Studies on Immunological Responses to Parasite Surface Antigen as a Potential Vaccine Candidate against Human Leishmaniasis and Estimation of Asymptomatic *Leishmania* Infection in the Endemic Areas

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CERTIFICATE

This is to certify that the thesis entitled "**Studies on Immunological Responses to Parasite Surface Antigen as a Potential Vaccine Candidate against Human Leishmaniasis and Estimation of Asymptomatic *Leishmania* Infection in the Endemic Areas"** and submitted by Himanshu Kaushal, ID No. 2010PHXF457P for award of Ph.D. Degree of the institute embodies original work done by him under my supervision.

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Abstract

At present, there is no licensed vaccine available against any form of human leishmaniasis, although, the development of an anti-*Leishmania* vaccine has been a long-term goal. Besides, for the sustainable elimination of VL, the presence of asymptomatic VL may prove an important impediment as they could play a vital role in maintaining transmission dynamics of *Leishmania* infection in the region. Here, we evaluated the recombinant native form of *La*PSA protein for its ability to induce cellular and humoral immune responses in active cases of VL and PKDL, healed VL (HVL) and naïve individuals, to assess its potential use as a vaccine candidate against human VL, caused by *L. donovani*. Further, using serological and molecular tools, we assessed *Leishmania* parasites in the blood samples of recruited healthy individuals living in two VL-endemic regions (Malda and Murshidabad) of the state of West Bengal, to understand the magnitude of asymptomatic *Leishmania* infection present in the region.

The study demonstrated *Leishmania*-specific cellular immune responses in terms of lymphoproliferation and cytokine profile upon PBMCs stimulation with *Leishmania* antigens i.e.; TSLA and *La*PSA, in the active cases of VL and PKDL and HVL individuals. Lymphoproliferation in response to TSLA in HVL and PKDL group showed heightened response whereas *La*PSA did not induce lymphoproliferation in either of *Leishmania* pre-exposed groups i.e., HVL or PKDL group. The cytokine profile upon *La*PSA and TSLA stimulation corroborated our lymphoproliferation data; *La*PSA did not induce significant pro-inflammatory cytokines response in either HVL or PKDL while TSLA elicited a strong protective cytokine response in the same group. Additionally, the significant activation of both $CD4^+$ and $CD8^+$ T cells along with high granzyme B production upon *in vitro* TSLA stimulation indicated for the first time the role of cytotoxic cells in resistance to *L. donovani* infection in HVL and PKDL groups. This finding indicated *Leishmania*-specific cell-mediated cytotoxicity as a part of the naturally acquired immunity developed in *Leishmania* pre-exposed groups (HVL and PKDL). In contrast and corroborating our cellular data, *La*PSA did not induce significant activation in either $CD8^+$ or $CD4^+$ T cells. Generalized cellular immunity based on the phenotype and proportion of the different peripheral blood lymphoid subsets revealed impaired cellular immunity in active VL while PKDL showed raised proportion of $CD3^+$ T cells.

Our approach to investigate the ability of a given antigen, for its ability to elicit cellular protective responses in different groups of human populations would be applicable to select vaccine candidates and prioritize antigens for the clinical development of a vaccine against leishmaniasis.

The other part of the study identified 22.35% asymptomatic individuals among the enrolled study population living in the two VL endemic districts of West Bengal. These could be the potential reservoir for *Leishmania* parasite and might play an important role in the disease transmission in the region. Besides, a qPCR method was more sensitive and specific than the serological method in assessment of asymptomatic VL individuals. Additionally, anti-TSLA or anti-*La*PSA IgG based detection of asymptomatic infection in healthy individuals may not be a good choice. Thus, deployment of combined molecular and serological methods could effectively help estimate the asymptomatic *Leishmania* infection in healthy individuals living in endemic regions, and may contribute to early case detection and treatment. Furthermore, the estimation of asymptomatic VL individuals in the endemic area will be useful to take appropriate measures for the sustainable elimination of VL from India.

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Abbreviations

AmpB	Amphotericin B
BSA	Bovine Serum Albumin
CBA	Cytokine Bead Array
CD	Cluster of Differentiation
Cm	Centimeter
CL	Cutaneous leishmaniasis
CMI	Cell mediated immunity
CTL	Cytotoxic T lymphocyte
DAT	Direct agglutination test
DCL	Diffuse cutaneous leishmaniasis
DNA/RNA	Deoxyribose/Ribose nucleic acid
dNTP	Deoxyribose nucleotide triphosphate
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS/FBS	Fetal Calf/Bovine Serum
FML	Fucose-mannose ligand
Foxp3	Forkhead box protein 3
HBSS	Hank’s balanced salt solution
HEPES	N-2-hydroxyethgylpiperazine-N-2 ethane sulfonic acid
Hr/hrs	Hour/hours
HRP	Horseradish peroxidase
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IM	Intramuscular
iNOS	Inducible nitric oxide synthase
ITS	Internal transcribed spacer
IV	Intravenous

KA	Kala-azar
kDNA	kinetoplastid DNA
Kb	Kilobase
LB	Luria-Bertani medium
LAMP	Loop-Mediated Isothermal Amplification
*La*PSA	*Leishmania amazonensis* Parasite surface antigen
*Li*ESAp	*Leishmania infantum* Excretory-Secretory Antigen particules
LDB	Leishman-Donovan bodies
LRR	Leucine Rich Repeats
LST	Leishmanin skin test
M	Molarity
mg	Milligram
MHC	Major histocompatibility complex
min/mins	Minute/Minutes
MIP	Macrophage inflammatory protein
ml	Millilitre
Mm	Millimeter
MPD	Muramyl dipeptide
mM	Millimolar
MPL-SE	Monophosphoryl lipid A in stable emulsion
N	Normality
$NaHCO_3$	Sodium bicarbonate
ng	Nanogram
NO	Nitric oxide
NRAMP1	Natural resistance associated macrophage protein 1
O/N	Overnight
^{O}C	Degree Celsius
OD	Optical Density
OPD	O-phenylenediamine
PBS	Phosphate Buffered Saline

PBST	Phosphate buffered saline with Tween-20
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
pg	Picogram
RFLP	Restriction fragment length polymorphism
RFM	Rifampicin
PHA	Phytohemagglutinin
PKDL	Post Kala-azar Dermal Leishmaniasis
PMNs	Polymorphonuclear neutrophils
RORγt	Retinoic acid-related orphan receptor gamma
rpm	Revolution per minute
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
SE	Standard error
SSG	Sodium stibogluconate
STAT	Signal transducer and activator of transcription
TAE	Tris acetate EDTA
TGF	Transforming growth factor
Th1	T helper-1 cell
Th2	T helper-2 cell
TNF	Tumor necrosis factor
TSLA	Total soluble *Leishmania* antigen
Tris	Tris (hydroxymethyl) amino acid
U	Unit
VL	Visceral Leishmaniasis
WHO	World Health Organization
Xg	Times gravity (centrifugal force)
μm	Micromole

Introduction

The leishmaniases are a group of diseases largely affecting people of economically underprivileged strata of society in the developing countries; posing the risk to over 350 million people worldwide (WHO 2010). The disease is caused by over 20 species of the genus *Leishmania (L)* (Ashford 2000) and transmitted by an infected female sandfly. The disease manifests in various clinical forms, ranging from self-healing cutaneous leishmaniasis (CL) to disfiguring mucosal lesions to the potentially fatal visceral form.

Visceral leishmaniasis (VL), commonly known as kala-azar is the most lethal form of leishmaniasis which proves fatal if diagnosed late or left untreated. It is caused by *L. donovani* complex comprising of *L. donovani and L. infantum*. VL has been reported from 62 countries, with approximately 0.5 million new cases annually, posing a risk to over 200 million people worldwide (Herwaldt 1999; Desjeux 2004). The Indian subcontinent and East Africa disease are endemic areas for the disease, and over 90% of global VL cases occur in six countries: Bangladesh, India, Ethiopia, Brazil, Sudan and South Sudan, (Alvar *et al.* 2012). In India, the state of Bihar and its adjoining areas, namely the state of Uttar Pradesh and West Bengal, are highly VL-endemic foci where periodic epidemics are common. The parasites are transmitted to human host by the bite of a female sandfly, wherein they rapidly invade macrophages and multiply inside phagolysosomes. The disease is clinically characterized by irregular bouts of fever, hepatosplenomegaly, pancytopenia, weight loss and anaemia and the disease condition may further get aggravated by secondary opportunistic infections.

Post kala-azar dermal leishmaniasis (PKDL), a dermal sequel of VL, develops in 5-15% of apparently cured VL individuals in the Indian subcontinent, as against 50-60%

in Sudan (Zijlstra *et al.* 2000; Ramesh *et al.* 2007; Mondal *et al.* 2010; Rahman *et al.* 2010). It is characterised by different clinical complications such as nodular, macular or maculopapular dermal lesions. In the Indian subcontinent, patients with PKDL based on their clinical presentations, can be categorized into two clinical subgroups: monomorphic PKDL, where the patients primarily have single clinical lesion type (either hypopigmented macules or papules); and polymorphic PKDL, cases with at least two different types of lesions (macular, papular and nodular) (WHO 2012). The polymorphic PKDL is predominant and represents 45-85% of Indian PKDL cases (Ganguly *et al.* 2010a; Singh *et al.* 2015). As transmission of VL in Indian subcontinent is anthroponotic, the patients with PKDL are considered durable reservoir of VL, especially during an inter-epidemic period (WHO 2010) which gives PKDL an added concern in the context of sustainable elimination of VL.

The current treatment against leishmaniasis is based on anti-parasitic drugs. Problems associated with drugs are logistics, transportation, a high financial burden which in the end imply low compliance and the subsequent pharmaco-resistance risks. Most of the treatments proposed (antimonial derivatives, amphotericin B) are inconvenient as they require inpatient procedures (which make the treatment a lot more expensive and so not always affordable), can be painful and lengthy. The oral anti-leishmanial drug, miltefosine was introduced in India in 2002, initially for the treatment of VL and subsequently recommended for PKDL treatment. This heralded the miltefosine era where the patients could take the drug orally and get cured. It proved to be a giant step in the management of PKDL (Ramesh *et al.* 2008a). However, similar to the

antimony era where *Leishmania* parasites showed a gradual increase in tolerance and resistance, miltefosine tolerance is gradually going up (Ramesh *et al.* 2015a).

As leishmaniasis primarily affects developing countries, strategies to control the disease should be affordable. Vaccination is seemed to be the most cost-effective mean, as for many other infectious diseases, it stands a suitable choice for the global control of *Leishmania* infection. Further, it has been estimated that if the people from 7 countries of Latin America: Ecuador, Mexico, Colombia, Peru Bolivia, Brazil, and Venezuela were given a vaccine formulation that would provide protection for 10 years, an approximately 41,000-144,000 CL cases could be averted, and that the estimated treatment cost would be lower than the current treatments cost (Bacon *et al.* 2013). Further, even a vaccine that has protective efficiency of 50% and provides 5 years of protection would still remain cost-effective (Bacon *et al.* 2013). A similar study for VL from the state of Bihar, India has also reiterated the cost-effectiveness of a vaccine against human VL (Lee *et al.* 2012). Thus, a vaccine solution appears to be the most appealing strategy for global control of leishmaniasis (Duthie *et al.* 2012).

The feasibility of vaccine development against leishmaniasis comes from the observation that the majority of the *Leishmania*-infected people do not get clinical symptoms and the previous episode of leishmaniasis provides lifelong protection against re-infection, once the infection is healed (von Stebut 2007). This is further supported by an ancient practice termed “leishmanization” practised in the Central Asia and Middle East where people were “vaccinated” using sharp tools or thorns to inoculate parasites into unexposed body parts to prevent the development of facial lesions through natural infection (Handman 2001).

The development of a vaccine to prevent leishmaniasis has been a long-term goal. Current molecular techniques have led to the development of recombinant antigen vaccines though none has reached clinical approval. LeIF, a recombinant antigen, showed promising results in experimental BALB/c model (Skeiky *et al.* 1998) and therefore, included in a multi-epitope subunit vaccine, Leish-111F, having a *L. major* homolog of eukaryotic thiol-specific antioxidant, *L. braziliensis* elongation and initiation factor and *L. major* stress-inducible protein-1 in combination with MPL-SE and has elicited protective responses in mouse models of VL and CL (Skeiky *et al.* 2002; Coler *et al.* 2007). These recombinant antigen based vaccines are still in experimental stage, none is currently ready for routine clinical use. On the other hand, attenuated parasites, although effective, have been unacceptable to the human population, due to lack of a clear genetic profile and chances of reversion. Several laboratories have engineered and investigated genetically mutant parasites that could point the way toward attenuated vaccines (Joshi *et al.* 2002; Burchmore *et al.* 2003; Selvapandiyan *et al.* 2006).

In recent times, there has been significant thrust on identifying newer recombinant antigens that can provide protection against *Leishmania* infection in experimental models. This is particularly because of the fact that the recombinant products can be made accessible in industrial-scale, reproducible and cost-effective, and the protective immune responses elicited by vaccination can be further potentiated and refined with suitable adjuvant formulation (Reed *et al.* 2009; Duthie *et al.* 2011). Recently, an effective canine vaccine against leishmaniasis was developed in France and has been licensed for commercial use under the name of CaniLeish® in Europe. This was based on the crude excreted-secreted antigen from promastigote culture supernatant of *L. infantum*

(*Li*ESAp), formulated with muramyl dipeptide (MDP). It was shown to elicit a durable Th1-mediated protective immunity against both natural and experimental canine VL (Lemesre *et al.* 2005, 2007). Native soluble *L. infantum* Promastigote Surface Antigen (ns*Li*PSA) has been identified as the active constituent of *Li*ESAp that reproduces the same level of protective immunity when used as a vaccine in dogs. The study demonstrated that sera of dogs vaccinated with *L. infantum* excreted/secreted antigens (ESA) in combination with MDP, recognized immunodominant antigens belonging to PSA protein family, from *L. infantum* ESA (*Li*PSA) and from *L. amazonensis* ESA (*La*PSA) (Bras-Gonçalves *et al.* 2014). The PSA is a naturally excreted-secreted protein belonging to the family of Promastigote surface antigen. Its main signature is a specific Leucine-Rich Repeats (LRR) known to be involved in protein-protein interactions and in pathogen recognition (Devault and Bañuls 2008). PSA is involved in parasite attachment and invasion of macrophages (Kedzierski *et al.* 2004) and also known to be present in both promastigote and amastigote stages (Handman *et al.* 1995a; Beetham *et al.*, 2003). PSA proteins are strongly over-expressed in metacyclic promastigotes suggesting an association with the virulence status of the parasites (Beetham *et al.* 2003). The presence of PSA family was demonstrated in several *Leishmania* species (Lohman *et al.*1990; McMahon-Pratt *et al.* 1992; Myung *et al.* 2002; Devault and Bañuls 2008). Above findings makes native soluble PSA protein ideal to be evaluated as a potential vaccine candidate against human VL.

Besides, the presence of asymptomatic VL cases in endemic areas could be an important hurdle towards VL elimination. Most of the *Leishmania*-infected human population do not develop into full-blown VL cases, and are considered asymptomatic

(Das *et al.* 2011; Ostyn *et al.* 2011; Stauch *et al.* 2011) and these cases could play an important role in maintaining transmission dynamics of *Leishmania* infection (Sharma *et al.* 2000). However, the actual estimate of asymptomatic cases in an endemic area is difficult to assess. A few studies have reported the presence of asymptomatic cases in high endemic areas of VL in Bihar, in the range of 10 to 34% (Topno *et al.* 2010; Das *et al.* 2011; Sudarshan *et al.* 2014) and the conversion rate to symptomatic VL was 17.85 per 1000 persons (Topno *et al.* 2010). These "asymptomatic carriers" could prove an important impediment towards VL elimination program.

In the present study, the recombinant native form of *L. amazonensis* PSA protein (*La*PSA), produced in a *L. tarentolae* expression system was evaluated for its ability to induce cellular and humoral immune responses in active cases of VL and PKDL, healed VL (HVL) and naïve individuals for its potential use as a vaccine candidate against human VL, caused by *L. donovani*. Furthermore, the study will enhance understanding of generalized cellular immunity based on different peripheral blood lymphoid subsets in all the four study groups examined simultaneously. Besides, the other goal of the study was to understand the magnitude of asymptomatic *Leishmania* infection in endemic regions of the state of West Bengal, India using serological and molecular analysis of *Leishmania* infection in blood samples of healthy individuals. This knowledge will help early case detection and treatment and help take appropriate measures for the sustainable VL elimination from India**.**

Review of Literature

Leishmaniasis

Leishmaniasis, a neglected tropical disease is caused by parasites of the genus *Leishmania* and transmitted via infected female sandflies (*Phlebotomus* and *Lutzomyia*). Depending on the species, the disease manifests in various clinical forms, ranging from self-healing cutaneous leishmaniasis (CL) to disfiguring mucosal lesions to fatal visceral form, visceral leishmaniasis (VL). It is a poverty-prone disease, affecting the poorest of the poor and has been linked to malnutrition, weakened immune system, displacement, illiteracy, poor housing, and the lack of resources. The disease is widely dispersed in 98 countries and 3 territories on 5 continents, with more than 350 million people at risk (WHO 2010, 2013). Approximately 1.3 million new cases occur annually, of which 0.3 million are of visceral and 1 million of cutaneous or mucocutaneous leishmaniasis. Of the 1.3 million estimated cases, only about 0.6 million are actually reported (Alvar *et al.* 2012). The true incidence is probably underestimated because the cases are not recognized and reporting is compulsory in only 33 of 98 affected countries (WHO 2013).

HIV infection appears to increase susceptibility to VL and affects its distribution. In year 2012, thirty-five endemic countries have reported cases of co-infection of HIV and VL. In places where there is insufficient access to antiretroviral therapy, the incidence of VL is increasing. In northern Ethiopia, the rate of co-infected cases has a rising trend from 19% during 1998–1999 to 34% during 2006–2007 (Alvar *et al.* 2008).

Types of Leishmaniasis

Depending on *Leishmania* species, the disease manifests into varied clinical complications ranging from self-healing CL to disfiguring mucosal lesions to the visceral

form. VL is most deadly form and is usually fatal within 2 years if left untreated. After treatment, VL may evolve into a dermal form known as post-kala-azar dermal leishmaniasis (PKDL), a potential reservoir of *Leishmania* infection and thus maintains transmission. CL, the most widely prevalent form, causes ulcers that recover itself. The mucocutaneous form particularly invades the mucous membranes of the upper respiratory tract, leading to mutilation of the soft tissues in the nose, mouth and throat. A summary of geographic distributions of different *Leishmania* species with their corresponding clinical presentations are summarized in **Table 2.1**.

Table 2.1 Summary of geographic distributions of different *Leishmania* species with their corresponding clinical presentations

Species	**Clinical manifestation**	**Geographical Distribution**
L. donovani	Visceral, PKDL	Old World: China, India, Bangladesh, Africa.
L. infantum	Visceral	Old World: North Central Asia, Northwest China, Uzbekistan, Middle East.
L. chagasi	Visceral	New World: South and Central America.
L. major	Cutaneous	Old World: Africa, Middle East, Northern Asia
L. mexicana	Cutaneous, Diffuse cutaneous, Mucocutaneous	New World: Southern Mexico, Belize, Northern Guatemala, Southern Texas.
L. amazonensis	Cutaneous, Mucocutaneous	New World: South and Central America.
L. braziliensis	Mucocutaneous, Cutaneous	New World: Throughout South America.
L. tropica	Cutaneous	Old World: India, South-West Asia, North and East Africa
L. panamensis	Mucocutaneous, Cutaneous	New World: Panama
L. venezuelensis	Cutaneous, Diffuse cutaneous	New World: Venezuela
L. guyanensis	Cutaneous, Mucocutaneous	New World: Brazil; Colombia; French Guiana

Cutaneous Leishmaniasis (CL)

This is the most common and widely prevalent form of leishmaniasis, which is also known as 'Oriental sore'. The incidence of over 90% of CL cases is from the Americas, the Mediterranean basin, the Middle East and Central Asia (**Fig 2.1**). The two-thirds of new CL cases are reported from 6 countries: Afghanistan, Algeria, Brazil, Colombia, Iran, and Syria. An estimated 0.7 to 1.3 million new cases occur annually worldwide. In India, CL is primarily reported in some pockets in the Thar Desert of the state of Rajasthan, the western part of the country and Pakistan border (Dogra *et al.* 1990). The disease is spreading to the new foci of CL such as Himachal Pradesh, where *L. donovani* and *L. tropica* are found to be the causative agents (Sharma *et al.* 2005).

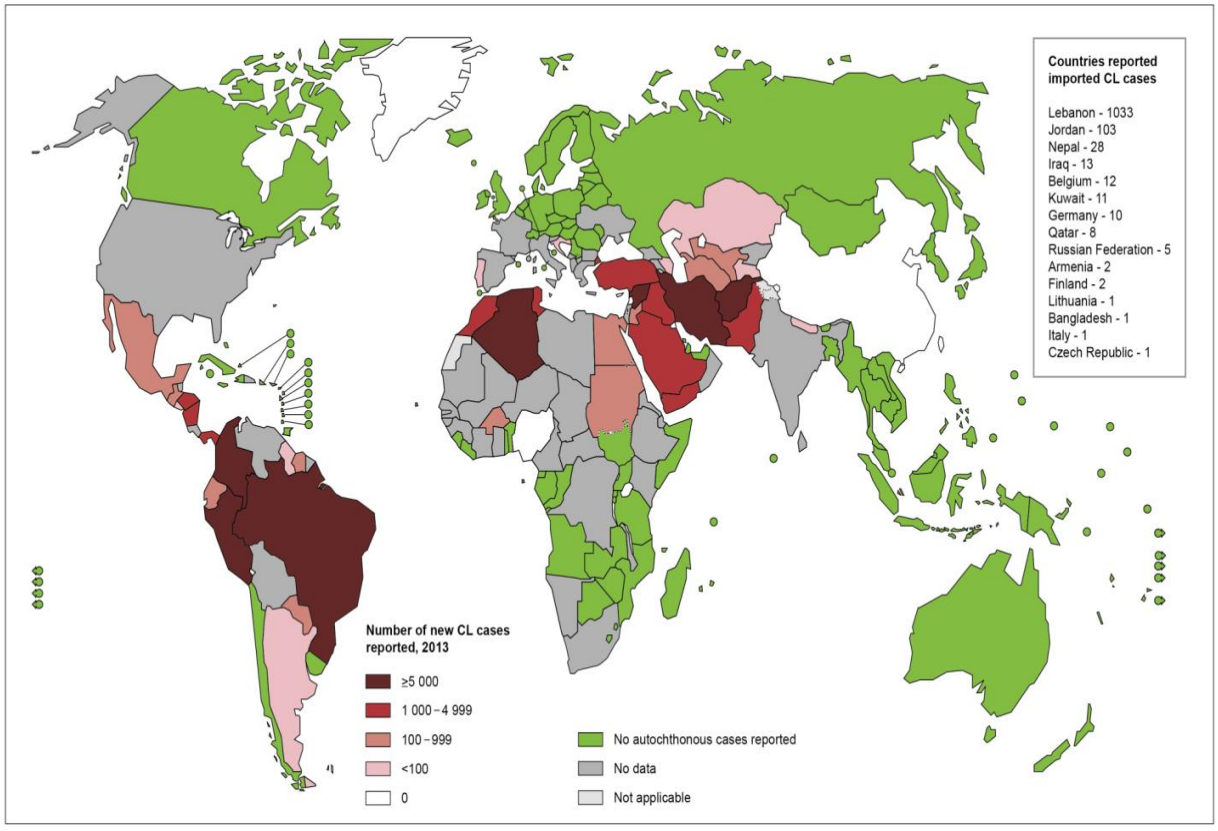

Figure 2.1: Status of endemicity of cutaneous leishmaniasis, worldwide, 2013. (Source: WHO; Map Production: Control of Neglected Tropical Disease (NTD) WHO; http://www.who.int/leishmaniasis/burden/Leishmaniasis_Burden_distribution_CL).

The simple cutaneous disease that manifests as a ulcerative or nodular lesion at or around the site of insect bite. These are usually found on exposed parts of the body such as face, forearms, and legs and evolve over weeks to months (**Fig 2.2**). The incubation period may be of few days or months. Gradually, the lesion size increases, becoming red, but without heat or pain. Healing of the lesion involves the recruitment of leukocytes, resulting to necrosis of the infected area of the tissues, and eventually the formation of a granuloma in the lesion.

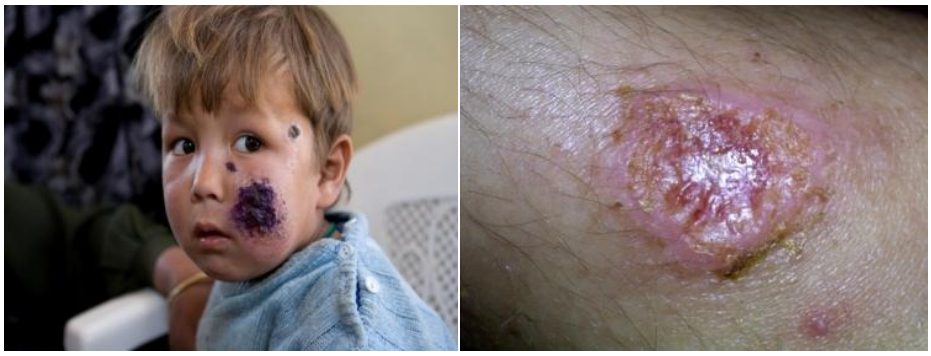

Figure 2.2: Clinical signs of Cutaneous Leishmaniasis. Patients with skin ulcers due to CL. (Source: WHO/C. Black; http://www.who.int/campaigns/world-health-day/2014)

Diffuse cutaneous leishmaniasis (DCL)

This is a progressive, chronic, polyparasitic form that develops in the presence of *Leishmania*-specific anergy and is clinically manifest as disseminated non-ulcerative cutaneous lesions, similar to lepromatous leprosy (**Fig 2.3**). Previously, DCL was restricted to Ethiopia and Kenya in Africa and the Dominican Republic and Venezuela in the western hemisphere. The previous report has shown DCL, caused by *L. tropica* in India (Khandelwal *et al.* 2011). It is mainly caused by *L. aethiopica* and *L. mexicana* species complex.

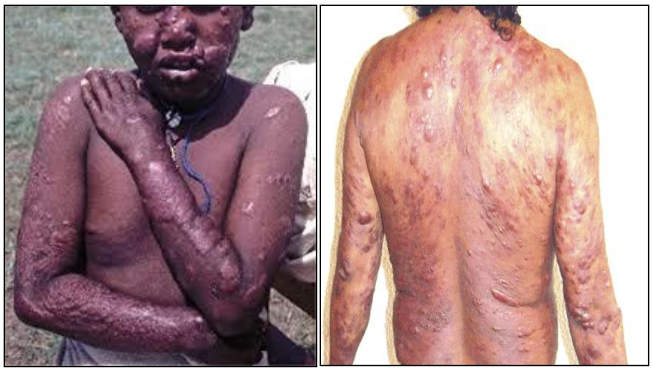

Figure 2.3: Patient with clinical symptoms of Diffuse Cutaneous Leishmaniasis. (Reproduced from otm.oxfordmedicine.com and Calvopina *et al*., 2006; *Am J Trop Med Hyg*. 75:1074-7).

Mucocutaneous Leishmaniasis (MCL)

This is also known as "espundia" that involves partial or total destruction of mucous membranes of the mouth, throat and nose, (**Fig 2.4**.) The underlying principle of pathogenesis of MCL is not well understood and probably is an outcome of a complex interplay of various host and parasite factors (de Oliveira & Brodskyn, 2012). The disease is often unresponsive to chemotherapy and the patients gradually die from secondary infections and malnutrition. MCL exists as zoonotic infection in which the *Leishmania* is transmitted from rodent to rodent and mammal by the sandfly vector *Lutzomyia* spp. The reservoir hosts include rodents, anteaters, sloths, opossums, and dogs. Humans are infected when they invade the forest habitats. The causative species of MCL in the new world are *L. braziliensis, L. mexicana, L. amazonensis, L. guyanensis and L. panamensis* and *L. aethiopica* (rare) in the old world. Approximately 90% of MCL cases occur in the Bolivia, Brazil, and Peru (WHO 2010).

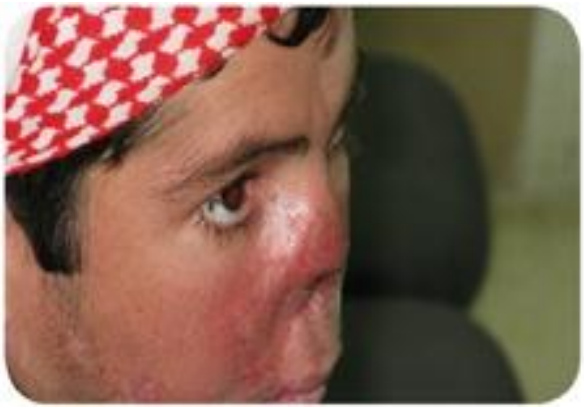

Figure 2.4: Patient with clinical symptoms of Mucocutaneous Leishmaniasis. (Source: http://www.who.int/leishmaniasis/clinical_forms_leishmaniases/en)**.**

Visceral Leishmaniasis (VL) or Kala azar (KA)

VL is the visceral form of leishmaniasis, caused by the *Leishmania (L.) donovani* complex: *L. donovani*, the causative species of VL in the Indian subcontinent and Africa; *L. infantum* (*L. chagasi)* responsible for VL in the Mediterranean Basin, Central and South America (WHO 2010). It is transmitted by sandflies as extracellular promastigotes form and replicates as intracellular, non-flagellated amastigotes in phagocytes in the mammalian host. The disease is also known by several other names as kala-azar, black fever, dum-dum fever, Burdwan fever, etc.

The epidemiology of leishmaniasis is diverse and complex. VL is endemic in the Indian subcontinent while Brazil and East Africa are also highly endemic regions for VL (**Fig 2.5**). Worldwide, the annual incidence of VL is around 0.2–0.4 million cases, 90% of which are reported from six countries: India, Bangladesh, Ethiopia, Brazil, South Sudan and Sudan (Alvar *et al.* 2012).

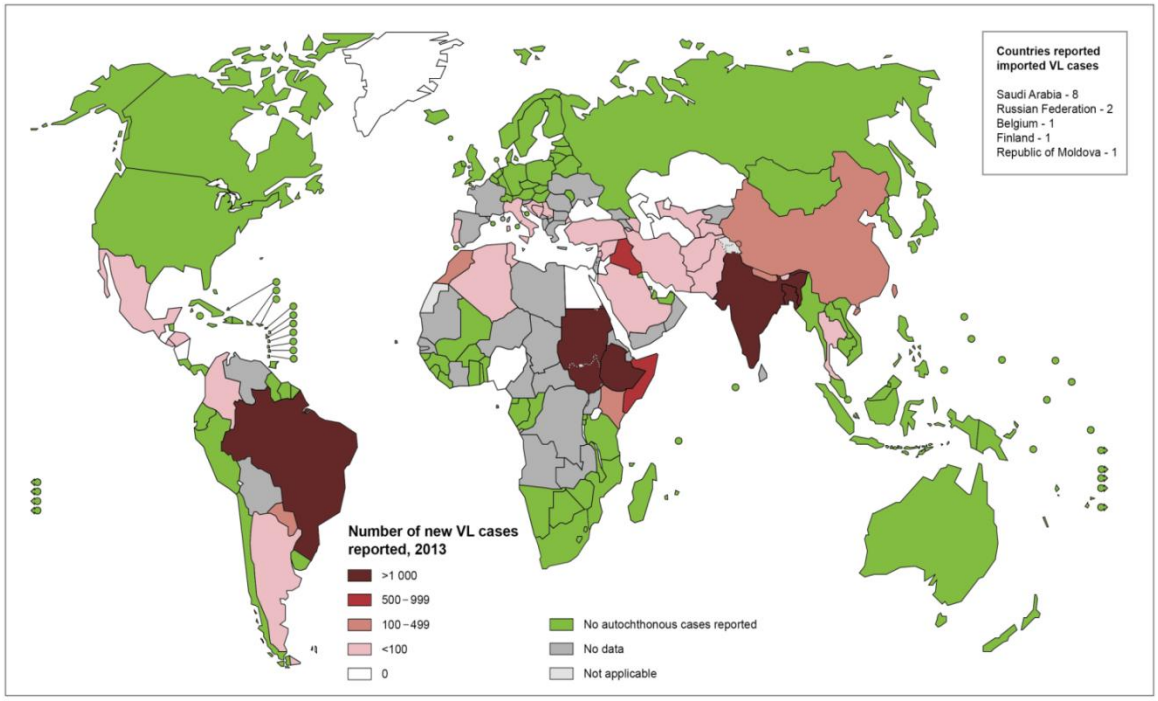

Figure 2.5: Status of endemicity of visceral leishmaniasis, worldwide, 2013. (Source: WHO; Map Production: Control of Neglected Tropical Disease (NTD) WHO; http://www.who.int/leishmaniasis/burden/Leishmaniasis_Burden_distribution_VL).

In WHO's South-East Asia Region, over 147 million people are at a risk of contracting this life-threatening disease, mainly in Nepal, Bangladesh and India, and recently sporadic cases has been reported from Bhutan and Thailand. Of the total 109 districts of the Indian subcontinent where VL is endemic, 52 are in India, 45 in Bangladesh, and 12 in Nepal (WHO 2015). In South East Asia, the target for VL elimination has been set to < 1 case/ 10,000 population/ year at the district or subdistrict level. This is due to the unique epidemiology of the disease, the availability of effective preventive measures and, more importantly, the strong commitment of the government in the region. The VL elimination programme has shown a remarkable achievement in the region by reducing the disease incidence trend and the case fatality rate (**Fig 2.6**).

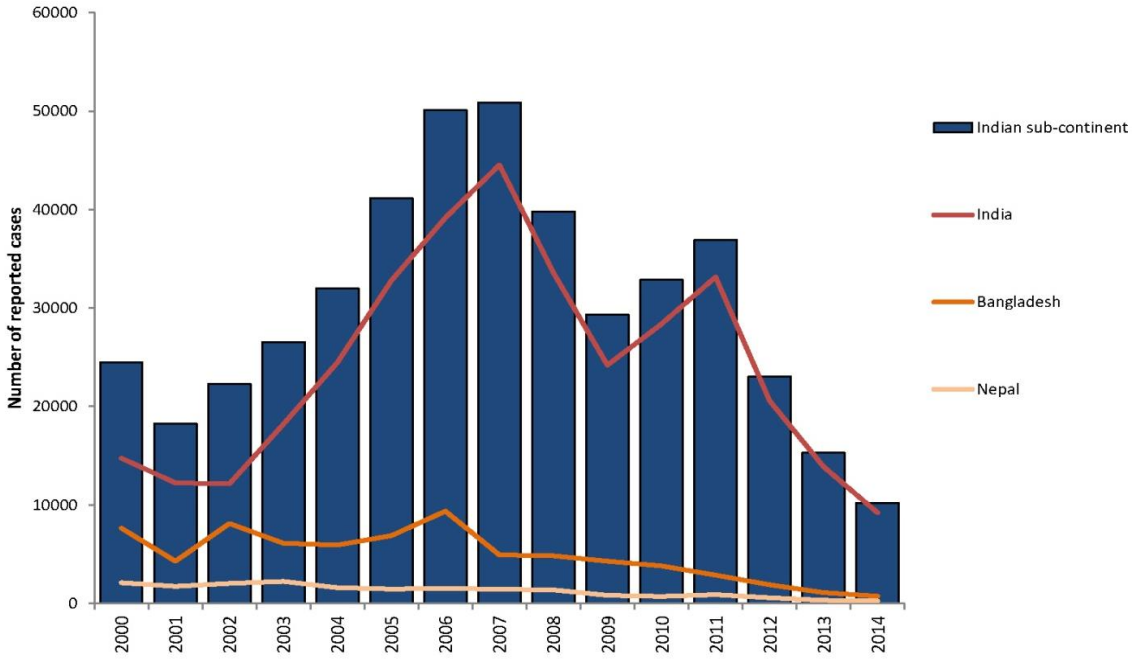

Figure 2.6: Visceral leishmaniasis in the WHO South East Asia region, 2000−2014. (Source: WHO/http://www.who.int/neglected_diseases/news/VL_SEARO_2000_2015)

Clinical features of VL typically include low-grade fever, pancytopenia, hepatosplenomegaly, weight loss and polyclonal hypergammaglobulinemia (Chappuis *et al.* 2007) and (**Fig 2.7**). The parasite invades and divides within macrophages and mainly target spleen, liver, bone marrow, and lymphoid tissue (Aggarwal *et al.* 1999; Boelaert *et al.* 2000). VL has been demonstrated to be an important opportunistic infection among patients with HIV-1 infection (Alvar *et al.* 1997; Desjeux and Alvar 2003). The incidence of HIV co-infection in India is also reported (Redhu *et al.* 2006). Patients with co-infection have higher parasite load and compromised immune responses, and they respond poorly to anti-leishmanial therapy including antimonials, amphotericin B (AmB) and others (Berman 2003). The course of co-infection is marked by a high relapse rate; prophylactic therapy is suggested to be useful in preventing relapses (Alvar *et al.* 1997).

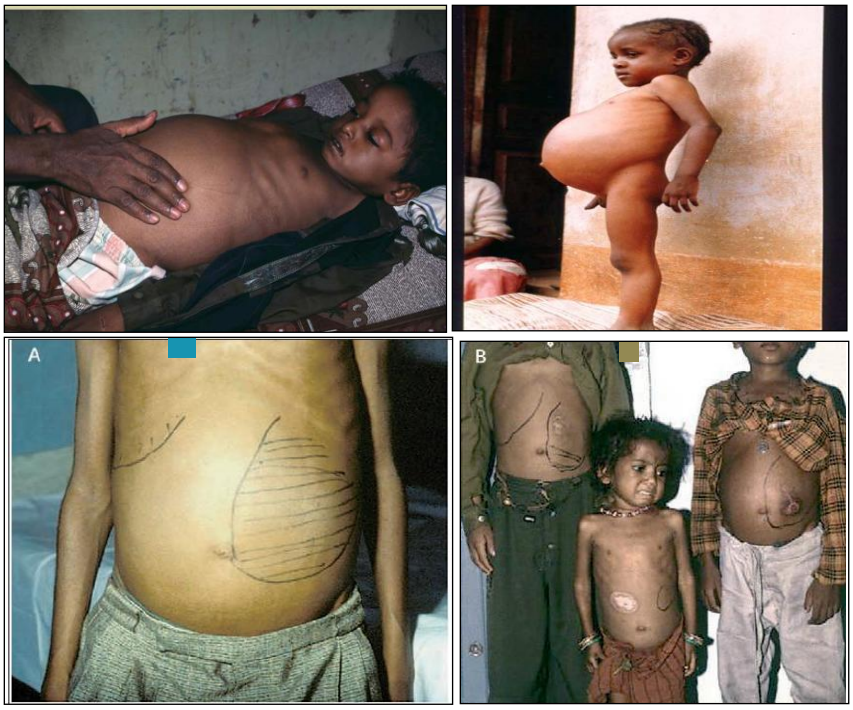

Figure 2.7: Clinical symptoms of VL. Hepatosplenomegaly and wasting are the main features (Reproduced from Murray *et al*., 2005; Lancet 366: 1561–77 and TDR report, February 2004).

Post kala-azar dermal leishmaniasis (PKDL)

PKDL, a dermal sequel of VL was first described by Brahmachari in 1922 in healed VL individuals with eruption and plaque in skin, which was confirmed by the demonstration of Leishman-Donovan bodies (LDB) in slit skin smear and termed "dermal leishmanoid" (Brahmachari UN 1922). Later, the disease was renamed as PKDL since eruptions follow the visceral disease, commonly called as kala-azar.

PKDL is reported in areas endemic for *L. donovani* in the Indian subcontinent, Sudan and its adjoining areas (Zijlstra *et al.* 2000; Ramesh *et al.* 2007), although sporadic cases have been reported from China, Japan, Iran and Iraq (Ono *et al.* 1998; Badirzadeh *et al.* 2013). In the Indian subcontinent, up to 15 % of apparently cured VL patients develop PKDL, against 50–60 % in Sudan (Zijlstra *et al.* 2000; Ramesh *et al.* 2007;

Mondal *et al.* 2010). The disease is characterized by different clinical presentations from simple hypopigmented macular form to more complicated lesions comprising of papular, nodular cutaneous lesions and/or polymorphic forms with mixed lesions. PKDL is not a life threatening disease, but more of a social stigma especially when lesions present on the exposed parts of the body. Importantly, in the Indian subcontinent, as the transmission of VL is anthroponotic, unattended cases of active PKDL considered a durable reservoir of *L. donovani* in VL-endemic areas, especially during an inter-epidemic period (WHO 2010).

Clinical features of PKDL

Patients with PKDL are healthy except for their skin rashes. Therefore, they may not always visit health centres promptly. Unlike VL cases, they do not have fever, splenomegaly or weight loss, and physically they look normal. However, a severe form of PKDL when present on the face can have significant clinical and social discomfort. As the initial diagnosis is based on clinical presentations, the differential diagnosis becomes very important.

In the Indian subcontinent, the polymorphic PKDL is the most commonly found: hypopigmented macules, nodules, indurated erythematous plaques and papules are also observed (WHO 2012). The typical clinical form is the lesion clustered around the mouth and chin, with discrete to no lesions in the rest of the body (**Fig 2.8**). The disease progresses most likely from the face to the other parts of the body. Approximately 20% of PKDL cases show mucosal involvement that affects the glans penis and oral cavity. PKDL with macular variant is important because it is most likely to be mistaken for vitiligo rather than leprosy since the degree of pigment loss is comparatively higher than

leprosy. Distribution of macular PKDL may be localized or extensive and the face may be partially or not affected (Ramesh and Singh 1999; Ramesh *et al.* 2008b). Notably, hypopigmentation may be seen on the entire skin with few areas of normal or hyperpigmented skin, notably at the flexures. A similar incidence of macular PKDL has been observed in other study (Saha *et al.* 2005). The other clinical manifestation of PKDL is of fibroid variety with plaques on the dorsa of fingers and toes.

Figure 2.8: Clinical presentations in PKDL. (A) Macular lesions on the legs (B) Discrete papules on the chin (C) Crops of nodules on chin and nose in a patient with PKDL in India. (Source: The Post Kala-azar Dermal Leishmaniasis (PKDL) atlas: a manual for health workers, WHO, 2012).

In Africa, PKDL is mainly concentrated in Sudan, and has been increasingly reviewed and reported (Musa *et al.* 2002; Zijlstra *et al.* 2003). In the Indian subcontinent, PKDL occurs in 5-15% of those treated for VL (WHO 2012), in contrast, PKDL appears in as high as 50% of patients following VL in Sudan (Musa *et al.* 2002). The distribution pattern of PKDL lesions in Sudan is similar to that of the Indian counterpart but differs in degree of ulceration that occurs in severe cases. The lesions typically arise first on the face as papules, and may remain confined to the face or spread to rest of the body parts: firstly, to the trunk and upper limbs, and subsequently to the rest of body parts. The

lesions are generally not itchy and symmetrical. The papules may get enlarge, and turn into plaques or nodules, or a combination of these; alternatively, the lesion may be macular. Maculopapular lesions are common; a micropapular form resembling measles may be seen (WHO 2012). Unlike PKDL in the Indian subcontinent, three grades of PKDL severity have been reported in Sudan (WHO 2012). A study on the Sudanese PKDL has implicated UV-B radiation of the sunlight with the severity of facial lesions and also, it appears to modulate the overall immune responses, promoting lesion development (Ismail *et al.* 2006).

PKDL is relatively common in HIV-positive individuals and often has atypical presentations, such as large nodular lesions. The typical clinical distribution i.e. the disease progression from face to other body parts is not always followed. The majority of these patients present florid disease with descriptions such as macules, disseminated miliary papules, papulo-erythematous eruption, nodules, and plaques, or a mixed picture (Rihl *et al.* 2006; Stark *et al.* 2006; Antinori *et al.* 2007). PKDL in co-infected patients has been reported to be caused by both *L. donovani* and *L. infantum/L. chagasi*; in most cases parasites were easily demonstrated (Stark *et al.* 2006; Antinori *et al.* 2007; Singh *et al.* 2015).

Morphology and Life cycle of *Leishmania* parasite

In the Indian subcontinent, the transmission of VL is anthroponotic and chiefly transmitted through the bite of the female sandfly, *Phlebotomus (P) argentipes*. The life cycle of *Leishmania* parasite is digenetic and found in two distinct forms (a) promastigotes: these forms are extracellular, elongated, flagellated and motile, ranges in size from 2µm × 2-20 µm (**Fig 2.9A**). This form is found in sandfly (b) amastigotes:

these are intracellular, aflagellated, round to oval and non-motile and its size ranges from 2-5μm (**Fig. 2.9B**). Amastigotes reside and multiplies by simple binary fission within the phagolysosomes of macrophages in reticuloendothelial system of the host (Handman 1999). As the parasite load increases inside, resulting into the physical disruption of the infected macrophages and delivering amastigotes into the ambient tissue where they are readily engulfed by macrophages and the cycle continues (**Fig 2.10**). Eventually, all organs containing macrophages and phagocytes get infected, especially the visceral organs such as spleen, liver and bone marrow.

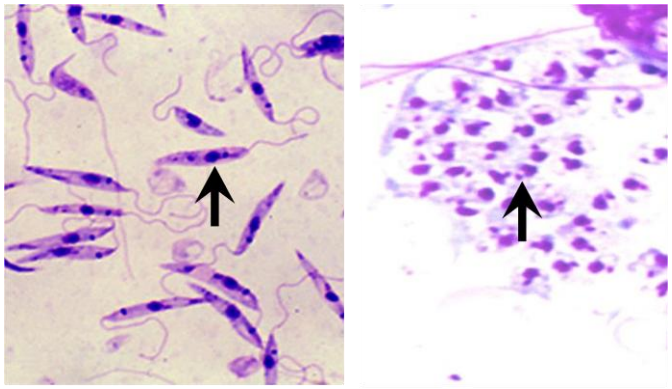

Figure 2.9: Two stages of *Leishmania* parasite; (**A**) Extracellular and the motile form called promastigotes each bearing a flagellum. (**B**) Intracellular and the non-motile stage called amastigotes (small dots) as seen in Giemsa-stained smear prepared from the spleen of *L. donovani* infected golden hamster.

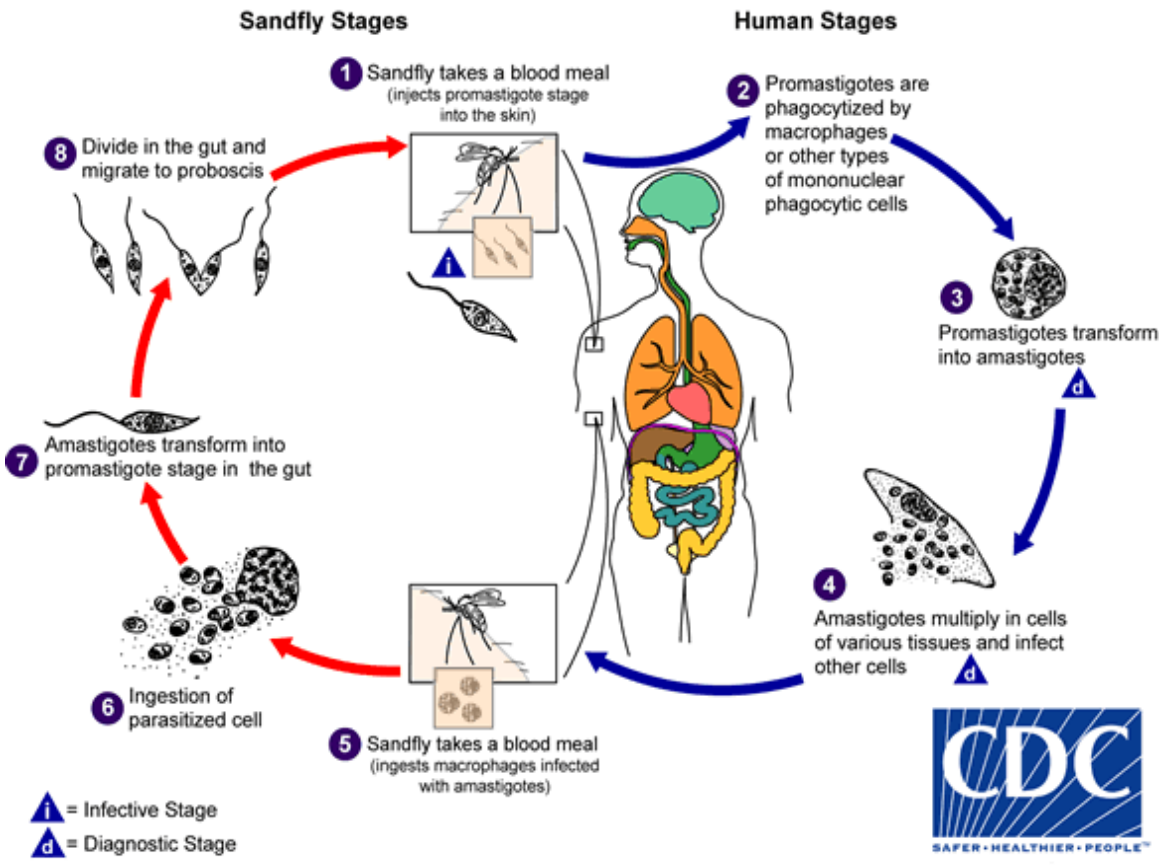

Figure 2.10: Life cycle of *Leishmania* parasite
(Source: CDC; http://www.cdc.gov/parasites/leishmaniasis/biology.html)

Leishmaniasis is transmitted by the infected female phlebotomine sandflies. The sandflies inject promastigotes during blood meals ❶. Promastigotes that reach the puncture site are phagocytosed by macrophages ❷ and other types of phagocytic cells. Inside these cells, promastigotes gets transformed into the amastigotes ❸, which divides by simple binary division and proceed to infect surrounding phagocytic cells ❹. Sandflies ingest the infected cells, while taking the blood meals, and get infected (❺, ❻). Sandflies, amastigotes get transformed to promastigotes, develop in the gut ❼ and migrate to the proboscis ❽

Vectors and *Leishmania* transmission

In the old world, VL is transmitted by the *Phlebotomus* (*P*) spp. and the vector in the new world is *Lutzomyia* spp. *P. argentipes* is the vector of VL in India (Kishore *et al.*

2006; Swaminath *et al.* 2006). There are 500 species of phlebotomine, of which 30 species of *Phlebotomus* belonging to 6 genera are the proven or suspected vectors for transmitting *Leishmania* parasites from animal to animal, animal to man, and man to man. In India, *P. argentipes* transmits the disease from man to man (Singh 2006). Sandflies are very minute in size (<3.5 mm) (**Fig 2.11**) and difficult to see them by naked eyes. They are most active in the twilight, evening and night hours from dawn to dusk. They are found in a very wide range of habitats, ranging from deserts to tropical rain forests. Female sandflies lay its eggs in the burrows of certain rodents, in cracks in house walls, in the bark of old trees, in animal shelters, in the ruined buildings, and in household rubbish.

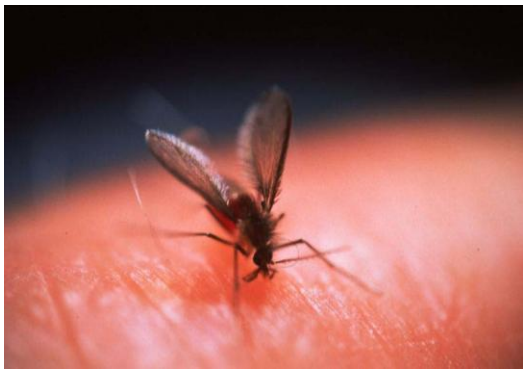

Figure 2.11: A sandfly vector of *Leishmania* parasites taking a blood meal through human skin. Source: WHO/S. Stammers

Modes of *Leishmania* transmission

The type of reservoir host and their surrounding environment determines the mode of transmission of the disease in the endemic areas. There are three distinct patterns of *Leishmania* transmission:

1. **Zoonotic transmission:** *Leishmania* parasites primarily circulate between animal host and sand flies in a primary environment and humans are only accidental hosts, e.g. *L. braziliensis* infection in Amazon forests.
2. **Anthropo-zoonotic transmission:** The parasite move between animals and humans. This mode of transmission is typically for secondary environments, e.g. *L. infantum* infection in Mediterranean region with stray and domestic dogs as the reservoir hosts.
3. **Anthroponotic transmission:** The parasites primarily circulate between humans in domestic environments, e.g. Infection with *L. donovani* in the East Africa and the Indian subcontinent.

Diagnosis of VL and PKDL

Direct demonstration of *Leishmania* amastigotes in clinical samples is the gold standard diagnostic method where tissue aspirates are feasible and microscopy and technical skills are available. The diagnostic sensitivity of spleen, bone marrow and lymph node aspirate smears is >95%, 55–97%, and 60%, respectively (Guerin *et al.* 2002; Sundar and Rai 2002; Tavares *et al.* 2003). Elsewhere, high anti-leishmanial immunoglobulin G in serum is the diagnostic standard, such as the direct agglutination tests (DAT) or other laboratory-based serological assays (Sundar and Rai 2002; Tavares *et al.* 2003; Desjeux 2004). DAT with freeze-dried antigen (Abdallah *et al.* 2004) and rapid detection of the anti-rK39 antibody with finger prick blood (Sundar *et al.* 1998) have advanced field serodiagnosis. In symptomatic patients, the sensitivity of anti-rK39 strip test is high viz 90–100% and, while specificity might vary by region, this test can be a safe substitute for invasive diagnostic procedures in Indian VL (Sundar *et al.* 2002b).

Testing urine samples for leishmanial antigen using KAtex kit is another approach (Boelaert *et al.* 2004; Sundar *et al.* 2005). In a well equipped laboratory, *Leishmania* parasites are isolated by culturing clinical samples, and the parasite DNA is detected by conventional PCR, from peripheral blood, bone marrow, serum and lesion tissue (Salotra *et al.* 2001; Tavares *et al.* 2003; Fissore *et al.* 2004). Quantitative PCR using fluorogenic probes allows the monitoring of the PCR products in a real-time i.e., during the amplification phase and in various studies it has been utilised for epidemiologic and diagnostic purposes as well as to assess parasite load in symptomatic, healed patients, and the asymptomatic cases (Selvapandiyan *et al.* 2005). It has been also found useful for assessment of parasite burden during chemotherapy in *Leishmania*/HIV-infected patients (Bossolasco *et al.* 2003; Mary *et al.* 2004).

PKDL diagnosis was initially relied only on the typical clinical presentations (macules, papules and nodules). The most common differential diagnoses are leprosy, vitiligo, sarcoidosis and secondary syphilis (Ramesh *et al.* 2015a). Its diagnosis is supported by the epidemiological background: the majority of cases report a history of VL, in a focus of anthroponotic transmission, and/or origin from an area endemic for VL. Diagnostic methods for VL generally lack specificity or sensitivity for PKDL diagnosis as (i) the parasite counts in biopsy specimens and skin smears is comparatively low, thus it requires long searches by routine microscopy (Singh 1968; el Hassan *et al.* 1992; Ghosh *et al.* 1995) (ii) serological tests may be positive due to the previous VL episode rather than the current PKDL (Haldar *et al.* 1981) however, serology can be useful when other diseases such as leprosy are considered in the differential diagnosis, or if a history of VL is not known; (iii) the leishmanin skin test (LST) may or may not be positive

(Ramesh and Mukherjee 1995) and (iv) cultures are often negative and, in the VL-endemic regions, are prone to contamination (Osman *et al.* 1998).

The parasite culture and slit skin smear examination are the two standard methods of diagnosis but have low sensitivity. In all types of PKDL, the demonstration of parasites is a bedside aid to diagnosis, and the nodular lesion is most likely to demonstrate *Leishmania* amastigotes that diminishes with less indurated skin lesions and is the lowest in the macular variant. Molecular methods utilising gene amplification techniques like PCR, is a reliable method of species-specific diagnosis. The methods have higher sensitivity than IHC or serological methods. Gene amplification is performed by targeting multicopy sequences like ribosomal RNA genes, kinetoplastid DNA (kDNA), miniexon derived RNA genes or genomic repeats (Salotra *et al.* 2002; Salotra and Singh 2006). A PCR assay based on kDNA developed in India detected the *Leishmania* parasite in 45 out of 48 PKDL cases with over 90% sensitivity (Salotra *et al.* 2001). Currently, different PCR assays are available such as QPCR, nested PCR, Loop-mediated isothermal amplification (LAMP) PCR, etc. that can detect *Leishmania* DNA in blood or on slit skin aspirates (Verma *et al.* 2013b). The LAMP assay in the diagnosis of PKDL was demonstrated to be rapid and reliable, with a sensitivity of 96.8% and specificity of 98.5% (Verma *et al.* 2013a). LAMP PCR has many advantages such as an easier to run kit is available, less sophisticated equipment, the temperature for DNA amplification is lower (62-65ºC), and the test run time is shorter (one hour). Also, the reading of test result is easy (color change and turbidity). However, it is difficult to decentralize the test further than district level. Parasitological confirmation using minimally invasive skin slit aspirate sample demonstrated to have equally reliable results

as with tissue biopsy (Verma *et al.* 2013b) and, therefore, should be encouraged, as the procedure will reduce discomfort and permanent scarring and thus motivate more patients to come forward for timely diagnosis and monitoring after treatment.

The main limitation is that the most of the molecular tests are unavailable or not performed in local primary health centres. The diagnosis of PKDL at primary health centres when *Leishmania* amastigotes are not seen often rests on the clinical manifestations and the response to anti-leishmanial therapy. In Africa, PKDL occurs frequently during or soon following treatment of VL, making the diagnosis comparatively easy and with low chance to be mistaken with other dermatoses, unlike the cases of PKDL from Indian subcontinent.

Treatment of VL and PKDL

For the past many decades, pentavalent antimonials has been the first line drug for VL and other drugs such as amphotericin B (AmB), miltefosine, and paromomycin remained as second-line therapies. During the 1980s, VL cases from Bihar, India developed resistance to pentavalent antimonials and isolates of *Leishmania* were found resistant to the drug (Croft *et al.* 2006; Singh *et al.* 2006). As a result, several formulations such as amphotericin B, miltefosine and paromomycin have been approved for treatment in the last decade. Amphotericin B has been the first-line treatment for VL in certain parts of Bihar where refractory to antimonials treatment reached >60% (Sundar *et al.* 2000). Amphotericin B, an antibiotic with efficient anti-leishmanial activity, was brought back in India for the treatment of refractory VL (Sundar and Rai 2005). AmB is administered at a dose of 0.75–1 mg/kg for 15–20 i.v. infusions either daily or on alternate days. Although it has shown high cure rates (>95%) in VL patients in India

(Sundar and Rai 2005), its adverse side effects such as high fever and rigors are common, besides, nephrotoxicity, hypokalemia, myocarditis are other uncommon adverse effects (Chappuis *et al.* 2007). Therefore, AmB treatment requires hospitalization and close monitoring for 4–5 weeks which escalates the treatment cost (Thakur *et al.* 1999). To overcome this, a highly effective lipid formulation of AmB (L-AmB) has been developed. This particular formulation leads to targeted drug delivery to macrophages of the affected organs such as spleen, liver and bone marrow without compromising the efficacy of the drug. These formulations have been successfully tested against VL in India, Brazil, Kenya and Europe, where HIV-co-infected cases were also included (Agrawal *et al.* 2005). L-AmB was administered in a single dose of 5mg and 7.5mg/kg with a cure rate (CR) of 91% and 90%, respectively (Sundar *et al.* 2001, 2010). Considering the preferential pricing and a single day of hospitalization, WHO has recommended single dose (10mg/kg) of L-AmB as the most suitable treatment regimen for VL in the Indian subcontinent (Sundar *et al.* 2003; WHO 2010).

Miltefosine, originally developed as an anti-tumor agent, is the first oral anti-leishmanial drug approved for treatment of VL in India way back in 2002. However, in Europe, it was primarily used for the treatment of VL-HIV co-infected cases, especially in those patients who are unresponsive to other treatments (Sindermann *et al.* 2004). It is used at a dose of 50–100 mg/day for 28 days and resulted in a long-term CR of 94% (Sundar *et al.* 2002a). The teratogenic potential of this drug requires that it should be used with caution in women of childbearing age. Its ease of use and applicability has shown remarkable results in containing *Leishmania* infection in the Indian subcontinent. However, after a decade of its use in the Indian subcontinent, the relapse rate got

doubled, and its efficacy declined (Ramesh *et al.* 2015b). Paromomycin is another antibiotic which has shown good anti-leishmanial activity. Sunder et al. demonstrated that Paromomycin sulfate equally effective as amphotericin B for the VL treatment in India and subsequently the drug was approved for the treatment of VL cases at a dose of 15 mg/kg for 21 days (Sundar *et al.* 2007). Sitamaquine is an 8-aminoquinoline drug and the study conducted in India obtained CR of 87%, but its application showed serious renal adverse effects (Jha *et al.* 2005). Presently, the combination therapy looks the only way to enhance treatment efficacy, lower the development of drug resistance, curtail treatment duration and perhaps make treatment cost-effective. Currently, the recommended treatment guidelines for VL in the Indian subcontinent region is either a single dose of 10 mg/kg of L-AmB or the combination of miltefosine and paromomycin each for 10 days (Sundar *et al.* 2010, 2011; WHO 2010). However, the combination of paromomycin with sodium stibogluconate (SSG) for 17 days is the recommended treatment in East Africa and Yemen, whereas a dose of 18–21 mg/kg L-AmB is the treatment of choice in the Middle East, Central Asia, Mediterranean Basin and South America (WHO 2010; Sundar and Chakravarty 2013).

In East Africa, the majority of PKDL lesions heal spontaneously within a year, so generally, treatment is not warranted. As there are no reports of antimony resistance in Sudan, sodium stibogluconate (SSG) is used to treat cases where the disease persisted for a year. In such cases, SSG (20mg/kg/day) for up to 2 months or a 20-day course of L-AmB at 2.5 mg/kg/day are the recommended regimen (Zijlstra *et al.* 2003; WHO 2010). As spontaneous healing is not a feature of Indian PKDL, its management requires long and stringent treatment regimen. In India, the recommended treatment options for PKDL

includes 60–80 doses AmB at 1 mg/kg for over 4 months or miltefosine for 3 months but the compliance is low (WHO 2010). Similar to the antimony era where *Leishmania* parasites showed a gradual increase in tolerance and resistance, miltefosine resistance has also been reported (Ramesh *et al.* 2015a). This calls for formulations of newer and effective oral drugs which can be used singly or in combination to combat resistance.

Host-pathogen Interaction

Leishmaniasis is an ideal example of complex host-parasite interaction. Upon infection, both polymorphonuclear neutrophils (PMNs) and macrophages migrate to the infection site and their interaction with the pathogens greatly influences the overall outcome of disease (Ribeiro-Gomes *et al.* 2004, 2007). However, a few studies suggested that comparatively more number of neutrophils are recruited at the infection site and phagocytose the parasite (Peters *et al.* 2008; Peters and Sacks 2009). Since neutrophils have a shorter lifespan, they serve as intermediate host and are considered to act as "Trojan horses" and facilitate silent entry into macrophages thereby avoids cell activation (Laufs *et al.* 2002; van Zandbergen *et al.* 2004). In fact, PMNs infected with *L. major* tends to delay their apoptosis and also secrets high levels of MIP-1β, a chemoattractant, that attract macrophages to the site of infection (van Zandbergen *et al.* 2004). Subsequently, the recruited macrophages phagocytose free parasites, as well as the infected/apoptotic PMNs and become the final destination for parasite multiplication as well as the effector cells responsible for the clearing parasites from the host.

The uptake of promastigotes by the host cells is a receptor-mediated process that initiates phagocytosis. Surface molecules of both parasite and macrophage have key roles in interaction between *Leishmania* and macrophages. For promastigotes, the complement

receptors 1, Mac-1, mannose-fucose receptor, and the fibronectin receptor present on macrophage surface play important roles in promastigote binding/attachment (Kane and Mosser 2000). However, ligation of the individual receptor does not activate macrophages, indicating multiple receptor ligations may be important for initiating the appropriate immune responses (Aderem and Underhill 1999). Further, the metacyclic promastigotes of *L. infantum/chagasi* use Mac-1 but not mannose-fucose receptor to enter macrophages, while the avirulent promastigotes use both these receptors to enter the cells. In fact, Mac-1 ligation by itself does not trigger respiratory burst through the activation of NADPH oxidase (Sehgal *et al.* 1993); in contrast, mannose-fucose receptor ligation has been demonstrated to induce inflammatory responses (Linehan *et al.* 2000). This is how metacyclic promastigotes avoid using detrimental mannose-fucose receptor route during their invasion of macrophages and enhance their chance of intracellular survival.

Additionally, the surface molecules such as lipophosphoglycan (LPG), GP63, and proteophosphoglycan (PPG) present on promastigote forms have been implicated in the initiation of phagocytosis and the parasite survival inside the host cells (Yao *et al.* 2003; Naderer and McConville 2007). These molecules serve as target sites for various opsonins such as mannose-binding protein (Green *et al.* 1994), complement component C3 (Brittingham and Mosser 1996) and galectins (Pelletier *et al.* 2003; Kamhawi *et al.* 2004). Also, the absence of a single surface molecule is not detrimental for attachment, as phagocytosis of LPG mutant parasites has demonstrated to be even better when compared to their wild counterparts (McNeely and Turco 1990). However, it significantly influences survival of these LPG mutant parasites (McNeely and Turco 1990), indicating

the molecules are important for subverting and inhibiting the host killing machinery. Once internalized into macrophage, promastigotes get transformed into aflagellated amastigotes. The *Leishmania* amastigotes replicate by simple binary fission, eventually rupturing macrophage and disseminating to other healthy cells. The internalization mechanism of amastigotes into the macrophages is not well defined. It has been shown that host IgG-coated *Leishmania* parasites could bind to Fc receptors present on macrophages, facilitating their uptake. This interaction and the subsequent entry into macrophages induce signaling pathways that inhibit killing and promote intracellular parasite survival (Miles *et al.* 2005).

Pathogenesis and Immune responses in VL

The pathology of *Leishmania* infection is determined not only by the causative *Leishmania* species but also by host immune and genetic factors (Saha *et al.* 2011; Choudhury *et al.* 2013). In VL-endemic areas, majority of the infected people with *Leishmania* parasites do not manifest clinical disease and develop an effective immune response. This indicates how the host genetic makeup influences the development of this disease (Jamieson *et al.* 2007; Jeronimo *et al.* 2007) in particular, natural resistance associated with macrophage protein1 (NRAMP1) plays an important role in susceptibility to VL (Bucheton *et al.* 2003). It is a cation transporter which effluxes out divalent cations such as iron from phagosome to the cytosol and creates ions deprived condition for pathogenic microorganisms. Besides, a number of other genes pertaining to cytokines, chemokines and their receptors, TNF-α (Karplus *et al.* 2002); IL4 (Mohamed *et al.* 2003); TGF-β (Frade *et al.* 2011); IL2 receptor (Bucheton *et al.* 2007); CXCR2 (Mehrotra *et al.* 2011), as well as mannan-binding lectin (Alonso *et al.* 2007) and the

Delta-like 1 ligand for Notch 3 (DLL1) (Mehrotra *et al.* 2012), have also been associated with the disease. However, it is not well known at what length polymorphism in these host immune response genes implicates to cause VL in human population.

Host immune responses play a pivotal role in the control and cure of infection as well as resistance to re-infection. Most of the experimental immunological data are from mouse models and the knowledge regarding immunopathology of human leishmaniasis is limited. *Leishmania* infection in human is characterized by the appearance of anti-leishmanial antibodies in the patients' sera. Strong anti-leishmanial antibody titers are observed in active VL (Neogy *et al.* 1987). Although it is known that Th1 cytokine, IFN-γ possibly upregulates isotypes IgG1 and IgG3 while Th2 cytokines, IL-4 and IL-5 stimulates the production of IgG4 in human (Abbas *et al.* 1996), the role of anti-leishmanial antibodies in VL patients towards protection or pathogenesis is still unclear. Previous reports demonstrated the role of IgG in aggravating *Leishmania* infection (Kima *et al.* 2000; Miles *et al.* 2005) via the formation of immune complexes. Binding of host IgG to the surface of amastigotes facilitate ligation of Fcγ receptors on macrophages. Subsequently, the macrophages secrete a high level of IL-10, which makes macrophages unresponsive to the protective effects of IFN-γ (Kane and Mosser 2001). It is known that activated T cells induce class switching of B cells to different IgG isotypes and are thus obligatory for humoral responses. In murine models, Th2/IL-4/IgG1 has been shown to be responsible for susceptibility to *Leishmania* infection (Bretscher *et al.* 1992; Afrin and Ali 1998). On the other hand, IgG1 and IgG3 classes of antibodies are known to possess complement-fixing and opsonizing activities (Burton *et al.*1986). Analysis of anti-*Leishmania* antibodies in sera of VL patients revealed raised levels of IgG, IgM and IgE

during the active phase of the disease (Ghosh *et al.* 1995). A successful cure corresponds to a decline, most significantly, in levels of IgE, IgG4 and IgG1 (Atta *et al.* 1998; da Matta *et al.* 2000). In paediatric VL, the anti-leishmanial antibody levels of subclass IgG3 and IgG4 have been shown to be significantly elevated (Ansari *et al.* 2008b).

The cellular immunity in active VL exhibit poor lymphoproliferative response upon stimulation with *Leishmania* antigens *in vitro* and fail to generate IFN-γ (Haldar *et al.* 1983). The lack of IFN-γ secretion by PBMCs likely to predict progression of the infection into fulminant VL (Carvalho *et al.* 1985, 1995). Additionally, VL cases test negative to leishmanin skin test during the active phase of the disease. However, the remarkable response is observed after 6–12 months following successful treatment (Reiner and Locksley 1995; Costa *et al.* 1999). On the other hand, lymphocytes from individuals cured of VL or with asymptomatic infection demonstrate a strong lymphoproliferative response and readily produce pro-inflammatory cytokines such as IFN-γ, IL-2 and IL-12 upon stimulation with *Leishmania* antigens *in vitro* (Carvalho *et al.* 1994). Therefore, both spontaneous and drug-induced cure is followed by protective cellular immunity (Cillari *et al.* 1995). In VL, despite the presence of high level of IFN-γ, the host fails to control *Leishmania* infection and could be due to the impaired response of IFN-γ-induced signaling mechanisms (Nandan and Reiner 1995; Hailu *et al.* 2004; Goto and Prianti 2009). The study by Dasgupta *et al* revealed the reason behind and demonstrated that there is a low expression of IFN-γ receptor-1 (IFN-γ R1) in mononuclear cells of VL patients which get restored following treatment (Dasgupta *et al.* 2003). Early and sustained secretion of IL-4 has been associated with Th2 responses in *Leishmania* infection. Further, high IL-4 and/or IL-13 levels have been associated with

active VL (Sundar *et al.* 1997; Nylén *et al.* 2007). However, there is elevated secretion of various cytokines and chemokines in VL patients, and most of the immunological response appears to be pro-inflammatory as indicated by the raised plasma protein levels of IL-1, IL-6, IL-8, IL-12, IL-15, IFN-γ-inducible protein-10 (IP-10), monokine induced by IFN-γ (MIG), IFN-γ and TNF-α (Ansari *et al.* 2006; Kurkjian *et al.* 2006). VL patients have raised levels of IL-10 in serum and high IL-10 mRNA expression in lesional tissue (Kenney *et al.* 1998; Ansari *et al.* 2006). Additionally, the study from our lab has demonstrated a strong correlation between parasite load and the level of IL-10, indicating IL-10 as a marker of disease severity (Verma *et al.* 2010).

Th17 and T regulatory (Treg) cells are now widely accepted subsets of T cells, with vital roles in induction and control of the inflammatory immune response. Both Treg and Th17 have a higher degree of plasticity in their differentiation decision as compared to Th1 and Th2 subsets, enabling an appropriate response to signals provided by their surrounding environment. Th17 cells, an independently regulated and pro-inflammatory subset of $CD4^+$ T lymphocytes, are known to secrete IL-17 and IL-22 (Bending *et al.* 2011), effectors of innate immunity. The stimulation of Th17 cells is characterized by neutrophils recruitment and, depending on their differentiation program, they may have either pathological or protective roles (Marwaha *et al.* 2012). Th17 cells have a protective role in human VL (Pitta *et al.* 2009) while they have been implicated in pathology and tissue necrosis in human MCL (Boaventura *et al.* 2010). Another immune cell, $CD4^+CD25^+$ T cells have been associated with both VL and PKDL (Saha *et al.* 2007) and the $FoxP3^-CD4^+$ T cells were demonstrated to be the important source of IL-10 mRNA expression in the spleen of VL patients (Nylén *et al.* 2007). Rai *et al*. indicated that

$CD4^+CD25^+FoxP3^+$ cells could play an important role in human VL as a key source of IL-10 (Rai *et al.* 2012). Recently, $CD4^+CD25^+FoxP3^+$ Treg cells have been shown to be the direct source of IL-10 and TGF-β during active VL (Bhattacharya *et al.* 2016), and this finding could open new avenues for immunotherapy of human VL.

Pathogenesis and immune responses in PKDL

The precise understanding of PKDL pathogenesis is still obscure and, importantly, the immunopathology varies between Sudanese and Indian PKDL. PKDL is considered to be immunological triggered, with the host, parasite, and drug, all perhaps contributing to the pathogenesis. Host's immunological characteristics seem to be important for the onset of PKDL. The host's role is implicated, based on: (1) relatively high occurrence of PKDL in immunocompromised individuals, (2) therapeutic efficacy of vaccines with high and extended immunogenicity, and (3) conversion of Leishmanin skin test (LST) associated with clinical cure from negative to positive (Musa *et al.* 2008).

Till now, no parasite strain has been implicated with either VL or PKDL in the endemic areas (Dey and Singh 2007; Subba Raju *et al.* 2008). Molecular studies on genetic typing revealed monomorphism between *L. donovani* isolates from VL and PKDL cases in India (Subba Raju *et al.* 2012). Further, the incidence of PKDL is much higher in areas endemic for *L. donovani* than in *L. infantum.* This higher incidence could be related to the parasite strain and/or to the epidemiology or due to anthroponotic transmission in *L. donovani* foci compared to the zoonotic transmission in *L. infantum* (WHO 2012; Bhattacharya and Dash 2016).

Drug characteristics may play role in PKDL onset. However, nearly 20% PKDL cases report no history of VL (Das *et al.* 2012; Desjeux *et al.* 2013; Ramesh *et al.* 2015a)

and, consequently, prior to receiving any drug for VL treatment. Majority of the PKDL cases reported so far have been treated with antimonials. Recent studies reported decline in the incidence of PKDL after the introduction of amphotericin B for VL treatment (Croft 2008; Thakur *et al.* 2008). It is well established that any anti-leishmanial drug can lead to PKDL but with varying proportions. PKDL after VL treatment with SSG are reported more frequently than with amphotericin B; however this may be because SSG has been the most commonly and extensively used drug over many decades. In recent times, PKDL cases have been reported after using miltefosine, amphotericin B and paromomycin for VL treatment (Das *et al.* 2009; Kumar *et al.* 2009; Pandey *et al.* 2012).

In South Asian cases of PKDL, one of the important effector cell implicated in pathogenesis are $CD3^+CD8^+$ T cells that have been found both in lesions and circulation (Rathi *et al.* 2005; Ganguly *et al.* 2008). The observation of high mRNA expression of FoxP3, CTLA-4, and CD25 at lesion site suggested the involvement of Treg cells (Ganguly *et al.* 2010b; Katara *et al.* 2011). Besides, an increased IL-10-expressing $CD3^+CD8^+$ T lymphocytes have been observed in circulation which gets restored to normal after the treatment (Ganguly *et al.* 2008). The cytokine profile at the lesion site shows enhanced expression of IL-10, TGF-β, IFN-γ, and TNF-α in both South Asian and Sudanese PKDL. However, the expression of IFN-γR and TNFR1 was lower in Indian PKDL which was restored following treatment (Ansari *et al.* 2006; Ansari *et al.* 2008a). Similar observations were made in Sudanese PKDL, where a genetic polymorphism was found in IFN-γR (Salih *et al.* 2007). In Sudanese PKDL, expression of IL-10 was considered as an important predictor of the onset of PKDL, particularly following VL (Zijlstra *et al.* 2003). Furthermore, high levels of IL-17, its transcription factor ROR-γt,

and IL-22, both in lesions and circulation indicated the involvement of Th17 cells in PKDL pathogenesis (Katara *et al.* 2012). The patients with PKDL in India demonstrated to have raised levels of IgG3 and IgG1, concomitant with increased serum levels of IL-10 (Ganguly *et al.* 2008). Their data further indicated the subclass order as IgG3> IgG1> IgG2> IgG4 in Indian PKDL (Ganguly *et al.* 2008). Furthermore, the polymorphic variant of PKDL has elevated IgG1 and IgG3 while PKDL with macular lesion showed only elevated IgG1 level; upon therapy, the levels of IgG1, IgG2 and IgG3 decreased only in polymorphic PKDL; whereas the level of IgE remains high in both groups but no marked difference occurred after treatment (Mukhopadhyay *et al.* 2012).

Vaccines for Leishmaniasis

Most of the individuals living in VL-endemic areas, either with subclinical infection or cured of VL, acquire immunity to re-infection from the same species (Nagill and Kaur 2011; Beaumier *et al.* 2013). This observation supports the possibility of a vaccine against leishmaniasis. Further evidence of the biological feasibility of vaccine development is indicated by an ancient practice termed "leishmanization" (Duthie *et al.* 2012; Beaumier *et al.* 2013). The development of an anti-*Leishmania* vaccine has been a long-term goal for both veterinary and human medicine. Admittedly, no vaccine is available against any form of human leishmaniasis though several vaccine formulations are in advanced stages of clinical trials.

Historically, CL has been the focus of vaccination attempts, possibly because it has been known since ancient times that those who recovered from skin lesions were protected from re-infections. Traditionally, some Kurdistani or Bedouin tribal societies exposed their babies' bottoms to sandfly bites to provide protection from facial lesions.

Another ancient practice is known as "leishmanization" practised in the Middle East and Central Asia where people were "vaccinated" using thorns or sharp tools to inoculate live parasites in an unexposed body part to prevent the risk of facial lesions through natural infection (Handman 2001).

Several attempts to develop anti-*Leishmania* vaccines based on whole, killed parasites were made during 1970s and 1980s. Subsequent trials with inactivated whole parasite vaccines were conducted in Ecuador (contained 3 strains of local parasites), Colombia (Biobras single strain *L. amazonensis*), Iran and Sudan (autoclaved *L. major* + BCG as an adjuvant: ALM + BCG) (Bahar *et al.* 1996; Sharifi *et al.* 1998; Momeni *et al.* 1999; Khalil *et al.* 2000; Armijos *et al.* 2003; Vélez *et al.* 2005). Except for the Ecuadorian trial, where a local vaccine was used, none of the trials demonstrated requisite protection (Noazin *et al.* 2009). Overall, the vaccine strategy involving the use of crude preparations cannot be optimally formulated to elicit effective immune responses and the results of clinical trials have been found inconsistent.

Vaccination strategies using attenuated *Leishmania* parasites are other attractive approach as they closely simulate the natural course of infection and elicit clinically protective immune responses. The use of attenuated vaccines is a promising vaccination strategy for leishmaniasis; however safety issues associated with their use as vaccines still need to be addressed. A few good prospects in the developments of attenuated vaccines include centrin and biopterin transporter knockout parasites. A loss of centrin from promastigotes forms did not affect its growth however these mutants were unable to survive as axenic amastigotes or in macrophages *in vitro* (Selvapandiyan *et al.* 2006) while the lack of biopterin transporter (BT1) showed lower infectivity (Papadopoulou *et*

al. 2002). Another approach has utilised non-pathogenic *Leishmania* species, similar to the use of BCG as a vaccine against tuberculosis infection (Azizi *et al.* 2009). A continuing synergy between immunological and molecular approaches will accelerate development of a sustainable vaccine for VL.

The second generation vaccine approach includes the use of the recombinant proteins with a wide range of adjuvants to enhance the immunogenicity of the potential vaccine antigens (Duthie *et al.* 2012; Beaumier *et al.* 2013). The production of these recombinant antigens can be made cost-effective, large-scale and reproducible. Further, the immune responses elicited by recombinant proteins can be potentiated and refined by formulation with appropriate adjuvant (Reed *et al.* 2009; Duthie *et al.* 2011). Several second generation vaccine candidates have been tested in murine and canine models with encouraging results, indicating these defined proteins can achieve protection against leishmaniasis. The fucose mannose ligand (FML) which is expressed throughout the life cycle of the parasite, in formulation with saponin, have been shown to be protective and safe in mouse and hamster models (Palatnik-de-Sousa *et al.* 1994; Santos *et al.* 2003). This formulation has now been licensed for veterinary use as Leishmune® following a series of canine VL field studies (da Silva *et al.* 2000; Parra *et al.* 2007). Another canine vaccine came into existence for use was Leish-Tec®. It is composed of recombinant A2-antigen of *Leishmania* amastigotes with saponin (Fernandes *et al.* 2008). The excretory/secretory proteins isolated from culture supernatants of *L. infantum* with MDP were tested in dogs experimentally infected with *L. infantum* (Lemesre *et al.* 2005). This vaccine formulation, *Li*ESAp-MDP, elicited significant, long-lasting protection against canine VL in a field trial in France with naturally infected dogs (Lemesre *et al.* 2007).

The *Li*ESAp-MDP formulation has now been licensed for commercial use under the name CaniLeish® for canine VL in Europe.

In recent times, there have been significant thrusts on identifying newer recombinant antigens that can provide protection against *Leishmania* infection in experimental models. Some antigens include kinetoplastid membrane protein (KMP)-11 (Carrillo *et al.* 2008; Agallou *et al.* 2011), sterol 24-c-methyltranferase (Goto *et al.* 2007), amastigote specific protein A2 (Ghosh *et al.* 2001), cysteine proteinase B (Rafati *et al.* 2006), *L. braziliensis* elongation and initiation factor (LeIF) (Skeiky *et al.* 1998), K26/HASPB (Stäger *et al.* 2000), *Leishmania*-activated C kinase (LACK) (Benhnini *et al.* 2009), promastigote surface antigen 2 (PSA-2) (Handman *et al.* 1995b), nucleoside hydrolase (Al-Wabel *et al.* 2007) and glycoprotein gp63 (Connell *et al.* 1993). Although most of these antigens have been evaluated for their immunogenicity and protective efficacy in animal models, very few have progressed to clinical trials in dogs, non-human primates or human preclinical studies (Kumar *et al.* 2010; Singh *et al.* 2012). A multisubunit recombinant *Leishmania* vaccine, Leish-111F, with an *L. major* thiol-specific antioxidant, *L. major* stress-inducible protein-1 and *L. braziliensis* elongation and initiation factor, with MPL-SE, has been demonstrated to be protective in mouse models of VL and CL (Skeiky *et al.* 2002; Coler *et al.* 2007). However, Leish-111F did not provide protection against canine VL caused by *L. infantum* in the field (Gradoni *et al.* 2005). Nevertheless, Leish-111F/MPL-SE formulation became the first second-generation vaccine to reach phase-I and phase-II clinical trials in patients with CL and ML in Brazil and Peru, healthy volunteers in South America, and individuals cured of VL

in India (Vélez *et al.* 2009; Llanos-Cuentas *et al.* 2010; Nascimento *et al.* 2010; Chakravarty *et al.* 2011).

Another biomolecule that could be utilized to develop a vaccine candidate is the parasite DNA. It has been extensively utilized as a vaccine delivery system, which opened a new arena of vaccinology. Here, target genes encoding the proteins are cloned into a mammalian expression vector, and injected either intradermally or intramuscularly to induce the Th1 responses, leading to a strong and protective cytotoxic T-cell responses. This approach is known for a while (Tang *et al.* 1992), and has several advantages, such as cost-effectiveness, the higher shelve life, sustained expression of the desired antigens and efficient generation of effective immune responses (Donnelly *et al.* 1997). Also, more than one antigen can be expressed by a single construct. The CpG motif of bacterial DNA provides the additional advantage of inducing an innate immune response to secrete IL-12, that can prime $CD4^+$ T cells to develop into Th1 cells (Gurunathan *et al.* 2000).

Challenges for sustainable elimination of VL

In South East Asia, the transmission of VL is known to be anthroponotic; therefore, PKDL has a major role as a reservoir of infection, especially during an inter-epidemic period (Addy and Nandy 1992; WHO 2010). In 1988, one study concluded that the presence of even as few as 0.5 % PKDL patients during an epidemic may cause VL to become endemic (Dye and Wolpert 1988). Another challenge is the perception of PKDL patients regarding the disease itself. They consider it as a durable, non-fatal, and problematic only if the lesions are of nodular variant. Therefore, they do not seek early diagnosis and treatment. The duration between the onset of clinical symptoms of PKDL

and the treatment is generally long that increases the risk of VL transmission to individuals residing in the same household or neighborhood. The other challenge is of PKDL diagnosis: The diagnosis of PKDL is intriguing and the disease is often misdiagnosed especially at primary health centres and/or private clinics, primarily as leprosy, a co-endemic dermatosis with high prevalence in the same areas. In addition, the treatment is long, costly, and frequently toxic. Increasing incidence of drug resistance is yet another impediment towards VL elimination.

The presence of asymptomatic VL cases in the endemic areas could be an important impediment towards VL elimination. A majority of the *Leishmania*-infected human population do not develop into full blown VL cases, are asymptomatic VL (Das *et al.* 2011; Ostyn *et al.* 2011; Stauch *et al.* 2011) and these cases could play a vital role in maintaining transmission dynamics of *Leishmania* infection (Sharma *et al.* 2000). However, the actual estimate of asymptomatic cases in the endemic area is difficult to assess. A few studies have reported the presence of asymptomatic cases in high endemic areas of VL in Bihar in the range of 10 to 34% (Topno *et al.* 2010; Das *et al.* 2011; Sudarshan *et al.* 2014) and the conversion rate to symptomatic VL was 17.85 per 1000 persons (Topno *et al.* 2010). It is imperative to shift focus on these "asymptomatic carriers" along with patients with PKDL or they might prove an important impediment towards VL elimination program.

Gaps in existing research

At present, there is no licensed vaccine available against any form of human leishmaniasis although several anti-leishmanial vaccine preparations have been tested in various experimental models with varying degrees of success. In 2011, an efficient canine vaccine under the trade name CaniLeish® was licensed in France. This was based upon *Li*ESAp, a crude excreted-secreted antigen obtained from promastigote culture supernatant of *Leishmania infantum*, formulated with MDP. It has been demonstrated to induce a long lasting Th1-mediated protection against both experimental and natural canine VL. Later, PSA protein was shown to be the active constituent of *Li*ESAp and also, the sera of dogs vaccinated with *Li*ESAp and MDP, recognized immunodominant antigens belonging to PSA protein family, from *L. infantum* ESA (*Li*PSA-54S) and from *L. amazonensis* ESA (*La*PSA-38S). Therefore, it became important to assess PSA protein for its potential use as a vaccine candidate against human VL.

Besides, in the VL-endemic areas, a majority of the *Leishmania*-infected human population does not develop into full-blown VL and are called asymptomatic VL. For the sustainable elimination of VL, these asymptomatic VL cases might prove an important hurdle as they could play a vital role in maintaining transmission dynamics of *Leishmania* infection in the region. Therefore, it is imperative to take up studies on asymptomatic VL, particularly, to understand the magnitude of *Leishmania* infection in VL-endemic areas. In this context, there are a few studies reported from Bihar however, none has been reported from adjoining VL-endemic state of West Bengal. This information will be useful to take appropriate health measures for the sustainable elimination of VL.

Aims and Objectives

The aim of the study is to evaluate the recombinant native form of PSA protein for its ability to induce cellular and humoral immune responses in active cases of VL and PKDL, healed VL (HVL) and naïve individuals, in order to assess its potential use as a vaccine candidate against human VL, caused by *L. donovani*. This PSA is derived from *L. amazonensis* and expressed in eukaryotic *L. tarentolae* expression system to obtain the recombinant native form of *L. amazonensis* PSA (*La*PSA) protein. Besides, the other goal of the study is to understand the magnitude of asymptomatic *Leishmania* infection in endemic zones of the state of West Bengal, India, by serological and molecular analysis of *Leishmania* parasites in blood samples of healthy individuals.

Specific Objectives

1. **Evaluation of cellular and humoral immune responses to recombinant *La*PSA in cases of VL, PKDL and healed VL**

In order to investigate the ability of recombinant *La*PSA antigen as a potential vaccine candidate against human VL, both humoral and cellular immune responses will be evaluated in cases of VL, PKDL, HVL and naïve individuals. The qualitative humoral responses to recombinant *La*PSA and Total Soluble *Leishmania* Antigen (TSLA) will be explored on sera from the different study groups. The specific cellular immune response will be determined by *in vitro* stimulation of PBMCs with parasite antigens (*La*PSA & TSLA), initially by the lymphoproliferative assay. The culture supernatant will be examined for cytokine profile and granzyme B level using flow cytometry. Also, using $CD69^+$ as a marker of activation, the percentage of activated $CD4^+$ and $CD8^+$ T cell populations upon *in vitro* TSLA and *La*PSA stimulation will be evaluated in different study groups. Further, to characterise generalised cellular immunity, the phenotype and

proportion of the different lymphoid subsets in peripheral blood will be examined in different groups under study using flow cytometry.

2. **Serological and molecular analysis of asymptomatic *Leishmania* infection in VL-endemic regions of West Bengal, India**

This study will help us to understand the magnitude of asymptomatic *Leishmania* infection in VL-endemic regions of West Bengal, India by the deployment of combined molecular (quantitative PCR) and serological (rK39 strip test) methods. The study will effectively estimate the asymptomatic *Leishmania* infection in healthy individuals living in endemic regions, and will contribute to early case detection and treatment.

Evaluation of cellular and humoral immune responses to recombinant LaPSA in cases of VL, PKDL and HVL

Introduction

Recovery from visceral leishmaniasis (VL) is generally associated with the development of a life-long immunity to re-infection. Moreover, a large majority of immunocompetent exposed individuals develop asymptomatic or subclinical infections rather than a severe form of leishmaniasis (symptomatic) and acquire a robust and durable immunity to re-infection. Resistance to infection is based on a Th1 dominant response with interferon (IFN)-γ production resulting in macrophage activation and parasite killing (Sacks and Noben-Trauth 2002; Mansueto *et al.* 2007; Nylén and Gautam 2010). In healed individuals, it has been shown that recovery and resistance to re-infection also correlate with the development of antigen-specific Th1 cell responses and IFN-γ production (Carvalho *et al.* 1995; Kharazmi *et al.* 1999; Sassi *et al.* 1999; Ajdary *et al.* 2000). Based on these data, one vaccine strategy developed against *Leishmania* infection has focused on the use of Th1 responses elicited in immune individuals by defined parasite antigens as indicators of protection.

Some *Leishmania* proteins have been characterized and evidence from studies in animal models indicated that variable levels of protection can be achieved using defined protein as vaccines (Coler *et al.* 2007; Kushawaha *et al.* 2011; Singh *et al.* 2012). However, significant differences exist in the innate and adaptive immunity of mice and humans (Mestas and Hughes 2004) thus, very few candidates have progressed beyond the experimental stage and designing an effective vaccine against leishmaniasis is still a matter of research. One of the candidate vaccine antigens is the Parasite Surface Antigen (PSA), which belongs to a unique *Leishmania* family of both membrane-bound and secretory proteins. It has a specific Leucine Rich Repeats that play important role in

protein-protein interactions and pathogen recognition (Devault and Bañuls 2008). It is an abundant glycolipid-anchored protein on the surface of promastigote form of most *Leishmania* species (Murray *et al.* 1989; Lohman *et al.* 1990; Hanekamp and Langer 1991; Murray and Spithill 1991; Symons *et al.* 1994; Beetham *et al.* 1997; Jiménez-Ruiz *et al.* 1998; Devault and Bañuls 2008). Also, a strong homology has been demonstrated among various PSA proteins derived from different *Leishmania* species (Bras-Gonçalves *et al.* 2014) (Table 4.1). A water soluble form released in promastigote culture supernatants of *L. major* (Symons *et al.* 1994) and of *L. infantum* (Jiménez-Ruiz *et al.* 1998) has also been described. PSA is involved in parasite attachment and invasion of macrophages (Kedzierski *et al.* 2004). It has been reported to induce Th1-mediated protection against murine leishmaniasis when used as a vaccine (McMahon-Pratt *et al.* 1993; Handman *et al.* 1995b). PSA is also specifically recognized by Th1 cells of humans with a history of self-healing CL (Kemp *et al.* 1998) and by human sera of VL patients (Boceta *et al.* 2000). This protein is present in both promastigote and amastigote stages (Handman *et al.* 1995a; Beetham *et al.* 2003). PSA proteins are strongly over-expressed in metacyclic promastigotes suggesting an association with the virulence of the *Leishmania* parasites (Beetham *et al.* 2003). The known role of PSA proteins in resistance to complement lysis further supports this hypothesis (Lincoln *et al.* 2004). The interest for the PSA is also supported by a few studies (Lemesre *et al.* 2005, 2007; Bras-Gonçalves *et al.* 2014) demonstrating that sera of dogs vaccinated with *L. infantum* excreted/secreted antigens (ESA) in combination with MDP (muramyl dipeptide) recognized an immunodominant antigens belonging to PSA protein family, from *L. infantum* ESA (*Li*PSA-54S; GenBank under Accession number: FJ974055) and from *L.*

amazonensis ESA (*La*PSA-38S; GenBank accession number: FJ974054). More importantly, the only vaccine available in Europe for canine VL is CaniLeish® that is based on native soluble PSA as its active constituent and formulated with an adjuvant, MDP. The vaccine is now licensed for commercial use. The present study was a part of European Commission project under FP7 (RAPSODI), a collaborative study between eight organizations from five countries, with the aim of identifying a human vaccine candidate targeting various *Leishmania* species that cause CL and VL. Additionally, these proteins belong to the same unique PSA family with well-known criteria constituting the PSA family unique signature (Devault and Bañuls 2008). Thus, all PSA might be considered as highly similar regarding their protein primary structure since they correspond to the same architecture depicted by a precise domain organization which is well conserved among PSA from different *Leishmania* species including PSA from *L. donovani* (Devault and Bañuls 2008). In the present study, the recombinant native form of *L. amazonensis* PSA protein (*La*PSA), produced in a *L. tarentolae* expression system was evaluated for its ability to induce cellular and humoral immune responses in active cases of VL and healed VL (HVL), Post Kala-azar Dermal Leishmaniasis (PKDL) and naïve individuals, for its potential use as a vaccine candidate against human VL, caused by *L. donovani*. The recombinant *L. amazonensis* PSA (*La*PSA) protein was prepared at Institut pour la Recherche et le Dévelopment (IRD), Montpellier, France and distributed to all other partners.

Table .4 1: Sequence homology (identity) of *La*PSA-38S (371 aa) protein with PSA protein from different species of *Leishmania*

Name	Protein size (aa)	*Leishmania* species	Identity	GenBank acc number
GP46/M2	476	*L. Amazonensis*	79.6% (270/339)	AAA29234
GP46	417	*L. chagasi*	62.6% (256/417)	AAB62271
PSAb	386	*L. donovani*	65.3% (258/395)	AAY96325
PSA	510	*L. infantum*	52.0% (131/252)	CAA71775
Surface antigen P2	384	*L. major*	50.5% (195/386)	CAA40329
PSA2	367	*L. mexicana*	80.3% (297/371)	XP_003873184
PSA-2	626	*L. tropica*	54.7% (139/254)	AAF80491

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Materials and Methods

Study population

Patients clinically diagnosed with PKDL (n=19) and active VL (n=12), admitted between Jan 2009 and April 2012 to Department of Dermatology or Medicine, Safdarjung Hospital, New Delhi, were included in the study. Active VL was diagnosed with the clinical features such as fever, splenomegaly with or without hepatomegaly, anemia, weight loss, and pancytopenia with positive rK39 immunochromatographic strip test. VL was confirmed by microscopic analysis of *Leishmania* amastigotes in bone marrow aspirates sample, and/or with a qPCR test for *Leishmania* DNA as reported earlier (Salotra *et al.* 2001; Verma *et al.* 2010). Active PKDL patients were clinically examined for the identification of characteristic lesions such as macular, papular, nodular or mixed/polymorphic forms. They were initially subjected to rapid rK39 test and

positive cases were further examined for confirmation of PKDL by demonstration of *Leishmania* amastigotes by direct microscopy in skin tissue/slit aspirates sample, and/or with a qPCR test for *Leishmania* DNA (Verma *et al.* 2010). In addition, we recruited cured VL individuals (n=19) with at least one-year duration following the treatment of VL and they were all found positive to rK39 strip test, however, one out of 16 was PCR positive. Besides, blood samples of healthy controls (n=19) that were, negative to rK39 strip test and *Leishmania* DNA by qPCR, were included in this study. Clinical features of the study groups are described in **Table 4.2.**

Table 4.2: Clinical features of the study groups

Feature	**Naive (n=25)**	**VL (n=25)**	**HVL (n=25)**	**PKDL (n=25)**
Age, years				
Mean ± SD	26.89±4.56	36.54±14.37	31.5±9.17	29.22 ± 10.05
Median (range)	28 (18-35)	35 (17-62)	30 (19-48)	27 (18-55)
Gender, M/F	15:10	19:6	21:4	18:7
Lesion types				
Polymorphic	-	-	-	16
Monomorphic	-	-	-	9
History of VL, % (number)	-	-	-	76% (19/25)
Interval b/w cure of VL and onset of PKDL, years				
Mean ± SD	-	-	-	11.88±4.9
Median (range)	-	-	-	11 (0.5-20)

NB: PKDL, post-kala-azar dermal leishmaniasis; VL, visceral leishmaniasis; HVL, healed visceral leishmaniasis**.**

Preparation of Total Soluble *Leishmania* antigen (TSLA)

Promastigotes of *L. donovani* (MHOM/IN/80/Ldd8Cl2) were harvested in stationary phase, washed and the pellet resuspended in the lysing solution (50 mM Tris/5 mM EDTA/HCl, pH7). After three cycles of freezing/thawing, the samples were subjected to three pulses of 20 seconds at 40 W with a sonicator, at the interval of one-minute. The sample was centrifuged at 5000 × g for 20 min at 4°C, and the supernatant was collected. Protein content was estimated using Bradford method. TSLA was aliquoted and stored at −80°C until further use.

Production and purification of *La*PSA recombinant protein

Recombinant native soluble *La*PSA was prepared as described earlier (Chamakh-Ayari *et al.* 2014). Briefly, promastigotes of *L. tarentolae* were transfected with the *L. amazonensis* PSA encoding gene, cloned and cultivated in chemically defined medium (CDM/LP) to produce recombinant *La*PSA. The culture supernatant containing *L. amazonensis* excreted secreted *La*PSA-38S, released by the parasite during its growth, was recovered at the late stationary phase of growth. The only proteins contained in the medium were of parasitic origin and were found in their native conformation because they were naturally excreted-secreted by *Leishmania* parasites. Excreted secreted PSA was purified from concentrated culture supernatant by Ni-NTA affinity chromatography. We received recombinant *La*PSA as a part of collaborative work from Institut pour la Recherche et le Développement (IRD), Montpellier, France.

Lymphoproliferative assay

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from the heparinized blood samples by density sedimentation (Ficoll-PaqueTM PLUS; GE Healthcare),

washed, resuspended in complete RPMI 1640 supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml). PBMCs at concentration of 1×10^6 cells/ml were cultured in triplicate in 96-well tissue culture plates (Axygen, Union city, CA, USA) and stimulated with TSLA (10 μg/ml) or *La*PSA (10 μg/ml) or PHA-M (10 μg/ml) for 120 hrs in humidified 37°C/5% CO_2 incubator. At 104–106 hrs incubation, 20 μL BrdU labelling solution was added and the samples re-incubated in humidified 37°C/5% CO_2 incubator for another 16–18 hrs. Lymphoproliferation was evaluated by commercially available kit (BiotrakTM cell proliferation ELISA system, version 2, GE Healthcare) using ELISA method. The proliferation index (PI) was calculated as the ratio of optical density (OD) of stimulated cultures and unstimulated cultures for each sample. Cell proliferation was considered significant when PI was above cut-off (mean + 3SD).

Estimation of cytokines and granzyme B in culture supernatant

PBMCs of VL, PKDL, HVL and naive individuals were incubated with TSLA and *La*PSA for 120 hrs as described above. The tissue culture plates were centrifuged and supernatants were collected and stored at −80°C until further use. The levels of cytokines (IFN-γ, TNF-α, and IL-10) and granzyme B were analysed by utilising cytometric bead array (CBA) flex sets (BD Biosciences) and measuring fluorescence by flow cytometry according to manufacturer's recommendations. Samples were acquired on a flow cytometer, BD FACSCalibur using BD CellQuest Pro software and the data were analysed using FCAP array software (BD Biosciences). The assay sensitivity for IFN-γ, TNF-α, IL-10 and granzyme B were 1.8, 1.2, 0.13 and 4 pg/ml respectively.

Phenotyping of the activated T cell populations

Freshly isolated PBMCs (10^6 cells/ml) from VL (n=6), PKDL (n=6), HVL (n=8) and naive (n=8) individuals, were incubated with TSLA (10 µg/ml) in a culture plate for 120 hrs at 37°C. Following incubation, the cells were harvested, and surface stained with fluorochrome-conjugated antibodies to CD3-FITC, CD4-PerCP-Cy5.5, CD8-PerCP-Cy5.5, and CD69-APC, along with appropriate isotype controls for 30 minutes at 4°C. After washing, the cells were resuspended in 0.5 ml staining buffer (BD Biosciences). Samples were acquired and analyzed on BD FACSCalibur using BD CellQuest Pro software on at least 10,000 events. For analysis, lymphocytes were gated using forward and side scatter properties and the frequencies of activated $CD8^+$ and $CD4^+$ T cells were acquired on $CD3^+$ T cells. Cell viability test using 7AAD staining of a limited number of samples confirmed that the gated lymphocytes were >99% viable for all the study groups.

Phenotyping the cells from whole blood lysate

To characterize cellular immunity we analyzed the phenotype and proportion of the different lymphoid subsets (T helper, T cytotoxic, NK and B cells) in peripheral blood of each individual of the study groups. 0.1 ml of well-mixed anticoagulated whole blood was surface-stained with appropriate fluorochrome-conjugated antibodies along with their corresponding isotype controls (BD Biosciences). Three set of sample tubes were prepared, one for T cell (CD3-PerCP, CD8-PE, CD4-FITC) and other for NK cells (CD45-PerCP, CD3-FITC, CD16+56-PE) and B cell (CD45-PerCP, CD3-FITC, CD19-PE). After incubation for 30 min at 4^0C in dark, 2ml of lysing buffer was added to each tube, vortexed and incubated at room temperature for 12 minutes. 2 ml of washing solution was added to each tube. The sample tubes were centrifuged at 200 x g for 5

minutes and the supernatant was aspirated out carefully. The cell pellets were suspended in 500 µl wash buffer and vortexed. Samples were acquired and analyzed on BD FACSCalibur using BD CellQuest Pro software.

Detection of anti-leishmanial antibodies in sera

TSLA and *La*PSA-specific antibodies were determined by ELISA. Briefly, 96-well polystyrene flat-bottom ELISA plates (NUNC MaxiSorp™) were coated with 100µl of TSLA (10µg/ml) or *La*PSA (10µg/ml) in bicarbonate buffer (pH 9.0) overnight at 4^{o}C. The plates were washed and blocked with 1% bovine serum albumin (BSA) for 2h at 37^{o}C and washed thrice with PBS containing 0.1% Tween 20 (PBST). The plates were incubated for 2h with sera (1:100) from active VL, PKDL, HVL or healthy controls. Wells were washed thrice with PBST and incubated with horseradish peroxidase-conjugated anti-human IgG (1:5000), IgG1, IgG3 (1:200), IgG2 (1:500), IgG4 (1:400) and IgM (1:800) for TSLA and IgG (1:5000), IgM (1:400) for *La*PSA for 2h at 37^{o}C. Plates were washed and Ortho-phenylene diamine substrate (SIGMA FAST OPD - ready-to-use substrate) was added to produce the color. The reaction was stopped on adding 1N H_2SO_4 and the absorbance was read at 492 nm in an ELISA reader (Tecan). Each reaction was carried out in triplicate.

Ethics statement

The study was approved by and carried out under the guidelines of the Ethical Committee, VMMC & Safdarjung Hospital, New Delhi. All patients provided the written consent for the sample collection and subsequent analysis.

Statistical analysis

Results are represented as mean±SE. Analysis of the data was done using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Statistical significance was determined by nonparametric Mann-Whitney test for two groups and by Kruskal-Wallis test followed by the post hoc Dunn multiple comparison tests for more than two groups. The correlation coefficient was calculated by Spearman rank test. The statistical tests were two-tailed and *P* values < 0.05 were considered significant.

Results

Lymphoproliferative response to *L. donovani* TSLA and *La*PSA

The specific cell-mediated immune response was analyzed in terms of their *in vitro* lymphoproliferation responses against TSLA and *La*PSA. The phytohemagglutinin (PHA) served as positive control in these experiments and the results of proliferation index (PI) are represented as mean ± SE. The lymphocytes from every individual out of the four study groups showed positive stimulation (Naïve PI, 10.30±0.838; VL, 7.73±0.955; HVL, 11.30±0.745; PKDL, 9.949±0.987). Every individual in HVL group responded positively to TSLA and the group mean (5.99 ± 0.497), was found significantly high ($P < 0.001$) when compared to the naive group (1.19 ± 0.064) or active VL (1.22 ± 0.124) **(Fig 4.1)**. However, TSLA induced variable proliferation in PKDL patients with PI, 3.065±0.565, (range 0.335-7.505). Fifty percent (8/16) cases showed PI values above cut off (=2) and the PKDL group mean was found significantly high ($P < 0.05$) compared to the naïve group. The VL patients did not respond to TSLA and the group means (1.222±0.124) was comparable to the naïve group (**Fig 4.1**). On the other hand, *La*PSA showed low PI values in HVL (1.49 ± 0.13), VL (1.04 ± 0.44) and PKDL

(1.17 ± 0.21) group and was comparable to naïve (1.11 ± 0.27). However, the HVL group showed marginally significant lymphproliferation (*P*=0.047) compared to VL (**Fig 4.1**). Positive cell proliferation was considered when PI was found ≥ 2 i.e., above cut off (Mean+3SD).

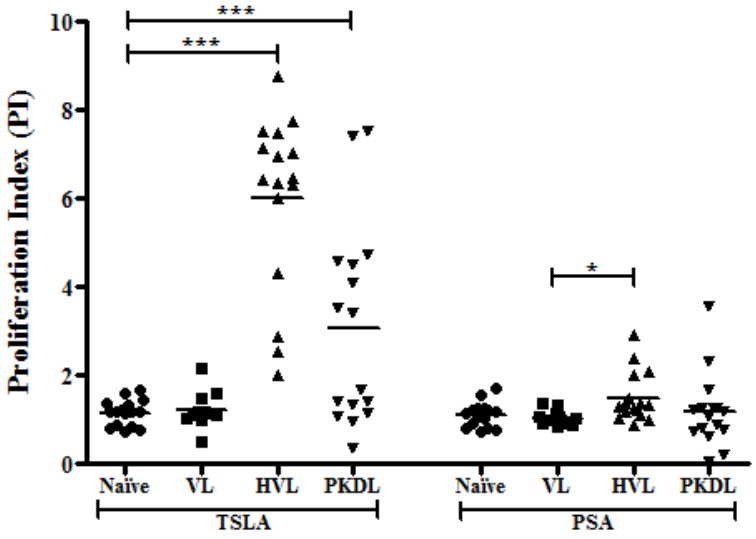


Figure 4.1: Comparative lymphoproliferative responses in different study groups *in vitro*. PBMCs from individuals with PKDL (n=16), VL (n=11), HVL (n=16) and from healthy group (n=16) were incubated with TSLA (10µg/ml) or *La*PSA (10µg/ml) for 120 hrs and lymphoproliferation was measured by BrdU incorporation for the last 12-14 hrs using Biotrak™ cell proliferation ELISA system. Horizontal lines indicate mean values. * $P < 0.05$, *** $P < 0.001$.

Cytokine profile upon TSLA and *La*PSA stimulation

PBMCs from all the study groups were examined for cytokine profile in response to TSLA and *La*PSA stimulation *in vitro* **(Fig 4.2 A-C)**. Against TSLA, the cellular immune response as judged from the secretion of pro-inflammatory (IFN-γ and TNF-α) cytokines were found significantly high in HVL and PKDL groups compared to the naïve or VL group. Results are represented as mean ± SE. Upon TSLA challenge, both HVL (738.44 ± 206.21) and PKDL groups (931.2±419.9) showed significantly high (*P* <

0.001) IFN-γ secretion compared to naive (2.104 ± 0.658) or active VL (5.845 ± 2.764) **(Fig 4.2 A)**. A similar observation was made for TNF-α upon TSLA stimulation, significantly high ($P < 0.001$) TNF-α secretion was observed in both HVL (37.71 ± 9.596) and PKDL (49.9 ± 24.37) groups compared to naïve (1.01±0.48) or VL (3.77±2.33) group **(Fig 4.2 B)**. For the anti-proinflammatory IL-10 cytokine, the measured values for HVL (2±0.633); PKDL (2.713±1) and VL (0.468±0.198) groups were found low and comparable to the naïve group against TSLA **(Fig 4.2 C)**.

Recombinant *La*PSA protein induced moderate but non-significant ($P > 0.05$) IFN-γ production in HVL (24.08±13.38) and PKDL (44.66±23.6) groups and were found comparable to naïve group (12.6±5.834) **(Fig 4.2 A)**. However, significant IFN-γ levels were observed in HVL and PKDL groups compared to active VL (mean±SE, 0.946±0.941). For TNF-α, similar observations were made with moderate, non-significant ($P>0.05$) TNF-α production upon *La*PSA stimulation, in PKDL (93±26.44) and naïve (157.6±51.99) group compared to active VL (29.74±12.51) group. However, TNF-α secretion was found marginally significant in HVL (115.5±28.99, $P< 0.05$) compared to VL, and was notably high for the naïve group. In contrast to TSLA stimulation, *La*PSA elicited high but comparable IL-10 production in HVL (72.95±13.84), PKDL (42.06±17.09) and active VL (22.2±10.7) and the naïve (77.04±21.62) group. However, the IL-10 level in HVL were marginally induced ($P < 0.05$) when compared to VL group **(Fig 4.2 C)**.

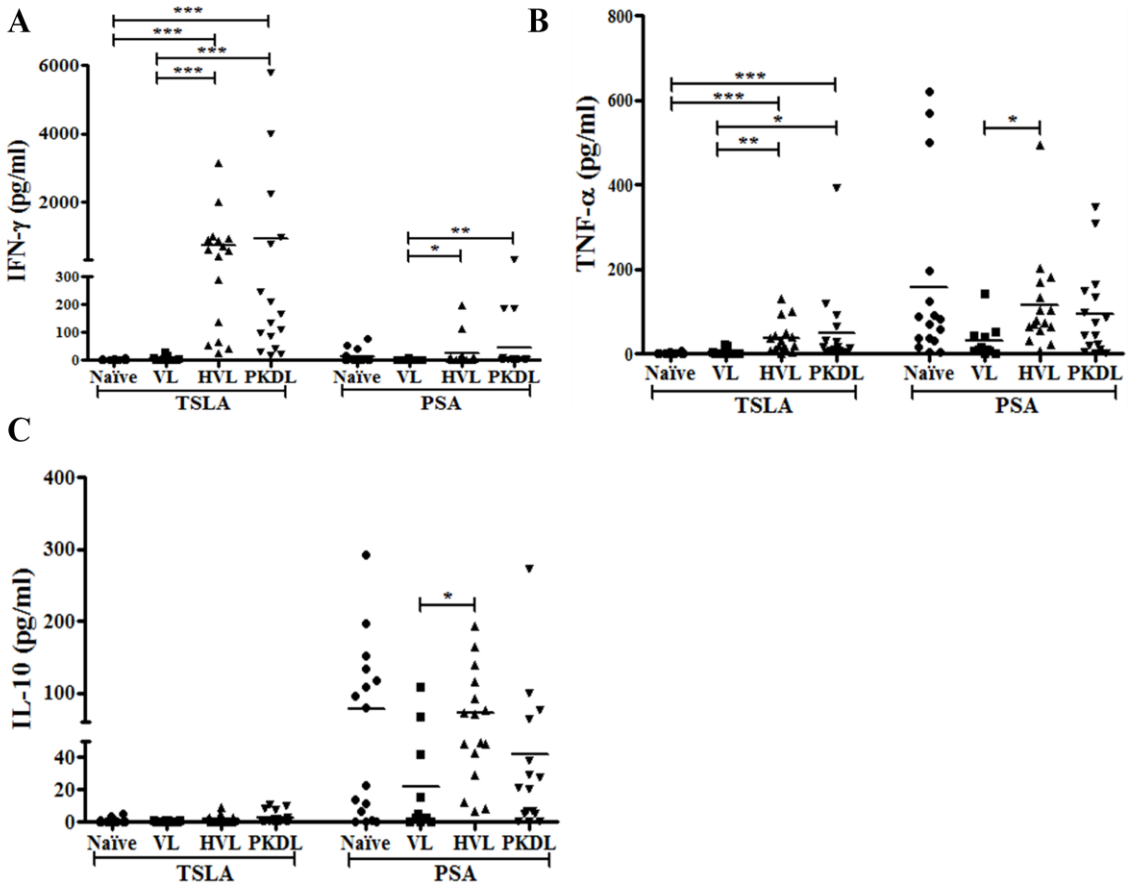


Figure 4.2: *In vitro Leishmania*-specific cellular immune responses in different study groups. PBMCs were isolated and incubated with TSLA (10µg/ml) and *La*PSA (10µg/ml) for 120 hrs. Cytokines (A) IFN-γ, (B) TNF-α and (C) IL-10 were measured in the culture supernatant of PKDL (n=16), VL (n=11), HVL (n=16) and from naïve healthy individuals (n=16). Horizontal lines indicate mean values. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Granzyme B analysis

Granzyme B, a serine protease expressed by the cytotoxic lymphocytes such as $CD8^+$, $CD4^+$ T cells, and Treg cells, induces apoptosis in target cells (Grossman *et al.* 2004; Vignali *et al.* 2008). Here, we evaluated granzyme B level to investigate whether individuals pre-exposed with *Leishmania* infection (HVL and PKDL groups) develop a

cytotoxic immune response upon re-exposure. So, granzyme B secretion after *in vitro* stimulation with TSLA and *La*PSA was estimated in the culture supernatant by CBA assay. The results are represented as mean ± SE. Upon TSLA stimulation, the granzyme B levels were found significantly high in HVL (1412.91 ± 441.62, $P< 0.001$) and PKDL group (272.1 ± 98.3, $P<0.01$) compared to naive (10.18 ± 4.369), with 11/12 (92%) HVL individuals and 8/14 (57.14%) PKDL cases showing values above the cut-off value (59.23 pg/ml) (**Fig 4.3**). However, the mean of VL group was found comparable to the naive ($P=0.371$). Upon *La*PSA stimulation, granzyme B production was found comparable among all the study groups. Against *La*PSA, the highest granzyme B secretion level was observed in HVL (64.39±34.76) followed by PKDL (63.85±28.71), naive (24± 43) and least in the VL group (2.7± 5.4) (**Fig 4.3**).

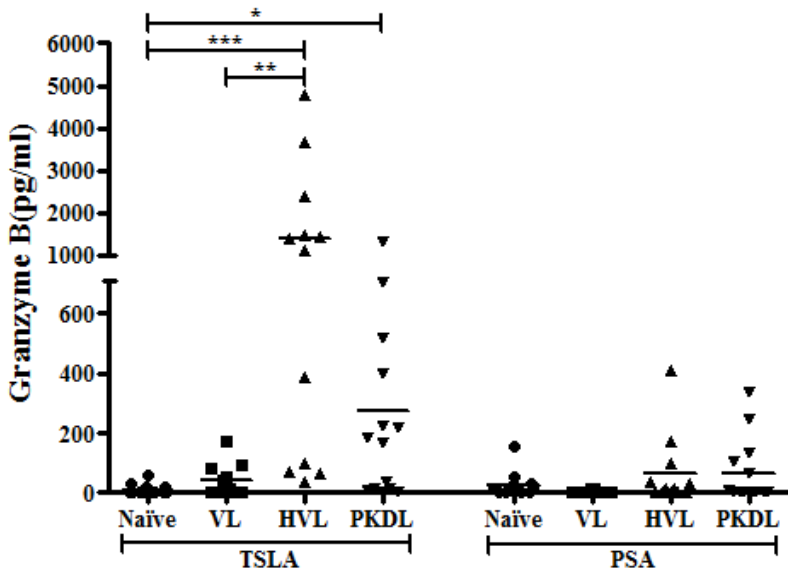


Figure 4.3: *Leishmania*-specific cell-mediated cytotoxic responses in different study groups. PBMCs were isolated and incubated with TSLA (10µg/ml) and *La*PSA (10µg/ml) for 120 hrs. Granzyme B level were analyzed in the supernatant of PKDL (n=14), VL (n=11), HVL (n=12) and naïve individuals (n=16) upon TSLA and *La*PSA stimulation, by using CBA. Horizontal lines indicate mean values. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Correlation of granzyme B level with proliferation index

The secretions of granzyme B upon TSLA stimulation were analyzed in PKDL, active VL, healed VL and healthy controls with their respective proliferation index. In HVL group, the level of granzyme B was found strongly correlated with the proliferation index, with correlation coefficient r= 0.895 (*P* = 0.0001) (**Fig 4.4**) whereas no correlation was observed in active VL, PKDL, and naïve controls. This result provides evidence that *Leishmania*-specific granzyme B-mediated cytotoxicity is part of the acquired cellular immunity developed in *Leishmania* immune individuals. Besides, we observed moderately significant correlation between PI and IFN-γ and TNF-α with r_s = 0.63 (*P* = 0.03) and r_s = 0.62 (*P* = 0.03) respectively in HVL group. However, we did not observe any significant correlation between granzyme B and IFN-γ level (r_s = 0.434, *P* = 0.158) in the culture supernatant.

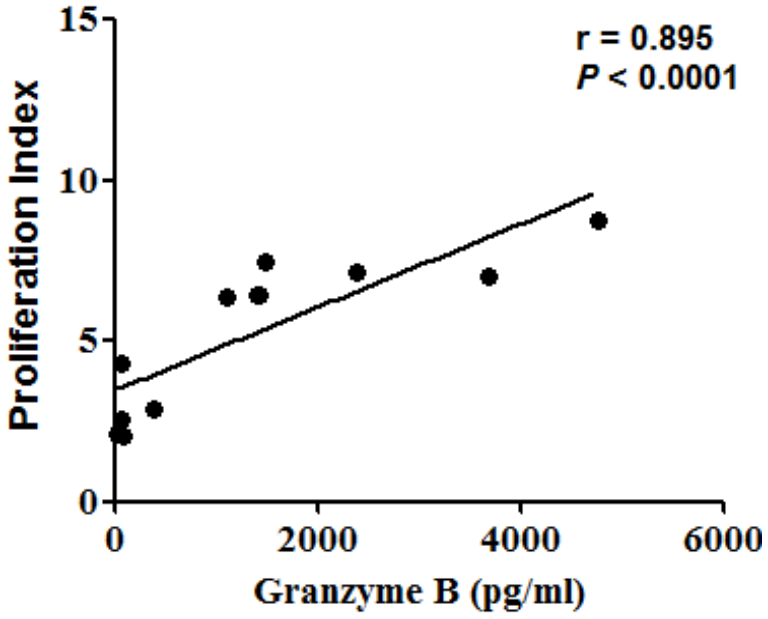


Figure 4.4: Comparative assessment of granzyme B level and proliferation index upon TSLA stimulation of PBMCs of HVL (n=12) group. The levels of granzyme B in culture supernatant upon TSLA stimulation were measured by CBA and stimulation index was determined by ELISA. The diagonal line represents the best-fit line.

Parasite load in PKDL tissues

Leishmania DNA was detected in tissue biopsy of all 19 PKDL patients. All PKDL cases were positive for qPCR with the mean value of parasite load as 5280 parasites/µg tissue DNA in the range of 10 to 24805 parasites/µg tissue DNA while all controls (n=20) were found negative. Comparative assessment of parasite load in the two clinical forms of PKDL revealed mean parasite load of 7582 parasites/µg tissue DNA in polymorphic (n=13), which was significantly higher (P = 0.003) as compared to the mean load of 293 parasites/µg tissue DNA in the monomorphic group (n=6). Scatter plot of parasite load in different clinical forms of PKDL is shown in **Fig 4.5.**

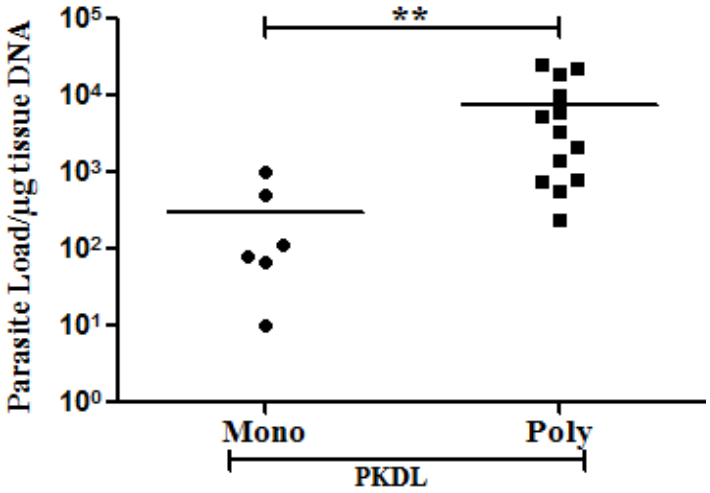


Figure 4.5: Parasite load in PKDL lesion tissues. Parasite load (parasites/µg tissue DNA) were determined in PKDL (monomorphic (macular), n=6; polymorphic (nodular), n=13) by real-time PCR. Statistical significance was determined by nonparametric Mann-Whitney test between polymorphic and monomorphic forms of dermal lesions in active PKDL cases. The horizontal line represents the mean parasite load. ** P < 0.01.

Correlation of immune response with parasite load

The secretion of IFN-γ, TNF-α, IL-10 and granzyme B upon TSLA stimulation were analysed in PKDL group (n=16) with respect to the parasite load. The level of IFN-

γ and TNF-α was significantly higher in cases with high parasite load in PKDL group and found significantly correlated (IFN-γ, r_s = 0.697, *P* = 0.002, and TNF-α, r_s = 0.629, *P* = 0.009) **(Fig 4.6).** No such correlation with parasite load was observed for IL-10 and granzyme B.

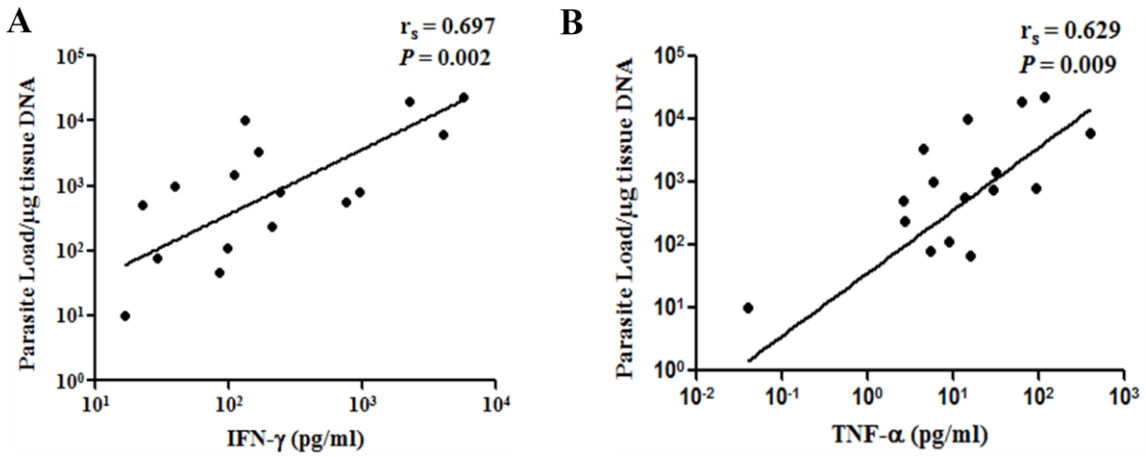


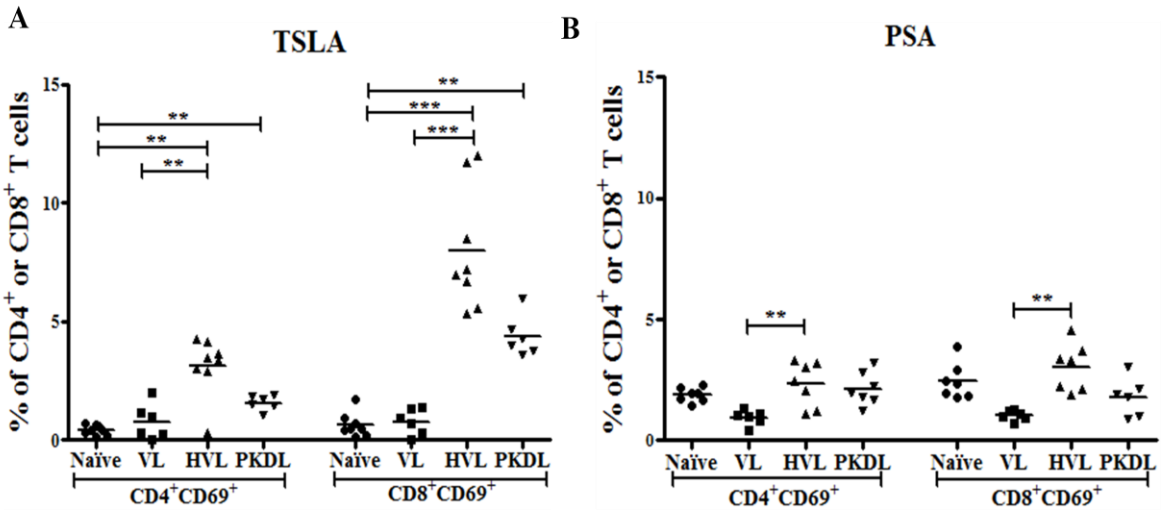
Figure 4.6: Comparative assessment of Th1 cytokines and parasite load in PKDL. The levels of (A) IFN-γ and (B) TNF-α in culture supernatant upon TSLA stimulation of PBMCs from patients with PKDL (n=16) were measured by CBA and parasite load in tissue lesions was determined by qPCR. The correlation was calculated by Spearman rank correlation test. The diagonal line represents the best-fit line.

Estimation of activated T lymphocytes

Using CD69 as a marker of activation (Arvå and Andersson 1999), we investigated the percentage of activated $CD4^+$ and $CD8^+$ T cell populations upon *in vitro* TSLA and *La*PSA stimulation in VL, PKDL, HVL and naive groups. The results are represented as mean ± SE. Upon TSLA stimulation, $CD8^+$ T cells showed a pronounced activation in both HVL and PKDL groups, with significantly higher percentage of $CD8^+CD69^+$ T cell population in HVL (7.99 ± 0.91, *P* < 0.001) and PKDL (4.348 ± 0.35, *P* < 0.01) groups compared to naive group (0.615 ± 0.176) **(Fig 4.7A)**. Similarly, there was also a significantly higher percentage of $CD4^+CD69^+$ T cell in HVL (3.118± 0.438,

P< 0.01) and PKDL groups (1.56 ± 0.133, *P* < 0.01) compared to naive group (0.412 ± 0.075). Active VL group did not show activation in either $CD4^+$ T cells (0.768 ± 0.303) or $CD8^+$ T cells (0.748 ± 0.218) and was comparable to the naïve group **(Fig 4.7A)**.

Upon *in vitro* PBMCs stimulation with *La*PSA, no study groups showed significant activation in either $CD4^+$ T cells or $CD8^+$ T cells compared to the naïve group **(Fig 4.7B)**. However, for HVL group, there was a significant activation in both $CD4^+$ T cells (0.768 ± 0.303) or $CD8^+$ T cells (0.748 ± 0.218) compared to VL group **(Fig 4.7B)**.



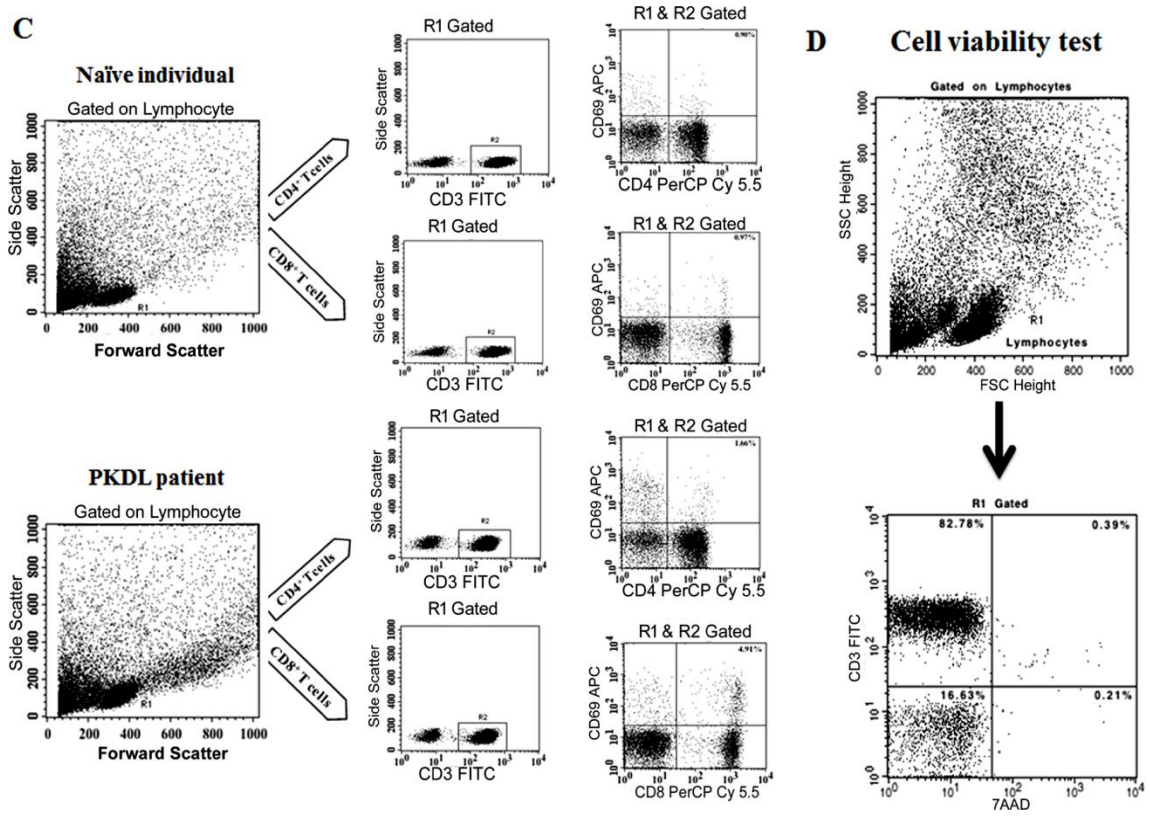


Figure 4.7: Estimation of activated T lymphocytes. (A & B) Percentage of TSLA and *La*PSA-activated $CD4^+$ and $CD8^+$ T cells in VL, PKDL, HVL and naive groups. PBMCs from VL (n= 6), PKDL (n= 6), HVL (n= 8) and naive individuals (n = 8) were incubated with TSLA (10µg/ml) and *La*PSA (10µg/ml) for 120 hrs at 37°C. The values of unstimulated cells were subtracted from TSLA or *La*PSA stimulated cells. (C) Data showing representative FACS analysis in one each from PKDL and naive individuals. For analysis, lymphocytes were gated using forward and side scatter properties and the frequencies of activated $CD8^+$ and $CD4^+$ T cells were acquired on $CD3^+$ T cells. (D) Cell viability test using 7AAD staining was done and the data show a representative FACS analysis in one of HVL individuals. Horizontal lines indicate mean values. $*P < 0.05$, $** P < 0.01$, $*** P < 0.001$.

Lymphocyte profile

Immunophenotyping of patients of active VL, PKDL and individuals of HVL and healthy controls groups were done to identify the proportion of peripheral T cells ($CD3^+$), helper T cells ($CD3^+CD4^+$), cytotoxic T cells ($CD3^+CD8^+$), NK cells ($CD3^-CD16^+CD56^+$) and B cells ($CD3^-CD19^+$). The results are represented as mean ± SE. The percentage of $CD3^+$ cells were significantly low in active VL (43.33±5.23, $P<0.05$) compared to HVL (60.66±2.19**)** and PKDL (62.16±2.25**) (Fig 4.8B)**. Similarly, the percentage of $CD4^+$ cells of total $CD3^+$ T cells were also found significantly low in active VL (37.16±2.5) compared to HVL (54.15±2.45, $P<0.001$), PKDL (53.66±2.66, $P<0.001$) and naïve controls (50.3±1.18, $P<0.05$) **(Fig 4.8C).** In contrast, the proportion of $CD8^+$ cells of total $CD3^+$ cells were found significantly high in active VL group (52.54±2.06, $P< 0.001$) compared to HVL (36.45±2.24), PKDL (36.45±9.79) and naive control (38.76±1.03) groups **(Fig 4.8D)**. Therefore, $CD4^+/CD8^+$ T cell ratio was found significantly low for VL (0.69±0.05, $P< 0.001$) compared to HVL (1.61±0.18), PKDL (1.51±0.14) and naïve controls (1.32±0.06) **(Fig 4.8E).** In patients with active VL, NK cells (6.23±1.07) were found significantly low compared to naïve controls (13.95±1.13, $P< 0.001$) and PKDL (12.37±1.54, $P< 0.05$) which were restored to normal in HVL (12.13±0.95, $P< 0.05$**) (Fig 3.8F)**. In contrast, the percentage of B lymphocytes in VL, HVL, and PKDL were found comparable to naïve controls **(Fig 4.8G)**.

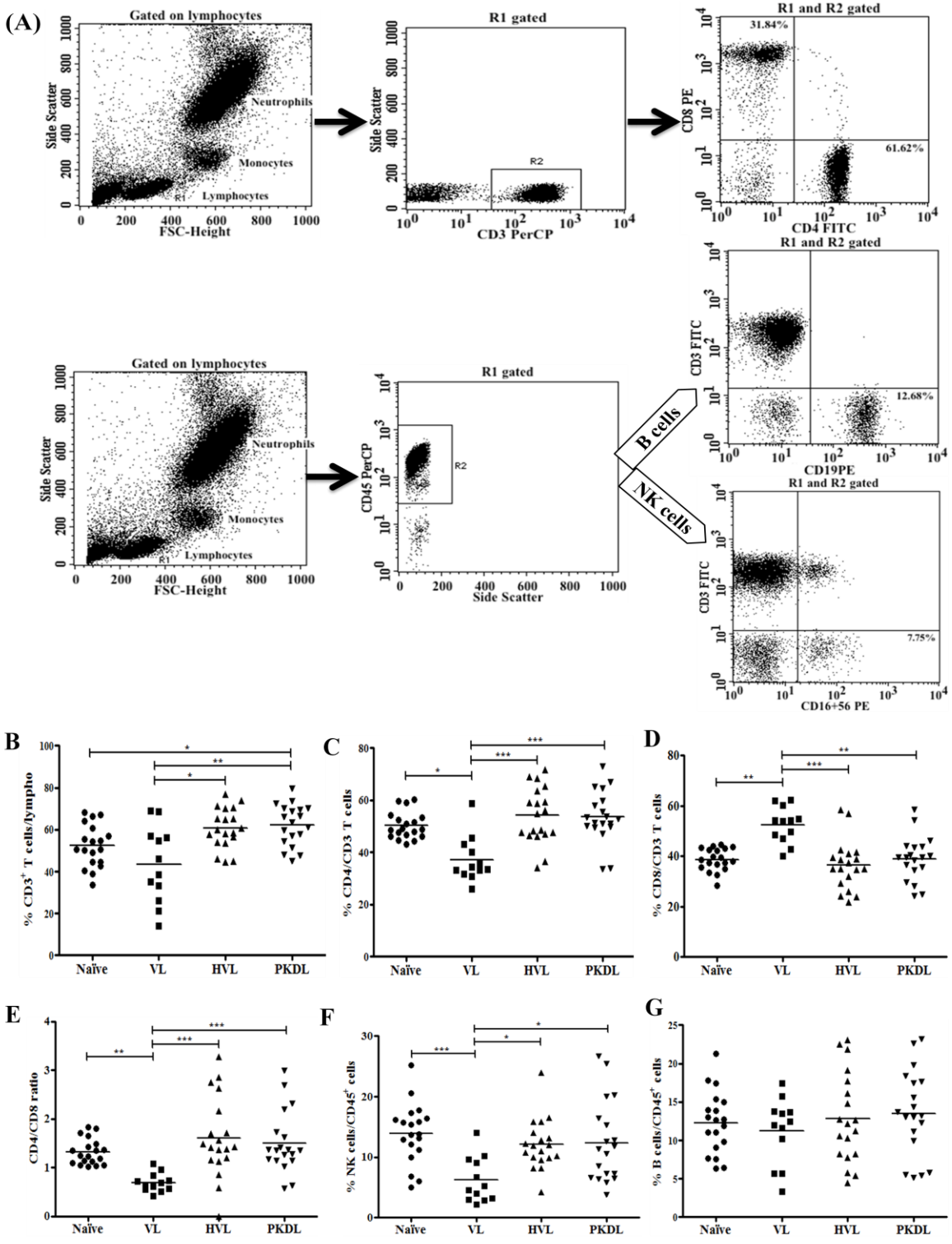


Figure 4.8: Flow cytometry analysis of lymphocyte subsets in peripheral blood. Blood from VL (n=12), PKDL (n=19), HVL (n=19) and naïve (n=19) were surface stained with appropriate antibodies as mentioned in material and methods section and analyzed using flow cytometry. (A) Data showing FACS analysis in one of the HVL

individual. Lymphocytes (R1) were gated on the basis of forward and side scatter and $CD3^+$ (B) and $CD45^+$ cells were gated on lymphocytes (R1). (C) $CD4^+$ and (D) $CD8^+$ cells were gated on $CD3^+$ cells with (E) ratio of CD4 and CD8 T cells whereas (F) NK and (G) B cells were gated on $CD45^+$ cells. Horizontal lines indicate mean values. *P < 0.05, ** P < 0.01, *** P<0.001.

Levels of total IgG and IgM to anti-leishmanial antigens, TSLA, and *La*PSA

Serum samples from VL (n=25), PKDL (n=25), HVL (n=25) and naive healthy controls (n=25) groups were initially tested for anti-leishmanial antigens (*La*PSA and TSLA) total IgG and IgM antibodies. The results are represented as mean ± SE OD_{492}. The anti-TSLA IgG level was significantly elevated in active VL (1.575±0.12, P<0.001), HVL (0.80±0.46, P<0.01) and PKDL (1.259±0.1, P<0.001) compared to naïve (0.078±0.01). In contrast, *La*PSA did not elicit significant humoral immune response in any of study group and the total anti-*La*PSA IgG levels in active VL (0.20±0.06), HVL (0.174±0.02) and PKDL (0.221±0.06) were found comparable to naïve group (0.118±0.02) (**Fig 4.9A**).

The anti-TSLA IgM level was significantly high in active VL (1.199±0.16, P<0.001) compared to naïve (0.318±0.04) and PKDL group (0.419±0.07) and decreased significantly in HVL (0.506±0.08, P<0.01). A similar trend was observed for anti-*La*PSA IgM, where significantly high IgM level was observed in active VL (1.099±0.16, P<0.05) compared to naïve group (0.526±0.1) which significantly decreased in HVL group (0.359± 0.05, P<0.01) (**Fig 4.9B**). However, the anti-*La*PSA IgM in PKDL (0.553±0.08) was found comparable to the naïve group.

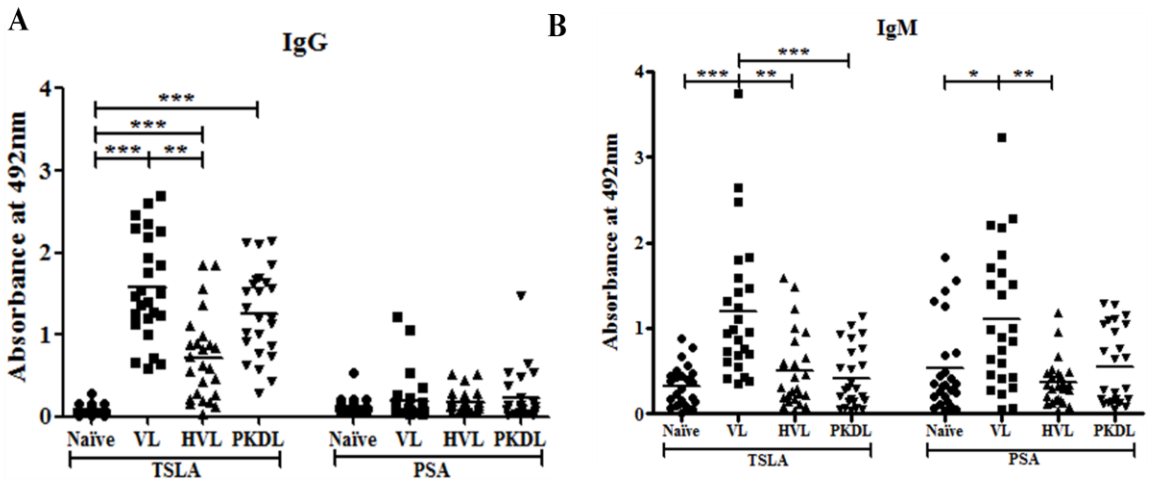


Figure 4.9: Comparison of anti-leishmanial (TSLA and *La*PSA) levels of (A) total IgG and (B) IgM in sera of different study groups. The mean±SE OD_{492} levels were determined by ELISA method. The bars represent the means for the different study groups. Data were analyzed between groups by nonparametric Kruskal-Wallis test followed by the post hoc Dunn multiple comparison tests. $P<0.05$ is considered statistically significant.*$P< 0.05$, **$P<0.01$, *** $P<0.001$.

Levels of anti-leishmanial IgG subtypes to TSLA and *La*PSA

We attempted to utilise the relative abundances of anti-leishmanial IgG subclasses to better discriminate the immunity among VL, PKDL, HVL and healthy control groups. Serum samples from patients with PKDL (n=25), active VL (n=25) and individuals from HVL (n=25) and naive groups (n=25) were tested for distribution of anti-TSLA and anti-*La*PSA IgG subclasses. The results are represented as mean ± SE OD_{492}. The levels of anti-TSLA IgG1 were significantly high in both VL (1.00± 0.04, $P < 0.001$) and PKDL (0.604± 0.04, $P < 0.001$) groups compared to naïve (0.023±0.01), that decreased significantly in HVL (0.194±0.03, $P< 0.001$) **(Fig 4.10A)**. For anti-*La*PSA IgG1, the level was significantly raised in active VL (0.074±0.02, $P< 0.05$) compared to naive group (0.018±0.01) and decreased after treatment in HVL (0.023±0.01). However, the level of anti-*La*PSA IgG1 in PKDL (0.023±0.01) was found comparable to the naïve

group **(Fig 4.10A)**. In contrast, the IgG2 levels to both TSLA and *La*PSA were found comparable among the different study groups **(Fig 4.10B).** The anti-TSLA IgG3 levels were significantly (P<0.001) elevated in active VL (0.063±0.01), PKDL (0.058±0.004) and HVL (0.044±0.002) compared to naïve group (0.017±0.001) **(Fig 4.10C)**. A similar trend was observed for anti-*La*PSA IgG3 level, the active VL group (0.045±0.007, P < 0.01) showed significantly high IgG3 levels compared to naïve group (0.017±0.002) **(Fig 4.10C)**. The anti-TSLA IgG4 level was minimal in HVL (0.028±0.002) and was comparable to naïve group (0.017±0.003), however, it was significantly (P < 0.05) elevated in both active VL (0.035±0.005) and PKDL group (0.034±0.003) compared to naïve group **(Fig 4.10D).** The anti-*La*PSA IgG4 levels were found comparable among the different study groups **(Fig 4.10D).**

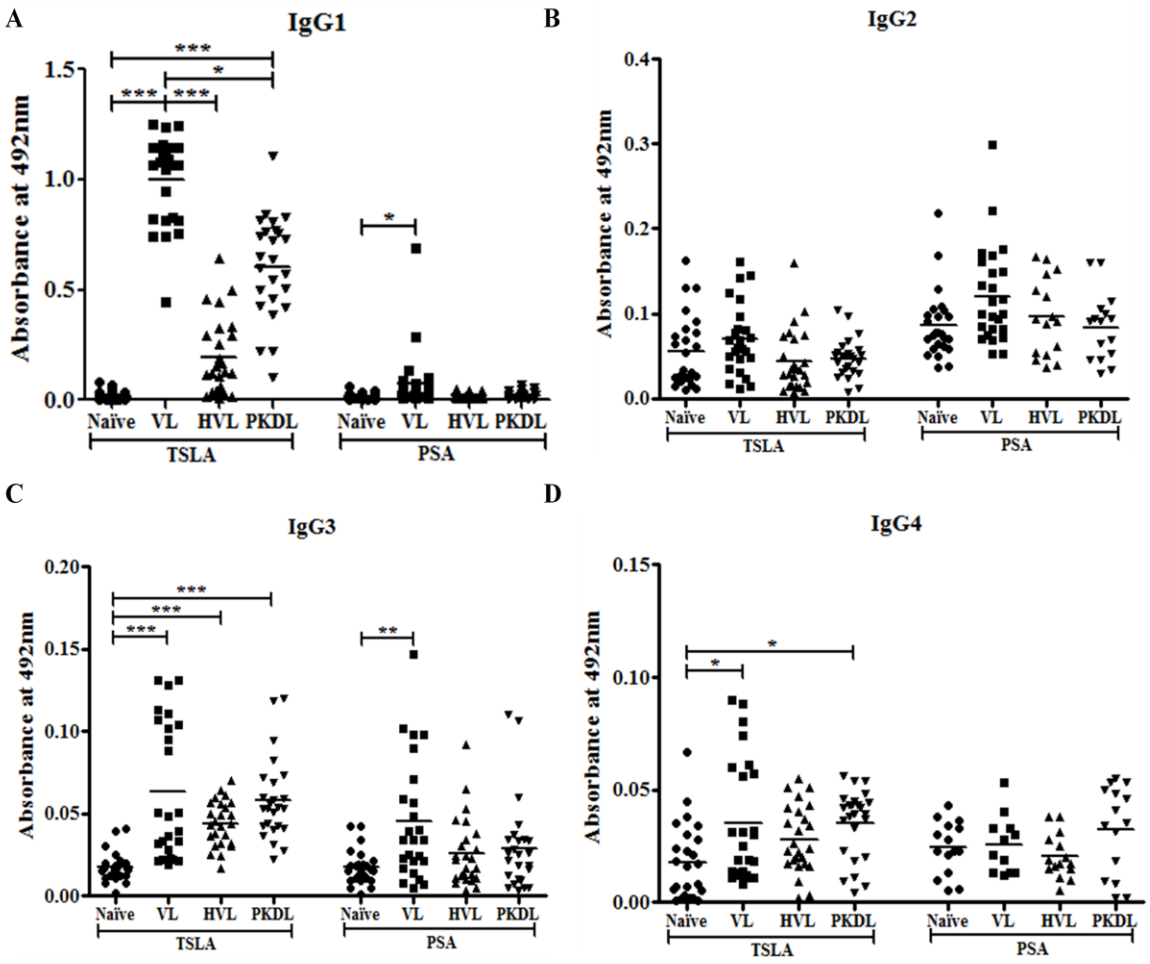


Figure 4.10: Comparison of anti-leishmanial (TSLA and *La*PSA) of IgG subclass (A) IgG1, (B) IgG2, (C) IgG3 and (D) IgG4 in sera of different study groups. The mean±SE OD_{492} levels were determined by ELISA method. The bars represent the mean for the different study groups. Data were analyzed between groups by the nonparametric Kruskal-Wallis test followed by the post hoc Dunn multiple comparison tests. $P<0.05$ is considered statistically significant.*$P < 0.05$, **$P < 0.01$, *** $P < 0.001$.

Discussion

Only a few vaccine candidates have progressed beyond the experimental stage in animal models of leishmaniasis (mice, hamsters). This is consistent with the difficulty in developing a suitable animal model that reproduces the features of the human disease. Direct extrapolation of results from experimental models to human disease is often controversial and the protective immune responses controlling experimental infection may not reflect those required to prevent leishmaniasis in endemic areas. The purpose of this study was to evaluate the ability of the recombinant *La*PSA antigen to elicit *in vitro* cellular immune response in PBMCs of previously exposed (immune) and non-exposed (naive) human subjects. The rationale is that antigens that elicit immune responses correlating with protection or cure (e.g. significant IFN-γ production in previously exposed and cured individuals) may be good candidates for prophylactic and/or therapeutic vaccines.

Previously it was demonstrated that naturally excreted/secreted antigens (ESA) purified from culture supernatants of *L. infantum* promastigotes formulated with an adjuvant were able to induce a long lasting Th1-mediated protection against experimental and natural canine VL (Beetham *et al.* 2003; Lemesre *et al.* 2005, 2007; Devault and Bañuls 2008). Interestingly, a native soluble *L. infantum* PSA has been identified as the active constituent of ESA that reproduces the observed protective immunity when used as a vaccine in dogs (Bras-Gonçalves *et al.* 2014). PSA is present in both promastigote and amastigote stages and is over-expressed in stationary phase parasites (Handman *et al.* 1995b; Beetham *et al.* 2003; Devault and Bañuls 2008). PSA isolated from *L. amazonensis* promastigotes as well as recombinant vaccinia viruses expressing this

protein have been demonstrated to elicit a protective immune response against infection with *L. amazonensis* in BALB/c mice (Champsi and McMahon-Pratt 1988; McMahon-Pratt *et al.* 1993). Moreover, PSA-2 polypeptides purified from *L. major* and plasmid DNA encoding PSA-2, but not recombinant PSA-2 purified from *E. coli*, provided a significant protection against *L. major* challenge in BALB/c mice (Handman *et al.* 1995b; Sjölander *et al.* 1998). The available knowledge regarding PSA protein makes it a good choice to be evaluated as a potential vaccine candidate against human leishmaniasis. In the present study, recombinant *La*PSA protein was produced in a non-pathogenic *L. tarentolae* recombinant expression system. This expression system allows the conservation of *Leishmania*-type post-translational modifications including glycosylation, phosphorylation and disulfide bond formation. Moreover, the *L. tarentolae* expression system, in addition to being non-pathogenic to mammals, is an ideal expression system to produce native proteins of *Leishmania* (Breitling *et al.* 2002).

We initially evaluated cellular immune response in terms of lymphoproliferation upon *La*PSA and TSLA stimulation *in vitro* in cases VL, PKDL, HVL and naive control groups. TSLA served as a positive control and as expected it induced significantly high lymphoproliferation in *Leishmania* immune (HVL) group and PKDL groups implying the presence of circulating *Leishmania*-specific memory T cells in these pre-exposed groups compared to that in unexposed individuals (naive). In contrast, recombinant *La*PSA did not show marked lymphoproliferation in either HVL or PKDL group compared to naïve. However, a small proportion in both HVL (18.7%) and PKDL (12.5%) groups showed PI value above cut-off (>2). Additionally, there was a marginal significant lymphoproliferation in HVL compared to VL group. The low PI value in active VL cases

may be due to immune dysfunction/anergy as reported earlier (Goto and Prianti 2009; Gautam *et al.* 2014).

The cytokine analysis constitutes an important part since they form a complex network of synergistic and antagonistic interactions, which not only induce but also control the immune response. IFN-γ produced predominantly by activated CD4 Th1 and CD8 cytotoxic T lymphocyte (CTL) and NK cells in response to IL-12 signaling, is an important activator of macrophages that enhances their microbicidal activity against intracellular pathogens (Gough *et al.* 2008; Mosser and Edwards 2008). It promotes NO production by inducing iNOS expression by infected phagocytes thus facilitating elimination of parasites and resolving *Leishmania* infection (Sacks and Noben-Trauth 2002). Another pro-inflammatory cytokine, tumor necrosis factor alpha (TNF-α), known to exert cytotoxic effects on pathogens, has been closely associated with VL pathogenesis, being low in active VL and getting restored after treatment (Peruhype-Magalhães *et al.* 2006). The cytokine profile upon *La*PSA and TSLA stimulation of PBMCs corroborates our lymphoproliferation data. The production of a significantly higher level of pro-inflammatory cytokines (IFN-γ and TNF-α) to TSLA was observed in both HVL and PKDL groups compared to naive or active VL group and is in accordance with earlier studies (Carvalho *et al.* 1985, 1995). The significant association between PI and IFN-γ and TNF-α in HVL group indicates the strong CMI in individuals with prior exposure to *Leishmania* antigens. As expected, no protective immune response was observed in naive individuals while the active VL group exhibited peripheral lymphocytes anergy (Goto and Prianti 2009). Furthermore, the level of pro-inflammatory cytokines (IFN-γ and TNF-α) was higher in cases with high parasite load for PKDL

group and showed a moderate positive correlation between levels of Th1 cytokines (IFN-γ and TNF-α) and parasite load. Corroborating the lymphoproliferative result, *La*PSA induced cytokine profile (IFN-γ and TNF-α) in HVL or PKDL comparable to naïve group which suggested no marked presence of memory cells against *La*PSA, therefore, recombinant *La*PSA may not be a promising vaccine candidate against human VL. IL-10, on the other hand, has a counter-regulatory role against IL-12 and IFN-γ and thus favors the survival of *Leishmania* parasites by inhibiting NO-mediated killing (Vouldoukis *et al.* 1997). We observed no significant difference in IL-10 level between different study groups against anti-leishmanial proteins (TSLA and *La*PSA) which could be either due to low detection for this cytokine or the dispersion of results. Previously it was shown that patients with PKDL in response to *L. donovani* antigens elicited pronounced IL-10 secretion (Saha *et al.* 2007) and showed a 9.6-fold rise in the percentage of IL-10-expressing $CD8^+$ T lymphocytes (Ganguly *et al.* 2008). Additionally, Ganguly *et al.* showed raised levels of IFN-γ and IL-10 in the serum of polymorphic PKDL cases compared to naïve or monomorphic groups (Ganguly *et al.* 2008). The reported peripheral cytokine levels corroborated with raised IgG1 and IgG3levels in polymorphic form, known to be driven by IL-10 (Brière *et al.* 1994; Fujieda *et al.* 1996).

There are mainly two mechanisms by which cytotoxic cells lyse their targets: the perforin-granzyme B pathway and death receptors (Fas/FasL) (Kägi *et al.* 1994; Bousoffara *et al.* 2004). The FasL-dependent pathway utilizes Fas surface receptor by Fas ligand expressed on the surface of the CTL and NK cells, which triggers Fas-mediated apoptosis in target cells. The chief mechanism used by cytotoxic cells to induce target cell death is through the granule exocytosis pathway and depends on the concerted action

of effector molecules contained in the cytolytic granules. These granules contain perforin, the pore-forming molecule, together with granule-associated enzymes. Among them, granzyme B is the most important effector molecule for target cell apoptosis (Lowin *et al.* 1994). It is unclear whether $CD8^+$ T cells contribute protection against *L. donovani* parasites through their cytotoxic activity. Limited studies have been conducted dealing with parasite-specific cell-mediated cytotoxicity in CL or MCL (Heusel *et al.* 1994; Barral-Netto *et al.* 1995; Brodskyn *et al.* 1997). To our knowledge, this is the first report of a *Leishmania*-induced granzyme B response in HVL or PKDL individuals to investigate whether individuals pre-exposed with *Leishmania* parasites develop a cytotoxic immune response upon re-exposure. We demonstrated significantly higher granzyme B level upon TSLA stimulation in *Leishmania* immune (HVL) and PKDL groups compared to the naive or VL group, with a strong association between PI and granzyme B level. Previous studies with viral infections have shown that granzyme B is predominantly secreted by $CD8^+$ T cells (McElhaney *et al.* 1996), although, the contributions of NK cells and $CD4^+$ cytotoxic cells have also been suggested (Griffiths and Mueller 1991; Sharp *et al.* 1992). Furthermore, we observed a significantly high percentage of $CD8^+CD69^+$ and $CD4^+CD69^+$ T cells in both HVL and PKDL individuals to TSLA. This is distinct from the report in cured CL individuals where a high $CD4^+CD69^+$ T cells were demonstrated to be responsible for the immunity to *L. major* infection (Chamakh-Ayari *et al.* 2014). Taken together, the elevated levels of both activated $CD4^+$ and $CD8^+$ T cells coupled with the high secretion of granzyme B upon *in vitro* TSLA stimulation suggested the role of cytotoxic activity in resistance against *L. donovani* re-infection in HVL and PKDL. On the contrary, recombinant *La*PSA produced

no cytotoxic activity in term of granzyme B production and showed no activation of either $CD4^+$ or $CD8^+$ T cells, corroborates lymphoproliferation and cytokine results, ruling it out of feasibility of being possible vaccine candidate for human VL.

Characterization of cellular immunity based on the phenotype and proportion of the different peripheral blood lymphoid subsets were done to understand generalized cellular responses. Most of the T cells derived from the PBMCs of the active VL patients were $CD8^+$ with lower $CD4^+$ proportion, while the situation is reversed in the HVL individuals with more numbers of $CD4^+$ than $CD8^+$ T cells which are in line with earlier studies on Kenyan VL (Koech 1987) and Iraqi VL (Taher *et al.* 2009) while another study on Indian VL reported contrary results (Nylén *et al.* 2007). For NK cells, a similar trend was observed with depressed NK cell proportion in active VL which restored to normal following VL treatment, supports an earlier study on Ethiopian VL (Cenini *et al.* 1993). Above observations suggest that these cells are mainly affected by *Leishmania* parasite and possibly responsible for the impaired cell-mediated immunity observed in human VL. In Indian PKDL, information pertaining to immunophenotype profile of peripheral blood is scarce, primarily because it occurs in a small subset of individuals recovered from VL. A previous study on PKDL cases from India reported a significant increase of $CD3^+CD8^+$ lymphocytes in peripheral blood, at both pre- and post-treatment stages (Ganguly *et al.* 2008). On the contrary, the present study demonstrated no major difference in the proportion of circulating $CD3^+CD8^+$ T cells in PKDL compared to naive group, an observation in line with an earlier study on Indian PKDL (Ghosh *et al.* 1995).

Analysis of humoral responses clearly showed that *La*PSA antigen did not induce a significant antibody response in individuals with either active VL or PKDL. These

results are in accordance with other studies describing reactivities of human VL sera to PSA antigen (Beetham *et al.* 2003). However, in our case, we cannot exclude that this low recognition may be due to the amino acids sequence divergence between *La*PSA and PSA from *L. donovani*. On the contrary, pronounced anti-TSLA IgG level was observed in active VL and PKDL which persisted with low intensity following VL treatment as seen in HVL group. Among the IgG subtypes, anti-TSLA IgG1 and IgG3 levels were found significantly raised in both VL and PKDL patients and decreased post-treatment and, therefore, anti-TSLA IgG1 and IgG3 levels can be utilised as a prognostic marker for PKDL and VL.

Conclusion

The study demonstrated *Leishmania*-specific cellular immune responses in terms of lymphoproliferation and cytokine profile upon PBMCs stimulation to *Leishmania* antigens i.e.; TSLA and *La*PSA, in the active cases of VL and PKDL and HVL individuals. Lymphoproliferation in response to TSLA in HVL and PKDL group showed heightened response whereas *La*PSA did not induce lymphoproliferation in either of *Leishmania* pre-exposed groups i.e., HVL or PKDL group. The cytokine profile upon *La*PSA and TSLA stimulation corroborated our lymphoproliferation data; *La*PSA did not induce significant pro-inflammatory cytokines response in either HVL or PKDL while TSLA elicited a strong protective cytokine response in the same groups. Additionally, the significant activation of both $CD4^+$ and $CD8^+$ T cells along with high granzyme B production upon *in vitro* TSLA stimulation indicated for the first time the role of cytotoxic cells in resistance to re-infection in HVL and PKDL groups. This finding indicated *Leishmania*-specific cell-mediated cytotoxicity as a part of the naturally

acquired immunity developed in *Leishmania* pre-exposed groups (HVL and PKDL). In contrast and corroborating our cellular data, *La*PSA did not induce significant activation in either $CD8^+$ or $CD4^+$ T cells. Generalized cellular immunity based on the phenotype and proportion of the different peripheral blood lymphoid subsets revealed impaired cellular immunity in active VL while PKDL showed raised proportion of $CD3^+$ T cells. Our approach to investigate the ability of a given antigen, for its ability to elicit cellular protective responses in different groups of human populations would be applicable to select vaccine candidates and prioritize antigens for the clinical development of a vaccine against leishmaniasis.

Serological and molecular analysis of asymptomatic Leishmania infection in VL-endemic regions of West Bengal, India

Introduction

In India, Visceral Leishmaniasis (VL) has been one of the major health issues in the state of Bihar, and the adjoining states such as West Bengal, Uttar Pradesh, and Jharkhand (Bora 1999). Sporadic incidences of VL cases have regularly been occurring in many districts since 1977 (Thakur 2000). Fifty-two districts in India, 45 in Bangladesh, 12 in Nepal and 4 in Bhutan are affected. In 2006, a few cases of VL were reported from Bhutan (Bhattacharya *et al.* 2010). In India, the most affected region for VL is the state of Bihar with 31 of 38 districts, followed by West Bengal with 12 of 20 districts, considered endemic for VL (Kesari *et al.* 2011).

In the Indian subcontinent, the transmission of VL is anthroponotic, hence, the cases of active VL and PKDL serve as a reservoir of *L. donovani*, especially during the inter-epidemic period (WHO 2010) therefore better management of the disease is needed. Additionally, a majority of the *Leishmania*-infected human population do not develop into full blown VL cases, and are considered asymptomatic (Das *et al.* 2011; Ostyn *et al.* 2011; Stauch *et al.* 2011) and such cases are considered important in maintaining transmission dynamics of *Leishmania* infection (Sharma *et al.* 2000). However, the knowledge of an exact number of the asymptomatic cases present in an endemic area is difficult to assess. Few studies have reported the presence of asymptomatic cases in the VL-endemic areas of Bihar (Topno *et al.* 2010; Das *et al.* 2011) in the range of 11 to 17%. Similarly, there could be a high probability of the presence of asymptomatic *Leishmania* infection in other parts of India where VL is known to be endemic such as the state of West Bengal, Uttar Pradesh, and Jharkhand. Identification and estimation of asymptomatic infections in VL-endemic areas will be useful for formulating appropriate

public health policies. Additionally, in view of the national goal of VL elimination by the end of the year 2020, which is not very far off, research to understand the magnitude of asymptomatic infection, is of paramount importance. The current study, to our best knowledge, is the first report from the state of West Bengal, India on serological and molecular analysis of *Leishmania* parasites in blood samples of healthy individuals living in VL-endemic regions, to understand the magnitude of asymptomatic *Leishmania* infection present in the region.

Materials and Methods

Study population

The study area was chosen based on the incidence of VL during last 3 years, as per the hospital records, in the state of West Bengal, covering both North and South Bengal, since this was a collaborative study with a group in Kolkata. The selected regions were the villages of Malda and Murshidabad districts, which are endemic for VL. Individuals living in these high endemic areas were taken as the study population. During the period between January 2014 and December 2014, a team of physician, technician and trained field investigators made 9 field trips in different villages and clinically examined individuals for the symptoms of VL such as fever for more than 2 weeks, an enlarged spleen and/or liver and general physical conditions. A house-to-house survey was undertaken for the identification of a cohort of asymptomatic individuals. A set of questionnaire was given to each to obtain basic information such as age, gender, nativity, details of the history of VL and/or PKDL, history of VL/PKDL in the family and/or neighborhood. Of 248 individuals examined, one had active VL and one active PKDL. These two cases were referred to the primary health center for the treatment and were

excluded from the study; the rest 246 individuals were included in the study. Peripheral blood samples (2-3 mL) were collected in heparinized tubes for serological and molecular analysis.

The study population was categorized into following three study groups.

Endemic healthy controls (EHCs): Healthy individuals who reported no history of VL and were negative by both qPCR and rK39 strip test.

Healed VL (HVL) group: Healthy individuals who reported a history of VL and completed full treatment regimen.

Asymptomatic group: Healthy individuals who were positive by qPCR and/or rK39 test.

The rK39 test using plasma sample

The rK39 strip test (Inbios International Inc, Seattle, WA, USA) is an immunochromatographic test used for screening individuals for the *Leishmania* infection. It is specific for antibodies to *L. donovani* complex in patients with VL (Sundar *et al.* 2006). The sensitivity of the test for detection of VL in India is 99% (95% CI: 95–100%) and its specificity is 89% (95% CI: 86–92%) (Goswami *et al.* 2003). The test is cost-effective, easy and reliable diagnostic tool for VL, but fails to discriminate between healed VL, asymptomatic and active cases of VL and PKDL. The test was carried out as per the manufacturer's instructions. One or two drops plasma were placed at the tip of the rK39 strip and two drops of chase buffer were added. The result of the test was observed after 10 minutes. Development of two visible bands (control and test) indicated the presence of anti-rK39 IgG.

DNA Isolation

Blood samples were collected in tubes with heparin. DNA was isolated using QIAamp DNA Blood mini kit (Qiagen, Germany) as per the manufacturer's instructions. In order to obtain high yield, the sample was digestion overnight with proteinase K in lysis solution (Qiagen). DNA was isolated from 200 µL of blood and eluted in 50 µL distilled water. All samples were processed and DNA was stored at -80^{0}C until use.

Real-time PCR assay

SYBR Green I based real-time PCR was used for the quantification of the target DNA sequence. A standard curve was generated using a 10-fold serial dilution of DNA obtained from *L. donovani* parasite corresponding to 10^4 to 0.1 parasites per reaction. DNA amplification was performed on a Prism 7000 sequence detection system (Applied Biosystems, USA). Standards, negative controls, and the samples were analyzed in triplicates for each run. A 10µl PCR reaction mixture was performed, containing 1µl DNA template, 5pmol each of forward and reverse primer and 1X SYBR Green I PCR Master mix (Applied Biosystems, USA). Cycling parameters were 50^{0}C for 2 min, 95^{0}C for 10 min, and 40 cycles at 95^{0}C for 15 sec and 60^{0}C for 1 minute. The threshold cycle (Ct) value for each sample was calculated by determining the point at which the fluorescence exceeded the threshold limit. The blood samples of 20 healthy individuals from non-endemic areas were taken for qPCR assay.

Preparation of TSLA and recombinant *La*PSA proteins

This was carried out as described in chapter 3.

Detection of anti-leishmanial antibodies in plasma

This was carried out as described in chapter 3

Ethics statement

The study was approved by and carried out following the guidelines of the Ethical Committee of National Institute of Pathology (ICMR), Safdarjung Hospital Campus, New Delhi. All patients or their guardians (in the case of minors) provided written informed consent for collection of samples and subsequent analysis.

Statistical analysis

The data were analyzed using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA). Statistical significance was determined by nonparametric Kruskal-Wallis test followed by the post hoc Dunn multiple comparison tests for more than two groups. The correlation was calculated by Spearman rank correlation test. Agreement between rK39 and qPCR tests was estimated using Cohen's kappa coefficient on SPSS software. The statistical tests were two-tailed and *P* values < 0.05 were considered significant.

Results

Clinical characteristics

Our study recorded 40.24% (n=99) male and 59.75% (n=147) female with the overall age in years, mean±SD, 30.14±17.24. The mean age of females (mean±SD 30.81±17.36) was comparable to that of male (mean±SD 29±17.24). The majority of cases belonged to the age group 19-44 years (47.15%, n=116), followed by pediatric cases aged $\leq$ 18 years (30.08%, n=74) and the group aged $\geq$ 45 years (22.76%, n=56)

(Table 5.1). In the study, 36.17% (n= 89) of individuals reported a history of VL and 63.82% (n=157) had no previous VL episode. Of all, 76.82% (n=189) of the study population had either household and/or neighborhood contacts of VL and/or PKDL. Of the total collected samples, 36.17% (n=89) of study population belonged to Malda district and the rest 63.82% (n= 157) to Murshidabad district.

Table 4.1: Age-Gender distribution of the study population

Age, years	Male		Female		Total	
	N (%)	Mean±SD	N (%)	Mean±SD	N (%)	Mean±SD
≤18	36 (36.36)	10.77 ± 17.34	38 (25.85)	10.73 ±17.41	74 (30.08)	10.75 ±17.31
19 – 44	42 (42.42)	30.33 ± 17.24	74 (50.34)	30.54 ± 17.33	116 (47.15)	30.46 ± 17.24
≥45	21 (21.21)	58.23 ± 17.45	35 (23.81)	53.2 ± 17.41	56 (22.76)	55.09 ± 17.41
Total	99 (40.24)	29.14 ± 17.24	147 (59.75)	30.81 ± 17.36	246	30.14 ± 17.24

Serological diagnosis

The rK39 strip test was positive in 10.97% (n=27) serum samples and the rest 89.02% (n=219) individuals were seronegative for the rK39 antigen (**Fig 5.1**). The majority of seropositive individuals, 81.48% (n=22) had reported a history of VL and were cured VL individuals except for 5 who were either household or neighborhood VL contacts. Among seronegative individuals, 67 had reported a history of VL, and the range of time lapse following VL treatment was 1 to 20 years, with mean±SD, 7.19±3.51 years.

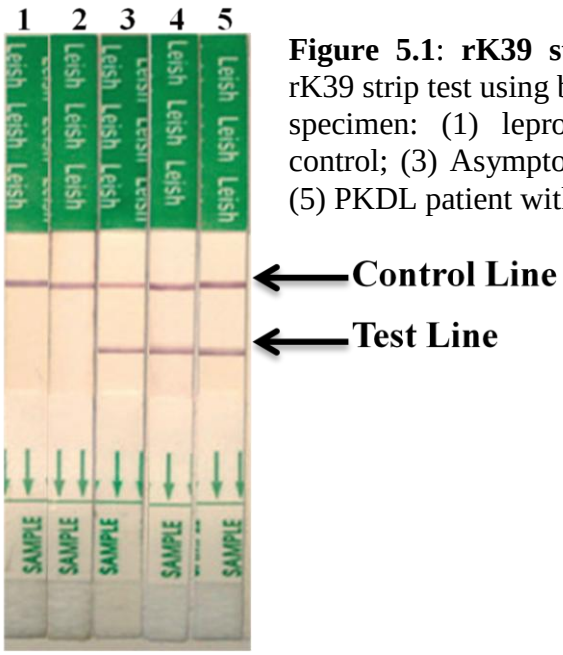


Figure 5.1: **rK39 strip test**. Lanes showing rK39 strip test using blood sample as diagnostic specimen: (1) leprosy control; (2) Healthy control; (3) Asymptomatic VL; (4) active VL; (5) PKDL patient with nodular lesion.

Sensitivity of Real-time PCR assay

Initially, the sensitivity of the assay was determined by a serial dilution of the known amount (10 ng –1fg) of DNA from *L. donovani*. The assay allowed a detection limit of 1 fg DNA, the detection limit of 0.01parasite per reaction. The standard curve was found linear over the range of 6 logs of DNA concentrations with a correlation coefficient (r^2) of 0.988 (**Fig 5.2**). The coefficient of variations of Ct values between the replicates was 3.92, 1.44, 0.41 1.37, 0.67 and 0.72 for the 6 different concentrations. A negative control (molecular grade water) with every PCR assay was taken for stringent measures to check contamination.

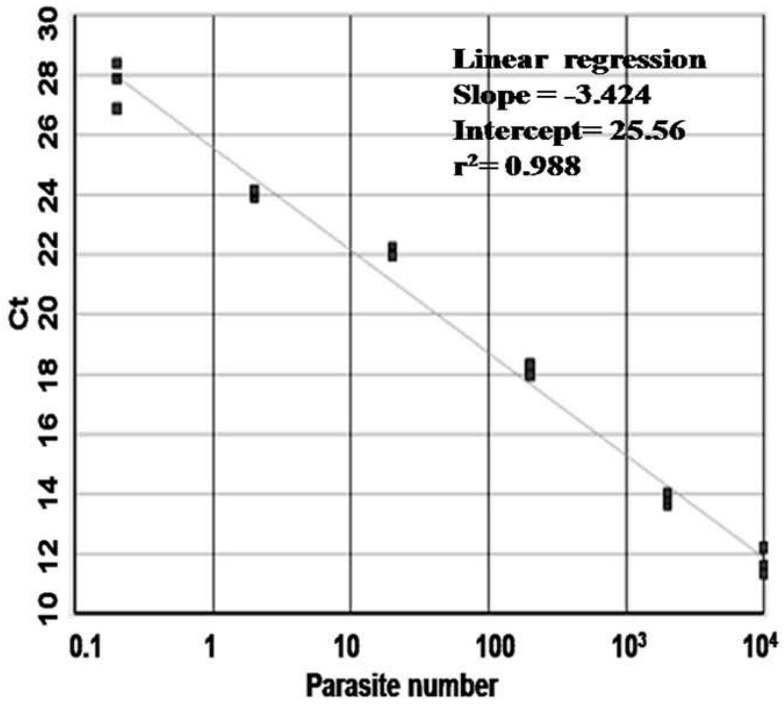


Figure 5.2: **Standard curve obtained using SYBR Green 1 based real-time PCR assay.** Amplification of *Leishmania* parasite DNA represented as the number of parasites per reaction.

Analysis of parasite load in blood samples

Of 246 healthy individuals, 21.54% (n=53) were found positive by qPCR for amplification of parasite DNA and therefore considered to have asymptomatic *Leishmania* infection. The median value of parasite load/ml of blood was 7.7 (range, 1-65). Among these, 12.82% (n=5) were exclusively exposed to household VL contacts and 27.11% (n=32) had neighborhood VL and/or PKDL contacts. Overall, 81.81% (45/53) individuals living in endemic areas had come in contact of VL and/or PKDL either in the household and/or neighborhood (**Table 5.2**). Among these qPCR positive individuals, 37.73% (n=20) had parasitemia levels below 5 parasites/ml, followed by 32.07% (n=17) individuals with parasitemia between 5-10 parasites/ml, 20.75% (n=11) individuals between 11-25 parasites/ml, 5.67% (n=3) individuals between 26-50 parasites/ml and the

rest 3.77% (n=2) with parasitemia above 50 parasites/ml **(Table 5.2)**. Besides, two healthy individuals were found negative by qPCR, but reacted positively to rK39 strip test; reported no history of VL and were considered asymptomatic. Additionally, we observed 8 overlapping cases that were found positive by both qPCR and rK39 test. Additionally, all the 20 individuals from the nonendemic area were found negative by qPCR. For the species identification, PCR-RFLP analysis targeting the internal transcribed spacer 1 (ITS-1) region was performed in 20 randomly selected samples. The species identified in all asymptomatic cases was confirmed to be *L. donovani*.

Table 5.2: Clinico-epidemiological characteristics of the study population living in VL-endemic regions of the state of West Bengal, India

		Household contacts, n (%)	Neighbour contacts, n (%)	Both, n (%)	None, n (%)	Total, n (%)	
% contacts of either VL and/or PKDL in different study groups	Asymptomatic	5 (12.82)	32 (27.11)	8 (25)	10 (17.54)	55 (22.35)	
	EHCs	28 (71.79)	59 (50)	10 (31.25)	21 (36.84)	118 (47.96)	
	HVL	6 (15.38)	27 (22.88)	14 (43.75)	26 (45.61)	73 (29.67)	
	Total	39 (15.85)	118 (47.96)	32 (13)	57 (23.17)	246	
Comparative analysis of rK39, PCR results		**rK39 strip test**					
	PCR	Positive	Negative	Total	kappa value, k		
	Positive	8 (29.62)	45 (20.54)	53 (21.54)	k = 0.089, *P* = 0.13		
	Negative	19 (70.37)	174 (79.45)	193 (78.45)			
	Total	27 (10.97)	219 (89.02)	246			
Analysis of parasite load in Blood Samples	Range of parasite load/ml blood from healthy individuals with or without history of VL from endemic and non-endemic areas, evaluated using qPCR technique						
	Positive	Negative	<5	5–10	11-25	26-50	>50
HVL (n = 89)	16	73	7	5	2	1	1
Rest (n =157)	37	120	13	12	9	2	1
NEHCs (n =20)	0	20					

NB: NEHCs, Non-Endemic Healthy Controls; HVL, Healed Visceral Leishmaniasis

Among the asymptomatic group, the mean age of male (mean±SD, 35.36±16.26) was comparable to that of female (mean±SD, 31.41±16.13). The majority of asymptomatic cases belonged to age group 19-44 years (60%, n=33), followed by the group aged ≥ 45 years (23.63%, n=13) and the pediatric cases aged ≤ 18 years (16.36%, n=9) (**Table 5.3**).

Table 5.3: Age-Gender distribution of the asymptomatic population

Age (yrs)	**Male**		**Female**		**Total**	
	N (%)	Mean±SD	N (%)	Mean±SD	N (%)	Mean±SD
≤18	4 (21.04)	13 ± 17.83	5 (13.88)	9 ±17.36	9 (16.36)	10 ±16.29
19 – 44	10 (52.63)	30.7 ± 16.03	23 (63.88)	29.52 ± 16.19	33 (60)	29.87± 16.2
≥45	5 (26.31)	62.6 ± 16.53	8 (22.22)	50.87± 16.57	13 (23.63)	55.31 ± 17.08
Total	19 (34.54)	35.36 ± 16.26	36 (65.45)	31.41 ± 16.13	55	32.78 ± 16.11

Levels of anti-leishmanial IgG to TSLA and *La*PSA

The host immune response was evaluated in terms of humoral anti-leishmanial IgG marker in different study groups. Serum samples from asymptomatic VL (n=25), HVL (n=21) and EHCs (n=25) groups were tested for the presence of anti-TSLA IgG and anti-PSA IgG and the results are represented as mean ± SD OD_{492}. Total anti-TSLA IgG were found significantly high in HVL (0.38±0.196, $P< 0.001$) compared to asymptomatic (0.04±0.058) and EHCs (0.04±0.027) **(Fig 5.3)**. However, anti-TSLA IgG level of the asymptomatic group was found comparable to EHCs group ($P > 0.05$). Additionally, every individual in HVL group and 12% (n=3) of the asymptomatic group had anti-TSLA IgG absorbance level above cut-off (0.111). On the other hand, *La*PSA did not elicit humoral immune response in any group and anti-*La*PSA IgG in HVL (0.01±0.024) and asymptomatic VL (0.01±0.022) were found comparable to EHCs 0.03±0.063) (**Fig 5.3)**

and therefore, anti-*La*PSA IgG and anti-TSLA IgG subclasses were not done. Against *La*PSA, none of the individuals across different study group showed absorbance value above cut-off (0.219). The cut-off value was calculated as mean+3SD of EHCs group.

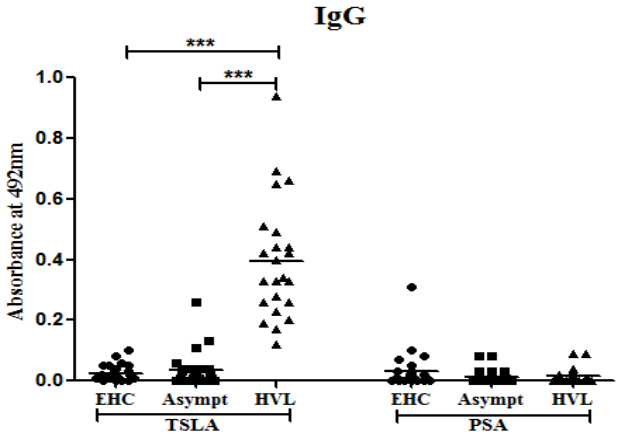


Figure 5.3: Comparison of anti-leishmanial (TSLA and *La*PSA) IgG in sera of EHCs, asymptomatic and HVL individuals. The mean±SD OD_{492} levels were determined by ELISA. The bars represent the means for the different groups. Data were analyzed between groups by the nonparametric Kruskal-Wallis test followed by the post hoc Dunn multiple comparison tests. $P<0.05$ is considered statistically significant. *** $P<0.001$.

Association of parasite load with anti-leishmanial immunoglobulin

The elevated level of parasite load in the asymptomatic group was correlated with anti-leishmanial IgG. We obtained a relatively weak negative correlation between parasite load and IgG (r = -0.229, $P = 0.27$) as represented in **Fig 5.4**.

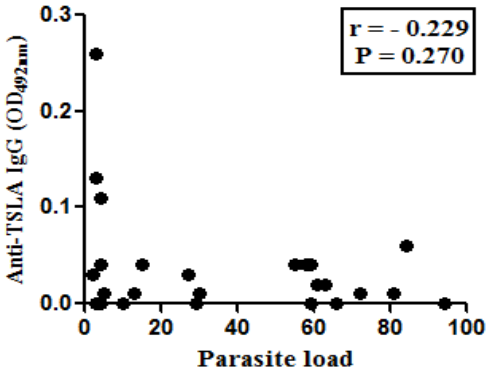


Figure 5.4: Correlation between parasite load and anti-leishmanial IgG level in the asymptomatic group. The correlation was calculated by Karl Pearson correlation test.

Discussion

In the Indian subcontinent, humans are the only known reservoir for the *Leishmania* parasites, therefore apart from the current control measures of VL, such as early diagnosis and treatment of active cases of VL and PKDL, and integrated vector management, correct assessment of asymptomatic infections in the endemic population needs attention. However, the assessment of the actual estimate of *Leishmania* infection in healthy individuals in an endemic area is difficult to assess due to various reasons such as non-availability of appropriate diagnostic methods in centers, the requirement of skilled personnel, the willingness of residents to participate in epidemiological studies and other practical issues.

The present study, to our best knowledge, is the first report from the state of West Bengal, India to assess the magnitude of the asymptomatic *Leishmania* infection in villages of Malda and Murshidabad districts. Herein, molecular and serological methods were combined to identify asymptomatic *Leishmania* infection in healthy individuals living in VL-endemic areas. Owing to simple, easy, field-friendly and cost-effective, we screened whole study population first with rk-39 strip test. The observed seropositivity at baseline was 3.25%, which was comparable to one report from Bihar (5.6%) (Das *et al.* 2011) but lower than that of two other studies conducted in India with seropositivity of 13.79% (Sinha *et al.* 2008) and 13.0% (Gidwani *et al.* 2009). These differences could be due to variations in size of the study population and/or endemic areas under study. Besides, we observed two seropositive healthy individuals who were negative by qPCR, could might be due to degradation and clearance of *Leishmania* DNA after infection, corresponding to the development of protective immunity (Sudarshan *et al.* 2014).

Using highly sensitive quantitative PCR method, the observed positivity at baseline was 21.54%, which was comparatively lower than one study (34.78%) (Sudarshan *et al.* 2014) but higher than other two studies were investigated using conventional PCR with 10% (Salotra and Singh 2006) and 7.2% (Das *et al.* 2011) from the state of Bihar, India and one from adjoining country, Nepal with 12.5% (Bhattarai *et al.* 2009). This higher positivity may be due to higher sensitivity of qPCR compared to that of standard PCR employed in the different studies. Notably, combining both molecular and serological methods have slightly increased the baseline prevalence rate of asymptomatic VL infection to 22.35% for the current study, which was comparable to one previous report 12-39% (Sundar 2003) while another study from Bihar reported a higher rate of 34.78% (Sudarshan *et al.* 2014). Yet another study from Bihar reported the lower prevalence rate of 9.8% (Das *et al.* 2011). The varied rates of asymptomatic infections could be multifactorial such as variation in sample size, geographical areas, the risk level of population, immune and socio-economic status (Alvar *et al.* 2006). Among molecular and serological methods, we observed poor agreement (k=0.089), the finding in line with two earlier reports (Ostyn *et al.* 2011; Sudarshan *et al.* 2014). Therefore, the deployment of combined molecular and serological methods rather than one, proved superior for screening the human population for the asymptomatic *Leishmania* infection and that positive serology results may not stand necessarily be true for a molecular method of *Leishmania* detection.

In the current study, a broad range of parasite load was observed in peripheral blood of the screened asymptomatic cases. The quantitative PCR assay was found positive in 21.54% of the total study population. The majority had high parasitemia and

so more likely to turn into full blown VL disease as compared to those with lower parasitemia, as reflected by the similar study (Sudarshan *et al.* 2014). Further, their routine follow-up is required to know the conversion rate of symptomatic cases or their status of infection. Besides, among individuals with a history of VL, 17.97% were found positive by qPCR, similar to two other reports, one from India 23% (Srivastava *et al.* 2013) and another one from Nepal 26.1% (Bhattarai *et al.* 2009). Generally, the parasite DNA does not often last longer than a year, but rarely may last for decades (Guevara *et al.* 1993). Persistence of *Leishmania* DNA and DAT antibodies has been demonstrated immediately after VL treatment both in Sudan and India (Zijlstra *et al.* 2001; Maurya *et al.* 2005) while during their follow-up DAT remained positive but PCR became negative in the majority of HVL cases. Considering the time lapse following VL treatment (1–20 years), the PCR-positive cases might either represent re-infections or relapses. Seronegative individuals having positive qPCR test could occur if the individual was bitten by a *Leishmania*-infected sandfly, either immune response has not yet developed or antibody levels are not high enough to be detectable by the methods employed. Similar findings were made by other investigators from India, where out of 1068 EHCs seronegative for *Leishmania* antigen, 31.8% (n=340) were found positive by qPCR (Sudarshan *et al.* 2014).

Besides, the host immunity to TSLA and *La*PSA were evaluated in terms of humoral response in different study groups to identify a marker for asymptomatic. As expected, elevated levels of anti-TSLA IgG were observed in HVL group compared to asymptomatic and EHCs whereas it was found comparable between asymptomatic and EHCs, and only 12% asymptomatic cases showed observable IgG level, indicating

TSLA, not a good choice for identifying asymptomatic cases of *Leishmania* infection in endemic area. Additionally, the weak negative correlation was observed between anti-TSLA IgG and parasite load for the asymptomatic group, implying that the humoral responses do not mirror the parasite load. On the other hand, *La*PSA did not elicit a humoral immune response in any group and the anti-*La*PSA levels across different study groups were found comparable. Therefore, neither TSLA nor *La*PSA could be a good choice for differentiating asymptomatic cases from HVL and EHCs for *Leishmania* infection in the VL-endemic areas.

There were some limitations in this study. First is the selection of study area as only two highly endemic districts were selected. Hence, the observed prevalence of the asymptomatic cases cannot be generalized to all VL-endemic areas. Secondly, not all villagers were willing to participate and so could not attend the camp for clinical examination and investigations. Third, the follow-up study of all the recruited individuals has not been included in the current study. Although the qPCR method is clearly more sensitive and specific than serological methods, neither approach is perfect. It is evident from our study as well from other studies (Das *et al.* 2011; Sudarshan *et al.* 2014) that some asymptomatic cases were found negative by qPCR but had positive serology test. Hence, a combination of both, qPCR to detect parasitemia, and serology to identify individuals with high titers may be an appropriate approach for early monitoring of *Leishmania* infection in the healthy population who might also pose a reservoir for disease transmission.

Conclusion

The study identified 22.35% asymptomatic individuals living in the two VL-endemic districts of West Bengal. These could be the potential reservoir for *Leishmania* parasite and might play an important role in the disease transmission in the region. Besides, a qPCR method is more sensitive and specific than the serological method in assessment of asymptomatic VL individuals. Additionally, anti-TSLA or anti-*La*PSA IgG based detection of asymptomatic infection in healthy individuals may not be a good choice. Thus, deployment of combined molecular and serological methods could effectively help estimate the asymptomatic *Leishmania* infection in healthy individuals living in endemic regions, and may contribute to early case detection and treatment. Furthermore, the knowledge of quantitative estimation of asymptomatic VL individuals in the endemic area will be useful to take appropriate measures for the sustainable elimination of VL from India.

Conclusions and future scope

Individuals recovered of VL or with asymptomatic *Leishmania* infections generally acquire a robust and durable immunity to re-infection. This resistance to infection is based on Th1 dominant cellular response with interferon (IFN)-γ secretion which eventually leads to macrophage activation and parasite killing (Sacks and Noben-Trauth 2002; Mansueto *et al.* 2007; Nylén and Gautam 2010). Based on these observations, one vaccine strategy targeting dominant Th1 responses elicited in immune individuals by defined parasite antigens has been utilized. One such vaccine candidate antigen is the Parasite Surface Antigen (PSA) that belongs to a unique *Leishmania* family of membrane-bound and secreted proteins. Further, it induces Th1-mediated protection against murine leishmaniasis when used as a vaccine (McMahon-Pratt *et al.* 1993; Handman *et al.* 1995b). It is also specifically recognized by Th1 cells of humans with a history of self-healing CL (Kemp *et al.* 1998). PSA is expressed at both promastigote and amastigote stages (Handman *et al.* 1995a; Beetham *et al.* 2003) and reported to be present in several *Leishmania* species (Lohman *et al.* 1990; McMahon-Pratt *et al.* 1992; Myung *et al.* 2002; Devault and Bañuls 2008). The choice of PSA as vaccine candidate is further supported by previous (Lemesre *et al.* 2005, 2007) and recent studies (Bras-Gonçalves *et al.* 2014) demonstrating that sera of dogs vaccinated with *L. infantum* excreted/secreted antigens in combination with MDP, recognized immunodominant antigens belonging to PSA protein family, from *L. infantum* ESA (*Li*PSA) and from *L. amazonensis* ESA (*La*PSA). Therefore, PSA stands a suitable choice to be evaluated as a vaccine candidate against VL, caused by *L. donovani*. The present study evaluated the recombinant native form of *La*PSA protein for its ability to induce cellular and humoral immune responses in active cases of VL and PKDL, HVL and naïve individuals for its potential use as a

vaccine candidate against human VL caused by *L. donovani*. The study demonstrated *Leishmania*-specific cellular immune responses in terms of lymphoproliferation and cytokine response in PBMCs upon stimulation by *Leishmania* antigens i.e.; TSLA and *La*PSA in cases of VL, PKDL and HVL. Lymphoproliferation in response to TSLA in HVL and PKDL groups gave heightened response whereas *La*PSA did not induce lymphoproliferation in either of the two *Leishmania* pre-exposed groups i.e., HVL or PKDL group. The cytokine profile upon *La*PSA and TSLA stimulation of PBMCs corroborated the lymphoproliferation data; *La*PSA did not induce significant pro-inflammatory cytokines production in HVL or PKDL while TSLA elicited a strong protective cytokine response in the same groups. Besides, our data on TSLA highlighted the presence of cytotoxic activity via granzyme B secretion and activation of the significantly high percentage of $CD8^+$ and $CD4^+$ T cells *in vitro* in both HVL and PKDL groups; revealing for the first time the role of cytotoxic cells in resistance to re-infection. However, *La*PSA did not induce significant activation in either $CD8^+$ or $CD4^+$ T cells. Analysis of humoral response showed that the *La*PSA antigen did not induce a significant antibody response in either active VL or PKDL group. Based on the findings of this study, the recombinant *La*PSA antigen does not hold promise as a potential vaccine candidate for human VL, caused by *L. donovani*. Further, generalized cellular immunity based on the phenotype and proportion of the different peripheral blood lymphoid subsets revealed impaired cellular immunity in active VL while PKDL showed a raised proportion of $CD3^+$ T cells. Besides, the present study is the first of its kind to investigate all the four groups of human population simultaneously.

The other goal of the study was to understand the magnitude of asymptomatic *Leishmania* infection in endemic regions of the state of West Bengal, India. This was based on serological (rk39 strip tests) and molecular (qPCR) analysis of *Leishmania* parasites in the peripheral blood of healthy individuals. The study identified 22.35% asymptomatic individuals living in the two districts of the state of West Bengal, endemic for VL. Further, the detection of asymptomatic *Leishmania* infection in healthy individuals using ELISA, neither anti-TSLA nor anti-*La*PSA IgG stands to be a good choice. Further, the study concluded that the deployment of combined molecular and serological methods rather than one effectively estimated the magnitude of asymptomatic *Leishmania* infection in healthy individuals living in VL-endemic regions.

Future scope

The study demonstrated that the recombinant *La*PSA does not hold promise as a potential vaccine candidate against human VL caused by *L. donovani*. Using four groups of human population simultaneously, the results with TSLA showed clear dominant protective cellular and humoral immune response in *Leishmania* immune group. The study streamlined approach for future evaluation of a protein antigen, for its cellular and humoral protective responses in human populations and thus will help prioritize antigen selection for clinical development of a vaccine against leishmaniasis. Besides, the study demonstrated for the first time the role of cytotoxic cells in resistance to *Leishmania* re-infection in HVL and PKDL. This knowledge could be exploited for the design and evaluation of future vaccine candidates against human leishmaniasis.

The other objective of the study identified 22.35% asymptomatic VL individuals living in the two major endemic districts namely Malda and Murshidabad of the state of

West Bengal, India. These could be the potential reservoir for *Leishmania* parasite and might lead to a high incidence of disease in the region. Therefore, close follow-up of such cases needs to be taken up. Furthermore, the knowledge of quantitative estimation of asymptomatic individuals in the endemic area will be useful to take appropriate measures for the sustainable VL elimination from India. Additionally, further work should consider understanding the underlying principles of protective cellular immunity in asymptomatic VL cases and further why the only small fraction of asymptomatic gets converted to symptomatic VL.

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Appendix

Appendix

Preparation of reagents

Phosphate Buffer Saline (PBS)

8gm of NaCl, 2gm of KCl, 1.44gm of Na_2HPO_4 and 0.2gm of KH_2PO_4 were dissolved in 800ml of DW. The pH was set to 7.4 with HCl. Final volume was made up to 1 liter and sterilized by autoclaving at 15lb/sq.in for 20 minutes and stored at room temperature.

Bicarbonate coating buffer

Dissolved 1.59g Na_2CO3 and 2.9g NaHCO3 in 1liter distil water; pH should be 9.6 without adjustment.

Blocking solution

Blocking solution was freshly prepared by adding 3g BSA, 20mg sodium azide and 100µl Tween-20 initially to 80ml of 1X PBS. After complete dissolution of BSA, the volume was made up to100ml.

Washing Buffer

Dissolve 1ml of Tween-20 in 1litre of freshly prepared 1X PBS, pH 7.4.

RBC lysis buffer

8.3g NH_4Cl and 1g KHCO3 was dissolved in 1litre distilled water. The pH of the solution was adjusted to pH7.2-7.4 and filtered with 0.22µm. The lysis buffer was stored at RT in dark until use.

Fixation buffer

To prepare 100ml of fixation buffer, 1ml of formaldehyde was added to 90 ml of 1x PBS and the volume were made up to 100ml.

Staining Buffers

1X PBS + 0.1% (w/v) sodium azide + 3% heat-inactivated fetal bovine serum. Adjust buffer pH to 7.2 – 7.4, filter (0.2 µm pore membrane), and store at 4ºC.

Preparation of Cytometer Setup Beads

50 µL of Cytometer Setup beads to three cytometer setup tubes labeled A, B and C was added. Then, 50 µL of FITC positive control detector was added to tube B. 50 µL of PE positive control detector was added to tube C. Tubes A, B and C were incubated

for 30 min., at RT and in the dark. 450 µL of wash buffer was added to tube A and 400 µL of wash buffer was added to tubes B and C.

Antibiotics

Ampicillin

100mg/ml Ampicillin stock was prepared in autoclaved distilled water and sterilized by filtration through 0.22 µm filter. 100µl aliquots were stored by freezing at –20°C.

Chloramphenicol

34 mg/ml chloramphenicol stock was prepared in ethanol and stored at –20°C. Chloramphenicol is required to ensure the presence of pLysS.

Stock solution of commonly used reagents

1M Tris

121.1gm of Tris base was dissolved in 800ml of distil water and pH set (6.8, 7.4, and 8.0) with concentrated HCl. Volume was made up to 1liter and autoclaved.

0.5M EDTA

186.1gm of disodium EDTA.-2H_2O was added in 800ml of dw, stirred vigorously on a stirrer, pH set to 8.0 with NaOH (~20 gm of NaOH pellets) and volume made up to1liter and autoclaved.

Sodium Phosphate (1M)

Monobasic

138gm of $NaH_2HPO_4.H_2O$ was dissolved in 800ml of distilled water and the volume made up to 1liter.

Dibasic

268gm of $Na_2HPO_4.7H_2O$ was dissolved in 700ml of distilled water and the volume made up to 1liter.

Publications and Presentations

Publications and Presentations

Research publications

1. **Kaushal H**, Bras-Gonçalves R, Avishek K, Deep DK, Petitdidier E, Lemesre JL, Papierok G, Kumar S, Ramesh V, Salotra P. Evaluation of cellular immunological responses in mono- and polymorphic clinical forms of Post Kala-Azar Dermal Leishmaniasis in India. ***Clin Exp Immunol***. **2016** Jul; 185(1):50-60.

2. Ramesh V, **Kaushal H**, Mishra AK, Singh R, Salotra P. Clinico-epidemiological analysis of Post kala-azar dermal leishmaniasis (PKDL) cases in India over last two decades: a hospital based retrospective study. ***BMC Public Health***. **2015** Oct 26; 15(1):1092.

3. **Kaushal H,** Bras-Gonçalves R, Negi NS, Lemesre JL, Papierok G, Salotra P. Role of $CD8^+$ T cells in protection against *Leishmania donovani* infection in healed Visceral Leishmaniasis individuals. ***BMC Infect Dis***. **2014** Dec 3; 14:653.

4. Chamakh-Ayari R, Bras-Gonçalves R, Bahi-Jaber N, Petitdidier E, Markikou-Ouni W, Aoun K, Moreno J, Carrillo E, Salotra P, **Kaushal H**, Negi NS, Areval o J, Falconi-Agapito F, Privat A, Cruz M, Pagniez J, Papierok GM, Rhouma FB, Torres P, Lemesre JL, Chenik M, Meddeb-Garnaoui A. *In vitro* Evaluation of a Soluble *Leishmania* Promastigote Surface Antigen as a Potential Vaccine Candidate against Human Leishmaniasis. ***PLoS One*.** **2014** May 2; 9(5):e92708.

5. Katara GK, Raj A, Kumar R, Avishek K, **Kaushal H**, Ansari NA, Bumb RA, Salotra P. Analysis of localized immune responses reveals presence of Th17 and Treg cells in cutaneous leishmaniasis due to *Leishmania tropica*. ***BMC Immunol.*** **2013** Nov 22; 14(1):52.

6. Khandelwal K, Bumb RA, Mehta RA, **Kaushal H**, Lezama-Davila C, Salotra P, Satoskar AR. A patient presenting with Diffuse Cutaneous Leishmaniasis (DCL) as a first indicator of HIV infection in India. ***Am J Trop Med Hyg.*****2011** Jul; 85(1):64-5.

7. Prasad N, Ghiya BC, Bumb RA, **Kaushal H**, Satoskar AA, Lezama-Davila CM, Salotra P, Satoskar AR. Heat, Oriental sore, and HIV. ***Lancet* 2011** Feb 12; 377(9765):610.

Chapter in Book

1. Poonam Salotra, **Himanshu Kaushal**, V Ramesh (2015). Containing Post Kala-Azar Dermal Leishmaniasis (PKDL): Pre-requisite for sustainable elimination of Visceral Leishmaniasis (VL) from South Asia. Chapter in "Kala Azar in South Asia; Current Status and Sustainable Challenges", 2nd edition, Ed, Eisei Noiri & T.K. Jha, Springer International Publishing AG, (Submitted)

Presentations/Abstracts published in Proceedings

1. **Himanshu Kaushal**, Rachel Bras-Gonçalves, Narender Singh Negi, Jean-Loup Lemesre, Gerard Papierok, Poonam Salotra. *In vitro* evaluation of protective role of $CD8^+$ T cells in individuals immune to *Leishmania donovani*. **Oral presentation** at 40th Annual Conference of Indian Immunology Society, IMMUNOCON 2013, November 15-17, 2013, held at UCMS, Delhi University, Delhi, India. P55.
2. **Himanshu Kaushal**, Venkatesh Ramesh, Narender Singh Negi, Poonam Salotra. Enhanced IFN-γ and TNF-α level in post kala-azar dermal leishmaniasis upon stimulation with *Leishmania* antigen. **Oral presentation** at 39th Annual Conference of Indian Immunology Society, IMMUNOCON 2012, November 9-11, 2012, held at BHU, Varanasi, India. P66.
3. Sophie Bonnel, Frédéric Vézilier, **Himanshu Kaushal**, Poonam Salotra, Julie Pagniez, Elodie Petitdidier, Rachel Bras Gonçalves, Jean-Loup Lemesre and Rapsodi Consortium. "Inter individuals variability of blood donor's Macrophages to get infected by *Leishmania donovani* promastigotes" Consortium in the international conference on neglected protozoan diseases held at Institut Pasteur, Paris, France on Sept 24, 2010, P2.

Biographies

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POSITIONS HELD

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22.01.2014–31.07.2015: NASI-Project SRF, National Institute of Pathology

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AWARDS AND SCHOLARSHIPS

1. Won Dr. S. Sriramachari Young Scientist Award for the year 2014 conferred by National Institute of Pathology (ICMR)
2. Qualified National Academy of Sciences, India (NASI)-Project SRF, 2014
3. Qualified Indian Council of Medical Research (ICMR)-SRF, 2011
4. Qualified Indian Council of Medical Research (ICMR)-JRF, 2008
5. Qualified joint CSIR-UGC test for Lectureship-NET, June 2008
6. Qualified Graduate Aptitude Test in Engineering (GATE), 2006 conducted by IIT, Kharagpur
7. Qualified All India Entrance Test for M.Sc. (Toxicology), 2004 conducted by Jamia Hamdard, New Delhi.

8. Qualified Bihar State Entrance Test for B.Sc. (Bio-Technology), 2000 conducted by Patna University, Bihar (Rank-07)
9. Received Sainik School Studentship (1989-1995) from Ministry of Defence, Govt. of India

MEMBERSHIP OF NATIONAL/INTERNATIONAL BODIES

1. **National**: Indian Immunology Society (Life Member)

SCIENTIFIC TRAININGS/ WORKSHOPS

2015: **Workshop on Research Methodology for Medical & Biomedical Scientists**, **NIP (ICMR), New Delhi -** Participated as member of scientific committee (organizer)

2014: **15th Indo-US Flow Cytometry Workshop at BD-JH** FACS™ **Academy, Jamia Hamdard, New Delhi -** Received two day training on advanced workshop on Multicolor Flow Cytometric Immunophenotyping

2013: **Shimadzu Analytical (India) Pvt. Ltd.**
Received two day training on Fundamental of HPLC with LC solution workstation

2012: Participated in **ICMR workshop** on "**Biomedical Communication**" held at the ICMR Hqrs., New Delhi

2009: **BD Biosciences Training Center, Gurgaon (India)**
Received three day training in Basic course on BD FACSCalibur

2009: **Dako Academy & DSS Training, New Delhi**
Received three day training on basics of Immunohistochemistry-A Diagnostic Tool

2006: Completed dissertation work on project entitled "Cytochrome P4501A1, Cytochrome P450 1A2 and NADPH oxidase gene polymorphisms in relation to chronic obstructive pulmonary disease" at **Institute of Genomics and Integrative Biology (IGIB), Delhi,** India

2002: **Institute of Animal Health & Production, Vetenary Council of India, Patna**
Received one month summer training on production & quality control of bacterial and viral vaccines

2001: **Sen Medical Research Centre, Patna** (Regd. Under Societies Act of 1860)
Received one month summer training on clinical laboratory techniques.

Brief Biography of the Supervisor

Personal Particulars

- Name Poonam Salotra
- Designation : Senior Deputy Director
- Place of work : National Institute of Pathology (ICMR), Safdarjung Hospital Campus, New Delhi – 110029, India.
- Tel. No: 011-26198402, 011-26166124
- Fax No: 011-26166124
- Email: salotra@vsnl.com, salotrap@icmr.org.in

Academic Qualifications

- B.Sc. Hons (Chemistry) from Delhi University in 1974.
- M.Sc. (Biochemistry) from P.G.I., Chandigarh in 1976.
- Ph.D. from V.P. Chest Institute, Delhi University in 1980.

Professional Appointments:

- Postdoctoral Fellow at Roche Institute of Molecular Biology, Nutley, U.S.A. (1980).
- Research Officer at Tuberculosis Research Centre, Madras (1982).
- Principal Investigator under Young Scientists scheme of Department of Science & Technology at Centre for Biotechnology, J NU, New Delhi (1991).
- Pool Research Officer (CSIR) at Centre for Biotechnology, JNU, New Delhi (1993).
- Senior Research Officer at Institute of Pathology (ICMR), New Delhi (1996).
- Assistant Director at Institute of Pathology (ICMR), New Delhi (2000).
- Deputy Director at Institute of Pathology (ICMR), New Delhi (2005 till date)

Research Interests:

- Worked mainly on the molecular basis of pathogenesis of infectious diseases such as Kala-azar, Anthrax, Tuberculosis and Cholera. Currently working on development of diagnostic tests and attenuated vaccines for kala-azar, mechanism of drug resistance

in Indian kala-azar, and characterization of immune responses in patients of kala-azar and post kala-azar dermal leishmaniasis.

Awards/Honors

1. Fellow of the National Academy of Medical Sciences, India (FAMS).
2. Fellow of The World Academy of Sciences (FTWAS).
3. Appointed by WHO as member of Expert Committee for Leishmaniasis control, TDR- WHO in 2010.
4. Awarded ORISE Fellowship by Centre for Biologics Evaluation and Research, FDA, USA in 2010.
5. Appointed Associate Editor of a BMC journal in 2009.
6. Elected Fellow of the National Academy of Sciences, India, in the year 2008.
7. Basanti Devi Amir Chand Award conferred by ICMR in 2007.
8. Prof. BK Aikat Award conferred by Indian Council of Medical Research conferred by ICMR in 2007.
9. Bill and Melinda Gates Global Health Series award for presenting work on "Drug resistance in Visceral Leishmaniasis" at Keystone Symposium in California, USA, 2007.
10. ICMR International fellowship for Senior Biomedical Scientists for the year 2006.
11. Awarded Courtesy Fellowship by CBER, FDA, USA in Sep 2005.
12. Awarded fellowship by National Foundation of Infectious Diseases, USA in 2005.
13. Granted ICMR Award for Excellent Research output in July 2004.
14. Silver Jubilee award by Indian Association of Medical Microbiology in 2003.
15. Awarded Courtesy Fellowship by CBER, FDA, USA in Dec 2003
16. Kshanika Oration Award, a National award for Eminent Woman Scientist, conferred by Indian Council of Medical Research in 2002.
17. National Science Talent Scholarship awarded by N.C.E.R.T. , New Delhi

Patents

1. US Patent No. 6,855,522, entitled "Species-specific PCR assay for detection of *Leishmania donovani* in clinical samples of kala-azar and PKDL" (2002).
2. Indian patent no. 243725 for "Live attenuated *Leishmania* vaccines" (2006).
3. US patent no. 7887812 for "Live attenuated *Leishmania* vaccines" (2011).
4. Indian patent filed (application no. 349/DEL/2014) for "Loop-mediated isothermal amplification (LAMP) assay for a reliable and rapid diagnosis of *Leishmania* infection" (2014).

Membership of professional associations

1. Society for Parasitology, India (Life member).
2. Society of Biological Chemists, India (Life member).
3. Association of Clinical Biochemists of India (Life member)
4. Indian Association of Medical Microbiologists, India (Life member
5. Indian Immunology Society, India (Life member).

Publications

Publications in peer-reviewed journals	106
Chapter in Books	08

Brief Biography of the Co-Supervisor

Personal Particulars

- Name Sanjeev Kumar
- Designation : Associate Professor
- Place of work : Dept. of Biological Sciences
  Birla Institute of Technology & Science, Pilani, Rajasthan
- Tel. No: + 91 1596 515 670
- Email: sanjeev@pilani.bits-pilani.ac.in

Academic Qualifications

- B.Sc. (Zoo, Bot, Chem) from Meerut University, Meerut 1991.
- M.Sc. (Biotechnology) from Guru Nanak Dev University, Amritsar in 1994.
- Ph.D. from Institute of Medical Sciences, BHU, Varanasi in 1999.

Professional Appointments:

Feb 2013 - Present	**Associate Professor**, Department of Biological Sciences, Birla Institute of Technology and Science (BITS), Pilani, Rajasthan, India.
June 2008 – Jan 2013	**Assistant Professor**, Department of Biological Sciences, Birla Institute of Technology and Science (BITS), Pilani, Rajasthan, India.
Dec 2003 - May 2008	**Research Fellow (*a Federal Full time equivalent, FTE, position)***, Laboratory of Malaria and Vector Research, National Institutes of Health (NIH), Rockville, MD, USA (*in the lab of Dr. Carolina Barillas-Mury*).
Dec 2000 - Nov 2003	**Post Doctoral Fellow**, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO, USA (*in the lab of Dr. Carolina Barillas-Mury*).
Feb – Nov, 2000	**INSERM foreign scientist**, Lymphoid cell differentiation and gene recombination laboratory, INSERM-CNRS Centre d'Immunologie Marseille-Luminy (CIML), Marseille, France (*in the lab of Dr. Pierre Ferrier*).

Research Interest:

Worked to understand the regulation of *Plasmodium* development by mosquito innate immune system. His previous research characterized two peroxidase enzymes in the gut of mosquito which modulates innate immunity against natural symbiotic bacteria. Gene silencing of these peroxidases also suppresses *Plasmodium* development (***Kumar et al, 2010. Science. 327. 1644-48***). Currently studying the combined effect of mosquito peroxidases and other innate immune molecules on *Plasmodium* development through gene silencing methodology. In addition, also developing ways to manipulate novel mosquito immuno-active molecules which can block *Plasmodium* development and subsequent transmission among humans.

Awards/Honors

1. Best oral presentation award for "Identification of plant secondary metabolites as potent mosquito-cidals" 4thcongress on insect science, April 16-17, 2015, Punjab Agriculture University, Ludhiana, India.
2. Received travel awards in recognition of outstanding achievements in XXIV International Congress of Entomology, South Korea.
3. Assortment of our Science research article as '**Hot Results for Scientific Community**' in '***Science* Express'** for rapid publication (2010).
4. NIH, Bethesda, USA employee special performance achievement award (2008).
5. NIH, Bethesda, USA employee special performance achievement award (2007).
6. NIH, Bethesda, USA employee special performance achievement award (2006).
7. NIH, Bethesda, USA employee special performance achievement award (2005).
8. NIH, Bethesda, USA employee special performance achievement award (2004).
9. NIH, Bethesda, USA research fellowship (2003-2008).
10. Outstanding achievement Post-doc category poster awards, Colorado State University, Fort Collins, CO, USA (2003).
11. Post-doctoral fellowship, Colorado State University (2000-2003).
12. Post-doctoral fellowship for foreign scientists by 'Institut National de la Santè et de la Recherche Médicale (***INSERM***)' France (Feb-Nov 2000).
13. Senior research fellowship, UGC, Government of India (1997-2000).

14. Junior research fellowship, UGC, Government of India (1995-1997).
15. *Gold Medal* for outstanding achievements during M. Sc degree, Guru Nanak Dev University (GNDU), Amritsar, India (1994).
16. Student fellowship during M. Sc. degree, Department of Biotechnology (DBT), Government of India (1992-1994).

Membership of professional associations

- The American Society of Tropical Medicine and Hygiene (ASTMH), USA.
- Keystone Symposia Society, USA.
- Society of Biological Chemists, India.
- Indian Society of Cell Biology, India.
- Indian Society of Parasitology

Publications

Publications in peer-reviewed journals 33