# Leishmaniasis immunopathology – impact on design and use of vaccines, diagnostics and drugs

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| Corresponding Author: | Simon Croft, PhD  
London School of Hygiene and Tropical Medicine Department of Global Health and Development  
UNITED KINGDOM |
| Corresponding Author Secondary Information: | |
| Corresponding Author's Institution: | London School of Hygiene and Tropical Medicine Department of Global Health and Development |
| Corresponding Author's Secondary Institution: | |
| First Author: | Paul M. Kaye, University of York |
| First Author Secondary Information: | |
| Order of Authors: | Paul M. Kaye, University of York  
Israel Cruz  
Albert Picado  
Katrien Van Bocxlaer  
Simon Croft, PhD |
| Order of Authors Secondary Information: | |
| Funding Information: | |
| Abstract: | Leishmaniasis is a disease complex caused by 20 species of protozoan parasites belonging to the genus *Leishmania*. In humans the it has two main clinical forms, visceral leishmaniasis (VL) and cutaneous or tegumentary leishmaniasis (CL), as well as several other cutaneous manifestations in a minority of cases. In the mammalian host *Leishmania* infect different populations of macrophages where they multiply and survive in the phagolysosomal compartment. The progression of both VL and CL depends on the maintenance of a parasite-specific immunosuppressive state based upon this host macrophage infection. The complexity and variation of immune responses and immunopathology in humans and the different host interactions of the different *Leishmania* species has an impact upon the effectiveness of vaccines, diagnostics and drugs. |
Leishmaniasis immunopathology – impact on design and use of vaccines, diagnostics and drugs

Paul M. Kaye¹, Israel Cruz²,⁴, Albert Picado², Katrien Van Bocxlaer¹, Simon L. Croft³

¹York Biomedical Research Institute, Hull York Medical School and Dept. of Biology, University of York, U.K.

²Foundation for Innovative New Diagnostics (FIND). Geneva, Switzerland.

³Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, U.K.

⁴National School of Public Health. Instituto de Salud Carlos III. Madrid, Spain

Correspondence: simon.croft@lshtm.ac.uk
1. Introduction

Leishmaniasis is a disease complex with two main clinical presentations – visceral leishmaniasis (VL, or kala-azar) and cutaneous leishmaniasis (CL). Both VL and CL have a worldwide distribution in nearly 100 endemic countries. Overall the number of potentially fatal VL cases has decreased during the past decade with an estimated annual incidence of less than 50,000 (2017 figures, https://www.who.int/leishmaniasis/burden/en accessed 01.10.19) with seven countries: Brazil, Ethiopia, India, Kenya, Somalia, South Sudan and Sudan having the major burden. The decrease in VL case numbers in the Indian subcontinent (ISC) is coincident with the introduction of a regional elimination programme launched in 2005, with improved access to diagnosis and treatment. The naturally cyclical pattern of transmission intensity of this anthroponotic disease may also contribute to this decrease in the ISC. In contrast, the number of cases in East Africa has not fallen and this endemic area is now a major focus for control programmes. The estimated worldwide annual incidence of CL is between 0.7 to 1.2 million cases [1] with Central and South America, the Middle East, Ethiopia and North Africa being the major endemic areas. Far less attention has been paid to the control and treatment of CL, a predominantly zoonotic and a slow self-curing disease (3 – 18 months in most cases) with disfigurement and stigma being the main corollaries of infection. In addition, there are rarer variants of CL: mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL), disseminated cutaneous leishmaniasis (DsCL), leishmaniasis recidivans (LR) and post-kala-azar dermal leishmaniasis (PKDL). With CL, these variant skin manifestations are often referred to collectively as the tegumentary leishmaniases (TL). PKDL is a perplexing clinical presentation which follows cure of VL (5-10.% cases in Asia, up to 50% cases in East Africa, [2,3]).

The protozoan parasites that cause VL and TL belong to at least 20 distinct species of the genus *Leishmania* (with new species being identified in the past decade) that are transmitted between mammalian hosts by female phlebotomine sandflies. Different species of *Leishmania* are largely responsible for the diverse clinical manifestations (Table 1) which are generated by the interplay between the parasite, host factors and vector biology. However, clinical cases reports suggest these
species-dependent clinical outcomes are not distinct. In the sand fly gut *Leishmania* parasites exist as flagellated promastigote forms, while in the mammalian host the parasites invade macrophages losing their flagellum and transforming into the intracellular amastigote. The amastigote multiplies and survives in the phagolysosomal compartment of the macrophage. It has long been known that promastigotes differentiate within the sandfly to generate infective “metacyclic” forms [4-6] and recent evidence suggests that there may also be heterogeneity in the intracellular amastigote form [7-9]. *Leishmania* parasites actively manipulate both of their hosts. In the sandfly, transmission efficiency of metacyclic promastigotes is enhanced by secretion of a proteophosphoglycan-rich, mucin-like gel (Promastigote Secretory Gel, PSG), which accumulates in the sand fly gut and mouthparts [10]. Mammalian infection is established in the skin following the inoculation of metacyclic promastigotes that possess a lipophosphoglycan coat that enables them to resist complement and attach to and invade host cells. Peptides in sand fly saliva (for example, maxadilan) cause vasodilation and erythema that also aids the establishment of the infection in macrophages in the dermal layer of the skin [11]. Early responses to infection involve neutrophil infiltration and invasion of resident macrophages. Progress of the disease depends on the parasite species and host responses. For both VL and CL, disease progression depends on the maintenance of a parasite-specific immunosuppressive state including host cell macrophages. A key difference between VL and CL is the propensity of VL-causing species (Table 1) to cause disease at systemic sites, invading macrophages of the liver, spleen and bone marrow, with minimal skin pathology. Restoration of macrophage function, either through the development of an appropriate immune response or therapeutic intervention, can lead to amastigote killing in macrophages by nitric oxide and oxygen radicals. Resolution of disease following the activation of macrophages is enhanced by T helper 1 (Th1) - like responses mediated through the interaction of antigen-presenting cells (e.g., dendritic cells, macrophages) with CD4+ and CD8+ T cells and subsequent secretion of pro-inflammatory cytokines, including interferon-γ (IFNγ) and tumor necrosis factor (TNF). In clinical forms of VL and in DCL, Th2 – like cell responses (including IL-4 and IL-13) are also evident [12,13] and inhibition of macrophage activation and immune dysregulation may be mediated through IL-10 and transforming growth factor-β (TGFβ) [14]. Immune responses and immunoregulatory pathways have been extensively defined in experimental inbred mice that show some but not all the characteristics of
human disease [15,16]. Immune responses directed against the parasite may also play a pathogenic role and contribute to tissue damage and clinical severity (see below).

The prevention, treatment and control of both CL and VL depends on drugs, diagnostics and vaccines, as described in recent comprehensive reviews [2,17]. The complexity and variation of immune responses and immunopathology in humans and the different host interactions of the *Leishmania* species has an impact upon the effectiveness of these tools (Fig. 1). Recent studies have significantly increased our understanding of these interactions and suggested new strategies for discovery and development, and such data are the focus of this review.

2. Key features of immunopathology in leishmaniasis

Virulence factors allow pathogenic microbes to establish infection, colonise, replicate and disseminate throughout the host, evade anti-microbial immunity and facilitate transmission. A plethora of *Leishmania* derived molecules have been described and reviewed elsewhere that have the cardinal properties of bona fide virulence factors [18]. Although some pathogens liberate virulence factors that directly cause tissue damage e.g. gangrene due to collagenases and phospholipases produced by *Clostridium perfringens*, similar factors have not yet been described for *Leishmania*. Rather, clinical symptoms in leishmaniasis are, for the most part, the result of inappropriate immune responses leading to collateral damage to host tissues. Hence, for its clinically relevant features, leishmaniasis represents a disease driven by immunopathology. Hence, the “appropriateness” of an inflammatory / immune response, defined by its ability to strike the delicate balance between removal of *Leishmania* parasites and the minimization of pathology, may be defined by its quality, quantity or kinetics as well as by the parasite species under study. Some of the key features of the immunopathology associated with leishmaniasis are described below.

*Macrophage activation:* Macrophage activation is a central tenet of anti-leishmanial immunity, and the capacity of macrophages to polarise their functions towards the generation anti-microbial effector molecules such as NO and O₂⁻ under the influence of type 1 cytokines (classical activation) or
towards the generation of arginine and polyamines under the influence of type 2 cytokines (alternate activation) is well established. However, the simple assumption that type I cytokines are “protective” and type 2 cytokines are “detrimental” is not always borne out by the literature. For example, IL-4, the prototypic Th-2 cytokine, plays an important early role in the generation of host protective CD4+ IFN\(\gamma\) Th-1 cells in murine models of CL [19] and IL-4R signalling is required for optimal protection in primary resistance and vaccine-induced immunity in experimental VL [20-22]. Similarly, chemotherapy with sodium stibogluconate (SSG) in models of VL also requires IL-4R signalling for optimal effectiveness [22]. More recent evidence indicates that cytokines influence macrophage metabolic states, with consequences for the amastigote’s access to required nutrients. Specific metabolic countermeasures may have evolved to combat these stresses and ensure long lived parasitism [23].

Tissue macrophages have two origins. Resident tissue macrophages originate from embryonic yolk sac-derived progenitors, fully differentiate to acquire their mature characteristics under the direction of tissue-specific transcription factors / tissue-derived cues and have the capacity of self-renewal. In contrast, relatively short-lived tissue macrophages originate from hematopoietic stem cells via monocyte precursors [24,25]. Resident tissue macrophages play many of the key roles in tissue homeostasis and function, and they have been identified as niches for survival of \textit{L. major} in the dermis [26] and \textit{L. donovani} in the liver and spleen [27] and it is likely some of their housekeeping properties are impacted on by infection. Monocytes and monocyte-derived macrophages [28-30], as well as dendritic cells [31] and neutrophils [32] may also be targets of infection and serve to regulate immunity and pathology though their relatively short life span may suggest that there is greater dynamics in the parasite populations they contain. Recent evidence suggests that the bone marrow becomes a target of collateral T cell-mediated damage during VL [33,34] but the consequences of this for the dynamics of \textit{L. donovani} infection in these diverse macrophage pools has yet to be fully established. As discussed below, it is also possible that each of these myeloid cell populations will have distinct relationships with anti-leishmanial drugs (see section 5), in addition to their roles in
tissue repair, remodelling and restoration of homeostasis, that are central to the processes underpinning clinical cure.

**Granulomatous inflammation:** Granulomatous inflammation is a focal mononuclear cell-rich response to poorly degradable foreign material that seeks to wall off the insult and/or focus immune responses. Granuloma form and function in experimental, canine and human leishmaniasis have been reviewed in detail elsewhere [35]. Granulomatous inflammation is a hallmark of VL and correlates well in experimental models with the degree of immune response. Failure of granulomatous inflammation may contribute to the severity of HIV-VL coinfection [36]. Although over-exuberant, granulomatous inflammation may have directly pathologic consequences, e.g. in schistosomiasis, this has rarely been described in VL [37]. In TL, granulomas are more variably observed, and may reflect the time of biopsy in relation to disease evolution and causative agent. For example, a recent study in Tunisia demonstrated that both granuloma formation and the extent of the dermal lymphocytic infiltrate were minimal in *L. major* zoonotic CL compared to sporadic CL due to *L. infantum* [38]. Granulomas have also been observed in a majority of cases of non-ulcerating atypical CL due to *L. infantum* in Honduras [39] and are a valuable diagnostic characteristic of CL caused by *L. donovani* in Sri Lanka [40]. Although vaccine induced immunity in a primate models of VL was accompanied with enhancement of granuloma formation [41], it is not known whether this is a pre-requisite for vaccine-induced protection. In human vaccine trials, histopathological responses to vaccination have not been studied to date, and the histopathologic response documented following leishmanisation was not documented [42]. The role of granulomatous inflammation in modifying the response to chemotherapy (see section 5 below) has not been formally addressed experimentally or clinically, largely because appropriate models do not exist and the invasive nature of tissue biopsies. It is possible that the intensity of granulomatous inflammation alters drug PK properties, and that macrophages at the core of such structures represent a “privileged” site for amastigotes, but this remains to be formally evaluated.
**Alterations in tissue architecture:** At homeostasis, each tissue has its own characteristic microanatomy, dedicated to performing its main function, be that of barrier or as a site of immune response induction. By definition, tissue homeostasis is disrupted during clinical disease and this has been most well characterised in the context of remodelling of lymphoid tissue architecture during experimental and canine VL. Immune tissues are highly compartmentalised, with discrete B and T cell zones organised on a framework of stromal cells and fed by lymphatics and blood vessels [43]. During normal immune responses, these tissues adapt to allow for clonal retention and proliferation, but these are usually short-lived changes. Leishmaniasis poses a long-lived insult to lymphoid tissues and these respond by exaggerated degree of remodelling that may negatively impact on immune function. In the spleen of mice, dogs and humans infected with *L. donovani / L. infantum*, these changes include loss of stromal elements (follicular dendritic cells and fibroblastic reticular cells), dissolution of the marginal zone, white pulp atrophy and various degrees of admixing of splenic leucocyte populations [44-46].

**Systemic changes in metabolic and immune pathways:** Transcriptomics has recently been applied to a variety of experimental models and to human samples in order to provide a more holistic view of the pathology associated with both VL and CL. In human VL, comparisons between the whole blood transcriptome of healthy endemic controls with active cases, drug-cured cases and asymptomatic individuals have been reported from Brazil [47] and India [48]. Transcriptomic analysis of the splenic, hepatic and blood response to infection over time has been performed in mice [49] and for spleen only in hamsters [50]. High level analysis of these various reports indicates (i) IFN signatures are a prominent feature of infection; (ii) Th2 responses are variably represented with a bias to more severe disease; iii) changes associated with cell cycle, lipid metabolism, angiogenesis and haematological disturbances are evident in active disease and reduced after treatment; iv) treated patients, at least over the time period of study, fail to fully return to a homeostatic state. Not surprisingly, each study also identified unique aspects to the transcriptomic profile. A comprehensive cross-organ analysis in the mouse revealed only a limited concordance of DE genes
between spleen, blood and liver and a clear lack of concordance across species [49]. Although such comparative studies require a degree of caution (due to variations in analytical approach), they serve as a reminder of the importance of further research on the pathophysiology of human disease.

**CL pathogenesis:** In contrast to VL, where studies on pathology have focused on systemic sites, CL immunopathology has largely focused on the skin, though many of the changes observed in chronic VL lymphoid tissue may also occur in the lymph nodes draining CL lesion sites. Precise mapping of the nature of the inflammatory lesion and its development over time has been reported in murine models of CL, most notably due to *L. major* infection, but also with *L. mexicana* and *L. braziliensis*, with an emphasis on identifying determinants of host protection (reviewed in [51]). More recent studies spurred on by clinical observations in human *L. braziliensis* infection [52] and the identification of *Leishmania* viruses in metastatic strains of *L. guyanensis* [53] have focused on determinants of excessive host pathology. In *L. braziliensis* infection, a pathogenic role for CD8+ T cells has now been firmly established. At a mechanistic level, highly cytotoxic CD8+ T cells are believed to induce the release of cellular DAMPS that trigger inflammasome activation that perpetuates the inflammatory cascade. These studies provide opportunities for novel forms of immunotherapy to minimise tissue damage [54]. Viral infections are well known as stimulators of the type I interferon response and it has been shown that type I interferons either driven by endogenous symbiont *Leishmania* viruses or through concomitant bystander viral infection can lead enhance the metastatic potential of *L. guyanensis* in mouse models [55]. These data require further substantiation in a clinical setting, however, but also pose questions regarding novel therapeutic approaches.

### 3. Vaccines

The case supporting development of a vaccine for leishmaniasis from a scientific and public health perspective has been made many times elsewhere [56]. Vaccine research has included the full range of techniques for antigen identification (from the use of serology through to computational prediction,
[57]) and almost all conceivable vaccine delivery strategies, including cellular vaccination with antigen-pulsed dendritic cells, [58,59] have been explored for one or other form of leishmaniasis in animal models. Barriers to successful development of a vaccine to date have included i) lack of translational funding; ii) lack of correlates of immunity, iii) over-dependence on animal models; and iv) lack of a coherent programme of advocacy. Nevertheless, recent years have seen excellent progress that has brought four candidate vaccines to or near to the clinic. These include a recombinant fusion protein delivered with strong Th1-inducing adjuvants (LEISHF3+ GLA-SE; [60]), a naked multi-epitope DNA vaccine (LeishDNAvax; [61]), an adenovirus-based vaccine (ChAd63-KH; [62]) and a live genetically attenuated vaccine (L. major/ L. donovani centrin [63]). Each has taken a different approach to vaccine antigen identification and vaccine delivery, providing a potential rich environment in the future for understanding the determinants of vaccine-induced immunity in humans.

Prophylactic vaccines by definition are used in non-infected individuals to prevent the development of disease. Hence, immunopathology associated with *Leishmania* infection does not play a determining effect on vaccine design or efficacy. This does not rule out, however, that a subset of the target population for vaccination in an endemic setting may harbour sub-clinical and undetectable infections (using the diagnostic tools discussed below) that nevertheless have some local pathologic consequence. More likely, however is the scenario whereby co-infections or other intrinsic (e.g. nutrition) or extrinsic (e.g. UV radiation) environmental factors impact on vaccine-induced lymphocyte activation, memory cell generation and/or effector and regulatory cell balance. Due attention should be paid to these possible factors when designing and evaluating future clinical trials.

In contrast, therapeutic vaccines require enhancement of the state of immunity in those already with leishmaniasis pathology. The extent to which ongoing pathology impacts the efficacy of therapeutic vaccines for CL is presently unknown and will require carefully designed clinical trials in which patients can be stratified according to pathologic criteria. In animal models of VL, therapeutic vaccination can overcome the immunosuppressive state induced by VL [64] and post-exposure
prophylactic vaccination in *L. infantum*-infected dogs was shown to reduce progression to symptomatic VL [65]. Prevention of PKDL by vaccination of previously treated VL patients poses an interesting challenge, given that the immune status of treated VL patients is likely to have been improved but not normalised [48]. A clinical trial to assess this approach is in development (clinicaltrials.gov; NCT04107961). Combination studies of vaccines deployed with additional immunomodulators to overcome pathology-induced immune regulation should be actively considered.

4. Diagnostics

As the leishmaniases are characterized by their clinical pleomorphism confirmation based solely on clinical grounds is a challenge. The wide variety of skin and mucosal lesions in CL involves an extensive differential diagnosis. In addition, diseases like malaria, infectious mononucleosis and malignancies, among others, present with fever, hepatomegaly and splenomegaly as does VL, often accompanied by cachexia and malnutrition in chronic courses.

Parasite demonstration by microscopy of Giemsa stained tissue smears is the gold standard in the diagnosis of leishmaniasis; this is not always accessible and has variable sensitivity. Thus laboratory confirmation rates before treating CL can be as low as 5% in some settings, with patients being put on treatment with potentially toxic drugs without receiving a confirmatory diagnosis [66]. For VL invasive tissue sampling is required, usually from bone marrow or spleen and to a lesser extent lymph node. This requires expertise and specialised facilities for managing potential complications, so VL suspects are usually referred to specialised treatment centres for diagnosis. Because of this WHO recommended that a strict case definition for VL, i.e. fever for more than 2 weeks plus hepatosplenomegaly and /or wasting; with this a positive serology enables treatment initiation [67].

**Diagnosis for case management**

A common feature in VL is a polyclonal hypergammaglobulinaemia with a marked increase in serum IgG level, including IgG with specificity for *Leishmania* [68]. Therefore, identifying anti-*Leishmania* specific antibodies is currently the cornerstone of VL diagnosis. Rapid diagnostic tests (RDTs) based
on the recombinant antigen rK39, from a *Leishmania infantum* strain from Latin America, are widely used and they show very good performance in the Indian subcontinent (ISC): 97% sensitivity (95% CI: 90.0 - 99.5), 90.2% specificity (95% CI: 76.1 - 97.7). In eastern Africa the specificity is similar 91.1% (95% CI: 80.3 - 97.3), but the sensitivity drops to 85.3% (95% CI: 74.5 - 93.2) [69]. This inferior performance requires that in eastern Africa it is necessary to include the direct agglutination test (DAT) in the diagnostic algorithm (Figure 2). This is a robust test based on whole *Leishmania* antigen and it has been extensively validated. It cannot be considered an RDT as it requires some degree of laboratory expertise and capacity, and results are obtained after an overnight incubation [70].

The peculiarities of the antibody response against specific *Leishmania* antigens are key in the performance of serological tests. VL elicits a different immune response and different levels of anti-*Leishmania* IgG across regions. rK39-based RDTs are based on a kinesin sequence from an American strain of *L. infantum* (syn. *L. chagasi*), this can explain the different performance of these tests in eastern Africa, where there is a high molecular diversity of the rK39 homologous sequences among regional *L. donovani* strains [71]. Another explanation for this is that there can be a different potency in the immune response, exemplified by the finding that anti-*Leishmania* IgG levels in VL patients from Sudan were significantly lower than in patients from India, independently of the geographic origin of the leishmanial antigen used to assess this [72]. A new RDT, based on the recombinant antigen rK28, may contribute to overcome this problem. The rK28 is a synthetic polyprotein containing rK39 repeats from a Sudanese *L. donovani* strain, flanked by HASPB1 repeats and the HASPB2 open reading frame from an Ethiopian strain [73]. Some preliminary studies claim that rK28 RDTs show better performance in eastern Africa than those using the rK39 antigen; however, data are not definitive, as studies comparing rK28 RDTs with the rK39 RDT IT-Leish (Bio-Rad), which is the one recommended in most eastern African guidelines for VL, are limited. These studies are either non-prospective or use just serum or plasma from well characterized controls and cases (confirmed by parasitology) [74-76]. A prospective large scale evaluation of an rK28 RDT will be conducted by the
AfriKADIA consortium in Ethiopia, Kenya, Sudan and Uganda; results will be available by the end of 2020 [https://www.afrikadia.org/].

Unlike in VL, CL is not characterized by an elevated production of anti-\textit{Leishmania} antibodies, and the level of these is also quite variable between the different forms of tegumentary leishmaniasis. Therefore, antibody detection tests are not useful in the diagnosis of these forms of leishmaniasis. Anti-\textit{Leishmania} antibodies are barely detectable in CL and to some extent these can be detected in ML and DCL but with limited and variable performance [67]. New opportunities for the serological diagnosis of CL may be brought by the detection of anti-\textit{α}-Gal antibodies, with promising preliminary results for Old World leishmaniasis, nevertheless this field requires further research [77].

In immunosuppressed HIV+ patients, antibody-detection tests have limited sensitivity and cannot be used to rule out VL [78,79]. Also, the detection of total anti-\textit{Leishmania} antibodies is not very helpful in the diagnosis of PKDL or VL relapses, as circulating antibodies from previous VL episodes remain for long periods even after successful treatment [80]. It seems however that exploring specific IgG types can be of help in assessing post-treatment cure and the risk of relapse, as well as in supporting the diagnosis of PKDL. The qualitative and quantitative detection of IgG1 in blood or serum/plasma samples, by means of ELISA or RDT using whole or recombinant rK39 leishmanial antigen, have shown the potential of IgG1 as a biomarker of post-chemotherapeutic relapse, as demonstrated with samples from Sudanese and Indian patients [81-83].

Antigen-detection tests can help in resolving some of the problems described above, these tests should be more specific and can distinguish active from past infections. Most of the approaches used up to date for the detection of leishmanial antigens in VL patients have targeted urine [84-86]. A latex agglutination test has shown a highly variable sensitivity (36%-100%) and specificity (64%-99%) across endemic regions and different groups of patients, including HIV-positive, thus this has not been fully implemented [69]. Different antigen detection ELISA tests have been developed, and they have shown high sensitivity and specificity in a preliminary study using samples from VL patients from different endemic regions, as well as utility in monitoring treatment efficacy [87]. Lack of further evaluation, the need to process the sample and the technical requirements of an ELISA tests
have precluded putting them into routine practice. A more practical approach would be to detect leishmanial antigens in blood or serum/plasma. A prototype immunochromatographic test showed high sensitivity (96%) and specificity (99%) in the diagnosis of VL in Chinese patients using blood or serum [88]; unfortunately, further validation of this test and its results in other settings has not been pursued.

A point-of-care (POC) tests for early diagnosis of CL is much needed to benefit patients and communities, its implementation will reduce morbidity and transmission (where this is anthropogenic). A target product profile (TPP) for a POC for CL was developed by consultation among experts with a consensus that this POC test should target *Leishmania* antigens [89]. A promising option for the point-of-care diagnosis of CL that fits this TPP is a RDT detecting the leishmanial amastigote antigen peroxidoxin, CL Detect™ Rapid Test (InBios International Inc., USA). This test has shown variable performance across endemic regions, but for CL due to *L. major* or *L. tropica* the sensitivity and specificity obtained in different studies in Tunisia, Morocco and Afghanistan is acceptable [90-92].

Nucleic acid amplification tests (NAATs) have shown good performance in the diagnosis of the different forms of leishmaniasis, both in immunodepressed and immunocompetent patients. Polymerase chain reaction (PCR)-based methods have proven that confirmatory diagnosis of VL is feasible using a less invasive sample such as peripheral blood, and with high sensitivity [93]. And this is especially relevant in CL, when chronic lesions have lower parasite loads that cannot be detected by microscopy [94,95]. A limitation to implement PCR-based diagnosis at the POC has been the requirement of well-equipped facilities and trained personnel, as well as the lack of standardisation, limiting the use of these tools to reference laboratories. Nevertheless, NAATs more amenable for use in limited resource settings have been recently developed, a commercial test based on the loop-mediated isothermal amplification (LAMP) of DNA, Loopamp™ Leishmania Detection Kit (Eiken Chemical Co., Japan), overcomes the problem of standardisation. Its performance using peripheral blood (97%-99% sensitivity), as demonstrated in a study in Sudan, enables its inclusion in the VL diagnostic algorithm, reducing the need for invasive tissue aspiration [76]. A diagnostics
evaluation study conducted in Afghanistan informed that an algorithm including CL Detect™ Rapid Test and Loopamp™ Leishmania Detection Kit would have a sensitivity of 93%, minimizing the number of patients that would need to be referred for parasitological confirmation [92]. Other studies evaluating non-commercial LAMP assays have also reported high sensitivities in the diagnosis of CL, VL and PKDL [96]. As such, the diagnosis and management of leishmaniasis would benefit from the implementation of accurate NAATs at the POC.

Diagnostics in the elimination and post-elimination of visceral leishmaniasis

WHO aims at eliminating VL as a public health problem in the Indian subcontinent (ISC) by 2020. The implementation of test-and-treat strategies, relying on the use of antibody-detection RDTs has been pivotal in moving towards this target [97]. This effort will need to be sustained in the next few years to avoid resurgence and move towards the interruption of *Leishmania* transmission by 2030 [98]. However, the current diagnostic tools may not be adequate to support this endeavour.

More specific tests will be needed in the near and post-VL elimination phases, since the positive predictive value of antibody-detection RDTs decreases in a context of low endemicity [97,99]. Only VL suspects with symptoms (e.g. fever, splenomegaly) for more than two weeks are tested by the current RDTs, which precludes early diagnosis and treatment of acute cases who remain infectious in the community. Reducing the time between onset of symptoms and diagnosis-treatment has been identified as a key parameter for achieving and sustaining elimination [100]. But current antibody-detection RDTs do not allow this, as they are not specific for acute VL and need to be interpreted along with a clinical case definition [69]. Detection of *Leishmania* antigens may be a more specific approach [101,102,87,88], and these can be detected earlier than anti-*Leishmania* antibodies [86,103]. Thus antigen-detection RDTs would allow diagnosis of VL cases without a time constraint or clinical case definition (e.g. < 2 weeks of fever); as described above several approaches for leishmanial antigens detection in clinical samples have been developed, but for different reasons these have not been implemented. A recent target product profile describes the features of an antigen detection RDT that can be used to assist in VL elimination [104]. Antigen detection tests would also allow diagnosing VL in groups of patients in which antibody detection tests underperform such as HIV-VL.
co-infected patients, relapses, as well as in PKDL. All these cases are gaining relevance in the last stages of the VL elimination in the ISC, as they are an important reservoir of *Leishmania* and are a serious threat to VL elimination [105,106,99].

Diagnosis of PKDL is a key factor in elimination efforts as well as in patient management, for PKDL treatments are long (up to 12 weeks). PKDL diagnosis is challenging as case confirmation requires microscopy and/or molecular tests in skin biopsies. As these diagnostic procedures are difficult to perform, diagnosis in endemic areas is often made clinically [107]. Antibody detection RDTs, which are part of the PKDL diagnosis algorithm in the ISC, have limited value and alternatives such as point-of-care molecular tests with promising performance (e.g. LAMP) should be evaluated [108,99].

There have been few if any studies directly seeking to evaluate the impact of pathology on the design and/or performance of diagnostic tests for VL or CL. Although there may be limitations in translating such knowledge, a consideration of the role of pathology in determining the specificity and sensitivity of current and future tests may be warranted. For example, perturbations in the regulation of antibody responses are linked to disease progression [109] and antibody and antigen half-lives will be affected by changes in plasma or lesion protein binding capacity, glomerular filtration rate (e.g. secondary to immune complex deposition [110]) and/or the degree of enteropathy [111]. Hence, each of the parameters highlighted in Fig. 3 will be influenced by the degree of pathology observed during the early pre-clinical, clinical and post-treatment phases of the disease. As above in the case of vaccine efficacy, these pathologic changes might occur directly as a consequence of ongoing or previous leishmaniasis, but equally so may reflect the indirect immunopathological consequence of coinfections or other immune insults.

5. Drugs

There has been considerable progress in the development of treatments for leishmaniasis over the past two decades. A number of new drugs have been registered for VL (miltefosine, the liposomal
formulation AmBisome, paromomycin) with some taking a lead role in disease control and elimination programmes [112,2]. At the same time there is an inadequacy of drugs for CL [113,114]. However, for the first time in history the pipeline of novel oral drugs for the treatment of VL and CL is more promising with five novel compounds for VL [115-117] and a renewed focus to find novel treatments for CL [118,119]. In this section, two aspects of the drug – immune response interaction will be discussed (i) immunosuppression and immunomodulation, and (ii) the impact of immunopathology on pharmacokinetics.

Immunosuppression and immunomodulation

Interactions between drugs and the immune response have been known, but poorly understood, since the early 20th century. It still provides a strong focus for research: a recent study on antibiotics that showed how host cell metabolites both reduce drug activity whilst also enhancing host cell phagocytic activity provides an example of such work [120]. For leishmaniasis, as for other infectious diseases, there have been two directions of study: (i) to understand why drugs do not work in patients and disease models and (ii) the development of a strategy to stimulate/modulate the immune response to overcome any immunosuppression or accelerate cure.

Drugs used for clinical treatment of VL have to work effectively in most endemic regions within the context of the immunosuppression provoked by this disease. With the more profound immunosuppression associated with HIV VL co-infections, the standard drugs become significantly less effective and only co-administrations have been shown to improve treatment [121]. The low efficacy of three of the drugs (liposomal amphotericin B, paromomycin and miltefosine) in East African VL [122,123] that are so effective in the Indian subcontinent, has not been explained. Clinical isolates from both regions show similar susceptibility to these drugs within standard in vitro assays, so the clear implication is that host factors (immunology, pathology, pharmacokinetics [PKs]) are the responsible for this difference. The potential importance of PKs has been illustrated by Dorlo and colleagues [124] who have determined different miltefosine exposures in adults and children in East Africa. There are further clinical and experimental observations, one confirming, one confounding: (a) several of the drugs that work effectively as VL treatments in immunosuppressed VL patients (ibid) have significant
dependence on cellular immune responses in mouse models of infection, either immuno-suppressed or deficient [125-127], (b) all the drugs, whether immune or non-immune dependent in these studies, have well described interactions with the immune system (see reviews by Dorlo (2012) for miltefosine [128], Cohen (2016) for amphotericin B [129] and Murray (2005) for pentavalent antimonials [130]). It is clear that more detailed studies are required to understand these interactions.

For CL, drugs work within the context of a slow self-cure immune response; exceptions include diffuse cutaneous leishmaniasis (DCL) where there is an absence of an effective cellular immune response and absence of effective treatment. The objectives for drug treatment, and combinations with immunomodulators, are to both reduce the parasite burden and at the same time accelerate self-cure.

Much of the area lacks clear clinical data due to the absence of robust clinical studies [113,114]. The central role of the macrophage in the survival of Leishmania parasite has been the driving force for most studies on immunomodulation. Have we progressed much further than a statement from Bernard Shaw’s play Doctor’s Dilemma (1906) “There is at bottom only one genuinely scientific treatment for all diseases, and that is to stimulate the phagocytes”? BCG, which has well characterised stimulatory interactions with macrophages [131], was the immunodulatory component of treatment with pentavalent antimonials and the recommended treatment for CL in Venezuela [132]. It was also used in combination with alum and pentavalent antimonials in clinical trials for the treatment of post-kala-azar dermal leishmaniasis [133]. Bacterial cell wall components, muramyl dipeptide and trehalose dimycolate have also been used in clinical studies [134] on CL and experimental VL [135]. Based on clearer understanding of cytokine-macrophage interaction both IFNγ [136] and GM-CSF [137] have been used in the treatment of both VL and CL. The rationale behind the potential application of GM-CSF for CL is threefold: (a) GM-CSF can promote proliferation, activation and differentiation of various myeloid cells (macrophages, dendritic cells and neutrophils) and their progenitors, (b) clinical trials have reported increased wound healing of diverse wound types (including CL) upon topical GM-CSF application, and (iii) CM-CSF is able to modulate the tissue immune response by increasing the levels of the anti-inflammatory cytokine IL-10.

Randomized controlled trials in Brazil have indicated that GM-CSF both after oral [138] and topical [139] administration reduced the healing time of CL lesions when given in combination with
intravenous antimonials. In addition to its anti-inflammatory role, IL-10 also inhibits IFNγ induced macrophage killing of *Leishmania* and Murray and colleagues [140] demonstrated in a model of VL that blockade of IL-10 using anti-IL-10R antibodies resulted in significant increase in antimonial drug activity. A clinical trial to evaluate combining AmBisome with anti-IL-10R antibodies in VL patients was proposed but later withdrawn due to unavailability of the required antibody (clinicaltrials.gov; NCT01437020).

Immunomodulator molecules that are also TLR agonists are being pursued for CL treatment. For example, CpG-D35, a D-type CpG TLR9 agonist, is currently undergoing Phase-I clinical trials after showing it was able to curtail lesion development upon administration to macaque monkeys infected with *L. major* [141]. Earlier studies indicated that this TLR9 agonist induces maturation of plasmacytoid dendritic cells and secretion of IFNα and -γ without direct activation of B cells [142]. A small molecule, the imidazoline imiquimod a TLR7 agonist, was shown by Matlashewski and colleagues to stimulate macrophage functions including signal transduction and NO production in experimental models, sufficient to kill intra-macrophage amastigotes [143]. Subsequent clinical studies using imiquimod as a topical adjunct therapy to pentavalent antimonials were inconclusive. Three of these randomized controlled trials (RCT) [144-146] showed no significant increased cure rate for the combination of imiquimod plus pentavalent antimonials versus antimonials alone. Efforts continue to find more active analogues [147]. A different approach was adopted by Smith et al (2000) with the small molecule, tucaresol (in clinical trials for sickle cell disease), an orally bioavailable compound that enhances T-helper-cell activity, with the induction of increased IL-2 and IFNγ levels in mice and humans and provides a costimulatory signal between macrophages and T-cells [148]. In mouse model of VL tucaresol gave a 60% reduction in parasite burden similar to that achieved by INFγ in the same model.

Other recent approaches have also added considerably to understanding drug-immune interactions and possible new routes forward to immunomodulation and successful cure. Long-term clinical research on South American CL [54] showed that NLRP3 inflammasome is activated by CD8+ T cell-mediated cytotoxicity and drives disease progression. Based on these observations, the group studied a number of small molecule inhibitors of the inflammasome, for example, MCC950 and the diabetes drug
glyburide. In mouse models of CL, they showed that mice treatment with compounds that inhibit NLRP3 inflammasome activation, MCC950 or glyburide, failed to develop the severe disease seen in untreated mice. In VL, more detailed knowledge of the immune response has also been exploited. Based on knowledge of the immunopathology of the spleen, the receptor tyrosine kinase inhibitor sunitinib maleate was shown to induce restoration of splenic microarchitecture. Although this drug did not possess inherent anti-leishmanial activity, it afforded a significant dose sparing effect for subsequently administered antimonials [149].

CL is also characterised by local inflammation. A combination of the anti-inflammatory agent, pentoxiphylline, with antimonial drugs has been shown to be an important adjunct therapy for some forms of CL and mucocutaneous leishmaniasis. In Brazil, randomized controlled trials (RCTs) have shown a synergistic effect of pentoxifylline in conjunction to antimonial therapy in ML patients [150] whereas this was not observed in patients suffering CL [151,152]. The difference in pentoxifylline efficacy as part of a combination therapy between New World CL and ML might be due to distinct differences in cytokine and macrophage populations present in the lesion [153] – an observation that merits further exploration as it could help elucidate immunomodulatory processes. It is worth noting that when tested in Old World CL, the combined approach of pentoxifylline with an antileishmanial agent showed enhanced healing in comparison to antileishmanial treatment only [154]. For further information on the activity of immunomodulators, see reviews by Dalton and Kaye (2010), Taslimi et al., (2018) and Adriaensen et al (2017) [149,155,156].

PK/PD relationships, immunopathology in drug monitoring and targets for host directed therapy.

Besides the direct involvement in the disease progression and outcome, the immunopathology also impacts drug pharmacodynamics (PD) and/or pharmacokinetics (PK). Impact on the latter has been shown in mice with CL, where the presence of parasites and the associated inflammation in the skin disturbed the cutaneous barrier function and physiology [157,158]. Interestingly, the permeation of hydrophilic compounds increased to a higher extent in comparison to lipophilic compounds which was hypothesized to be in part due to the inflammatory hydrophilic environment in CL skin. Whilst these
findings are particularly important for topical drug administration where the drug is applied locally, increased drug levels were also observed in *Leishmania*-infected skin upon systemic drug administration [159,160] where they were associated with enhanced capillary leakiness and increased macrophage infiltration both of which could potentially be attributed to local inflammation. How *Leishmania* infection alters the skin tissue microenvironment in humans is yet unknown and is subject to further research.

During the development of VL, the liver, a major drug metabolism site for drugs, undergoes morphological and functional changes; hence, it seems evident that VL impacts drug pharmacokinetics. In VL infected mice for example, the no-observed-adverse-effect level of miltefosine is approximately 25 mg/kg (QD, for 10 consecutive days), whereas in experimental CL 35mg/kg is still well tolerated (experimental data, unpublished). Other studies also demonstrated a deterioration of the capacity of host hepatic microsomal membranes to metabolise xenobiotics in VL infected mice and hamsters [161,162]. Whilst *in vitro* studies suggest no involvement of cytochrome P450 isoenzymes in the metabolism of miltefosine [163], the enzymes are involved in the metabolism and clearance of many xenobiotics and reduced activity could potentially lead to reduced drug efficacy (metabolites are active) or toxicity when increased amounts linger around in the systemic circulation. For amphotericin B, a significantly lower concentration of drug was found in liver and spleen of VL-infected mice as compared to uninfected mice, which was hypothesised to be due to a reduced phagocytic activity in infected macrophages [155, 157]. These findings were not investigated in humans but it seems plausible that the PK of certain drugs is altered by the disruption of the liver function as observed in VL. Pathology is also known to affect drug ADME (absorption, distribution, metabolism and excretion) processes by alterations in protein binding which is important given that hypoalbuminemia is observed in both human and experimental VL. Low serum albumin levels have been associated with alterations in the degree of protein binding of highly protein-bound drugs such as miltefosine and thus could potentially impact its PK-PD relationship [164,165].

Another phenomenon which remains unexplored in VL patients and experimental models is the influence of granuloma formation on drug PK and thus efficacy. In tuberculosis, a granuloma forms a
shielded lesion compartment that harbours bacteria and makes it difficult for drugs to permeate [166]; hence, the long and intense treatment regimen composed of a cocktail of four different drugs. Whilst difference in the extent of granulomatous inflammation are clear between leishmaniasis and TB [35], the altered local cellularity of a granuloma may nevertheless be of importance.

Of further importance is the impact of the pathology on drug PD which involves processes best reflected by “what the drug does to the body”. Most chemotherapeutics exert their activity by stimulating or inhibiting enzymes that are involved in pathways essential for parasite survival. Recent research describes the presence of different metabolically active parasite populations in CL lesions [8]. Kloehn et al (2015) measured semi-quiescent L. mexicana parasites in non-healing CL lesions in mice [7]. This reduced metabolic state is characterised by low transcription rates and protein turnover and might thus contribute to a reduced drug efficacy.

**Summary**

Here we have illustrated how the complexity and variation of human immune responses and immunopathology and the different host interactions to twenty *Leishmania* species has an impact upon the effectiveness of vaccines, diagnostics and drugs (Fig. 1). Although rodent models have proved to be a useful tool for experimental studies on these interactions, well described limitations of (a) absence of models for important species (*L. aethiopica, L. tropica, L. braziliensis*), (b) differences between mouse and human immune responses (for example, to TLR agonists, [167,164] and serological indicators, and (c) differences in pharmacokinetics between mouse and humans [168,165], underpin most of this work. The application of transcriptomics, genomic data bases and methodologies to determine pharmacokinetics in infected tissues (not just plasma) in future studies in human subjects must now be used to inform future work so tools for treatment, control and elimination can be used with more understanding and effectiveness.
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<table>
<thead>
<tr>
<th>Main Leishmania species</th>
<th>Clinical presentations</th>
<th>Main Reservoir</th>
<th>Countries or regions found</th>
</tr>
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<tbody>
<tr>
<td><em>L. donovani</em></td>
<td>VL, PKDL</td>
<td>Humans</td>
<td>India, Bangladesh, Ethiopia, Sudan, South Sudan</td>
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<tr>
<td><em>L. tropica</em></td>
<td>CL, LR, rarely VL</td>
<td>Humans</td>
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<td><em>L. aethiopica</em></td>
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<td>Hyraxes</td>
<td>Ethiopia, Kenya</td>
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<td>CL</td>
<td>Rodents</td>
<td>North Africa, Middle East, Central Asia, West Africa</td>
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<td><em>L. infantum</em> [L. chagasi]</td>
<td>VL, CL</td>
<td>Dogs, humans</td>
<td>China, South Europe, Brazil, South and Central America</td>
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<td><em>L. braziliensis</em></td>
<td>CL, MCL, DCL, LR</td>
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<td><em>L. mexicana</em></td>
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VL = Visceral Leishmaniasis; PKDL = Post-Kala-Azar Dermal Leishmaniasis; CL = Cutaneous Leishmaniasis; MCL = Mucocutaneous Leishmaniasis; DCL = Diffuse Cutaneous Leishmaniasis; DsCL = Disseminated Cutaneous Leishmaniasis; LR = Leishmaniasis Recidivans
Leishmaniasis immunopathology – impact on design and use of vaccines, diagnostics and drugs

Paul M. Kaye¹, Israel Cruz², Albert Picado², Katrien Van Bocxlaer¹, Simon L. Croft³

¹York Biomedical Research Institute, Hull York Medical School and Dept. of Biology, University of York, U.K.

²Foundation for Innovative New Diagnostics (FIND). Geneva, Switzerland.

³Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, U.K.

⁴National School of Public Health. Instituto de Salud Carlos III. Madrid, Spain

Correspondence: simon.croft@lshtm.ac.uk
Abstract

Leishmaniasis is a disease complex caused by 20 species of protozoan parasites belonging to the genus Leishmania. In humans it has two main clinical forms, visceral leishmaniasis (VL) and cutaneous or tegumentary leishmaniasis (CL), as well as several other cutaneous manifestations in a minority of cases. In the mammalian host *Leishmania* parasites infect different populations of macrophages where they multiply and survive in the phagolysosomal compartment. The progression of both VL and CL depends on the maintenance of a parasite-specific immunosuppressive state based around this host macrophage infection. The complexity and variation of immune responses and immunopathology in humans and the different host interactions of the different *Leishmania* species has an impact upon the effectiveness of vaccines, diagnostics and drugs.

Key Words

Leishmaniasis, immunopathology, vaccines, diagnostics, drugs
1. Introduction

Leishmaniasis is a disease complex with two main clinical presentations – visceral leishmaniasis (VL, or kala-azar) and cutaneous leishmaniasis (CL). Both VL and CL have a worldwide distribution in nearly 100 endemic countries. Overall the number of potentially fatal VL cases has decreased during the past decade with an estimated annual incidence of less than 50,000 (2017 figures, https://www.who.int/leishmaniasis/burden/en accessed 01.10.19) with seven countries: Brazil, Ethiopia, India, Kenya, Somalia, South Sudan and Sudan having the major burden. The decrease in VL case numbers in the Indian subcontinent (ISC) is coincident with the introduction of a regional elimination programme launched in 2005, with improved access to diagnosis and treatment. The naturally cyclical pattern of transmission intensity of this anthropogetic disease may also contribute to this decrease in the ISC. In contrast, the number of cases in East Africa has not fallen and this endemic area is now a major focus for control programmes. The estimated worldwide annual incidence of CL is between 0.7 to 1.2 million cases [1] with Central and South America, the Middle East, Ethiopia and North Africa being the major endemic areas. Far less attention has been paid to the control and treatment of CL, a predominantly zoonotic and a slow self-curing disease (3 – 18 months in most cases) with disfigurement and stigma being the main corollaries of infection. In addition, there are rarer variants of CL: mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL), disseminated cutaneous leishmaniasis (DsCL), leishmaniasis recidivans (LR) and post-kala-azar dermal leishmaniasis (PKDL). With CL, these variant skin manifestations are often referred to collectively as the tegumentary leishmaniases (TL). PKDL is a perplexing clinical presentation which follows cure of VL (5-10.% cases in Asia, up to 50% cases in East Africa, [2,3]).

The protozoan parasites that cause VL and TL belong to at least 20 distinct species of the genus *Leishmania* (with new species being identified in the past decade) that are transmitted between mammalian hosts by female phlebotomine sandflies. Different species of *Leishmania* are largely responsible for the diverse clinical manifestations (Table 1) which are generated by the interplay between the parasite, host factors and vector biology. However, clinical cases reports suggest these
species-dependent clinical outcomes are not distinct. In the sand fly gut *Leishmania* parasites exist as flagellated promastigote forms, while in the mammalian host the parasites invade macrophages losing their flagellum and transforming into the intracellular amastigote. The amastigote multiplies and survives in the phagolysosomal compartment of the macrophage. It has long been known that promastigotes differentiate within the sandfly to generate infective “metacyclic” forms [4-6] and recent evidence suggests that there may also be heterogeneity in the intracellular amastigote form [7-9]. *Leishmania* parasites actively manipulate both of their hosts. In the sandfly, transmission efficiency of metacyclic promastigotes is enhanced by secretion of a proteophosphoglycan-rich, mucin-like gel (Promastigote Secretory Gel, PSG), which accumulates in the sand fly gut and mouthparts [10]. Mammalian infection is established in the skin following the inoculation of metacyclic promastigotes that possess a lipophosphoglycan coat that enables them to resist complement and attach to and invade host cells. Peptides in sand fly saliva (for example, maxadilan) cause vasodilation and erythema that also aids the establishment of the infection in macrophages in the dermal layer of the skin [11]. Early responses to infection involve neutrophil infiltration and invasion of resident macrophages. Progress of the disease depends on the parasite species and host responses. For both VL and CL, disease progression depends on the maintenance of a parasite-specific immunosuppressive state including host cell macrophages. A key difference between VL and CL is the propensity of VL-causing species (Table 1) to cause disease at systemic sites, invading macrophages of the liver, spleen and bone marrow, with minimal skin pathology. Restoration of macrophage function, either through the development of an appropriate immune response or therapeutic intervention, can lead to amastigote killing in macrophages by nitric oxide and oxygen radicals. Resolution of disease following the activation of macrophages is enhanced by T helper 1 (Th1) - like responses mediated through the interaction of antigen-presenting cells (e.g., dendritic cells, macrophages) with CD4+ and CD8+ T cells and subsequent secretion of pro-inflammatory cytokines, including interferon-γ (IFNγ) and tumor necrosis factor (TNF). In clinical forms of VL and in DCL, Th2 – like cell responses (including IL-4 and IL-13) are also evident [12,13] and inhibition of macrophage activation and immune dysregulation may be mediated through IL-10 and transforming growth factor-β (TGFβ) [14]. Immune responses and immunoregulatory pathways have been extensively defined in experimental inbred mice that show some but not all the characteristics of
human disease [15,16]. Immune responses directed against the parasite may also play a pathogenic role and contribute to tissue damage and clinical severity (see below).

The prevention, treatment and control of both CL and VL depends on drugs, diagnostics and vaccines, as described in recent comprehensive reviews [2,17]. The complexity and variation of immune responses and immunopathology in humans and the different host interactions of the *Leishmania* species has an impact upon the effectiveness of these tools (Fig. 1). Recent studies have significantly increased our understanding of these interactions and suggested new strategies for discovery and development, and such data are the focus of this review.

2. Key features of immunopathology in leishmaniasis

Virulence factors allow pathogenic microbes to establish infection, colonise, replicate and disseminate throughout the host, evade anti-microbial immunity and facilitate transmission. A plethora of *Leishmania* derived molecules have been described and reviewed elsewhere that have the cardinal properties of bona fide virulence factors [18]. Although some pathogens liberate virulence factors that directly cause tissue damage e.g. gangrene due to collagenases and phospholipases produced by *Clostridium perfringens*, similar factors have not yet been described for *Leishmania*. Rather, clinical symptoms in leishmaniasis are, for the most part, the result of inappropriate immune responses leading to collateral damage to host tissues. Hence, for its clinically relevant features, leishmaniasis represents a disease driven by immunopathology. Hence, the “appropriateness” of an inflammatory / immune response, defined by its ability to strike the delicate balance between removal of *Leishmania* parasites and the minimization of pathology, may be defined by its quality, quantity or kinetics as well as by the parasite species under study. Some of the key features of the immunopathology associated with leishmaniasis are described below.

*Macrophage activation:* Macrophage activation is a central tenet of anti-leishmanial immunity, and the capacity of macrophages to polarise their functions towards the generation anti-microbial effector molecules such as NO and O$_2^-$ under the influence of type 1 cytokines (classical activation) or
towards the generation of arginine and polyamines under the influence of type 2 cytokines (alternate activation) is well established. However, the simple assumption that type 1 cytokines are “protective” and type 2 cytokines are “detrimental” is not always borne out by the literature. For example, IL-4, the prototypic Th-2 cytokine, plays an important early role in the generation of host protective CD4+ IFNγ+ Th-1 cells in murine models of CL [19] and IL-4R signalling is required for optimal protection in primary resistance and vaccine-induced immunity in experimental VL [20-22]. Similarly, chemotherapy with sodium stibogluconate (SSG) in models of VL also requires IL-4R signalling for optimal effectiveness [22]. More recent evidence indicates that cytokines influence macrophage metabolic states, with consequences for the amastigote’s access to required nutrients. Specific metabolic countermeasures may have evolved to combat these stresses and ensure long lived parasitism [23].

Tissue macrophages have two origins. Resident tissue macrophages originate from embryonic yolk sac-derived progenitors, fully differentiate to acquire their mature characteristics under the direction of tissue-specific transcription factors / tissue-derived cues and have the capacity of self-renewal. In contrast, relatively short-lived tissue macrophages originate from hematopoietic stem cells via monocyte precursors [24,25]. Resident tissue macrophages play many of the key roles in tissue homeostasis and function, and they have been identified as niches for survival of L. major in the dermis [26] and L. donovani in the liver and spleen [27] and it is likely some of their housekeeping properties are impacted on by infection. Monocytes and monocyte-derived macrophages [28-30], as well as dendritic cells [31] and neutrophils [32] may also be targets of infection and serve to regulate immunity and pathology though their relatively short life span may suggest that there is greater dynamics in the parasite populations they contain. Recent evidence suggests that the bone marrow becomes a target of collateral T cell-mediated damage during VL [33,34] but the consequences of this for the dynamics of L. donovani infection in these diverse macrophage pools has yet to be fully established. As discussed below, it is also possible that each of these myeloid cell populations will have distinct relationships with anti-leishmanial drugs (see section 5), in addition to their roles in
tissue repair, remodelling and restoration of homeostasis, that are central to the processes underpinning clinical cure.

*Granulomatous inflammation:* Granulomatous inflammation is a focal mononuclear cell-rich response to poorly degradable foreign material that seeks to wall off the insult and/or focus immune responses. Granuloma form and function in experimental, canine and human leishmaniasis have been reviewed in detail elsewhere [35]. Granulomatous inflammation is a hallmark of VL and correlates well in experimental models with the degree of immune response. Failure of granulomatous inflammation may contribute to the severity of HIV-VL coinfection [36]. Although over-exuberant, granulomatous inflammation may have directly pathologic consequences, e.g. in schistosomiasis, this has rarely been described in VL [37]. In TL, granulomas are more variably observed, and may reflect the time of biopsy in relation to disease evolution and causative agent. For example, a recent study in Tunisia demonstrated that both granuloma formation and the extent of the dermal lymphocytic infiltrate were minimal in *L. major* zoonotic CL compared to sporadic CL due to *L. infantum* [38]. Granulomas have also been observed in a majority of cases of non-ulcerating atypical CL due to *L. infantum* in Honduras [39] and are a valuable diagnostic characteristic of CL caused by *L. donovani* in Sri Lanka [40]. Although vaccine induced immunity in a primate models of VL was accompanied with enhancement of granuloma formation [41], it is not known whether this is a pre-requisite for vaccine-induced protection. In human vaccine trials, histopathological responses to vaccination have not been studied to date, and the histopathologic response documented following leishmanisation was not documented [42]. The role of granulomatous inflammation in modifying the response to chemotherapy (see section 5 below) has not been formally addressed experimentally or clinically, largely because appropriate models do not exist and the invasive nature of tissue biopsies. It is possible that the intensity of granulomatous inflammation alters drug PK properties, and that macrophages at the core of such structures represent a “privileged” site for amastigotes, but this remains to be formally evaluated.
Alterations in tissue architecture: At homeostasis, each tissue has its own characteristic microanatomy, dedicated to performing its main function, be that of barrier or as a site of immune response induction. By definition, tissue homeostasis is disrupted during clinical disease and this has been most well characterised in the context of remodelling of lymphoid tissue architecture during experimental and canine VL. Immune tissues are highly compartmentalised, with discrete B and T cell zones organised on a framework of stromal cells and fed by lymphatics and blood vessels [43]. During normal immune responses, these tissues adapt to allow for clonal retention and proliferation, but these are usually short-lived changes. Leishmaniasis poses a long-lived insult to lymphoid tissues and these respond by exaggerated degree of remodelling that may negatively impact on immune function. In the spleen of mice, dogs and humans infected with L. donovani / L. infantum, these changes include loss of stromal elements (follicular dendritic cells and fibroblastic reticular cells), dissolution of the marginal zone, white pulp atrophy and various degrees of admixing of splenic leucocyte populations [44-46].

Systemic changes in metabolic and immune pathways: Transcriptomics has recently been applied to a variety of experimental models and to human samples in order to provide a more holistic view of the pathology associated with both VL and CL. In human VL, comparisons between the whole blood transcriptome of healthy endemic controls with active cases, drug-cured cases and asymptomatic individuals have been reported from Brazil [47] and India [48]. Transcriptomic analysis of the splenic, hepatic and blood response to infection over time has been performed in mice [49] and for spleen only in hamsters [50]. High level analysis of these various reports indicates (i) IFN signatures are a prominent feature of infection; (ii) Th2 responses are variably represented with a bias to more severe disease; (iii) changes associated with cell cycle, lipid metabolism, angiogenesis and haematological disturbances are evident in active disease and reduced after treatment; (iv) treated patients, at least over the time period of study, fail to fully return to a homeostatic state. Not surprisingly, each study also identified unique aspects to the transcriptomic profile. A comprehensive cross-organ analysis in the mouse revealed only a limited concordance of DE genes
between spleen, blood and liver and a clear lack of concordance across species [49]. Although such comparative studies require a degree of caution (due to variations in analytical approach), they serve as a reminder of the importance of further research on the pathophysiology of human disease.

**CL pathogenesis:** In contrast to VL, where studies on pathology have focused on systemic sites, CL immunopathology has largely focused on the skin, though many of the changes observed in chronic VL lymphoid tissue may also occur in the lymph nodes draining CL lesion sites. Precise mapping of the nature of the inflammatory lesion and its development over time has been reported in murine models of CL, most notably due to *L. major* infection, but also with *L. mexicana* and *L. braziliensis*, with an emphasis on identifying determinants of host protection (reviewed in [51]). More recent studies spurred on by clinical observations in human *L. braziliensis* infection [52] and the identification of *Leishmania* viruses in metastatic strains of *L. guyanensis* [53] have focused on determinants of excessive host pathology. In *L. braziliensis* infection, a pathogenic role for CD8+ T cells has now been firmly established. At a mechanistic level, highly cytotoxic CD8+ T cells are believed to induce the release of cellular DAMPS that trigger inflammasome activation that perpetuates the inflammatory cascade. These studies provide opportunities for novel forms of immunotherapy to minimise tissue damage [54]. Viral infections are well known as stimulators of the type I interferon response and it has been shown that type I interferons either driven by endogenous symbiont *Leishmania* viruses or through concomitant bystander viral infection can lead enhance the metastatic potential of *L. guyanensis* in mouse models [55]. These data require further substantiation in a clinical setting, however, but also pose questions regarding novel therapeutic approaches.

### 3. Vaccines

The case supporting development of a vaccine for leishmaniasis from a scientific and public health perspective has been made many times elsewhere [56]. Vaccine research has included the full range of techniques for antigen identification (from the use of serology through to computational prediction,
and almost all conceivable vaccine delivery strategies, including cellular vaccination with antigen-pulsed dendritic cells, [58,59] have been explored for one or other form of leishmaniasis in animal models. Barriers to successful development of a vaccine to date have included i) lack of translational funding; ii) lack of correlates of immunity, iii) over-dependence on animal models; and iv) lack of a coherent programme of advocacy. Nevertheless, recent years have seen excellent progress that has brought four candidate vaccines to or near to the clinic. These include a recombinant fusion protein delivered with strong Th1-inducing adjuvants (LEISHF3+ GLA-SE; [60]), a naked multi-epitope DNA vaccine (LeishDNAvax; [61]), an adenovirus-based vaccine (ChAd63-KH; [62]) and a live genetically attenuated vaccine (L. major/ L. donovani centrin [63]). Each has taken a different approach to vaccine antigen identification and vaccine delivery, providing a potential rich environment in the future for understanding the determinants of vaccine-induced immunity in humans.

Prophylactic vaccines by definition are used in non-infected individuals to prevent the development of disease. Hence, immunopathology associated with Leishmania infection does not play a determining effect on vaccine design or efficacy. This does not rule out, however, that a subset of the target population for vaccination in an endemic setting may harbour sub-clinical and undetectable infections (using the diagnostic tools discussed below) that nevertheless have some local pathologic consequence. More likely, however is the scenario whereby co-infections or other intrinsic (e.g. nutrition) or extrinsic (e.g. UV radiation) environmental factors impact on vaccine-induced lymphocyte activation, memory cell generation and/or effector and regulatory cell balance. Due attention should be paid to these possible factors when designing and evaluating future clinical trials.

In contrast, therapeutic vaccines require enhancement of the state of immunity in those already with leishmaniasis pathology. The extent to which ongoing pathology impacts the efficacy of therapeutic vaccines for CL is presently unknown and will require carefully designed clinical trials in which patients can be stratified according to pathologic criteria. In animal models of VL, therapeutic vaccination can overcome the immunosuppressive state induced by VL [64] and post-exposure
prophylactic vaccination in *L. infantum*-infected dogs was shown to reduce progression to symptomatic VL [65]. Prevention of PKDL by vaccination of previously treated VL patients poses an interesting challenge, given that the immune status of treated VL patients is likely to have been improved but not normalised [48]. A clinical trial to assess this approach is in development (clinicaltrials.gov; NCT04107961). Combination studies of vaccines deployed with additional immunomodulators to overcome pathology-induced immune regulation should be actively considered.

4. Diagnostics

As the leishmaniases are characterized by their clinical pleomorphism confirmation based solely on clinical grounds is a challenge. The wide variety of skin and mucosal lesions in CL involves an extensive differential diagnosis. In addition, diseases like malaria, infectious mononucleosis and malignancies, among others, present with fever, hepatomegaly and splenomegaly as does VL, often accompanied by cachexia and malnutrition in chronic courses.

Parasite demonstration by microscopy of Giemsa stained tissue smears is the gold standard in the diagnosis of leishmaniasis; this is not always accessible and has variable sensitivity. Thus laboratory confirmation rates before treating CL can be as low as 5% in some settings, with patients being put on treatment with potentially toxic drugs without receiving a confirmatory diagnosis [66]. For VL invasive tissue sampling is required, usually from bone marrow or spleen and to a lesser extent lymph node. This requires expertise and specialised facilities for managing potential complications, so VL suspects are usually referred to specialised treatment centres for diagnosis. Because of this WHO recommended that a strict case definition for VL, i.e. fever for more than 2 weeks plus hepatosplenomegaly and/or wasting; with this a positive serology enables treatment initiation [67].

**Diagnosis for case management**

A common feature in VL is a polyclonal hypergammaglobulinaemia with a marked increase in serum IgG level, including IgG with specificity for *Leishmania* [68]. Therefore, identifying anti-*Leishmania* specific antibodies is currently the cornerstone of VL diagnosis. Rapid diagnostic tests (RDTs) based
on the recombinant antigen rK39, from a *Leishmania infantum* strain from Latin America, are widely used and they show very good performance in the Indian subcontinent (ISC): 97% sensitivity (95% CI: 90.0 - 99.5), 90.2% specificity (95% CI: 76.1 - 97.7). In eastern Africa the specificity is similar 91.1% (95% CI: 80.3 - 97.3), but the sensitivity drops to 85.3% (95% CI: 74.5 - 93.2) [69]. This inferior performance requires that in eastern Africa it is necessary to include the direct agglutination test (DAT) in the diagnostic algorithm (Figure 2). This is a robust test based on whole *Leishmania* antigen and it has been extensively validated. It cannot be considered an RDT as it requires some degree of laboratory expertise and capacity, and results are obtained after an overnight incubation [70].

The peculiarities of the antibody response against specific *Leishmania* antigens are key in the performance of serological tests. VL elicits a different immune response and different levels of anti-*Leishmania* IgG across regions. rK39-based RDTs are based on a kinesin sequence from an American strain of *L. infantum* (syn. *L. chagasi*), this can explain the different performance of these tests in eastern Africa, where there is a high molecular diversity of the rK39 homologous sequences among regional *L. donovani* strains [71]. Another explanation for this is that there can be a different potency in the immune response, exemplified by the finding that anti-*Leishmania* IgG levels in VL patients from Sudan were significantly lower than in patients from India, independently of the geographic origin of the leishmanial antigen used to assess this [72]. A new RDT, based on the recombinant antigen rK28, may contribute to overcome this problem. The rK28 is a synthetic polyprotein containing rK39 repeats from a Sudanese *L. donovani* strain, flanked by HASPB1 repeats and the HASPB2 open reading frame from an Ethiopian strain [73]. Some preliminary studies claim that rK28 RDTs show better performance in eastern Africa than those using the rK39 antigen; however, data are not definitive, as studies comparing rK28 RDTs with the rK39 RDT IT-Leish (Bio-Rad), which is the one recommended in most eastern African guidelines for VL, are limited. These studies are either non-prospective or use just serum or plasma from well characterized controls and cases (confirmed by parasitology) [74-76]. A prospective large scale evaluation of an rK28 RDT will be conducted by the
AfriKADIA consortium in Ethiopia, Kenya, Sudan and Uganda; results will be available by the end of 2020 [https://www.afrikadia.org/].

Unlike in VL, CL is not characterized by an elevated production of anti-\textit{Leishmania} antibodies, and the level of these is also quite variable between the different forms of tegumentary leishmaniasis. Therefore, antibody detection tests are not useful in the diagnosis of these forms of leishmaniasis. Anti-\textit{Leishmania} antibodies are barely detectable in CL and to some extent these can be detected in ML and DCL but with limited and variable performance [67]. New opportunities for the serological diagnosis of CL may be brought by the detection of anti-α-Gal antibodies, with promising preliminary results for Old World leishmaniasis, nevertheless this field requires further research [77].

In immunosuppressed HIV+ patients, antibody-detection tests have limited sensitivity and cannot be used to rule out VL [78,79]. Also, the detection of total anti-\textit{Leishmania} antibodies is not very helpful in the diagnosis of PKDL or VL relapses, as circulating antibodies from previous VL episodes remain for long periods even after successful treatment [80]. It seems however that exploring specific IgG types can be of help in assessing post-treatment cure and the risk of relapse, as well as in supporting the diagnosis of PKDL. The qualitative and quantitative detection of IgG1 in blood or serum/plasma samples, by means of ELISA or RDT using whole or recombinant rK39 leishmanial antigen, have shown the potential of IgG1 as a biomarker of post-chemotherapeutic relapse, as demonstrated with samples from Sudanese and Indian patients [81-83].

Antigen-detection tests can help in resolving some of the problems described above, these tests should be more specific and can distinguish active from past infections. Most of the approaches used up to date for the detection of leishmanial antigens in VL patients have targeted urine [84-86]. A latex agglutination test has shown a highly variable sensitivity (36%-100%) and specificity (64%-99%) across endemic regions and different groups of patients, including HIV-positive, thus this has not been fully implemented [69]. Different antigen detection ELISA tests have been developed, and they have shown high sensitivity and specificity in a preliminary study using samples from VL patients from different endemic regions, as well as utility in monitoring treatment efficacy [87]. Lack of further evaluation, the need to process the sample and the technical requirements of an ELISA tests
have precluded putting them into routine practice. A more practical approach would be to detect leishmanial antigens in blood or serum/plasma. A prototype immunochromatographic test showed high sensitivity (96%) and specificity (99%) in the diagnosis of VL in Chinese patients using blood or serum [88]; unfortunately, further validation of this test and its results in other settings has not been pursued.

A point-of-care (POC) tests for early diagnosis of CL is much needed to benefit patients and communities, its implementation will reduce morbidity and transmission (where this is anthropogenic). A target product profile (TPP) for a POC for CL was developed by consultation among experts with a consensus that this POC test should target *Leishmania* antigens [89]. A promising option for the point-of-care diagnosis of CL that fits this TPP is a RDT detecting the leishmanial amastigote antigen peroxidoxin, CL Detect™ Rapid Test (InBios International Inc., USA). This test has shown variable performance across endemic regions, but for CL due to *L. major* or *L. tropica* the sensitivity and specificity obtained in different studies in Tunisia, Morocco and Afghanistan is acceptable [90-92].

Nucleic acid amplification tests (NAATs) have shown good performance in the diagnosis of the different forms of leishmaniasis, both in immunodepressed and immunocompetent patients. Polymerase chain reaction (PCR)-based methods have proven that confirmatory diagnosis of VL is feasible using a less invasive sample such as peripheral blood, and with high sensitivity [93]. And this is especially relevant in CL, when chronic lesions have lower parasite loads that cannot be detected by microscopy [94,95]. A limitation to implement PCR-based diagnosis at the POC has been the requirement of well-equipped facilities and trained personnel, as well as the lack of standardisation, limiting the use of these tools to reference laboratories. Nevertheless, NAATs more amenable for use in limited resource settings have been recently developed, a commercial test based on the loop-mediated isothermal amplification (LAMP) of DNA, Loopamp™ Leishmania Detection Kit (Eiken Chemical Co., Japan), overcomes the problem of standardisation. Its performance using peripheral blood (97%-99% sensitivity), as demonstrated in a study in Sudan, enables its inclusion in the VL diagnostic algorithm, reducing the need for invasive tissue aspiration [76]. A diagnostics
evaluation study conducted in Afghanistan informed that an algorithm including CL Detect™ Rapid Test and Loopamp™ Leishmania Detection Kit would have a sensitivity of 93%, minimizing the number of patients that would need to be referred for parasitological confirmation [92]. Other studies evaluating non-commercial LAMP assays have also reported high sensitivities in the diagnosis of CL, VL and PKDL [96]. As such, the diagnosis and management of leishmaniasis would benefit from the implementation of accurate NAATs at the POC.

**Diagnostics in the elimination and post-elimination of visceral leishmaniasis**

WHO aims at eliminating VL as a public health problem in the Indian subcontinent (ISC) by 2020. The implementation of test-and-treat strategies, relying on the use of antibody-detection RDTs has been pivotal in moving towards this target [97]. This effort will need to be sustained in the next few years to avoid resurgence and move towards the interruption of *Leishmania* transmission by 2030 [98]. However, the current diagnostic tools may not be adequate to support this endeavour.

More specific tests will be needed in the near and post-VL elimination phases, since the positive predictive value of antibody-detection RDTs decreases in a context of low endemicity [97,99]. Only VL suspects with symptoms (e.g. fever, splenomegaly) for more than two weeks are tested by the current RDTs, which precludes early diagnosis and treatment of acute cases who remain infectious in the community. Reducing the time between onset of symptoms and diagnosis-treatment has been identified as a key parameter for achieving and sustaining elimination [100]. But current antibody-detection RDTs do not allow this, as they are not specific for acute VL and need to be interpreted along with a clinical case definition [69]. Detection of *Leishmania* antigens may be a more specific approach [101,102,87,88], and these can be detected earlier than anti-*Leishmania* antibodies [86,103]. Thus antigen-detection RDTs would allow diagnosis of VL cases without a time constraint or clinical case definition (e.g. < 2 weeks of fever); as described above several approaches for leishmanial antigens detection in clinical samples have been developed, but for different reasons these have not been implemented. A recent target product profile describes the features of an antigen detection RDT that can be used to assist in VL elimination [104]. Antigen detection tests would also allow diagnosing VL in groups of patients in which antibody detection tests underperform such as HIV-VL.
co-infected patients, relapses, as well as in PKDL. All these cases are gaining relevance in the last stages of the VL elimination in the ISC, as they are an important reservoir of *Leishmania* and are a serious threat to VL elimination [105,106,99].

Diagnosis of PKDL is a key factor in elimination efforts as well as in patient management, for PKDL treatments are long (up to 12 weeks). PKDL diagnosis is challenging as case confirmation requires microscopy and/or molecular tests in skin biopsies. As these diagnostic procedures are difficult to perform, diagnosis in endemic areas is often made clinically [107]. Antibody detection RDTs, which are part of the PKDL diagnosis algorithm in the ISC, have limited value and alternatives such as point-of-care molecular tests with promising performance (e.g. LAMP) should be evaluated [108,99].

There have been few if any studies directly seeking to evaluate the impact of pathology on the design and/or performance of diagnostic tests for VL or CL. Although there may be limitations in translating such knowledge, a consideration of the role of pathology in determining the specificity and sensitivity of current and future tests may be warranted. For example, perturbations in the regulation of antibody responses are linked to disease progression [109] and antibody and antigen half-lives will be affected by changes in plasma or lesion protein binding capacity, glomerular filtration rate (e.g. secondary to immune complex deposition [110]) and or the degree of enteropathy [111]. Hence, each of the parameters highlighted in Fig. 3 will be influenced by the degree of pathology observed during the early pre-clinical, clinical and post-treatment phases of the disease. As above in the case of vaccine efficacy, these pathologic changes might occur directly as a consequence of ongoing or previous leishmaniasis, but equally so may reflect the indirect immunopathological consequence of coinfections or other immune insults.

5. Drugs

There has been considerable progress in the development of treatments for leishmaniasis over the past two decades. A number of new drugs have been registered for VL (miltefosine, the liposomal
formulation AmBisome, paromomycin) with some taking a lead role in disease control and elimination programmes [112,2]. At the same time there is an inadequacy of drugs for CL [113,114]. However, for the first time in history the pipeline of novel oral drugs for the treatment of VL and CL is more promising with five novel compounds for VL [115-117] and a renewed focus to find novel treatments for CL [118,119]. In this section, two aspects of the drug – immune response interaction will be discussed (i) immunosuppression and immunomodulation, and (ii) the impact of immunopathology on pharmacokinetics.

**Immunosuppression and immunomodulation**

Interactions between drugs and the immune response have been known, but poorly understood, since the early 20th century. It still provides a strong focus for research: a recent study on antibiotics that showed how host cell metabolites both reduce drug activity whilst also enhancing host cell phagocytic activity provides an example of such work [120]. For leishmaniasis, as for other infectious diseases, there have been two directions of study: (i) to understand why drugs do not work in patients and disease models and (ii) the development of a strategy to stimulate/modulate the immune response to overcome any immunosuppression or accelerate cure.

Drugs used for clinical treatment of VL have to work effectively in most endemic regions within the context of the immunosuppression provoked by this disease. With the more profound immunosuppression associated with HIV VL co-infections, the standard drugs become significantly less effective and only co-administrations have been shown to improve treatment [121]. The low efficacy of three of the drugs (liposomal amphotericin B, paromomycin and miltefosine) in East African VL [122,123] that are so effective in the Indian subcontinent, has not been explained. Clinical isolates from both regions show similar susceptibility to these drugs within standard in vitro assays, so the clear implication is that host factors (immunology, pathology, pharmacokinetics [PKs]) are the responsible for this difference. The potential importance of PKs has been illustrated by Dorlo and colleagues [124] who have determined different miltefosine exposures in adults and children in East Africa. There are further clinical and experimental observations, one confirming, one confounding: (a) several of the drugs that work effectively as VL treatments in immunosuppressed VL patients (*ibid*) have significant
dependence on cellular immune responses in mouse models of infection, either immuno-suppressed or deficient [125-127], (b) all the drugs, whether immune or non-immune dependent in these studies, have well described interactions with the immune system (see reviews by Dorlo (2012) for miltefosine [128], Cohen (2016) for amphotericin B [129] and Murray (2005) for pentavalent antimonials [130]). It is clear that more detailed studies are required to understand these interactions.

For CL, drugs work within the context of a slow self-cure immune response; exceptions include diffuse cutaneous leishmaniasis (DCL) where there is an absence of an effective cellular immune response and absence of effective treatment. The objectives for drug treatment, and combinations with immunomodulators, are to both reduce the parasite burden and at the same time accelerate self-cure. Much of the area lacks clear clinical data due to the absence of robust clinical studies [113,114].

The central role of the macrophage in the survival of *Leishmania* parasite has been the driving force for most studies on immunomodulation. Have we progressed much further than a statement from Bernard Shaw’s play Doctor’s Dilemma (1906) “There is at bottom only one genuinely scientific treatment for all diseases, and that is to stimulate the phagocytes”? BCG, which has well characterised stimulatory interactions with macrophages [131], was the immunodulatory component of treatment with pentavalent antimonials and the recommended treatment for CL in Venezuela [132]. It was also used in combination with alum and pentavalent antimonials in clinical trials for the treatment of post-kala-azar dermal leishmaniasis [133]. Bacterial cell wall components, muramyl dipeptide and trehalose dimycolate have also been used in clinical studies [134] on CL and experimental VL [135]. Based on clearer understanding of cytokine-macrophage interaction both IFNγ [136] and GM-CSF [137] have been used in the treatment of both VL and CL. The rationale behind the potential application of GM-CSF for CL is threefold: (a) GM-CSF can promote proliferation, activation and differentiation of various myeloid cells (macrophages, dendritic cells and neutrophils) and their progenitors, (b) clinical trials have reported increased wound healing of diverse wound types (including CL) upon topical GM-CSF application, and (iii) CM-CSF is able to modulate the tissue immune response by increasing the levels of the anti-inflammatory cytokine IL-10.

Randomized controlled trials in Brazil have indicated that GM-CSF both after oral [138] and topical [139] administration reduced the healing time of CL lesions when given in combination with
intravenous antimonials. In addition to its anti-inflammatory role, IL-10 also inhibits IFN\(\gamma\) induced macrophage killing of \textit{Leishmania} and Murray and colleagues [140] demonstrated in a model of VL that blockade of IL-10 using anti-IL-10R antibodies resulted in significant increase in antimonial drug activity. A clinical trial to evaluate combining AmBisome with anti-IL-10R antibodies in VL patients was proposed but later withdrawn due to unavailability of the required antibody (clinicaltrials.gov; NCT01437020).

Immunomodulator molecules that are also TLR agonists are being pursued for CL treatment. For example, CpG-D35, a D-type CpG TLR9 agonist, is currently undergoing Phase-I clinical trials after showing it was able to curtail lesion development upon administration to macaque monkeys infected with \textit{L. major} [141]. Earlier studies indicated that this TLR9 agonist induces maturation of plasmacytoid dendritic cells and secretion of IFN\(\alpha\) and \(\gamma\) without direct activation of B cells [142]. A small molecule, the imidazoline imiquimod a TLR7 agonist, was shown by Matlashewski and colleagues to stimulate macrophage functions including signal transduction and NO production in experimental models, sufficient to kill intra-macrophage amastigotes [143]. Subsequent clinical studies using imiquimod as a topical adjunct therapy to pentavalent antimonials were inconclusive. Three of these randomized controlled trials (RCT) [144-146] showed no significant increased cure rate for the combination of imiquimod plus pentavalent antimonials versus antimonials alone. Efforts continue to find more active analogues [147]. A different approach was adopted by Smith et al (2000) with the small molecule, tucaresol (in clinical trials for sickle cell disease), an orally bioavailable compound that enhances T-helper-cell activity, with the induction of increased IL-2 and IFN\(\gamma\) levels in mice and humans and provides a costimulatory signal between macrophages and T-cells [148]. In mouse model of VL tucaresol gave a 60% reduction in parasite burden similar to that achieved by INF\(\gamma\) in the same model.

Other recent approaches have also added considerably to understanding drug-immune interactions and possible new routes forward to immunomodulation and successful cure. Long-term clinical research on South American CL [54] showed that NLRP3 inflammasome is activated by CD8\(^+\) T cell-mediated cytotoxicity and drives disease progression. Based on these observations, the group studied a number of small molecule inhibitors of the inflammasome, for example, MCC950 and the diabetes drug
glyburide. In mouse models of CL, they showed that mice treatment with compounds that inhibit NLRP3 inflammasome activation, MCC950 or glyburide, failed to develop the severe disease seen in untreated mice. In VL, more detailed knowledge of the immune response has also been exploited. Based on knowledge of the immunopathology of the spleen, the receptor tyrosine kinase inhibitor sunitinib maleate was shown to induce restoration of splenic microarchitecture. Although this drug did not possess inherent anti-leishmanial activity, it afforded a significant dose sparing effect for subsequently administered antimonials [149].

CL is also characterised by local inflammation. A combination of the anti-inflammatory agent, pentoxiphylline, with antimonial drugs has been shown to be an important adjunct therapy for some forms of CL and mucocutaneous leishmaniasis. In Brazil, randomized controlled trials (RCTs) have shown a synergetic effect of pentoxifylline in conjunction to antimonial therapy in ML patients [150] whereas this was not observed in patients suffering CL [151,152]. The difference in pentoxifylline efficacy as part of a combination therapy between New World CL and ML might be due to distinct differences in cytokine and macrophage populations present in the lesion [153] – an observation that merits further exploration as it could help elucidate immunomodulatory processes. It is worth noting that when tested in Old World CL, the combined approach of pentoxifylline with an antileishmanial agent showed enhanced healing in comparison to antileishmanial treatment only [154]. For further information on the activity of immunomodulators, see reviews by Dalton and Kaye (2010), Taslimi et al., (2018) and Adriaensen et al (2017) [149,155,156].

PK/PD relationships, immunopathology in drug monitoring and targets for host directed therapy.

Besides the direct involvement in the disease progression and outcome, the immunopathology also impacts drug pharmacodynamics (PD) and/or pharmacokinetics (PK). Impact on the latter has been shown in mice with CL, where the presence of parasites and the associated inflammation in the skin disturbed the cutaneous barrier function and physiology [157,158]. Interestingly, the permeation of hydrophilic compounds increased to a higher extent in comparison to lipophilic compounds which was hypothesized to be in part due to the inflammatory hydrophilic environment in CL skin. Whilst these
findings are particularly important for topical drug administration where the drug is applied locally, increased drug levels were also observed in *Leishmania*-infected skin upon systemic drug administration [159,160] where they were associated with enhanced capillary leakiness and increased macrophage infiltration both of which could potentially be attributed to local inflammation. How *Leishmania* infection alters the skin tissue microenvironment in humans is yet unknown and is subject to further research.

During the development of VL, the liver, a major drug metabolism site for drugs, undergoes morphological and functional changes; hence, it seems evident that VL impacts drug pharmacokinetics. In VL infected mice for example, the no-observed-adverse-effect level of miltefosine is approximately 25 mg/kg (QD, for 10 consecutive days), whereas in experimental CL 35mg/kg is still well tolerated (experimental data, unpublished). Other studies also demonstrated a deterioration of the capacity of host hepatic microsomal membranes to metabolise xenobiotics in VL infected mice and hamsters [161,162]. Whilst *in vitro* studies suggest no involvement of cytochrome P450 isoenzymes in the metabolism of miltefosine [163], the enzymes are involved in the metabolism and clearance of many xenobiotics and reduced activity could potentially lead to reduced drug efficacy (metabolites are active) or toxicity when increased amounts linger around in the systemic circulation. For amphotericin B, a significantly lower concentration of drug was found in liver and spleen of VL-infected mice as compared to uninfected mice, which was hypothesised to be due to a reduced phagocytic activity in infected macrophages [155, 157]. These findings were not investigated in humans but it seems plausible that the PK of certain drugs is altered by the disruption of the liver function as observed in VL. Pathology is also known to affect drug ADME (absorption, distribution, metabolism and excretion) processes by alterations in protein binding which is important given that hypoalbuminemia is observed in both human and experimental VL. Low serum albumin levels have been associated with alterations in the degree of protein binding of highly protein-bound drugs such as miltefosine and thus could potentially impact its PK-PD relationship [164,165].

Another phenomenon which remains unexplored in VL patients and experimental models is the influence of granuloma formation on drug PK and thus efficacy. In tuberculosis, a granuloma forms a
shielded lesion compartment that harbours bacteria and makes it difficult for drugs to permeate [166]; hence, the long and intense treatment regimen composed of a cocktail of four different drugs. Whilst difference in the extent of granulomatous inflammation are clear between leishmaniasis and TB [35], the altered local cellularity of a granuloma may nevertheless be of importance.

Of further importance is the impact of the pathology on drug PD which involves processes best reflected by “what the drug does to the body”. Most chemotherapeutics exert their activity by stimulating or inhibiting enzymes that are involved in pathways essential for parasite survival. Recent research describes the presence of different metabolically active parasite populations in CL lesions [8]. Kloehn et al (2015) measured semi-quiescent L. mexicana parasites in non-healing CL lesions in mice [7]. This reduced metabolic state is characterised by low transcription rates and protein turnover and might thus contribute to a reduced drug efficacy.

**Summary**

Here we have illustrated how the complexity and variation of human immune responses and immunopathology and the different host interactions to twenty Leishmania species has an impact upon the effectiveness of vaccines, diagnostics and drugs (Fig. 1). Although rodent models have proved to be a useful tool for experimental studies on these interactions, well described limitations of (a) absence of models for important species (L. aethiopica, L. tropica, L. braziliensis), (b) differences between mouse and human immune responses (for example, to TLR agonists, [167,164] and serological indicators, and (c) differences in pharmacokinetics between mouse and humans [168,165], underpin most of this work. The application of transcriptomics, genomic data bases and methodologies to determine pharmacokinetics in infected tissues (not just plasma) in future studies in human subjects must now be used to inform future work so tools for treatment, control and elimination can be used with more understanding and effectiveness.
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Conflicts of Interest

The authors have no conflict of interest.

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<table>
<thead>
<tr>
<th>Main <em>Leishmania</em> species</th>
<th>Clinical presentations</th>
<th>Main Reservoir</th>
<th>Countries or regions found</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. donovani</em></td>
<td>VL, PKDL</td>
<td>Humans</td>
<td>India, Bangladesh, Ethiopia, Sudan, South Sudan</td>
</tr>
<tr>
<td></td>
<td>Fatal within two years (VL). PKDL lesions may follow drug treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. tropica</em></td>
<td>CL, LR, rarely VL</td>
<td>Humans</td>
<td>East Mediterranean, Middle East, North Africa</td>
</tr>
<tr>
<td></td>
<td>Self-healing within 12-18 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. aethiopica</em></td>
<td>CL, DCL, DsCL</td>
<td>Hyraxes</td>
<td>Ethiopia, Kenya</td>
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<tr>
<td></td>
<td>Self-healing (except DCL) within 2-5 years.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. major</em></td>
<td>CL</td>
<td>Rodents</td>
<td>North Africa, Middle East, Central Asia, West Africa</td>
</tr>
<tr>
<td></td>
<td>Self-healing within 3-12 months.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. infantum</em></td>
<td>VL, CL</td>
<td>Dogs, humans</td>
<td>China, South Europe, Brazil, South and Central America</td>
</tr>
<tr>
<td>[<em>L. chagasi</em>]</td>
<td>Can be fatal within two years (VL), most adults asymptomatic</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. braziliensis</em></td>
<td>CL, MCL, DCL, LR</td>
<td>Dogs, Humans, Rodents</td>
<td>South America</td>
</tr>
<tr>
<td></td>
<td>May self-heal within six months.</td>
<td>&lt; 5% progress to MCL.</td>
<td></td>
</tr>
<tr>
<td><em>L. amazonensis</em></td>
<td>CL, DCL, DsCL</td>
<td>Marsupials, Rodents</td>
<td>South America</td>
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<td></td>
<td>Not well described.</td>
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<td><em>L. guyanensis</em></td>
<td>CL, DsCL, MCL</td>
<td>Marsupials, Sloths</td>
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<tr>
<td></td>
<td>May self-heal within six months.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. mexicana</em></td>
<td>CL, DCL, DsCL</td>
<td>Rodents, Marsupials</td>
<td>South America</td>
</tr>
<tr>
<td></td>
<td>Often self-healing within 3-4 months.</td>
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<td></td>
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</tbody>
</table>

VL= Visceral Leishmaniasis; PKDL = Post-Kala-Azar Dermal Leishmaniasis; CL = Cutaneous Leishmaniasis; MCL = Mucocutaneous Leishmaniasis; DCL = Diffuse Cutaneous Leishmaniasis; DsCL = Disseminated Cutaneous Leishmaniasis; LR = Leishmaniasis Recidivans
Figure 4
LEVELS OF TARGET ANALYTES IN VL

- Onset of symptoms
- DNA / Antigen
- IgG1
- Total IgG
- Detection threshold

TIME FROM INFECTION

Months

Years

Detection threshold
Clinical VL suspect
Fever ≥ 2 weeks with splenomegaly or wasting
(rule out malaria)

NEW case*
Perform a RDT (rK39**)

Positive
VL treatment

Negative
DAT

Positive
≥ 1:3200
VL treatment

Borderline 1:400 – 1:1600
Re-test in 1 week with DAT OR perform tissue aspirate microscopy

Negative 1:100 – 1:200
Search for other diagnosis and treat or refer

If DAT not available

DAT

Positive
VL treatment as relapse

Negative
Search for other diagnosis and treat or refer

Previous VL case
Perform tissue aspirate microscopy
(lymph node/ bone marrow/spleen)

Positive
VL treatment

Negative

Microscopy

VL diagnostic algorithm. Source: National guidelines from Ethiopia, Kenya, Sudan and Uganda

* Parasitological diagnosis may also be conducted in tertiary hospitals and research centres

**National guidelines in Sudan also consider the use of rK28 RDTs
Key facets of leishmaniasis pathology

- Macrophage function
- Granulomatous Inflammation
- Immune regulation and metabolism
- Tissue remodeling and damage

Vaccines
- Prophylactic
- Preventative
- Therapeutic

Diagnostics
- Antibody detection
- Antigen detection
- DNA

Drugs
- ADME
- PD
- Immunomodulators
Response to Reviewers

We thank the reviewer for comment and corrections. We have:

Added 5 new references in the immunology/vaccine sections which refer to the DC issues raised by the reviewer. Our aim was not to produce a full review on all aspects on immune regulation but to focus on issues indicated in the title of the manuscript.

We have added an additional paragraph on molecular diagnostics with an additional 5 references; this does add to the manuscript.

Apologies that the Table 1 was omitted from the first submission. This was the corresponding author’s error.