CHAPTER 2

Immunogenicity and efficacy of a multi-antigen MIDGE vector based DNA vaccine against experimental cutaneous leishmaniasis model in C57BL/6 mice.
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INTRODUCTION:

Different species of *Leshmania* genus differ in many parasite properties- tissue tropism, capacity for immune-evasion and persistence, temperature sensitivity etc. This leads to different dissemination properties- a) visceralizing species colonize liver, spleen and bone marrow (manifesting as Kala-azar); b) some species reside in multiple cutaneous sites, nasal and oropharyngeal mucosa causing cutaneous leishmaniasis. The cutaneous leishmaniasis can be categorized further depending on clinical manifestation: ulcerative skin lesions developing at the site of the sandfly bite (localised cutaneous leishmaniasis [LCL]); multiple non-ulcerative nodules (diffuse cutaneous leishmaniasis [DCL]); and destructive mucosal inflammation (mucosal leishmaniasis). Cutaneous leishmaniasis is endemic in more than 70 countries worldwide, and 90% of cases occur in Afghanistan, Algeria, Brazil, Pakistan, Peru, Saudi Arabia, and Syria. Surveillance data indicate that the global number of cases has increased during the past decade, as documented in Afghanistan, Bolivia, Brazil, Colombia, Peru, and Syria. The global burden of cutaneous leishmaniasis is likely to be underestimated as many infections are symptomless or misdiagnosed.

The most common agent of cutaneous leishmaniasis in the Old World is *L. tropica* and *L.major*, in the New World *L. mexicana* and *L.braziliensis*. It has been well recognized that there is a clinical and histological spectrum in cutaneous leishmaniasis [492]. The spectrum and its variability are dependent on a number of factors such as the type and duration of clinical lesion, strain of organism, geographic location, size of the initial inoculum, host immunity etc [492]. The disease manifestation depends on the species of *Leishmania*- i.e. *L. major*, *L. tropica* mainly occupy lesions in the skin whereas *L. braziliensis* occupies and destroys mucous membrane. There are differences in *L. tropica* and *L. major* caused pathology as well. *L. tropica* infection in Middle-East results in a single dry ulcer [493] but *L. major* infection causes multiple weeping lesions [494].
There is an evolution of lesions as they progress from a papule/nodule, into a soft, boggy, crusted plaque/nodule. The lesion then breaks open, after a varying period of 3-4 months into a well circumscribed ulcer, which heals slowly over a period of 3-12 months. Occasionally, the healing does not take place and the lesion may then persist as a chronic yellow-brown firm nodule [495].

Studies have shown that resistance or susceptibility to \textit{L. major} infections in mice is dependent on the type of CD4+ helper T cell (Th) subset that is induced [496, 497]. Healing in resistant mice infected with \textit{L. major} is associated with the development of Th1 cells that produce IFN-\gamma, a crucial cytokine that is necessary for activation of macrophages for the production of nitric oxide (NO) the key effector molecule for destroying intracellular amastigotes [498, 499]. Both the initial development and maintenance of Th1 cells in \textit{Leishmania} infected mice is dependent on the presence of IL-12 produced by infected dendritic cells [500]. In contrast, in the highly susceptible BALB/c mice, there is an early IL-4 production by a unique population of CD4+ T cells that promotes the development and expansion of Th2 cells [500]. Another key cytokine that has been shown to regulate disease outcome is IL-10. IL-10 gene deficient mice are highly resistant to \textit{L. major} [166] and overexpression of IL-10 renders resistant mice susceptible [501]. IL-10 mediates its effect by blocking the activating effects of IFN-\gamma on infected macrophages thereby preventing the production of parasiticidal NO [317]. In addition to deactivating macrophages and inhibiting intracellular parasite killing, IL-10 also directly inhibits the development of Th1 cells and their production of IFN-\gamma [502, 503]. Both macrophages [504] and CD4+ Th2 cells [316] have been shown to be important sources of IL-10 in \textit{Leishmania}-infected mice.

The main problems in treating cutaneous leishmaniasis are that clinical diagnosis is difficult in the absence of microscopy at the basic health-care level, and pentavalent antimonial drugs can have serious, although usually reversible, side-effects (e.g. musculo-skeletal pains, renal failure, hepatotoxicity, and cardiotoxicity) [90] and are of variable efficacy against mucosal leishmaniasis [505]. Drugs and medical attention because of the side-effects make treatment expensive, and reports on patients nonresponsive to the drugs either because of
drug-resistant parasite strains [506] or to immunosuppression (i.e., caused by HIV) [507] are increasing.

Moreover, the invasiveness of the standard treatment protocol (i.e., a lengthy course of intramuscular or intravenous injections) means that many patients fail to complete their full course of treatment [508]. Although treatment is available, the drugs are very expensive, toxic (cause over 20% morbidity in treated patients) and there is increasing incidence of drug-resistant Leishmania strains. Hence, to reduce systemic toxic effects, economic cost, and poor treatment compliance, most research in the past decade has focused on the development of alternative dosage schedules, modes of delivery (i.e. parenteral vs local, or topical vs oral), or treatments.

The rationale for vaccine development is provided by the evidence that most individuals that had leishmaniasis or symptomless infection are resistant to subsequent clinical infections. Attempts at vaccination against cutaneous leishmaniasis can be traced back hundreds of years. Following the realization that most cutaneous leishmaniasis usually present as benign ulcers that heal spontaneously and is accompanied by protection from reinfection, the ancient Middle Easterners started the practice of deliberately exposing uninfected individuals to sand fly bites or to infectious materials obtained from active lesions. This practice known as leishmanization has been used for centuries and a more standardized form of leishmanization is still in use in certain areas [359]. Substantial effort has been spent in developing a leishmania vaccine, an effort that has so far remained fruitless. However, for several basic and logistic problems (e.g., difficulties in maintaining parasite virulence, risk of unacceptable lesions in some recipients), leishmanisation is not currently recommended by WHO. Its use is restricted to a few countries (e.g., Uzbekistan), notably as an evaluation method of new leishmaniasis vaccines. Several vaccination trials in humans using killed Leishmania parasites yielded very disappointing results [352]. In murine studies, several experimental vaccines are effective, but many of them rely on IL-12, or components that induce IL-12, as adjuvant [439, 440]. However, as with killed Leishmania vaccines, the protection wanes with time. In order to harness the desirable attributes of leishmanization (parasite persistence
and durable immunity) without the potential safety concerns [509, 510], vaccination strategies involving attenuated parasites with defined genetic mutations to remove virulence have been taken.

These observations highlight the need of a new vaccine for cutaneous leishmaniasis. In this chapter I have again chose the LEISHDNAVAX vaccine formulation which was described at length in the previous chapter to evaluate against CL. With this aim the multivalent DNA vaccine, LEISHDNAVAX, was assessed for its preventive efficacy against *L. major* infection in murine model. We have used C57BL/6 mice and experimental challenge with *L. major* to evaluate the immunogenicity and efficacy of the vaccine in this model. Our studies reveal that the vaccine formulation induces both humoral and cellular immunity but only partially protects the animals from experimental infection.
MATERIALS AND METHODS:

Animals:
Female C57BL/6J mice (Charles River, UK) were maintained at the Royal Veterinary College (RVC), London, under specific pathogen free conditions. The age of mice when receiving the first immunization was 8-10 weeks. All the animal experiments had been reviewed and approved by the responsible institutional ethics committees.

Immunization:
Groups of mice received PBS, LEISHDNAVAX or vector control by intradermal (i.d.) injections (25 µl, base of tail) using 29G needles (BD Microfine Plus Insulin Syringes, single use). Animals were immunized three time at 14 day intervals.

LEISHDNAVAX is an equimass mixture of five MIDGE-Th1 vectors (in PBS), each encoding a distinct Leishmania antigen: KMP11, TSA, P74, CPA or CPB. The vector control was non-expressing human IL-2 encoding MIDGE-TH1 (MCV1.4-hIL-2 ATG -Th1). Both LEISHDNAVAX and vector control were administered at a dose of 100µg total DNA / injection (20µg DNA per antigen, respectively).

Parasite challenge:
To assess protective efficacy of LEISHDNAVAX groups of mice were challenged by s.c. injection (pre-shaved rump) of $5 \times 10^6$ L. major (MHOM/SA/85/JISH118) stationary phase promastigotes in 100 µl Schneider's medium (Sigma) without serum using 25G needles. Stationary phase of promastigotes was confirmed by daily counting of promastigote cultures. Infected mice were monitored for lesion development and lesions measured with a digital Vernier Caliper in 2 directions; one direction in parallel to the spine, the other direction at a perpendicular angle. The mean of the two diameters was taken as the mean lesion diameter for that lesion. Mean diameters for a group of mice were based on mean diameters of individual lesions.
Immunogenicity - preparation of peptides pools:
Stock solutions of peptides in DMSO (300 µg in 15µl; 20 mg/ml) were stored at -80ºC in a temperature monitored freezer. Peptide pools were prepared to achieve peptide concentrations in pools for use at 5 µg/ml/peptide in 100 µl final assay volume with a maximum final DMSO concentration of 1%. The peptide pools spanned the entire length of the five antigen sequences in LEISHDNAVAX. The pools termed KMP-11, TSA, CPA-1, CPA-2, CPB, P74-1 and P74-2 each were mixtures of 19, 38, 35, 34, 30, 44 and 44 peptides, respectively, in cell culture grade DMSO (Sigma). Working solutions of peptide pools were prepared in RPMI 1640 medium without serum by rapid dilution of peptide pools into medium under vortexing. Fifty µl of peptide pool / stimulation were added to each well of U-bottom 96-well plates (BD Falcon). The actual DMSO concentration in assays was kept constant in all stimulations. DMSO used to adjust concentrations was taken from sealed ampules freshly opened for this purpose. Negative and positive controls (DMSO and ConA at 5µg/ml respectively) were included.

The composition of the peptide pools is given in the appendix.

IL-2 ELISA. The spleen cells were plated in triplicate at 5x10^5 cells per well and stimulated with peptide pools as mentioned above. Plates were kept in humidified CO₂ incubator at 37ºC for 24 hours. After that the supernatant was collected and IL-2 was measured with an ELISA kit (BD-Pharmingen) as per the manufacturer’s instruction.

IgG ELISA. IgG and subclass IgG1 and IgG2a ELISAs were carried out on serum samples as described in the protocol on the secure website. Briefly plates were coated with recombinant proteins at a concentration of 5µg/ml in PBS and a volume of 100µl / well added. Washing steps were carried out with PBS and blocking with 5% BSA in PBS. Serum and detection antibody dilutions were added in assay diluent (5% BSA in PBS) and plates developed by addition of TMB substrate. Colour development was stopped after 15-20 minutes by addition of stop reagent and absorbance read at a wavelength of 450nm in a SPECTRAmax GEMINI Microplate Spectrofluorometer. To detect antigen
specific whole IgG in C57BL/6J mice, the sera dilution used was 1:250 and detection antibody (anti-mouse whole IgG peroxidase, Sigma) was used at a dilution of 1:3000. Subclass IgG2a and IgG1 ELISAs were carried out with sera dilutions of 1:100 and both detection antibodies (anti-mouse polyclonal IgG1:HRP and IgG2a:HRP, Serotec) used at a 1:1000 dilution.

**Statistical analysis.** Statistical level of significance between different groups was determined with the two-tailed Student $t$ test using GraphPad Prism (Version 6) software. Results with $p<0.05$ were considered statistically significant.
RESULTS:

Monitoring cellular immune responses of T cells - IL-2 production

Immunization with LEISHDNAVAX at 20µg per antigen dose in C57BL/6J mice was done three times at 14 days intervals. 8 days after final immunization spleen cells were isolated from PBS, vector control and LEISHDNAVAX injected animals and were restimulated with peptide pools corresponding to the coded antigens. In these experiments only the splenocytes of the vaccine inoculated groups responded to antigen recall by secreting IL-2, a cytokine that serves as marker for T-cell activation and also triggers T-cell proliferation. PBS or vector control injected mice did not respond to antigen recall. The KMP-11 pool stimulated the least amount of IL-2. All other pools stimulated appreciably more amounts of IL-2 with CPB producing the highest response (20-fold increase compared to control). There was no significant difference in IL-2 production between the vector control and PBS control groups, excluding antigen specific IL-2 production in these groups (Figure Ch2-1).

Figure Ch2-1: IL-2 production by T cells from mice immunized LEISHDNAVAX. C57BL/6J mice were immunized with different doses of DNA vaccine as summarized in Fig. 3A, and splenocytes were prepared 8 days after of the last immunization and cultured in the presence of peptide pools corresponding to sequence of coded antigens. The peptides for CPA and P74 were divided into two separate pools. The final concentration of each constituent peptide in the pools was 5 µg/ml. The T cells responses to the peptide pools were correlated with the IL-2 levels in the culture supernatant measured by ELISA. Statistical significance between vaccine injected and PBS injected groups are indicated by asterisk. “*” - p<0.0001, “**” - 0.0001<p<0.05
Monitoring humoral immune responses - IgG ELISAs

Humoral responses following immunization were assessed by detecting the presence of antigen specific antibodies in sera of different groups of mice. Sera was collected from the three different groups of mice (PBS, vector control and LEISHDNAVAX injected respectively) and presence of antibodies specific to the 5 coded antigens KMP-11, TSA, CPA, CPB and P74 was measured by ELISA. Antibodies against all five recombinant antigens were detected in the sera of LEISHDNAVAX injected C57BL/6J mice (Figure Ch2-2A). The highest level of antibody production was seen against CPB. This was followed by the TSA and CPA specific antibody levels. Antibodies towards the KMP-11 and P-74 were least abundant.

In addition to the detection of whole IgG, sera from C57BL/6J mice were analysed for detection of antigen specific IgG2a and IgG1 antibodies. Subtyping of antigen specific antibodies in C57BL/6J mice reveals that there is almost exclusive production of IgG2a antibodies in the LEISHDNAVAX injected mice. There was no significant increase in antigen specific IgG1 antibodies following immunization in any of the groups. Data is represented as Ig2a/IgG1 ratio in Figure Ch2-2B where higher values in vaccinated group indicate a Th1 biased response in contrast to PBS control.

Figure Ch2-2: Antibody responses induced by LEISHDNAVAX in C57BL/6 mice. Animals were immunized with the indicated doses of the DNA vaccine, PBS or vector control, sera prepared 8 days after the final immunization and tested for specific antibodies by ELISA with recombinant KMP11, TSA, CPA, CPB and P74. A) Experimental scheme. B) Total IgG induced against the 5 antigens depending on vaccine dose. C) IgG2a/IgG1 ratio of antigen specific antibody levels induced with the highest vaccine dose compared to PBS injection. “*” indicated statistical significance at p<0.0001 between vaccine and PBS injected groups.
Lesion development – C57BL/6J mice

Three different groups of mice received PBS, vector control or LEISHDNAVAX (20 µg/antigen dose) 3 times at 14 day gaps. 10 days after the last immunization the animals were challenged with *L. major* parasites. After that development of skin lesions at the site of parasite inoculation was monitored for 25 days. During the measurement of lesions the appearance of skin was noted as well. In some cases no lesions were measurable, but a difference in skin colour or other slight changes in the skin condition around the injection site noted. If possible these sites were also measured for size. Additionally it was noted if lesions were papular / nodular (raised with distinct borders) or flat.

Analysis was based on two parameters: 1) The number of animals / group which developed lesions and 2) the mean lesion diameter including standard deviation for each group (PBS treated, LEISHDNAVAX treated and vector control treated). Mice which had to be killed during the analysis due to animal welfare regulations were kept in the analysis as mice which developed lesions and their lesion size included as the last measured size in all subsequent time points.

When all measurements (and corresponding mice) were included in the analysis a maximum of 85% of mice developed lesions by day 20 post infection in the PBS group. This number decreased to 79% over the following days (Day 25 post infection), which is in line with the healing characteristics of this mouse model. Maximum of 77% of animals in the vector control injected mice developed clear lesions. There was no significant difference in the percentage of animals with lesions between the PBS and vector control groups. Whereas, in LEISHDNAVAX treated group only 21% of animals were recorded to have lesions by day 15 post infection. This number increased to 42% animals with lesions at 25 days post infection. Thus it appears that although not completely, but LEISHDNAVAX offers some degree of resistance to lesion development in cutaneous leishmaniasis model in C57BL/6 mice.
A second analysis was undertaken by measuring the size of the lesions in animals from different groups. Lesions were included in the analysis if they were papular / nodular (i.e. raised with distinct borders) or the skin showed definite scars and re-epithelialisation as sign of healing. All animals which did not meet these criteria (i.e. without clearly demarcated lesions) were excluded from the analysis. In the PBS group, the mean lesion diameter among all the animals reached 8.3 mm at day 25 post-infection, increasing from 7.5mm on day 15. In the vector control group also the mean lesion diameter reached 8.9mm at the day 25. At this time point the mean lesion size in the LEISHDNAVAX treated group was 7.7mm. The results of lesion size show no statistical significance between any of the three groups. Hence it is evident that the even in the LEISHDNAVAX immunized group, the animals that do develop clear lesions are unable to check its growth.
and hence the disease pathogenesis. In the LEISHDNAVAX immunized group, some of the animals limit the emergence of skin lesions following parasite challenge. But those that cannot do the same, their skin lesions grow to the same extent as the control groups receiving PBS and vector DNA.

Figure Ch3-4: Mean lesion diameter among different vaccination groups. It contains pooled data points from 2 independent experiments.
DISCUSSION:

The mechanism of resistance to cutaneous leishmaniasis has been characterized over the years, both the role of innate as well as adaptive immune system. Following the identification of distinct mouse CD4+ T helper cell subsets by Mosmann et al. [511], it was demonstrated that IFN-γ production by CD4+ T cells was associated with healing of *L. major*-infected C57BL/6 mice, while IL-4 production was associated with susceptibility in the BALB/c mice [511]. Scott *et al.* demonstrated that adoptive transfer of polarized T cell clones can change the outcome of *L. major* infection: Th1 clones were “protective” while Th2 clones were “non-protective” [178]. Holaday *et al.* (1991) further confirmed this finding by transferring Th1-like or Th2-like cell lines into SCID mice, which resulted in the recipient mice becoming resistant or susceptible, respectively [512]. Thus, the balance of Th1/Th2 cytokines determines disease outcome in mouse model of CL: healing in resistant mice is associated with the development of CD4+ Th1 cells that produce IFN-γ whereas susceptibility is associated with an early IL-4 production by CD4+ T cells that promotes the development and expansion of Th2 cells [513].

Although the involvement of Th1 response and its contribution to disease resistance is well established, there is still no universally acceptable, safe, and effective vaccine against human leishmaniasis. Several vaccination trials in humans using killed *Leishmania* parasites yielded very disappointing results [352]. In murine studies, several experimental vaccines are effective, but many of them rely on IL-12, or components that induce IL-12, as adjuvant [439, 440]. However, as with killed *Leishmania* vaccines, the protection wanes with time. These studies suggest that we need to know more about the requirements for maintenance of anti-*Leishmania* immunity in order to better define the correlates of protection.

Several *Leishmania* protein antigens have been used as subunit vaccine candidates against leishmaniasis. Vaccination with Leish-111f, a recombinant polyprotein vaccine that contains thiol-specific antioxidant (TSA), *L. major* stress inducible protein 1 (LmST11) and *L. major* elongation initiation factor (LeIF) was shown to protect against both visceral and CL [415]. Phases 1 and II clinical
trials for Leish-111f vaccine have been completed and show that the vaccine is safe and immunogenic in both healthy and adult patients with mucocutaneous and CL [514]. This vaccine has also been used therapeutically in combination with sodium stilbogluconate for treatment of mucosal leishmaniasis [514] and in combination with meglumine for the treatment of human CL [515]. A more recent study showed that the polyprotein comprising of kinetoplastid membrane protein 11 (KMP11), Sterol 24-c-methyltransferase (SMT), A2, and cysteine proteinase B (CPB) given with monophosphoryl lipid A (MPL-SE) as adjuvant was able to protect mice against visceral and CL caused by *L. infantum* and *L. major*, respectively [515].

DNA vaccine is also attractive for cutaneous leishmaniasis since the induction of Th1 responses is also a general property of DNA vaccines [439, 465]. DNA vaccines are relatively simple to produce, administer and under appropriate conditions, are highly immunogenic. This is because they present a protein that is usually correctly folded and structurally similar to the native protein. As a result, such vaccines are able to elicit high antibody and T cell (including CD4+ and CD8+) immune responses. The gene encoding gp63 was the first to be used as a DNA vaccine. Mice immunized with plasmid expressing gp63 protein developed strong Th1 responses as well as significant resistance to infection with *L. major* [516]. More recently, a comparative study evaluating different DNA vaccine candidates including gp63 showed that protection was transient, and immunized mice would eventually develop lesions similar to those observed in controls [517]. LACK is the most extensively studied DNA vaccine against both cutaneous and visceral leishmaniasis. Protective immunity against *L. major* was also achieved following delivery of LACK in a minimalistic, immunogenically defined gene expression (MIDGE) vector with lower doses of DNA required for protection [469].

There is an in-depth understanding of the immunobiology of leishmaniasis and studies in this area of research have helped shed lights into the factors that regulate the induction, maintenance, and loss of cell-mediated immunity in infectious diseases. Therefore, it is very frustrating and disappointing that despite this enormous wealth of information, there is currently no generally and globally
acceptable, effective, and efficacious vaccine against the disease in humans. The reasons for this failure are many, but primarily related to the obvious differences between mouse and human immune systems. In addition, the use of different vaccination protocols (nature of adjuvants, frequency of vaccination, and/or boost, time before challenge) and arbitrary markers or correlates of protection in murine studies have complicated the situation [518]. Researchers tend to select vaccination protocols that most likely will yield desirable results in mice studies, which are unrealistic in clinical settings and/or “real world” environment. Therefore, it is important for researchers in this field to set standards for vaccination studies, such as the time from immunization to challenge and the minimum duration of immunity before any experimental vaccine and/or vaccination protocol is deemed protective. In addition, most of the vaccination studies (particularly CL) utilize BALB/c mice, which do not mimic the clinical disease in humans. The CL in the C57BL/6 mice more closely resembles the human disease and hence it is imperative that vaccination studies be conducted in this strain of mice.

The candidate vaccine LEISHDNAVAX was designed to include antigens that are conserved among parasites that cause visceralizing and cutaneous forms of the disease. Here we have established the immunogenicity of the vaccine in C57BL/6J mice and the fact that it is also able to partially protect against *L. major* infection. From the results it seems that further studies are required to optimize this vaccination protocol and formulation so mice are completely protected against virulent challenge. But this vaccine is optimized for expression in humans and as discussed previously, optimization of the vaccine formulation is best done during the course of clinical trials in humans due the inherently different immunogenetics between humans and laboratory animal models. This study serve as proof-of-concept that LEISHDNAVAX vaccine formulation elicits immune response in C57BL/6 mice and this immune activation can offer a low degree of resistance to development of lesions following *L. major* infection.