in a BALB/c mouse model. Mice treated with the same doses of the OX40L purified by the three resins showed a significant delay in tumour growth compared to the controls injected with PBS. Mice treated with the OX40L purified by MBI resin showed a significant delay in tumour growth compared with mice injected with OX40L purified by other resins suggesting a greater biological activity for the product purified by using the MBI resin. Better biological activity for OX40L purified by MBI may be due to less harsh conditions of purification; in MBI resin since the samples were loaded at pH 5.2 (less acidic) and eluted at pH 9.5 (basic) whereas in MEP and protein A sepharose resins the samples were loaded at pH 8 or 7 and eluted at4.5 or 3 (highly acidic). It has been observed that proteins at low pH are denatured and their tertiary or even secondary structures are changed [Poklar et al., 1997]. Then, it is possible that the elution of the OX40L at very low pHs affects its biological activity by altering the molecular structure of the protein. Further studies are needed to confirm these findings.

6.3.3 Effect of OX40L in leishmania infection

The role of OX40-OX40L co-stimulation in leishmania infection is not fully understood. Some studies showed that blocking the OX40L by the administration of anti-OX40L mAb abrogated the progressive disease in BALB/c mice. This was accompanied by reduction of Th2 cytokines and anti-*L. major* IgG1 [Akiba et al., 2000]. In addition, studies on *L. major* in OX40-deficient mice have suggested a less essential role for OX40 co-stimulation in immunity to leishmania [Pippig et al., 1999]. In another study, it was shown that constitutive OX40-OX40L interactions in OX40L deficient mice converted the normally resistant C57BL/6 strain, into a susceptible status following *L. major* infection due to an extraordinary elevated Th2 response [Ishii et al., 2003]. This was in agreement with studies showing that the OX40-OX40L co-stimulation augments the differentiation of T cells toward Th2 by up regulating expression of IL-4 and IL-13 and suppression of IFN-γ [Delespesse et al., 1999; Flynn et al., 1998; Ohshima et al., 1998; Tanaka et al., 2000]. On the other hand, other studies have suggested a mutual role for OX40-OX40L co-stimulation affecting both Th1 and Th2 immune response. Ito et al [Ito et al., 2006] showed that OX40L strongly inhibited IL-10 production and abrogated the suppressive function of IL-10-producing CD4+ type 1 regulatory T cells. Also, OX40L-deficient mice show a reduction of both T helper type 1 (Th1) and Th2 cytokines [Murata et al., 2000].

Furthermore, the Th1 and Th2-cytokine production by DCs including IL-2, IFN-γ, IL-10, and IL-13 were inhibited by an anti-rat OX40L mAb [Satake et al., 2000]. Stimulation of DC through OX40L enhanced their maturation and up regulated the production of Th1 cytokines such as IL-2, IL-12 and IFN-γ, and increased the expression of other co-stimulatory molecules [Ohshima et al., 1997; Wang et al., 2004]. Similar results were obtained when DCs were transfected with the OX40L gene where the transfected DCs were capable of polarization of naive CD4+ T cells toward Th1. Vaccination of tumor-bearing mice using OX40L mRNA-cotransfected DCs resulted in significant enhancement of therapeutic anti-tumour immunity due to in vivo priming of Th1-type T-cell responses [Dannull et al., 2005]. The potency of OX40L in generating CTL activity against tumour has also been shown in mouse model [Ali et al., 2004b; Assudani et al., 2006].

In *L. donovoni*, recent studies have shown that the OX40L fusion protein (OX40L-FC) has a therapeutic effect on leishmania either alone or when co-administered with anti-leishmania drugs through enhancing CD4+ T cell activity [Zubairi et al., 2004]. To the best of my knowledge, there is no report of administration of OX40L on cutaneous leishmaniasis either in therapy or prophylactic immunisation and the present study for the first time reports the effect of OX40L on *L. mexicana* infection. The therapeutic effects of MM1 (OX40L-IgG purified by protein A sepharose and produced by Xenova plc) was tested in a challenge experiment using *L. mexicana* infected BALB/c mice. Mice received two doses of 100µg MM1, which was previously shown to be effective against tumour progression [Ali et al., 2004b], on day 3 and 7 after the infection. Mice receiving MM1 generated an inflammatory reaction a few days after the injection of the OX40L, which was gradually dampened and finally disappeared 3 weeks later. This inflammatory reaction could be due to the high dose of OX40L in MM1 product. There was a significant delay in the growth of developing lesions in mice receiving OX40L compared to controls injected with PBS and the size of lesions in the group receiving MM1 was significantly smaller than that of injected with either PBS. 40% of mice given MM1 remained lesion free for two months, when experiments were terminated. The efficacy of the OX40L purified by MBI resin was also determined by the administration of 500µg of the product (the effective dose against tumour progression see 6.2.4) resulting in a significant delay in lesion progression in mice treated with the OX40L compared to those receiving PBS; 33% of the OX40L treated mice remained free of lesion after two months and did not experience major inflammatory reaction as shown for mice treated with MM1.

The results clearly indicate the high therapeutic effect of OX40L in *L. mexicana* infection. The positive therapeutic effect of OX40L demonstrated in this study was in agreement with that of Zubairi [Zubairi et al., 2004]. The effect of the method of purification on the affectivity of OX40L in leishmania infections is now being carried out in our lab. We failed to detect CTL activity against DCs loaded with SLA in the mice challenged with *L. mexicana* promastigotes and then treated with MBI-purified OX40L-IgG. Determination of CTL activity soon after treatment with OX40L-IgG and also the role of other T cell subsets in the healing process needs to be further studied. Immunisation of SLA in combination with MM1 revealed that OX40L did not enhance the immunogenicity of SLA. In addition, injection of MM1 alone before the initiation of infection did not result in any effect on the infection. The effect of OX40L on the enhancement of the immunogenicity of other types of vaccines and increasing the efficacy of methods of immunisation, such as gene gun immunisation, needs to be further investigated since it provides new opportunities for developing new vaccine strategies.

Chapter 7 Discussion

7.1 Leishmania Immunity Induced by immunisation

Leishmania is an intracellular parasite affecting a large group of people in the world and developing a potent vaccine has always been a goal for leishmania vaccine researchers. It is well known that leishmania infected individuals develop a long-term immunity to the parasite after the infection [Khamesipour et al., 2006], rationalizing the efforts made for developing a potential vaccine for prevention or cure of leishmania infections. Vaccines produced against leishmania parasites are now classified into two main groups: old and new generation vaccines. The old vaccines were based on using either live or killed parasites as a vaccine, which have demonstrated a lack of sufficient immunity or difficulty in the standardisation of the vaccine [Khamesipour et al., 2006; Sharifi et al., 1998]. New generation of leishmania vaccines are mainly based upon using a single or a combination of immunogenic genes rather than a "cocktail" of proteins such as killed parasites. The genome of Leishmania, which is approximately 35 Mb containing approximately 8500 genes, was sequenced in 2002. It is believed that these genes are probably translated into more than 10 000 proteins [Almeida et al., 2002]. Different leishmania proteins have been used as vaccine candidates in which only a few immunogenic proteins have been found and tested in animal models using different methods of immunisation.

In the present study, the immunogenicity of different vaccines including leishmania autoclaved vaccine, soluble leishmania antigens and DNA vaccines were compared. Although the protective potential of Autoclaved *L. major* (ALM) along with BCG against *L. donovani* in animal models was shown in some studies [Dube et al., 1998], other studies did not confirm this type of immunisation for cutaneous leishmaniasis particularly in human [De Luca et al., 1999; Khalil et al., 2000; Momeni et al., 1999]. Therefore, the protective immunity of autoclaved parasite is still questionable. In our experiments, the autoclaved parasite did not induce significant immunity to protect BALB/c mice against the infection. Also, the evaluation of IgG1 and IgG2a in the serum of immunised mice suggested the absence of Th1 immune response. Injection of SLA containing *L. mexicana* gp63 protein also failed to induce better immunity than autoclaved parasites and none of the SLA immunised mice remained lesion free. Analysing the antibody levels and types induced by the SLA immunisation showed that SLA produced high levels of both IgG2a and IgG1 indicating the presence of both Th1 and Th2 immune responses respectively.

These results were similar to those of Sharma [Sharma et al., 2006] suggesting a mixed Th1/Th2 immune response induced by SLA.

Dendritic cells (DCs) have been proposed to play an important role as adjuvants in vaccination and immunotherapy [Jack et al., 2007]. In leishmania vaccination studies, higher levels of immunity has been reported using SLA-loaded and/or IL-12-transfected DCs [Ahuja et al., 1999; Ghosh et al., 2003]. In contrast, our results demonstrated that DCs loaded with SLA, although capable of inducing significant levels of both Th1 and Th2 responses measured by increases in IgG2a and IgG1 respectively, failed to protect BALB/c mice from the infection.

The immunity induced by *L. mexicana* gp63 cDNA using two different methods of immunisation, intramuscular injection of the DNA and gene gun immunisation, was investigated and compared with those of autoclaved parasite or SLA immunisation. It was shown that DNA immunisation with *L. mexicana* gp63 cDNA resulted in higher immunity to the parasite; the immunised mice exhibited a significant delay in lesion formation and some of the mice remained free of lesions. This clearly confirms the feasablity of new strategies in using single or combinated antigens than a “cocktail” of antigens for leishmania vaccination. Although there are reports showing that the alteration of the method or the route of immunisation results in alteration of the immunity [Jaafari et al., 2006; Russell & Alexander, 1988], this was the first time that two different methods of DNA immunisation were compared in a leishmania vaccination model. Immunisation using the gene gun gave higher levels of immunity to the parasite. In contrast with reports showing a Th2-bias immunity for gene gun [Scheiblhofer et al., 2007; Schirmbeck & Reimann, 2001], in this study, all mice immunised by the gene gun demonstrated a stronger Th1-type immune response, which was accompanied by a sharp increase in IgG2a in the early stages of immunisation. The sharp rise in mouse IgG2a occurred short time after the immunisation, which was not accompanied by increase in IgG1 level. This may be due to the differences between antigens used for immunisation suggesting the need for testing different antigens with different methods. Mice immunised intramuscularly with DNA demonstrated lower levels of protection, which was accompanied by a gradual increase in IgG2a.

Comparison of the results obtained from the immunisation with the SLA and the DNA demonstrates that the immunisation with the SLA resulted in a mixed Th1/Th2 immune response, which was similar to that induced during the course of the infection in non-immunised mice [Rolao et al., 2007]. Although it was reported that the initial Th2 immune

response may play a role for raising Th1 immune response in *L. donovoni* [Mazumdar et al., 2004], results of this study showed that there was no correlation between the initial rise in Th2 immune response and immunity to *L. mexicana*; SLA induced little immunity compared to that of the DNA vaccination, which did not induce Th2-type immune response in the early phases of the immunisation. Instead, it seems the early activation of a Th1 immune response is crucial for the induction of immunity to the parasite. Results clearly showed that immunisation by gene gun induced a sharp activation of Th1 immune response soon after the initiation of immunisation. On the other hand intramuscular injection of the DNA induced a gradual increase in Th1 immune response, which peaked a few weeks after the initiation of immunisation.

The use of DCs as an adjuvant (DCs pulsed with SLA) to promote immunity did not significantly alter the immunogenicity of the SLA derived from *L. mexicana*, which was in contrast with the results obtained in *L. donovoni* model by Ahuja and Ghosh [Ahuja et al., 1999; Ghosh et al., 2003]. This might demonstrate the species-dependency of immunogenicity of SLA, suggesting differences in immunity between leishmania species and this should be taken in consideration in developing vaccine strategies.

Irradiated CT26 tumour cells transfected with *L. mexicana* gp63, as a surrogate antigen, did not induce strong immunity against the parasite, which may suggest the immune domination of tumour antigens. However, this approach requires further study in order to gain insight into the mechanisms involved.

7.2 CTL activity induced by L. mexicana gp63 and SLA

The role of cytotoxic T lymphocytes in immunity to leishmania is still not clearly identified. CD8+ T cells are reported as an important source of IFN-γ during leishmania infection in mice [Lehmann et al., 2000] and it has also been shown that depletion of CD8+ T cells in mice vaccinated with LACK DNA at the time of vaccination abrogated the control of infection, indicating a significant role for these cells in this model [Gurunathan et al., 2000b].

In the present study the role of CTLs in immunity against leishmania induced by DNA and SLA was investigated. The results showed that gene gun immunisation with a gp63 cDNA construct, which was capable of inducing high levels of Th1-type immunity and significantly protect the mice from the infection, also induced long-term CTL activity that could be detected against tumour cells expressing the gp63 protein. However, mice infected with *L. mexicana* did not show significant CTL activity, suggesting that the CTL

activity was only induced by immunisation. This was compatible with the results obtained by Gurunathan where DNA vaccination encoding LACK resulted in the production of IFN-γ by CD8+ T cells, which induced protective immunity against *L. major* in mice; in this system no protection was observed in CD8+ T cell depleted mice, although they did not measure CTL activity *in vitro* [Gurunathan et al., 2000b]. On the other hand, immunisation of mice with DCs loaded with SLA induced a high level of CTL activity but this was not correlated with protection. Similar CTL activity has recently been obtained in our lab by immunisation with SLA + IFA [Fathia & Ali, personal communication]. These results are similar to those obtained by Mendonca where they immunised 43 Brazilian volunteers with a vaccine made of whole antigens derived from killed promastigotes of five American dermotropic Leishmania strains. In this study peripheral blood mononuclear cells were obtained one year after vaccination and tested in a proliferation assay against *L. braziliensis* antigens; the majority of the responding cells were of a CD8+ T cell subtype [Mendonca et al., 1995]. De Luca also reported the predominance of CD8+ over CD4+ T cells among the leishmania-reactive cells after administration of a vaccine composed of whole antigens of killed *L. amasonensis* promastigotes [De Luca et al., 1999]. Similar involvement of CD8+ T cells in the clearance of *L. donovoni* was also reported by Ahmed [Ahmed et al., 2003]. The discrepancy in the CTL activity and protection induced by immunisation with *L. mexicana* gp63 DNA and SLA has only been reported in the present study and needs further investigation to determine the mechanism underlying the role CTL activity in leishmania protection; however, the difference between the antigens used and the methods of immunisation may represent important parameters.

To clarify aspects of the role of CTLs in immunity to leishmania the effect of leishmania infection on the expression of the MHC class I in DCs was investigated. The results clearly showed that only infection with live leishmania parasites effectively down regulated the expression of MHC molecules in DCs; treatment with autoclaved parasite, SLA or transfection of DCs with *L. mexicana* gp63 cDNA did not reduce MHC expression. The effect of down regulation of MHC class I by the parasite on the immunogenicity of vaccines is not clear and reqires further investigations. Down regulation of MHC class I is a known strategy for tumour escape from CTL-mediated immunity [Khanna, 1998].

Down regulation of HLA-C molecules was also reported in *herpes simplex virus* and *human cytomegalovirus* infections whereby viral genes interfered with the expression of MHC class I molecules [Huard & Fruh, 2000]. In parasitic infections, it has been shown that *T. cruzi*, effectively inhibits the up-regulation of MHC class I molecules induced by

LPS on the surface of human DCs [Van Overtvelt et al., 2002]. Similar observations were also reported during *P. falciparum* malaria infection [Brustoski et al., 2005]. In leishmania infection, whether down regulation of MHC class I has any impact on the role of CD8+ T cells in immunity to leishmania needs to be determined. Perhaps leishmania vaccine candidates are potent enough to prevent infection if the expression of MHC class I molecules is not altered or down regulated by the parasite. Therefore, a better understanding of the mechanisms by which the parasite down regulates the expression of the MHC class I molecule is essential, for the design of future vaccine strategies.

7.3 Peptide immunisation

Immunogenic synthetic peptides have been widely used for identification of both B and T cell epitopes responsible for protection against parasites including eimeria, malaria, schistosoma and leishmania with different outcome [Dobano & Doolan, 2007; Jardim et al., 1990; LoVerde et al., 2004; Spitzer et al., 1999; Talebi & Mulcahy, 2005]. In leishmania, most of the studies on synthetic peptides were carried out on long-sequence peptides [Jardim et al., 1990], which were not clearly defined as MHC class I or II, or even both. However, in most of these studies the role of CD8+ T cell epitopes was not identified. Therefore, in order to evaluate the role of MHC class I epitopes in immunity to leishmania, the immunogenicity of MHC class I epitopes derived from *L. mexicana* gp63 vaccine was investigated (gp63 protein/Ag). Preliminary results to detect CTL activity induced by *L. mexicana* gp63 DNA immunisation indicated the presence of immunogenic MHC class I epitopes derived from this proteins. The SYFPEITHI data base is a known web-based algorithm, which has successfully been used in other studies for prediction of HLA and mouse MHC class I restricted epitopes [Dong & Sui, 2005; Harpur et al., 1993]. MHC class I peptides were predicted by "SYFPEITHI" software in two different models; mouse MHC class I and human HLA-A2 epitopes were defined and tested in BALB/c and HHDII transgenic mouse models respectively. None of the peptides predicted for mouse MHC class I, which were tested in BALB/c mice, showed significant immunogenicity but three out of the four peptides predicted for human HLA-A2 were immunogenic and were able to induce significant CTL activity detected by standard 4-hour cytotoxicity assay. The immunogenic peptides were tested to determine whether they were natural processed using DNA immunisation. It was shown that two of the immunogenic peptides (RLAAAGAAV & AAAGAAVTV) induced significant levels of IFN-γ indicating the possibility that they are naturally processed within the cell. Finally, the immunogenicity of the peptides was

tested in the protection studies against the live parasite. None of the immunogenic peptides could protect HHDII mice from the infection. As the peptides used in this study were restricted to MHC class I molecules, the lack of protection in mice immunised with the immunogenic peptides may suggest the need for additional activation of CD4+ T cells. In addition, the lack of immunogenicity of peptides predicted for mouse MHC class I, which were tested in BALB/c mice compared with the peptides predicted for human HLA-A2 indicates either differences between the immune system in the two strains of mice (BALB/c and HHD II) or the lack of accuracy of SYFPEITHI software for prediction the immunogenic peptides in BALB/ mice.

7.4 Application of OX40L in Leishmania infection

Combination therapy is a new approach that has recently been applied in the treatment of leishmaniasis. Different materials, such as cytokines, have been used in combination with drugs or vaccines in order to enhance their efficacy [Michel et al., 2006]. OX40L, a co-stimulatory molecule expressed on APCs, has recently been used to enhance the immunity in tumour mouse models [Ali et al., 2004b]. In leishmania, only a few studies have been carried out with a discrepancy of results. Early studies showed that blocking the OX40-OX40L interaction resulted in the abrogation of progressive disease in BALB/c mice, possibly due to blocking the Th2 immune response [Akiba et al., 2000]. Later studies revealed that administration of the OX40L after challenge of susceptible mice with leishmania resulted in healing and a reduction in parasite burden [Zubairi et al., 2004]. Collectively, these results indicate that the OX40L may play a mutual role in inducing Th1 or Th2-type immune responses.

In agreement with the study of Zubairi [Zubairi et al., 2004], our results showed that the administration of the OX40L, after the initiation of the infection, induced a significant delay in the disease, which was accompanied by a reduction in the lesion size. Surprisingly, application of the OX40L purified by two different resins, MBI or protein A sepharose, resulted in different potency of healing; injection of Protein A Sepharose purified OX40L on day 3 and 7 of parasite inoculation resulted in a delay in progression of the lesion along with the production of scar tissue while MBI purified OX40L showed more delay in lesion progression and far less scar formation. Almost no protection, and even exacerbation of the infection, was observed when OX40L purified by protein A sepharose resin was administered after immunisation of mice with SLA, while injection of OX40L purified with the MBI resin still induced a significant delay in lesion progression

(data not shown). The main difference between the methods of purification was the pH conditions under which the OX40L samples were loaded and eluted. Therefore the conditions in which the OX40L is purified has a direct effect on the biological activity/integrity of the OX40L. This could explain the different behaviour of OX40L preparation and the discrepancy in the results obtained in the pilot studies. The difference in the biological activity of the OX40L purified by different resins was also confirmed by the administration of the OX40L against CT26 tumour cells (see chapter 6).

7.5 Future work

The data presented in this study have demonstrated new opportunities to develop leishmania vaccine strategies where further studies are required:

1- The data presented demonstrates that SLA is capable of inducing both Th1 and Th2 immunity along with high levels of CTL activity in immunised mice. The antigens present in the SLA could be fractionated and assessed for their individual ablity to induce Th1 or CTL activity important in generating immunity to the parasite. Active fractions could be analysed by mass spectrometry and used to search data bases to discover the identity of the protein and gene.

2- Gene gun immunisation was shown to be the most effective in inducing immunity to the leishmania parasite compared to other methods of immunisation. Gene gun immunisation with *L. mexicana* gp63 could be used in combination with other immunogenic vaccines such as SLA or viral vaccines. Intramuscular injection of DNA or electroporation injection in prime-boost studies and this would form the basis of an extensive vaccine program.

3- Our results indicate the presence of CTL activity following both gene gun and SLA immunisation, which is a key factor for developing an effective immunity. For immunisation with the DC + SLA, CTL activity occurred but was not correlated with protection. Further study to understand the mechanisms behind these contradictory results is required, including the use of MHC class I knock out mice or administration of anti-MHC class I antibody together with the gene gun immunisation.

4- The down-regulation of the MHC class I molecules in DCs induced by the parasite is another finding that could be extended to include other cell lines, particularly macrophages. It is important to know if the down-regulation of the MHC class I plays a role in decreasing the immunogenicity of the vaccines. Moreover, it is important to define the mechanisms used by the parasite to down-regulate the MHC class I molecules; preventing this action by the parasite may lead to enhancing immunity. The effect of infection with other species of leishmania parasites in down regulating MHC class I expression also needs to be determined. The effect of leishmania infection on cytokine production and expression of other co-stimulating molecules by DCs or other cells such as macrophages needs to be determined at the gene and protein expression levels. Our preliminary results showed that infection with *L. mexicana* did not downregulate the expression of MHC class II (data not shown),

which is in agreement with other studies. However, this needs to be confirmed by further studies.

5- In this study MBI and MEP were used to optimise the purification of the OX40L-IgG fusion protein. It was shown that those resins have the potential to purify the OX40L-IgG fusion protein from B9B8E2 cell culture supernatant. The potency of MBI and MEP resins in purification of other fusion proteins should be investigated. Furthermore, the use of OX40L in vaccine studies is fairly a new approach in leishmania research and the potency of the OX40L in enhancing the immunogenicity of gene gun immunisation and other methods of immunisation should be further investigated.

Reagent Apendix

Reagents

Culture Media	Company
DMEM	Bio Whittaker, Europe
1640 RPMI	Bio Whittaker, Europe
Schneider	Sigma

Supplements added to Culture Media	Company
Foetal Calf Serum (FCS)	Bio Whittaker, Europe
glutamine synthetase (GS) and 10%	IRH Biosciences
Bio-FCS (FCS without bovine IgG)	Autogen Bioclear, UK Ltd.
2-mercaptoethanol	Bio Whittaker, Europe
Penicillin/Streptomycin	Bio Whittaker, Europe
HEPES buffer	Bio Whittaker, Europe
Fungizone	Bio Whittaker, Europe
Geniticin (G418)	Bio Whittaker, Europe

Other Reagents	Company
Trypsin	Gibco, UK
Versene	Gibco, UK
Heparin	Sigma, UK
DNAase	Sigma, UK
Collagenase	Calbiochem, UK
Trypan Blue	Sigma, UK
Lipopolysaccharide	Sigma, UK

Molecular Grade Chemicals	Company
Molecular Grade Water	Sigma, UK
Absolut Ethanol	BDH, UK
Isopropanol	Sigma, UK
RNA Stat 60	AMS Biotechnology, UK

Chloroform	Sigma Aldrich
Agarose	Bioline
Tryptone	Oxoid
Yeast	Oxoid
Bacteriological Agar	Oxoid
Sodium Chloride	Sigma
Kanamycin	Sigma
Tetracyclin	Sigma
Ampicillin	Sigma
Phenol-Chloroform IsoAmyl Alcohol	Sigma
Absolute Ethanol	BDH
α-Chymotrypsin	Sigma Aldrich
Aprotinin	Sigma Aldrich
BSA	Sigma
Sucrose	BDH Lab Supplies
PBS tablets pH 7.2-7.4	OXOID
Acetic Acid	Fisher Scientific Ltd
Tween 20	Promega
Sodium azide	Sigma
Trypan Blue	Sigma
Ethidium Bromide	Sigma
Sodium Chloride	Fisher Scientific Ltd
Sodium Hydroxide	Fisher Scientific Ltd
Tris	Fisher Scientific Ltd

Other Reagents	**Company**
EMLA Anaesthetic Cream	Astra Zeneca, UK
Chromium 51	Amersham
Incomplete Freunds adjuvant [Rafati et al.]	Gibco

T cell Media

Ingredients	Quantity
Complete RPMI	500 ml
10% FCS (by volume)	50 ml
Glutamine	5 ml
20 mM HEPES	10 ml
50 μM 2 Mercaptoethanol	500 μl
50U/ml Penicillin/Streptomycin	5 ml
0.25 μg/ml Fungizone	500 μl

BM-DC media

Ingredients	Quantity
Complete RPMI	500 ml
10% FCS (by volume)	25 ml
Glutamine	5 ml
20 mM HEPES	10 ml
50 μM 2 Mercaptoethanol	500 μl
50U/ml Penicillin/Streptomycin	5 ml
0.25 μg/ml Fungizone	500 μl

PBS-BSA wash for FACS

Ingredients	Quantity
PBS tablets	10/litre
BSA	0.1% (1g/litre)
Sodium Azide	0.02% (0.2 g/litre)

RIP Buffer

Reagent	gm/500 ml	mM

Sodium Chloride	4.38	150
Tris	3.027	50
EDTA, anhydrous	0.931	5

Western Blot Lysis Buffer

Ingredients	Quantity
RIP Buffer	5 ml
Igepal	50 µl
Deoxycholate acid	25 mg
10% SDS	50 µl
500 mM Benzamidine	10 µl
100 mM PMSF	5 µl
200 mM Sodium Valproate	25 µl
1 M Sodium Fluoride	5 µl

Other Buffers

Buffer	**Composition**
PBS	1 tablet dissolved in 100 ml distilled water
PBA	PBS 0.1% (w/v) BSA 0.02% (w/v) Sodium Azide
TBS	10mM Tris 150nM NaCl pH 7.4
1 x TAE Freshly prepared from 10x TAE	40 mM Tris Acetate 1 mM EDTA

RT-PCR Enzymes, Restriction Enzymes and Reagents

Reagent	**Company**
M-MLV-RT	Promega
Oligo dT Primers	Promega
RNasin Inhibitor	Promega
Taq Polymerase	Bioline
T4 Ligase Enzyme	Promega
EcoRI Restriciton Enzyme	Promega
BamHI Restriciton Enzyme	Promega
HindIII Restriciton Enzyme	Promega
Pfu Polymerase	Promega
Phusion Taq polymerase	Finnzyme
pcDNA3 plasmid	Invitrogen
SYBR Green Master Mix	Biorad
dNTPs	Bioline
DNA ladder (1Kb plus)	Invitrogen
10X Reaction Buffer	Promega
Magnesium Chloride	Promega

Antibodies and Kits

Cell line/Antibody	**Source/Manufacturer**
Goat anti-mouse FITC	Sigma
HB54 (HLA-A2.1)	Hybridoma
Rabbit Anti Goat-HRP antibody	DAKO
CD80	Cambridge Biosciences
CD40	Hybridoma FGK-45
CD25-FITC	Serotec
CD11c	Hybridoma

Rabbit anti-mouse IgG1	Serotech
Rabbit anti-mouse IgG2a	Serotech
goat anti-rabbit IgG – HRP	Serotech
Streptavidin – HRP	Zymed, USA
Goat Anti-Mouse – HRP	DAKO
Anti-Rat FITC	Serotec
Anti-hamster FITC	Serotec
Anti-Goat FITC	Sigma
Mouse IFN-γ ELISA kit	R&D Systems, UK
Mouse IL-4 ELISA kit	R&D Systems, UK
Mouse CD8: Dynabeads	Dynal, Europe
MBI resin	Pall Biosepra Ltd, France
MEP resin	Pall Biosepra Ltd, France
Protein A	Sigma

Laboratory Plastic ware, glass ware and sharps

Item	**Company**
T25 and T75 tissue culture flasks	Sarstedt, UK
50 ml screw top tubes	Sarstedt, UK
10 ml and 5 ml pipettes	Sarstedt, UK
20 ml Universal tubes	Sterilin UK
Centrifuge Tubes (15ml)	Sarstedt, UK
Bijou tubes (7 ml)	Sterlin, SLS, UK
FACS tubes	Elkay, UK
10 ml syringes	Becton Dickenson
BD Microlance 3 needles	Becton Dickenson
24 well and 6 well flat bottom culture dishes	Sarstedt, UK
96 well round bottom plates	Sarstedt, UK
Pasteur pipettes	Sarstedt, UK
1.5 ml eppendorf tubes	Sarstedt, UK
0.5 ml eppendorf tubes	Sarstedt, UK
1.2 ml Cryovials	TPP, UK

Pipette tips < 1ml	Sarstedt, UK
96 well ELISA plates	Costar, UK
Petri dishes	Sterilin UK
25 ml Pipettes	Sarstedt, UK
10 ml Pipettes	Sarstedt, UK
5 ml Pipettes	Sarstedt, UK
Haemocytometer	Weber
96 well plate harvester filters	Perkin Elmer
Scalpels	Swann Morton Ltd.
PCR Tubes	Micronic Systems
0.2µm Filters	Sartorius, UK
Realtime PCR tubes	Strategene, Germany
0.5 – 10µl tips	Sarstedt, UK
20 – 200µl tips	Sarstedt, UK
200 – 1000µl tips	Sarstedt, UK

Electrical Equipment

Equipment	**Manufacturer**
Refrigerated centrifuge	Mistral 1000, MSE
Flow Cytometer	Beckman Coulter
Clenz	Beckman Coulter
Isoton	Beckman Coulter
Liquid Nitrogen Freezer	Forma Scientific
-80°C Freezer	Ultima II, Revco
Class II safety cabinets	Walker
37°C incubator	Forma Scientific
96 well plate harvester	Packard
Light microscope	Olympus
96 well plate reader	Tecan
Top count scintillation counter	Packard
Drying Cabinet	Scientific Laboratory Supplies Ltd

PCR Thermal Cycler	Hybaid, Germany
Water Baths	Grant Instruments
Real Time PCR Thermal Cycler	Bio-rad
Microscope	Nikon
Power Packs	Bio-rad
Electrophoresis gel tanks	Bio-rad
Microwave	Matsui
UV Spectrophotometer	Sanyo
Transilluminator	Ultra Violet Products
Whirlimixer	Scientific Industries

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