CHAPTER 4

Vaccination with Leishmania Soluble Antigen and Immunostimulatory Oligodeoxynucleotides Induces Specific Immunity and Protection Against Leishmania donovani Infection
1.0 Introduction

*Leishmania* are protozoan parasites that cause leishmaniasis. Worldwide 10-15 million people are infected with 2 million new cases each year and 1/10 of world population is at risk of infection. (Baily *et al*., 1994, Herwaldt., 1999). In a recent survey, 88 countries have been declared as leishmaniasis-endemic (WHO., 1997, TDR News., 1998). *L. donovani* causes the severe, often fatal disease visceral leishmaniasis (VL). The drugs that are commonly used for the treatment of VL are pentavalent antimonials. Lately there has been an increase in the cases of leishmaniasis refractory to antimonial treatment. Both human and mice develop protective immunity after recovery from VL. While the development of an anti-leishmanial vaccine appears feasible, it remains to be accomplished.

Several candidate vaccine molecules have already been evaluated in the mice model for leishmaniasis. Considerable attention and efforts toward vaccination against leishmaniasis have focused almost exclusively on localized cutaneous disease (Alexander., 1982, Campos-Neto *et al*., 2001, Howard *et al*., 1982, Howard *et al*., 1984, Mayrink *et al*., 1979, Mayrink *et al*., 1986). Purified antigens, subcellular fractions and avirulent parasites have been used as potential vaccine candidates for cutaneous leishmaniasis (Handman., 2001). DNA vaccines have also been reported to induce protective immunity in a variety of experimental mouse models of infection through MHC class I and class II-restricted T-cell responses (Gurunathan *et al*., 1997, Sukumaran *et al*., 2003, Sjolander *et al*., 1998). While a substantial progress has been made no acceptable antileishmanial vaccine exists against this infection. Presently there is no immunoprophylactic regimen for leishmaniasis although several human trials using killed leishmanial promastigotes with *Mycobacterium bovis* BCG are going on and initial results look promising (Cabrera *et al*., 2000).

The mouse models of infection with the protozoan parasite *L. major* have helped to define the Th1/Th2 model. Resistant C57BL/6 mice develop protective Th1 responses that control infection, whereas susceptible BALB/c mice are unable to control infection due to an aberrant Th2 response (Julia *et al*., 1996, Launois *et al*., 1997). Treatment and vaccination that control the disease in susceptible mice invariably promote Th1 responses over Th2 responses (Sukumaran *et al*., 2003, Diego-Miralles *et al*., 1994, Kemp *et al*., 1993). There is no clear-cut immunological regime for *L. donovani*. In mice infected with *L. donovani*, the differential production of Th1 and Th2 does not control the cure rate, although Th1 response correlates with resistance to
infection, the Th2 response does not determine susceptibility (Kaye et al., 1991). In human disease, there is a trend that both Th1 and Th2 responses against leishmaniasis coexist in humans who have been cured of visceral leishmaniasis (Kemp et al., 1993, Karp et al., 1993).

Certain sequences of bacterial DNA and immunostimulatory CpG DNA can modulate immune response towards Th1 type (Halpern et al., 1996, Klinman et al., 1996, Kreig et al., 1995, Sato et al., 1996). The vertebrate immune system can be activated by a variety of microbial components. Among these component CpG dinucleotide in particular base context are found in bacterial DNA. Once recognized CpG motifs activate a wide variety of innate immune responses. (Kreig et al., 1995, Klinman et al., 1999). This stimulatory capacity could be transferred to single-stranded oligodeoxynucleotides (ODN) containing specific sequences called as CpG-ODN. These CpG-ODN directly activate monocyte, macrophages and dendritic cells to secrete Th1 like cytokines (e.g. IL-12, TNF-α and IL-6 etc) and express increased level of cell surface costimulatory molecules (Klinman et al., 1996, Kreig et al., 1995, Ballas et al., 1996, Cowdery et al., 1996, Messina et al., 1991, Tokunaga et al., 1984, Weiner et al., 1997). These CpG-ODNs have been used as adjuvants with a range of protein vaccines and they facilitate a Th1 mediated response, where as conventional protein vaccines alone normally result in Th2 response with high titers of neutralizing antibodies and poor cellular immunity (Weiner et al., 1997, Chu et al., 1997, Davis et al., 1998, Lipford et al., 1997, Roman et al., 1997, Sun et al., 1998, Rhee et al., 2002, Zimmerman et al., 1998). The potent Th1 like immune activation by CpG ODNs suggests a possible utility for vaccination against leishmaniasis.

The role of CpG ODN as a prophylactic vaccine adjuvant has been studied using leishmanial proteins in combination with CpG ODN and has been reported to confer some protection after a challenge with L. major and L. donovani (Rhee et al., 2002, Walker et al., 1999, Stacey et al., 1999, Tewary et al., 2004). CpG ODN is reported to be more potent and durable than IL-12 protein in terms of immune and biological effects in susceptible BALB/c mice after infection with L. major (Rhee et al., 2002)

In view of the potent in vivo effects on the cellular immune response elicited by CpG ODN, we have examined the protective efficacy of immunostimulatory DNA or CpG motifs as immune adjuvants when administered with Leishmania donovani soluble antigen in a murine model system. We have also tested the effect of CpG-ODN
in combination with the soluble antigen on humoral and cellular responses in BALB/c mice model of L. donovani infection.

2.0 Materials and Methods

2.1 Animals

Female BALB/c mice (4-6 weeks old) were obtained from the National Institute of Nutrition, Hyderabad, India. All mice were used at ages ranging from 6-8 weeks. Animals were used in accordance to the Institutional guidelines and the relevant committee duly approved the use of animals for this work.

2.2 Parasite culture

Leishmania donovani strain AG83 (MHOM/IN/1983/AG83) was maintained in BALB/c mice. Amastigotes were isolated from spleens, and then transformed to promastigotes in Medium 199 (Sigma, USA) supplemented with penicillin (100 units/ml Sigma, USA), 100 µg/ml streptomycin (Sigma, USA), and 30% fetal calf serum (FCS) (Biological Industries, Israel). Freshly transformed promastigotes were cultured at 22°C in M199 medium and 10% heat-inactivated FCS and were used for infecting BALB/c mice.

2.3 Source of soluble antigen

Soluble antigen was prepared from stationary phase L. donovani AG83 promastigotes. Cells were harvested by centrifugation at 4000 x g for 15 minutes at 4°C and the pellet was washed once with phosphate buffered saline (PBS), pH 7.2. The pellet was resuspended in PBS containing protease inhibitors and lysed by sonication with 6 pulses of 30 seconds each. The lysate was centrifuged at 17000 x g for 15 minutes at 4°C and the supernatant was used as the soluble antigen (SA) (Dole et al., 2000).

2.4 Oligodeoxynucleotides

A non CpG-ODN 1911 (5' TCCAGGACTTTCCTCAGGTT 3') and a CpG-ODN 1826 (5' TCCATGACGTTCCTGACGTT 3') were used in the present study. All oligonucleotides were synthesized with a nuclease resistant phosphorothioate backbone. (Microsynth, Switzerland). The ODNs were resuspended in 10 mM Tris, pH 7.0 /1 mM EDTA and stored at -20°C.

2.5 Immunization of mice and assessment of parasite load

Female BALB/c mice, 6-8 weeks of age, were used for all immunizations (n=5 per group). Injections were given at midpoint of left thigh muscle (Plate. 1) For intramuscular injection 50 µg of soluble antigen (SA) and 10 µg each of ODN in
phosphate buffered saline (PBS) was used. SA was adsorbed on alum. Control mice were injected with alum diluted with PBS (final volume 100 µl). For the vaccination studies, cell proliferation, cytokine production and antibody response, mice were immunized twice at two-week interval with alum, SA, CpG-ODN 1826 alone, SA plus CpG-ODN 1826, ODN 1911 alone and SA plus ODN 1911. Mice were bled 2 weeks following the final injection and sera from the mice in each group were pooled. For the vaccination and protection studies, mice were injected with alum, SA, CpG-ODN 1826 alone, SA plus CpG-ODN 1826 and a booster were given at 4-week interval. The mice were then challenged 2 weeks after the final boost. For the challenge experiment, 1x10⁸ stationary phase promastigotes of L. donovani were injected intravenously via the tail vein in 100 µl of PBS per mouse.

After 4 weeks and 8 weeks of infection, mice were euthanized and liver and spleen touch biopsies were microscopically examined after fixing and staining the slides with Giemsa. In order to quantitate levels of infection, Leishman Donovan units (LDU) were calculated as: (number of amastigotes /number of tissue nuclei) x weight of tissue in milligrams. Assessment of protection was performed using ten mice per group. Five mice from each group were sacrificed for determination of parasitemia both at 4 weeks and 8 weeks. The experiments were repeated twice with similar results.

2.6 Spleen cell proliferation assay

Mice were immunized twice at two-week interval with 50 µg SA, 10 µg of each of the ODN's either alone or in combination with SA (in 100 µl of PBS). Two weeks after the final injection, spleens were removed from mice under aseptic conditions on a sterile dish containing RPMI 1640 media. Single cell suspensions were prepared by grinding the spleen with disk bottom of the plunger of a 10 ml syringe. RPMI 1640 media (5-10 ml) was added to the suspension and the contents were mixed well. The dish was kept undisturbed for two minutes and the clear supernatant was pipetted out slowly. Cells were pelleted by centrifugation at 4°C at 250 x g (Sorvall RC-5 centrifuge, HB-4 rotor) for 10 min. Washing the pellet once with 0.9% ice-cold ammonium chloride lysed erythrocytes. The remaining cells were resuspended to a density of 2.5x10⁶ cells/ml in RPMI 1640 containing 10% FCS and then divided into 200 µl aliquots (5x10⁵ cells) in 96-well plates. Cells were incubated in the presence or absence of SA (5 µg/ml) and incubated for 3 days at 37°C in an atmosphere containing 5% CO₂. Proliferation was measured by incorporation of 1 µCi of ³H-thymidine over the last 16 hours of the culture. Amounts of incorporation of ³H were measured by
liquid scintillation counter. All assays were performed in triplicate, with five mice representing each group.

2.7 Cytokine assays

The concentrations of IFN-γ, IL-4 and IL-12 in culture supernatants were determined as described previously (Walker et al., 1999). Briefly spleen cells were isolated from mice coinjected with CpG-ODN 1826 or SA or a combination of SA and CpG-ODN 1826, ODN 1911, ODN 1911 plus SA two weeks after the final booster and resuspended in RPMI 1640 medium supplemented with 10% FCS. Cells were then incubated in 96 well flat bottomed plate (Nunc, Roskilde, Denmark) at a density of 5x10⁵ cells per well and cultured with SA (5 μg/ml). After 48 h supernatants were collected and diluted serially, and cytokine concentrations were quantitated using enzyme linked immunosorbent assay (ELISA). The assay was performed using Opt EIA kit (Pharmingen, San Diego, CA) according to the manufacturer’s instructions.

2.8 Measurement of Soluble antigen-specific antibody response

In brief, 96-well plates (Immulon-4, Nunc) were coated with 0.1 μg of SA in 50 μl of carbonate buffer, pH 9.2 and incubated overnight at 4°C. The plates were washed thrice with PBS containing 0.05% Tween 20 (PBST) and then blocked by incubation for 2 h at 37°C in 200 μl of 5% milk in PBST. Plates were then washed thrice with PBST. Mouse sera (100 μl) diluted to 1:300 to 1:30,000 in PBST were added to the wells (except for experimental blanks which were incubated with 5% milk in PBST) and incubated at 37°C for 2 h. The plates were washed thrice with PBST. The plates were then incubated with 100 μl of 1:2000 dilution of horseradish peroxidase conjugated anti mouse IgG (BioRad, Hercules, CA) in PBST for 1 h at 37°C and washed again. Color was developed by incubating with 100 μl aliquot of o-phenylenediamine (5 mg/10 ml in 50 mM citrate-phosphate buffer pH 5.2) and 10 μl of 30% H₂O₂ for 30min. The reaction was then stopped with 50 μl of 2 N H₂SO₄. The absorbance at 490 nm was determined using a model 7520 Microplate reader (Cambridge Technology).

Serum Ig isotypes were assayed by enzyme-linked immunosorbent assay (ELISA) using 100 ng of SA per well, isotype-specific secondary antibodies (biotinylated rabbit antimouse IgG2a, IgG2b and IgG1) and streptavidin-horseradish peroxidase (Pharmingen, San Diego, CA). Antibody levels were determined by comparison to a standard curve generated using a pooled antiserum prepared from infected mice with high antibody titre (Klinman et al., 1999, Stacey et al., 1999).
2.9. Statistical analysis

Results from the different treated groups were compared by student’s t-test. P values of <0.05 were considered statistically significant.

3.0 Results

3.1 Splenocyte Proliferation in mice co injected with SA and ODN

Splenocytes proliferation in mice injected with alum, ODN 1911, SA plus ODN 1911, CpG-ODN 1826 and CpG-ODN 1826 plus SA, was investigated in vitro as described in the materials and methods section. Splenocytes from mice injected with ODN’s did not exhibit increased proliferation of cells when compared with the soluble antigen group. However co-administration of CpG-ODN 1826 and soluble antigen augmented splenocyte proliferation in vitro by 1.76 fold when compared to mice injected with soluble antigen alone (P< 0.001). Co-administration of the control ODN 1911 (which has composition matched to 1826 but lacks CpG dinucleotide motif) resulted in only 1.03 fold increase in splenocyte proliferation over the group treated with soluble antigen (P<0.05) (Plate. 2). This could probably be due to a non-specific response to the phosphorothioate background. Non CpG-ODN has been reported to induce B cell proliferation and splenomegaly (Stacey et al., 1999).

3.2 Isotype response by coinjection of ODN and SA

Antibody isotype profile provides a convenient marker of Th1 and Th2 CD4+ T cell differentiation (Hemmi et al., 2001). To compare IgG isotypes in non protective vaccinated mice, sera was collected from mice two weeks after the second immunization and were assessed for IgG1 and IgG2a and IgG2b. Immunization with SA antigen alone gave a mixed Th1/Th2 response with more anti-SA antibodies of IgG1 (Th2) than IgG2a (Th1) isotype (Plate. 3). Control ODN without CpG motifs (1911) did not affect the isotype profile of sera from mice injected with ODN 1911 and SA antigen. In contrast, mice injected with SA antigen plus CpG-ODN 1826, the majority of antibodies were IgG2a (Plate. 3).

3.3 Cellular response to SA and ODN in vaccinated mice

Susceptible BALA/c mice were coinjected intramuscularly with CpG-ODN 1826 or SA or a combination of SA and CpG-ODN 1826, ODN 1911, ODN 1911 plus SA. Two weeks later, spleens were removed and the levels of IL-4, IL-12 and IFN-γ production in splenocytes from mice immunized with alum, ODN 1911, SA + ODN 1911, CpG-ODN 1826 and CpG-ODN 1826 plus SA were assayed in vitro. The splenocytes were incubated in vitro in the presence of soluble antigen (5μg/ml). The
cytokines levels were measured by cytokine ELISA. The levels of IFN-γ, IL-12 and IL-4 produced in splenocytes from mice immunized with soluble antigen are shown in Plate. 4A, B and C respectively. There was an enhanced production of IFN-γ (3.5 fold) (Plate. 4A) and IL-12 (2.1 fold) (Plate. 4B) in mice injected with soluble antigen and CpG-ODN 1826 when compared to groups where injections were given individually. However there was no significant difference in the IL-4 levels in mice injected with either SA or CpG-ODN-1826 (Plate. 4C). Non CpG-ODN did not result in enhanced production of IFN-γ (Plate. 4A). However co-administration of ODN 1911 with SA showed an increase in IL-12 levels when compared to group where injection was given individually. This increase is probably due to the SA. Thus immunization with soluble antigen and CpG-ODN led to preferential production of IFN-γ and IL-12 in spleen cells stimulated with soluble antigen in vitro. It appears immunization schedule preferentially induces Th-1 type immune responses.

3.4 Antibody response in mice coinjected with ODN and Soluble antigen.

BALB/c mice were immunized with CpG-ODN 1826 or ODN 1911 with or without Soluble antigen (SA) (50 μg/mouse) antigen. Mice were immunized twice at two-week interval with PBS/alum, SA, CpG-ODN 1826, SA + CpG-ODN 1826, ODN 1911 and SA + ODN 1911. Mice were bled 2 weeks following the final injections and sera from the mice in each group (n=5) were pooled.

Relative antibody titers in groups immunized with soluble antigen were nearly 7,659 and CpG-ODN 1826 combined with soluble antigen had a relative titer of 7,968. Relative antibody titers in animals receiving ODN without CpG motifs (1911) alone or with alum were not significantly different from those in respective control groups (Plate. 5).

3.5 Protective immunity after immunization with CpG-ODN and Leishmania soluble antigen.

Since the in vitro splenocyte proliferation and cytokine analysis data suggested that CpG-ODN 1826 resulted in IFN-γ dominant response as compared to ODN 1911, we decided to determine whether co-injection of CpG-ODN 1826 with SA would confer protective immunity. Alum by itself did not result in decreased parasite load when compared with mice injected with PBS alone. Therefore alum was used as a control for all the experiments involving protection studies. BALB/c mice vaccinated with soluble antigen, CpG-ODN 1826 alone or soluble antigen plus CpG-ODN 1826 resulted in 42%, 33%, 55% inhibition respectively of parasite load in liver after 4
weeks (Plate. 6A.). Parasite load was also checked in the spleen. The parasite load decreased by 40%, 31%, 60% in BALB/c mice vaccinated with either soluble antigen, CpG-ODN 1826 alone or soluble antigen plus CpG-ODN 1826 respectively (Plate. 6B). The inhibition of parasite load after 8 weeks was 40%, 35% and 59% in liver and 46%, 34% and 61% in spleen in BALB/c mice vaccinated with either soluble antigen, CpG-ODN 1826 alone or soluble antigen plus CpG-ODN 1826 respectively. A comparison of the SA vaccinated and SA plus CpG-ODN 1826 vaccinated mice shows a modifying role of the ODN on the immune response to SA. Soluble antigen specific antibody titer showed a significant increase 4 weeks after parasite challenge, with relative titer 7953 in sera from mice immunized with SA and the titer of 8893 in sera from mice injected with a combination of SA plus CpG-ODN 1826. Thus, antibody titers were still maintained even after 8 weeks of infection (Plate. 7)

4.0 Discussion

Earlier studies have demonstrated that plasmid DNA immunostimulatory sequences promote Th1 responses (Chu et al., 1997). The ability of CpG-ODN to induce both innate and adaptive cellular immune response has made it a potential treatment and/or prophylactic vaccine adjuvant, respectively for disease requiring cellular immunity. The strong activating effect of CpG DNA on B cells, as well as the induction of cytokines that could have indirect effects on B cells via T-helper pathway, suggests utility of CpG ODN as a vaccine enhancer (Kreig et al., 1995, Klinman et al., 1999). In the present study, we demonstrate that coinjection of immunostimulatory phosphorothioate-modified ODN as an adjuvant with a leishmanial soluble antigen can modulate leishmanial specific immunity towards a protective response in mice.

This study demonstrates that the effects of immunostimulatory ODN as an adjuvant were partial and limited, as protection was only 33% in mice 4 weeks after challenge. The greater efficacy of the leishmanial soluble antigen plus CpG ODN was observed. CpG-ODN had an in vivo impressive effect in modulating the response to vaccination with soluble antigen. Administration of soluble antigen alone or with CpG-ODN resulted in ~40% and ~60% reduction in the parasite load in liver at 8 weeks after challenge respectively.

Earlier observations have also shown that susceptible BALB/c mice when immunized with leishmania soluble antigen with or without CpG-ODN as adjuvant and then challenged with L. major metacyclic promastigotes showed a highly significant reduction in swelling when compared to soluble antigen-vaccinated mice and enhanced
survival when compared to unvaccinated mice (Walker et al., 1999, Stacey et al., 1999). CpG ODN, when used as a vaccine adjuvant with either a recombinant protein or heat killed leishmanial antigen, can induce long-term protection against an intracellular infection in a CD8+ -dependent manner (Rhee et al., 2002). Individual mice immunized with ODNs and Leishmania lysate demonstrated leishmania specific lymphocyte proliferation and IFN-gamma protection in response to parasites in vitro (Walker et al., 1999). The mechanism by which CpG ODN mediate their function in vivo is by enhancing antigen presenting cells (APC) function through toll-like receptor 9, thereby augmenting both the activation and maturation of dendritic cells (DC) as well as the induction of proinflammatory cytokines (Hemmi et al., 2001). CpG ODN induction of IL-12, IL-18 and other soluble mediators from activated DC is likely to result in a more physiological cognate interaction between the DC and T cells, resulting in both a qualitative and quantitatively different type of CD4+ and CD8+ T cell response (Rhee et al., 2002).

It is well established that CpG ODN are potential inducers of Th1 responses, which is consistent with our findings that mice vaccinated with ODN plus soluble antigen had striking enhancement of the production of IFN-gamma and IL-12. Use of CpG-ODN along with soluble antigen resulted in almost additive effect in the production of cytokines in vitro. Immunization with SA antigen alone gave a mixed Th1/Th2 response with more anti-SA antibodies of IgG1 (Th2) than IgG2a (Th1) isotype. In contrast, in mice injected with SA antigen with CpG-ODN 1826, the majority of antibodies were IgG2a.

Thus the immunostimulatory effect of CpG-ODN can be utilized to create a protective vaccine when given in combination with specific antigen from L. donovani in mice model system. Our results in murine L. donovani model, shows promising role of ODN as adjuvants in combination with Leishmania specific antigen and can be exploited to create a protective vaccine. Additionally, CpG-ODN may be very useful for eliciting an enhanced cellular and humoral response and increasing the efficacy of individual antigens that induce partial but incomplete protection. Further studies would be required to evaluate the effect of different doses of CpG-ODN to further improve the efficacy of the present vaccine for visceral leishmaniais in a murine model.
0 DAY — Intramuscular injection of alum, 50μg SA, adsorbed on alum, 10 μg of 1826, 10 μg of 1911, 1826+SA and 1911+SA

4 WEEKS LATER

Protection study

Intramuscular injection of alum, 50μg SA, adsorbed on alum, 10 μg of 1826 and 1826+SA

2 WEEKS LATER

Immunization study

Intramuscular injection of alum, 50μg SA, adsorbed on alum, 10 μg of 1826, 10 μg of 191 1826+SA and 1911+SA

2 WEEKS LATER

Challenge with 10⁸ L donovani AG83 promastigotes intravenously

2 WEEKS LATER

1. Sera isolated for analysis of humoral response
2. Spleen isolated for analysis of cell-mediated response

4 AND 8 WEEKS LATER

A. Sera isolated for analysis of humoral response
B. Parasite load checked in liver and spleen

Analysis

Humoral response

Cell-mediated response

ELISA

Western blot

Splenocyte proliferation, RT PCR for cytokine analysis

Plate: 1

Scheme for immunization and protection studies in BALB/c mice.
Plate: 2

Splenocyte proliferation in mice coinjected with CpG-ODN and soluble antigen. Mice were immunized twice at two-week interval with 50 μg SA, 10 μg of each of the ODN’s either alone or in combination with SA (in 100 μl of PBS). Spleens were collected as described in Materials and Methods. Two weeks following the final injection, splenocytes were stimulated with SA and thymidine incorporation was determined. Delta CPM represents the difference in counts compared with the corresponding non-stimulated cells.
Relative anti-leishmanial IgG1, IgG2a and IgG2b levels in immunized mice before challenge. Mice were bled two weeks after the final injection. Antibody levels are expressed relative to a standard pool as described in Materials and Methods. Mean and standard error of Ig levels measured in triplicates on at least five animals per group are shown. Immunization was done using CpG-ODN 1826, ODN 1911, SA, or a combination of CpG-ODN 1826 plus SA and ODN 1911 plus SA.
IFN-γ levels in the spleens of immunized mice. BALB/c mice were immunized twice at two-week interval with SA, 10 μg of each of the ODN’s either alone or in combination with SA (in 100 μl of PBS). Spleens were collected two weeks following final injection. Splenocytes were stimulated with SA and concentration of released IFN-γ in the culture supernatants was determined. The data is represented as the mean ± SE. Each sample was examined in triplicate and these results are representative of two experiments.
**Plate: 4B**

**IL-12 levels in the spleens of immunized mice.** BALB/c mice were immunized twice at two-week interval with SA, 10 μg of each of the ODN's either alone or in combination with SA (in 100 μl of PBS). Spleens were collected two weeks following final injection. Splenocytes were stimulated with SA and concentration of released IL-12 in the culture supernatants was determined. The data is represented as the mean ± SE. Each sample was examined in triplicate and these results are representative of two experiments.
Plate : 4C

IL-4 levels in the spleens of immunized mice. BALB/c mice were immunized twice at two-week interval with SA, 10 µg of each of the ODN’s either alone or in combination with SA (in 100 µl of PBS). Spleens were collected two weeks following final injection. Splenocytes were stimulated with SA and concentration of released IL-4 in the culture supernatants was determined. The data is represented as the mean ± SE. Each sample was examined in triplicate and these results are representative of two experiments.
Plate: 5

Relative anti-leishmanial antibody levels in mice following immunization with Soluble antigen (SA) with or without ODNs. Relative antibody levels were determined by comparing to a pooled standard. BALB/c mice were immunized twice at two-week interval with 50 µg recombinant SA, 10 µg of each of the ODNs either alone or in combination with SA (in 100µl of PBS). Mice were bled 2 weeks following the final injections and sera from the mice in each group were pooled. Plate. 5 represents relative antibody titres before challenge. These results are representative of two replicate experiments and triplicates were used for each sample.
Infection levels of vaccinated mice following challenge with *L. donovani*. BALB/c mice were immunized with alum, CpG-ODN 1826, SA and SA plus 1826 twice at four weeks intervals. Two weeks following the final injection, the mice were challenged intravenously with $1 \times 10^8$ *Leishmania donovani* promastigotes. Four and eight weeks after challenge infection, mice were killed and Leishman Donovan Units (LDU) was calculated from liver (A) and spleen smears (B). The mean LDU ± SE is shown ($n = 5$ mice per group). Means which differ significantly from that of the corresponding alum control are indicated by + ($P < 0.005$), ++ ($P < 0.0005$), xx ($P < 0.0001$) or x ($P < 0.001$).
Relative antibody levels in vaccinated mice following challenge with *L. donovani*. BALB/c mice were immunized with PBS/Alum, CpG-ODN 1826, SA and SA+1826 twice at four week intervals. 2 weeks following the final injection, the mice were challenged i.v. with $1 \times 10^8$ *Leishmania donovani* promastigotes. Four and eight weeks after challenge infection, mice were killed and sera from the mice in each group were pooled. Plate. 7 represents relative antibody titres after challenge. These results are representative of two replicate experiments and triplicates were used for each sample.