CHAPTER 6

ASSESSMENT OF IMMUNOGENICITY OF ADHT & TPR USING PBMCS/LYMPHOCYTES OF CURED *LEISHMANIA* INFECTED PATIENTS AS WELL AS HAMSTERS

6. Assessment of immunogenicity of ADHT & TPR using PBMCs/lymphocytes of cured *Leishmania* infected patients as well as hamsters

6.1 Introduction

A major factor contributing to healing in leishmaniasis is the development of strong cell mediated immune response (Howard & Liew, 1984). The measures of cell-mediated immunity are *Leishmania* specific lymphoproliferation and the stimulation of T-cells to produce macrophage activating factor, including IFN-γ which, in turn, activate macrophages to kill the intracellular parasites. The lymphocyte transformation test (LTT) is a well established *in vitro* method for investigating antigen specific cellular immune responses. It has been reported earlier that upon stimulation with SLD and its subfractions, a T-cell response develops in cells (lymphocytes) from exposed or individuals infected with *Leishmania* and cured using anti-leishmanials (Garg et al., 2005a; Kumari et al., 2008b). Since, the Th1 stimulatory proteins cloned earlier in this study- LdADHT and LdTPR, are derived from the sub-fraction of SLD belonging to 89.9– 97.1kDa range (Kumari et al., 2008c), it would be worthwhile to assess whether these proteins alone could induce equivalent or even better cellular immune responses than SLD in *Leishmania* infected and cured hamsters.

In the preceding chapter, the cloning, expression and purification of the Th1 stimulatory proteins viz. rLdADHT and rLdTPR was described. These recombinant proteins were subjected to re-assessment of their immunogenicity in comparison to SLD using PBMCs/lymphocytes of cured *Leishmania* infected patients as well as hamsters.

6.2 Materials & Methods

6.2.1 Parasites

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 chapter 4.

6.2.2 Animals

Laboratory-bred golden hamsters (*Mesocricetus auratus*, 45–50g) from the Institute's animal house facility were used as experimental host and maintained as described in chapter 4.

6.2.3 Preparation of soluble *L. donovani* promastigote antigen

Soluble *L. donovani* promastigote antigen (SLD) was prepared as per method described elsewhere (Scott *et al.*, 1987c) and modified by Choudhry et al (Choudhry et al., 1990). Briefly, promastigotes were harvested from 3 to 4 day old cultures and washed thrice by centrifugation at 3000 X g for 10 minutes each. The washed cells were finally suspended in phosphate buffer saline (PBS) and then sonicated (Soniprep-150) for two periods of 1.5 min each in ice, (separated by an interval of 3 min) at medium amplitude. The sonicated sample was rapidly frozen and thawed 4 times using liquid nitrogen and left at 4°C for one hour for complete extraction of soluble antigen. The suspension was then centrifuged at 4,000 X g for 20 min at 4°C. The supernatant so obtained was finally ultra centrifuged at 40,000 X g for 30 min. After assessing protein contents by Bradford method, the antigen was distributed in small aliquots and stored at -80°C.

6.2.4 Patients and isolation of peripheral blood mononuclear cells (PBMCs)

The study groups for human samples were as follows:

[1] Eight treated cured patients (6 males and 2 females, age ranging from 10–40 years) were taken from hyper-endemic areas of Bihar. All the patients had received complete course of amphotericin B and had recovered from VL. Samples were collected from 2 months to 1 year after the completion of treatment. Diagnosis was established in all cases by demonstration of parasites in splenic aspirates and found negative at the time of study.

[2] Six endemic household contacts (4 males and 2 females, age range-15 to 45 years) that neither showed clinical symptoms nor received any treatment for Kala-azar were chosen.

[3] Six infected patients (3 males and 3 females, age range- 5 to 40 years) showing clinical symptoms of Kala-azar were identified for the study.

[4] Six normal healthy donors (3 males and 3 females, age range 25–30 years) from non-endemic areas, without any history of leishmaniasis, served as negative control.

The study was approved by the Ethics committee of the Kala-azar Medical Research Centre, Muzaffarpur (Protocol # EC-KAMRC/Vaccine/VL/2007-01).

Heparinized venous blood (10ml each) was collected from all the study subjects and peripheral blood mononuclear cells (PBMCs) were isolated from blood by Ficoll-Hypaque density gradient centrifugation (Histopaque 1077, Sigma, USA) as described by Garg et al. (Garg et al., 2005a). A final suspension of 1×10^6 cells/ml was made in complete RPMI medium (cRPMI) after determining cell viability by trypan blue staining method. These were used for various immunological assays.

6.2.5 Treatment of *L. donovani* infected hamsters and isolation of mononuclear cells (lymph node cells)

Approximately 20 hamsters, infected with 10^7 amastigotes intracardially, were assessed one month later for parasitic burden by splenic biopsy as described in chapter 4, section 4.5.2. The animals harbouring >25–30 amastigotes/100 macrophage cell nuclei were then treated with antileishmanial drug-Miltefosine (Zentaris, Germany) at 40 mg/kg bodyweight daily for 5 days. The animals were reassessed for complete cure by splenic biopsy performed on day 30 post-treatment. Mononuclear cells/lymphocytes were separated from lymph nodes of cured, infected as well as normal hamsters following the protocol of Garg et al. (Garg et al., 2005a) and a suspension of 10^6 cells/ml was made in cRPMI. These cells were used for lymphoproliferative assay and for the estimation of NO production.

6.2.6 Immunological assays

6.2.6.1 Assessment of Lymphocyte proliferative responses (LTT) in cured/exposed patients and hamsters

Lymphocytes suspension (1×10^6 cells/ml) of cured/exposed patients and normal, infected (30 days p.i.) and cured hamsters was cultured in 96-well flat bottom tissue culture plates (Nunc, Denmark). This assay was carried out as per protocol described by Garg et al. (Garg et al., 2005a) with some modifications, wherein XTT (Roche diagnostics) was used instead of ^3H thymidine. About 100µl of predetermined concentration (10µg/ml) of Phytohaemagglutinin (PHA, Sigma, USA) for Patient's PBMCs, Concanavalin A (Con A) for hamster's lymphocytes, as well as two recombinant proteins and SLD were added to the wells in triplicate. Wells without stimulants served as blank controls. The plates were incubated at 37°C in a CO_2 incubator with 5% CO_2 for 3 days in the case of the mitogens, and for 5 days in the case of the antigens. Eighteen hours prior to termination of experiment, 50 µl of XTT

was added to 100 µl of supernatants of each well and absorbance measured at 480 nm with 650 nm as reference wavelength.

6.2.6.2 Nitrite production in macrophages of hamsters

Isolated lymphocytes from all the three study groups of hamsters viz. Normal, infected (30 p.i.) and cured, were suspended in culture medium and plated at 10^5 cells/well and stimulated for 3 days in case of Lipopolysaccharide (LPS, Sigma, USA) and 5 days in case of antigens (recombinant proteins, SLD) at 10 µg/ml. The presence of NO was assessed in the culture supernatants of cured hamster peritoneal macrophages (Garg et al., 2005a) after the exposure with supernatant of stimulated lymphocyte's. The supernatants (100µl) collected from macrophage cultures 24 h after incubation was mixed with an equal volume of Griess reagent (Sigma, USA) and left for 10 min at room temperature. The absorbance of the reaction was measured at 540 nm in an ELISA reader (Ding et al., 1988). The nitrite concentration in the macrophages culture supernatant samples was extrapolated from the standard curve plotted with sodium nitrite.

6.2.6.3 Assessment of Cytokine levels- IFN-γ/IL-12/IL-10 in lymphocytes of cured/endemic patients

Culture of PBMCs (1×10^6 cells/ml) from human patients was set up in 96-well culture plates and recombinant proteins were added to triplicate wells at a concentration of 10 µg/ml. The level of IFN-γ, IL-12 and IL-10 was estimated by ELISA kit (OptEIA set, Pharmingen) after 5 days of incubation with antigen using supernatants. Based on the standard curves generated using recombinant cytokines provided in the kit, the results were expressed as picograms of cytokine/ml. The lower detection limits for cytokines were: 4.7 pg/ml for IFN-γ, 7.8 pg/ml for IL-12p40 and 7.0 pg/ml for IL-10.

6.2.6.3.1 Solutions used for cytokine assay:

1. **Antigen coating buffer (Carbonate Bicarbonate buffer):**

   0.398 g of Na_2CO_3 and 0.933 g $NaHCO_3$ were dissolved in 250 ml of TDW and pH was adjusted at 9.4-9.6.

2. **Wash buffer (PBS-T):**

NaCl	8.0 g

KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
Tween-20	0.5 ml

The above reagents were dissolved in 1000 ml of TDW and the pH was adjusted at 7.2.

3. **Blocking Buffer:**

   4 g of BSA was dissolved in 100 ml of PBS.

4. **Secondary antibody diluents:**

   1 g of BSA and 0.05 µl of Tween-20 were dissolved in 100 ml PBS.

5. **Substrate buffer (Citrate phosphate buffer):**

   Substrate buffer was provided with kit.

6. **Substrate solution:**

   20µl of Tetramethylebenzidine (TMB) and 4µl of H_2O_2 were mixed with 10 ml of citrate phosphate buffer.

Procedure for assessment of IFN-γ/ IL-12p40/IL-10 level:

This was standardized using OptEIA set enzyme-linked immunosorbent assay kit (Pharmingen, San Diego, California) in supernatants of antigen primed lymphocytes.

1. ELISA plate (NUNC maxisorp microtitre plates) was coated with 100 µl of capture antibody (anti-human IFN-γ/ IL-12p40/IL-10, coating dilution – 1:250) diluted in coating buffer.

2. The plate was incubated overnight at 2-8°C or for 2 hr at 37°C.

3. Wells were washed several times with wash buffer (PBS-T).

4.200µl of blocking buffer was added to each well for blocking of non-specific binding site and incubated for 1hr at RT.

5. Blocking buffer was aspirated from wells.

6.100 µl/well of recombinant human IFN-γ/IL-12p40/IL-10, at different dilutions was added (for IFN-γ, 300, 150, 75, 37.5, 18.8, 9.4, 4.7 and for IL-12 and IL-10, 500, 250, 125, 62.5, 31.3, 15.65 and 7.8 pg/ml) and sample supernatant were added in duplicates.

7. The plate was sealed with adhesive cover and incubated for 1hr at 37°C.

8. Wells were washed 3 times by forcefully dispensing wash buffer.

9. Wells were blotted on paper towels.

10. Thereafter, 100µl of HRP-conjugated anti-human IFN-γ/IL-12p40/IL-10, (2.5µg/ml) was added in each well.

11. Plates were incubated for 1hr at 37°C.

12. Wells were washed 3 times as in step 3.

13.100µl of TMB substrate reagent (prepared just before use) was added to each well.

14. The plate was incubated for 30 min at RT.

15. Reaction was stopped by adding 100µl of stop solution (2N-H_2SO_4) per well.

16. Optical density was recorded at a wavelength of 450 nm in micro plate ELISA reader.

17. A standard curve was constructed by plotting the mean absorbance for each standard on their vertical (Y) axis verses the corresponding standard IFN-γ/IL-12p40/IL-10, concentration on the horizontal (X) axis.

18. Level of IFN-γ/IL-12p40/IL-10, in sample was calibrated by using the standard curve.

6.3 Results:

6.3.1 The recombinant Th1 stimulatory proteins (rLdADHT, and rLdTPR,) induced lymphoproliferative and NO responses in normal/infected/cured hamsters:

The cellular responses of lymph node cells of cured hamsters were assessed by XTT against the mitogen Con A as well as SLD and recombinants proteins. The responses were compared with that of normal as well as *L. donovani* infected groups that served as controls. The normal, infected control as well as cured *Leishmania* infected group had shown significantly higher proliferative responses (mean OD 2.416 ± 0.188, 2.316 ± 0.088 and 2.571 ± 0.521 respectively) against Con A as compared to *L. donovani*-infected group indicating the procedural sensitivity. The results of the proliferative response of lymphocytes against rLdADHT showed significantly higher stimulation in cured/infected hamsters (mean OD 1.762± 0.120 and 1.125 ± 0.078) than SLD (mean OD 1.077 ± 0.118 and 1.449 ± 0.056). The difference was statistically significant (p<0.01). On the other hand the proliferative response of rLdTPR was lesser than that of SLD as evident in the figure and also was not significant at all **(fig. 6.1).**

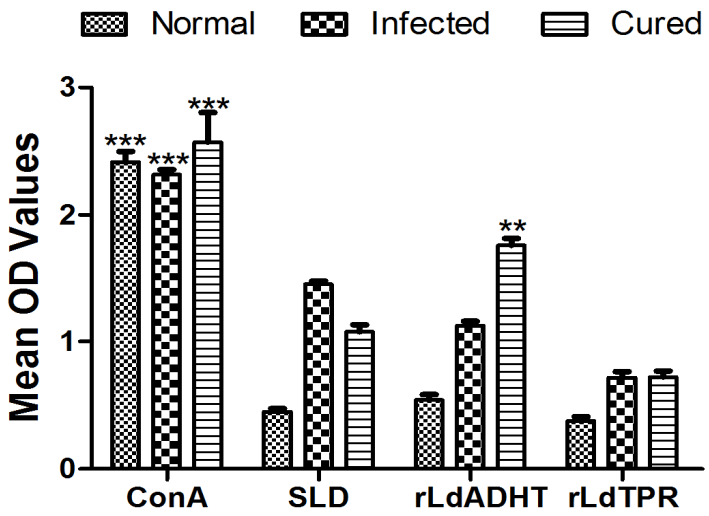

Figure 6.1 : Lymphoproliferative response of mononuclear cells of lymph nodes from normal, *L. donovani* infected and treated cured hamsters in response against two recombinant proteins/SLD. Significance values indicate the difference between the SLD and the recombinant proteins' stimulation (*, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$).

NO-mediated macrophage effector mechanism is known to be critical in the control of parasite replication in the animal model hence its production in peritoneal macrophages of cured hamsters, was studied after 24 h of incubation in the presence of recombinant proteins and SLD. For comparison, NO production in mitogen (LPS) stimulated and unstimulated cells served as positive and negative controls respectively. NO production was recorded to be ~1-1.5 fold higher against rLdADHT and while it was lesser in case of rLdTPR in comparison to SLD. The difference between rLdADHT protein and SLD was significant (p<0.001) (**Fig. 6.2**).

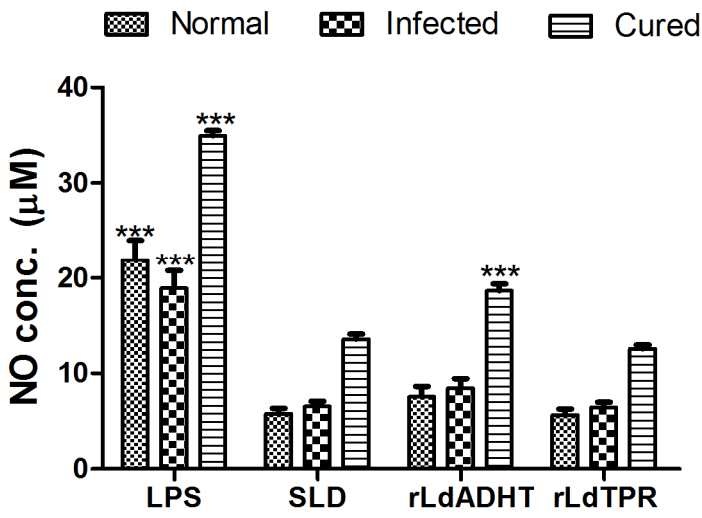

Figure. 6.2 : NO production by macrophages of hamsters, in response to the recombinant proteins/SLD stimulation. Significance values indicate the difference between the SLD and the recombinant proteins' stimulation (*, p<0.05; **, p<0.01; and ***, p<0.001).

6.3.2 The recombinant proteins (rLdADHT and rLdTPR) stimulate PBMCs from *Leishmania* infected cured/endemic contacts to proliferate and to express a predominant Thl Cytokines Profile:

The cellular responses (LTT and cytokine levels) were further validated in PBMCs of cured/infected patients, and endemic controls. Individual donors in each study group were found to elicit different responses. Proliferation and cytokine responses of recombinant proteins viz. rLdADHT and rLdTPR in PBMCs from patients with active VL/cured/endemic were compared with SLD. Endemic control, cured and infected patients exhibited higher

mean OD values against PHA, i.e.2.268 ± 0.226, 2.711 ± 0.310 and 1.844 ± 0.190 respectively. PBMCs from all the cured, active VL patients and endemic contacts proliferated in response to rLdADHT with mean OD values of 1.143±0.150, 0.537±0.085 and 0.704 ± 0.177 which were higher values than SLD (mean OD values of 0.604±0.72, 0.316±0.095 and 0.409±0.098) respectively. The difference was highly significant (p<0.01). In case of rLdTPR, the stimulation was greater than that of SLD and lesser as compared to the other recombinant protein rLdADHT and was also statistically significant (p<0.05).The results demonstrated that out of two, only rLdADHT was potent T cell antigens as recognized by a majority of *L. donovani*–infected/cured/endemic individuals in different stages or manifestations of infection (**Fig. 6.3**).

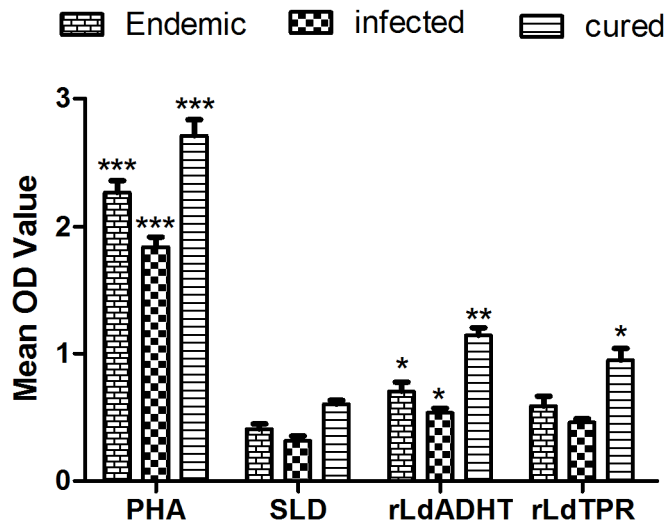

Figure.6.3 : Lymphoproliferative response of PBMCs from individuals of Cured VL patients and Endemic controls against the recombinant proteins/SLD stimulation. Significance values indicate the difference between the SLD and the recombinant proteins' stimulation (*, p<0.05; **, p<0.01; and ***, p<0.001).

To validate the Th1/Th2 stimulatory potential of the recombinant proteins-rLdADHT and rLdTPR, the estimation of cytokine levels viz. IFN-γ, IL-12p40 and IL-10 was further carried out in PBMCs from cured/infected patients as well as in endemic contacts. Optimum stimulation of IFN-γ and IL-12p40 responses were noticed against both rLdADHT and rLdTPR in all the cured patients and endemic contacts. The level of IFN-γ was found to be

higher in cured patients ranging from 773 to 2150 pg/ml (rLdADHT) and 754 to 1945 pg/ml (rLdTPR) and in endemic contacts it was in the range of - 332 to 1553 pg/ml and 417 to 1280 pg/ml in comparison to SLD (105 to 705 and 165 to 591 pg/ml). Similarly, IL-12 level was observed to be elevated in cured patients ranging from 823 to 1470 pg/ml and 733 to 1201 pg/ml as well as in endemic contacts -844 to 1262 pg/ml and 742 to 1160 pg/ml in contrast to SLD (178 to 541 and 122 to 488 pg/ml). On the contrary, very low level of IL-10 cytokines against all the two recombinant proteins was detected in cured (7- 27 pg/ml and 8 to 32 pg/ml) and also in endemic contacts (23 to 68 pg/ml and 13 to 73 pg/ml) as compared to SLD (239-436 and 66-291 pg/ml) (**Fig. 6.4**). Moreover, no or little detectable amount of IFN-γ and IL-12p40 level was observed with the lymphocytes of the healthy individuals as well as infected patients against the recombinant proteins (data not shown). PBMCs of cured/endemic contacts generated a mixed Th1/Th2 cytokine profile against SLD wherein high levels of IL-10 and very little level of IFN-γ, and IL-12p40 were noticed in response to SLD in cured/endemic contacts. Overall, the cytokine responses of the cured *Leishmania* patients as well as endemic contacts were better against rLdADHT as compared to rLdTPR.

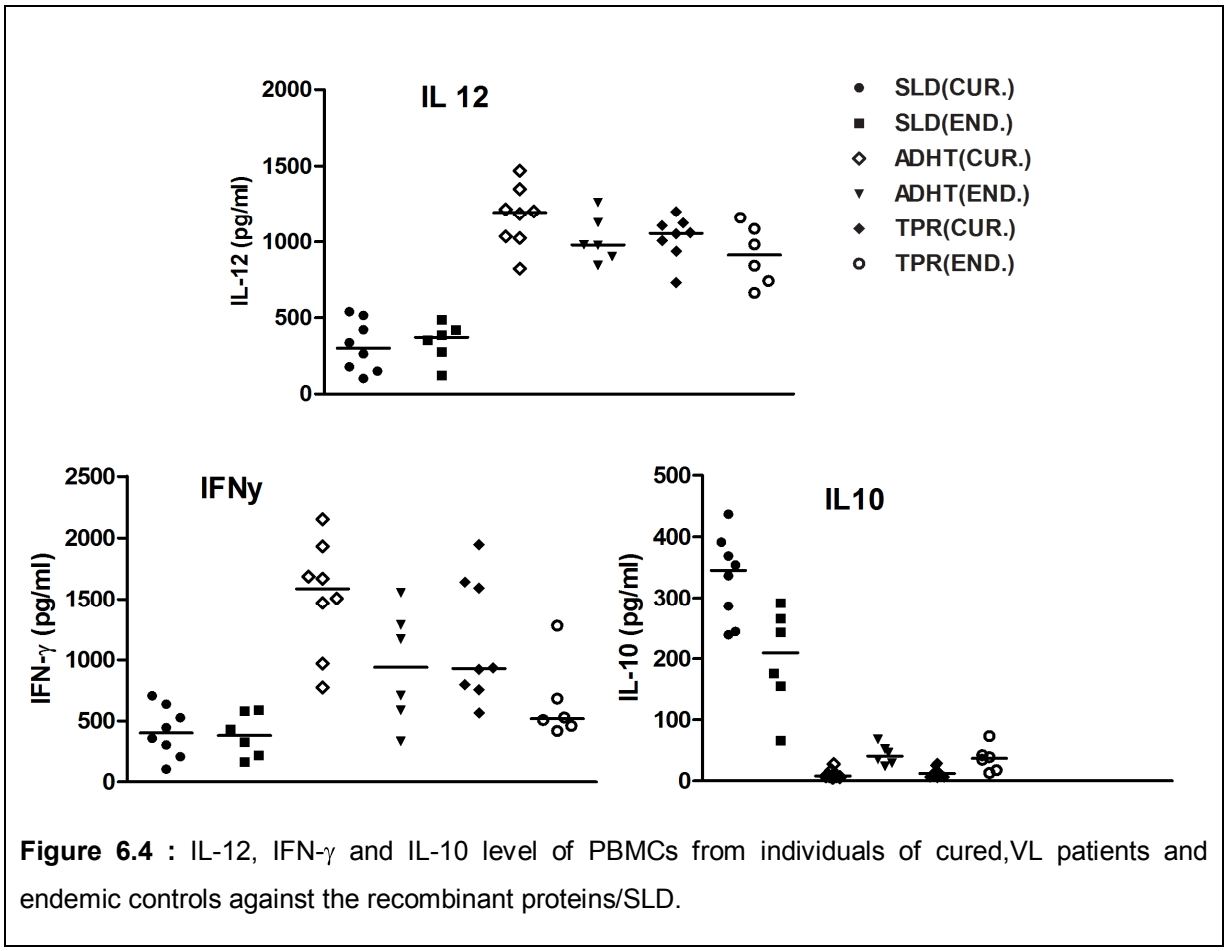

Figure 6.4 : IL-12, IFN-γ and IL-10 level of PBMCs from individuals of cured,VL patients and endemic controls against the recombinant proteins/SLD.

6.4 Discussion

In VL, Th1 immune responses play an important role in controlling the disease hence, T-cell stimulatory antigens are thought to be good vaccine targets. Consequently, search of such antigens in *Leishmania* parasite which elicit cellular responses in PBMCs/lymphocytes of cured *Leishmania* patients/hamsters was made. In earlier studies from our laboratory, while carrying out classical activity based fractionation and sub-fractionation of soluble *L. donovani,* we observed that the sub-fraction ranging from 89.9-97.1 kDa elicited significant Th1 stimulatory response in PBMCs/lymphocytes of cured Leishmania patients/hamsters. Further proteomic characterization of this sub-fraction revealed a number of proteins (Kumari et al., 2008c). that were identified as drug target(s) in other organisms Among these ADHT and TPR were developed as recombinant proteins and were eventually reassessed for their immunogenicity using PBMCs of cured *Leishmania* patients and lymphocytes of cured *Leishmania* infected hamsters.

The existence of human analog to the rodent Th1 and Th2 subsets has been disputed. The reason for reluctance in accepting human Th1 and Th2 is probably that the dichotomy in the human system is not as clear as in rodent particularly murine cells. Besides, the infection pattern also does not simulate the human profile, as it is self-limiting in murine VL. On the other hand, systemic infection of the hamster with *L. donovani* results in a relentlessly increasing visceral parasite burden which closely mimic active human VL (Melby et al., 1998a). Hence, analysis of cellular immune response of the recombinants proteins were carried out using hamsters' lymphocytes as well as macrophages that have been cured with Miltefosine in order to correlate the observations made with the human lymphocytes. All the cured hamsters showed good LTT and NO response to rLdADHT as compared to rLdTPR. Successful vaccination of humans and animals is often related to T-cell stimulation with mitogen and antigens *in vitro* (Armijos *et al.*, 2003; Melby *et al.*, 1998a). Further, it is well established that recovery from *Leishmania* infection, relies on induction of Th1 response (Liew, 1991a; Liew, 1991b) with production of IFN-γ, IL-12 and enhanced expression of nitric oxide synthase (Assreuy et al., 1994). Therefore, in the absence of cytokine reagents for hamsters, nitric oxide assay was used to indirectly estimate the IFN-γ response, as NO is up regulated by IFN-γ.

Although lymphocyte proliferation has been widely used to analyze T-cell function, the documentation that the CD4+ population is heterogeneous as regard to the cytokine profile secreted, indicates that cytokines should be measured to determine whether an immune response would be protective or deleterious. It is well known that in human and experimental leishmaniasis immunity is predominantly mediated by T lymphocytes (Sacks & Noben-Trauth, 2002) which also participate in the immune response to *L. donovani* infection by producing different cytokines. Th1 cells produce IFN-γ and are involved in cell-mediated immunity, while Th2 cells produce IL-4, IL-5 and IL-10 and are more involved in humoral immunity.

The characterization of the cellular immune response was first performed in cured *Leishmania* infected hamsters and then the responses of both of the proteins were validated in endemic non immune donors (household contacts without any clinical symptoms), VL patients and in immune patients of VL that were cured with amphotericin B following the similar protocol as described earlier (Garg et al., 2005a; Tripathi et al., 2006), who have demonstrated the development of a good T-cell response, when cells from these individuals are stimulated with different fractions of *Leishmania* antigen. (Burns et al., 1991) and (Russo et al., 1991), have also shown the induction of lymphocyte proliferation and IFN-γ production by some of the recombinant antigens viz. gp63 in subjects cured of visceral form and in patients with cutaneous or mucosal leishmaniasis.

The most commonly used method for assessing cellular proliferation is measurement of [3^{H}]thymidine incorporation into DNA, but due to its being radioactive, we herein used a relatively non-hazardous protocol- XTT (sodium 3'-[1-phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate. This assay is based on the extracellular reduction of XTT by NADH produced in the mitochondria via trans-plasma membrane electron transport and an electron mediator. Reduction of XTT produces a water-soluble formazan which dissolves directly into the culture medium, accompanied by a measurable shift in wavelength with a corresponding change in color.

The significant lymphoproliferative responses were observed against rLdADHT among the two recombinant proteins and this was supported by the high levels of IFN-γ, IL-12p40 stimulated by this protein. The cellular responses of rLdADHT was stronger than SLD whereas that of rLdTPR was lesser or equal to that of SLD. Of particular interest is the observation that IL-12, which is produced primarily by phagocytic cells in response to

infection with intracellular pathogens or in response to microbial products, can promote the development of Th1 cells (Heinzel et al., 1993; Sypek et al., 1993) and augments cytotoxicity (Bloom, 2005; Ghalib *et al.*, 1995; Heinzel *et al.*, 1993). Once secreted, IL-12 is a strong stimulator of IFN-γ production by T and NK cells. On the other hand, the level of IL-10 against both of the proteins was found to be suppressed in cured patients as well as in endemic contacts in this study. IL-10 is thought to promote intracellular infection, including human VL, by disabling Th1 -type immune responses and/or deactivating parasitized tissue macrophages (Carvalho *et al.*, 1994; Holaday *et al.*, 1993).

Thus, it may be summarised that the results obtained herein strongly indicate that rLdADHT protein contained dominant Th1 stimulatory property as compared to rLdTPR. The data further suggest that this protein have the potential of being developed as vaccine as this might have some potent epitopes readily recognized by T-cells from both treated/cured as well as endemic individuals presumably sensitized with *L. donovani*. Hence, individuals who control parasite load successfully either following treatment in the case of human patients/hamsters or due to adequate immunity, as in endemic contacts, exhibit good T cell reactivity to the *Leishmania* antigen. This finding leads to the first step of antigen selection in experimental models as well as in human being, in order to evaluate ability to induce protection against leishmanial infection.