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The immunological, environmental, and phylogenetic perpetrators of metastatic leishmaniasis

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Abstract

Cutaneous leishmaniases have persisted for centuries as chronically disfiguring parasitic infections affecting millions of people across the subtropics. Symptoms range from the more prevalent single, self-healing cutaneous lesion to a persistent, metastatic disease, where ulcerations and granulomatous nodules can affect multiple secondary sites of the skin and delicate facial mucosa, even sometimes diffusing throughout the cutaneous system as a papular rash. The basis for such diverse pathologies is multifactorial, ranging from parasite phylogeny to host immunocompetence and various environmental factors. Although complex, these pathologies often prey on weaknesses in the innate immune system and its pattern recognition receptors. This review explores the observed and potential associations among the multifactorial perpetrators of infectious metastasis and components of the innate immune system.

Keywords

cutaneous leishmaniasis; metastatic leishmaniasis; post-kala-azar dermal leishmaniasis; *Leishmania* RNA virus; pattern recognition receptor; Toll-like receptor

An ancient and emerging disease

Leishmaniases have persisted for centuries as life-threatening and disfiguring parasitic diseases affecting millions of people across the subtropics. Currently, 98 countries are listed as having endemic disease, amounting to an estimated 12 million cases with 2 million more each year [1]. Human disease is caused by sp. of *Leishmania* protozoan parasites and is cycled among hosts through the bite of a female sand fly vector. Symptoms range from single self-healing cutaneous lesions to fatal visceralization or chronic metastatic dissemination throughout the skin. However, despite its prevalence, persistence, and conspicuous symptoms, the disease remains largely uncontrolled, with few new treatment options and no comprehensively effective vaccine. Migration and densification of populations in subtropical regions are compounding with global warming and a growing

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HIV-positive (immunodeficient) demographic to class leishmaniasis as a serious emerging global threat [2]. Further, growing local and international instability has fuelled major outbreaks in new populations that spread quickly among the vulnerable of conflict zones, living in densely packed and poorly insulated shelters. These unsettled populations pose a risk of widening leishmanial geography during resettlement, as was the case after the Sudanese Civil War, the Gulf and Iraq wars, and currently among Syrian refugees [3,4].

The centuries of geographically isolated evolution have allowed each *Leishmania* spp. to develop intricate pathways of immune evasion, creating various symptomatic outcomes, and enabling parasites to persist under astounding immunological pressure, even existing as life-long infections after symptomatic resolution [5].

A common route of entry – widely different outcomes

Leishmania is generally transmitted through the bite of an infected sand fly. However, from this common origin, the same sp. can cause widely different outcomes. In most instances, disease is ‘asymptomatic’, without any obvious pathology, although still able to support life-long infection. The presence of persistent parasites in asymptomatic infections is a double-edged sword – on the one hand, potentially conferring immunity to superinfection, but on the other hand, creating the dangerous likelihood of reactivation, which is often associated with a more severe symptomatic outcome. In infections for which pathology is overt, outcomes can again vary widely. Localized cutaneous leishmaniasis (LCL) occurs in many cases, which can persist as chronic open lesions or resolve into hyperpigmented scars. For the more severe forms of leishmaniasis, pathology is not limited to the infection site but instead progresses in various ways that can be divided into metastatic leishmaniasis, diffuse CL (DCL) or a systemic visceralization (VL) that has an important cutaneous complication, post-kala-azar dermal leishmaniasis (PKDL). These forms can also appear following seemingly ‘asymptomatic’ infections without a prior cutaneous presentation.

Surprisingly, little is known about the basic mechanisms of symptomatic divergence. This review aims to assemble the current knowledge on the immunological, environmental, and phylogenetic perpetrators of persistent and metastatic outcomes, which significantly complicate the diagnosis, treatment, and control of leishmaniasis. We also use this opportunity to propose new potential risk factors that are supported by anecdotal evidence with the hope to stimulate much-needed further research.

Symptomatic outcomes of cutaneous leishmaniasis

Human infections are generally caused by species of two major *Leishmania* subgenera, namely *Leishmania* (*Leishmania*) and *L. (Viannia)*. Although *L. (Leishmania)* is found worldwide, the majority of infections occur in the Paleotropics (Eurasia and Africa), where common infecting species are *Leishmania major*, *Leishmania tropica*, *Leishmania aethiopica*, and *Leishmania donovani*. Species of the *Viannia* subgenus, by contrast, are exclusively endemic in the Neotropics (the Americas), with common infections being caused by *Leishmania braziliensis*, *Leishmania panamensis*, and *Leishmania guyanensis*. Depending on the infecting species and the immune response in a susceptible host, *Leishmania* parasites can induce two major pathologies: VL or CL.

Although VL, or 'kala-azar' (see Glossary), is the most serious form of the disease, it is relatively rare, contributing to only 10% of all leishmaniasis worldwide. *Leishmania* parasites are mostly dermotropic, where even VL is often followed by the diffuse and difficult-to-treat cutaneous lesions of PKDL. This review focuses on the various forms of persistent CL.

Localized cutaneous leishmaniasis

CL is endemic in numerous regions of the subtropics. It is most frequently caused by *L. major*, *L. tropica*, and *L. aethiopica* in the Paleotropics, whereas in the Neotropics it is caused by *Leishmania mexicana* and *Leishmania amazonensis*, or *L. (Viannia) braziliensis*, *L. (Viannia) panamensis*, and *L. (Viannia) guyanensis* [6]. Although CL generally manifests as a lesion localized at the inoculation site (LCL), its various physical presentations and immunopathologies have complicated diagnosis and scuttled attempts of forming a universal therapeutic or vaccination strategy. Globally, lesions can vary from a small self-healing ulceration to granulomatous nodules and large, seeping, erythematous wounds. Chronic infection and inflammation can last for several months or years and often leads to significant tissue damage and permanent, hyperpigmented scarring. In certain cases, the infection metastasizes to sites beyond the inoculation and can be referred to as metastatic leishmaniasis, by analogy to tumor cell metastasis.

Metastatic leishmaniasis

Metastatic complications occur across all regions where leishmaniasis is endemic but are particularly prevalent and aggressive in *L. (Viannia)* infections of the Neotropics [7]. They may also present with various symptoms, seemingly dependent on differences in the immune response elicited by the various metastatic parasite species (Box 1). Particular symptomatic outcomes can be grouped geographically (Figure 1), where, for example, the Paleotropics hosts many forms of nonulcerative, papular, and herpetiform leishmaniasis spreading within a small radius of the primary lesion, whereas the Neotropics is better known for large ulcerative and granulomatous lesions, which often occur at sites distant from the primary lesion. Indeed, certain Neotropical parasites have a specific tropism for the delicate mucosal tissues of the nose and face, creating a particularly disfiguring disease known as mucosal leishmaniasis. The inflammation in the nasal mucosa is inordinately potent when contrasted with the sometimes undetectably low number of parasites. Lesion biopsies often reveal no infection. This phenomenon emphasizes the major role of the immune response in disease pathology and the potential of immunomodulatory agents in antileishmanial therapy. However, the immune involvement is diverse and opposing responses have been blamed for the various forms of infectious metastasis. For example, the mostly Paleotropical recidivans CL is described as a symptomatic reactivation of infection within the scars of a healed lesion; it is characterized by a potent cell mediated response in multiple nodules, which spread and coalesce to form significant tissue damage. Conversely, a total lack of a cell mediated response has been seen in DCL, where infection diffuses into hundreds of immunologically anergic nodules throughout the skin [8].

Post-kala-azar dermal leishmaniasis

PKDL is an important dermal complication of Paleotropical VL, occurring in 10–20% of VL patients in India and up to 60% in Eastern African states such as Sudan and Ethiopia [9]. Although treatment is essential in Indian PKDL, African disease is generally self-healing. In most cases, patients feel otherwise well and present with a range of slowly evolving painless macular or papular rashes over large body surfaces, generally radiating from facial mucosa to large surfaces of the trunk and limbs. Nevertheless, the persistence of this seemingly harmless rash may play a more insidious role in the life cycle of its causative agent, *L. donovani*: functioning as a reservoir phase to shelter the parasite during the interseasonal periods of the sand fly. Indeed, the disease is mostly anthroponotic in these regions and only a few isolated studies have identified a potential animal reservoir. Despite the fact that the disease is caused by the same parasite, slight differences are seen between presentations in Africa and India. African PKDL patients have a higher tendency for ulcerative nodular forms, whereas Indian patients more commonly experience hypopigmented and maculopapular rashes with large plaque formations (Figure 1B). Interestingly, PKDL occurs almost exclusively in patients that were previously cured of VL and may appear up to 20 years after the initial infection; the average inter VL–PKDL period is 6 years on the Indian subcontinent and less than 1 year in Africa [9]. Although PKDL in Sudan can appear concurrently with, or sometimes in the absence of, VL, in general the disease is highly uncommon in patients who have not yet presented with VL and received treatment. Thus, certain therapies are actually considered a significant risk factor for developing the disease [10]. Because the therapies often restore or boost the patient's inflammatory T helper 1 (Th1) immune response, PKDL is widely accepted as being an immunologically mediated disease (Box 2).

Importantly, the appearance of any one of these forms of leishmanial metastasis can occur several months or years of the initial infection and often appear after the resolution of the initial infection with seemingly no predictive factors. The next part of this review describes a few circumstantial risk factors that may identify patients at higher risk of developing metastatic disease.

Metastatic risk factors

The mechanisms behind metastatic potential are currently unknown. Although immunological and environmental vulnerabilities in the host and various parasite phylogenies have been linked to the onset of infectious metastasis (Figure 2), there is no consensus on which of these factors is essential. Conflicting evidence is probably an indication that the process is multifactorial and dependent on complex interactions among parasite, host, and environmental factors, including genetic and nongenetic factors.

Metastatic risk factors in the parasite

Phylogeny and polymorphisms

Geographical boundaries remain the clearest delineation among symptomatic outcomes, and because they parallel parasite-specific endemic regions, it is often assumed that disease

outcomes branch with parasite phylogeny. So far, comparative analysis across the genomes of LCL, metastatic CL, and VL *Leishmania* spp. has not revealed a universal 'metastatic gene' [11–13]. Nevertheless, these studies cannot yet include the effect of differential protein expression achieved through changes in gene regulation, copy number, single nucleotide polymorphisms, or the presence of pseudogenes [14]. Although many of the differentially expressed genes are 'hypotheticals' of unknown function, some may have putative virulence activity. For instance, metastatic *L. braziliensis* is known to carry supplementary copies of NADPH-dependent fumarate reductase and a homolog of glutathione peroxidase (as well as having lost a trypanothione synthase-like protein): all are important enzymes in the detoxification of oxidative stress. Whether these have any physiological importance is unknown. Hopefully, the rapidly growing database of *Leishmania* genomes (www.tritrypdb.org) will soon provide more insight into the role of parasite phylogeny in symptomatic outcome.

Although differences at the genetic level are poorly identified, various physiological differences have already been described, where metastatic parasites seem to have improved survival capabilities attributable to improved resistance to oxidative stress [15] and antileishmanial drugs such as antimony [16,17]. Interestingly, some studies have found heterogeneity among parasites isolated from the metastatic and primary lesions within the same patient. The most striking example was found in PKDL, where visceralizing parasites had significant genetic differences to those found later in the skin during PKDL [18,19]. Further, the cutaneous parasites showed an upregulation of certain surface proteins associated with virulence [20], implying that metastasis is a result of divergent parasite evolution or that, rather than metastasis, patients are actually reinfected with a variant, for which their existing immunity to previous infection acts as a susceptibility factor for the onset of PKDL.

Overall, findings support the hypothesis that infectious metastasis arises from low levels of persistent infection, where slowly dividing or 'dormant' parasites are possibly reactivated by antimony treatment or some similar type of immunological stress [16,17]. Indeed, parasites can often be detected in histologically 'normal' mucosal tissues of LCL patients [21], thereby indicating that this state of dormancy is probably achieved through a tightly controlled immunological tolerance rather than by transforming into a specialized 'dormant' morphology, for which there is no substantial evidence. Convincing support of immune tolerance is seen repeatedly where disruptions to immune functioning initiate symptomatic reactivations, such as after organ transplants or herpes zoster infection [22]. So far, some links to reactivation have been found in variations of parasite immunogenicity, where the concentration or combination of certain pattern recognition receptor (PRR) ligands is able to determine the course of infection. A striking example of this arises from a surprising nongenetic source, discussed below.

Nongenetic factors

We recently provided evidence that cytoplasmic pathogens of the *Leishmania* parasite can influence the course of leishmaniasis. Here, strains of *L. guyanensis* were found to be infected by a cytoplasmic virus, *Leishmania* RNA virus (LRV). These LRV-bearing

parasites repeatedly metastasized in a hamster model of infection, in contrast to their LRV-negative counterparts [23,24]. The process was shown to be immunologically mediated, where the viral double-stranded RNA (dsRNA) provoked a potent inflammatory response after engaging endosomal Toll-like receptor 3 (TLR3), resulting in the production of interferon (IFN)-β, which inflamed and worsened leishmanial lesions in mice as well as prolonging parasite survival. This situation of improved parasite survival in spite of a potent inflammatory response is reminiscent of what is observed in human mucosal leishmaniasis patients. This variant of LRV (LRV1) has since been found in various isolates of Neotropical metastatic cutaneous leishmaniasis (MCL) from species of *L. guyanensis* and *L. braziliensis* [25]. Further, a depletion of LRV1 in genetically identical *L. guyanensis* clones confirmed the role of LRV1 in disease severity [23]. Similar to other Totiviruses, which are generally neither shed nor infectious and thus inherited only vertically or during genetic exchange, the relationships between the LRVs closely parallel the relationships between the parasite species within which they reside. Recently, confirmation of the presence in *L. aethiopica* of a new LRV variant of the single (and exceptional) LRV2 isolate of *L. major* [26], indicates that LRV infection may have a much wider global reach. As described in Box 1, *L. aethiopica* is one of the few Paleotropical species causing metastatic complications such as mucosal leishmaniasis and DCL. So far, however, LRV has only been proven to act as a virulence factor in murine models of *L. guyanensis* infections. Its clinical role in *L. braziliensis* and *L. aethiopica* infections is yet to be defined. Unfortunately, no reliable clinical or epidemiological data exist to assess the correlation between LRV presence and metastatic leishmaniasis. Current reports show that infectious metastasis for *L. braziliensis* is not exclusively associated with LRV presence [27]. Additionally, LRV has not yet been detected in *L. panamensis*, which can also cause mucosal leishmaniasis. These facts suggest that infectious metastasis is a complex multifactorial process, in which the host and its environment also play a major role.

Metastatic risk factors in the host

Although fair assumptions can be made on host risk factors from approximations in animal models, the lack of large-scale or systematic human studies leaves many questions regarding the effect of human-specific or ‘real-life’ factors, such as risks stratified with socioeconomic status, ethnicity, genetics, or occupation. The lack of these studies are probably a major contributing factor to the conflicting data found in studies on leishmanial metastasis.

Immunology and environment

Leishmaniasis has established itself as an emerging opportunistic infection in HIV-positive patients, where its occurrence is now used as a clinical indicator for performing an HIV test. Indeed, HIV predisposes the onset of less common leishmanial complications such as VL and diffuse metastatic infection by 1000-fold [2]. Similarly, the immunological dysfunction associated with severe malnutrition can also be exploited by leishmaniasis and stands as the most prevalent cause of immunodeficiency worldwide [28]. Equally, metastatic leishmanial complications have been associated with sudden immune-reconstitution such as after highly active antiretroviral therapy (HAART) [29] or Th1-boosting antimonial therapy [30], thus showing that diverse fluctuations in immunocompetence can influence CL. As

immunocompetence also determines the composition of the host microbiome, a recent study has elegantly linked disruptions in skin commensals to the control of CL [31]. This study, however, did not investigate leishmanial metastasis, but we can postulate the potential importance of these potently immunomodulatory ‘co-infections’, especially considering the metastatic influence of the nested co-infection, LRV.

Local changes in skin immunity after physical injuries have already been shown to predispose metastatic reactivations. Here, secondary lesions were observed to develop in the scar tissue of unrelated wounds [32]: a phenomenon implying that, similar to cancer, metastatic sites are immunologically ‘seeded’ before the establishment of secondary lesions by local changes in the immune microenvironment. These local variations in immunological status remain as an interesting and underdeveloped avenue of study in leishmaniasis. The most common and potent facilitators for these local changes are the PRRs, which are at the frontline of innate pathogen recognition. Each PRR has an immunological arsenal specific to a range of signature pathogen ligands. Thus, concomitant infections stimulating a variety of PRRs induce a range of signaling cascades that not only blend together to create a unique immunological microenvironment but are also able to synergize or inhibit each other. Further, it has been shown that even a previous infection in the host can alter subsequent PRR activity, inducing homotolerance and heterotolerance or hyperactivity [33]. The potent effect of TLR3 signaling on disease severity, exemplified in the case of LRV nested co-infection, sheds light on the possible importance of the number, timing, and magnitude of innate immune responses in the evolution of leishmaniasis. Consequently, the mechanism whereby HIV infection acts as an aggravating factor in metastatic leishmaniasis may be far more complex than a simple collapse of the CD4 compartment. Indeed, *Leishmania* survive better in cells exposed to HIV and vice versa [34,35]. This creates a situation where PRR crosstalk as well as genetic polymorphisms in these PRRs (Box 3) should be considered as key parameters of leishmanial pathogenesis.

Pattern recognition receptors and *Leishmania*

The *Leishmania* parasite has several molecular patterns, which are sensitively detected by the innate PRRs of the host. The host cell of the obligate intracellular stage of *Leishmania* is the macrophage, a potent immune phagocyte notoriously coated and lined with various PRRs, and so it is clear that the parasite does not go by undetected. Indeed, inoculation results in a rapid initiation of inflammatory signaling cascades, where the collateral tissue damage of the resultant hyperinflammation is at the root cause of disease pathology. Three major families of signaling PRRs have been identified: the TLRs, the nucleotide-binding domain, leucine-rich repeat containing receptors (NLRs), and the retinoic acid-inducible gene 1 like receptors (RLRs). So far, the TLRs are the most extensively described.

Toll-like receptors

In *Leishmania* infection, the role of certain TLRs has been well documented and recently reviewed [36,37]. In most instances, studies were based on the common adaptor molecule MyD88, where deficient mice showed that TLRs play a generally protective role against *Leishmania* infection. This MyD88-dependent protection was mostly attributed to TLR2 and TLR4 signaling that have been repeatedly noted as beneficial across a broad range of

Leishmania species [38–40]. Interestingly, however, the MyD88 pathway is not exclusive to TLRs, and thus these protective roles may also be explained by MyD88-dependent components of IL-1/IL-18 signaling, as will be discussed in the next section. Nevertheless, further protective roles were found for endosomal TLRs (sensing nucleic acids) through studies on the Unc93B1 chaperone protein: involved in the translocation of TLRs from the endoplasmic reticulum to endosomes [41]. Among the endosomal TLRs, TLR9 seems crucial to pathogenesis, because TLR9-deficient mice are rendered more susceptible to *L. major* [42,43], *L. braziliensis* [44], and *L. guyanensis* [45]. However, as mentioned previously, it is difficult to make generalizations about TLRs from these studies as *Leishmania* may engage multiple TLRs with intersecting pathways that are then able to either synergize with or reduce the efficacy of the co-stimulated pathway. For example, *L. panamensis* induces TLR1, TLR2, TLR3, TLR4, and TLR9 transcription in primary macrophages [46]. The TLR4 ligand in *L. panamensis* remains to be determined, however, as *Viannia* spp. lack the P-8 proteoglycolipid complex that is generally responsible for leishmanial TLR4 stimulation. Further, the classic TLR2 ligand, LPG, is also expressed in significantly lower quantities (10–20-fold less) in *Viannia*. It is possible that its activation and upregulation could be attributed to crosstalk, where nuclear factor-κB (NF-κB) activation, a common target of many PRRs, is known to upregulate TLR2.

Protection via TLRs is usually linked to the production of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), C-X-C motif chemokine 10 (CXCL10), and chemokine ligand 5 (CCL5). Importantly, however, these same immunological mediators of protection have also been blamed for the extreme inflammation associated with mucosal and metastatic leishmaniasis. The model of LRV-dependent metastasis is one such example where TNF-α-mediated hyperinflammation not only induces tissue damage that worsens disease outcome but is also favorable to parasite survival [47], indicating that the magnitude as well as the combination of TLR signaling is important in determining disease outcome [48]. Immunological crosstalk has already been described among many TLRs [33]. TLR7 stimulation, for instance, can induce inhibitory heterotolerance on concomitantly stimulated TLR2, TLR4, and TLR9 pathways [49]. Further, TLR3 has shown inflammatory synergy with TLR9 and TLR2 [50,51], a phenomenon that may account for the hyperinflammatory response seen in TLR3 stimulatory *Leishmania* infections carrying LRV. These studies propose that TLR stimulation should be investigated in a multidimensional manner, taking into account not only the incidence of their stimulation but also the concomitantly stimulated receptors and the chronological order in which they are stimulated.

NLRs and RLRs

Unlike the TLRs, NLRs are exclusively intracellular, enabling stressed, damaged, or infected cells to directly sense and respond to internal danger signals. Although the signals and activation complexes are very different from those of the TLR pathway, some family members (NOD1 and NOD2) can directly engage the common TLR nuclear factor, NF-κB, producing many of the same proinflammatory cytokines. Most NLR family members, however, signal through the formation of an inflammasome, a multiprotein complex that induces inflammation via caspase-1-mediated cleavage and activation of pro-IL-1β and pro-

IL-18 [52]. The RLRs, by contrast, detect foreign cytoplasmic DNA and RNA to induce an antiviral immune response. The RLRs include RIG-I and MDA5, which work via interferon regulatory factor 3 (IRF3) and IRF7 by utilizing the mitochondrion-localized adaptor protein MAVS [53].

Because NLRs and RLRs are able to detect both pathogen-associated molecular patterns (PAMPs) as well as cellular danger signals (which are undoubtedly present in the destructive inflammatory environment of a typical leishmanial lesion), we expect to find a significant role for NLRs and RLRs in the evolution of disease. Despite this great potential, however, studies on their involvement in leishmaniasis are sparse. Thus far, there is no report on a possible role of RLRs in leishmaniasis and only a handful on NLR activation. The first study describing the role of NLRs in leishmaniasis correlated the clearance of various New World *Leishmania* species to an NLRP3-dependent production of nitric oxide (NO) and IFN-γ [54]. The authors showed that the resultant active IL-1β induced by NLRP3 signaling carried out this response in an autocrine manner, where the IL-1 receptor and its MyD88 adaptor protein were necessary and sufficient to trigger inducible nitric oxide synthase (iNOS). Interestingly, immunological cross-talk from TLR4 can increase the inactive IL-1β precursor, thus facilitating the NLRP3 response by ‘priming the system’ [55]. Indeed, TLR4 is an essential component of initiating parasitotoxic oxidative stress in *L. major* infection. Although these studies note NLRP3 inflammasome activation, they did not identify the stimulating ligand. In the ulcerative environment of a typical leishmanial lesion, we expect many cellular danger signals with the potential of NLRP3 stimulation. An example of such activation has been described, whereby nucleotides released by macrophages infected with *L. amazonensis* engaged the purinergic receptors (P2Y2 and P2Y4), often found upstream of the NLRP3 inflammasome. This drastically reduced parasite burden and even induced apoptosis of the host cell [56]. TLR crosstalk can also have a deleterious effect on this parasitotoxic inflammasome, for instance, IFN-β, such as is produced in response to TLR3 engagement, is known to disrupt oxidative parasite killing via the upregulation of superoxide dismutase (SOD1) [57]. This molecule decreases caspase-1 activation, which is an essential part of NLRP3 inflammasome function [58]. SOD1 upregulation is seen particularly in Neotropical *Leishmania* spp., where infectious metastasis (and IFN-β inducing LRV1) is endemic. Interestingly, IFN-β is described as having many contradictory roles in leishmaniasis (Box 4).

The only other study on the inflammasome in leishmaniasis was a preliminary effort to profile the transcription of inflammasome components in a macrophage system of infection. Here, the authors quantified the mRNA of two inflammasomes (NLRP3 and NAIP5), as well as some common inflammasome adaptor/effector molecules (IPAF, ASC, caspase-1, IL-1β, and IL-18) in macrophages infected with *L. major* and found a significant upregulation for all the above-mentioned components except NAIP5 [59]. Again showing the potential for such studies in leishmaniasis. Anecdotal evidence widely supports a role for NLRs in leishmaniasis, for instance, a common NRLP3 stimulant, poly (lactic-co-glycolic) acid (PLGA), is an effective adjuvant in a KMP11-based antileishmaniasis vaccine [60].

The virus-recognizing RLRs would probably have the highest potential to play a role in *Leishmania* infected with the dsRNA virus, LRV. However, it is also likely that the length of the dsRNA genome of LRV may not fit the size restrictions for RLR activation.

Concluding remarks

Infectious metastasis in CL is a complex, multifactorial process involving various risk factors. Much research is still needed to definitively identify the exact causes of this disfiguring complication and, further, to gauge their relative contributions in the pathogenesis of disease. Outstanding questions for further research are proposed in Box 5. So far, anecdotal evidence has short-listed some candidate 'metastatic risk factors' in parasite phylogeny, host immunocompetence, and environmental influences, which were summarized here. The geographical isolation of metastatic outcomes insinuates that parasite-intrinsic factors are the overriding determinants of the complication. Indeed, metastatic parasites seem to have an increased resistance to oxidative stress and antileishmanials, prolonging their survival even in the more potently inflammatory environment that they tend to induce. Nevertheless, the small genetic differences between metastatic and nonmetastatic parasites make the presence of a 'metastatic gene' unlikely. This indicates that increased parasite survival is probably attributable to nongenetic factors or to a more complex situation of immune evasion in the host. A strong candidate for such a nongenetic, immunomodulatory metastatic factor is the presence of the highly immunogenic dsRNA virus within the cytoplasm of metastatic strains of *L. braziliensis*, *L. guyanensis*, and *L. aethiopica* parasites. This potently pathogenic role for an innate antiviral pathway strongly implicates PRRs and coinfecting pathogens in metastatic leishmaniasis. Already, encouraging studies have shown the substantial influence of the host skin microbiome and HIV viral particles in leishmanial lesion formation and parasite survival. Therefore, further studies on PRR polymorphisms and crosstalk in metastatic disease have great potential to reveal associations and ultimately stand as evidence for the use of innate immunomodulators in the treatment and prevention of metastatic disease.

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Glossary

Damage- (or Danger-) associated molecular patterns (DAMPs) molecules that are generally present due to a noninfectious threat to cellular integrity and are capable of initiating signaling cascades, through PRRs such as NLRs and TLRs. Some examples are proteins released during DNA damage such as cytosolic DNA and RNA fragments, or nucleotides such as ATP.

	Fragments of damaged tissue such as hyaluronan, uric acid, and heparin sulfate have also been described as potent DAMPs
Highly active antiretroviral therapy (HAART)	a combination of at least three drugs proven to suppress HIV replication, thus prolonging and improving the quality of life for individuals with HIV. A combination is used to avoid the development of drug resistance
Immune reconstitution inflammatory syndrome (IRIS)	a condition developing during immune recovery from a major immunosuppressive event (e.g., HIV infection, or VL) in which the immune system responds to previously acquired antigens with an overwhelming level of inflammation that paradoxically worsens the disease
Kala-azar (KA)	a Hindi term for ‘Black Fever’, describing the unexplained cutaneous discoloration associated with end-stage VL, where parasites fatally (if left untreated) infest the liver, spleen, and bone marrow
***Leishmania* RNA virus (LRV)**	a cytoplasmic double-stranded RNA (dsRNA) virus residing within some strains of the *Leishmania* parasite, which may act as a virulence factor in metastatic leishmaniasis
Nucleotide-binding oligomerization domain receptors (NLRs)	also called Nod-like receptors, are intracellular sensors of PAMPS and DAMPs able to cooperate with TLRs to regulate or potentiate the inflammatory and apoptotic response
Pathogen-associated molecular patterns (PAMPs)	small molecular motifs common among certain pathogen groups that are recognized by PRRs in cells of the innate immune system and generally produce a cytokine signaling cascade. Some examples include bacterial lipopolysaccharide (LPS, specific to TLR4), flagellin (TLR5), dsRNA (TLR3), and unmethylated CpG DNA (TLR9)
Retinoic acid-inducible gene 1 like receptors (RLRs)	also called RIG-1-like receptors, are cytoplasmic RNA helicase enzymes, which recognize viruses by binding their dsRNA. RIG-1, MDA5, and LGP2 are the currently described members of this PRR family
T helper cell subsets (Th1/Th2/Th17/Treg)	subsets of a CD4 T cell lineage, which promote various types of immune response. Th1: cell mediated cytotoxic response (via IFN-γ). Th2: B cell mediated antibody response (via IL-4, IL-5, and IL-13). Th17: antifungal response (via IL-17A). Treg: anti-inflammatory response (via IL-10)
Toll-like receptors (TLRs)	are PRRs able to detect a range of pathogenic patterns on the plasma membrane (TLR1, TLR2, TLR4, TLR5, and TLR6) or within the endosomal compartment (TLR3, TLR7/8, and TLR9)

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Box 1. Species-specific immunopathologies of metastatic leishmaniasis

L. braziliensis

Mucosal leishmaniasis is most frequently observed with *L. braziliensis*, where metastasis occurs in 5–10% of CL patients. Although the manifestations of this disease can be complex, mucosal leishmaniasis generally metastasizes directly to the nasal mucosa with a limited number of secondary skin lesions. It most commonly affects the nasal septum, although infections have also been noted in the cartilaginous turbinates as well as the larynx. Mucosal lesional biopsies commonly reveal an unregulated hyperinflammatory response, where proinflammatory cytokines such as IFN-γ and TNF-α are produced without the dampening influence of IL-10 and transforming growth factor-β (TGF-β) [61,62]. Patients also produce significant quantities of inflammatory chemokines (CXCL10 and CCL4), resulting in the recruitment of intralesional $CD8^+$ T cells [39]. Recently, the cytotoxic enzymes (granzyme B and perforin) produced by these intralesional $CD8^+$ T cells were shown to mediate tissue damage in ulcerative CL [63,64]. However, it is also likely that tissue destructive enzymes such as matrix metalloproteases play important roles, although knowledge on their specific actions in metastatic leishmanial pathologies is sparse [65–67].

L. panamensis

More rarely, mucosal leishmaniasis can be caused by *L. panamensis*, a species that is endemic in western South America (Bolivia, Peru, and Colombia) and has also been found in Brazil. In Colombia, *L. panamensis* causes 55–80% of CL cases, 5% of which progress into mucosal leishmaniasis. Mucosal lesions due to *L. panamensis* have a tendency to be less destructive and less severe than those induced by *L. braziliensis*. Immunopathology in *L. panamensis* infection consists of a mixed Th1/Th2 immune response with high numbers of T regulatory cells [68]. Elements of this observation were recently replicated in a BALB/c murine model, which also revealed IL-13 and IL-4Ra as mediators of parasite persistence [69].

L. guyanensis

The clinical picture is slightly different for *L. guyanensis*, which is possibly the most prevalent parasite species in the Amazon basin. Although mucosal leishmaniasis has been described for *L. guyanensis*, the more common presentation is a large number of secondary lesions, which are nonulcerated, granulomatous, and display a weak response to treatment [70]. In general, *L. guyanensis* are less sensitive to antimonials than *L. braziliensis* [17] and parasites isolated from secondary lesions are more resistant to oxidative stress [15]. The intralesional immune response involves a strong role for T regulatory cells, which have been shown to underlie the chronicity of infection [71].

L. aethiopica

In the highlands of Ethiopia, there is a high incidence of CL caused by *L. aethiopica*. Generally, the cutaneous lesions are able to self-heal but persistent infections commonly metastasize to other parts of the body. Two major clinical presentations have been described: mucocutaneous leishmaniasis and DCL. DCL patients are poor responders to

antimony treatment and are anergic to parasite antigens, whereas mucocutaneous patients develop chronic inflammation and are sensitively reactive to parasite antigens. These clinical presentations are independent of parasite genotype [72] but may still be dependent on extranuclear parasite factors such as LRV, which has recently been discovered in the region [26].

Box 2. Species-specific immunopathologies of PKDL

Interestingly, the onset of PKDL is strongly associated with the successful treatment of VL in which the restoration of the patient's antileishmanial immune response reduces the visceral parasite load and results in symptomatic resolution [30] (Figure I). Antimonial therapy carries a specific risk of developing PKDL. However, cases have also been reported after treatment with amphotericin B and miltefosine [73]. Although the sudden shift from a Th2 response during VL to Th1 after treatment has been implicated in numerous studies, specifically being seen through the restoration of leishmanin skin test (LST) positivity [29], the mechanism seems to rely on a more complex underlying immune response. Indeed, high serum IL-10 and upregulated intralesional IL-6 and TNF-α are predictive of, and essential to, the development of PKDL [9]. These high levels of local inflammatory markers could be compensatory for the malfunctioning IFN-γ signaling pathway, because although intralesional IFN-γ levels are high, its signaling seems to be corrupted by the low expression of the IFN-γ receptor [74]. The first line antileishmanials used for VL are known to induce or rather restore such cytokine environments and may thus explain the association of antileishmanial therapy to the onset of PKDL. Indeed, PKDL has recently been recognized as a form of paradoxical IRIS that emerges as a new disease entity following successful VL treatment and immune recovery [29].

Box 3. Host PRR polymorphisms – a potential risk factor in metastatic leishmaniasis

PRRs have been almost completely overlooked in studies relating genetic polymorphisms to symptomatic variants of leishmaniasis. For instance, there is no study yet correlating PRR polymorphisms and susceptibility or resistance to *Leishmania* infection, although it is likely to be a key parameter in the control of parasite burden. Various genetic polymorphisms, however, have revealed immune susceptibilities for cytokines and chemokines in the pathology of metastatic leishmaniasis [75–79,88]. A few potential candidates are suggested below.

TLR polymorphisms

Numerous TLR polymorphisms have already been identified in humans and associated with the development of various common inflammatory diseases [89]. In the context of LRV recognition, TLR3 polymorphisms could play a major role in leishmanial metastasis. Several polymorphisms of TLR3 have been associated with human disease [80,90,91]. For instance, an intronic polymorphism, elevating TLR3 expression, was correlated with improved viral clearance in hepatitis C [92]. TLR3 polymorphisms have been similarly linked to common coinfections of leishmaniasis. For example, natural resistance to HIV-1 was associated with a common polymorphism (Leu412Phe) of TLR3, where the presence of the 412Phe allele was further associated with increased production of inflammatory cytokines [93]. Interestingly, the distribution of this allele varies between Eurasia and Africa, being almost absent in the latter group [94].

NLR polymorphisms

NLRs have also been associated with several diseases. For example, NLRP3, was originally named Cryopyrin for its association with a class of inflammatory diseases induced by low temperatures. Since then, similar pathologies have been mapped to this gene and are now commonly known as the Cryopyrin-associated periodic syndromes. Symptoms are mediated by hyperactive NLRP3 and a resultantly high level of the proinflammatory cytokine IL-1β. Surprisingly, loss-of-function polymorphisms have also been associated with chronic inflammatory conditions. An example of which is genetically defective NLRC2 expression, now strongly associated with Crohn’s disease [95,96]. This, however, could be explained by the fact that NLRC2 is essential in controlling gut bacteria through its recognition of a component of bacterial cell wall, muramyl dipeptide [97]. This reduction in microbial control could subsequently drive the inflammation associated with the development of Crohn’s disease. Because NLRC2 is especially expressed in the skin, this polymorphism results in a similar environment of proinflammation through disruptions of the cutaneous microbiome, and thereby influences the local inflammatory microenvironment in leishmaniasis. Therefore, much potential lies in the study of PRR polymorphisms in leishmanial pathogenesis.

Box 4. The contradictory roles of IFN-β in leishmaniasis

Type I interferons, such as IFN-β, were originally discovered and defined by their ability to 'interfere' with viral infections and, for a long time, it was the sole function to which they were attributed. In recent years, more diverse roles have been added, ranging from antineoplastic agents to regulators of the immune system. They are currently found to have various and sometimes contradictory roles in viral, bacterial, and protozoan infections [98,99]. For leishmaniasis, the roles of IFN-β seem to be particularly conflicting, where IFN-β treatment has been demonstrated to play both protective and detrimental roles during *L. major* infection by differentially modulating iNOS expression, depending on the dose and timing of its administration [100]. For example, low-dose IFN-β treatment in a murine model was noted as having a significantly protective effect against progressive CL, restoring natural killer cell cytotoxic activity, increasing lymphocyte proliferation, and upregulating parasitotoxic nitric oxide [101]. However, high concentrations of this proinflammatory cytokine increased *L. braziliensis* parasite load and worsened disease in a murine model of *L. amazonensis* infection: an observation analogous to the correlation of hyperinflammation with increased parasite infectivity in metastatic disease. Parasites evidently benefited from an IFN-β-mediated upregulation of superoxide dismutase in human macrophages, thereby detoxifying the oxidative radicals used to kill intracellular infections [57], whereas destructive inflammation was caused by an extreme recruitment of inflammatory monocytes [102].

Box 5. Outstanding questions

- Large-scale epidemiological studies are needed to understand the geographical extent and clinical relevance of the LRV nested coinfection in various *Leishmania* species.
- A continued and more robust search for parasite-intrinsic metastatic factors could be achieved through broad-ranging comparative studies at the genetic and protein levels.
- Genome-wide association studies on parasites and on leishmaniasis endemic human populations could reveal any immunological susceptibilities, which predispose leishmanial metastasis.

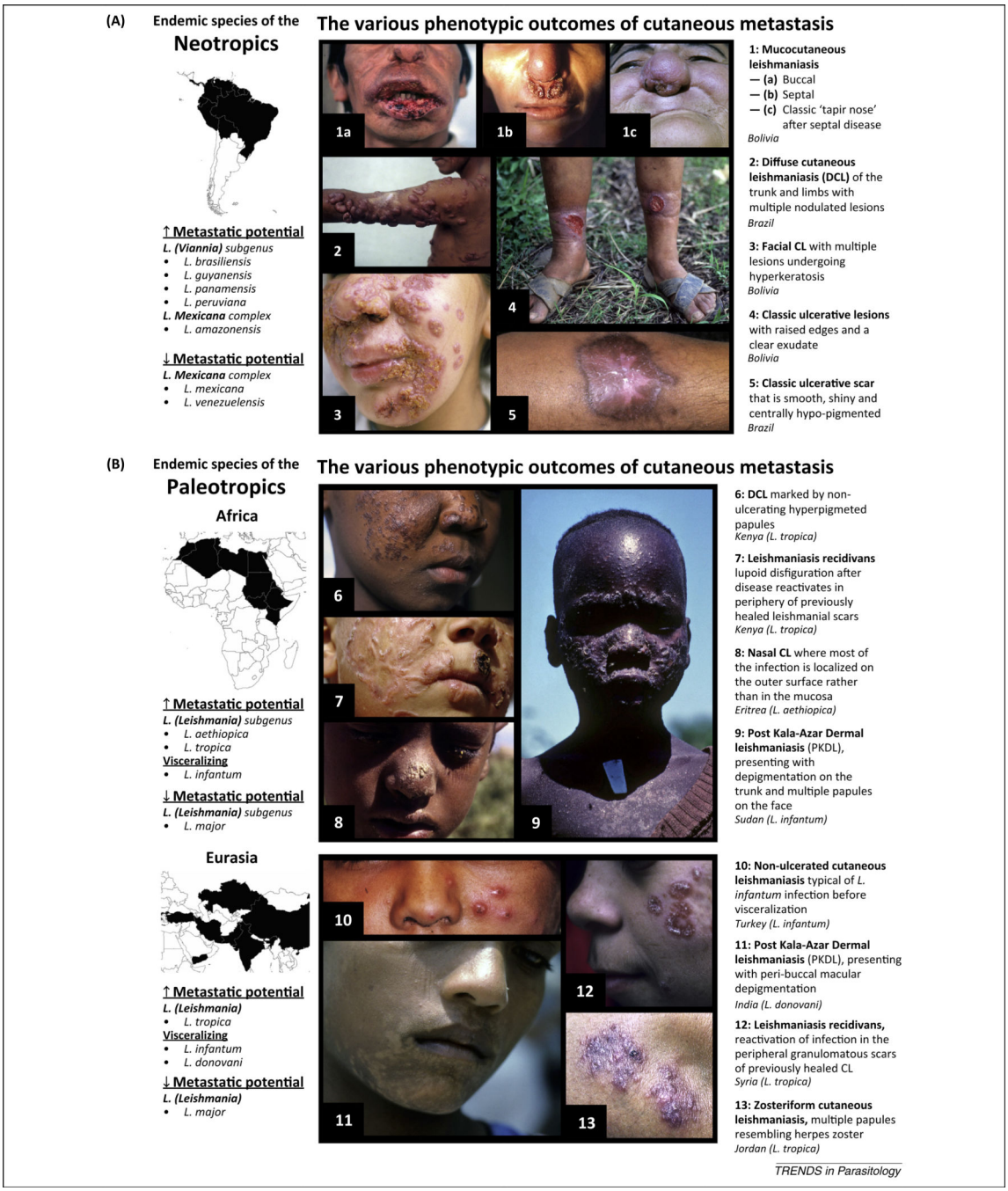

Figure 1.

Geographical context of the various outcomes of metastatic leishmaniasis. Metastatic cutaneous leishmaniasis (MCL) occurs across all *Leishmania* endemic regions but is much more prevalent in the Neotropics **(A)**. Neotropical infections are mostly caused by the variously 'metastatic' species of the *Leishmania (Viannia)* subgenus. Species with high metastatic potential cause some form of MCL in approximately 20% of their infections. Neotropical MCL has a particular predilection for facial mucosa (1a–c) and are known for forming large ulcerative lesions (4–5) or, less frequently, diffuse granulomatous disease (2–

3). The Paleotropics **(B)** have a much lower incidence of classic MCL. Post-kala-azar dermal leishmaniasis (PKDL), however, can occur in as many as 60% of all infections. MCL presentations vary from diffuse papular infections (6) to the rare leishmaniasis recidivans (12). PKDL presentations differ slightly between Eurasia and Africa, where India sees more progressive hypopigmented and macular rashes (11), whereas African PKDL has higher incidences of self-healing papular and ulcerative forms (9). All figures were kindly provided by Dr Philippe Desjeux. Geographically representative images were chosen from a global photographic catalog depicting the various symptomatic outcomes of cutaneous leishmaniasis.

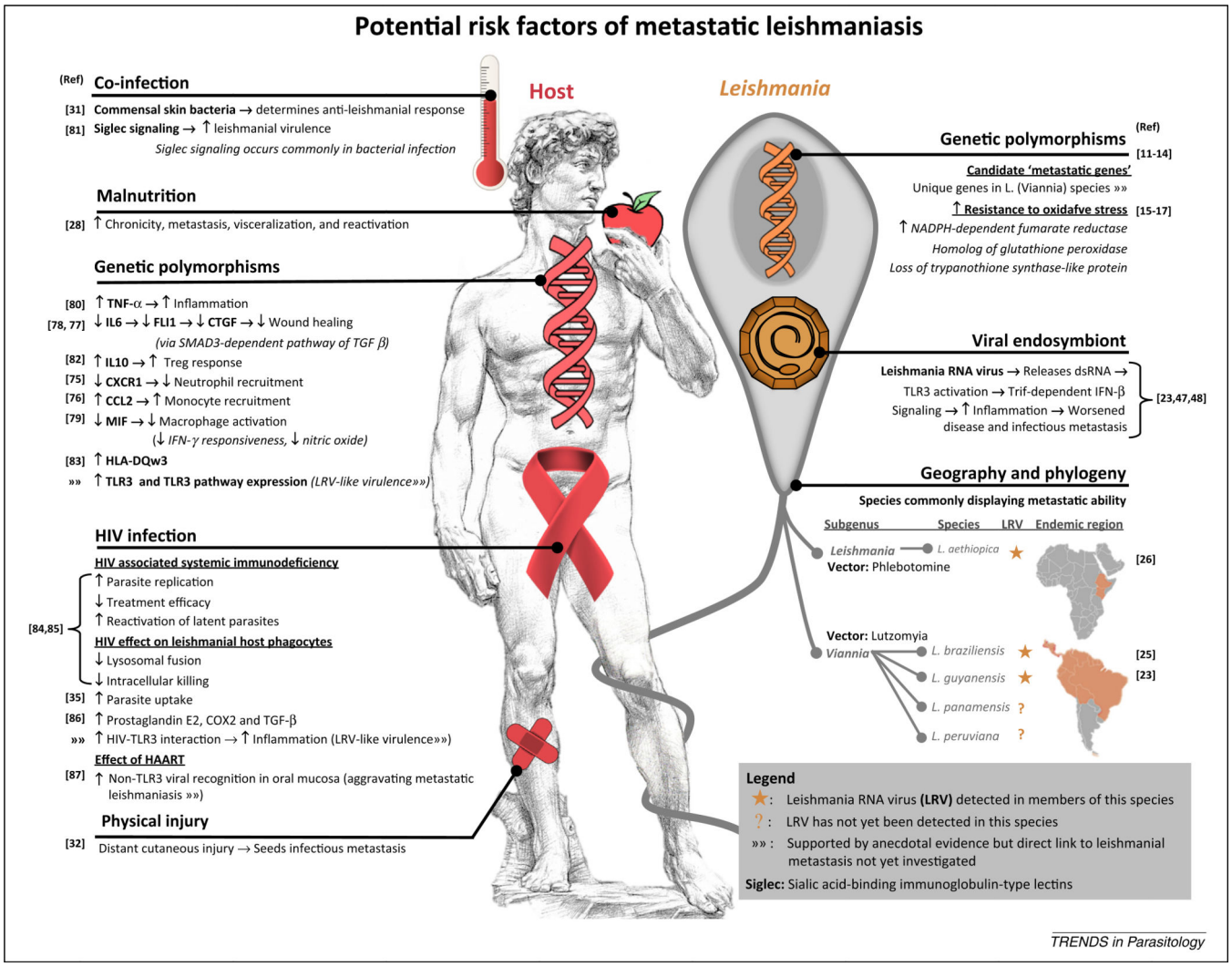

Figure 2.

The potential risk factors of metastatic leishmaniasis. A summary of the anecdotal or direct evidence for risk factors that may predispose metastatic complications in cutaneous leishmaniasis [11–17,23,25,26,28,31,35,47,48,75–87].

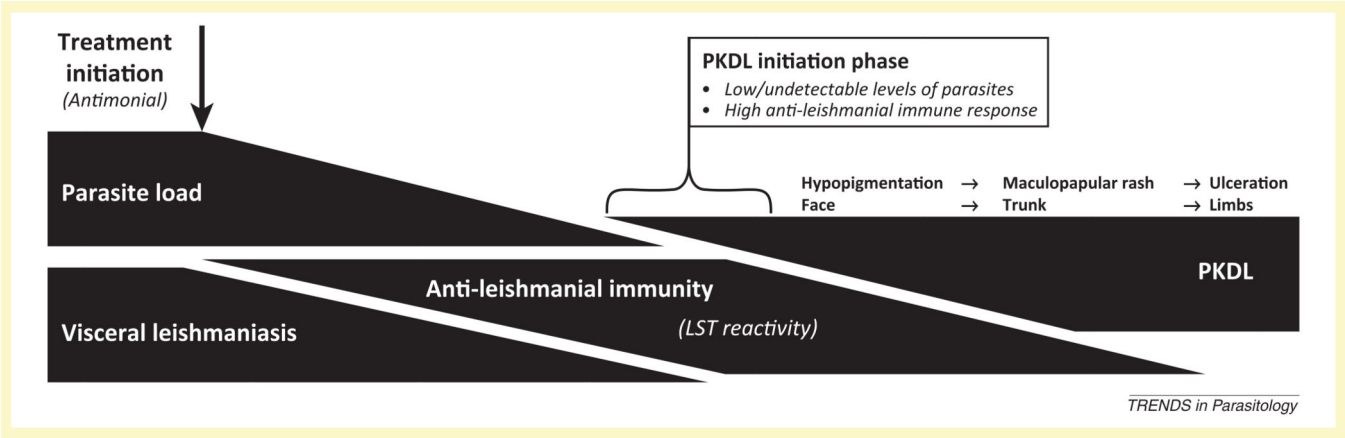

Figure I.

The clinical context of post-kala-azar dermal leishmaniasis (PKDL) development. Visceral leishmaniasis (VL) is marked by high visceral parasite loads and is often associated with a weak or negative result for the leishmanin skin test (LST). The absence of LST reactivity is indicative of low cellular immunity against leishmanial antigens. VL is often successfully treated with antimonials resulting in a reduction of parasite load and recuperation of antileishmanial response. This stage of disease resolution is often where PKDL is initiated. PKDL develops in three clinically relevant stages, spreading as a hypopigmented rash from the periorificial regions of the face to the periphery as a maculopapular or ulcerative rash.