CHAPTER

7

PROPHYLACTIC EFFICACY OF RECOMBINANT PROTEIN AGAINST *LEISHMANIA* CHALLENGES

7. Prophylactic efficacy of recombinant protein against *Leishmania* challenges

7.1 Introduction

In the previous chapter it was demonstrated that out of two, only one proteins viz. rLdADHT had the strong ability to yield optimum cellular responses. Thus, this protein was further evaluated for their prophylactic potential against the virulent dose of *Leishmania* parasites. Vaccination has been an effective tool against various infectious agents, particularly- viral and bacterial and the vaccines are potentially safer, cheaper and more efficacious as prophylactics than drugs, however, only limited progress has been made in designing vaccines against *Leishmania*. Though much effort was concentrated against cutaneous forms of Leishmaniasis, considerable attempts have been made, during the last decade, by several different groups to develop vaccines against visceral leishmaniasis.

So far, the only successful vaccine trials carried out were against canine VL wherein two dog vaccines yielded very satisfactory results in Phase III trials: the Fucose-Mannose ligand (FML) along with saponin (Borja-Cabrera *et al.*, 2002; da Silva *et al.*, 2000) and the *L.infantum* antigen proteins excreted–secreted from promastigotes (LiEASAP), together with muramyl dipeptide (MDP) adjuvant (Lemesre et al., 2005; Lemesre et al., 2007). Another vaccine –the glycoprotein enriched preparation of *L. donovani* promastigotes- FML (Palatnik *et al.*, 1989), antigenic for human (Palatnik-de-Sousa et al., 1995) and dogs (Cabrera et al., 1999), was formulated with *Quillaja saponaria* saponin and passed Phase I-III trials to become the Leishmune® licensed vaccine in Brazil (Parra et al., 2007). Very recently, the Leish-F1+MPL-SE vaccine has entered clinical trials against VL in humans (Chakravarty *et al.*) 2011).

The first and foremost step in developing vaccines is the selection of antigen, and as discussed in earlier chapters that our group (provide references) while in search of such antigens identified several proteins as Th1 stimulatory. ADHT and TPR were identified as immunogenic among them. In this chapter, prophylactic potential of ADHT ,which was most potential among the two giving optimum cellular responses, was evaluated in hamsters against *Leishmania* challenges..

7.2 Materials & Method

7.2.1 Parasite

The *L. donovani* clinical strain 2001 was procured from a patient admitted to the Kala-azar Medical Research Centre of the Institute of Medical Sciences, BHU, Varanasi and maintained *in vitro* and *in vivo* as described in earlier chapter.

7.2.2 Animals

Laboratory-bred golden hamsters (*Mesocricetus auratus*, 45–50 g) from the Institute's animal house facility were used as experimental host and maintained as described in chapter 4. They were kept in a limited access area at a controlled room temperature with food and water *ad libitum*.

7.2.3 Vaccination schedule and assessment of parasitic burden

Experiments were performed on four groups of hamsters (12-15per group), wherein groups 1–3 served as controls and group 4 as the main experimental group as described herewith: group 1, unvaccinated and unchallenged (normal control); group 2, unvaccinated and challenged (infected control); group 3, Bacillus Calmette Guerin (BCG) alone; and group 4, vaccinated with recombinant proteins+BCG (vaccinated group). The hamsters of Group 4 were immunized i.d on the back with rLdADHT (50µg/50µl per animal) along with equal volume of BCG (0.1mg/50µl per animal) in emulsified form and group 3 was given BCG only. Fifteen days later a booster dose of half of the amount of recombinant protein along with BCG was given i.d to all the hamsters Group 4 and only BCG to group 3.

Twenty-one days after the booster dose, all the vaccinated and unvaccinated control groups were challenged intracardially with 10^8 late log phase promastigotes of *L. donovani*. The prophylactic efficacy of rLdADHT was assessed in spleen, liver and bone marrow of three vaccinated hamsters on necropsy at different time intervals, i.e. on days 0, 45, 60, 90, 120 post-challenge (p.c.). Peritoneal exudates cells, inguinal lymph nodes and blood were also collected at these time points to obtain cells and sera for evaluation of cellular and antibody responses (Samant et al., 2009). The criterion of prophylactic efficacy was the assessment of parasite load as the number of amastigotes/1000 cell nuclei in each organ in comparison to the unvaccinated controls and the percentage inhibition (PI) was calculated by the following formula (Singh et al., 2009):

$$PI = \frac{(\text{No. of parasite count from infected control} - \text{No. of parasite from vaccinated group})}{\text{No. of parasite count from infected control}} \times 100$$

7.2.4 Immunological assays

7.2.4.1 DTH

DTH was performed by injecting 50µg/50µl of SLD in PBS intradermally into one footpad and PBS alone into the other one of each of the vaccinated and unvaccinated controls. The response was evaluated 48 h later by measuring the difference in footpad swelling between the two with and without SLD for each animal (Bhowmick et al., 2007).

7.2.4.2 LTT assay

LTT assay was carried out as described in previous chapter.

7.2.4.3 NO production

NO production was carried out as described in previous chapter.

7.2.4.4 Quantification of mRNA cytokines and inducible NO synthase (iNOS) in hamsters by Real time-PCR

7.2.4.4.1 RNA isolation

For RNA isolation, all the glasswares, eppendorfs and tips should be treated with 0.1% Diethyl pyrocarbonate (DEPC) for at least 12 hrs at 37°C and then autoclaved for 15 min. DEPC is a strong but not absolute inhibitor of RNase, and autoclaving after DEPC treatment is done to remove traces of DEPC as it might modify purine residues in RNA by carboxymethylation. Total RNA was isolated from spleen by using Trizol method (Sigma, USA) as per manufacturer's protocol. Briefly, spleens from different experimental groups were homogenized using Trizol and the mixture was kept for 5-20 min at RT followed by addition of 200µl of chloroform/ml of Tri reagent. The content of the tube was mixed by inverting the tube up-down for 15sec and kept aside at RT for 10 min. The tubes were centrifuged at 12000Xg for 15 min at 4°C and the aqueous layer obtained was transferred to a fresh tube. Equal volume of isopropanol was added to each tube, mixed and kept for 10min at RT and centrifuged for 10 min at 12000Xg at 4°C. The pellet obtained was washed in 70% DEPC-ethanol, air-dried for 10min and dissolved in 20µl of RNase free water (DEPC water) and stored at -70°C.

7.2.4.4.2 Formaldehyde gel electrophoresis

Agarose (0.5gm) was dissolved in 5ml of 10XMOPS (pH 7.0) and 36.5ml of water by boiling and kept for cooling to 55°C. Then 8.5 ml of formalin was added to the gel mix and gel was cast and kept covered. The RNA sample (6μl) was prepared in Formamide (12.5μl and MOPES [MOPS (pH 7.0) 0.1 M, 40 mM Sodium acetate, 5 mM EDTA (pH 8.0), DEPC water (0.1%) upto 1 L]. The sample was denatured by heating at 65°C for 15min and immediately chilled on ice. Then 2.5 μl of 6Xgel loading buffer with EtBr was added to each sample. Samples were resolved on 1% agarose gel in 1X MOPS buffer. RNA was treated with DNAse (1U/ μl) before cDNA synthesis.

7.2.4.4.3 cDNA synthesis and amplification

The cDNA was synthesized using the Revertaid H Minus first strand cDNA synthesis kit as per manufacture's protocol (Fermentas). Revertaid H Minus has a point mutation that completely eliminates the RNase H activity, thereby resulting in higher yields of full length cDNA as degradation does not occur during the synthesis. Briefly 0.1- 5μg total RNA, 1μl of random hexamer primer (0.2μg/μl) and DEPC water (11 μl) was incubated at 70°C for 5 min and chilled on ice. After a brief spin, 4μl of 5X reaction buffer was added along with 1μl of Ribolock RNase inhibitor (20u/μl), 2μl of 10mM dNTP mix and 1μl of Revertaid H Minus M-MuLV Reverse transcriptase (200u/μl) was added. The contents were mixed gently by centrifugation and incubated for 5 min at 25°C followed by 60 min at 42°C and finally the reaction was stopped by heating at 70°C for 5 min.

7.2.4.4.4 RT - PCR

qRT-PCR was performed to assess the expression of mRNAs for various cytokines and iNOS in splenic cells. For real-time PCR, primers were designed using Beacon Designer software (Bio-Rad) on the basis of cytokines and iNOS mRNA sequences available on PubMed (Melby et al., 1998a) (**Table-**7.1). qRT-PCR was conducted as per the protocol described earlier (Samant et al., 2009). Briefly, it was carried out with 12.5 μl of SYBR green PCR master mix (Bio-Rad), 1 μg of cDNA, and primers at a final concentration of 300 nM in a final volume of 25 μl. PCR was conducted under the following conditions: initial denaturation at 95°C for 2 min followed by 40 cycles, each consisting of denaturation at 95°C for 30 s, annealing at 55°C for 40 sec., and extension at 72°C for 40 sec. per cycle using the iQ5 multicolor real-time PCR system (Bio-Rad). cDNAs from infected hamsters were used as

"comparator samples" for quantification of those corresponding to test samples whereas in vaccination studies. All quantifications were normalized to the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT). A no-template control c-DNA was included to eliminate contaminations or nonspecific reactions. The cycle threshold (CT) value was defined as the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value (background noise). Differences in gene expression were calculated by the comparative CT method (Samant et al., 2009). This method compares test samples to a comparator sample and uses results obtained with a uniformly expressed control gene (HPRT) to correct for differences in the amounts of RNA present in the two samples being compared to generate a ΔCT value. Results are expressed as the degrees of difference between ΔCT values of test and comparator samples in the form of fold expression.

Table 7.1: Sequences of forward and reverse primers of hamster cytokines used for quantitative real time RT-PCR.

S.N.	Primer	Primer sequence
1	HGPRT Forward	5' GATAGATCCACTCCCATAACTG 3'
	Reverse	5' TACCTTCAACAATCAAGACATTC 3'
2	TNFα Forward	5' TTCTCCTTCCTGCTTGTG 3'
	Reverse	5' CTGAGTGTGAGTGTCTGG 3'
3	IFNγ Forward	5' GCTTAGATGTCGTGAATGG 3'
	Reverse	5' GCTGCTGTTGAAGAAGTTAG 3'
4	IL-12 Forward	5' TATGTTGTAGAGGTGGACTG 3'
	Reverse	5' TTGTGGCAGGTGTATTGG 3'
5	TGFβ Forward	5' ACGGAGAAGAACTGCTGTG 3'
	Reverse	5' GGTTGTGTTGGTTGTAGAGG 3'
6	IL-4 Forward	5' GCCATCCTGCTCTGCCTTC 3'
	Reverse	5' TCCGTGGAGTTCTTCCTTGC 3'
7	IL-10 Forward	5' TGCCAAACCTTATCAGAAATG 3'
	Reverse	5' AGTTATCCTTCACCTGTTCC 3'
8	iNOS Forward	5' CGACGGCACCATCAGAGG 3'
	Reverse	5'AGGATCAGAGGCAGCACATC 3'

7.2.4.5 Determination of antileishmanial antibody responses in hamsters:

The level of IgG antibody and its isotypes in sera samples of hamsters of different experimental groups was measured as per protocol by Samant et al (Samant et al., 2009) with slight modifications. Briefly, 96-well ELISA plates (Nunc) were coated with rLdADHT (0.2 µg/100 µl/well) overnight at 4°C and blocked with 1.5% BSA at room temperature for 1 h. Sera was used at a dilution of 1/100 for IgG, IgG1, and IgG2 and kept for 2 h at room temperature. Biotin-conjugated mouse anti-Armenian and Syrian hamster IgG, IgG1 and biotinylated anti-Syrian hamster IgG2 (BD Pharmingen) were added for 1 h at room temperature at 1/1000 dilutions and were further incubated with peroxidase-conjugated streptavidin at 1/1000 (BD Pharmingen) for 1 h. Finally, the substrate O-phenylenediamine dihydrochloride (Sigma-Aldrich) was added and the plate was read at 492 nm in a spectrophotometer (Spectra$_{Max,}$ U.S.A.).

7.2.4.6 Post-challenge survival

Survival of hamsters belonging to group 4 was checked until day 180 p.c. in comparison to the normal hamsters (group 1). Animals in all of the groups were given proper care and were observed for their physical conditions until their survival period. Survivals of individual hamsters were recorded and mean survival period was calculated.

7.2.4.7 Statistical analysis

Results were expressed as mean ± S.D. Two sets of experiments were performed for vaccination studies and in each experiment 12–15 animals were used. The results (pooled data of three independent experiments) were analyzed by one-way ANOVA test and comparisons with control data were made with Dunnett's or Tukeys post-test which ever appropriate using Graph Pad Prism software program. One-way ANOVA statistical test was used to assess the significance of the differences among various groups and a P value of < 0.05 was considered significant.
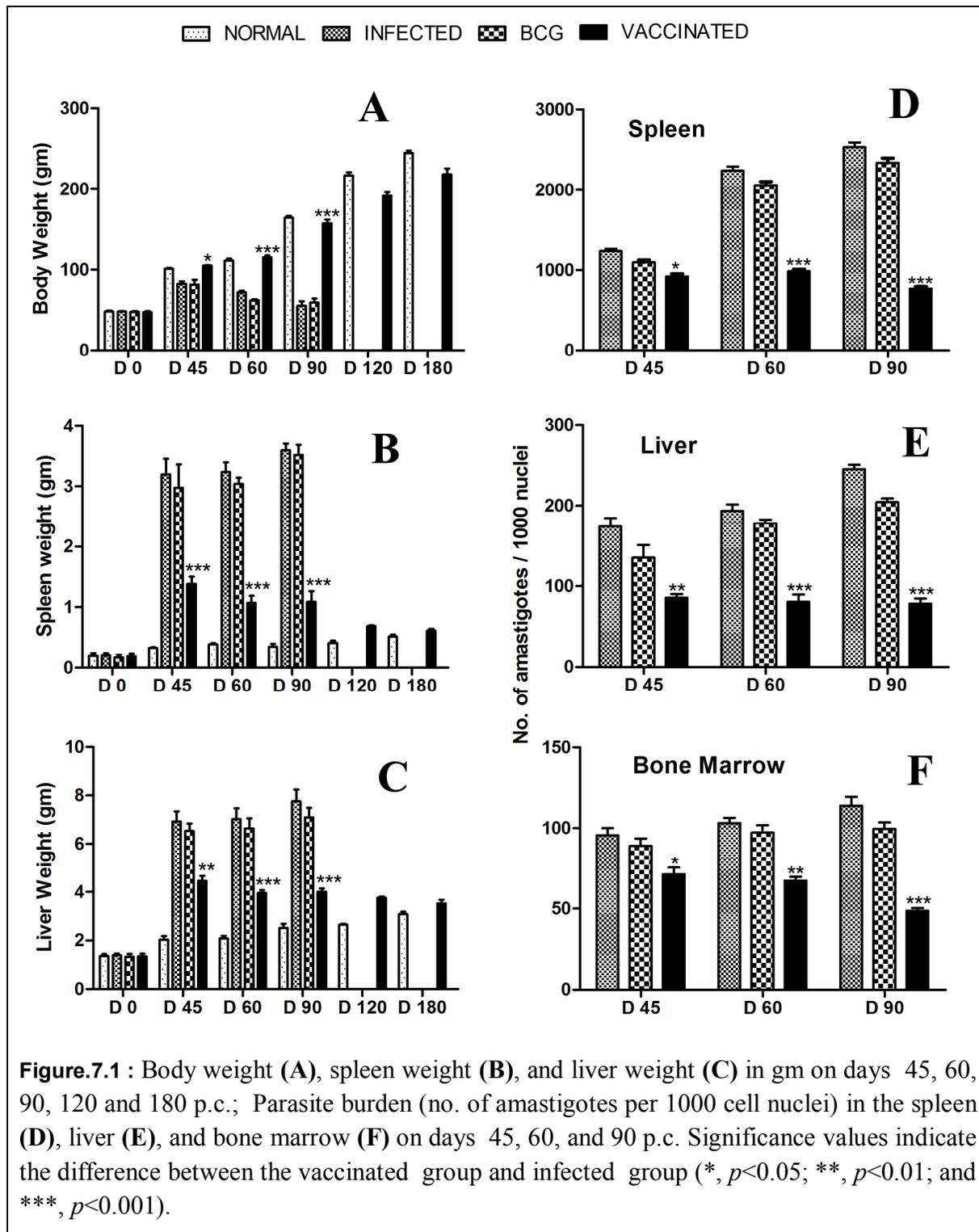
7.3 Results

Encouraged with the abilities of rLdADHT to generate a Th1 type profile, as described in the last chapter, these were further evaluated for their prophylactic efficacies as recombinant protein along with BCG in hamsters against lethal challenge of *L. donovani*. The effect of this protein in vaccinated hamsters was assessed by gross changes in their body weights, parasitic burden in visceral organs (spleen, liver and bone marrow) and alterations in immunological profile generated thereof.

7.3.1 Assessment of parasitic burden in hamsters vaccinated with rLdADHT+BCG and challenged with *L. donovani*
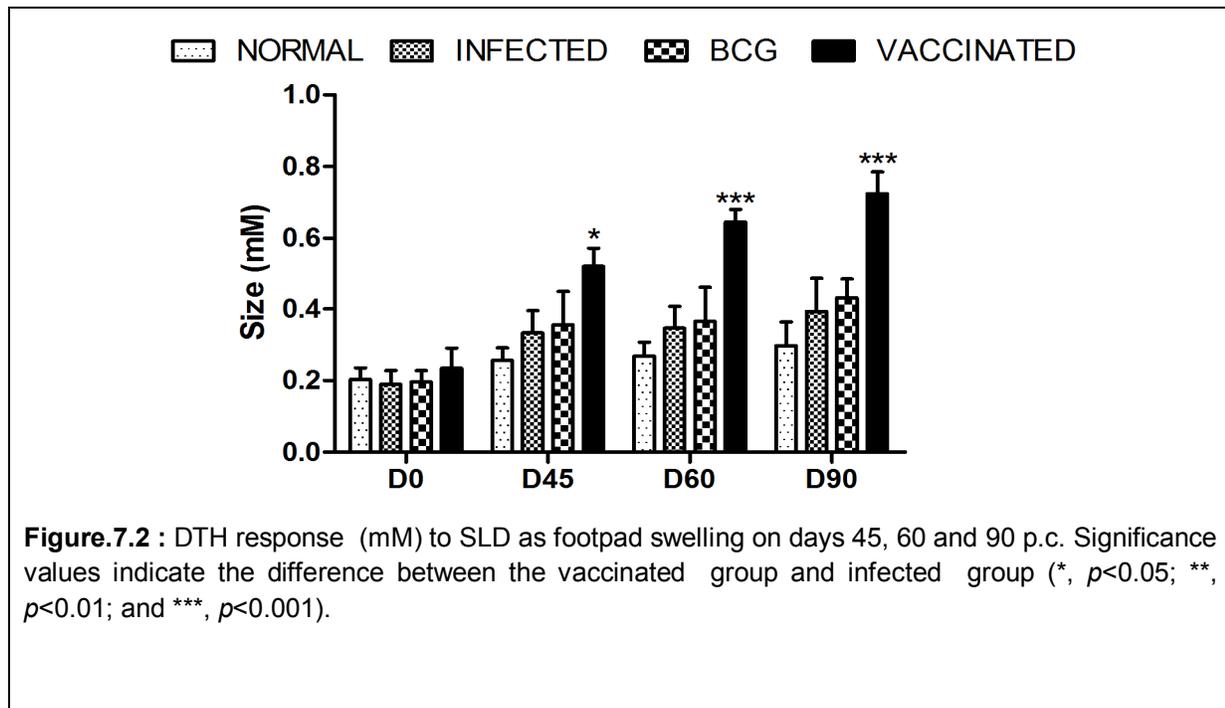
The hamsters, vaccinated with rLdADHT+BCG, were found to be optimally protected from the *L. donovani* challenge. These animals gained substantial weight as compared to those immunized with BCG alone as well as to unimmunized infected animals, when observed simultaneously on days 45, 60, 90 and 120 post challenge (p.c.). A significant ($p<0.001$) reduction in parasite load (~70%) was observed in spleen, liver and bone marrow of rLdADHT+BCG vaccinated animals on day 90 p.c. alongwith an absence of hepato-splenomegaly that is normally associated with the challenge infection (**Fig.** 7.1D-F). On the other hand, no protective effect was seen when hamsters were immunized with BCG alone. There was progressive increase in parasite load in these hamsters as well as unimmunized ones and they succumbed to virulent *L. donovani* challenge within 2–3 months (**Fig.** 7.1). A positive correlation of parasite loads with splenomegaly and hepatomegaly was observed among the experimental and control groups.

All the hamsters vaccinated with rLdADHT+BCG survived longer after the lethal challenge of *L. donovani* and remained healthy until the termination of the experiment up to 6 months post-infection. Moreover, in liver and bone marrow, parasite loads decreased sharply after day 45 p.c. and parasites were almost absent by day 180 p.c. in the vaccinated group.

Figure.7.1 : Body weight **(A)**, spleen weight **(B)**, and liver weight **(C)** in gm on days 45, 60, 90, 120 and 180 p.c.; Parasite burden (no. of amastigotes per 1000 cell nuclei) in the spleen **(D)**, liver **(E)**, and bone marrow **(F)** on days 45, 60, and 90 p.c. Significance values indicate the difference between the vaccinated group and infected group (*, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$).
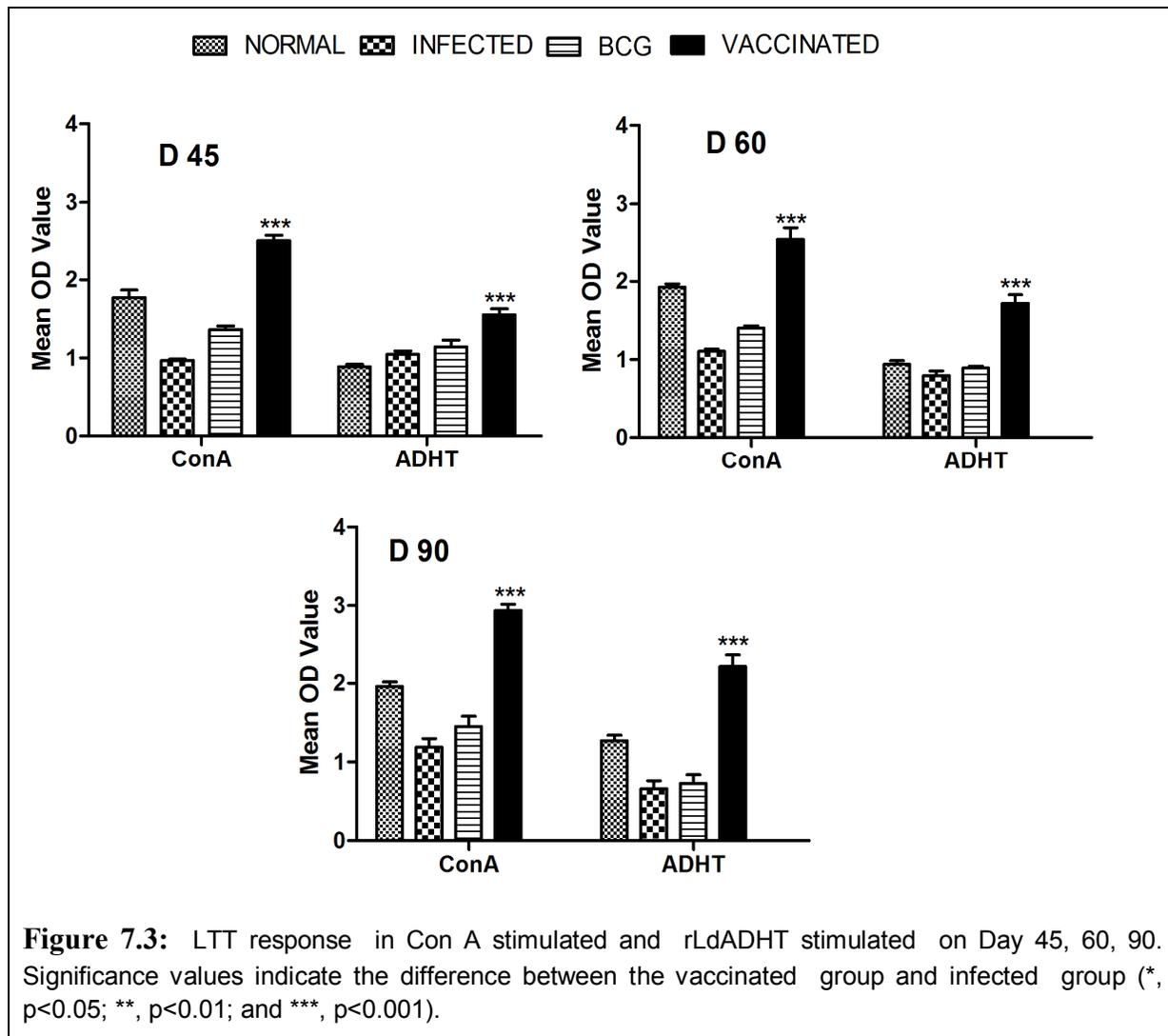
7.3.2 Immunological Responses (DTH, mitogenic and *Leishmania*-specific cellular responses)
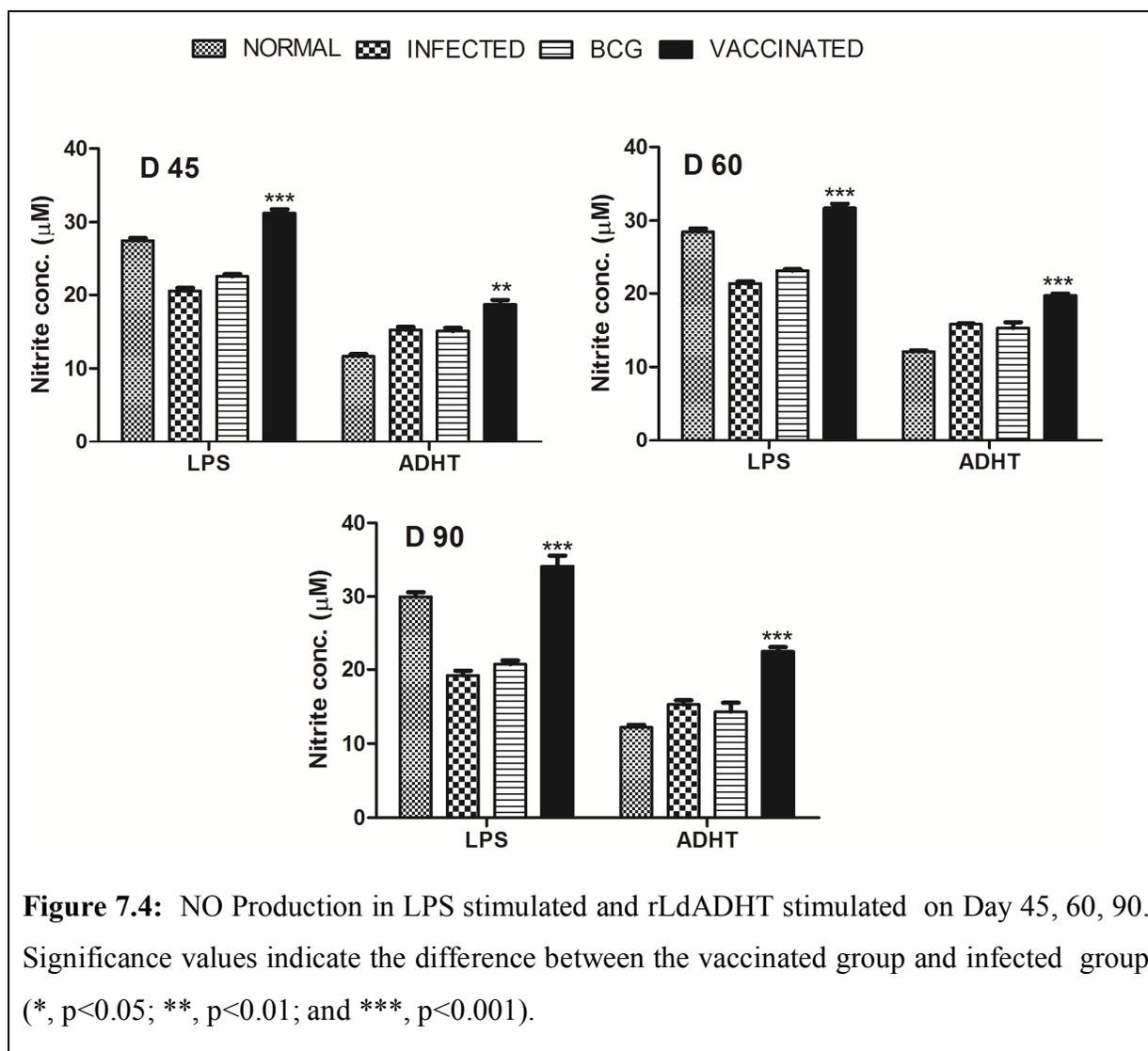
Delayed Type Hypersensitivity (DTH), an index of cell mediated immunity *in vivo*, and an antigen specific *in vitro* T-cell proliferation assay revealed the status of cellular responses generated in vaccinated animals. It was therefore of interest to see the DTH and proliferative responses elicited by vaccinated and challenged animals. The hamsters vaccinated with rLdADHT+BCG displayed significant DTH responses, which increased progressively and was higher than those of the control groups ($p<0.001$) at all the time points for the duration of the experiments for up to 90 days (**Fig.** 7.2).

Figure.7.2 : DTH response (mM) to SLD as footpad swelling on days 45, 60 and 90 p.c. Significance values indicate the difference between the vaccinated group and infected group (*, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$).

Con A induced lymphoproliferation (**Fig.** 7.3) in vaccinated animals was observed to be similar to the normal ones throughout the entire p.c. period (days 45, 60 and 90 p.c.) whereas it was lower in other control groups. On the other hand, there was lesser proliferative response in animals vaccinated with BCG alone as well as unvaccinated infected control (**Fig.** 7.3).

Figure 7.3: LTT response in Con A stimulated and rLdADHT stimulated on Day 45, 60, 90. Significance values indicate the difference between the vaccinated group and infected group (*, p<0.05; **, p<0.01; and ***, p<0.001).

NO is the critical killing effector molecule against leishmaniasis produced by IFN-γ stimulated and inducible NO synthase induced classical macrophages. Lymphocyte-mediated activation of macrophages to produce NO for leishmanicidal activities was found to differ between control and experimental groups of hamsters. Macrophages isolated from naïve hamsters, when incubated with stimulated supernatants of lymphocytes from rLdADHT+BCG vaccinated hamsters, produced significant amount of NO ($p<0.01$) than the other control groups on day 45 p.c. Further increase in NO level was observed by days 60 and 90 p.c. (**Fig.7.4**).

Figure 7.4: NO Production in LPS stimulated and rLdADHT stimulated on Day 45, 60, 90. Significance values indicate the difference between the vaccinated group and infected group (*, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$).

7.3.3 Estimation of mRNA cytokines in rLdADHT+BCG vaccinated hamsters as well as in control groups

It is well established that the cytokine milieu at the initiation of infection is critical in determining disease outcome (Reiner *et al.*, 1994; Seder *et al.*, 1993). So to understand the interplay between the disease healing inflammatory cytokines IFN-γ and IL-12 and disease associated cytokines IL-10 and IL-4, the expression of these cytokines as well as the level of iNOS transcript was investigated by qRT-PCR. As the reagents for cytokine estimation in hamsters is commercially unavailable, herein quantitative real time PCR (qRT-PCR) was used for assessing the expression of cytokine mRNAs in the experimental groups.

The mRNA expression of Th1 and Th2 cytokines viz. IFN-γ, TNF-α, IL-12, TGF-β, IL-4, IL-10 and iNOS in hamsters of rLdADHT+BCG vaccinated group was evaluated on days 45 and 90 p.c. and compared to animals infected with *L. donovani* as well as normal control groups. An increase in the expression levels of Th1 type of cytokines was observed on days 45 and 90 p.c. wherein, a significantly high level of IFN-γ ($p<0.001$) was observed on day 90 p.c. in comparison to the infected controls (~2.5 folds). There was a progressive increase in the expression levels of TNF-α ($p<0.01$) and IL-12 ($p<0.05$) mRNA transcripts but in case of iNOS, it was moderately increased on day 45 p.c. and these cytokines were highly significant on day 90 p.c. ($p<0.001$ for iNOS, IL-12 and $p<0.01$ for TNF-α).

On the other hand, an extreme down-regulation in the expression levels of Th2 type cytokines was observed in the vaccinated group as compared with the infected ones at both time points i.e. on days 45 and 90 p.c. Among these, the expression of TGF-β, IL-10 and IL-4 were significantly down-regulated ($p<0.001$) at day 90 p.c. (**Fig.**7.5).The data for day 60 cytokine has not presented here because no clear changes have been observed at the level of mRNA transcript.
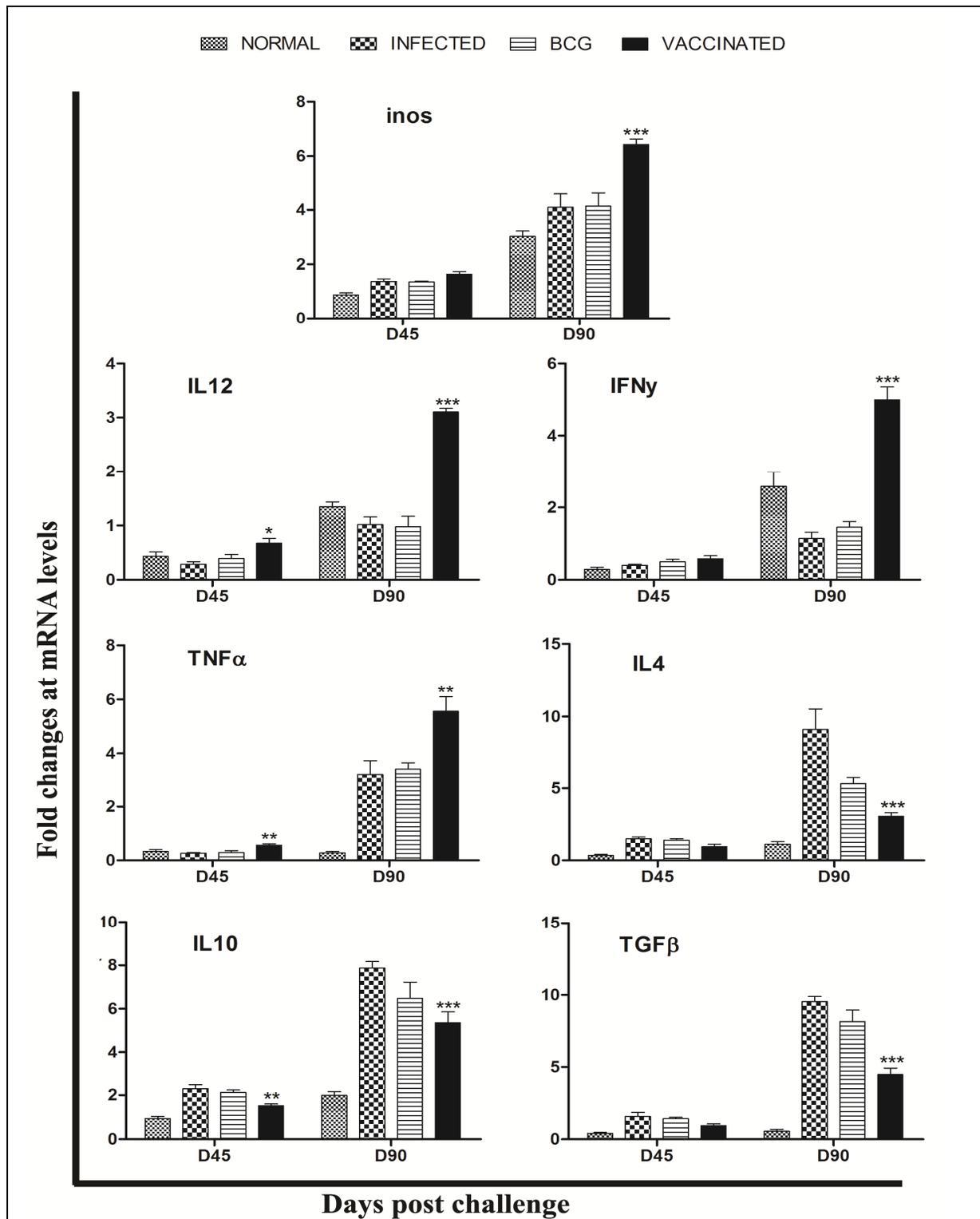
Figure 7.5: Splenic iNOS and cytokine mRNA expression profile analysis of normal and vaccinated hamsters on days 45 and 90 p.c. by quantitative Real-time -PCR. Significance values indicate the difference between the vaccinated group and infected group (*, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$).

7.3.4 Estimation of antibody response in rLdADHT+BCG vaccinated hamsters

Active VL is known to be associated with the production of high level of Leishmania specific antibody which is observed prior to detection of parasite specific T-cell response. In kala-azar, antibody levels correlate with intensity of infection harbored by the host. The serum levels of Leishmania-specific IgG and its isotypes (IgG1 and IgG2) from all of the experimental groups were assessed by ELISA. The anti-Leishmania IgG and IgG1 were elevated progressively with time to a high level in all the experimental groups except vaccinated group (**Fig.** 7.6).. In contrast, rLdADHT + BCG vaccinated animals were the only group that showed a significant increase in the level of IgG2 by 2- to 3 fold over the others (p<0.001) (**Fig**. 7.6). As a measure of CMI, the elevation of IgG2 was consistent with the development of effective immune responses.
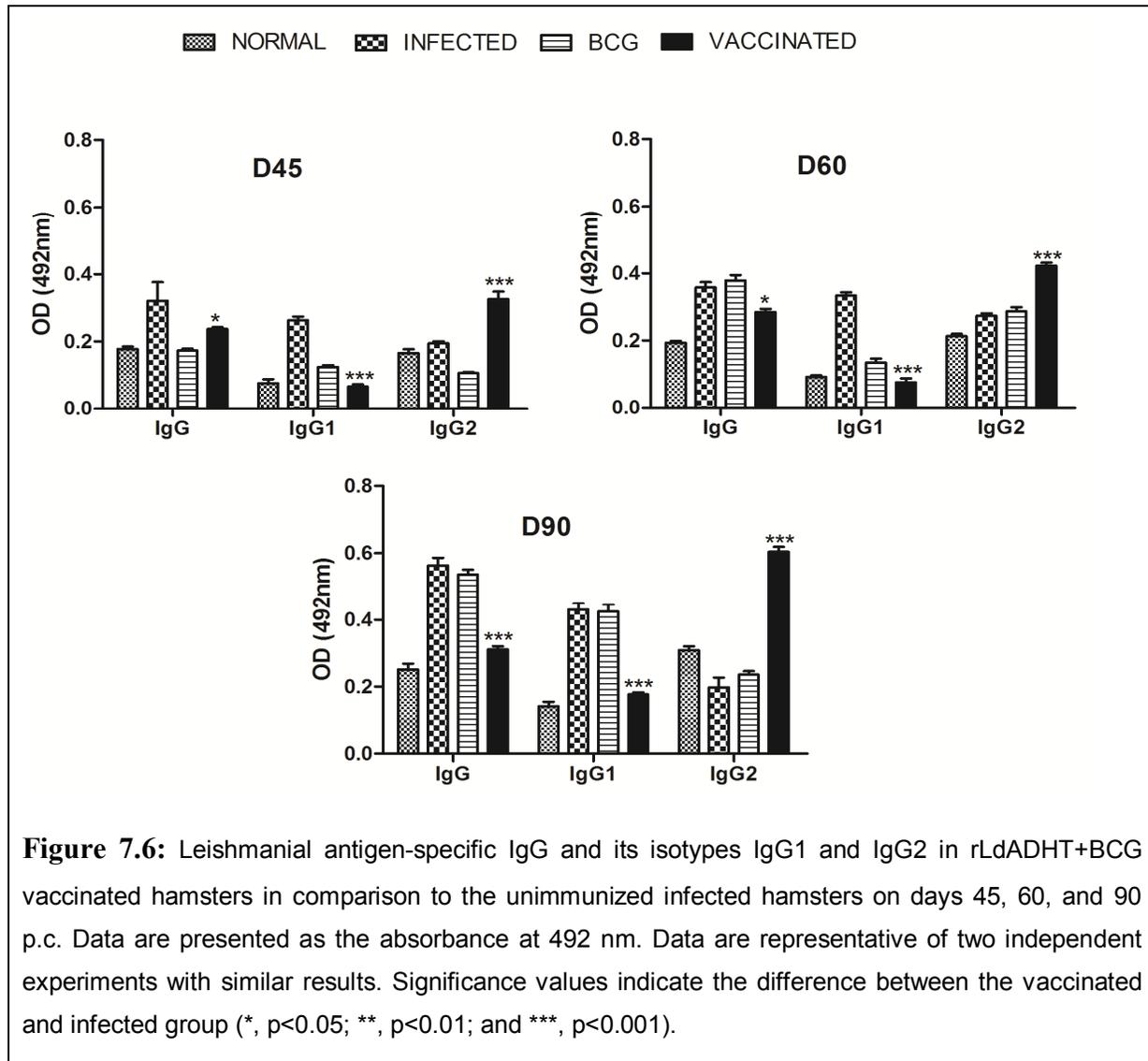

Figure 7.6: Leishmanial antigen-specific IgG and its isotypes IgG1 and IgG2 in rLdADHT+BCG vaccinated hamsters in comparison to the unimmunized infected hamsters on days 45, 60, and 90 p.c. Data are presented as the absorbance at 492 nm. Data are representative of two independent experiments with similar results. Significance values indicate the difference between the vaccinated and infected group (*, p<0.05; **, p<0.01; and ***, p<0.001).

7.4 Discussion:

In the present global scenario of VL, identification of immunostimulatory antigens of *L. donovani* for vaccine development against VL due to severe immunosuppression is becoming almost necessary. For a realistic assessment of efficacy of a vaccine against VL, proper animal model in which the vaccine has to be tested is important. The hamster is a good model for VL, as it develops a progressive, lethal disease which very closely mimics the disease in humans and as such has been used for vaccination studies (Garg *et al.*, 2005b; Melby *et al.*, 1998a). For vaccination studies BCG had been used here as an adjuvant (Khalil et al., 2000; Misra et al., 2001) to the recombinant proteins since it has been reported that it activates macrophages inducing NO (MacMicking et al., 1997; Nozaki et al., 1997) and elicits long lasting cellular and humoral immune responses (Warren et al., 1986). Also, BCG, licensed for human use and effective against intracellular pathogens, was extensively used in clinical trials of vaccination against CL and VL (Noazin et al., 2008).

The results presented here, revealed that vaccination with rLdADHT+BCG offered considerably good prophylactic efficacy to the tune of ~70% . The parasite load in the vaccinated hamsters (rLdADHT) decreased progressively reaching a negligible level by day 120 p.c., rendering them difficult to discern demonstrating their strong protective immunogenic potentials. In addition, an increase in body weight and decrease in the weight and sizes of visceral organs- spleen and liver also supported the data.The rLdADHT+BCG vaccinated animals survived 6 months. The longer survival of the hamsters vaccinated with former suggest its superiority over the later as a potential vaccine candidate against VL.

The immune mechanism(s) responsible for protection in the hamster vaccine model or any other leishmanial vaccine model has not been yet defined. Active VL is known to be associated with the absence of parasite-specific cell-mediated immune response (Carvalho *et al.*, 1985; Carvalho *et al.*, 1981). A major factor that is believed to contribute in healing of leishmaniasis is the development of strong cell mediated immune (CMI) responses like T-cell responses, NO production, and DTH responses (Afrin *et al.*, 2002; Ali & Afrin, 1997). One measure of CMI is *Leishmania*-specific LTT, which almost always accompanies control of parasite growth and healing. Successful vaccination of humans and animals is often related to T-cell stimulation with mitogens and antigen *in vitro* (Armijos *et al.*, 2004; Melby *et al.*, 1998a; Melby *et al.*, 1998b). Most of the assays in this study were done on days 45, 60 and 90 p.c. as the hamsters of most of the experimental groups survived by this time. It appeared

that all the group of hamsters vaccinated with rLdADHT and challenged with *L. donovani* have a specific active T-cell response that was severely impeded in their respective non-immunized infected and healthy control hamsters. There was a significant but comparable lymphoproliferative responses after challenge in all the groups of vaccinated hamsters. However, it is not LTT itself that is behind the primary effector mechanism of immunity, but rather the stimulation of *Leishmania*-specific T-cells to produce macrophage activating factor, including IFN-γ which in turn activate macrophages to kill the intracellular parasites (Scott et al., 1987a). Similarly, the antigen stimulated cells from the vaccinated hamsters (of all the recombinant proteins) produced a remarkable level of NO. The generation of NO in these cultures also support the view regarding the upregulation of iNOS by Th1-cell associated cytokines and confirms that NO-mediated macrophage effector mechanism is critical in the control of parasite replication in the animal model (Armijos et al., 2004).

DTH has been shown to be absolutely dependent on the presence of memory T-cells. Both the CD4+ and CD8+ fractions of cells have been shown to modulate an immune response. Contemporary debate regarding the reaction is focused on the role of the Th1 and Th2 cells (Mosmann & Coffman, 1989). It has been postulated that the Th1 cell is the "inducer" of a DTH response since it secretes IFN-γ, a potent stimulator of macrophages, while the Th2 cell is either not involved or act as a downregulator of the cell mediated immune response. Notably, the vaccinated hamsters' elicited strongest DTH reaction among all the experimental groups suggesting a correlation between CMI responses and immunity to infection in this model. Low level of parasite-specific DTH responses in infected and vector control animals can be correlated with disease progression in hamsters. These animals became resistant to *Leishmania* challenge on immunization with the recombinant proteins alongwith BCG and expressed strong DTH response even after pathogenic challenge. The presence of a comparable existence of Th1 and Th2 clones producing both IFN-γ and IL-4 obtained from patients cured of VL encouraged to assess whether the protective response which was utmost elicited by vaccination in hamsters can reflect this feature of clinical findings (Kemp, 2000; Kemp et al., 1999; Kemp et al., 1993). IFN-γ, a signature cytokine of Th1-type response that has a dominant effect on macrophage microbicidal responses and other effector killing mechanisms, was found to be moderately expressed (Melby et al., 2001a), along with low TNF-α production in infected hamsters. Transcripts of IFN-γ and TNF-α, often reported to act in concert to activate iNOS for the production of NO (Liew et al., 1990b), showed

manifold increase in all the immunized groups of hamsters. It is also suggested that TNF-α is one of the primary agents to stimulate macrophage to produce NO (Liew et al., 1990a). High TNF-α levels present in the sera of active VL patients with IL-10-mediated suppressed T-cell response may not be effective and beneficial for the patient (Barral-Netto *et al.*, 1991; Ho *et al.*, 1992; Pisa *et al.*, 1990). A higher concentration of TNF-α might be required to mount a NO generating signal as observed during a protective response in vaccinated hamsters. In infected animals mRNA transcripts of TGF-β, IL-4 and IL-10 were highly up regulated whereas the expression of mRNA for IL-12 and IFN-γ was found to be down regulated. Transcripts of IL-12 showed manifold increase in the immunized groups of hamsters. IL-10, a key macrophage deactivating Th1 suppressive cytokine, is reported to have a definitive association with an acute phase of VL during which a progressive increase of IL-10 transcripts in tissues was generated but IL-10 mRNA was not detectable after successful chemotherapy (Ghalib et al., 1993; Melby et al., 1998a). Commensurate with these results an extreme down-regulation of IL-10 mRNA levels was observed in all the groups of vaccinated hamsters. Similar results were observed in case of IL-4 cytokine which was down-regulated in vaccinated hamsters as compared to infected control hamsters (Noben-Trauth et al., 2003). As observed by Basu et al (Basu et al., 2005), here also moderate IFN-γ transcript production from infected hamsters could be correlated with the clinical findings in which presence of IL-10 as well as IFN-γ was reported in patients with acute VL whereby only IL-10 levels decreased remarkably with disease cure (Karp et al., 1993). All of these findings together with the present study indicate a clear-cut proportion of IFN-γ requirement and immunosuppressive cytokine like IL-10, which can dictate the outcome of the disease. There are reports that primary Th1 cell-mediated antileishmanial events induced in IL-10 deficient mice require IFN-γ that is largely induced by IL-12 (Basu *et al.*, 2005; Murray *et al.*, 2000). In the present study, IL-12 was completely down-regulated in *L. donovani* infected hamster, whereas high levels of IL-12 mRNA transcripts were found in all the vaccinated hamsters with highest expression of IL-12 in animals vaccinated with rLdADHT+BCG. The synergism of IL-12 with IFN-γ might have an additional paramount effect on leishmanicidal activities of *L. donovani* in this tissue (Basu et al., 2005). The authors in their study have inferred that high expression of IL-10 in infected hamsters might have a profound suppressive effect on these Th1-like cytokine-induced responses and thus infection continues unabated. TGF-β, a pleiotropic cytokine with diverse functions, is known to be expressed at a moderate level even in normal hamsters (Basu *et al.*, 2005; Melby *et al.*, 2001a; Melby *et al.*, 1998a). TGF-β

is also known to inhibit the activities of immune cells and was found to be down-regulated in vaccinated hamsters compared with the infected controls. Unlike the findings of (Basu *et al.*, 2005), Apart from diminished cellular responses, VL is associated with the production of high level of *Leishmania* specific antibody which is observed prior to detection of parasite specific T-cell response (Ghose et al., 1980). The serum levels of leishmanial antigen specific IgG and its isotypes (IgG1 & IgG2) of all the experimental and control groups were assessed by ELISA. The anti-*Leishmania* IgG and IgG1 in all the groups except the vaccinated group were elevated progressively with time to a higher level whereas the level of IgG2 was significantly prominent in all the vaccinated groups by 1- to 2-fold over the other control groups. As a measure of CMI, the elevation of IgG2 is consistent with the development of effective immune response. These observations further support the views that protection against leishmaniasis is induced by a strong T-cell response and almost undetectable amounts of antibodies (Bretscher *et al.*, 1997; Scott *et al.*, 1987a). This pattern was also seen in clinical as well as experimental VL (Dube et al., 1998; Fragaki et al., 2001).

Finally, unlike mice where IL-4 and IL-12 direct IgG subclass switching of IgG1 and IgG2a, respectively, such distinct IgG classes remain obscure in hamsters (Bhowmick *et al.*, 2007; Rodrigues Junior *et al.*, 1992). It is believed that hamster IgG1 and IgG2 correspond to mouse IgG1 and IgG2a/IgG2b, respectively. It has been well-established that IgG and IgG1 antibodies increase in titre with the *L. donovani* loads (Basu et al., 2005). The apparent level of these antibodies is thus consistent with the decreasing parasite loads seen in vaccinated group. The significant increase in the IgG2 levels only in vaccinated animals is indicative of enhanced CMI.

7.5 Conclusions:

In a nut shell this study demonstrated considerably good prophylactic efficacies of one recombinant protein -rLdADHT against experimental VL as all the vaccinated hamsters continued to survive upto 6 months post challenge. The vaccinated hamsters of both the groups demonstrated a surge in IFN-γ, TNF-α, and IL-12 levels along with extreme down-regulation of IL-4, IL-10 and TGF-β. The observation was well supported by the rise in the level of *Leishmania* specific IgG2 which is indicative of enhanced CMI. Surprisingly,

contrary to some previous reports, inducible NO synthase was actively synthesized by macrophages of the protected as well as infected hamsters.

These results, therefore, suggest that though both the antigens are immunogenic, rLdADHT has the potential of being effective vaccine candidate. Future work should however be directed towards the identification and synthesis of immunogenic/immunodominant epitopes/regions of this protein which would generate specific T-cell response. Also, the immunodominant regions of this protein may be cloned and fused together to form a fusion protein expressing the specific epitopes of these proteins. The fusion protein construct can also be administered in the form of DNA vaccine. More importantly, this construct may exert absolute protection in hamsters against challenge infection with *L. donovani*. In addition, the fact that rLdADHT constructs could induce unambiguous protection as single molecules, a cocktail composed of these constructs shall conceivably be a better vaccine because specific immunity will be generated against an increased number of parasite epitopes. This is a desirable condition, because a vaccine containing a broad range of different protective epitopes is unlikely to suffer from major histocompatibility complex related unresponsiveness in heterogeneous outbred populations, such as humans and dogs. Further, since these proteins are highly conserved among *Leishmania* spp, due to their having more than 80% homology to *L. infantum,* a closely related *L. donovani* as well as to *L. major*- causative agent for cutaneous Leishmaniasis, they could be also evaluated for their cross-protective properties.