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Leishmania Vaccine Development: Exploiting the Host-Vector-Parasite Interface

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Summary

Visceral leishmaniasis (VL) is a disease transmitted by phlebotomine sand flies, fatal if untreated, and with no available human vaccine. In rodents, cellular immunity to *Leishmania* parasite proteins as well as salivary proteins of the sand fly is associated with protection, making them worthy targets for further exploration as vaccines. This review discusses the notion that a combination vaccine including *Leishmania* and vector salivary antigens may improve vaccine efficacy by targeting the parasite at its most vulnerable stage just after transmission. Furthermore, we put forward the notion that better modeling of natural transmission is needed to test efficacy of vaccines. For example, the fact that individuals living in endemic areas are exposed to sand fly bites and will mount an immune response to salivary proteins should be considered in pre-clinical and clinical evaluation of leishmaniasis vaccines. Nevertheless, despite remaining obstacles there is good reason to be optimistic that safe and effective vaccines against leishmaniasis can be developed.

Keywords

Leishmania; vaccine; sand fly; saliva; adjuvant

1. Introduction

Leishmaniasis is a complex of parasitic diseases caused by several different species of *Leishmania.* The parasites are transmitted to and from animals or humans through the bite of infected phlebotomine sand flies leading to an intracellular infection in the mammalian host, predominantly within macrophages (Figure 1) (1, 2). Together with parasites and other

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parasite-derived molecules, such as the promastigote secretory gel (PSG) (3), an infected sand fly injects saliva, composed of biologically active components, mostly proteins, with anti-hemostatic and immunomodulatory properties that help the insect to acquire a blood meal and consequently benefits *Leishmania* parasite establishment in mammalian hosts (4).

The main clinical manifestations of the disease include visceral leishmaniasis (VL), also known as kala azar, cutaneous leishmaniasis (CL), and mucosal leishmaniasis (ML). Post-kala-azar dermal leishmaniasis is considered a complication of VL in areas where *Leishmania donovani* is endemic. Fortunately for vaccine development the parasite species are highly related antigenically, raising the potential for developing cross-protective vaccines. In addition, some of the vector salivary gland components are also conserved among vector species.

VL, the most serious form of leishmaniasis, is primarily caused by parasites of the *Leishmania donovani* complex, including *L. donovani* and *L. infantum* (5, 6). VL is characterized by fever, wasting, anemia, hepato-splenomegaly, and a depressed immune response (7). VL has a very high fatality rate in the absence of treatment. After malaria, VL is the second most common parasitic cause of death and is prevalent in 47 countries with about 200 million people at risk and an estimated annual incidence of approximately 500,000 cases (8, 9). In South Asia and East Africa, VL is anthroponotic and caused by *L. donovani* (10). In the Mediterranean region, and in Central and South America, the disease is zoonotic and caused by *L. infantum* (= *L. chagasi*) where the main reservoir is dogs (11). Recently, cats and leporids have also been considered as possible alternative reservoirs of VL in certain foci in the Mediterranean region (12). Ninety percent of VL cases occur in five countries – India, Bangladesh, Nepal, Sudan, and Brazil – most often in remote regions without ready access to medical care. In the state of Bihar where more than 90% of all the cases in India are reported, high prevalence of asymptomatic persons and clinical VL cases has led to increased transmission of VL (8).

In this opinion piece, we present an additional consideration for vaccine development, i.e. the inclusion of vector components and the relevance of immunity to vector components in endemic populations.

2. Development of a Vaccine for Leishmaniasis

Efforts to develop a safe, effective, and practical vaccine for one or more leishmanial diseases have been driven by a largely unmet need. Measures such as vector control by insecticide spraying or impregnated bed nets have been insufficient, impractical, or difficult to sustain. Adding to the problem are issues with the drugs used to treat VL that are toxic (pentavalent antimonials, amphotericin B), or expensive (AmBisome) (8, 13–15). Similar issues exist for CL, particularly for the virulent *L. braziliensis* (CL, ML) and *L. tropica* (CL) (16).

Development of effective defined vaccines for the leishmaniases should be possible for the following reasons:

- Unlike many other protozoa, *Leishmania* do not undergo significant antigenic variation.
- *Leishmania* have a single host cell, the macrophage, although it interacts with neutrophils and dendritic cells. There are ample immunologic data on enhancing macrophage killing of pathogenic organisms.
- A single morphologic form, the amastigote, is associated with pathology in the mammalian host.
- Although there are many distinct species of *Leishmania*, most pathogenic species share many important antigens.
- Safe and effective adjuvants that preferentially induce T_H1 responses are now available.
- Both parasite and vector proteins may have protective capabilities

2.1 Brief History of Vaccination

There is evidence for natural immunity in human leishmaniasis. Sero-epidemiological studies indicate that the majority of infections with *L. donovani* or *L. infantum*, the causative agents of VL, remain asymptomatic with natural immunity to disease being the norm rather than the exception (7, 17, 18). Individuals cured from certain forms of CL are resistant to subsequent development of CL disease (though this is not true for all forms, particularly CL caused by *L. braziliensis*) (19). There are clearly parasite, as well as host factors that may influence the development of immunity.

The history of leishmaniasis vaccine development and a description of tried vaccine candidates have been reviewed (20). Much as in the case of small pox, early attempts at vaccination against CL involved the use of live parasite inoculation, leading to self-healing lesions and subsequent immunity. However, this approach was only used for CL, particularly *L. major* (21). Attempts to modernize the approach included using killed *Leishmania* preparations, with only partial and sporadic success in clinical studies (22–25). Experience, generally failures, with developing CL vaccine candidates has led scientists to question how to best mimic natural infection, and indeed how to do so for not only different forms of CL but for VL as well. One approach is obviously to continue efforts on attenuated live vaccines (26, 27), including genetic knockout strains (28), but manufacturing and regulatory issues will likely add additional barriers to the development of a commercially viable vaccine. Despite setbacks, past vaccine studies have been instructive. In this article, we present evolving concepts; which may lead to practical vaccine approaches that take aspects of natural infection into consideration.

2.2 New Approaches to Vaccine Development: Antigen Components

Immunity to the intracellular *Leishmania* parasites appears to be dominated by appropriate $CD4^+$ T cell responses, particularly those with T_H1 dominant profiles, characterized by the production of IFNγ, IL-2 and TNFα by polyfunctional CD4+ T cells (29–31). Negative effects on immunity to leishmaniasis have been associated with excess production of IL-10 and TGF-β (32–36). Understanding the positive and negative aspects of immunity are

important considerations for vaccine development, including both antigen and adjuvant selection. Several parasite proteins have been evaluated as vaccine candidates in a variety of animal models, including mice, hamsters, dogs, and non-human primates. A summary of *Leishmania* antigens tested in experimental studies was recently reviewed (20).

Several *Leishmania* protein antigens induce potent antibody and T cell responses, yet it is clear that they are unable to induce protection in animal models, regardless of adjuvant formulation or delivery method used. Indeed, it appears that the majority of immunodominant parasite components, such as the repeat antigens represented by k39 (37), are not able to induce protection.

It has been appreciated that phlebotomine sand fly vectors of *Leishmania* can greatly affect the immune response to the parasite as well as the development of disease (3, 4, 38–40). This has rarely been considered in vaccine development. Two important paradigms establishing the influence of vector-derived factors on transmission and disease progression of leishmaniasis should be of interest to vaccinologists. The first concerns the heightened virulence of *Leishmania* transmission by vector bites resulting in enhanced disease pathology. Currently, this is mostly attributed to the immunomodulatory properties of saliva and the PSG (3, 4, 40). Other mechanical and behavioral factors such as the site of parasite deposition and altered feeding behavior of infected flies (41) may also contribute to the observed virulence of vector-transmitted leishmaniasis. Importantly, this virulence overrides protection provided by vaccines tested against needle-injected parasites (42) revealing a weakness in current preclinical evaluation of the efficacy of most vaccines. The second paradigm involves the use of such vector-derived factors to protect against *Leishmania* infections. Indeed, immunity generated against salivary proteins or PSG protected rodents against vector-transmitted leishmaniasis (3, 4, 40, 43) demonstrating their potential as vaccines against leishmaniasis.

The concept of vector salivary proteins as vaccines against human leishmaniasis has been gaining traction in recent years. Evidence to date indicates that salivary molecules inducing a T_H1-delayed type hypersensitivity response in immunized animals create an adverse immunological environment at the bite site that impacts the co-deposited parasites, controlling disease and promoting *Leishmania*-specific immunity (Figure 2). Importantly, there is no cross reaction between vector salivary proteins and parasite antigens. The immunological environment (T_H1) developed at the bite site to the salivary protein promotes a protective T_H1 response against the parasite (Figure 2). These sand fly salivary proteins have emerged as candidates for a vaccine or components of a vaccine against leishmaniasis. Table 2 lists the three most promising candidates to date: **PdSP15**, a 15 kDa salivary protein member of the family of small odorant binding proteins from *P. duboscqi* (4), the vector of *L. major* in Sub-Saharan Africa, as a candidate antigen against cutaneous leishmaniasis (39); **LJM19**, an 11 kDa salivary protein with unknown function, and **LJL143**, a 38 kDa salivary protein with anti-coagulant activity (44), both from saliva of the sand fly *Lutzomyia longipalpis,* the vector of *L. infantum* in Latin America, as candidates against visceral leishmaniasis (38).

PdSP15 protected non-human primates against vector-transmitted parasites (39). Rhesus macaques immunized with PdSP15, a major component of the saliva of this insect, showed a significant reduction in lesion pathology, a decrease in parasite number, and the induction of *Leishmania*-specific immunity. Importantly, individuals from a CL endemic area in Mali recognized the PdSP15 salivary protein suggesting this molecule is also immunogenic in humans (39). Mice immunized with PpSP15, the homologue of PdSP15 in *Ph. papatasi*, the vector of *L. major* in the Middle East and North Africa, were also protected against CL (45). PdSP15 also shares homology with PsSP15 from *Ph. sergenti*, a principal vector of *L. tropica* (39) and as such is a good candidate antigen for a vaccine against Old World CL. LJM19 protected hamsters against the fatality of VL caused by *L. infantum* (38) and against CL caused by *L. braziliensis* infection when challenged together with *Lu. Longiplapis* saliva (46). LJL143 is immunogenic in dogs, was detrimental to the parasites *in vitro* (47). Importantly, these salivary proteins are mostly unique to sand flies and do not have human homologues (39, 48).

2.3 Vaccine Development: Inclusion and Selection of Adjuvant

Clinical grade adjuvants that induce preferential and durable T_H1 responses in experimental animal models and in humans are now available. Recombinant proteins often induce only weak T cell responses without the inclusion of adjuvant. Distinctively, salivary antigens induce a robust T cell response in mice without adjuvant (49). Specific adjuvant molecules may directly activate innate immune receptors, for example, Toll-like receptors (TLRs) (50, 51). Other formulation systems may include both delivery and immunostimulatory components. Thus, adjuvants may be broadly classified into three groups of delivery systems: immunomodulatory molecules, particulate formulation and combinations of the former two classes (combination systems) (Table 3).

In leishmaniasis vaccine development, a synthetic toll-like receptor 4 (TLR-4) ligand, Glucopyranosyl Lipid A (GLA) is in clinical development (52). When formulated in a stable oil-in-water nano-emulsion (SE), the GLA-SE adjuvant system induced antigen-specific T_H1 immune responses that are associated with vaccine or protective efficacy in several animal models of infection, including tuberculosis, malaria, and influenza (53–55).

A clinical study that evaluated LEISH-F3, a fusion of nucleoside hydrolase (NH) and sterol 24-c-methyltransferase (SMT), combined into a fusion construct, combined with GLA-SE was safe and immunogenic. T cell cytokines and T_H1 biased Ab responses were seen in subjects immunized with protein/adjuvant, but not protein alone, demonstrating the need for adjuvant (56). This result, along with the results of several clinical studies with GLA-SE in nearly 1000 human subjects demonstrates the potential for a safe and efficacious adjuvant that can practically be applied to development of a human vaccine against leishmaniasis. This, along with the potential of using a sand fly protein as adjuvant, perhaps alone or in combination with GLA-SE, opens exciting and practical development opportunities.

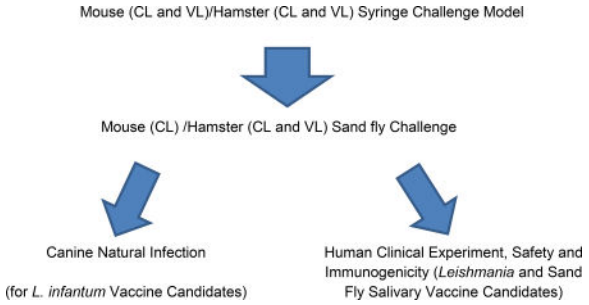
2.4 New Approaches to Vaccine Development: Formulating Parasite and Vector Salivary Antigens

Considering that both parasites and salivary proteins are co-deposited at the bite site, and that immunity to a sand fly salivary protein promotes protective immunity against the parasite in rodents and non-human primates (39, 45), it is possible that a combination of antigens from different sources may be complementary. Recently, a study provided evidence that combining a salivary molecule and a recombinant *Leishmania tarentolae* parasite confers a stronger protection against CL compared to the efficacy of each antigen alone (57). For this combination approach, the best immunization strategy consisted of priming with a sand fly salivary antigen and boosting with the salivary antigen and *L. tarentolae* (57). It is likely that the salivary antigen rapidly recalled an immune response to itself during the boost resulting in a T_H1-type environment against *L. tarentolae* parasites that favored the development of a protective immunity (57). Importantly, the immunity generated against a salivary protein and a *Leishmania* antigen is likely to be amplified when the immunized animals encounter an infective bite. Additionally, this immune response will be localized to the skin interface when the parasites are at their most vulnerable stage, in low numbers and transitioning from the insect to the vertebrate host.

2.5 New Paradigms for Vaccine Design and Evaluation: Modeling Natural Transmission

The use of challenge models that mimic natural transmission has been a neglected aspect of vaccine development for vector-borne diseases. Nevertheless, developing such models is vital to vaccine assessment. In addition to the pathogen itself, a vector inoculum is complex comprising a multitude of vector-derived factors of pathogen and vector origin. In leishmaniasis, the infected sand fly salivates into the bite site in the skin and egests molecules alongside the parasites that modulate the bite site and participate in enhancement of disease (3, 4, 40). Sand flies are telmophagic feeders that lacerate skin capillaries creating a shallow pool of blood and lymph mixed with tissue into which the parasites are deposited. The site of parasite deposition in the skin, the first barrier to infection and an immunologically rich compartment, most likely influences the nature of the early host immune response. This response is further modulated by vector-derived factors that are egested by the fly into the bite site together with the parasites. These include a plethora of salivary proteins to facilitate feeding (58). Studies, reviewed in (4, 59) have established the immunomodulatory properties of several of these proteins, all of which promote *Leishmania* establishment. The infected sand fly also egests a proteophosphoglycan-containing PSG that modulates the environment to the benefit of the parasites (3). Additional non-biological factors such as the modified persistent feeding behavior of infected flies (41) also contribute to the complexity and virulence of a vector-challenge. We are therefore proponents of incorporating a vector-challenge study at the final stages of preclinical testing of a vaccine. The need for this additional selection step has been validated by the failure of a vaccine considered efficacious after a needle-initiated infection when challenged by infected sand flies (42). We have developed vector-transmission models for both CL (39, 60) and VL (61). These models provide a more stringent evaluation of vaccines and a more accurate readout of their expected performance under field conditions. We are currently using these models to prioritize promising vaccines for further development.

Proposed Sequence of Testing Vaccine Candidates—



2.6 Clinical Development: A New Strategy to Demonstrate Vaccine Efficacy in the Field

A vaccine cannot be useful if not used, not used if not approved, and not approved without demonstration of safety and efficacy in field studies. Following research efforts to demonstrate pre-clinical rationale, early clinical studies may involve, for example antigen and adjuvant dose finding to optimize safety and immunogenicity. Such studies may be critical, as recent studies have led to an improved understanding of how to increase potency and efficacy of adjuvanted proteins by lowering the dose, a concept described in leishmaniasis more than 20 years ago (62). We have recently demonstrated that a lower dose (2ug) of a recombinant adjuvanted tuberculosis vaccine candidate was more potent than a higher dose (10ug) when used to immunized *Mycobacterium tuberculosis*-exposed individuals (Coler et al., unpublished),

Classically, testing vaccine candidates is performed in naïve animals. However field testing of VL vaccine candidates will likely be conducted in adults in endemic areas, and these individuals have been exposed to vector salivary proteins through sand fly bites. Following natural challenge via a sand fly bite, the parasites will most likely encounter a site that is already primed to salivary proteins as well as by the injected vaccine. The same applies to a vaccine that includes a salivary molecule where the salivary antigen injected during vaccination will encounter a site naturally primed to sand fly salivary proteins. In exposed populations, therefore, the first injection of a salivary antigen may act as a boost of a preexisting immune response. Even if testing solely a *Leishmania* antigen, the immunological environment will be different (from naïve animals or naïve healthy individuals) due to the immune response to salivary proteins induced by a sand fly bite (in sand fly exposed individuals in endemic areas). The presence of immune responses to sand fly salivary proteins in humans living in endemic areas is well documented (4). Exposure to sand fly bites is known to generate specific antibodies to sand fly salivary proteins (63–65) but more importantly individuals experimentally or naturally exposed to sand fly bites develop a cellular immune response to these salivary proteins (66, 67). It was recently shown that a T_H1 response at the bite site was developed in individuals from endemic areas in Mali after exposure to small number of uninfected sand flies (68) suggesting that the immunological site for any given vaccine to be tested has to be considered also in the context of the immune response in the skin to vector salivary proteins.

These scenarios are often neglected when designing or testing vaccines against leishmaniasis and may be also of consequence for other vector-borne diseases including malaria and

dengue. Using animal models previously exposed to vector bites as part of pre-clinical testing may provide a more stringent approach to vaccine evaluation and also provides insights into the early immunological events following an infection event in vaccinated animals. In addition to previous exposure to vector salivary proteins, individuals living in endemic areas may harbor subclinical *Leishmania* infections. The prevention of disease in individuals with asymptomatic infections raises an interesting vaccine development issue, as rather than inducing de novo T cell responses, a vaccine candidate may act as a boost to an immune response primed by asymptomatic infection. This may affect the choice of adjuvant for example, as well as dose of both antigen and adjuvant components.

Using VL as an example, the desired effects of a vaccine would be to prevent clinical disease and to prevent or decrease infection to reduce the human reservoir. Demonstrating prevention of infection may be possible in areas with high rates of transmission, but will require surveillance with sensitive and specific tools to monitor asymptomatic infection and progression from negative to positive during one or more transmission seasons (69, 70). Although effective VL diagnostics for detecting disease have been widely available for years (71–76), only recently has the promise of sensitive and specific tools for monitoring early and low level infection been demonstrated (77).

Rather than attempting to demonstrate prevention of infection during initial stages of vaccine development, a more practical approach to evaluating VL vaccines will be to perform clinical studies in individuals with latent *L. donovani* infection to prevent progression to disease. In India and Bangladesh, it has been estimated that 5–7% of recently infected individuals may progress to acute VL within 12–18 months. This raises the possibility of demonstrating vaccine efficacy in relatively small clinical studies, using development of disease (and potential stabilization or decrease of parasite replication in asymptomatic individuals) as an endpoint(s).

Regardless of the approach used to evaluate vaccine efficacy, vaccine recipients are likely to have been exposed (repeatedly) to sand fly proteins or to both sand fly and parasite proteins, and these factors should be considered in the approach to prevention of infection and to prevention of disease progression, respectively, in pre-clinical modeling.

3. Expert commentary and five-year view

There is every reason to be optimistic that safe and effective vaccines against leishmaniasis can be developed. Many of the developmental hurdles can be overcome with a transition from first generation vaccines to defined vaccines that induce long-lasting protection. As we learn more about the appropriate interplay between host and parasite, host and vector, vector and parasite we should be able to design safe and effective vaccine candidates that can be delivered at low cost. Critical to this is, for the first time, availability of a safe and effective T cell adjuvant formulation not controlled by big pharmaceutical companies that can be produced in large volume at low cost. Sand fly components with both antigen and adjuvant activity have been identified and are now undergoing process development for clinical testing, which may be performed in conjunction with other promising clinical stage vaccine candidates, such as Leish F3. Perhaps of critical importance will be the design of clinical

trials that will enable a more direct and affordable path toward demonstration of efficacy and ultimate approval. These trials will take into account the realization that we will be in most cases boosting, not priming, an immune response capable of preventing disease development in healthy individuals previously exposed to both parasite and vector. Identification of these individuals is now feasible with new diagnostic tools, enabling targeting of a vaccine to the most susceptible population.

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Abbreviations used

IFNγ	interferon gamma
IL	interleukin
TLR	Toll-like receptor
VL	visceral leishmaniasis
T_H1	T helper 1
PSG	Promastigote secretory gel

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Reference annotations

* Of interest

** Of considerable interest

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Key Issues

- Leishmania delivered into the skin of humans by sand bites are accompanied by immunomodulatory vector-derived factors forming a complex infectious inoculum that enhances the virulence of transmitted parasites. Importantly, the immune response to vector-derived factors has a negative impact on parasite establishment.
- Certain Leishmania and sand fly salivary antigens induce a cellular immune response that protects animals against leishmaniasis. Combining antigens form these two sources may produce a more effective vaccine as recently demonstrated in rodent models.
- Leishmania vaccines are tested in naïve animals neglecting the fact that individuals living in endemic areas are constantly exposed to sand fly bites. Potentially, these individuals may react differently to a Leishmania vaccine as they concurrently mount an immune response to vector saliva.
- New models for testing Leishmania vaccines are needed that account for virulence of natural transmission as well as pre-existing confounders including an underlying immunity to vector saliva in target populations.

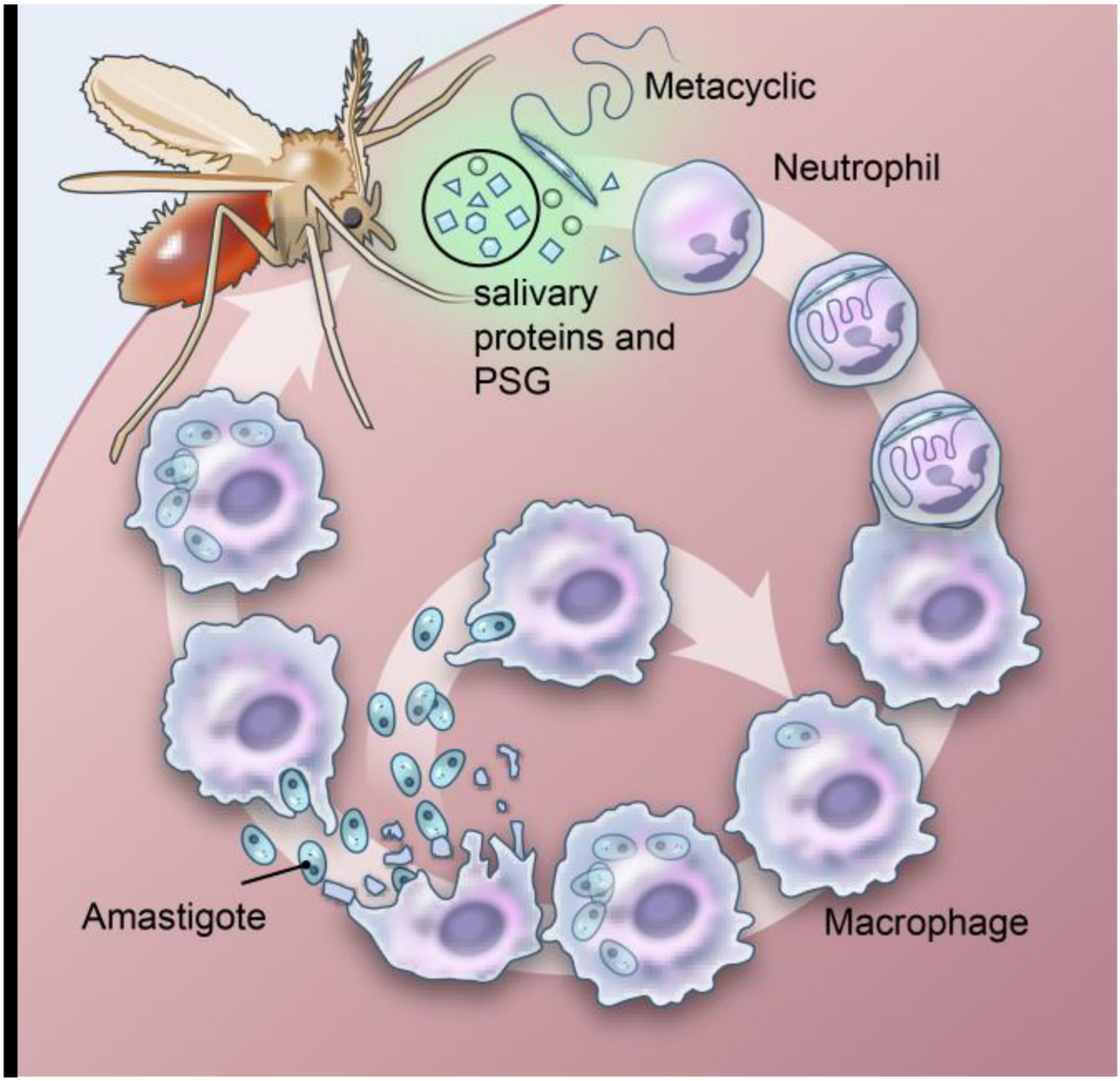

Figure 1.

Leishmania species are inoculated into the skin together with vector-derived factors after an infected sand fly bite where they infect macrophages and form skin lesions (cutaneous forms) or migrate, typically to the spleen, liver, and bone marrow (visceral forms).

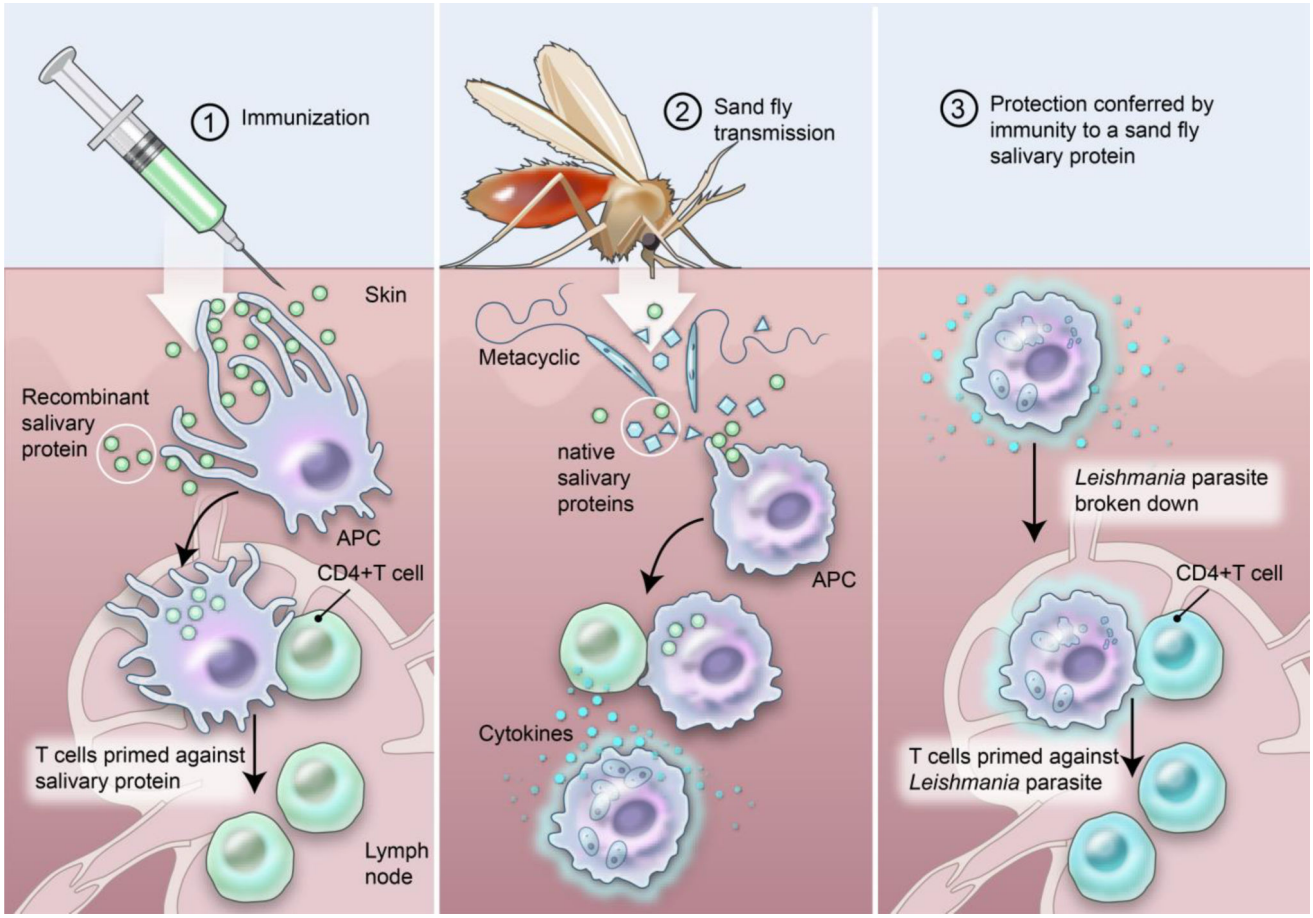

Figure 2.

(1) Immunizing with sand fly salivary proteins induces a T_H1 delayed type hypersensitivity response specific to the salivary protein (2) recalled to the bite site after the sand fly deposits parasites and salivary proteins while feeding where it activates nearby macrophages and (3) promotes *Leishmania*-specific immunity.

Table 1

Examples of Parasites Causing Human Leishmaniases

Parasite	Diseases	Distribution
L. donovani	visceral leishmaniasis, post-kalaazar dermal leishmaniasis Human	India, Nepal, Bangladesh, Sudan, Ethiopia
L infantum	visceral leishmaniasis Human, Canine	Brazil, Mediterranean Region
L. major	cutaneous leishmaniasis Human	Middle East
L. tropica	cutaneous leishmaniasis Human	Middle East
L. braziliensis	cutaneous leishmaniasis, mucocutaneous leishmaniasis Human	Brazil

Table 2

The three most promising sand fly salivary vaccine candidates to date

Vaccine Candidate	Origin	Protection	Animal Model	Homology	Potential vaccine coverage
PdSP15	*Ph. duboscqi*	CL[1]	NHP	*P. Papatasi*[1] *P. sergenti*[1]	MENA and Sub-Saharan Africa
LJM19	*Lu. longipalpis*	VL[2,3], CL[4]	Hamsters	*Lu. intermedia*[5] *Lu. ayacuchensis*[5]	Latin America
LJL143	*Lu. longipalpis*	VL (*in vitro*)[6]	Dogs	Ubiquitous[5]	Global

[1] Oliveira et al., 2015;

[2] Gomes et al., 2008;

[3] da Silva et al., 2001;

[4] Tavares et al., 2011;

[5] Abdeladhim et al., 2015;

[6] Collin et al., 2009

Table 3

List of Adjuvants

Adjuvant	Class	Mechanism of action	Type of immune response
Aluminum mineral salts	Particulate formulation antigen depot	NACHT, LRR and PYD domains-containing protein 3 (NALP3), immunereceptor tyrosine-based activation motif (ITAM), antigen delivery, Interleukin-1 secretion, Inflammasome, necrosis	Antibody, T helper 2
Lipid A analogues (for example, Monophosphoryl Lipid A (MPL,) Glucopyranosyl Lipid A (GLA))	Immunomodulatory molecule	Toll-like receptor 4	Antibody, T helper 1
Imidazoquinolines (for example Imiquimod, R848)	Immunomodulatory molecule	Toll-like receptor 7 Toll-like receptor 8	Antibody, T helper 1
CpG oligodeoxynucleotides (CpG ODN)	Immunomodulatory molecule	Toll-like receptor 9	Antibody, T helper 1, T helper 2, CD8+ T cells
Saponins (for example, QS21)	Immunomodulatory molecule	Unknown	
Virosomes	Particulate formulation	Antigen delivery	Antibody, T helper 1, T helper 2
GLA-SE (GLA, stable emulsion)	Combination	Toll-like receptor 4	Antibody, T helper 1