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## Visceral leishmaniasis: An immunological viewpoint on asymptomatic infections and post kala azar dermal leishmaniasis

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### ABSTRACT

Elimination of visceral leishmaniasis is a priority programme in Indian subcontinent. The World Health Organization has set a new target to eliminate kala-azar by the year 2020 as previous target elimination year (2015) has passed. The elimination programme has successfully curbed the rate of infection in endemic regions; however, there are still few challenges in its route. The current drug control regime is extremely limited and comprises only two (amphotericin B and miltefosine) drugs, which are also susceptible for parasites resistance. Moreover, these drugs do not produce sterile cure, and cured patients may develop post kala-azar dermal leishmaniasis even after a decade of cure leaving behind a potent source of parasitic reservoirs for further disease transmission. A significant proportion of endemic population remain seropositive but asymptomatic for many years without any clinical symptom that serve as latent parasitic reservoirs. The lack of tools to identify live parasites in asymptomatic infections and their association in disease transmission, parameters of sterile cure along with post kala-azar dermal leishmaniasis progression remain a major threat in its elimination. In this review, we discuss the potential of host immune inhibitory mechanisms to identify immune correlates of protective immunity to understand the mystery of asymptomatic infections, sterile cure and post kala azar dermal leishmaniasis.

## 1. Introduction

A protozoan parasite of the genus *Leishmania* causes a vector-borne disease leishmaniasis, which is prevalent in more than 98 countries at present. Out of 53 described species of *Leishmania* parasites, 20 are known to cause human pathogenesis that are spread by approximately 30 species of sand flies[1]. The infection of *Leishmania* in the human may produce three discrete clinical manifestations, i.e., cutaneous leishmaniasis, mucocutaneous leishmaniasis and visceral leishmaniasis (VL), which are caused by different species and differ in their immunopathology, degree of

morbidity and mortality.

VL (kala-azar), caused by *Leishmania donovani* (*L. donovani*), is a latent threat to more than 147 million people living in disease endemic South East Asia region of the Indian subcontinent. The estimates indicate about 100 000 cases per year that includes 15 000 reported cases. Out of five VL-affected countries (India, Bangladesh, Nepal, Thailand, and Bhutan of this region), India contributes more than 80% of reported cases whereas in Bhutan and Thailand reports are sporadic. In India, Bihar is the most VL-endemic state, with 90% of the Indian VL cases reported there[2]. *Leishmania infantum* causes VL in North Africa and Southern Europe while in Latin America the VL causing species is *Leishmania chagasi* and *L.*

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*donovani* is responsible for VL infections in East Africa[3].

The goal of elimination of VL from the Indian subcontinent by 2015, as set by World Health Assembly in 2005, has now passed[4]. The set target was to bring one VL case in 10 000 individuals among endemic residents at district level, which is not achieved yet. The WHO road-map target has now set a new goal for regional elimination of VL by or before 2020 from five countries India, Nepal, Bangladesh, Bhutan and Thailand[5]. The challenges in parasites and sand fly control measures along with changing epidemiology of VL in disease endemic countries are major threat in its elimination[6-8]. Further, the migration of infected but asymptomatic individuals from endemic regions has resulted in new infections in non-endemic regions[9]. Recently new cases have been reported from mountain areas in Nepal, which has almost eliminated the disease in Terai regions[10]. In southern Sudan, population migration to the area of greater sand fly exposure has led to an increased incidence of VL cases[11]. In India, new cases have been reported from various non-endemic regions, too[12]. The risk of disease transmission is further compounded by dormant parasitic reservoirs responsible for the resurgence of VL in both endemic and non-endemic regions[13].

## 2. Control measures and their limitations

Soon after identification of *Leishmania* parasite in 1901, the pentavalent antimony compounds were used to treat VL cases, which were also considered as true antileishmanials[14,15]. VL was controlled by such treatment until antimony resistant parasites appeared in early 1980s in Indian subcontinent[16,17] and treatment failure is now reached up to 65% in few endemic areas[18-20]. In early 1980s, an antimicrobial drug pentamidine (1983) was used in antimony refractory cases and cured 99% of patients initially however, within 2 decades its efficacy declined to approximately 70% patients leading to its abandonment in VL treatment[20-22].

During 1990–1998, an antifungal amphotericin B and an anticancer (miltefosine) became the first choice drugs to treat VL cases[23,24]. At present, single dose AmBisome, a liposomal formulation of amphotericin B, is a drug of choice for VL elimination programme and approved by WHO as preventive measure[25]. At present, these drugs are effective with satisfactory results but reports indicate that the parasites are developing of resistance to amphotericin B[26,27] and miltefosine[28-31].

In spite of significant knowledge on host-parasite relationships and immunobiology, the accurate parameters of protective immunity are not identified; therefore, a vaccine candidate either preventive or prophylactic is far from reality. Both, native and recombinant vaccine antigens such as gp63, gp46, m2, PSA2, TSA, LACK, LmsT1, and Leish111f, have been evaluated but all have failed to achieve long lasting protective immunity[32-35]. Among all vaccination approaches the live attenuated parasites have shown to produce the required magnitude of protective immunity in various animal models but clinical trials on humans are awaited [36,37].

## 3. Immunobiology of VL: Immune response and lacunae in the knowledge

All type of *Leishmania* infections begin with the entry of flagellated metacyclic promastigotes form of parasite in the blood stream, which are transmitted by female sand flies. *Leishmania* has developed various evasion strategies to counter innate and adaptive immunities for its survival and proliferation in mammalian host but precise mechanisms are not identified. After infection, neutrophils and macrophages are quickly recruited under the skin, at the site of bite, to protect host in early stages[38,39]. *Leishmania* induces phagocytosis without eliciting oxidative burst in macrophages, which is followed by induction of disease exacerbating anti-inflammatory cytokines [like interleukin (IL-4), IL-10 and transforming growth factor (TGF)- $\beta$  ] production[40-42]. *Leishmania* also down regulates a divalent cationic transporter; natural resistance associated with macrophage protein 1 (NRAMP1) now referred as solute carrier family 11a member 1 (SLC11A1), on phagolysosomes for its proliferation in macrophages. This transporter creates  $Fe^{2+}$ ,  $Mn^{2+}$ , and  $Zn^{2+}$  deprived environment inside the phagolysosomes by pumping them out as a host protective strategy that are required for growth and proliferation[43].

Adaptive immunity in VL is characterized by mixed Th1/2 immune responses. The parasite alters the phenotypic differentiation of antigen experienced  $CD4^+$  T cells into Th2 phenotype. The Th2 cytokines like IL-4 and IL-10 are known to be responsible for disease outcome whereas Th1 cytokines like IL-2, IL-12, and interferon- $\gamma$  (IFN- $\gamma$ ) confer disease resistance[44-46]. Some recent studies indicate the role of IL-17, IL-21, IL-22, and IL-27 in disease resistance and susceptibility[46,47]. Recently it has been observed that skin-resident  $CD4^+$  T cells also play a significant role in parasite clearance during early days of infection in a nitric oxide and reactive oxygen species dependent manner[48]. Interestingly, these  $CD4^+$  T cells are also long lived and establish residence in the absence of persistent parasites, similar to central memory T cells hence may play substantial role in vaccine induced immunity but requires more studies. The present immunological information seems to be inadequate to classify and explain various clinical and subclinical states of VL infections. This necessitates identification of new correlates of host immunity for a better understanding on sterile cure, post kala-azar dermal leishmaniasis (PKDL) progression, asymptomatic infections and development of a vaccine candidate.

## 4. Immune inhibitory mechanisms: Perspective of immune tolerance in VL

The immune suppression mechanisms are mainly controlled by two types of inhibitory processes (extrinsic and intrinsic), which are mediated by various pro- and anti-inflammatory cytokines production by activated cells. The extrinsic mechanisms involve recruitment of specialized effector cells such as T regulatory cells

(Tregs), which produce inhibitory (anti-inflammatory) cytokines (mainly IL-10) to control activated immune response. The intrinsic mechanisms involve expression of specialized receptors on the surface of innate and adaptive immune cells that deliver inhibitory signals via immunoreceptor tyrosine based inhibitory motifs and non-inhibitory motifs after ligand interaction[49,50]. Some of these receptors are PD1, CTLA4, BTLA, LAG3, TIM3, CD47, CD200, CD200R, and CD300, which are known to regulate activation states of both, myeloid and lymphoid cells, and have been found to alter pathophysiology of various infectious and non-infectious diseases[51–53]. These receptors after interaction with their ligands activate Src homology region 2 domain-containing phosphatase-1/2 (SHP1/2) and Src homology region 2 domain-containing inositol phosphatases (SHIP), which dephosphorylate activated proximal and distal kinases of activated cells in order to control their functions[50,54].

In VL, the production of IL-10 has been found to be correlated with VL pathogenesis but its source is not identified, yet. Surprisingly the increased IL-10 production in VL is not associated with Tregs, the cells of extrinsic mechanism[55]. Very little knowledge is available on the role of intrinsic mechanisms of immune inhibition on macrophages dysfunction, poor antigen presentation by dendritic cells, CD4<sup>+</sup> T cells phenotypic differentiation and functionality in VL. The literature indicates (discussed later in this review) that these mechanisms either lead to total or partial exhaustion of activated T cells through specific receptor ligand mediated effector mechanisms. Activation of these receptors has been linked to alter phenotypic differentiation of activated T cells, inhibition of their multifunctional abilities and production of inhibitory cytokines[51]. The determinants of host immunity that alter or determine active phenotypes of antigen experienced CD4<sup>+</sup> T cells are not known. Therefore, delineation of intrinsic mechanisms may help to understand immunobiology of VL pathogenesis and to identify parameters of protective immunity. Based on nature of *Leishmania* parasite and its dominance over host immunity, we understand that the CD200, CD200R and CD300 linked mechanisms may help to delineate the mechanisms associated with various clinical and subclinical states of VL.

CD200 is a type Ia membrane protein with extracellular immunoglobulin superfamily domain, a single transmembrane region and a short cytoplasmic tail with no signalling motifs. It is widely expressed on myeloid, lymphoid cell lineage and non-immune cells as well[56]. Its receptor, CD200R, is differentially expressed on T cells, B cells, NK cells and cells of myeloid origin[56–58]. The 67 aa long cytoplasmic tail of CD200R has three tyrosine residues of which the distal residue is located in a phosphotyrosine binding domain recognition motif, NPxy. After phosphorylation by Src kinases, NPxy binds to phosphotyrosine binding domain containing downstream of tyrosine kinase 1 and 2 adaptor protein, which results in further recruitment of Src homology region 2 domain containing inositol phosphate and RAS p21 protein activator 1[59]. The Src homology region 2 domain-containing inositol phosphatase eventually dephosphorylate phosphotydylionositol 3 phosphate

and RAS p21 protein activator 1 leading to the deactivation of Ras related kinases, which eventually inhibit Akt pathway related effector molecules required in cellular growth, differentiation and function.

The CD200–CD200R interaction may negatively or positively regulate activated cells towards an auto-regulatory process for their self-inactivation by controlling both, pro- and anti-inflammatory cytokines production, and also maintain required balance between immune response and immune tolerance[60,61]. Studies suggest that CD200 down regulates macrophage effector functions, inhibit antigen specific T cell response in various tumors[62–64]. Recently, Rygiel *et al.* observed that the external and internal tumor antigens up regulate CD200–CD200R axis, which result in altered immune tolerance and increased frequency of Treg/Th17 cells[49]. Further, the CD200 blockade results in antigen specific Th1 response and significant decrease in Tregs in various cancers that further suggest an important role of this axis in regulation of T cell functions[65–67]. Although there is not much data on its role in pathogenic infections, studies reveal that this axis controls exacerbated inflammation during viral and bacterial infections and the lack of CD200 signal exacerbate disease pathology[58,68,69]. In herpes (Kaposi's sarcoma-associated herpesvirus) infection CD200 and its viral analogue OX2 inhibit antigen specific T cells, IFN- $\gamma$  production and target killing ability of the cytolytic granule component, CD107a, to cell surface along with inhibited Akt phosphorylation[70]. During influenza infection, CD200<sup>-/-</sup> mice results in severe pathology in spite of adequate immune response that was dependent on the presence of T cells since T cell depletion yielded in resolution of pathological symptoms[68,71]. Although these studies did not analyze the functional characteristics of antigen experienced T cells, this suggested the loss of protective ability and acquisition of disease promoting ability of T cells. Further, in herpes simplex virus-I infection CD200 blockade suppressed Th1 type response and up regulated Tregs production suggesting the role of axis in T cell function and differentiation[72]. The only study in *Leishmania* by Cortez *et al.* has shown that *Leishmania amazonensis* induces CD200 expression and suppresses macrophage activation via inducible nitric oxide synthase inhibition that eventually leads to increased parasite growth[73].

Similarly, the other immune inhibitory receptor, CD300, is known to play significant role in various diseases like cancer and sepsis[74]. The human CD300 family is a family of seven membrane receptors and all of them have an extracellular immunoglobulin V like domain. The inhibitory receptors of this family have long immunoreceptor tyrosine-based inhibition motif for their adaptor proteins[75]. These receptors are expressed on both lymphoid and myeloid lineages[76]. CD300 recognizes phosphatidylserine and phosphatidylethanolamine, exposed on the outer leaflet of dead and activated cells, and delivers inhibitory signals to activated cells through SHP1/2 phosphatases[75]. The role of CD300 receptors are not studied in any parasitic diseases yet. Since *Leishmania* expresses plenty of phosphatidylserine in the outer leaflet of cytoplasmic membrane[77], the same molecule to which CD300 is known to

bind, it would be worthy to explore CD300 role in VL pathogenesis. Therefore, on these backgrounds it would be worthy to delineate their role in VL pathogenesis in the context to existing challenges in VL such as asymptomatic infections, sterile cure and PKDL progression.

## 5. Challenges in VL elimination

The existing lacunae in the knowledge on VL affected subjects are a serious threat in its elimination. The drug control regime is very limited and also believed to be inefficient to produce sterile cure as successfully cured patients have shown to carry live parasites and remain seropositive for prolonged year[78,79]. The immunological information is not adequate to define parameters of protective immunity that is why a vaccine candidate is far from reality.

In VL the sterile protection or cure is a debatable issue, but there are plenty of reasons and evidences to believe that sterile protection can be achieved. After successful treatment a person develop substantial protective immunity and considered to be protected from reinfection or relapse that suggest such possibilities. In all endemic regions there are asymptomatic individuals who remain seropositive for many years without developing disease. It is not well understood whether they carry live parasites and help in disease transmission. Further, a majority of these individuals turn to seronegative in due course of time, albeit they live in endemic regions suggesting a possibility of resistant development in such individuals. The identification of immune correlates of disease resistance and susceptible will also help to monitor VL progression in non-endemic regions.

Along with altered functionality and exhaustion, the proliferation of T cells (CD4 and CD8) is also highly compromised during active VL, though the associated mechanisms are not well known. An ideal magnitude of immune response is required for generation and proliferation of effector T and B cells, which eventually leads to effective establishment of memory T and B cells[80]. Although it is practically difficult to establish the magnitude of required immune response for sterile protection, it is achievable as this has already been established in malaria infection[81]. The factors to establish protective threshold of immune response, either host or parasite, are needed to be identified in *Leishmania* infections too. Certainly, there are some factors in VL as well, which protect few endemic individuals from infections as evidenced by conversion of their seropositive to seronegative status as well as prolong onset of disease. Nevertheless, some seropositive endemic individuals develop VL, which may be due to failure of protective immune response in presence of excessive parasite load after successive sand fly bites or other unknown factors. Substantial knowledge on the mechanisms by which *Leishmania* modulates host immune response, as discussed above, in its favour is available but how it manipulates host immune tolerance mechanisms is still unknown. It has already been established that for a stable protection and long term immunity a perfect balance of immune response and immune tolerance

mechanisms is essentially required[82]. It is quite possible that *Leishmania* induces immune tolerance mechanisms to control antigen experienced T and B cells phenotypic differentiation, proliferation and functions soon after infection. Since our knowledge on these mechanisms in VL is very limited therefore, their understanding may provide an insight and can divulge to identify correlates of protective immunity.

The nature of VL pathogenesis is one of the biggest hurdles in finding such parameters but a comparative and comprehensive study on immune tolerance and immune response mechanisms in various clinical and subclinical states of VL may provide such information. We have tried to summarize the possible pathologies in VL affected subjects, which are based on both, and scientific evidences (Table 1).

## 6. Mysteries of VL

### 6.1. Mystery of asymptomatic infections: Do they carry live parasites?

Asymptomatic infections are those who remain seropositive for many (up to 10–12) years without developing into active disease[83,84], and are more prevalent in VL endemic regions[85]. Of these, 10%–20% of healthy endemic population without past history of VL, show seroreactivity with parasitic antigens, and 20%–25% display polymerase chain reaction positivity in Indian subcontinent[86]. Reports from the other endemic regions also confirm the existence of parasitic DNA in all VL causing species in asymptomatic individuals[87–90]. Although majority of seropositive asymptomatic individuals convert in seronegative status in due course of time, these are known to contribute in disease progression in non-endemic regions[42,91–93]. A report from Bangladesh also confirms that approximately 80% of asymptomatic individual contribute in disease transmission as compared to 8%–10% of VL and PKDL[85].

The conversions of asymptomatic infections to symptomatic VL also indicate the persistence of parasites in these individuals albeit various host and parasite factors may be additional contributors[94]. The reported ratios of conversion are 4:1 in Kenya[87] and Bangladesh[95], 1.2:4 to 11:1 in Africa[96] between 6.5:1 to 18.5:1 in Brazil[97] and 8.9:1 in India and Nepal[91,98]. Interestingly, these numbers clearly suggest that a large number of individuals do not turn into active disease and also turn to seronegative status, which is suggestive of protective immune response generation in such individuals.

The factors that play role in transition of asymptomatic to symptomatic pathologies are largely unknown. Few studies indicate that host genetic association and development of clinical symptoms is linked to *NRAMP1*[99], *TNF- $\alpha$*  [100], *IL-4* and interferon- $\gamma$  receptor (*IFNGR1*)[101], *TGF  $\beta$  1*, *IL-8*[102], C-X-C chemokine receptor 1 (*CXCR1*) and C-X-C chemokine receptor 2 (*CXCR2*)[103], *IL-2R  $\beta$*  [104], Delta-like1 (*DLL1*)[105], and mannan binding lectin[106] genes.

The extrinsic factors such as host immune compromised status, age and nutritional status are also considered to play an important role in asymptomatic to symptomatic conversion[107,108].

In addition, increased serum levels of IFN- $\gamma$ , C reactive protein, nitric oxide, and IL-12 has also been found to provide resistant to asymptomatic subjects[109,110]. This indicates that disease resistant endemic subjects develop protective immune response after sand fly bite that protects them from pathogenesis. On contrary, the increased levels of IL-10, IL-4 and TNF- $\alpha$  in these cases have been found to be associated with the development of disease susceptibility suggesting a breach in protective immune response due to various unidentified host and parasitic factors[95,111]. However, these findings are more or less similar to VL immunology hence cannot be used to frame a clear picture of parameters of protective immunity in asymptomatic subjects.

### 6.2. Disease susceptible asymptomatic infections: The carrier of parasites

Among asymptomatic infections, the first group comprises those individuals who remain seropositive for many years (>1 years) without developing clinical symptoms. None of the study, so far, either provides or discusses the reason of persistent antibody in these individuals. Along with protective immune response, it seems that

these individuals may either have strong pool of long lived memory B cells, which constantly secrete antibodies, or they get constant *Leishmania* exposure but do not develop VL due to protective immune response[112,113]. In these cases, the strong generation of cell mediated immunity, sufficient number of activated T cells with retained multi-functionality to produce pro-inflammatory cytokines, specific B cells population, and a greater pool of memory T and B cells protect them from future infections and VL pathogenesis. These individuals clearly suggest that they produce required threshold of immune response, which is sufficient to counter a fresh bite of infected sand flies and prevent them from active disease for a longer duration. Probably they also do not carry live parasite due to strong immune response generation that efficiently eliminates invading infections but they show seropositivity probably because of long lived plasma cells. However, the possibility of live parasites in such cases cannot be overruled due to constant seropositive nature of such individuals. A detailed and comparative study on immune tolerance and immune response mechanisms in these individuals can provide the correlates of protective immunity, which can be further used to engineer a vaccine candidate or can be used to discriminate between clinical and subclinical states of VL.

The second group comprise those seropositive asymptomatic individuals who remain positive for certain period but develop VL in due course of time (<1 years). Studies indicate that 1.5%–23%

**Table 1**

A perceptible classification of various categories of *L. donovani* affected individuals in disease endemic regions.

Infected individuals	Classification	Manifestations	Probable reasons	Outcome
Seropositive but asymptomatic individuals in endemic regions	True resistant cases	Turn seronegative in due course of time.	Strong CMI. Large pool of memory T cells. Short lived plasma cells.	Do not carry live parasites.
	Transitional cases	Remain seropositive from months to years without clinical symptoms.	Possess required immune response to keep them protected. Substantial generation of CMI after leishmanial infection. They show seropositivity for a prolonged period even in absence of live parasites due to long lived plasma cells.	They may carry live parasites for a transient period.
	True sensitive cases	Show signs and symptoms of VL pathogenesis soon after parasite inoculum.	Insufficient generation of CMI at the time of parasite inoculum. Poor or compromised immune status. Breach in immunity.	Carry live parasites.
Cured VL individuals	Sterile cure	Turn seronegative in due course of time. No relapse	Establishment of strong CMI after treatment. Sufficient memory T cells reservoir to protect future infections. Generation of short lived Plasma cells.	Do not carry live parasites.
	Non sterile cure	Remain seropositive after completion of treatment. May develop in PKDL	Insufficient generation of CMI after completion of treatment. Breach in established CMI due to compromised immune status.	Carry live parasites.

VL: visceral leishmaniasis; CMI: cell mediated immunity; PKDL: post kala-azar dermal leishmaniasis. A prospective and detailed study of both, immune response and immune tolerance mechanisms can provide factors related to diseases resistance, susceptibility and parameters of protective immunity, asymptomatic to symptomatic conversions, post kala-azar dermal leishmaniasis (PKDL) progression which could also be exploited to develop a targeted vaccine.

of asymptomatic infections develop sign and symptoms of VL within 1 year [83,91,93,98,114–117]. The rate of asymptomatic to symptomatic VL conversion has been linked to amount of antibodies in their blood. The individuals with high (up to 40 times) antibody titre are more susceptible for asymptomatic to symptomatic conversion [114,116,118]. These cases further provide evidence that they carry live parasites throughout the period before appearance of active disease. The detection of parasitic DNA in about 25% of such asymptomatic carriers who had no past history of VL further confirm such possibility [86,89,119]. The low titre individuals suggest the possible existence of required immune response threshold that protects them for a quite long time from active disease as compared to those with high antibody titre. A breach in immune status either by parasitic (successive sand fly bite as they live in endemic zone) or their compromised immune status (due to other infections or poor nutritional status) may lead to appearance of VL symptoms [108,120]. Therefore, such asymptomatic cases may be classified as true asymptomatic carriers who may transmit disease. This may be due to the breach in protective immune response threshold by either parasitic or compromised host immunity. Although it will be very hard to monitor these cases, such individuals may help in identification of host immune factors responsible in disease resistance and susceptibility.

### 6.3. Disease resistant asymptomatic infections: An array of hope to identify parameters of sterile cure and protective immunity

The third asymptomatic category comprises those individuals of endemic regions who turn seronegative in due course of time [91]. The spontaneous conversion of seropositive into seronegative status varies from 33%–86% [91,98,121,122]. However, within a year among individuals with high antibody titre, this conversion is as low as 6.3% as observed in a study conducted in Bangladesh [95]. These individuals provide further concrete evidence that there is a requirement of threshold immune response level to protect host from parasite. These individuals probably achieve desired threshold of immune response after the parasite exposure, which protect them from future disease onset by effectively clearing live parasites. The probable reasons of their seronegativity can be understood as they either do not encounter *Leishmania* infected sand fly bite (not possible as they live in endemic region) or host immunity efficiently/immediately eliminates infection. They probably do not carry live parasites, and can be considered as true resistant cases. Such a condition also support that sterile cure is achievable in VL. In addition, successfully cured patients are supposed to be resistant for recurrence/reinfection, and very small percentage (about 4%) of treated patients relapse that further confirms the existence of protective immune response post cure [27,79,123]. The immune biology of resistant asymptomatic infections may be associated with strong cell mediated immunity, a large repertoire of memory T and B cells along with short lived plasma cells (they do not show seropositivity

for a longer period). Focussed studies on these individuals may reveal the sought parameters of protective immunity to develop a prophylactic vaccine candidate.

### 6.4. Active VL individuals: The sufferers

The fourth category of VL comprises those individuals who develop VL soon after infection or in due course of time. However, the current available tools are not adequate to predict the exact time line for development of VL symptoms in *Leishmania* infected individuals. They harbour live parasites and require medical intervention. The possible reasons of VL pathogenesis may be the insufficient generation of cell mediated immunity at the time of infection due to their compromised immune status or other unknown factors. Various socioeconomic, environmental factors and their genetic integrity may also be associated factors for breach in the immunity as discussed above [124–126]. However, the early onset of disease in some (chronic VL cases) endemic individuals, as compared to seropositive asymptomatics, further suggests the possibility of protective host traits and hence requires more studies.

### 6.5. PKDL ambiguity

PKDL is an unusual presentation of VL causing *Leishmania* species, which is characterized by discrete clinical symptoms like hypo pigmented macular, papular and nodular lesions with presence of live parasites. Out of three VL causing species, *L. donovani* infection results in more PKDL cases followed by *Leishmania infantum*. PKDL cases are very rare in *Leishmania chagasi* infections, which suggest parasitic role in PKDL development albeit the host or parasitic parameters responsible in changing the properties of parasite, i.e., viscerotropic to dermatotropic are not identified, yet. An estimated 30% of *Leishmania* affected individuals develop signs and symptoms of PKDL without developing VL or any past history of VL [127,128]. Interestingly, about 15% of successfully cured VL cases also develop PKDL symptoms within a year in Indian subcontinent [127,129], whereas in Sudan the conversion rate is 50%–60% [130]. Considerable cases of PKDL resolve spontaneously suggesting protective immune response [128]. The increasing incidence of PKDL in endemic regions, post amphotericin B era including miltefosine, warrants more studies on its etiological factors [127,131,132]. In addition, in certain disease endemic regions PKDL cases (versus VL) are gradually increasing as the disease may develop even after 10 years post cure [133]. A recent study also reports increased numbers of PKDL case in Indian states of Bihar, West Bengal and eastern Uttar Pradesh [134]. So far the role of PKDL in disease transmission is not clear but xenodiagnostic and culture studies have demonstrated that these individuals carry live parasites and therefore may be involved in spread of the disease [78,134–136].

The immunological presentation of PKDL is similar to VL but dissociated between the visceral organs and skin. However, dominance of Th2 cytokines especially IL-10 over Th1 is more

prominent in PKDL. The host or parasite factors that enable *Leishmania* to appear and survive in the skin are not clear. As PKDL lesions occur in sun exposed areas, the compromised dendritic cells under UV light have been linked to Th2 type immune response [137,138]. The increased level of IL-10 secreting CD3<sup>+</sup> CD8<sup>+</sup> Tregs and IL-10, TGF- $\beta$  by keratinocytes has also been linked to PKDL [139]. The clinical manifestation of PKDL is also associated to host cell mediated immunity as in acute PKDL it is way higher than chronic cases suggesting its protective role in PKDL development. Few studies also indicate the role of M2 type macrophages, metalloproteinases, and Th17 response in development and persistent of PKDL. However, it is still not clear that why there is so much variability in PKDL development among asymptomatic infections and cured VL cases; moreover, the current tools are insufficient to understand this puzzle.

## 7. Concluding remarks

We have made significant progress in VL diagnostic, epidemiology, and understanding on the role of innate immune mechanisms in VL pathogenesis such as a test of cure (sterile vs. nonsterile), quantitative test to measure parasites load in different VL cases (asymptomatic, PKDL, fresh and cured cases), correlates of protective immunity and PKDL development, and prevention is still required. On the basis of current knowledge and available tools, it is very difficult to discriminate individuals among resistant, sterile, asymptomatic, who will develop active disease or develop PKDL or remain asymptomatic or become sterile. Finding such knowledge and identification of parameters of protective immunity will not only help to discriminate such individuals in disease endemic regions but also will help to design new prophylactic control measures and disease monitoring. A comprehensive and comparative study on immunological responses considering all aspects of host immune response and immune tolerance mechanisms of chronic, cured, PKDL and asymptomatic VL infections may help to identify parameters of protective immunity. However, this will be a multi-centric task, therefore focused and collaborative work is required to solve the remaining challenges of visceral leishmaniasis.

## Conflict of interest statement

The authors declared that they have no conflict of interest.

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