CHAPTER I

REVIEW OF LITERATURE
Introduction

Post kala-azar dermal leishmaniasis (PKDL) is a chronic, dermal sequel of visceral leishmaniasis (VL) or kala-azar that occurs in 10-20% and up to 60% of patients treated for VL, in the Indian subcontinent/South Asia and Sudan respectively [Mondal and Khan, 2011]. PKDL was first described almost 90 years ago in Sudan by Christopherson and in India by Sir U.N.Brahmachari in 1922, when he initially termed the disease as ‘dermal leishmanoid’ [Zijlstra et al. 2001, Brahmachari, 1922]. PKDL is an enigmatic disease for researchers and clinicians owing to the complex nature of the disease in terms of diagnosis and treatment. PKDL is caused mainly by *L. donovani*, however involvement of *L. infantum* and *L. chagasi* have also been reported [Antinori et al. 2007, Desjeux and Ramesh 2011]. Patients with PKDL hold special importance in South Asia as VL is anthropoontic in transmission and therefore these patients are considered as the only reservoirs of VL [Thakur et al. 2008]. So, it should be stressed that the kala-azar elimination programme will not be effective until we can eliminate PKDL. To eliminate PKDL it is necessary to explore the cause of the disease which to date is still not clear as in South Asia, only 10%-20% of patients with VL eventually develop PKDL.

Epidemiology

In India, PKDL was reported to be 32 per 10,000 in 1954 [Majumdar TD, 1969]; later the situation improved with good vector control done for malaria elimination and effective reduction in the sand fly population stopped the transmission. This anti-malaria campaign showed good results and the incidence rate declined to 2.5 per 10,000 population in 1964 [Ramesh V and Mukherjee A, 1995]. Sadly this improvement was lost in subsequent years as there was a slack in vector control and there was resurgence with resulted in the prevalence rate increasing to 48.2 per 10,000 in 1989 [Ramesh V and Mukherjee A, 1995, Rai et al. 1989].

The incidence of VL and PKDL generally runs a parallel course which signifies chronicity of infection [Napier LE and Krishnan KV, 1933]. In a recent epidemiological study in Bangladesh, it was noted that the incidence of PKDL showed a steep rise (from 1 case per 10,000 in 2002-2004 to 21 cases per 10,000 in 2007), notably at a time when the incidence of VL was declining following a peak in 2004-05 [Rahman et al. 2010]. As there was a lag period between cure from VL and onset of PKDL, it signified that PKDL echoes the epidemic of kala-azar and this echo can persist well after the epidemic. In the last 25 years, in India the incidence of VL peaked in 1992 and smaller peak in 2007 [WHO, accessed on 6th of January]. Therefore extrapolating from the Bangladesh experience, incidence of PKDL in India may well see a sharp rise in the years to come.
The clinic-epidemiological aspects of PKDL are important for our proper understanding of the transmission dynamics, as also for defining VL control strategies. All major epidemiological studies with PKDL across Sudan, India, Nepal and Bangladesh have reported no sex biasness [Figure 1.1, Zijlstra et al. 2003, Mondal et al. 2010, Rahman et al. 2010, Uranw et al. 2011, Das et al. 2012]. However, studies from our group showed a predominance of male patients [Ganguly et al. 2008, Ganguly et al. 2010a]. Similarly, reports from PKDL endemic areas also suggested that patients treated with SAG during their VL are more likely to develop PKDL. In India, 73% of patients with PKDL were treated with SAG while in Sudan, Bangladesh and Nepal, 100% were treated with SAG for VL [Zijlstra et al. 2003, Mondal et al. 2010, Rahman et al. 2010, Uranw et al. 2011, Das et al. 2012]. There are epidemiological differences between PKDL in Sudan vs. South Asian PKDL summarised in Table 1 [Zijlstra et al. 2003, Mondal et al. 2010, Das et al. 2012].

**Table 1: Epidemiological differences between Sudanese and South Asian PKDL**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Sudanese PKDL</th>
<th>South Asian PKDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag period (gap between cur of VL – development of PKDL)</td>
<td>0-6 month</td>
<td>2-10 years, can be up to 40 years</td>
</tr>
<tr>
<td>Prevalence rate</td>
<td>50-65% of patients cured of VL, 4.8/100 individuals</td>
<td>10-20% of patients cured of VL, 4.8/1000 individuals</td>
</tr>
<tr>
<td>Age group</td>
<td>4-8 years</td>
<td>Adults (20-28 years)</td>
</tr>
</tbody>
</table>

**Figure 1.1: Global distribution of post-kala-azar dermal leishmaniasis, 2005–2010**

PKDL serves as the only reservoir of VL in the inter-epidemic period [Zijlstra et al. 2003] and therefore an upsurge can mean a real threat to the kala-azar elimination program which aims to bring down the annual incidence of VL to less than one per 10,000 population at the district or sub-district level by the end of year 2015 [www.who.int/tdr/publications/documents/kala_azar_indicators.pdf]. Thus, the presence of PKDL in the community could well initiate another outbreak of VL as highlighted previously in West Bengal, India where the epidemic in 1980 was traced to a PKDL patient who developed PKDL lesions in 1976 [Addy and Nandy, 1992]. Taken together, PKDL is a disease of epidemiological significance and elimination of PKDL must be achieved for a VL free world.

Clinical manifestations and pathological features of PKDL

Patients with PKDL are apparently healthy except for the cosmetic problem i.e. skin lesions. Clinically these lesions are classified as hypopigmented macules, papules and nodules along with erythematous plaques. Macules are present in any part of the body and are usually symmetrical in nature whereas papules and nodules appear more commonly on exposed areas like the face, ear lobes etc. [Singh et al. 2011]. The distribution of dermal lesions is also different between India and Sudan, papular or nodular lesions are more common while polymorphic cases (macules along with papules or nodules) predominate in India. In contrast, macular cases are highest in Bangladesh [Mondal et al. 2010]. Lymphoadenopathy is a common finding in Sudanese PKDL in contrast to India [Zijlstra et al. 2003]. The histopathological features of PKDL in India comprises absence of granuloma formation, heavy infiltration of plasma cells followed by lymphocytes and macrophages while in Sudan, plasma cells are absent in almost all cases and akin to Indian PKDL, granuloma formation is rare [Rathi et al. 2005a, Ismail et al. 2006a]. Cellular infiltration is always higher in nodular patients than macular PKDL. Interestingly, distinct pathological features exist within different ethnic variants of PKDL as in the nodular form of the disease, epidermal atrophy or thinning occurs in Indian PKDL whereas Sudanese PKDL is characterized with epidermal thickening or acanthosis [Rathi et al. 2005a, Ismail et al. 2006a]. The distribution of lesional CD4⁺ and CD8⁺ T cells is different between Sudanese and Indian PKDL as in Indian PKDL there is a preponderance of CD8⁺ T cells while in Sudanese PKDL, CD4⁺ T cells predominate [Rathi et al. 2005b, Ismail et al. 2006a]. Collectively, these variations indicate that the pathophysiology of the disease is possibly different in Sudan and India which would impact upon the disease management.
Chapter I: Review of Literature
Available diagnostics and treatment strategies

Diagnosis of PKDL is largely dependent on a prior history of VL and clinical suspicion, but absence of a prior history of VL occurs in 10-15% cases [Singh et al. 2011, Salotra and Singh 2006]. Till date, diagnostic methods applied to detect PKDL cases are parasitological, serological and PCR based with each having their own pro and cons. Demonstration of L.D bodies in skin slit smear or culture of parasites from biopsies are still considered as a gold standard in diagnosis of PKDL but both methods have low sensitivity, particularly for the macular variant [Salotra et al. 2003]. Another method for parasite identification using *Leishmania* specific monoclonal antibody G2D10 (raised against *Leishmania gerbelli*) was used [Beena et al. 2003] which showed better results than the haematoxylin and eosin staining (H&E method) as it had sensitivity close to 90% [Salotra et al. 2003].

Among the serological tests available, rk39 based ELISA or immunochromatographic strip test is still the best method, rarely there may be a misdiagnosis [Das NK et al. 2011]. The major problems with the serological tests are (1) firstly, immunocompromised and macular groups of patients give low sensitivity with either DAT or ELISA or immunochromatographic strip based methods and (2) secondly, one cannot exclude the possibilities of false positive tests because antibodies can be stable up to many years within patients with a prior history of VL [Chappuis et al. 2007]. To resolve this problem, an assay based on IgG avidity against rk16 antigen was developed by Redhu *et al.*; however in their study, they tested only 8 PKDL patients and the assay needs to be validated [Redhu et al. 2006].

To circumvent all these problems detection of antigen through a molecular approach is considered as a better option and in the recent past, multiple *Leishmania* DNA based PCR approaches (targeting 18S rRNA, SSU rRNA, mini exon gene repeat, kinetoplast maxi and mini circle, Internal transcribed spacer sequence specific DNA etc) are being used as a diagnostic tool for PKDL [Singh et al. 2011, Salotra and Singh 2006]. In addition to these methods, a quantitative real time PCR to measure parasite load has been evaluated [Katara *et al.* 2011]. Although PCR based approaches have proven to be effective for parasite detection in immunocompromised patients, it failed to provide any information regarding parasite viability. To measure parasite viability, a new method known as quantitative nucleic acid sequence based amplification (QT-NASBA) using single stranded RNA of parasite is being started, but its effectiveness is yet to be proven [Singh et al. 2011].

Treatment of PKDL is another challenge to clinicians due to absence of prognostic markers for detecting early cure. Although our group tried to establish serological markers (particularly *Leishmania* specific IgG, IgG1 and IgG3), it was found to be effective only in polymorphic PKDL, not in macular PKDL [Ganguly *et al.* 2008]. For treatment Sodium stibogluconate or SAG is still used at a dose of 20 mg/kg body weight/day *i.m* for 4 month in
areas where there is no antimony resistance (with a cure rate of 64%-92%) and 2-3 month in Sudan (cure rate 95%). Due to the resistance issue in India, alternative strategies are also being applied which includes miltefosine (100 mg/day p.o for 2 months [Ganguly et al. 2010a] or 50 mg thrice daily for 2 months [Ramesh et al. 2011]. Sudanese PKDL are treated with alternative drugs such as liposomal amphotericin B (2.5 mg/kg body weight/day i.v for 20 days) with a success rate of 85% [Musa et al. 2005]. Amphotericin B deoxycholate has been found to be superior compared to SAG in India with a cure rate of almost 100% [Thakur et al. 1997]. In addition to conventional antileishmanials, immunotherapy based on sodium stibogluconate with alum precipitated autoclaved L. major and BCG, in Sudanese PKDL also showed promising results with a cure rate of 87% but needs to be evaluated further [Musa et al. 2008].

**Immune mechanisms of PKDL**

The precise immune mechanisms of PKDL are still obscure and interestingly, the immunobiology is different in Sudanese and Indian PKDL and therefore information from one is not extrapolatable to the other [Ganguly et al. 2010b]. In Sudanese PKDL, the disease associated immune involvement mimics the scenario of immune reactivation after cure from VL because of the shorter time lag between cure from VL and development of PKDL. PBMCs from Sudanese PKDL patients react and proliferate following induction with *Leishmania* antigen, secrete more IFN-γ while IL-10 was produced primarily from CD4+ T cells [Ismail et al. 1999]. Lesional immunology of Sudanese patients was also a mixed Th1/Th2 type as IL-10 and IFN-γ were expressed. Furthermore, an association in IFN-γ receptor polymorphism was found which probably accounted for the presence of parasites despite the presence of high levels of IFN-γ in the lesions [Salih et al. 2007]. A high parasite load in lesions of Sudanese patients also showed high levels of IL-10 which counteracted the effect of IFN-γ, although no polymorphism was found in the IL-10 promoter region [Farouk et al. 2010]. In Sudanese PKDL, expression of IL-10 from keratinocytes was considered as a key factor and a predictor for development of PKDL, particularly after cure from VL [Zijlstra et al. 2003]. Therefore it plays an important immunoregulatory role in patients with of Sudanese PKDL which is associated with altered levels of HLA-DR, ICAM-1 expression and CD4^+^T cells in the lesions [Ismail et al. 2006a].

In contrast to Sudanese PKDL, immunological studies are better defined in Indian PKDL; as here the disease is more chronic than the Sudanese variant due to the larger gap between cure of VL and onset of the disease, many differences occurs regarding the immunopathological mechanisms [Ganguly et al. 2010b]. In Indian PKDL, CD8^+^ T cells plays a more important role than the conventional CD4^+^ T cells and it was found that CD8^+^ T cells predominated in the lesions and circulation [Rathi et al. 2005a, Ganguly et al. 2008].
Regulatory T cells play an important role in the lesional immunology of Indian PKDL as evident by elevated mRNA expression of FoxP3, CTLA-4 and CD25 [Ganguly et al. 2010a, Katara et al. 2011]. Our group also showed that in addition to lesional immunology, systemic immune changes also took place in Indian PKDL as evident by increased antigen induced IL-10 synthesis in circulating CD8+ T cells along with their decreased antigen induced proliferative capacity. We further showed that these cells were anergic in nature as they lost the co-stimulatory CD28 molecule on their surface [Ganguly et al. 2008, Ganguly et al. 2010a]. There was a mixed pro and anti-inflammatory cytokine response in lesions of PKDL patients; despite the higher levels of IFN-γ and TNF-α, the expression of IFN-γR and TNFR1 were lowered in patients with PKDL and increased after treatment (Ansari et al. 2006a and 2008a). In terms of humoral response, increased levels of IgG, IgG1 and IgG3 were considered as hallmarks of PKDL [Ganguly et al. 2008]. Recently, Katara et al. (2012) reported raised levels of IL-17, its transcription factor ROR-γt and IL-22 in lesions and circulation (plasma and lymphocytes). Taken together, immunological studies conducted so far indicate that PKDL is not a localized disease but involves systemic immunity which necessitates further evaluation. Figure 1.2 summarises our current knowledge of the local immune response seen in patients with PKDL.
Role of monocytes/macrophages in leishmaniasis

Leishmania-macrophage interactions are exemplary of the evolutionary battle for survival between host and parasite. To initiate infection, Leishmania invade macrophages via a multitude of ligand-receptor interactions and exploit non-self recognition systems for accessing host cells. To sustain infection, it is mandatory that Leishmania parasites establish themselves in macrophages, but considering the potent antimicrobial functions of macrophages, the subject of how Leishmania survive remains a subject of intense research [Saha et al. 2011].

Parasite entry mechanisms into macrophages determines the outcome of the infection

The initial interactions between parasite ligands and their respective host cell receptors on the cell membrane are crucial for parasite survival. Importantly, different types of Leishmania utilize different strategies for entering into the host which possibly influences the degree of variation in the clinical manifestation of leishmaniasis [Reviewed in Dey et al. 2007]. Most studies have revealed that Leishmania gains entry into the macrophages by either an antibody dependent or complement mediated phagocytic pathway; however other pathways are also known to be involved [Reviewed by Kima 2007]. The role of antileishmanial IgG in this regard is well known, in that Leishmania coated with IgG enters into the macrophages, triggers IL-10 mediated suppression of host immunity which then results in disease progression [Miles et al. 2005]. Similarly, Leishmania utilizes its Lipophosphoglycan (LPG) and gp63 for complement mediated entry into the macrophages. Both LPG and gp63 protect the parasite from complement mediated lysis by shedding off the membrane attack complex (MAC) from the parasite membrane and converts C3b to iC3b which mediates promastigote uptake by binding to the macrophage complement receptors CR1 and CR3. Similarly, gp63 contains a Ser-Arg-Tyr-Asp motif that mimics fibronectin and allows the parasite to enter into macrophages via fibronectin receptors [Reviewed in Dey et al. 2007, Kima 2007]. Other evidence suggests that the mannose-fucose receptor, C-reactive protein receptor, receptor for advanced glycosylation end products (RAGE) are also occasionally involved [Reviewed in Stafford et al. 2002].

Another important molecule implicated in internalization of the parasite is phosphatidyl serine (PS) particularly in Leishmania amazonensis as it displays PS to create an apoptotic “eat me” signal; what follows is silent internalization into macrophages via PS receptors which then induces IL-10 and TGF-β from macrophages [Reviewed in Wanderley and Bacinsky, 2010]. In this regard, the role of neutrophils is also important as Leishmania uses apoptotic neutrophils as a ‘Trojan horse’ for gaining silent entry into macrophages followed by production of high TGF-β to create an anti-inflammatory milieu, beneficial for survival of Leishmania [Reviewed in Laskay et al. 2008]. Another pathway exploited by
Leishmania for entry into macrophages and subsequent immune subversion, was the caveolin mediated mechanism that involves cholesterol enriched lipid rafts. Using this strategy L. chagasi was shown to perturb the lipid rafts which ultimately caused disruption of intracellular signaling and host antigen presentation [Reviewed in Lodge and Descoteaux 2006, Chakraborty et al. 2005, Rub et al. 2009, Reviewed in Bhardwaj et al. 2010].

Subversion of macrophage signaling for survival and disease progression

Following internalization, Leishmania parasites are rapidly converted into amastigotes and start manipulation of the macrophage effector functions by modulating important signalling pathways. It does so mainly by its gp63 and an exosome containing either gp63 or elongation factor 1α (EF-1α) [Reviewed in Silverman and Reiner, 2011]. These Leishmania derived proteins activate various macrophage protein tyrosine phosphatases (PTPs) including Src homology 2 domain containing tyrosine phosphatase 1 (SHP-1) and PTP-1B, protein phosphatases (PP1 and PP2A) which are involved in inactivation of mitogen activated protein kinases (MAPKs) and interferon-γ signalling. This ultimately results in downregulation of the macrophage microbicidal arsenal, including TNF-α, reactive nitrogen and oxygen species (NO, ROS, Saha et al. 2011). In addition to changing the macrophage phenotype, it dampens the immune response of monocyte-derived dendritic cells and CD4+ T lymphocytes. Similarly, Leishmania in mice secreted cysteine proteinases such as cathepsin B which are important as its deletion leads to impaired virulence [Bart et al. 1997]. In addition to this, Leishmania amastigotes, through activation of host SHP-1, PP1 and PP2A inactivate Toll like receptor pathways (TLRs), Janus Kinase 2 (JAK-2) mediated and protein kinase C (PKC) signaling pathways resulting in parasite persistence and disease progression [Reviewed in Bhardwaj et al. 2010]. Shio et al. (2012) proposed that L. major amastigotes blocked the nuclear translocation of nuclear factor kappa B (NF-κB) whereas L. mexicana is known to degrade NF-κB by its cysteine proteinases and inhibit LPS induced IL-12 production from macrophages.

Suppression of superoxide and nitric oxide generation in leishmaniasis

Leishmania amastigotes but not promastigotes are known to be potent suppressors of superoxide generation [Reviewed by Kima 2007]. Within the macrophages, superoxide is the product of a multisubunit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex that contains membrane associated gp91phox and p22phox, four cytosolic components, p47phox, p67phox, p40phox and the GTPase Rac. During phagocytosis or upon activation, the membrane- associated and the cytosolic components assemble, resulting in the reduction of NADPH to NAD⁺ resulting in production of O₂⁻ from oxygen [Van Assche et al. 2011]. Leishmania inhibits superoxide generation by different strategies; infection with L.
pifanoi induced haeme oxygenase 1 (HO-1) which prevented the maturation of gp91phox and blocked assembly of NADPH oxidase [Pham et al. 2005]. On the other hand, L. donovani inhibited the classical PKC pathway by inducing ceramide synthesis within host macrophages which resulted in blockade of phosphorylation of p47phox that in turn led to a defective phagosomal recruitment of p47phox and p67phox and decreased generation of superoxide [Reviewed in Bhardwaj et al. 2010, Majumder et al. 2012]. L. donovani also inhibited generation of superoxide either by attenuation of inositol phosphate accumulation and Ca^{++} release or by disrupting the phagosomal lipid raft integrity, inhibition of phagosomal maturation by preventing F-actin to G-actin polymerization [Reviewed in Van Assche et al. 2011, Bhardwaj et al. 2010].

Nitric oxide or NO becomes an extremely important radical in leishmaniasis because it protects against intracellular amastigotes and appears to be secondary to generation of ROS during phagocytosis. Studies have revealed that whereas phagocytosis and ROS generation is completed within 2 hrs, synthesis of NO by inducible nitric oxide synthase (iNOS) takes 4-6 hrs to achieve detectable levels [Wang et al. 2009]. Phagosomal amastigotes are known for their ability to reduce production of NO in infected macrophages. The mechanism by which *Leishmania* decreased NO production is also diverse, akin to inhibition of NADPH oxidase. *L. amazonensis* enters into macrophages via PS receptors and thus induces generation of IL-10 and TGF-β which in turn blocks induction of iNOS and therefore the production of NO [Wanderley et al. 2006]. On the other hand, a wide spectrum of *Leishmania* strains are known to inhibit IFN-γR mediated JAK-STAT signaling either by induction of PTPs [Blanchette et al. 1999] or by proteasome mediated degradation of signal transduction and activators of transcription 1 [STAT-1, Forget et al. 2005]. In a study by Bhardwaj et al. (2005), downregulation of IFN-γR expression by *L. mexicana* and *L. major* was observed. On the contrary, Sen et al. (2012) showed that in mouse macrophages infected with *L. donovani*, the expression and ligand affinity of IFN-γR remained unaltered. It is attributed to quenching of membrane cholesterol by parasites that causes disruption of the macrophage lipid raft and as it is responsible for defective assembly of IFN-γR1 and IFN-γR2 on the macrophage membrane, defective IFN-γ mediated signaling occurs along with impaired generation of NO [Sen et al. 2011]. Induction of IL-10, TGF-β within infected macrophages and IL-4, IL-13 from T cells is known to induce arginase-1 in macrophages which competes for L-Arginine, the key substrate for iNOS mediated production of NO [Iniesta et al. 2002]. In a recent study Osorio et al. (2012) showed that in experimental VL, *L. donovani* itself induces phosphorylation of STAT-6 within macrophages to activate arginase-1, causing immune suppression without the requirement of Th2 type cytokines. In Indian PKDL, it has been found that the lesional expression of IFN-γR is downregulated at presentation which suggested that despite having high levels of IFN-γ, the parasites can reside within the dermis.
Chapter I: Review of Literature

[Ansari et al. 2006a], However information regarding the generation of NO which is downstream of IFN-γ signaling is unexplored and warrants to be studied.

**Role of TLR pathways in leishmaniasis**

TLRs are hallmarks of cellular receptors that recognize pathogen associated molecular patterns (PAMPs) and participate in innate responses to infections. Since TLR recognition is often associated with the production of pro-inflammatory cytokines and generation of free radicals, it is unquestionably important to determine the implications of TLR signaling during *Leishmania* infections. A few *Leishmania*-derived molecules have been reported to activate TLRs and majority of studies to date have focused on the activation of TLR-2, TLR-4 and TLR-9 [Reviewed in Tuon, 2008, Faria et al. 2012].

Early reports showed that the MyD88 dependent pathways are required for the development of host protective IL-12 mediated Th1 response against *L. major* in C57BL6 mice, as MyD88⁻ knockout mice infected with *L. major* developed a non protective Th2 response [Muraille et al. 2003]; later it was found that LPG interacted directly with TLR-2 [Becker et al. 2003]. The role of TLR-2 mediated signaling by LPG stimulation proved to be parasite strain specific and also influenced the outcome of infection [Veer et al. 2003]. However, studies with *L. donovani* revealed that TLR-2 mediated pathways are indispensable for development of a strong Th1 response and moreover, treatment with immunomodulatory antileishmanials up regulated their TLR-2 expression [Bhattacharya et al. 2010, Kar et al. 2011, Mukherjee et al. 2012]. The infection of human THP-1 derived macrophages with *L. donovani* in vitro suppressed TLR-2 and TLR-4 stimulated IL-12 release, with an increase in IL-10 production, through parasite dependent contact and suppression of p38-MAPK phosphorylation [Chandra and Naik, 2008]. On the contrary, studies with *L. braziliensis* or *L. amazonensis* using TLR-2 knockout mice showed completely different results, as TLR-2 was shown to be necessary for lesion development [Vargus-Inchaustegui et al. 2009]. A similar result was observed with purified LPG from *L. major* indicating that TLR-2-LPG interaction negatively regulated anti-leishmanial responses [Srivastava et al. 2013]. On the other hand, impaired resistance to *L. major* was reported in TLR-4 deficient mice compared to wild type controls. An enhanced arginase mediated response was observed which helped in proliferation of parasites within macrophages and indicated that TLR-4 signaling could enhance the microbicidal activity of macrophages harboring parasites. Miltefosine is reported to increase the expression of TLR-4 and downstream genes responsible for either secretion of host protective pro-inflammatory cytokines or generation of free radicals in human VL [Mukherjee et al. 2012].
Role of co-stimulatory molecules in leishmaniasis

Co-stimulatory pathways play a key role in leishmaniasis as they are involved in the interaction between antigen presenting cells (APC) and T cells. Amongst the co-stimulatory molecules implicated in the pathogenesis of leishmaniasis, CD40 and CD80/86 are the most extensively studied [Tuladhar et al. 2011]. An important co-stimulatory molecule that determines the outcome of macrophage-Leishmania interactions is CD40 as the CD40-CD40L interaction helps to increase the Th1 immune response [Bhardwaj et al. 2010]. With regard to Leishmania infection, CD40 mediated MAPKs have been reported to promote parasite survival by modulating the expression of IL-10 and IL-12 in macrophages [Mathur et al. 2004], The CD40 of macrophages interacts with CD40L of T cells and pass the signal onwards to produce IL-12 via p38MAPK and NF-κB. The released IL-12 then binds to IL-12 receptors present on macrophages, increases their production of IFN-γ, which by acting on infected macrophages induces parasite killing. However, this CD40-CD40L interaction has been proposed to exert a dual effect, as when CD40 signaling is associated with depletion of cholesterol and TRAF-6, it causes activation of ERK1/2, higher levels of IL-10 follow along with decreased levels of IL-12p40. Conversely, if the CD40 signalosome is associated with normal levels of cholesterol and TRAF-2/3/5, it causes p38 MAPK activation which is accompanied with increased leishmanicidal IL-12 p40 and accompanying proinflammatory responses [Rub et al. 2009].

A number of studies have indicated that co-stimulatory molecules B7-1/B7-2 can differentially influence the development of Th1 or Th2 cells in leishmaniasis [Reviewed in Tuladhar et al. 2011]. Studies with L. major have implicated B7-2 in development of Th2 pathway and hence susceptibility, however B7-1 did not influence CL [Brown et al. 1986] while in vitro studies using human cells where L. major infected macrophages were used to prime peripheral blood leukocytes indicated a decrease in IFN-γ and IL-5 upon blocking of the B7 pathway with anti CD86 or cytotoxic T lymphocyte antigen 4 Ig [CTLA4-Ig, Brodskyn et al. 2001]. Similarly, when peripheral blood mononuclear cells from cutaneous leishmaniasis (CL) patients were treated with Leishmania antigen, a decrease was noted in the production of TNF-α, IL-10 and IFN-γ on blocking B7-CD28/CTLA4 using CTLA4-Ig [Favali et al. 2005]. Studies in a VL model have also suggested a modulatory role for B7 co-stimulation in the outcome of infection. Blockade of B7-1 but not B7-2 was shown to enhance T cell responses in a L. chagasi infection model [Gomes et al. 1998]. In both BALB/c and C57BL/6 mice infected with L. donovani, administration of anti CTLA4 MAb led to better granuloma formation [Zuabairi et al. 2004]. But in contrast to the L. chagasi model of infection, it has been observed that blockade of B7-2:CTLA4 but not B7-1:CTLA4 led to parasite clearance from liver [Zuabairi et al. 2004]. From these studies it appears that this pathway, especially B7-2 is important in shaping the immune response to Leishmania and
differential effects of B7 molecule could probably be exerted due to different types of parasite and also the host cell.

**Role of alternative activation of macrophages in leishmaniasis**

Macrophages count as one of the most pleiotropic cells of the immune system, exhibiting a plethora of biological functions. T cells and their secretory cytokines influence the heterogeneity and state of activation of macrophages. Amongst the macrophages, classically activated form (M1) stimulated by IFN-γ and Th1 cytokines or alternatively activated type (M2) which are stimulated by IL-4, IL-13, IL-10 and TGF-β are best characterized [Martinez and Gordon, 2009]. Now in leishmaniasis, macrophages triggered with IFN-γ can control infection through activation of iNOS. However *Leishmania* inhibits production of IL-12, yet induces release of IL-10 and TGF-β. This imbalance underlies a shift from a Th1 to Th2 immune response and reflects susceptibility to disease [Scharton-Kersten et al. 1995]. Indirect evidence suggests that M2 macrophages emerge in a Th2 immune environment, facilitating the dissemination of *Leishmania* parasites in the host. Recent studies by Osorio et al. (2012) indicated that *Leishmania* itself can switch on the M2 macrophage-like phenotype in infected macrophages by direct contact through phosphorylation of STAT-6 followed by induction of arginase-1. Another study by Ehrchen et al. (2007) clearly showed that Vitamin D₃, a potent inducer of M2 polarization, contributes to infection with *L. major*. The role of IL-4/IL-13 dependent M2 in susceptibility to *L. major* infections has been proved recently using macrophage/neutrophils specific IL-4Ra deficient mice in a non-healing BALB/c background. Despite sustained development of Th2 cytokines, these mice show significantly delayed disease progression due to enhanced M1 induction and leishmanicidal functions [Holscher et al. 2006]. IL-4Ra mediated mechanisms were shown to be dependent on arginase-1, counter regulating NO production by competing for the common substrate L-Arginine and inducing the production of polyamines thus favouring parasite replication [Kropf et al. 2005, Holscher et al. 2006].

As polarization of macrophages towards M2 type is a conventional feature of many chronic infectious diseases [Reviewed in Noel et al. 2004, Raes et al. 2007] and studies regarding M2 polarization in leishmaniasis is limited, examination of M2 polarization in human leishmaniasis is clearly warranted. PKDL is one of the most chronic forms of leishmaniasis known and so we took up this challenge to study whether polarization of monocytes/macrophages occurs in patients with PKDL or not.
Why does PKDL develop in some but not all individuals having a previous history of VL?

Information regarding the etiopathogenesis of PKDL is limited and therefore, no consensus has emerged to explain what factors influence the normally viscerotropic *L. donovani* parasite to become dermatotropic during PKDL. Herein, we critically evaluate a number of hypotheses to explain the development of PKDL proposed to result from (i) use of antimonials (ii) UV-induced skin damage (iii) re-infection (iv) organ specific failure of T cell memory and/or (v) genetic susceptibility. These points will enable researchers and clinicians to address the unresolved yet pertinent issues pertaining to PKDL and yield important advances to facilitate elimination of leishmaniasis.

**Hypothesis 1: a role for antimonial drugs**

Epidemiological data and clinical reports have strongly suggested a link between administration of sodium antimony gluconate (SAG) and subsequent development of PKDL [Croft, 2008]. Surveillance data has indicated that following treatment with drugs other that SAG, PKDL does appear, but with a far lower frequency *vis a vis* SAG [Thakur et al. 2008, Pandey et al. 2012]. Most studies on PKDL in South Asia and Sudan have reported administration of SAG to patients during their kala-azar period, suggesting that SAG could well influence the development of PKDL. It is interesting however that SAG can and is still being used for the treatment of PKDL, albeit at a higher dose and for a prolonged period (20 mg/kg/day for 4 months) than required for the treatment of VL [10 mg/kg/day, 3 weeks, Zijlstra et al. 2003]. Before incriminating SAG for the development of PKDL, it is necessary to examine whether patients treated for VL with other anti-leishmanial drugs such as amphotericin B, miltefosine and paromomycin develop PKDL with similar frequency, especially as there is now a long history of usage of miltefosine and amphotericin B to treat patients with VL in India, consequent to the rise in antimony resistance [Sundar et al. 2002]. Thakur et al. reported that amphotericin B at a dose of 20 mg/kg body weight for treatment of VL effectively minimized development of PKDL, whereas in individuals treated with lower doses of amphotericin B (15 mg/kg body weight), there was appearance of PKDL [Thakur et al. 2008]. Patients who received miltefosine, paromomycin and a combination therapy of amphotericin B/miltefosine have rarely been found to develop PKDL [Das et al. 2009, Kumar et al. 2009, Pandey et al. 2012]. Collectively, available data strengthens our notion that SAG directly or indirectly influences the incidence of PKDL, but definitive evidence may require another decade, as in India, PKDL can even develop 20-40 years after cure from VL [Kumar et al. 2009, Ganguly et al. 2010b]. This leads to another speculation that anti-leishmanial drugs at lower doses and shorter duration can eliminate the parasite from the viscera, not from
the skin, which requires a higher dose. Hence PKDL may be regarded not only as a drug-related but a dose-related phenomenon.

In addition to epidemiological evidence, immunological data too supports this hypothesis as levels of TGF-β and IL-10, factors that support parasite persistence, remained high even after completion of treatment with SAG however, this was not so with amphotericin B [Figure 1.3, Saha et al. 2007]. Furthermore, in an in vitro model, treatment of THP1 macrophages with SAG caused elevation of two anti-inflammatory, disease-sustaining molecules namely hemeoxygenase-1 (HO-1) and glutathione [Figure 1.3, Fadili El et al. 2008]. This was corroborated in patients with PKDL who had raised circulating levels of glutathione and expression of HO-1 [DM; unpublished]. This was corroborated in patients with PKDL who had raised circulating levels of glutathione and expression of HO-1 [MC, personal communication]. Furthermore, impairment of host peroxisome function during VL is not restored by SAG, further implicating its influence on development of PKDL [Figure 1.3, Gupta et al. 2009]. Additionally, the failure of SAG to cause a sterile cure has fuelled the hypothesis of drug-induced genetic alterations in residual parasites, as it has been suggested that SAG-resistant parasites have an enhanced degree of ‘fitness’ [Vanaerschot et al. 2011]. Another pertinent observation is that PKDL-causing strains expressed higher levels of PSA2 and gp63, molecules associated with dermatotropism; concomitantly, they had a decreased expression of A2, which is linked with enhanced viscerotropism [Figure 1.3, Salotra et al. 2006, McCall & Matlashewski 2012]. Given our recent understanding of the plasticity of the Leishmania genome [Rogers et al. 2011], future studies aimed at characterising PKDL strains from patients with various drug histories are clearly warranted.
Hypothesis 1: PKDL is a drug induced disease

Patients with VL when treated with SAG harbour resident parasites in the skin; these parasites are possibly genetically altered by SAG such that their viscerotropic ability is impaired while their dermatotropic ability is enhanced resulting in development of PKDL rather than systemic disease. Alongside, SAG modulates the host immune status such that the host susceptibility to develop PKDL is enhanced.

Hypothesis 2: a role for UV light

Lesions of PKDL consistently appear predominantly on sun exposed areas particularly the face, ear, arms etc. rather than unexposed areas like the scalp and chest, which has fuelled the hypothesis that exposure to UV light may play a contributory role in the pathogenesis of PKDL [Figure 1.4, Ismail et al. 2006b]. This role of UV light is further highlighted by the characteristic presence of photosensitivity in patients with PKDL. The potent immunosuppressive property of UV light is linked to its ability to damage epidermal Langerhans antigen presenting cells (E-LCs) and also inhibit contact hypersensitivity and alloantigen responses [Clydesdale, 2001]. The recurrence of papulo-nodules was observed in a patient initially treated for PKDL (SAG, 4 months) followed by psoralen + UVA (PUVA) for treatment of remnant hypopigmented macules [MC, personal communication]. The UVB light (280-320 nm) induced immunosuppression can operate either through its chromophore urocanic acid or via modulation of vitamin D [Figure 1.4, Amerio et al. 2009] or via modulation of vitamin D [Hart et al. 2011]. During keratinisation, trans-urocanic acid produced from histidine released from filaggrin undergoes trans to cis UV-induced photoisomerisation which then impacts on all dermal cell types, especially E-LCs and by...
reducing their number and morphology, collectively attenuates their antigen presenting property [Noonan and DeFabio 1992, Gibbs and Norval 2011]. Langerhans cells from UV irradiated skin have been shown to have reduced expression of MHC class II, along with a loss of co-stimulatory molecules, CD80 and CD86, translating into impaired antigen presentation [Figure 1.4, Weiss et al. 1995, Simon et al. 1991]. Additionally, by impacting on keratinocytes and lymphocytes, cis-urocanic acid modulates a vast array of cytokines that range from pro-inflammatory TNF-α to anti-inflammatory and immunosuppressive IL-10 [Amerio et al. 2009]. In general, 40% of normal adults are UVB sensitive when tested for impairment of hapten-induced contact dependent hypersensitivity, and if extrapolated to PKDL, it might account for some but not all individuals developing PKDL [Ismail et al. 2006b]. Studies have suggested that lesional patterns often mirror the clothing habits of individuals which strongly suggested a link between exposure to UV light and pathogenicity of PKDL [Musa et al. 2002]. However, no such study has been undertaken in India, but as sparing of the vault of axilla and groin (photo-protected sites) occurs in Indian PKDL, it goes in favour of UV light having a contributory role in disease pathogenesis.

The role of UV and Vitamin D₃ along with its dihydroxylated form (1α,25(OH)₂D₃) as potent immunomodulators has been reported [Figure 1.4, Baekke et al. 2010, Hart et al. 2011] wherein UV enhances the synthesis of Vitamin D, with 7-dehydrocholesterol absorbing UV and being converted into Vitamin D by a series of enzymatic and non-enzymatic reactions. The circulating Vitamin D₃ upon binding to Vitamin D binding protein (VBP) enters immune cells, such as monocytes-macrophages; upon further hydroxylation by CYP27B1, the active 1α,25(OH)₂D₃ is formed which then forms a complex with VDR. The complex then translocates into the nucleus and after binding to the Vitamin D response elements (VDRE), induces synthesis of antibacterial peptides such as LL-37 and immunomodulatory factors including TGF-β and arginase [Griffin et al.2003, Whitcomb et al. 2012] . The impact of Vitamin D₃ on the immune system appears dichotomous as on the one hand it induces synthesis of potentially host protective antibacterial peptides from macrophages, while on the other hand it inhibits TLR induced activation of macrophage, downregulates co-stimulatory molecules and pro-inflammatory cytokines along with induction of TGF-β and IL-1. Additionally, its ability to upregulated the arginase pathway would imply that it facilitates alternative activation of macrophages [Figure 1.4, Martinez et al. 2009]. Vitamin D₃ also helps in the activation of regulatory T cells by promoting IL-10 secretion, which by virtue of their ability to suppress the local immune response supports parasite persistence. In Indian PKDL, an increased presence of FoxP3, a key molecular marker of Tregs has been reported [Ganguly et al. 2010b, Katara et al. 2011]. Similarly in Sudan, at disease presentation, a preponderance of Tregs has been identified in peripheral blood and lesions which is decreased with treatment [El-Hassan AM, personal
Chapter I: Review of Literature

Indeed in B6.Vdr<sup>−/−</sup> mice, resistance to *L. major* infection is further enhanced, while treatment with 1<sub>α</sub>,25(OH)<sub>2</sub>D<sub>3</sub> led to a VDR-dependent inhibition of macrophage killing, induction of arginase and down regulation of iNOS [Ehrchen et al. 2007]. Logically, a strong case between exposure to UV and PKDL in terms of dermal phenotypic changes exists, but does not necessarily constitute an argument for causation. Nevertheless, there is sufficient data to warrant further exploration of the role of UV light in the etiopathogenesis of PKDL, potentially feasible in animal models.

### Hypothesis 2: Pathogenesis induced by UV light

This diagram summarizes the potential role of UV light in pathogenesis of PKDL via enhanced secretion of cisturocanic acid and vitamin D<sub>3</sub> It translates into a reduced number of epidermal Langerhans cells (E-LCs) with altered morphology. They also have a decreased expression of MHCII, CD80 and CD86 along with increased IL-10; this stimulation of Th2 cells subsequently activates dermal dendritic cells (dDCs) which leads to an increased presence of regulatory T cells allows for parasite persistence. Similarly vitamin D<sub>3</sub> contributes by enhancing the activation of regulatory T cells via IL-10 secretion.

**Figure 1.4: Hypothesis 2: Pathogenesis induced by UV light**

A hallmark of many infectious diseases is persistence of the pathogen even after clinical cure as evident in tuberculosis, viral infections (e.g. herpes) and protozoan diseases, such as trypanosomiasis [Mendonca et al. 2004]. The underlying immunological mechanisms include impaired T cell activity, modulation of host antimicrobial responses and enhanced synthesis of immunosuppressive cytokines [Mendonca et al. 2004]. In leishmaniasis, evidence
of parasite persistence after clinical cure exists and in areas where leishmaniasis is endemic, recurrence has been attributed to parasite persistence and/or re-infection. Logically, the optimal approach would be to characterize these parasites and compare them with the parental type [Aebischer et al. 1993, Schubach et al. 1998]. In a mouse model, Aebischer et al. (1993) showed that parasites that persisted retained characteristics of the parental clone; however, analogous studies in humans are logistically not possible, in view of the parasite density being insufficient for establishment of parasite isolates [Schubach et al. 1998, Mendonca et al. 2004]. As Indian strains from VL and PKDL have genetic heterogeneity, the axis tends to tilt in favour of re-infection [Dey & Singh 2007]. However, to truly dissect this issue, parasites would need to be isolated from a patient suffering from VL and compared with a strain isolated from the same individual when PKDL develops, which in practical terms is near impossible owing to the long and unpredictable lag period between VL and onset of PKDL. Such studies are feasible in Sudan where the onset of PKDL following VL is much shorter and should be undertaken. In Sudan, PKDL is likely to be due to parasite persistence as patients with VL post-treatment when moved to a non endemic area still developed PKDL [El Hassan AM, personal communication]. Another challenge to the re-infection hypothesis is why should genetically different strains exclusively cause PKDL, and not re-emergence of VL? An important factor possibly preventing re-emergence could be due to the fact that VL generates a strong systemic memory response but the failure of tissue specific immunity allows parasites to multiply in the skin.

**Hypothesis 4: Tissue specific T cell memory**

Recovery from *Leishmania* infection is associated with development of a strong memory immune response and in humans this induces lifelong protection in the majority of individuals [Figure 1.5, Gollob et al. 2005]. However, under certain conditions, such as immunosuppression, this infection-induced immunity may be impaired, rendering the host susceptible to infection or reactivation of latent parasites [Desjeux 1999]. Tissue sites are capable of influencing qualitative and quantitative aspects of host immunity, as evident in models of infectious disease including leishmaniasis [Engwerda & Kaye 2000]. In this context, PKDL poses an interesting challenge as organ specific deficits in immunity apparently occur in the skin following the acquisition of systemic protective immunity [Figure 1.5].

To confer memory, important players are central memory T cells (CM T cells, a CD62Lhi, CD45RBhi, and CCR7hi phenotype) and effector memory T cells (EM T cells, a CD62Llow, CD45RBhi-lo, and CCR7low phenotype), the latter being more functionally relevant than the former [Gollob et al. 2005], the latter being more functionally relevant than the former [Okwor and Uzonna 2008]. In India, patients with PKDL have a reduced frequency of
circulating CD62L⁻CD4⁺ T cells i.e. a high percentage of CD4⁺62L⁺ indicating a presence of EM T cells [MC, personal communication]. Importantly, these CD4⁺62L⁻ T cells are also considered as homing T lymphocytes, necessary for effector T cell function at the disease site and should logically cause resolution of the disease [Zaph et al. 2004]. Therefore, it follows that in Indian PKDL, this higher frequency of EM T cells should facilitate disease resolution. However, this is not the case, as in the dermal lesions there is a notable absence of CD4⁺ cells [Rathi et al. 2005b] thus allowing for development of PKDL. This hypothesis would hold if studies with the self resolving Sudanese PKDL variant showed a memory response. Indeed, in Sudanese PKDL, the proportion of memory CD4⁺ T cells is high at the lesional site and systemic circulation [Figure 1.5, Ismail et al. 2006a], which is in sharp contrast with the non resolving Indian PKDL variant [Rathi et al. 2005b]. In Indian PKDL, antigen specific recall responses as measured by response to *Leishmania* antigen or PHA stimulation in circulating CD4⁺ T cells was intact but CD8⁺ T cells were functionally impaired [Ganguly et al. 2010a]. Additionally, in Sudanese PKDL, parasite antigens persist within macrophages and epithelial cells as lesional cytotoxic T cells are suppressed owing to an enhanced production of IL-10 by T reg cells in blood and lesions at presentation, which decreases sharply with treatment [El Hassan AM, personal communication]. Therefore, it is necessary that the functional ability of these CD8⁺ memory T cells (in terms of perforin/granzyme production) be established.

For homing of memory CD4⁺ T cells, increased expression of the cutaneous lymphocyte antigen (CLA) is important for self resolution as observed in localized self resolving CL while the lack of CLA expression in DCL accounts for its non healing outcome [Diaz et al. 2002]. Taken together, it may be envisaged that in PKDL, an aberrant expression of CLA prevents these CD4⁺ EM T cells from homing to the dermal lesions and stimulating host immunity. It has been proposed that granuloma formation in the skin offers protection against leishmaniasis as evidenced in CL [Tuon et al. 2010]. Importantly, in PKDL, granuloma formation was scanty and as Indian PKDL was accompanied with a substantial decrease in the proportion of CD4⁺ T cells [Ismail et al. 1999, Rathi et al. 2005a, Rathi et al. 2005b, Ismail et al. 2006a], it strengthened the hypothesis that impaired tissue specific immunity supports disease persistence.
Hypothesis 4: Organ specific memory T cell response

In patients with self limiting PKDL (like in Sudan), good effector memory T cells in the skin renders self resolution whereas in Indian PKDL an impaired EM T cell response accounts for non self healing disease progression.

EM T cells - Effector memory T cells

Hypothesis 5: Host Genetic susceptibility factors:

Hypotheses 2-4 have focussed on different aspects of host immunity, wound healing and regeneration, all of which can potentially be regulated by the host genotype. Various forms of leishmaniasis have been subject to intense genetic analysis in murine models and humans [Lipoldova & Demant 2006, Foote & Handman 2005, Blackwell 1996, LeishGen consortium et al. 2013], but data pertaining to patients with PKDL remains preliminary and is restricted to a total of three studies all of which were performed in Sudan, thus leaving the conundrum unresolved as to why some, but not all patients with VL develop PKDL. A significant association between IFN-γR polymorphism and development of PKDL has been reported [Figure 1.6, Mohamed et al. 2003, Salih et al. 2007]. Although a critical balance between IFN-γ vs. IL-4 and/or IL-10 secreting cells is essential in leishmaniasis [Alexander & Bromabcher, 2012] no correlation was found between IFN-γ or IL-10 promoter polymorphism and disease susceptibility [Salih et al. 2007, Farouk et al. 2010]. Furthermore, Blackwell et al. have proposed that failure to respond adequately to IFN-γ during active disease occurs due to compromised function of IFNGR1 that tilts the balance in favour of the parasite promoting cytokine IL-10, culminating in localization of parasites in the skin Blackwell et al. [2004]. Indeed, in Indian PKDL, expression of IFN-γ at the mRNA and protein level is lowered as compared to healthy controls thus strengthening this hypothesis [Ansari et al., 2006a].

In Sudan, PKDL tends to develop in patients with VL having higher levels of plasma CRP but it remains unexplored whether genetic polymorphisms in CRP genes or its promoter
correlates with individuals having a higher propensity to develop PKDL [Gasim et al. 2000]. Study of innate immunity genes, especially those associated with complement activation such as Ficolin-2 (encoded by FCN-2 gene) are associated with an enhanced susceptibility to CL [Kilpatrick & Chalmers, 2012, Assaf et al. 2012] while Mannose binding lectin 2 was associated with increased susceptibility to VL [MBL2, Alonso et al., 2007]. Another important candidate gene for Leishmaniasis is the slc11a1 (solute carrier family 11a, that functions as a proton/divalent cation antiporter present on the endosomal membrane of phagocytes, formerly NRAMP1) whose polymorphism is associated with increased susceptibility to VL [Figure 1.6, Mohamed et al. 2004, Blackwell et al., 2004]. Notably, as no studies have so far included patients with PKDL from the Indian subcontinent, they are clearly warranted to help establish susceptibility determinants.

![Figure 1.6: Hypothesis 5: Genetic susceptibility of the host](image)

**Figure 1.6: Hypothesis 5: Genetic susceptibility of the host**

A hypothetical model indicating the possible interplay of genes that contributes towards host susceptibility in PKDL. SLC11A1 enhances macrophage activation in terms of its antimicrobial activity (e.g. iron sequestration from parasites) and its polymorphism (SLC11A1P) can render the host susceptible to VL. On the other hand, individuals who additionally have polymorphism in IFN-γR gene (IFN-γR*) are more susceptible to develop PKDL as the reduced functionality of IFN-γR gene allows the immune deactivating properties of IL-10 to predominate.

Conclusions:

PKDL is a perplexing disease, notable for a shift in parasite tropism after the onset of apparent cure from VL and also for the geographical variation in clinical presentation. None of the various hypotheses discussed above provides a fully satisfactory explanation for PKDL, though some more than others would appear to provide suitable explanations for PKDL at least in some geographic settings. The lack of mouse models of PKDL may have hampered
the formal testing of some of the hypotheses discussed above, but has helped focus attention on how best to analyze the available clinical material. Studies of parasite genotype/phenotype are clearly a high priority, and need to be intimately linked to state of the art functional histopathology. Creative clinical studies on this challenging disease are sure to yield new insight into the complexity of the *Leishmania*-host interaction in the years to come. To address the lacunae in our present knowledge on PKDL, particularly in India, the key objectives of my work were as follows:

1. **Estimation of the humoral responses in patients with PKDL in terms of their antileishmanial immunoglobulin distribution (total Ig, IgM, IgG, IgE and IgG subclasses along with IgG avidity) as compared with healthy controls.**
   
   This was aimed to confirm the antileishmanial humoral response status in a large study population. Accordingly, we checked the antileishmanial Ig levels among polymorphic and macular variants of PKDL patients. We also validated the therapeutic response of Indian PKDL patients to Miltefosine vis-à-vis Sodium antimony gluconate (SAG). We have also tried to measure the degree of IgG avidity in different clinical groups of PKDL and correlated with disease duration.

2. **Evaluation of cytokine response in patients with PKDL from plasma and cultured PBMCs as compared to healthy controls**
   
   Circulating levels of cytokines were measured, in terms of major pro-inflammatory cytokines (TNF-α, IL-6, IL-1β, and IL-8) and anti-inflammatory cytokines (IL-4, IL-10, IL-13 and TGF-β). The cytokine levels were compared between polymorphic and macular variants of PKDL as also between patients who received SAG vis-à-vis Miltefosine. Additionally, we assessed the ability of PBMCs to secrete cytokines with or without antigen stimulation.

3. **Evaluation of monocyte activation status in patients with PKDL**
   
   A detailed analysis of monocyte functions was attempted in this study. Parameters studied included CD14/CD16 distribution along with expression of co-stimulatory molecules (CD80, CD86 and CD40), activation and adhesion markers (HLA-DR, CD23 and CD54). Furthermore, expression of Toll like receptors (TLR-2 and TLR-4), generation of nitric oxide (NO), reactive oxygen species (ROS), intracellular thiol levels and intracellular cytokines (IL-6, IL-1β, IL-8, IL-12p40, TNF-α).

4. **Assessing the polarization of circulating monocytes and lesional macrophages towards alternative activation in patients with PKDL**
   
   Considering the complexity and plasticity of monocytes/macrophages and the fact that its relevance to the PKDL immune scenario is not known, this study examined polarization of circulating monocytes and/or dermal macrophages towards alternative activation. We have assessed the expression of arginase-1 and mannose receptor, two
most potent markers associated with alternative activation along with Peroxisome proliferator activator-γ (PPAR-γ) in monocytes and macrophages. In search of the signaling involved in this switch we have measured plasma vitamin D$_3$ and associated gene expression (CYP27B1, vitamin D receptor and cathelicidin or LL-37). We also have measured plasma iron, ferritin, transferrin, intramonocyte iron level, expression of transferrin receptor along with Haeme oxygenase-1 (HO-1) and CD163 to check whether the modulation of iron was also involved or not. Finally we confirmed the regulatory and immunosuppressive nature of these monocytes by measuring the intracellular IL-10 and TGF-β.