ADDIS ABABA UNIVERRSITY
COLLEGE OF HEALTH SCIENCE
DEPARTEMENT OF MICROBIOLOGY, IMMUNOLOGY AND PARARSITOLOY

IMMUNOGENICITY OF LEISHMANIA DERIVED ANTIGENS IN PERIPHERAL BLOOD MONONUCLEAR CELLS USING BLOOD OBTAINED FROM PREVIOUSLY TREATED VISCERAL LEISHMANIASIS PATIENTS OF SOUTHWEST ETHIOPIA.

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A THESIS SUBMITTED TO DEPARTMENT OF MICROBILOGY, IMMUNILOGY AND PARASITOLOGY, COLLEGE OF HEATLH SCIENCES, ADDIS ABABA UNIVERRSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MEDICAL PARASITOLOGY.

June, 2015
Addis Ababa, Ethiopia

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Contents

Acknowledgement i

Contents ii

List of tables iv

List of figures v

Abbreviations vi

Abstract: vii

1. Introduction 1

1.1 Background 1

1.2 Statement of the problem 3

1.3 Rationale of the study 5

2. Literature review 6

3. Objectives 10

3.1. General Objective 10

3.2. Specific Objectives 10

3.3. Hypothesis 10

4.2 Population 11

4.2.2 Source population 11

4.2.3 Study population 11

4.3 Study design 11

4.4 Sample size and sampling technique 11

4.5 Variables 12

4.5.1 Dependent variables 12

4.5.2 Independent variables 12

4.5.3. Exclusion criteria 12

4.6 Data collection 12

4.6.1 Data collection instrument 12

4.7 Specimen Collection 13

4.8. PBMC isolation and culture 13

4.10 Cell culture of PBMCs and antigen stimulation 13

4.11 Cytokine asssays 16

4.12 Statistical Analysis 16

4.13 Data Quality Assurance 17
4.14 Dissemination of Results 17
4 .15 Ethical consideration........ 17
5.2 Cytokines response to PHA, LSA and leishmania derived antigens........ 19
5.2.1 TNF-alpha response to leishmania derived antigens 19
5.2.2 IFN-gamma response to leishmania derived antigens........ 23
5.2.3 IL-10 response to leishmania derived antigens........ 27
5.3 Comparison of cytokine responses among the candidate vaccine antigens 32
5.3 Stool examination. 32
6. Discussion........ 33
7. Limitation of the study........ 36
8.Conclusions and Recommendations 37
8.1 Conclusions........ 37
8.2 Recommendations........ 38
9.References........ 39
10. Annexes........ 42
Annex I: Questionnaire 42
Annex II፡ Protocol for Isolation and Quantitation of PBMCs........ 44
Annex III: - ELISA procedure 45
Annex IV:-Formal-Ether Concentration technique procedure........ 46
Annex V- Information Sheet and consent form 47
ለ. የስምምነት ማረጋጫ ቅጽ 49

List of tables

Table 1:-Leishmania-derived potential candidate vaccine antigens tested ex vivo in PBMC of previously treated VL patients. 14

Table 2:- Demographic and clinical characteristics of all study participants in Arba Minch Hospital LRTC (Leishmaniasis Research and Treatment centre), SNNPR, 2014. 18

Table 3 :-TNF-alpha response to different leishmania-derived antigens measured in pg/ml in experiments involving drug-cured pateints (n=18). 19

Table 4 :-FN-gamma response of PBMCs derived from drug-cured VL patients after stimulation with different leishmania derived antigens. 23

Table 5 :-IL-10 response of PBMCs derived from drug-cured VL patients after stimulation with different leishmania derived antigens. 27

List of figures

Figure 1:- TNF-alpha production by peripheral blood mononuclear cells in response to PHA, LSA and 9 leishmania-derived antigens examined in drug-cured patients (n=18), endemic healthy controls (n=3), and non-endemic healthy controls (n=2)... 20

Figure 2:-shows TNF-alpha production differnce among drug-cured (n=18), endemic (n=3) and non-endemic healthy controls (n=2) to PHA, LSA and 9 leishmania derived antigens. Statistical differences between groups was determined using the non-parametric, Mann-Whitney test. 21

Figure 3 :-IFN-gamma production by peripheral blood mononuclear cells in response to PHA,LSA and 9 leishmania derived antigens examined in drug-cured patients (n=18), endemic healthy controls (n=3), and non-endemic healthy controls (n=2) 24

Figure 4:- shows IFN-gamma production among drug-cured (n=18), endemic (n=3) and non-endemic healthy controls (n=2) to PHA, LSA and 9 leishmania derived antigens.Statistical differences between groups was determined using the non-parametric, Mann-Whitney test. 25

Figure 5 :- IL-10 production by peripheral blood mononuclear cells in response to PHA, LSA and 9 leishmania derived antigens in drug-cured patients (n=18), endemic healthy controls (n=3), and non-endemic healthy controls (n=2). 28

Figure 6:- IL-10 production among drug-cured (n=18), endemic (n=3) and non endemic healthy controls (n=2) to PHA, LSA and 9 leishmania derived antigens... 29

Figure 7:-IL-5 production by peripheral blood mononuclear cells in response to PHA,LSA and 9 leishmania derived antigens examined in drug-cured patients (n=18), endemic healthy controls (n=3), and non-endemic healthy controls (n=2). 31

Abbreviations

CL = Cutaneous leishmaniasis

Dc = Drug cured

EHC = Endemic healthy control

GIPLS = Glycoinositol phospholipids

HC = Healthy Controls

IMP = Integral membrane protein

LDA = Leishmnia donovani amastigote

LDP = Leishmnia donovani promastogote

LPG = Lipophosphoglycan

LPGAP = Lipophosphoglycan associated protein

MoAb = Monoclonal antibody

NEHC =Non-Endemic Healthy Controls

NSP = Non- membranous soluble protein

PBMC = Peripheral blood mononuclear cells

PPD = Purified protein derivatives

SAF2 = Sub fraction two

SLA = Soluble leishmania antigens

VL = Visceral leishmaniasis

WE = Whole cell extract

ZCL = Zoonotic cutaneous leishmaniasis

ZVL = Zoonotic visceral leishmaniasis

Abstract:

Background:-Visceral leishmaniasis is a public health problem and it is fatal if untreated. Disease burden is associated with poverty, lack of effective, affordable, and minimally toxic treatments. An effective vaccine is needed to combat this disease.

Method: - Peripheral blood mononuclear cell-based assays were set-up in blood samples obtained from 5 healthy controls and 18 drug-cured VL patients (DC-VL), who are putatively immune to re-infection. For PBMCs stimulation nine *Leishmania* derived antigens were selected, and together with whole promastigote lysate antigen and Phytohaemagglutinin (PHA), and assessment of immunogenicity was carried out by cytokine assays using ELISA kit. The assay aimed at measuring IFN-gamma, TNF-alpha, IL-10 and IL-5 in culture supernatants of PBMCs stimulated in vitro.
The association of cytokine production with immunogenicity difference of antigens was determined by using non-parametric tests (Mann Whitney & Tukey's Multiple Comparison tests) found in Graph Pad Prism version 6.

Result: - Drug-cured visceral leishmaniasis (VL) patients develop protection mediated by Th1-type cellular responses against new infections. IFN-gamma, TNF-alpha and IL-10 production were higher in drug-cured groups, but neither drug-cured nor healthy controls produced IL-5. The data suggest the mounting of Th1 type responses upon cure from visceral leishmaniasis, while small amounts of IL-10 were also measurable. NS, S, PNS and NSL were more immunogenic than other antigens as determined by levels of IFN-gamma, TNF-alpha and IL-10.

Conclusion:-This study indicates that T cell recall ability of humans cured from VL by chemotherapy was conspicuous and related to Th1 type immune response. Further, the data confirm that high levels of IFN-gamma and TNF-alpha in the presence of low levels of interleukin-10 (IL-10) could be proxy indicators of protective immunity in drug-cured visceral leishmaniasis patients. Among tested antigens NS, NSL, PNS and S were more immunogenic and elicited high levels of IFN-gamma and TNF-alpha in PBMCs of drug-cured patients, with supernatant levels ranging from16 to1826 mg/ml.

Key words: Antigen immunogenicity, visceral leishmaniasis

1. Introduction

1.1 Background

Visceral leishmaniasis (VL) also known as kala-azar, is a disseminated protozoan infection caused by eukaryotic intracellular parasites belonging to the *Leishmania donovani* complex. Two parasite species are responsible for VL. *L. infantum* in Europe, the Middle East and North Africa and *L. chagasi* in Latin America, which are transmitted zoonotically with dogs serving as reservoir hosts, while *L. donovani* in the Indian subcontinent as well as East Africa is transmitted between humans(Chappus F *et al.*, 2007).

Leishmania is transmitted by the bite of infected *Phlebotomine* sand flies that inoculate highly infective metacyclic promastigotes into the mammalian host, where they differentiate inside macrophage phagolysosomes into the replicative amastigote form. The remarkable resistance of Leishmania to hydrolytic environments, encountered in both the insect and mammalian hosts, is conferred by a dense surface glycocalyx, formed by related glycoinositol phospholipids (GIPLs) and lipophosphoglycan (LPG), and proteins such as the parasite surface protease gp63, proteophosphoglycans and gp46 (Joseph L *et al.*, 2004).

Zoonotic VL caused by *L. infantum* is a re-emerging zoonosis which represents a complex epidemiological cycle in the new world. Domestic dogs serve as a reservoir host for potentially fatal human infection, and where dog culling is the only measure for reservoir control. Lifelong immunity to VL has motivated the development of prophylactic vaccines against the disease but very few antigens have progressed beyond the experimental stage(Amrita D and Nahid A, 2012).

Acute visceral leishmaniasis in humans and mice is associated with the production of specific and nonspecific circulating antibodies (Ghose A *et al.*, 1980, Joseph L *et al.*, 2004). At the same time, there is repression of cell-mediated immunity, which is associated with an inability to generate gamma interferon (IFN-γ) and interleukin-2 (IL-2); and decreased production of IL-1 and tumor necrosis factor-α (TNF-α) (Carvalho *et al.*, 1985). These immunological defects are restored after recovery from infection, and both humans and mice are resistant to re-infection by *L. donovani*.

Epidemiologic studies of human visceral leishmaniasis suggest that up to 85% of infected individuals may spontaneously control infection(Evans T *et al.*, 1992). This is related to the simultaneous production of IFN-γ, IL-2 and TNF-α by a particular subset of CD4 T cells (Darrah P *et al.*, 2007) and/or by the balance between pro-inflammatory IFN-γ and TNF-α and regulatory IL-10(Stober C *et al.*, 2005). Therefore, in the analysis of human immune responses to known and novel antigens, the ability to stimulate production of IFN-γ, TNF-α and IL-10 in cured VL cases compared to active VL cases would provide some insight into their potential as vaccine candidates(Omprakash S *et al.*, 2012a).

Interferon gamma (IFN-γ) is an important Th1 cytokine, which is crucial for the control of intracellular infections. The production of IFN-γ is controlled by a number of monocyte/macrophage-derived cytokines, among which, interleukin-12 (IL-12) is a potent inducer of IFN-γ production when used as single stimulus. While IL-18 is known to synergistically enhance the effect of IL-12 on IFN-γ release, IL-15 has also been implicated as an important co-stimulus for IFN-γ production (Hailu A *et al.*, 2004).

Increased levels of mRNA encoding IL-10, IL-2 and IFN-γ in bone marrow aspirates (Christopher L *et al.*, 1993) and lymph nodes (Ghalib H *et al.*, 1993) from African patients have been observed during active disease, whereas after healing, mRNA for IFN-γ and IL-2 were readily detectable (Christopher L *et al.*, 1993). Furthermore, the neutralization of IL-10 with specific monoclonal antibody (MoAb) restores T-cell proliferation and IFN-γ production in peripheral blood mononuclear cells (PBMC) obtained from acute VL patients; and IL-12 shifts the responses towards a Th1 enhancing IFN-γ production (Ghalib H *et al.*, 1995).

Early classical experiments established that CD4+ T cells are crucial for resistance, whereas CD8+ T cells seem to participate more in generation of immune memory and as effectors cells for parasite elimination (Awasthi A and Saha B, 2004). However, recent studies have suggested that CD8+ T cells may be involved in the clearance of primary infection. Regarding immune responses in acute human visceral leishmaniasis, the cytokine profile is typified by high production of IL-4 and IL-10 and low IL-2 and IFN-γ production (Khalil E *et al.*, 2005a).

1.2 Statement of the problem

Visceral leishmaniasis is a serious public health problem, accounting for enormous morbidity and mortality. The estimated annual global incidence of VL is 200,000 to 400,000, and 90% of these cases occur in India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil (Evans T *et al.*, 1992). And approximately 1–1.5 million cases of cutaneous leishmaniasis occur worldwide each year. However, this is probably an underestimate, as many cases are not diagnosed(Evans T *et al.*, 1992) .

In Africa, the worst affected region is southern Sudan with an estimated average of 15,000-20,000 cases per year (Alvar J *et al.*, 2008). The most important VL endemic areas in Ethiopia are found in the northwest (Metema-Humera lowland), which accounts for approximately 60% of the cases, and in the southwest (Lake Abaya, Omo river plains and Segen and Woito valleys). In recent years VL has spread to the highlands of Libo-Kemkem district (south Gondar), claiming the lives of hundreds of patients (Chappus F *et al.*, 2007).

Most of infected humans are subclinical or asymptomatic, and this asymptomatic infection is associated with strong cell-mediated immunity (Alvar J *et al.*, 2012). Only a small percentage of infected individuals develop severe disease and patients who recover from VL display resistance to re-infection. It is believed that direct interaction between parasite antigens and host immune cells shape the subsequent pathogenic or protective immune responses (Murray H, 2001).

Immunological abnormalities could be found in monocyte and T-cell functions, such as diminished production of TNF-α and IL-1 and absence of delayed-type hypersensitivity to *Leishmania* antigen-stimulation and a decreased capability of T-cells to activate macrophages and kill *Leishmania*. By the antigen specific lymphocyte population, plasma levels of IFN-γ, IL-12 p40, IL-18, IL-15, interferon-gamma inducible protein (IP-10) and monokine induced by IFN-γ are markedly elevated in symptomatic VL patients as compared to individuals with asymptomatic infection, and significant decrease of plasma levels of IFN-γ and all mediators has been observed after treatment of such patients. In these patients, production of type I cytokines is not depressed, but there appears to be unresponsiveness to the stimuli of type 1 cytokines (Malla N and Mahajan R, 2006).

Leishmania vaccine development has proven to be a difficult and challenging task and is hampered by an inadequate knowledge of disease pathogenesis, the complexity of immune responses needed for protection, and the cost of vaccine development. The burden of VL is concentrated in resource poor nations and a lack of political will and investment in reseach further aggravates the situation. However, the rise of biotechnology industries in endemic countries, such as India, may provide an impetus for VL vaccine development and investment.

A recent clinical trial in India assessed the safety and immunogenicity of the LEISH-F1+MPL-SE vaccine(J. Chakravarty, 2011).

1.3 Rationale of the study

Kala-azar is one of the public health problems in Ethiopia which affects 4500-7000 persons annually. People cured from visceral leishmaniasis develop protection mediated by Th-1 type response against new infection, and such protective immunity is associated with antigen specific cell mediated immune response, which can be measured by lymph proliferative responses and delayed type hypersensitivity reaction and production of Th-1 cytokines like IFN-γ and IL-2.

A vaccine is direly needed for protection against VL caused by *L. donovani* and *L. infantum* as well as against the many species that cause mutilating cutaneous disease. However, to date, no vaccine has been approved for use against human leishmaniasis. However, efforts to discover protective antigens are ongoing, and several antigens with a potential prophylactic and therapeutic potential are under investigation.

This study was initiated to assess immunogenicity of candidate vaccine antigens in patients with past history of VL, specifically to assess immunogenicity of the antigens among south Ethiopian VL patients. Drug-cured VL patients are expected to be naturally protected when no relapses or secondary episodes of VL have occurred. The study will assist to select more potent antigens for further evaluation in larger studies.

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2. Literature review

Several experimental studies in murine models of cutaneous leishmaniasis (CL) have established a clear-cut dichotomy between Th1-mediated protection and Th2-mediated disease susceptibility (Sacks D and Noben-Trauth N, 2002). Th1 responses contribute to resistance and protection in visceral disease. The majority of human leishmania infections are subclinical or self-limited often leading to the development of long-term immunity and protection against re-infection (Kharazmi A *et al.*, 1999).

A study was conducted in India to assess immune response of drug- cured visceral leishmaniasis patients by whole blood stimulation using kinetoplastid membrane protein-11 (KMP11), sterol 24-c-methyltranferase (SMT), A2, cysteine proteinase B (CPB), 10 K26/HASPB, 11 and nucleoside hydrolase (NH) (Rajiv K *et al.*, 2010). Among 15 post-treatment individuals with a history of kala-azar, 12 showed soluble leishmania antigen (SLA)-specific stimulation of IFN-γ production (Kamlesh G *et al.*, 2011)(> 20 pg/mL), but only 1 of 5 healthy individuals responded to SLA. Significantly higher IFN-γ production was found in the cured group than in the non-exposed group (P = 0.03). Although both mean and median values of TNF-alpha were higher in the cured group, it was not statically significant (*P* = 0.96). The IL-10 was detected in 3 of the cured individuals and 1 of the non-exposed. Only 2 study participants showed IL-2 levels above the detection limit; none of the IL-4 and IL-5 levels were above the detection limit (Rajiv K *et al.*, 2010). IL-2, IL-4, IL-5, and TNF-alpha did not clearly differentiate between the cured and non-exposed groups, with levels ranging from low to undetectable(Rajiv K *et al.*, 2010).

Among candidate vaccine antigens tested, the largest number of cured subjects recognized cysteine proteinase B, leading to heightened IFN-γ responses, followed by sterol 24-c-methyltransferase. These two antigens were the most immunogenic and protective in a murine VL model, indicating a relationship between T cell recall responses of humans cured from VL and protective efficacy in an experimental model(Rajiv K *et al.*, 2010).

In another study conducted in India to assess cytokine resonance to novel antigen in whole blood assay using blood obtained from VL cured patients, antigen specific cytokines were released in response to novel vaccine antigens. Maximal IFN-γ (>=20 ng/mL above background) production was observed at 24 and 72 hours post stimulation with 55–87.5% of the novel antigens (R71, L37, N52, J41 and M22). From five leishmania antigens (R71, L37, N52, J41 and M22), L37 exceptionally elicited the highest sustained IFN-γ level at day 6 post stimulation. TNF-alpha responses in cured VL and EHC groups paralleled responses of IFN-γ, but no IL-10 responses were found(Omprakash S *et al.*, 2012a).

Five Leishmania antigens (R71, L37, N52, J41 and M22), among 11 putative candidate vaccines, stimulated potent pro-inflammatory recall responses in exposed but protected individuals (cured VL patients and EHC) in the absence of regulatory IL-10, providing potential immunotherapeutic or vaccine target(Omprakash S *et al.*, 2012a).

From 15 cured subjects with a history of kala- azar from India showed SLA-specific IFN-γ production. However, the SLA specific IFN-γ production in 11 of the 13 individuals with active VL disease was unlikely(Kamlesh G *et al.*, 2011). Persons recovered from ZVL produced significantly higher levels of IFN-γ against Leish A (mean ± SE = 2.66 ± 0.61 IU/mL) than persons in the ZCL area (P = 0.029)(Mohammed H *et al.*, 2012). Significantly increased levels of IFN-γ was produced in patients with active VL upon stimulation with SLA (80% [28/35]), but there was no significant difference in IFN-γ production when compared with cured VL. Only 1 of the 27 patients (3.8%) suffering from another febrile illness secreted IFN-γ above the baseline level(Omprakash S *et al.*, 2012b).

The frequencies of IFN-γ and IL-10-producing cells were significantly higher among individuals with a history of VL (P=0.026 and P=0.018, respectively). The frequency of IL-4-producing cells was also higher, but the difference was not statistically significant (P=0.054)(Kemp K *et al.*, 1999).The mixed production of IFN-γ and IL-4 is in line with the response observed in Leishmania-reactive $CD4^+$ T cell clones isolated from cured visceral leishmaniasis patients stimulated by crude leishmania antigens (LDP and LDA) or LPGAP, which can be either Th2, Th1, or Th0 and weak proliferative response (Kurthals J *et al.*, 1994).

The immune response in non-membranous soluble protein (NSP) stimulated blood taken from India showed low IFN-γ levels (47·0 ± 25·7 & 35·2 ± 14·3 pg/ml) in non endemic controls groups, whereas endemic control and cured patients showed significantly higher interferon gamma($P < 0·01$) against IMP (315·24 ± 185·67 & 356·3 ± 213·4, respectively) and NSP (255·6 ± 94·7 & 294·29 ± 107·6, respectively)(Garg R *et al.*, 2005).
The frequency of positive responses among the exposed Indian subjects was higher (median 7.1, range 1.0 - 74.3), and it was significantly higher than among healthy controls [median 1·2 (range 0·5–1·6) $P < 0·0001$], none of whom showed a positive response. Drug-cured individuals [median 8·1 (range 5–74·3)] and endemic contacts [median 6·4 (range 1–41)] had comparable responses. IFN-γ levels in the WE-stimulated supernatants were significantly higher in the exposed group [median 188 pg/ml (range 1–913)] compared to healthy controls [median 3·6 pg/ml (range 1–28) $P < 0·0001$]. The levels of IL-12 and IL-10 in the exposed group [median 1355 pg/ml (range 59– 2718); median 364 pg/ml (range 1–1100), respectively]) were also significantly higher than in controls [IL-12: median 93·2 pg/ml (range 2·1–623·8); IL-10: median 3·6 pg/ ml (range 2·1–28·1);$P <0·0001$]. IL-4 was not detectable in any of the control or study group samples (Tripathi P *et al.*, 2005).

In a study carried out (conducted) in Sudan to investigate the expression of CD4 and CD8 among the cytokine-producing cells, most of the IFN-γ was found in CD4+ cells; with 75% - 97% of the IFN-γ producing cells expressing CD4. The remaining IFN- γ containing cells were either CD8+ (between 2% and 17%) or CD4-CD8- (0-8%). Similarly, most IL-4 was found in CD4+ cells, as 77-97% of IL-4 containing cells were CD4+. CD8+ cells constituted 3-20%, whereas 0-7% was CD4-CD8-. Thus IFN-γ and IL-4-producing cells were CD3+; and among these, the cytokines were mainly found in CD4+ cells rather than in CD8+ cells(Kemp K *et al.*, 1999).

Another study in Sudan, in a vaccine study using Alum-precipitated autoclaved *Leishmania major* (Alum/ALM) ± bacille Calmette–Guérin (BCG), spontaneous IFN-γ production by PBMCs (>1000 pg/ml) was seen in two volunteers in pre-vaccination samples (P=0·001), with no significant spontaneous production at days 45 and 90. *L. donovani* antigen-induced IFN-γ production in the pre-vaccination sample was seen in five volunteers (mean level 1189+-23 pg/ml; P=0·001), while their levels were 731+-668 and 816+-514 at days 45 and 90, respectively(Khalil E *et al.*, 2005b).

The level of IFN-γ in cured patients stimulated by sub-fractions SAF2 followed by SAF3 was positive in 9 out of 10 cured patients though it was not significant (534 pg/ml with range of 133–856 pg/ml for SAF2; and 180 pg/ml with a range of 111–285 pg/ml for SAF3). On the other hand, low levels of IL-10 against MAF2 and MAF3 was found in cured patients (13.89 pg/ml with range of 4.02–44.03 pg/ml for MAF2 and 12.41 pg/ml with range of 5.32– 28.93 pg/ml for MAF3 (Kumari S *et al.*, 2012).

The majority of VL patients produced robust antigen-specific IFN-γ and TNF-alpha responses; and that IL-10 secretion is indeed a signature cytokine response distinguishing active VL from cured or sub clinically infected "immune" individuals (Omprakash S *et al.*, 2012b). Therefore, in the analysis of human immune responses to known and novel antigens, the ability to stimulate production of IFN-γ, TNF-α and IL-10 in cured VL would provide some insight into their potential as vaccine candidates.

3. Objectives

3.1. General Objective

- To determine the immunogenicity of leishmania derived antigens by ex vivo peripheral blood mononuclear assay in blood samples of drug-cured VL patients.

3.2. Specific Objectives

- To determine the types of cytokines (in terms of the Th1 and Th2 profile) produced by putatively immune drug-cured VL patients after in vitro stimulation of PBMCs by candidate vaccine antigens.
- To compare immunogenicity of several leishmania-derived candidate vaccine antigens.

3.3. Hypothesis

Drug-cured VL patients from southwest Ethiopia who have not experienced relapse of VL, 6 months or above after treatments are putatively immune, and blood samples obtained from those patients when stimulated by candidate vaccine antigens, will produce cytokine profiles consistent with immune protection.

4. Method and Materials

4.1. Study area and period

The study was conducted in Arba Minch Hospital (Leishmaniasis Research and Treatment centre, LRTC) south west Ethiopia from May 2014 to January 2015.

Arba Minch (Amharic, " forty springs") is a zonal city in southern Ethiopia located in the Gamo Gofa Zone of the southern Nations, Nationalities, and Peoples Region about 500 kilometers south of Addis Ababa, located at elevation of 1285 meters above sea levels. It is the largest town in Gamo Gofa zone and the second town in SNNPR next to Hawassa. It is surrounded by Arba Minch Zuria woreda.

4.2 Population

4.2.2 Source population

The source population was all previously treated visceral leishmaniasis patients who had completed treatment before 6 months of the study period.

4.2.3 Study population

The study population was randomly selected previously treated visceral leishmaniasis patients who had treatment at least 6 months before, did not experience relapse, and who were able to give consent.

4.3 Study design

Case series experimental study involving drug-cured VL patients.

4.4 Sample size and sampling technique

Sample size calculation was carried out using the statistical package PASS (Power Analysis & Sample Size) 12. For this purpose, we used a two sample T-test allowing unequal variance. Based on 90% power & alpha 0.05; and assuming the cytokine levels in drug-cured patients to be increased compared to healthy controls and further taking a 1:4 allocation of healthy controls to DC-VL patients, a sample size of 20 individuals (16 DC-VL and 4 HCs) was arrived at.

After a further allowance of 10% for experimental errors/failures, the total sample size was 23 (5 HCs and 18 DC-VL patients). We selected the study subjects randomly from previous records of patients available in Arba Minch. All patients were residents of Konso district.

4.5 Variables

4.5.1 Dependent variables

Cytokine production
Antigen immunogenicity

4.5.2 Independent variables

Age
Sex
Ethnicity
Treated VL (Drug-cured)
Time of treatment finished
Type of drugs patient used

4.5.3. Exclusion criteria

Those who had known immunosuppressive disease, cancer (leukemia or lymphoma), gastro-intestinal bypass surgery, diabetes mellitus, renal failure, recent major surgery, pregnancy, lactation, allergic reaction, any febrile disease, children under 5 and VL patients who have not finished treatment before 6 months were not included in the study. Verification of this depended on medical records and interview.

4.6 Data collection

4.6.1 Data collection instrument

Before starting data collection, each participant was informed about the objective of the study, and consent sought. A semi-structured questionnaire was used to capture socio-demographic and clinical data by interviewing the participants.

4.7 Specimen Collection

Blood specimens were collected in heparinized test tubes, using sterile syringes. 20 milliliter of heparinzed venous blood from forearm was collected from each of the eighteen drug-cured visceral leishmaniasis patients and 5 healthy individual using sterile syringes and needles. Samples were collected on site in Konso and transported to Arba Minch Leishmaniasis Research and Treatment Centre for PBMCs isolation and culture.

In addition to blood, 2 mg of stool was collected, preserved in 10% formol-saline until processed by formol- ether concentration method. This was carried out to document the possible helminthic infections that could possibly affect the Th profile of the immune response, and could possibly confound the results of the study in which protection is likely to be associated with Th1 responses.

4.8. PBMC isolation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Hypaque density gradient centrifugation. The cells were washed, and resuspended in RPMI 1640 supplemented with 10% heat-inactivated bovine calf serum, 20 U/ml penicillin and 20 mg/ml streptomycin, and seeded into 24-well multidish plates (detail in Annex II). Each well of the 24-well microtitre plate contained 1.5 – 2.0 x 10^6 cells.

4.10 Cell culture of PBMCs and antigen stimulation

PBMCs were stimulated with PHA (5 mg/ml) and 20 mg/ml of other leishmania derived antigens and cultured at 37^oC in a humidified atmosphere with 5% CO2 for 18 hr (in case of PHA) and 3 days (other leishmani derived antigens). Unstimulated cells were maintained in control wells in each of the 24-well microtitre plates.

Upon completion of the incubation, 1 ml of cell supernatant from each well was collected and kept frozen until analyzed. Supernatants were transferred to LRDL in Addis Ababa at Addis Ababa University Faculty of Medicine,Microbiology,Immunology and Parasitology Department.

Table 1:- Leishmania-derived potential candidate vaccine antigens tested ex vivo in PBMC of previously treated VL patients.

Name	Description	Size	Source	Species conservation	Immuneresponses (References)
NH	Nucleoside Hydrolase	34Kda	L. donovani	100% *L.infantum* 96%*L.major* (300/314) 93% *L. mexicana* (293/314) 84%*L. braziliensis* (264/314)	- Protects mice against VL whenvaccinated subcutaneously with saponin (Aguilar-be *et al*., Infect Immun, 2005)
SMT	Sterol 24-c-methyltransferase	40Kda	L. infantum	99.7% *L.donovani* (352/353) 97%*L.major* (341/353) 94% *L. mexicana* (330/353) 86%*L. braziliensis* (302/353)	Protects mice against VL and CLwhenvaccinated subcutaneously with MPL-SE (Goto *et al*., Vaccine, 2007; Goto *et al*., Vaccine, 2009)
A2	Mature amastigote-specific gene composed of10aa repeats (aa23-236)	20Kda	L. donovani	99% *L. infantum* (235/236) 18%*L.major* (44/236) 83% *L. mexicana* (205/246) n/a *L. braziliensis*	Protects mice against VL whenvaccinated subcutaneously with heat-killed *P. acnes* (Ghosh *et al*., Vaccine, 2001) Protects mice against VL whenvaccinatedasan adenovirusconstruct (Resende *et al*., Vaccine, 2008)
P21	“p21” antigen	21Kda	L. infantum	100% *L. donovani* 99% *L. mexicana*	- *L. major* homolog induces a proliferative response in

	protein			(189/191) 97% *L. major* (186/191) 93%*L. braziliensis* (178/191)	leishmanial parasite-specific T-cell lines derived from an *L. braziliensis*-infected donor (Probst *et al*., J Immunol, 2001)
LeIF	Leishmania eukaryotic Initiation Factor	25Kda	L. major	100% *L. infantum* 100% *L. mexicana* 97% *L. braziliensis*	Leishmania protein that induces an IL-12-mediated Th1 cytokine profile (Skeiky et al, J Immunol, 1998) - In combination with TSA and LmSTI-1, protects mice against CL and VL when vaccinated with MPL-SE (Coler *et al*., Infect Immun, 2002; Coler *et al*., Infect Immune, 2007)

Note

NH = N

SMT = S

A2 = A

P21 = P

LeIF = L

NS = Fusion of N, S

NSA = Fusion of N, S, A

NSL = Fusion of N, S,L

PNS = Fusion of P, N, S

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4.11 Cytokine asssays

Cytokine levels in supernatants from the PBMC cultures stimulated with the antigens: PHA, LSA, S, LeIF, N, P, NS, PNS, NSA and NSL were measured by enzyme-linked immunosorbent assay (ELISA) kit (e-Bioscience, Affymetric campany, USA). Each of the antigen-stimulated PBMC experiments were controlled by wells of the microtitre palte containing uvnstimulated cells.The OD values were converted to pg/ml using non-linear four parametric logistic curve. The results were expressed as picograms (pg) of cytokine/ml, based on the standards provided in the kit. The detection limit of the assays was 4 pg/ml for IFN-gamma, TNF alpha and IL-5; and 2pg/ml for IL-10.

4.12 Statistical Analysis

Because data were not normally distributed, statistical differences ($P<0.05$) between groups were determined using non parametric unpaired Mann-Whitney tests. Tukey's multiple comparisons test was used to compare the cytokine profiles among the 9 candidate vaccines.

Plots were generated using GraphPad Prism 6, and statistical analyses were performed using GraphPad Prism 6 and SPSS software version 17. Nominal P-values are presented throughout (i.e. without correction for multiple testing).

Data were summarized in tables and figures. Levels of cytokines were quantified, and comparisons performed between the various antigens and between the experimental (patients) and control groups. Cytokine profiling was carried out in an array of 11 antigens and 4 cytokines. The cytokine concentrations in supernatants PBMCs culture stimulated by individual leishmania-derived candidate vaccine antigens were measured by ELISA using commercially available kits.

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All comparison involves mean and median values of cytokines. Independent variables such as age, sex, ethnicity, type of treatment, and duration after treatment were used to dichotomize patients into groups and to analyze the data by group-wise comparison using non-parametric statistic available in SPSS version 17 and Graphpad Prism version 6.

4.13 Data Quality Assurance

Site assessment and pre-test of the assays were done prior to data collection so as to optimize the experimental set up. This was done in samples obtained from 5 patients and 3 healthy controls. Data collection was done after the data collectors received on-site training. The data were checked for completeness and representativeness prior entry.

4.14 Dissemination of Results

The results was presented to the Department of Microbiology, Immunology and Parasitology; College of Health Sciences-AAU and will be discussed with potential collaborators. Finally the result will be submitted for publication in a peer reviewed journal.

4 .15 Ethical consideration

The study protocol was approved by the Research Ethics Committee of Addis Ababa University, Department of Microbiology, Immunology and Parasitology and Patients were asked to sign the informed consent. All the information obtained from the study subjects were coded to maintain confidential.

5. Results

5.1 Socio demographic and clinical characteristics

In this study, 23 male study subjects were participated. Of these subjects, 18 were among drug-cured VL patients, and 5 were control subjects (three endemic healthy controls, and two non-endemic healthy controls). The median age of the study subjects was 15, 20, and 24 for drug-cured, endemic and non-endemic controls respectively. All drug-cured study participants were living in rural areas. Ten of the 18 (55.6 %) patients were previously treated with sodium stibogluconate (Table 2).

Table 2:- Demographic and clinical characteristics of all study participants in Arba Minch Hospital LRTC (Leishmaniasis Research and Treatment centre), SNNPR, 2014.

Characteristics	**Drug-cured patients**	**Endemic healthy controls**	**Non-endemic healthy controls**
Sex			
Male	18	3	2
Female	0	0	0
Median age (yrs)	15	20	24
Educational status			
Illiterate	10	1	0
Primary	7	2	0
Secondary	1	0	0
Diploma	0	0	0
Degree and above	0	0	2
Type of drug taken by patients			
AmBisome	2	NA	NA
Glucantime	2	NA	NA
SSG	10	NA	NA
SSG+PM	4	NA	NA=Not Applicable

5.2 Cytokines response to PHA, LSA and leishmania derived antigens

The supernatant of PHA, LSA and nine defined antigens (NS, PNS, NSL, S, P, Leif, N, S and NSA) stimulated PBMCs were assayed for TNF-alpha,IFN gamma,IL-10 and IL-5 production**.** The level of cytokines produced by PBMCs in response to leishmania antigens stimulation were determined by subtracting background levels measured in the non-stimulated (PBS) samples.

Concentrations of TNF-alpha, IFN-gamma, and IL-10 measured in supernatants of PHA (an antigen non-specific activator of T cells) stimulated PBMCs were above the detection levels (>4pg/ml) in both the experimental and control subjects. On the other hand, the levels of TNF-alpha and IL-10 in LSA stimulated PBMCs of two drug- cured patients and two healthy controls were below the detection level.

PHA induced IL-5 responses in PBMCs of drug-cured patients, concentrations were very low, albeit above the detection levels; with mean and median values of 31.3 and 28.2 pg/ml respectively. In healthy controls, the cell supernatant IL-5 response to PHA and LSA was below the detection limits(< 4pg/ml).

5.2.1 TNF-alpha response to leishmania derived antigens

In all study subjects, the level of TNF-alpha was above the detection limits in PBMCs stimulated by S, NS, PNS and NSL.The mean and median value of TNF-alpha concentration measured from in vitro stimulated PBMCs obtained from drug-cured individuals in all tested antigens except in cases of A and NSA were above the detection levels (Table 2).

Table 3 :-TNF-alpha response to different leishmania-derived antigens measured in pg/ml in experiments involving drug-cured pateints (n=18).

parameter	PHA	LSA	LeIF	N	S	P	NS	PNS	A	NSA	NSL
Mean	668.7	257.4	11.2	79.8	359.9	148.1	670.6	321.1	1.7	13.1	394.5
SEM	58.7	72.8	6.2	34	69.3	66.9	119.3	48.9	1.0	3.5	26.0
Median	614.7	128.8	0.0	19.9	405.5	16.2	557.4	360.3	0.0	8.7	403.2
SD	249.1	308.8	26.4	144.3	294.1	183.9	506.2	207.6	4.4	14.9	110.3
Min	422.9	0.0	0.0	0.0	2.26	0.0	55.8	23.7	0.0	0.0	137.3
Max	1374	1076	89.6	595	1034	1034	1374	596.8	14.2	44.2	687.8

Only four of the 18 drug-cured individuals with a history of kala-azar had showed LeIf and A specific stimulation of TNF-alpha production (> 4 pg/ml). The TNF-alpha response to A was below the detection level (< 4pg/ml) for all healthy controls. Similarly the TNF-alpha response for P simulated PBMCs was below the detection level in 7 drug-cured individuals and 4 healthy controls (Figure 1).

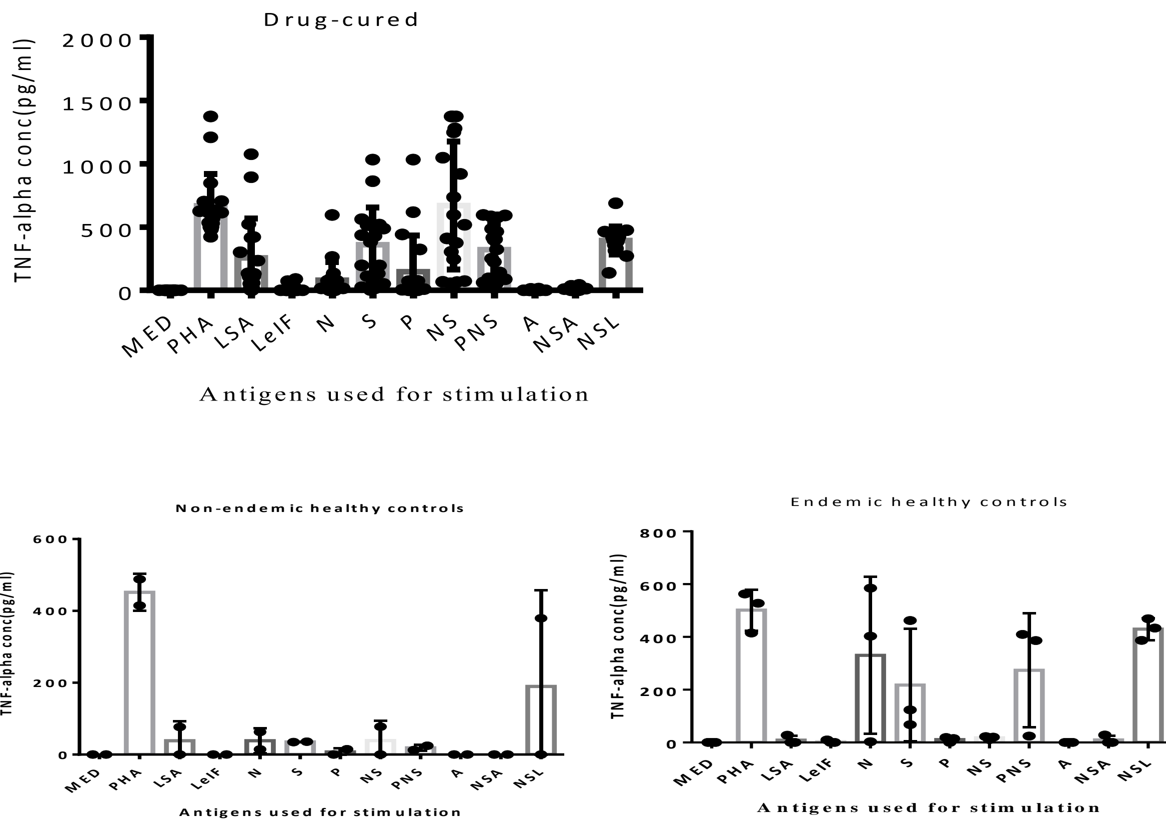


Figure 1:- TNF-alpha production by peripheral blood mononuclear cells in response to PHA, LSA and 9 leishmania-derived antigens examined in drug-cured patients (n=18), endemic healthy controls (n=3), and non-endemic healthy controls (n=2).

Although both the mean and median value of TNF-alpha response against LeIF, N, S, P, A, NSA and NSL were higher in drug-cured group, the median difference between drug-cured and healthy controls were not statistically significant (p_value > 0.05). TNF-alpha response against PHA was significantly different between drug-cured group and non-endemic healthy controls with *P value=0.0421*.

Similarly in NS and PNS stimulated PBMCs of drug-cured VL patients, TNF-alpha responses were higher than non-endemic healthy; and the difference was statistically significant, (P-value =0.0053 and 0.0211 respectively. However,TNF-alpha response against 9 leishmania derived antigens between endemic healthy and drug-cured groups was not significant except in the case of NS (P-value=*0.0015*) (Figure 2).

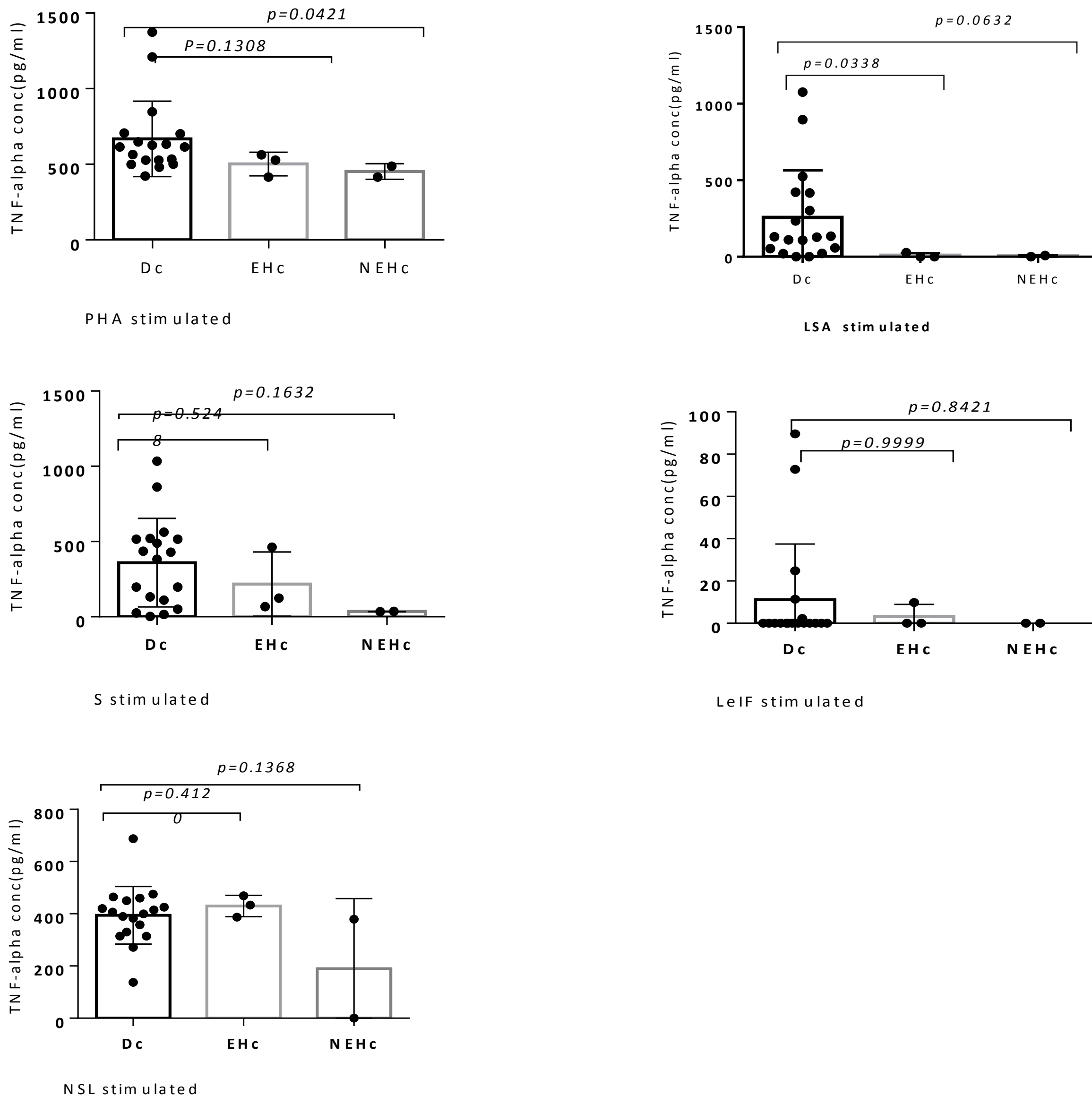
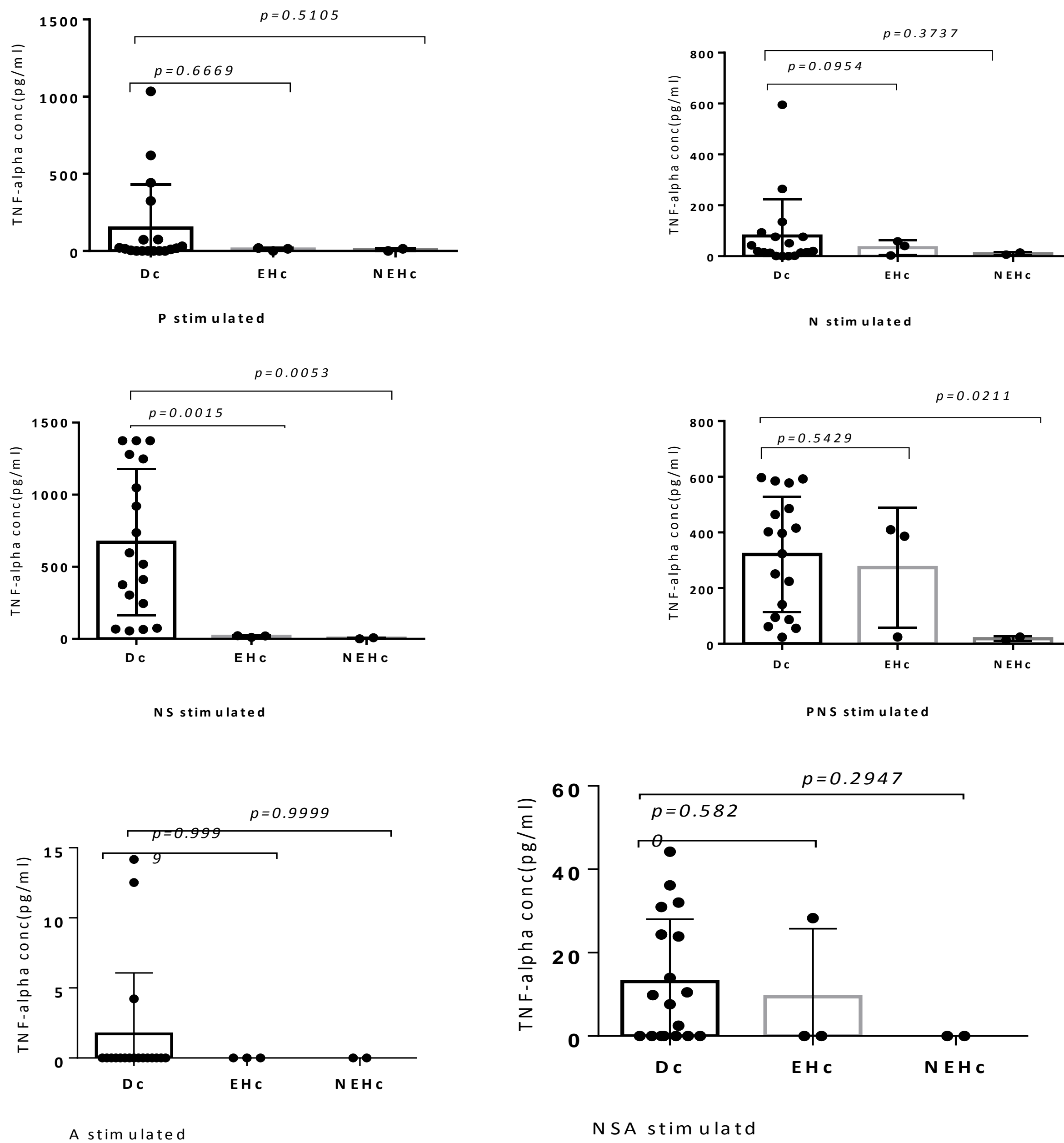


Figure 2:-shows TNF-alpha production differnce among drug-cured (n=18), endemic (n=3) and non-endemic healthy controls (n=2) to PHA, LSA and 9 leishmania derived antigens. Statistical differences between groups was determined using the non-parametric, Mann-Whitney test.Bar indicates median value.



NB: Dc = drug-cured, Ehc = Endemic Healthy controls, NEHc = Non-Endemic Healthy controls

Figure 2:-shows TNF-alpha production differnce among drug-cured (n=18), endemic (n=3) and non-endemic healthy controls (n=2) to PHA, LSA and 9 leishmania derived antigens. Statistical differences between groups was determined using the non-parametric, Mann-Whitney test.

5.2.2 IFN-gamma response to leishmania derived antigens

IFN-gamma production by drug-cured and healthy controls in response to 9 leishmania derived antigens was analyzed. The mean and median values of IFN-gamma were above detection limits in both drug-cured and endemic healthy controls. On the other hand, IFN-gamma response to A, LeIF, N, NS, PNS, NSA, and P were below 20 pg/ml in PBMCs derived from non-endemic controls. The pattern of IFN-gamma response was higher in drug-cured VL patients, with the mean and median values against all tested antigens were being above detection levels (Table 4).

Table 4:- IFN-gamma response of PBMCs derived from drug-cured VL patients after stimulation with different leishmania derived antigens.

parameter	PHA	LSA	LeIF	N	S	P	NS	PNS	A	NSA	NSL
Mean	1468.9	1431.8	139.7	352.6	399.7	127.1	553.3	349.6	13.5	207.4	886.5
SEM	45.8	103.9	83.1	132.9	130.8	60	140.9	94.9	8.9	71.4	133.7
Median	1534	1525.5	24.2	76.1	49.4	46.8	193.9	151.4	6.6	135.7	975.3
SD	194.5	441.2	352.4	564.1	555.1	254.6	597.7	402.9	37.8	303.1	567.2
Min	1065	195.8	4.4	8.4	7.8	7.1	5.1	15.4	0	0	31.8
Max	1691	1886	1487	1741	1447	1098	1826	1323	124	1263	1666

Endemic healthy controls' IFN-gamma response to LSA was similar to drug-cured individuals with the mean and median value of 220 pg/ml and 306 pg/ml respectively. But it was very low in non-endemic healthy controls with median value of 27 pg/ml.

The IFN-gamma response was above the detection level (>4pg/ml) for all drug-cured patients PBMCs' stimulated by leishmania derived antigens (LeIF, N, S, P, NS, PNS, and NSL). But it was below detection levels in PBMCs of seven drug-cured individuals and three healthy controls stimulated by antigen A (Figure 3).

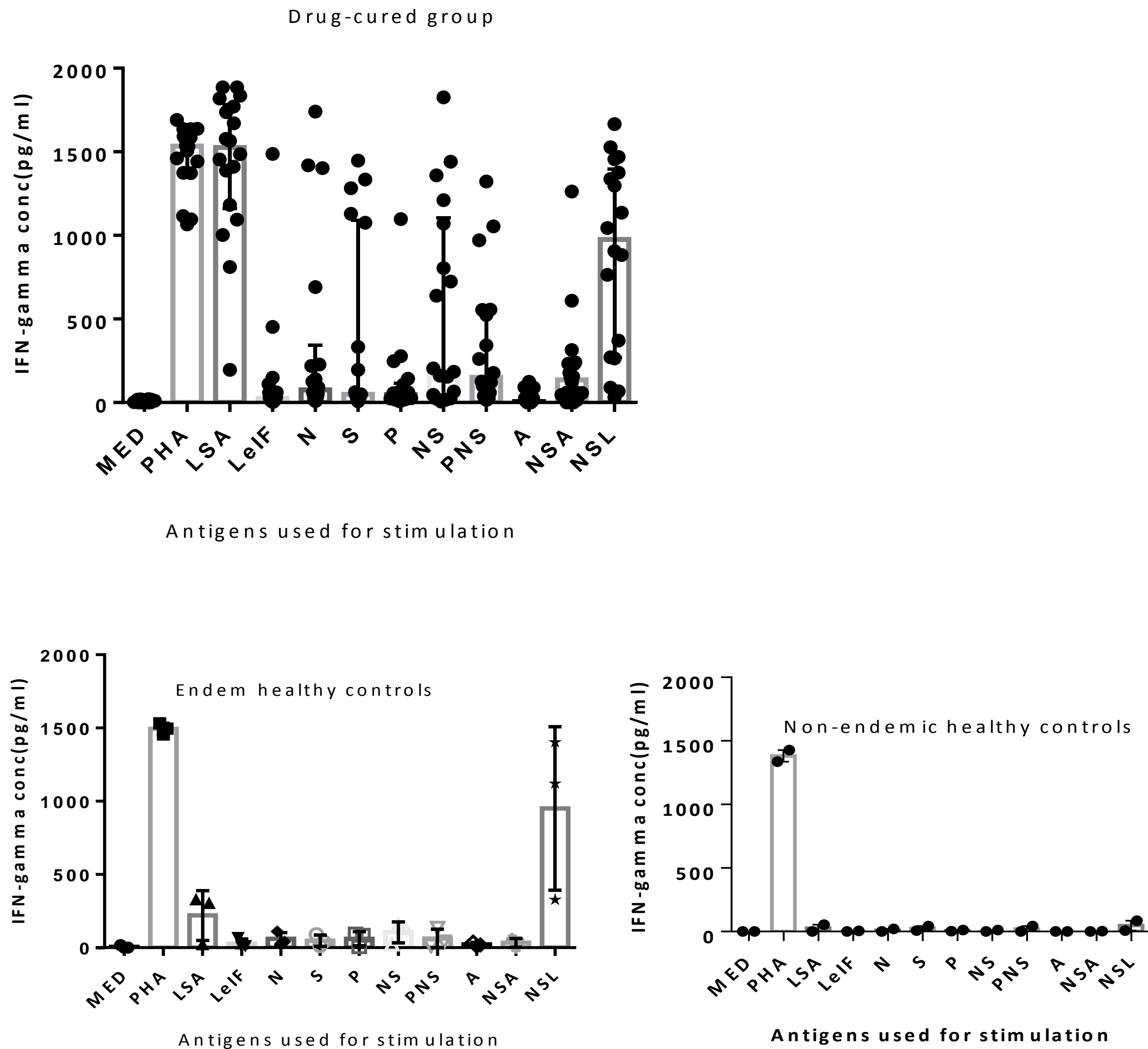


Figure 3 :-IFN-gamma production by peripheral blood mononuclear cells in response to PHA,LSA and 9 leishmania derived antigens examined in drug-cured patients (n=18), endemic healthy controls (n=3), and non-endemic healthy controls (n=2) .

As shown in Figure 3 above , the IFN-gamma production by drug-cured patients in response to PHA, LSA, S, NSL and NS was higher relative to other antigens stimulations. Except for PHA and NSL stimulation, the IFN-gamma production by PBMC of endemic healthy controls were very low. Similarly, the IFN-gamma response of PBMCs from non-endemic healthy controls to all leishmania derived antigens was below the detection limits.

By group wise comparison, IFN-gamma response to diferent antigens (LSA, N, P, NS and NSL) was significantly different between drug-cured and non-endemic healthy controls with P-values of 0.015,0.0316, 0.0211, 0.0211 and 0.0316 respectively (Figure 4).

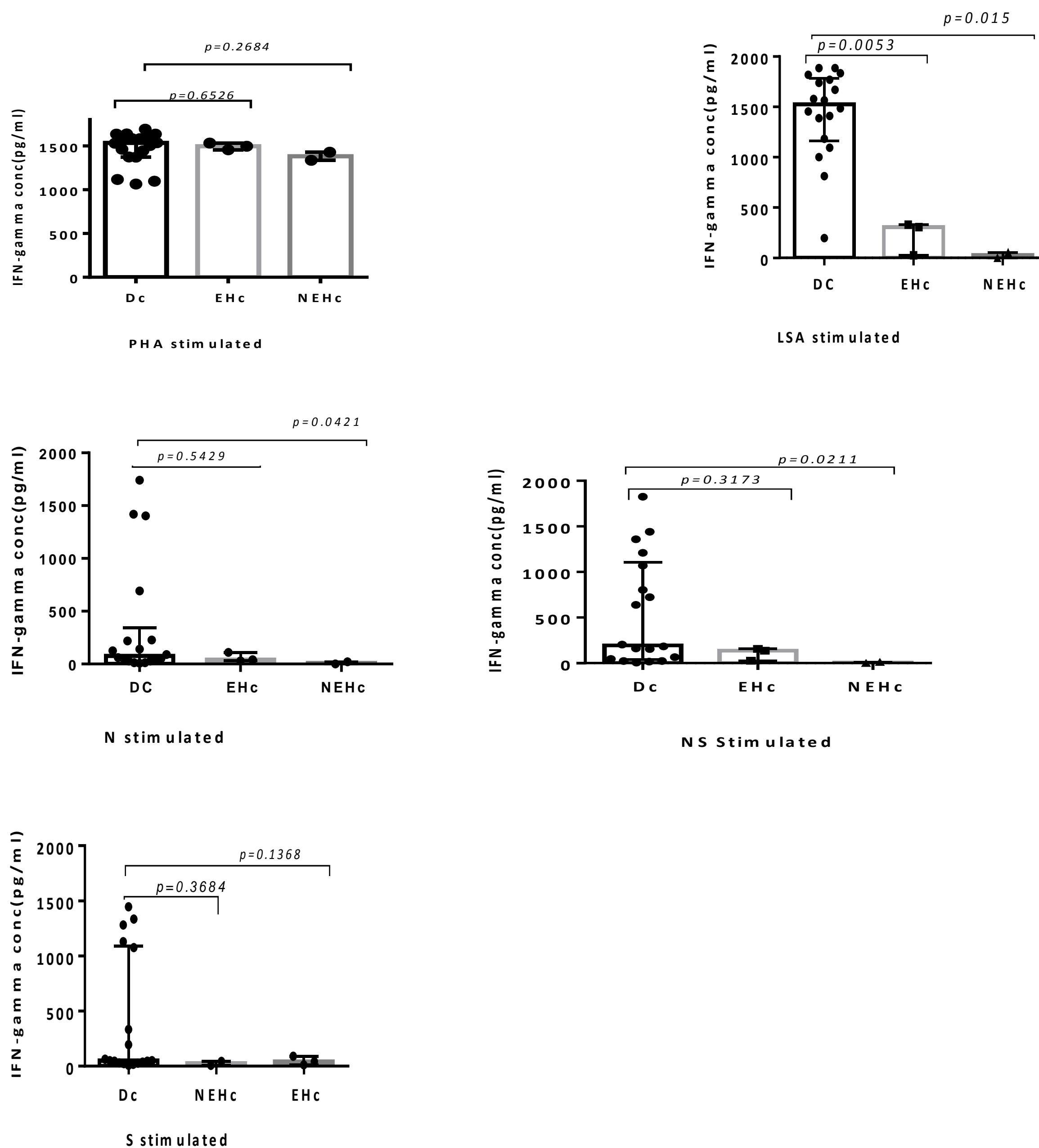


Figure 4:- shows IFN-gamma production among drug-cured (n=18), endemic (n=3) and non-endemic healthy controls (n=2) to PHA, LSA and 9 leishmania derived antigens.Statistical differences between groups was determined using the non-parametric, Mann-Whitney test.

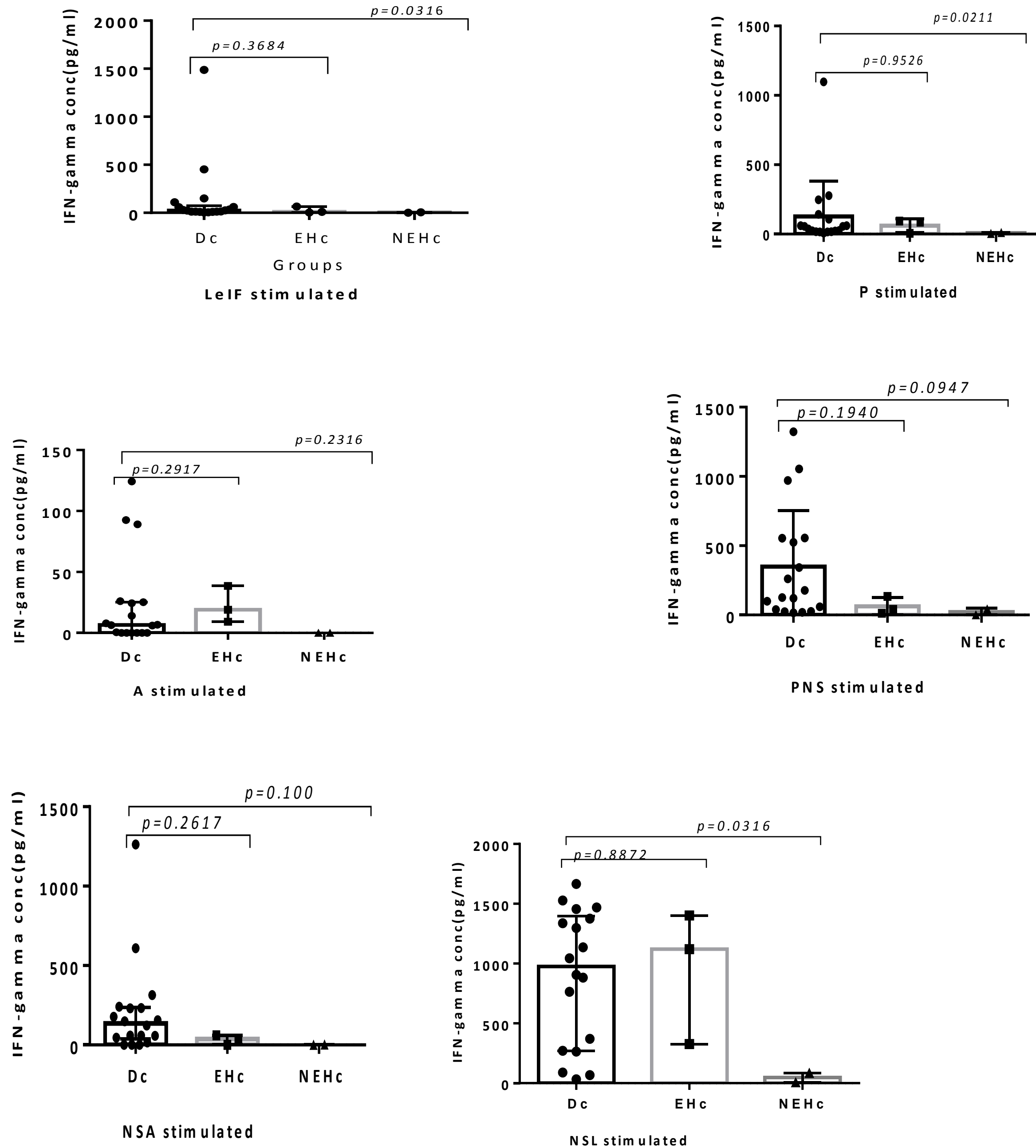


Figure 4: shows IFN-gamma production among drug-cured (n=18), endemic (n=3) and non-endemic healthy controls (n=2) to PHA,LSA and 9 leishmania derived antigens.Statistical differences between groups was determined using the non-parametric, Mann-Whitney test.

5.2.3 IL-10 response to leishmania derived antigens

The IL-10 responses of drug-cured VL patient's PBMCs stimulated with NS, PNS and NSL was high (Table 5); whereas it was > 20pg/ml in endemic healthy controls only in response to PNS and NSL stimulation. In non-endemic healthy controls, IL-10 response against NS, NSL, LSA, Leif, A and NSA was below detection level (< 2pg/ml).

Table 5 :-IL-10 response of PBMCs derived from drug-cured VL patients after stimulation with different leishmania derived antigens.

Parameter	PHA	LSA	LeIF	N	S	P	NS	PNS	A	NSA	NSL
Mean	297.6	53.1	3.9	27.2	59.6	45.0	221.2	69.8	2.9	8.9	169.1
Median	330.5	46.3	0.02	19.5	31.4	7.1	69.7	42.3	0.0	6.1	171.9
SD	103.3	48.2	6.1	24.8	72.1	81.3	264.8	72.1	7.7	8.4	100.8
Min	107.6	0.0	0.0	0.0	0.0	0.0	4.3	0.4	0.0	0.0	14.9
Max	414.	151.8	18.7	97.8	263.6	256.5	845.5	259.5	32.4	25.4	334.9

The IL-10 response to LeIF in 13 drug-cured VL patients and 15 of drug-cured patients to antigen A were below the detection level. As shown in figure 5, antigen A, Leif and NSA were less antigenic; and thus, IL-10 concentrations in supernatants of PBMC cultures stimulated with Leif, A and NSA were very low.

The IL-10 response against all leishimania derived antigens in healthy controls was very low(< 2pg/ml) except for PNS and NSL. IL-10 responses in two drug-cured patients whose PBMCs were stimulated with antigens N and S were below the detection levels. Similarly the IL-10 production in response to antigens P and PNS in five drug-cured VL patients was below the detection levels.

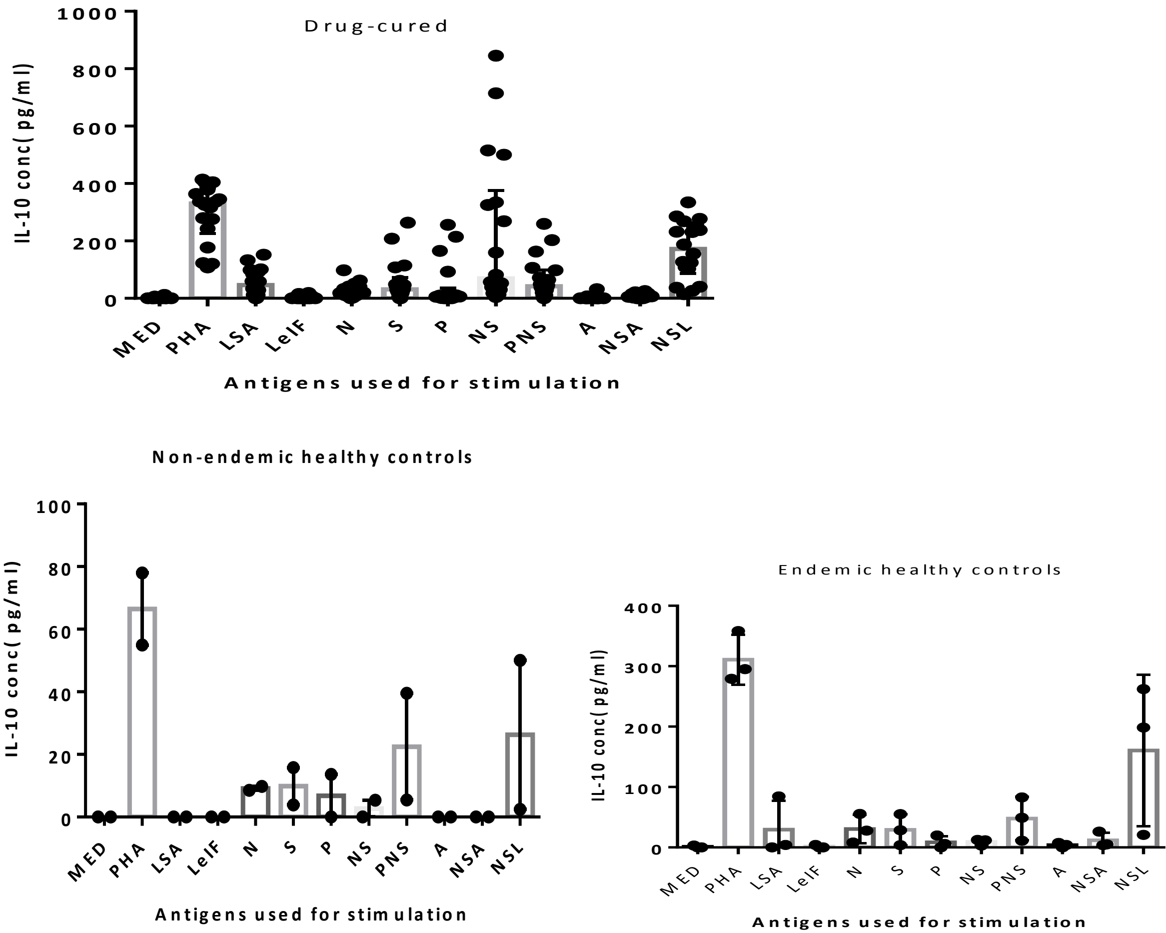


Figure 5 :- IL-10 production by peripheral blood mononuclear cells in response to PHA, LSA and 9 leishmania derived antigens in drug-cured patients (n=18), endemic healthy controls (n=3), and non-endemic healthy controls (n=2).

Even though the mean and median value of IL-10 concentrations in response to diferent leishmania derived antigens was higher in drug cured patients than healthy controls, the differences were not statisticaly significant with the exception of NS stimulated PBMCs (Figure 6).

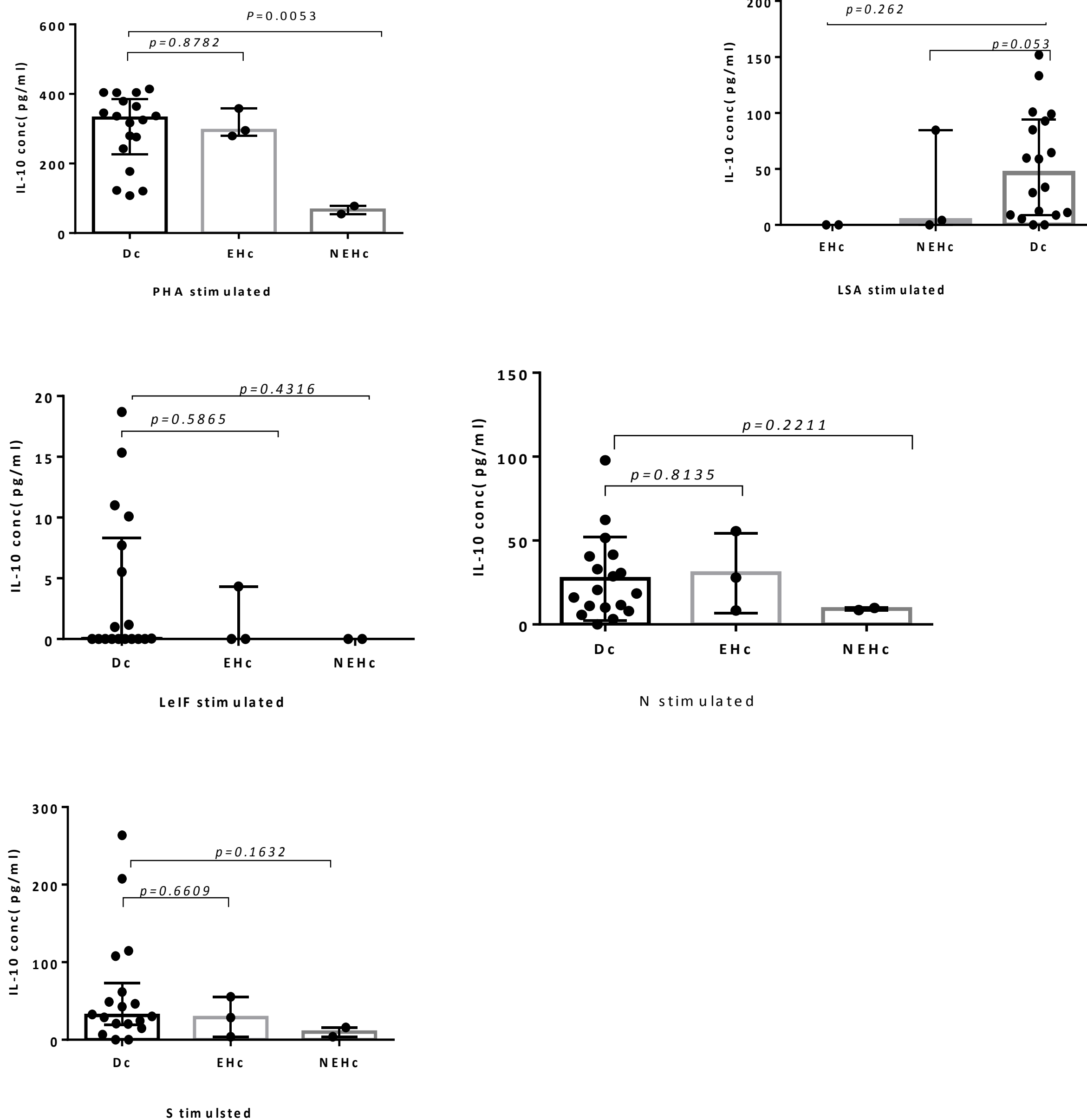


Figure 6:- IL-10 production among drug-cured (n=18), endemic (n=3) and non endemic healthy controls (n=2) to PHA, LSA and 9 leishmania derived antigens

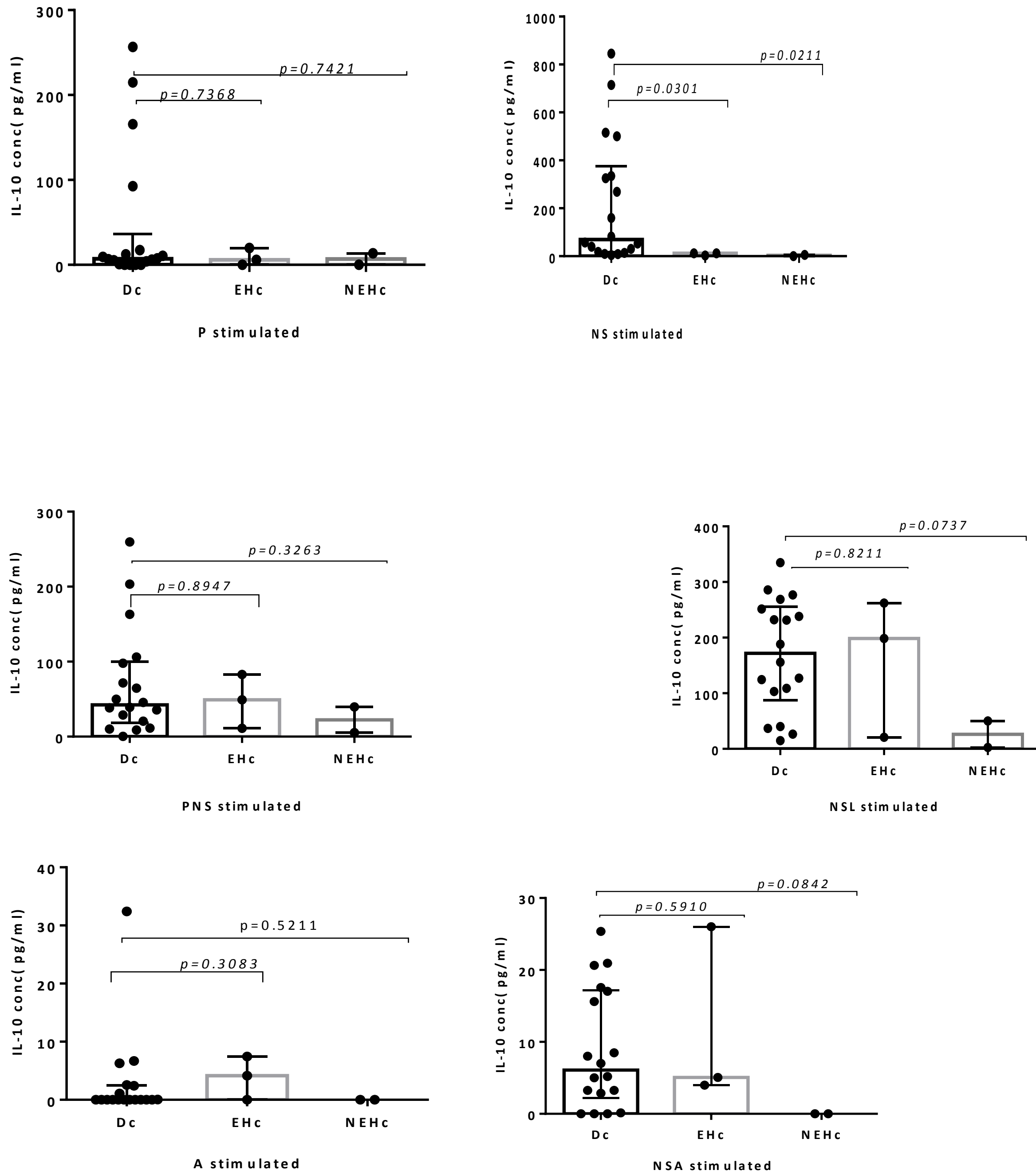


Figure 6:- IL-10 production among drug-cured (n=18), endemic (n=3) and non endemic healthy controls (n=2) to PHA, LSA and 9 leishmania derived antigens.

5.2.4 IL-5 response to leishmania drived antigens

Phytohaemagglutinin (PHA)-stimulated PBMCs of all drug-cured patients, two endemic healthy controls produced IL-5 above the detection levels (> 4 pg/ml). The IL-5 response to LSA stimulated PBMCs for seven drug-cured VL patients and to N, PNS and NSL for one drug cured individual, was above the detection limit (Figure 7). Both endemic and non-endemic healthy control's PBMCs IL-5 response to different leishmania derived antigens were below detection levels. Most of the candidate vaccine antigens did not induce IL-5 production in drug-cured VL patients and healthy controls.

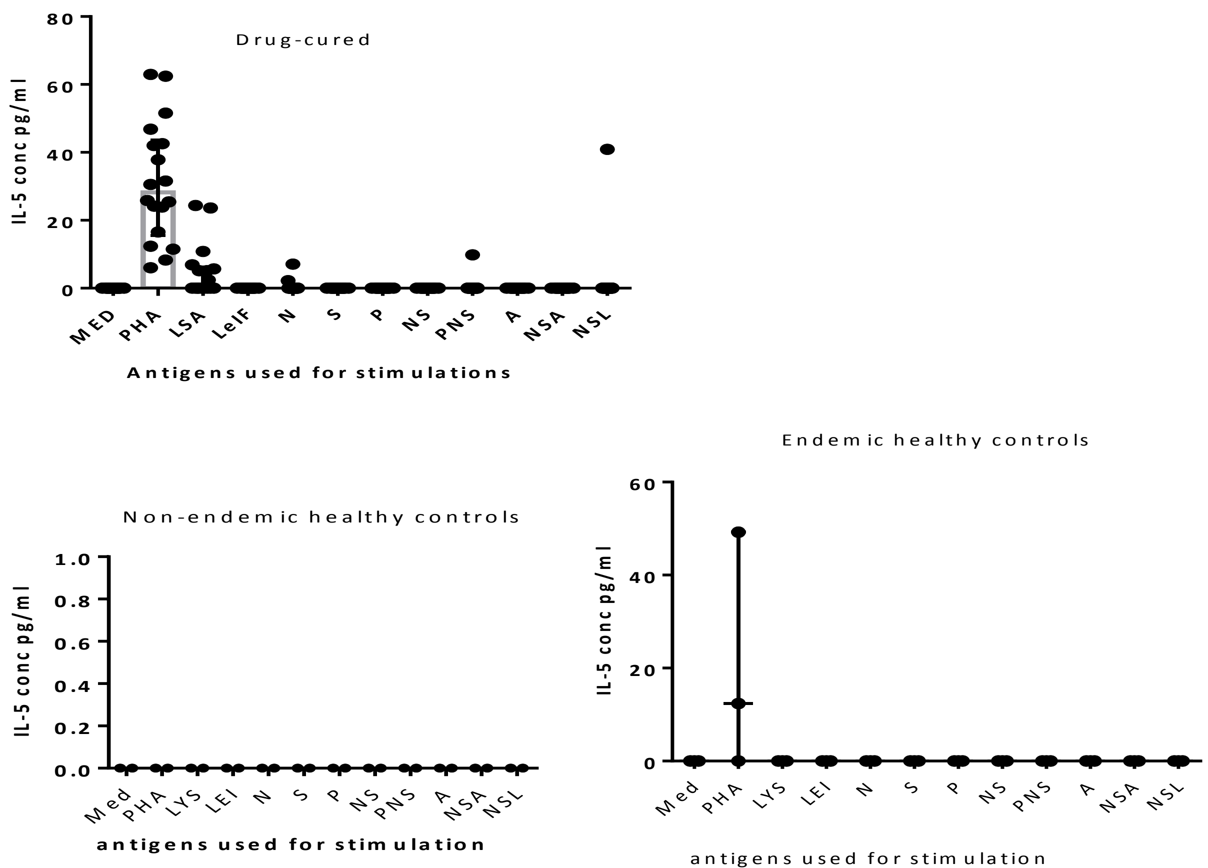


Figure 7:-IL-5 production by peripheral blood mononuclear cells in response to PHA,LSA and 9 leishmania derived antigens examined in drug-cured patients (n=18), endemic healthy controls (n=3), and non-endemic healthy controls (n=2).

5.3 Comparison of cytokine responses among the candidate vaccine antigens

PBMCs of drug cured patients responded significantly to NS by way of heightened TNF-alpha responses followed by NSL, S and PNS. NS elicited significantly stronger (*P*=0.001) TNF-alpha responses than all other leishmania derived antigens tested in this study.

Significantly higher production of TNF-alpha (*P* = 0·01) was also observed in the drug-cured patients' PBMC cultures stimulated with NSL compared to those stimulated with N, LeIF, A and NSA. However, in the same group no significant differences of TNF alpha were seen among those stimulated by NSL, S, P, and PNS.

The IFN-gamma responses were significantly stronger (*P*=0.04) in response to NSL and NS compared to LeIF, N, S, P, PNS, A and NSA stimulation.

There was also IL-10 responses in drug-cured patients' PBMCs stimulated by the NS and NSL; which were significantly higher *P* (=0.01) than those elicited by LeIF, N P, PNS, A and NSA, but were not significantly different from those elicited by N and S.

5.3 Stool examination.

In addition to cytokine assays we did stool examination by formol- ether concentration method but no ova of parasites seen in the stool of all study subjects. This was carried out to rule out confounding results by Th2 skewed responses associated with helminthic infection.

6. Discussion

At different times, animals have been used to test protective efficacy of VL vaccine candidates and different antigens. To date only a few have advanced to human clinical trials (**Malcolm S. Duthie**, 2012).

The purpose of this study was to assess the potential abilities of 9 VL vaccine candidates to elicit protective cellular immune responses by peripheral blood mononuclear cells of drug-cured VL patients and healthy controls.

In the present study, we could demonstrate that most of the antigens ingestigated induced proinflamatory cytokines (IFN-gamma/TNF-alpha) and moderate amount of IL-10 response. The balance between pro-inflammatory IFN-γ/TNF-α and regulatory IL-10, have been variously shown to be predictive of vaccine outcome (Stober C, 2005).

In a study conducted in India to assess immunogenecity of Kmp11 antigen in human and murine cells infected *in vitro*, and in mice *in vivo*, clearance of *Leishmania* parasites required IFN-gamma (Rhea N. Coler, 2007).
In our study, PBMCs derived from drug-cured VL patients and healthy controls,when stimulated in vitro with PHA (non-specific T cell stimulator), IFN-gamma,TNF-alpha and IL-10 responses were found to be above detection levels. However, the responses to PHA stimulation of PBMCs were more pronounced in drug-cured VL patients compared to healthy controls as cured patients are likely to have more circulating memory cells; hence higher mean and median values of cytokine concentrations were measured..

PBMCs of drug-cured VL patients, stimulated with LeIf, produced low level of TNF-alpha response, with mean and median values of 11.2 pg/ml and 0.0 pg/ml respectively, which were also below the detection levels in healthy controls. However LeIf in combination with TSA and LmSTI-1 protected mice against CL and VL when vaccinated with MPL-SE (Rhea N. Coler, 2007) showing the potenitial of LeIF as Th1 type and aduvants and as atherapeutic and prophylactic vaccine antigen for leishmaniasis when used with other leishmnaia antigens.(Yasir A, 2014).

Though mean and median values of TNF-alpha were higher in drug-cured patients than healthy controls, statistically significant difference between groups were seen only in the case of NS and PNS (p=0.0053 and 0.0211 respectively). This was similar to the study in India, whereby the in vitro whole blood response to SLA in drug-cured patients was not different from non exposed individuals(23).

IFN-gamma response to seven antigens (LeIF, N, S, P, NS, PNS and NSL) were above detection limits in all of the 18 drug-cured individuals. But only in 11 (61.1%) drug-cured individuals, IFN-gamma response against antigen A was above detection levels. Antigen A is predominantly comprised of multiple copies of a 10 amino acid repeat sequences. The protective response generated by recombinant A2 protein immunization was associated with a mixed Th1/Th2 response, production of IFN-gamma, and an anti-A2 humoral response. These results indicate that immune response to *L. donovani* infection by A2 immunization did not appear to be biased towards either Th1 or Th2 (Anirban Ghosh, 2001).

In an Indian study, IFN-gamma response of drug-cured persons in response to LSA was significantly higher than in the non-exposed group (P = 0.03) (23), which is similar to our finding, whereby the difference was significant (p=0.015) between drug-cured and non-exposed groups. In addition to the response against LSA, significantlly higher responses were observed in drug-cured patients' in response to antigen P (p=0.0211), NS (p=0.0211) and NSL (p=0.0316).

There were 10 (55.5%) drug-cured patients whose PBMCs responded with TNF-alpha when stimulated with antigen NSA; and only two (11.1%) of the drug-cured patients responded likewise in response to antigen A. In contrast, TNF-alpha levels were below the detection limits in all endemic and non-endemic healthy controls in response to antigens A and NSA.

TNF-alpha and IFN-gamma modulate the immune system synergically. The capability of cured subjects to simultaneously express these two cytokines may be beneficial for protection against new infections(Rhea N. Coler, 2007). These two cytokines synergize in inducing NADPH oxidase, the product of which, superoxide, is involved in killing *Leishmania* parasites in human macrophages, as is nitric oxide in mice (Marco A. Cassatella, 2010). On the other hand, those antigens that elicit strong IFN-gamma and TNF-alpha production may also produce IL-10. IL-10 is generally associated with immune suppression during active VL, negatively regulating Th1 responses.

The responses of drug-cured of VL patients in our study depict that, in response to some of the candidate vaccine antigens, PBMCs produced IFN-gamma and TNF-alpha, but also IL-10 when stimulated with N, S, NS, PNS and NSL. Similar results were demonstrated against KMP-11 antigen (Rhea N. Coler, 2007).

Except PHA induced PBMCs of drug-cured VL patients, IL-5 response against different antigens were below detection limits. This was similar to the study conducted in India whereby neither IL-4 nor IL-5 levels were above the detection limit in response to six antigens (Hailu A *et al.*, 2004). In contrast,IL-15 has also been implicated as an important co-stimulus for IFN-γ production (Hailu A *et al.*, 2004).

Among the 9 candidate vaccine antigens tested, we had found that LeIF, A, N, S, P, NS, PNS, NSA and NSL as well as LSA and PHA elicited IFN-γ responses in a high percentage of drug-cured VL patients (61.1%–100%) than NEHc (0-50%) subjects. Further, we also specifically found that antigens PHA, LSA, NS, NSL, PNS, and S elicited more IFN-γ, TNF-α and IL-10 responses in PBMCs of drug-cured VL patients than healthy subjects. These results show that cured VL patients have retained memory cells that could easily respond to antigen encounters, and that the responses are primarily Th1 (IFN-gamma) and proinflammatory (TNF-alpha and IFN-gamma) even though IL-10 responses were also clearly notable.

Drug-cured VL patients are protected against subsequent clinical disease by producing Th1 cytokines. In India, infected asymptomatic individuals who are resistant to developing active VL disease produced Th1 cytokines(Omprakash S *et al.*, 2012a).

Our findings indicate that NS, PNS, NSL and S were the more immunogenic antigens eliciting significant IFN gamma,TNF alpha and moderate amount of IL-10 but not IL-5. Based on several published works, those antigens resulting in pro-inflammatory IFN-γ and TNF-α and regulatory IL-10 production are generally considered as good vaccine candidates(Stober C *et al.*, 2005). Infact IFN gamma is crucial to control intracellular infection, while TNF-alpha modulates the immune response synergetically with IFN gamma.IL-10 is immoprtant to regulate immune function.

7. Limitation of the study

Since most antiges used for PBMCs stimulations were novels,it was difficult to characterize the nature of antigens(Candidate vaccines).

Un able to see other cytokines profile, we only analyzed four cytokine (TNF-alpha, IFN-gamma, IL-10 And IL-5).

8.Conclusions and Recommendations

8.1 Conclusions

In this study we have shown that PHA, LSA, NS, PNS, NSL, S, N, A, LeIF, A, NSA and P elicited a Th1 like immune response in PBMCs obtained from drug-cured VL patients as evidenced by production of high amounts of IFN_gamma and TNF-alpha. In both healthy and drug-cured individuals, these antigens induced the production of IFN-gamma, TNF-alpha and IL-10, but not IL-5. It, therefore, appears that certain Leishmania antigens may be able to elicit a dominant Thl cytokine profile as well as inhibit the production of Th2 cytokines.

Among the antigens used for PBMCs stimulation;NS, NSL, PNS and S were more immunogenic. Especially NS was the most potent antigen.

The antigens NS, NSL, PNS and S stimulated PBMCs of all drug-cured patients inducing the production of IFN-gamma and TNF-alpha.This suggests PBMCs' response to NS, NSL, PNS and S may be associated with protective immunity.

Therefore, in the analysis of PBMCs responses to different leishmania derived antigens, the ability to stimulate production of IFN-gamma, TNF-alpha and IL-10 in cured VL cases would provide some insight into their potential as vaccine candidates.

8.2 Recommendations

This study analyzed 4 cytokines only (IFN-gamma, TNF-alpha, IL-10 and IL-5. Further studies on other cytokines profiles is recommended.

Most of antigens we used for PBMCs stimulations are novel antigens. So futher studies on immunogenecity and protective efficacy in experimental animals are recommended as next steps.

9.References

Alvar J, Aparcio P, Assefa A, Denboer M, Canavate C, Dedeet G, Gradoni L, Terhorst R, Lopezvelez R & Moreno J, 2008 The relation ship between leishmaniasis and AIDS the second 10 years. *Clin. Microbiol,* 21**,** 334-359.

Alvar J, Velez I, Berne C, Herrero M, Desjeux P, Cano J, Jannin J & Denbor M, 2012. Leishmaniasis world wid and global estimates of its incidence. . *PLOS one* 7**,** e35671.

Amrita D & Nahid A, 2012. Vaccine development against leishmania donovani. *Inf. Dis and Immunol,* 3**,** 1-19.

Anirban Ghosh, W.W.Z., Greg Matlashewski, 2001. Immunization with A2 protein results in a mixed Th1/Th2 and a humoral response which protects mice against *Leishmania donovani* infections. 2002**,** 59-66.

Awasthi A & Saha B, 2004. Immune response to leishmania infection. . *India. J. Med. Res* 119**,** 238-258.

Carvalho, Badaro R, Reed S, Jones T & Johnson S, 1985. Absence of gamma interferon and interlukin- 2 during active vicseral leishmaniasis. *J. Clin. Invest,* 76**,** 2066-2069.

Chappus F, Sundar S, Hailu A, Ghalib H, Rijal S & Peeling R, 2007. Visceral leishmaniasis: what are the needs to diagnosis,treatment and control? *Nat Rev mcrobiol,* 5**,** 873-882.

Christopher L, Sayda H, Thomas A, Maria M, Anwar M, Faisal A, Mohammed H, Franklin A, Thomas B & David L, 1993. In vivo cytokine profile in patient with kala-azar marked elevation of both interlukine -10 and interferon gamma. . *J. Clin. Invest,* 94**,** 1644-1648.

Darrah P, Patel D, De Luca P, Lindsay R & Davey F, 2007. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat Med* 13**,** 843-850.

Evans T, Teixeira M, Mcaculiff I, Vasconcelos W, Souse A, Lima J & Pearson R, 1992. Epidemiology of visceral leishmaniasis in North east Brazil *J. infect. Dis,* 166**,** 1124-1132.

Garg R, Gupta S, Tripathi P, Naik S, Sundar S & Dube A, 2005. Immunestimulatory cellular responses of cured leishmania infected patients and hamesters against the integral membrane proteins and non-membranous soluble proteins of arecent clinical isolate of leishmania donovani . *Clin. EXP. Immunol,* 140**,** 149-156.

Ghalib H, Pluvezam M, Skeiky Y, Siddig M, Hashim F, Hassan A, Russo D & Reed S, 1993. Interlukin -10 production correlate with pathology in huma n eishmania donovani infections. *J . Clin. Invest,* 92**,** 324-329.

Ghalib H, Whittle J & Kubin M, 1995. Interlukin -12 enhance Th-1 type response in human leishmania donovani infections. *J. Clin. Invest,* 154**,** 4623-4629.

Ghose A, Haldar J, Pal S, Mishra B & Mishra K, 1980. Serological investigation in Indian kal-azar. *Clin. EXp. Immunol,* 40**,** 318-326.

Hailu A, Vanderpoll T, Berhe N & Piet A, 2004. Elevated plasma level of gamma interferon and gamma interferon inducing cytokines,and interferon gamma CXC chemokine receptors. . *Am. J. Trop. Med. Hyg,* 71.

J. Chakravarty, S.K., S. Trivedi Et Al. 2011. “A clinical trial to evaluate the safety and immunogenicity of the LEISHF1+ MPL-SE vaccine for use in the prevention of visceral leishmaniasis,” *Vaccine,. Clin Exp Immunol,* 29**,** 3531-3537.

Joseph L, Jin S, Salvatore J, Henry W, Petr A, Gurdial S, Steven A & Gerald F, 2004. Asub set of NKT cells is activated during leishmania donovani infection by CD 1d -bounded lipophosphoglycan. *J. Exp. Med,* 200**,** 895-904.

Kamlesh G, Stephen J, Rajiv K, Marleen B & Shyam S, 2011. Interferon gamma release assay(modified Quanti FERON)as apotenitial marker of infection for leishmania donovani,aprof of concept study. *PLOs. Neg.Trop. Dis,* 5**,** e1042.

Kemp K, Kemp M, Kharazmi A, Ismail A, Kurtzhals J & Theander T, 1999. Leishmania specific T-cells expressing interferon gamma and interlukin-10 up on activation are expanded in individuals cured of visceral leishmaniasis. *Clin. EXP. Immunol,* 116**,** 500-504.

Khalil E, Ayed N, Musa A, Ibrahem M & Mukhtar M, 2005a. Dichotomy of protective cellular immune response to human visceral leishmaniasis. . *Clin. EXP. Immunol,* 140**,** 349-353.

Khalil E, Ayed N, Musa A, Ibrahem M, Mukhtar M, Zijlstra E, Elhassan I & Smith P, 2005b. Dichotomy of protective cellular immune responses to human visceral leishmaniasis. . *Clin. EXP. Immunol,* 140**,** 349-353.

Kharazmi A, Kemp K & Ismail A, 1999. T-cell immune response in human leishmaniasis. *Immunol. Lett,* 65**,** 105-8.

Kumari S, Misra P, Tandon R, Samant M, Sundar S & Dube A, 2012. Leishmania donovani immunstimulatory cellular response of membrane and soluble protein fractions of splenic amastigotes in cured patients and Hamster. *PLOS one,* 7**,** e30746.

Kurthals J, Hey A, Jardim A, Kemp M, Schaefer K, Odera E, Christensen C, Githure J & Oalfson R, 1994. Dichotomy of the human T cells response to leishmania antigens absent or Th2 like response to gp63 and Th1 like response to lipophosphoglycan associated protein in cells from cured visceral leishmaniasis patients. *Clin. EXP. Immunol,* 96**,** 416-421.

Malcolm S. Duthie, V.S.R., Franco M. Piazza, and Steven G. Reed, 2012. The Development and Clinical Evaluation of Second-Generation Leishmaniasis Vaccine**s**. *NIH Public Access,* 30**,** 134–141.

Malla N & Mahajan R, 2006. Pathology of vissceral leishmaniasis some recent concepts. *Indian. J. Med. Res,* 123**,** 267-274.

Marco A. Cassatella, L.H., Bice Perussia, and Giorgio Trinchieri, 2010. Tumor Necrosis Factor and Immune Interferon Synergistically Induce Cytochrome b-245 Heavy-Chain Gene Expression and Nicotinamide-Adenine Dinucleotide Phosphate Hydrogenase Oxidase in Human Leukemic Myeloid Cells. *Plos*.

Mohammed H, Stephen L, Haideh D, Farhad R, Soheilo A, Aliakbar S, Mohammed A, Mehdi M, Zohreh H & Farrokh M, 2012. Assessment of interferon gamma levels and Leishmania skin test Results in person Recoved leishmaniasis. *Am. J. Trop. Med. Hyg,* 87**,** 70-75.

Murray H, 2001. Tissue granuloma structure -function in experimental visceral leishmaniasis. . *Int. J. Exp. pathol,* 82**,** 249-67.

Omprakash S, Carmel B, Abhishek K, Jenefer M & Shyam S, 2012a. Cytikine Response to Novel Antigens in an indian population Living in an Area Endemic for visceral leishmaniasis. *PLOS .Neg.Trop. Dis,* 6**,** e1874.

Omprakash S, Kamlesh G, Rajiv K, Susanne N, Stephen L, Marleen B, David S & S, S., 2012b. Reassessment of immune correlates in human visceral leishmaniasis as defined by cytokine release in whole blood. *J. ASM.Org,* 19**,** 961-966.

Rajiv K, Yasuyuki G, Kamlesh G, Karen D, Cowgill S & Steven G, 2010. Evaluation of Ex vivo Human immune response Against candidates antigens for avisceral lleishmaniasis vaccine. *Am. J. Trop. Med. Hyg,* 82**,** 808-813.

Rhea N. Coler, Y.G, Lisa Bogatzki, Vanitha Raman, and Steven G. Reed, 2007. Leish-111f, a Recombinant Polyprotein Vaccine That Protects against Visceral Leishmaniasis by Elicitation of CD4 T Cells. *American Society for Microbiology,* 75. Sacks D & Noben-

Trauth N, 2002. The immunology of suceptibility and resistance to leishmania major in mice. *Nat. Rev. Immunol,* 2**,** 845-58.

Stober C, Lange U, Roberts M, Alcami A & Blackwell J, 2005. IL-10 from regulatory T cells determines vaccine efficacy in murine Leishmania major infection. *J Immunol* 175**,** 2517-2514.

Stober C, L.U, Roberts Mtm,Alcami a,Bllackwell Jm, 2005. IL-10 from regulatiry T cells determine vaccine efficacy in murine ;eishmania major infection. *J .Immunology,* 75**,** 2517-2514.

Tripathi P, Ray S, Sundar S, Dube A & Naik S, 2005. Identification of leishmania donovani antigens stimulating cellular immune response in exposed immune individuals. *Clin. EXP. Immunol,* 143**,** 380-388.

Yasir a, M.K, David K, Monamaris M, Jeffery a, Jhon K, Pamela J, Et Al, 2014. LeIF: Arecombinant Leishmania protein that induce an IL-12 mediated Th1 cytokine profile. *The Journal of Immunology* 161**,** 6171-6179.

10. Annexes

Annex I: Questionnaire

Patient code-------------

Part I. socio demographic and clinical questions

Q	Questions	Response	Skip
101	Age	--------years	
102	Sex	1.male 2.female	
103	Resident	1.urban 2.rural	
104	Ethnicity	----------------	
105	Educational status	1.not yet 4.diploma 2.elementary 5.degree 3.secondary 6.master And above	
106	Time of treatment ended	1. 6 months 2. 7m-1 year 3. 13 m- 2 years 4. 2 years and above	
107	Type of drug used	1. Amphotercin B 2. Paromomycin 3. Others	

መጠይቅ

የበሽተኛው ምስጢር ቁጥር----------

ቁጥር	መጠይቅ	መልስ	የሚቀጥለው
101	ዕድሜ	--------	
102	ፆታ	1.ወንድ 2.ሴት	
103	መኖሪያ ቦታ	1.ከተማ 2.ገጠር	
104	ብሔር	----------------	
105	የትምህርት ደረጃ	1. ያልተማረ 2.አንደኛ ደረጃ 3.ሁለተኛ ደረጃ 4.ዲፕሎማ 5.ዲግሪ 6.ሁለተኛ ዲግሪ ና ከዚ በላይ	
106	መድሐኒት መውሰድ የጨለሱበት ጊዜ	1.ስድስት ወር 2. 7 ወር- ከ አንድ አመት 3.ከ 13 ወር - 2 አመት 4.ሁለት አመት ና በላይ	
107	የወሰዱት የመድሐኒት አይነት	1.አምፎተርሲን ቢ 2.ፓሮሞማይሲን 3. ሶዲየም ስቲቦ ግሉኮኔት 3.ሌላ	

Annex II: Protocol for Isolation and Quantitation of PBMCs

1. Pipette 20 ml blood in to 50ml centrifuge tube and mix with 20 ml of RPMI
2. Pipette 5 ml of ficol in to 15 ml centrifuge tube
3. Gently homogenize the blood sample and slowly pipette 8 ml blood on top of Ficoll (slant tube when dispensing blood). Be careful not to mix blood with ficoll.
4. Centrifuge at 1900rpm with no brake for 20 minutes at room temp. You will get four layers (plasma, PBMCs, ficoll and erythrocytes plus granulocytes from top to bottom respectively).
5. Using a sterile pipette collect the interphase b/n plasma and ficoll (collecting as little Ficoll and plasma as possible). The interphase is considered to contain most of the PBMCs.
6. Wash the interphase with RPMI (fill centrifuge tube)
7. Centrifuge at 1800rpm for 10 minutes at room temp 2x.
8. Use the PBMCs for cell count
9. Dilute 10 µl of the suspended PBMC in 90µl of trypan blue and count the cells using Fast-Read Counting Slide (Fast Read-102) by Trypan Blue exclusion method and calculate the viability of PBMCs. Viable cells appear colorless where as non-viable cells stain blue as they are permeable to trypan blue.

Total PBMC/ml of suspension = Average N^{o} of cells X DF (10) X Vol. of the chamber (10^4)

$$\text{Viability} = \frac{\text{Total viable PBMCs}}{\text{Total PBMCs}} \times 100$$

10. Centrifuge at 1900rpm for 8min and pour away the supernatant.
11. Finally cell supernatant was mix with12 ml of RPMI complet media .

Reference (Instructions of *Ficoll-Paque Plus*: 71-7167-00 AD, 2002-06, p1-p4(Amersham Biosciencies) "Instruction manual" *Leucosep*® 227290, Greiner Bio-one.

Annex III: - ELISA procedure

Bring all reagents to room temperature (20 - 25°C) before use.

1. Coat nunc Maxisorp ELISA pate with 100 microliter/well of capturing antibody in coating buffer diluted(48 microliter of capture antibody to 12 ml of 1x coating buffer,accordnig to affymetrix,eBioscience,Lot E12422-1631).seal the plate and incubate over night at 4^0c.
2. Aspirate wells and wash 3-5 timeswith >300microliter wash buffer.allowing time for soaking 1minute.Blot plate on absorbent paper to remove any residual buffer.
3. Dilute 1 part 5x concentrated assy Diluents with 4 parts distled water(10 ml of ELISA Diluent to 40 ml of distled water,Lot: E00010-1656).block well with 200 micro liter/well of 1x assay diluent.Incubate at room temperature for 1 hr.
4. Optional :Aspirate and wash at least once with wash buffer.
5. Add 200 micro liter/well of top standard concentration to the appropriate wells. Add 100 micro litter of diluent from B1 - B2 to H1-H2 .Transfer 100 microliter satandard to B1-B2 and mixt it and transfer100 microliter/well to H1-H2 and finally discard it
6. Add 100 microliter/well of samples to apropriate wells.Incubate at roomtemperature for 2 hr.
7. Repeat step 2
8. Add 100 microliter/well of Detction antibody.and icubate at room temperature for 1 hr
9. Repeat step 2
10. Add 100 microliter/well of enzyme .Incubate at room temperature for 30 minutes
11. Aspirate wells and wash at least 5 times with >300microliter wash buffer.Allowing time for soaking 1minute.Blot plate on absorbent paper to remove any residual buffer
12. Add 100 microliter/well of substrate .Incubate at room temperature for 15 minutes
13. Add 50 micro liter/well of stop solution.
14. Read the plate at 450 nm using a plate reader, following the instructions provided by the instrument's manufacturer.

Annex IV:-Formal-Ether Concentration technique procedure

1. Using a stick, emulsify 2 gm of stool in 4 ml of 10% Formol water in a tube
2. Add a further 3-4 ml of 10% formol water, cap the tube and mix well by shaking
3. Sieve the emulsified feces and collect the suspension in a beaker
4. Transfer the suspension to centrifuge tube and add 3-4 ml of diethyl ether or ethyl acetate
5. Mix the tube for 1 minute centrifuge at 3000 rpm for 1 minute
6. Discard the ether, fecal debris and formol water
7. Tap the bottom of the tube and mix the sediment
8. Add a drop of normal saline in a clean slide, add a piece of specimen and cover it with cover slide
9. First examine microscopically using 10 x objectives to give good contrast and use the 40x objective to identify cysts and ova of parasites.
   (Cheesbrough, 1998).

Annex V- Information Sheet and consent form

A. Information sheet

Greeting, my name is Gebreselassie; I came from Addis Ababa University Department of Microbiology, Immunology and Parasitolgy to conduct research entitled with "Immunogenicity of **Leishmania derived antigens in PBMCs using blood obtained from previously treated visceral leishmaniasis patients**". The aim of this research is to determine the immunogenicity of leishmania derived antigens in vitro PBMCs assays and to identify the types of cytokine produced against leishmania derived antigens. The aim of the form is to make the study participants clear about the purpose of research work, data collection procedures and get permission to undertake the research.

Thes tudy will conduct by taking 20 ml of blood to determine antigen immunogenecity and types of cytokine production by drug-cured patients. In addition,1mg of stool needed to detect intestinal parasites.

The study is consent-based, voluntary, confidential, private. Other than a general serial code, your name and other identification aspects are not going to be recorded. Every thing you are going to tell and we will take get kept strictly confidential and private.

When you participate in the study there mightn't be an immediate benefit to you except those who are infected by intestinal parasites will be treated by antihelmenthic drug. However the study finding may be used to further development of vaccine against leshmania. When we take blood you may get simple discomfort because of syringe puncture of your arm.

If in case you want to know more information about the research and its undertakings, contact

Name of investigator: - Gebreselassie Demeke

Phone no: 0924338828, mail;gebredemeke@yahoo.com

Name of Advisor: - Professor Asrat Hailu

Phone no: 0911480993,

B.Consent form(English version)

Based on the purpose and objectives of the study, therefore, you are rightfully eligible to participate . I would like to ask you set of specific questions. I will be grateful if you can spend some time with me.

Now, I can only start asking you the set of specific questions after I have confirmed your willingness. I kindly ask you to take active part and contribute to the study.

Are you willing to participate in the study?

Yes---------Continue

No---------Give thanks to the participant and go to the next participant.

(Please mark with "✓" in the box provided to confirm respondent's permission.

Consent given ☐ code___________Signiture: _____________Date_______

Data collector's name____________________________signature ________Date_______

Supervisor's name_______________________________signature ________Date_______

ለ. የስምምነት ማረጋጫ ቅጽ

ጥናቱ በሚሰራበት ምክንያትና ዓላማ መሰረት እርስዎ ናሙና እንዲሰጡ ተመርጠዋል። ዘርዘር ያሉ እና በክፍል የተከፋፈሉ ጥያቄዎችን ልጠይቅዎት እወዳለሁ። ጥቂት ጊዜዎትን ቢሰጡኝ ምስጋናዬ የላቀ ነው።

ናሙና ልወስድ የምችለው እርስዎ ፈቃደኛ መሆንዎትን ካረጋገጥኩ በኃላ ብቻ ነው። በጥናቱ ንቁ ተሳትፎ በማድረግ አስተዋጽኦ እንዲያደርጉ በትህትና እጠይቀዎታሉ።

በጥናቱ ለይ ለመሳተፍ ፈቀደኛ ነዎት? (በተዘጋጀው ሳጥን የራይት (✓) ምልክት ያድረጉ)

የለም፣ ለመሳተፍ ፍቃደኛ አይደለሁም ☐

(ምስጋና በማቅረብ መጠይቁን ያቁሙ)

አዎ፣ ለመሳተፍ ፍቃደኛ ነኝ ኮድ_☐_ ፊርማ፡ _____ቀን

(ተሳትፏቸውን በማድነቅ መጠይቁን ይቀጥሉ)

የናሙና ሰብሳቢ ስም፡ ________________ ፊርማ፡ ____________ቀን

የሱፐርቫይዘሩ ስም____________________ ፊርማ፡ ____________ቀን

Declaration

I the undersigned declare that this MSc. thesis is my original work and it has not been presented for a degree in any other university. All source materials used for the thesis have been duly acknowledged.

Investigator: Gebreselassie Demeke (Bsc.)

Signature: _________________ Date of submission: _________________

Advisor: Professor Asrat Hailu

Signature: ____________________ Date: _____________________