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Virulence Mechanisms And Vaccine Development Strategies Against Leishmaniasis: An Evolving Battle

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Abstract

Leishmaniasis, a neglected tropical disease, is caused by more than 20 species of Leishmania and transmitted primarily by sand flies. It manifests in various forms, including cutaneous, mucocutaneous, visceral, and post kala-azar dermal leishmaniasis (PKDL). Visceralleishmaniasis (VL) is the most severe, affecting organs like the liver and spleen, while cutaneous and mucocutaneous forms cause skin ulcers and mucosal lesions, respectively. Leishmania parasites have evolved sophisticated virulence factors, such as lipophosphoglycan(LPG), GP63 protease, and elongation factor 1α (EF1α), to evade the host immune system and survive within macrophages. These virulence factors are critical targets for vaccine development. Second-generation vaccines have focused on recombinant antigens like gp63 and KMP-11, while third-generation vaccines explore DNA-based strategies targeting key Leishmania antigens. Despite promising results in preclinical studies, challenges such as immune evasion, genetic diversity, and variable host responses hinder universal vaccine efficacy. Continued research into virulence mechanisms and innovative vaccine approaches is essential to developing effective, safe, and widely applicable vaccines against leishmaniasis.

Keywords: Leishmaniasis, PKDL, Virulence factor, gp63, Vaccine, Leishmanial antigens

1. Introduction

Leishmaniasis is a multifaceted neglected tropical disease (NTD) transmitted by vector,presenting in forms such as cutaneous or CL, muco-cutaneous or MCL, visceral or VL, and post kala-azar dermal leishmaniasis or PKDL. This disease is caused by more than twenty species of Leishmania, a known intracellular parasite. Visceral leishmaniasis (VL) is the mostsevere form, targeting vital organs like the spleen and liver, and can be deadly if not treated this hepato-splenomegaly. In contrast, CL and MCL are less in severity, with CL typically causing self-healing ulcers and MCL resulting in disfiguring lesions of the oropharyngeal mucosa.[1] The primary mode of transmission is through bites from female sand flies belonging to the Phlebotomine and Lutzomyia genera, though transmission can also occur, albeit rarely, through blood transfusions, shared needles, or from mother to child during birth.[2]

According to the World Health Organization (WHO), leishmaniasis is the second most significant neglected tropical disease after malaria, with more than a million new cases reportedannually. The disease has a long history, with evidence dating back to 2500–1500 B.C., including detailed descriptions by historical figures such as Alexander Russel, and the clinicalidentification of visceral leishmaniasis by Sir William Leishman and Charles Donovan in 1903.[3]

Today, leishmaniasis is found in tropical and subtropical regions, along with parts of southern Europe, impacting around 90 countries. WHO data shows that the disease is widespread, with the exception of Australia and Antarctica. The annual occurrence of cutaneous leishmaniasis is estimated to be between 700,000 and 1.2 million cases, while the incidence of visceral leishmaniasis has declined to an estimated 100,000 to 400,000 cases per year.[4]

This review explores the intricate mechanisms by which various Leishmania species evade thehost's immune system, focusing on the role of key virulence factors and their contribution to the pathogenesis of leishmaniasis. Additionally, it examines the current advancements in vaccine development, highlighting the potential of targeting these virulence factors to create more effective immunotherapies. The review also delves into the broader therapeutic landscape, discussing novel strategies in combating the disease. By addressing the challenges posed by immune evasion and genetic diversity among Leishmania species, this review aims to provide a comprehensive understanding of both the obstacles and opportunities in the fight against leishmaniasis.

2. Life Cycle of Leishmania parasite, its Vector, and Epidemiology

Leishmania is a protozoan parasite belonging to the Order Trypanosomatida and Class Kinetoplastae. Its life cycle is digenetic, includes both a mammalian host and an insect vector.The parasite has two primary forms: the elongated, motile promastigote (10–20 μm in size) and the oval, non-motile amastigote (3–7 μm in diameter). In the sand fly vector, *Leishmania* exists as promastigotes, which differentiate into metacyclic promastigote form (infective form). These metacyclic promastigotes are heavily transmitted to mammalian hosts during the sand fly's blood meal.[5] Once inside the host, the promastigotes are engulfed by phagocytic cells, such as macrophages, where they transform into the amastigote form and multiply intracellularly. When an infected mammal is bitten by a sand fly, the amastigotes are ingested and convert back into promastigotes in the insect's midgut. These promastigotes multiply by binary fission, migrate to the sand fly's salivary glands, and are passed on to another mammalian host during the next bite. The sand fly's saliva contains an anticoagulant that facilitates transmission by preventing blood clotting at the bite site. Once inside the host, promastigotes are quickly taken up by immune cells, reverting to amastigotes, which proliferate and eventually cause cell lysis. Released amastigotes can be either ingested by another sand fly during a blood meal or phagocytosed by other macrophages, perpetuating the parasite's life cycle. This cycle results in the infection of neighboring cells and tissues in CL, and in organs like the liver, spleen and bone marrow in VL.[1]

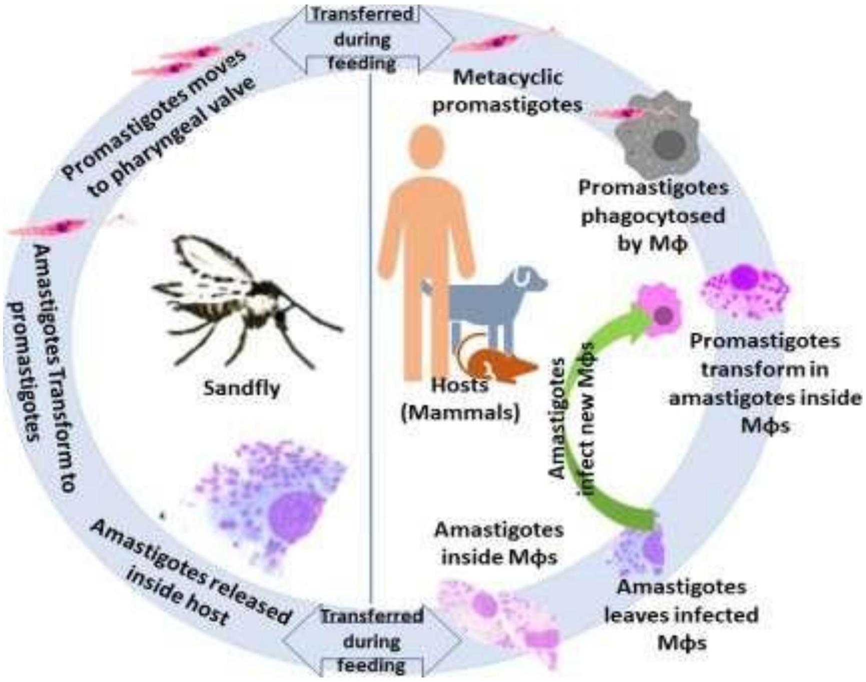

Figure 1: Life cycle of Leishmania spp.

2.a. Visceral Leishmaniasis

Visceral leishmaniasis, which affects the liver, spleen, and bone marrow, is the most severe kind of leishmaniasis. It is sometimes referred to as Kala-azar or black fever. VL can be lethal if untreated. *L. donovani* and *L. infantum* are the principal causes of VL in the Old World, but *L. chagasi* is the predominant culprit in the New World. The disease manifests with symptomsincluding high to low fever, loss of weight, anemia, hypergammaglobulinemia, and hepatosplenomegaly, largely due to the high parasite load in the affected organs. The overproduction of adrenocorticotropic hormone in VL patients can lead to darkening of skin, which is why the disease is called Kala-azar in India. Although many cases are asymptomaticinitially, symptoms can develop years later, particularly in immunocompromised individuals. The increasing prevalence of HIV along with VL co-infection is a significant emerging concern.

2.b. Post Kala-Azar Dermal Leishmaniasis (PKDL)

After treatment for VL, some patients develop a form of skin lesion known as post kala-azar dermal leishmaniasis (PKDL), which serves as reservoir for the parasite. PKDL manifests as macules, papules, and nodules, primarily around the mouth, which can then be spread to otherbody parts of the infect. It is most common in occurrence where *Leishmania donovani* causes VL, such as Sudan and India, with prevalence rates of approximately 50% and 5–10%, respectively. While PKDL is uncommon in infections caused by *Leishmania infantum*, it can occur in immunocompromised individuals. The onset of PKDL typically occurs 2 to 3 years after VL treatment in India, but it may appear within zero to six months in Sudan. Recent studies suggest a connection between PKDL and a Th1 immune response, particularly involving interferon-gamma and IL-10 in the blood of patients treated with VL.[6,7]

2.c. Cutaneous and Mucocutaneous Leishmaniasis

Cutaneous leishmaniasis, CL, is the most prevalent form of leishmaniasis and is generally lesssevere than VL, often resolving on its own. Chronic ulcers at the locations of insect bites are the hallmarks of CL. These ulcers can leave long-lasting scars, which can cause psychologicaldiscomfort, cosmetic problems, and social disgrace. Depending on the Leishmania species involved and the host's immunological response, the severity of the symptoms varies. *L. major*, *L. aethiopica*, and *L. tropica* are responsible for CL in regions such as the Mediterranean, Central Asia, and the Middle East, while in South America, species like *L. braziliensis*, *L. panamensis*, *L. guyanensis*, *L. mexicana*, and *L. amazonensis* are more common. CL also affects canines in South America, with *L. braziliensis* and *L. chagasi* being the typical causativeagents. According to the World Health Organization (WHO), in 2017, over 95% of new CL cases were reported from Brazil, Iran, Colombia, Afghanistan, Syria, Iraq, and Algeria.[8]

Mucocutaneous leishmaniasis (MCL) is a more severe and less common form of cutaneous leishmaniasis, primarily caused by *Leishmania braziliensis*, *L. amazonensis*, and *L. mexicana*. MCL is marked by destructive lesions in the mucous membranes of the nose, throat, and mouth, frequently resulting in severe disfigurement. The condition arises from an exaggerated immuneresponse that targets the host's own tissues. MCL is life-threatening if not diagnosed and treatedearly, with endemic cases occurring in regions such as Bolivia, Brazil, Ethiopia, and Peru.[9]

3. Virulence Factors of the Parasite

The parasite leishmania has evolved complex defense mechanisms against the host's immune system, relying on various virulence factors to survive and proliferate within macrophages. Lipophosphoglycan (LPG), a significant part of the parasite's surface glycocalyx, is one important virulence factor. LPG mostly contributes in the early phases of infection, helping theparasite infiltrate host cells and elude immunological reactions. Promastigotes in the sand fly vector express LPG, which changes as the parasite transforms into the infectious metacyclic form. Following transmission to the mammalian host, LPG helps the parasite enter macrophages, frequently via integrin receptor p150/95 and complement receptors like CR3.[10]

LPG also plays a crucial role in protecting the parasite during the transformation frompromastigotes to amastigotes inside macrophages. It prevents the fusion of phagosomes with lysosomes, creating a favorable environment for the parasite's survival. Additionally, LPG helps the parasite avoid destruction by reactive oxygen species and lysosomal enzymes. The inclusion of disaccharide repeats such as galactose-mannose in LPG is critical for blocking lysosomal enzymes, whereas LPG's capacity to prevent NADPH oxidase assembly in phagosomes dramatically reduces the host's innate immunological response.[4]

LPG's importance as a virulence factor was confirmed when genetically modified *Leishmania*strains lacking LPG showed reduced infectivity in both *in vitro* and *in vivo* models. LPG's rolein delaying the appearance of endosomal markers and preventing vacuolar acidification furtherunderscores its significance in the parasite's survival strategy. While LPG is not present in amastigotes, it preconditions the immune environment throughout the early stages of infection,allowing amastigotes to thrive within the host.

Given its critical role in *Leishmania* virulence, LPG has been identified as a potential vaccinetarget. Studies have shown that LPG can induce immune-suppressive signals, such as PD-1 expression on CD8+ T cells, although its low immunogenicity has limited its success as a vaccine candidate. Despite these challenges, LPG remains a key focus in the development of therapeutic interventions against leishmaniasis.[11]

3.a. GRP94

The identification of LPG3, a truncated form of lipophosphoglycan (LPG) in *Leishmania donovani*, revealed its structural similarity to the mammalian ER chaperone GRP94. Although LPG3/GRP94 in Leishmania performs similar activities to its mammalian equivalent, such as protein assembly, secretion, and antigen presentation, it has a distinct and essential role in the parasite. Without impacting N-glycosylated protein production or promastigote proliferation, GRP94 deficiency in Leishmania leads to abnormalities such reduced surface GPI-anchored proteins, suggesting that the parasite's developmental stage rather than stress controls GRP94'sactivity. In *Leishmania infantum*, GRP94 orthologs are highly immunogenic, suggesting that GRP94 also helps modulate the host's immune response during infection. Thus, LPG3 is essential for *Leishmania* metabolism, functioning differently from its mammalian equivalent.[11,10]

3.b. Arginase

Arginase, an enzyme central to the urea cycle, also plays a vital part in the virulence of *Leishmania*. It transforms L-arginine into L-ornithine and urea, allowing the parasite to bypassthe host's immune system. Specifically, *Leishmania* uses its own arginase to reduce the availability of L-arginine for nitric oxide production, thus mitigating the toxic effects of nitricoxide, a key immune defense. Localized in glycosomes, arginase supports amastigote survival within macrophages by maintaining the L-arginine pool. Studies confirm that *Leishmania* strains lacking arginase show reduced virulence and survival, both in vitro and *in vivo*.[12]

3.c. EF1α

Elongation Factor 1α (EF1α) is another key virulence factor in *Leishmania*, crucial formanipulating host macrophage signaling. Unlike its human counterpart, *Leishmania* EF1α lacks a 12-amino acid loop, making it a unique target for drug development. Secreted by parasitophorous vacuoles, EF1α binds to the host's SHP-1 phosphatase, causing the deactivation of macrophages and inhibiting their ability to produce nitric oxide in response to IFN-γ. This emphasizes the function of EF1α in immune evasion and its possible therapeutic target.[13]

Additionally, EF1α plays a role beyond virulence by facilitating the export of other leishmanialantigens from parasitophorous vacuoles to the host cell cytosol via exosomes. Its involvement in these processes has highlighted its potential as a vaccine candidate. Studies have shown thatcationic liposomal EF1α can induce a strong immune response, including delayed-type hypersensitivity and long-term defensive memory in both CD4+ and CD8+ T cells, especially in *Leishmania donovani*-challenged BALB/c mice. These findings underscore EF1α's importance in Leishmania pathogenesis and its promise as a target for future therapeutic interventions.[4]

3.d. Proteases

Proteases, a kind of enzyme that degrades proteins or peptides, serve critical roles in the life cycles of many organisms, including Leishmania species. These enzymes are classified according to the amino acids in their active sites, including serine, threonine, aspartyl, metallo-, and cysteine proteases. Aspartyl, serine, metallo-, and cysteine proteases have all been intensively investigated for their function as virulence factors in Leishmania. For example, the production of active aspartyl protease in Leishmania mexicana promastigotes is required for parasite multiplication, but its role in influencing the host immune response is unknown.[14]

3.d.1. GP63

GP63, first identified in 1980, is a key surface protease of *Leishmania*, initially recognized as a major surface antigen (MSA). This 60–66 kDa Zn-dependent metalloprotease, also known asleishmanolysin, is part of the metzincin class and is characterized by the HExxHxxGxxH motif. While GP63 expression decreases during the transformation from promastigotes toamastigotes, its reduced levels in amastigotes are offset by the lower expression of LPG, whichensures GP63 remains functional and accessible.[15]

GP63 plays a multifaceted role in *Leishmania* virulence. It cleaves a wide range of substrates,including C3b into C3bi, facilitating the parasite's evasion of the complement system and promoting its safe entry into macrophages via C3bi opsonization. Additionally, GP63 helps in the parasite's attachment to macrophages through the fibronectin receptor, contributing to the degradation of extracellular matrix components and abetting penetration and dissemination, particularly in *Leishmania mexicana*.[16]

One of GP63's most crucial functions is its ability to modulate host immune responses. By degrading key signaling proteins and transcription factors, like NF-κB and AP-1, GP63 helps the parasite evade the host's inflammatory responses. It also inhibits the activation of the inflammasome complex, thereby preventing the production of IL-1β, a pro-inflammatory cytokine, which are essential for clearing intracellular parasites. Moreover, GP63's role in suppressing the macrophage immune signaling pathway, particularly through the stimulation of protein tyrosine phosphatases (PTPs) like SHP-1, further underscores its significance in *Leishmania* survival within the host.[17]

3.d.2. Cysteine Proteases (CPs)

CPs are significant targets for drug development and vaccine design due to their crucial roles in the species of *Leishmania* parasite's life cycle. These enzymes, similar to papain proteases,which are categorized into three main types: CPA, CPB another is CPC. First two types resemble cathepsin L, while later is similar to cathepsin B. Interestingly, single nucleotide polymorphisms (SNPs) in the genes encoding these proteases in *L donovani* and *L major* influence whether the parasite will be dermatotropic or viscerotropic.[18]

Research using murine models infected with *L mexicana* species, such as *L. amazonensis, L. mexicana*, and *L. pifanoi*, has demonstrated the vital role of CPs in virulence. Furthermore, CPexpression levels and parasite virulence have been found to be strongly correlated in both human cell lines and hamsters infected with L. infantum and *L. chagasi*. Notably, CPs are moreabundantly expressed in *L. amazonensis* amastigotes than in promastigotes, emphasizing theirimportance in the survival of the parasite inside hosts system.[19]

Infectivity studies with *L. mexicana* mutants lacking CPA, CPB, and CPC revealed that CPB,in particular, plays a crucial role in virulence. CPB-deficient parasites exhibited reduced virulence, which was fully restored only when multiple copies of the cpb gene were introduced.This underscores the significance of CPB in modulating immune responses, particularly in inducing IL-4 production, which is vital for the parasite's survival in hosts like BALB/c mice. CPB also assists *Leishmania major* in establishing infection by suppressing IFN-γ and IL-12 production, which are key components of the host's immune defense. Additionally, CPB cleaves essential inflammatory transcription factors and MHC-II proteins, preventing presentation of antigen and dampening the Th1 type of immunological responses.[20] Interestingly, the COOH-terminal extension (CTE) of CPB has been shown to possess immune- regulatory functions, with the CTE domain alone capable of inducing Th2 cytokines. This implies that Leishmania infection and immunological regulation depend on both the active site and the CTE domain. In addition to CPB, CPC has also been investigated as a potential DNA vaccine against visceral leishmaniasis; it has been shown to elicit robust immune-protective responses and to significantly lower the parasite load.[21,22]

3.d.3. Serine Protease

Oligopeptidase B (OPB) is a serine protease of *Leishmania* that plays a prime part in the virulence of parasite. OPB is upregulated during the differentiation of amastigotes and aids in their protection by interacting with host molecules like enolase and plasminogen. OPB- deficient *L. donovani* parasites have been shown to induce extensive gene dysregulation in hostmacrophages, suggesting OPB's significant role in modulating host-pathogen interactions. Similarly, *L. major* strains lacking OPB demonstrated impaired development of metacyclic promastigotes, crucial for infecting macrophages.[23] Another serine protease, subtilisin (SUB), is also upregulated during the amastigote stage of *L. donovani*. Studies have shown that animals injected with parasite fractions containing active SUB were more susceptible to infection, while those treated with SUB inhibitors exhibited reduced susceptibility, highlighting the protease's role in virulence.[24]

3.e. Deubiquitinases (DUBs)

Deubiquitinases (DUBs) are critical enzymes in *Leishmania* that regulate the parasite's life cycle through reversible post-translational modifications. Ubiquitination, a conserved process in eukaryotes, involves tagging proteins for degradation, and DUBs remove these tags to fine-tune protein regulation. There are 20 DUBs in *Leishmania mexicana* that are known to be involved in protein trafficking, autophagy, and DNA repair. Studies using CRISPR-Cas9 technology have identified specific DUBs, such as DUB2, as essential for parasite survival andinfection establishment. Additionally, DUBs like Otubain (OTU) have been linked to host immune signaling, suggesting their importance in parasite virulence and their potential as therapeutic targets.[25,26]

3.f. Heat Shock Proteins (HSPs)

Leishmania parasites produce heat shock proteins (HSPs) to cope up with the temperature shift and acidic conditions encountered when transitioning from the sand fly to the mammalian host.These HSPs, particularly HSP100 and HSP78, play crucial roles in parasite survival. HSP100 is an amastigote-specific protein that aids in exosomal trafficking, essential for parasitevirulence. HSP78, another clpB family member, is critical for managing heat and pH stress, and its absence leads to non-viable parasites. HSP78 is a possible target for chemotherapy since it also functions to inhibit pro-inflammatory responses in host macrophages. Small HSPs (sHSPs), such as HSP20, P23, and HSP23, also contribute to *Leishmania* survival by assisting in protein folding and providing resistance against antimonial drugs. HSP23, in particular, is crucial for amastigote stage differentiation and plays a role in drug resistance, highlighting its significance in the parasite's lifecycle.[27]

3.g. A2 Protein

The amastigote-specific protein known as A2 in *Leishmania donovani* is essential to the parasite's survival in host macrophages. The A2 gene is linked to the viscerotropism of the parasite and is primarily expressed in those that cause VL. A2 is essential for the transformationfrom promastigotes to amastigotes, enabling the parasite to withstand the harsh conditions of the host's phagosomal vacuoles. Silencing the A2 gene results in significantly reduced virulence, while overexpression leads to increased parasite burden in experimental models. A2has shown promise as a vaccine candidate, eliciting protective immune responses in mice against *L. donovani* challenges.[28]

3.h. Protein Tyrosine Phosphatases (PTPs)

In Leishmania, PTPs regulate crucial life cycle events including differentiation and the onset of illness. The deletion of the LdPTP1 gene reduces the parasite's capacity to survive in host species, even though it is not necessary for promastigote survival. Owing to the structural resemblance between Leishmania PTP1 and human PTP1B, both enzymes have been recognized as possible therapeutic targets, providing a promising path for the creation of novelleishmaniasis therapeutics.[29,30]

4. Virulence Factors in Vaccine Development

Virulence factors are molecules produced by pathogens, such as *Leishmania*, that enable them to cause disease by evading the host immune system, promoting infection, and enhancing their survival within the host. These factors are critical in the pathogenesis of leishmaniasis and represent valuable targets for vaccine development. By focusing on these virulence factors, vaccines can be designed to specifically neutralize or inhibit the mechanisms that *Leishmania*uses to establish infection, thereby offering a strategic approach to combating the disease.[31] Below, I provide detailed descriptions of key virulence factors that have been explored in the development of vaccines against leishmaniasis.

4.1. Second-Generation Vaccines

Second-generation vaccines for leishmaniasis focus on the use of recombinant or synthetic antigens, peptides, genetically modified organisms (such as bacteria, viruses, or Leishmania species), and purified native fractions from parasites. Numerous Leishmania proteins have been identified as potential vaccine candidates based on their abundance and localization on the parasite surface.[32,33]

4.1.a. gp63

gp63 is a crucial 63-kDa glycoprotein found in Leishmania promastigotes surface, playing a significant role in influencing host cell signaling and aiding the parasite's survival within macrophages. Liposomal formulations using protein have been utilized as potent adjuvants in gp63-based vaccines. For example, purified gp63 antigen from *L. donovani* when encapsulated in positively charged liposomes has shown to induce protective responses against VL in experimental mice model. The immunized mice demonstrated increased levels of IFN-gamma and a decrease in IL-4. Other formulations using different liposomal compositions have also resulted in a strong immunological response characterized by augmented IgG2a and IFN-γ production.[17,20]

Further, peptide-based vaccines derived from gp63, designed to target specific human HLA molecules, have shown strong immunogenicity in various transgenic mouse models. These formulations successfully generated long-lasting immune responses, including high levels of IgG, without significant toxicity.[34]

4.1.b. Leishmania Homolog for Receptors of Activated C Kinase (LACK)

It has been discovered that the LACK antigen, which is produced from *L. infantum*, protects BALB/c mice against *L. major*, particularly when IL-12 is given. Subcutaneous immunization with recombinant Lactobacillus lactis that expresses LACK and secretes IL-12 has been showno significantly lower the parasite burden and postpone the onset of the illness. This tactic stimulated CD8+ cytotoxic T cells and produced a particular Th1 response. Additionally, a protective Th1 response against *L. major* infection was elicited by oral immunization with L. lactis that co-expressed IL-12 and LACK.[35,36]

Another strategy comprised combining a CpG oligonucleotide adjuvant with *L. tarentulae* that were genetically modified to express KMP-11, EGFP, and LACK. This led to a significant reduction in the parasite burden in a mouse model.[35]

4.1.c. Kinetoplastid Membrane Protein-11 (KMP-11)

KMP-11 is expressed in promastigotes as well as in amastigotes form of *Leishmania* and has shown potential as a vaccine candidate. When transfected into bone marrow-derived macrophages or dendritic cells, KMP-11 induced a robust CD8+ cytotoxic T lymphocyte response[37]. Ex vivo studies have shown that KMP-11, when combined with CpG oligonucleotides, could significantly reduce parasite load in visceral organs in a murine model of VL, primarily by enhancing Th1 and Th17 responses and promoting the production of IFN-γ.[38]

KMP-11 encapsulated in biodegradable nanoparticles has also demonstrated a reduction in parasitic burden, likely due to the induction of a pro-inflammatory immune response driven bymacrophages. In another study, a subunit vaccine combining recombinant form of LACK along with KMP-11 and CpG oligonucleotide adjuvant, was shown to elicit a more effective Th1 type of immunological response in mice model than a prokaryotic form of subunit vaccine.[39]

4.1.d. Fucose Mannose Ligand (FML)

In canine models, the FML glycoprotein in combination with saponin have shown to protect against zoonotic VL (92-97%). FML-pulsed dendritic cells have also been effective in conferring Th1-mediated protection against *L. infantum*. Moreover, the addition of natural immunomodulatory compounds like glycyrrhizin to FML-based vaccines has enhanced their efficacy by increasing NO, TNF-α, and IL-12p70 production while reducing IL-10 levels.[40]

In a mouse model challenged with L. infantum, a new vaccine called Chimaera A, which is manufactured with MPL-A, has been found to generate a significant Th1 response characterized by IFN-γ, IL-2, and lower levels of IL-4 and IL-10, in addition to a decreased burden of splenic parasites.[41]

4.1.e. Hypothetical, In Silico Protein Constructs, and Other Proteins

Innovative vaccines are also being developed using in silico modeling and hypotheticalproteins. For instance, a vaccine combining proteins from *L. braziliensis*, such as LbHyp and EiF5a, has shown to induce protection against VL. Another candidate, LiHyT, combined with saponin, has elicited a strong immune response in BALB/c model, with increased levels of protective cytokines (IL-12 and IFN-γ along with GM-CSF).[42] Additionally, recombinant enolase and a peptide-based vaccine constructed using molecular docking techniques have shown promising results in terms of inducing Th1 responses and strong binding to immune receptors like TLR-2.

4.1.f. Vaccines Against Canine Leishmaniasis

L. infantum is the primary cause of canine leishmaniasis (CanL), and successful treatment of the illness is critical to preventing the spread of zoonotic VL. Several CanL vaccines are available, including Leishmune®, Leish-Tec®, CaniLeish®, and LetiFend®.[43] The first approved vaccination against canine VL was Leishmune®, which provided 92-95% protection in field testing. However, production and marketing were halted in 2014 due to regulatory non-compliance. Currently, Leish-Tec®, CaniLeish®, and LetiFend® are the primary vaccines used for CanL prevention, with reported efficacies ranging from 68.4% to 80%[44]. Leish-Tec® is a recombinant protein A2-based vaccine that has shown partial protection in dogs against *L. chagasi*. CaniLeish®, the first European CanL vaccine, uses purified secreted proteins from *L. infantum* with an adjuvant to induce a Th1 response. LetiFend®, licensed in 2016, consists of a recombinant protein composed of multiple antigenic fragments from *L. infantum*.[45,46]

Research into new vaccine strategies continues, with recent studies focusing on using sand fly salivary proteins, novel antigen combinations, and advanced immunoinformatics approaches to develop more effective vaccines against CanL.

4.2. Third-Generation Vaccines

Third-generation vaccines, primarily DNA vaccines, offer a flexible and effective approach for inducing strong Th1 immune responses against leishmaniasis. Among the most studied DNA vaccine candidates are gp63, LACK, and ORFF, which have shown considerable promise in experimental models.[47]

DNA vaccines are favourable because of their simplicity of manufacture, stability, and capacity to elicit humoral and cellular immune responses. These reactions can be amplified by altering the vector or introducing immunomodulators such CpG motifs, GM-CSF, Flt3 ligands, or cytokine genes. The notion of heterologous prime-boost techniques, which entail priming with a DNA vaccine and then boosting with protein antigens or vectors, has been used successfullyagainst Leishmania.

4.2.a. gp63-Based DNA Vaccines

The first DNA vaccine for leishmaniasis expressed the gp63 gene in Salmonella typhimurium,stimulating a CD4+ Th1 response but without generating a detectable delayed-type hypersensitivity (DTH). The effectiveness of this approach was enhanced by including additional antigens such as gp46 and cysteine proteinase B (CPb), and by using adjuvants likealuminum phosphate and plasmids encoding CD40L.[32]
A multi-epitope vaccination containing gp63, KMP-11, and amastin was utilised in a prime- boost method against VL, in which the immune response was stimulated with a DNA vaccineand subsequently boosted with a protein antigen or vector. This technique showed significant protective benefits.[47,48]

4.2.b. Other DNA Vaccine Strategies

Different Leishmania antigens have been used to generate a number of additional DNA vaccines. For example, aluminium phosphate and NH36 DNA, a crucial part of FML, together generated a strong Th1 response that protected against L. chagasi, L. mexicana, and L. amazonensis.[15]

By eliciting particular CD4+ Th1 responses, plasmids expressing antigens such as TSA, LmSTI1, and LACK have also been demonstrated to shield mice against infection with L. major. Specifically, these antigen-containing plasmid combinations have shown complete protection against the infection.[49]
Leishmania histones have proven to be excellent immunogens, with plasmids encoding histones H2A, H2B, H3, and H4 from *L. infantum* inducing strong Th1 responses in murine models. These vaccines have been effective in preventing lesion development and reducing parasite burdens in various models.[46]

4.2.c. KMP11-Based DNA Vaccines

DNA vaccines with KMP11 encoding have demonstrated great promise; they are frequently paired with additional antigens such as HASPB. In L. infantum-infected mice, for instance, a recombinant lentiviral vaccination encoding a KMP11-HASPB fusion protein generated a potent IFN-γ response and decreased parasite burden. Similar to this, a strong Th1 response was elicited by a pEGFP-N1-KMP11-gp96 DNA vaccine, which provided protection against *L. major*.[50,51]

4.2.d. Hemoglobin Receptor (HbR)-Based Vaccines

Leishmania utilizes HbR-mediated endocytosis to obtain heme, making HbR a promising targetfor vaccines. In mice and hamsters, DNA vaccines encoding the whole length of HbR or its N- terminal domain have demonstrated complete protection against VL and a marked decrease inhepatic and splenic parasite loads.[52] Leishmania antigens (KMP-11, TSA, CPA, CPB, and P74) are encoded by plasmids used in the DNA vaccine LEISHDNAVAX, which has also demonstrated protective effectiveness in mice against L. donovani infection.[53,54]

4.2.e. Emerging DNA Vaccine Approaches

The creation of recombinant vaccines such as LEISH-F2 and LEISH-F3+ is one recent development. These vaccines, when paired with TLR4 antagonists, have produced potent CD4+ mediated T cell responses and shown therapeutic efficacy in CL models. RNA vaccinesthat express F2 and F3+ sequences have further boosted CD4+ and CD8+ T cell responses, providing protection against L. donovani.[55–57]

It has been demonstrated that in mice infected with *L. mexicana*, DNA vaccines that target the LmxMBA gene and lack transmembrane sections decrease lesion size and parasite burden. LACK-based DNA vaccines have demonstrated protective effects against *L. major* and *L. infantum* in mice and Beagle dogs. A combination of LACK with other antigens like TSA has further increased survival rates and reduced disease progression, correlating with strong IFN-γ responses and reduced IL-4 levels.[55,58]

4.2.f. Advanced Vaccine Constructs and Trials

In phase I human trials, a new vaccine called ChAd63-KH, which uses a replication-defective simian adenoviral vector carrying a synthetic gene combining KMP-11 and HASPB, has produced high IFN-γ production and activated dendritic cells. In Sudanese individuals with chronic PKDL, phase IIa studies showed strong immunological responses and little side effects.[59,60]

The recombinant protein L. infantum pyridoxal kinase (PK) when administered as a DNA vaccine led to a considerable reduction in parasite burdens and triggered a protective Th1 response marked by elevated levels of IFN-γ, IL-12, GM-CSF, and particular IgG2a antibodies.[61]

4.2.g. Enhancing DNA Vaccines with Immunomodulatory Drugs

An inventive tactic involves boosting the effectiveness of a DNA vaccine made up of four plasmids (LACK, TRYP, PAPLE22, and KMPII) using the immunomodulatory medication sirolimus (SIR). substantially though the DNA vaccination by itself considerably decreased the parasite burden, SIR added to the mix offered substantially more protection.[62] These third- generation DNA vaccines represent a significant advancement in the fight against leishmaniasis, offering promising strategies for both prevention and treatment through the induction of robust and tailored immune responses.

5. Conclusion

The development of vaccines against leishmaniasis has progressed significantly, with second- and third-generation vaccines showing promise in preclinical studies. These vaccines, particularly DNA-based ones targeting virulence factors such as gp63, KMP-11, and hemoglobin receptors, have demonstrated the ability to induce robust immune responses and reduce parasite burdens in animal models. However, despite these advances, significantchallenges remain. The parasite's complex immune evasion strategies, genetic diversity among species, and variability in host immune responses complicate the development of a universally effective vaccine.

The strategic targeting of *Leishmania* virulence factors in vaccine development offers a promising approach to controlling and potentially eliminating leishmaniasis. However, furtherresearch is needed to address the challenges associated with vaccine development, particularlyin enhancing the immunogenicity and efficacy of these vaccines across different *Leishmania* species and host populations. Ongoing clinical trials and research into novel vaccine candidateswill be crucial in achieving this goal. In conclusion, while progress has been made, the fight against leishmaniasis will require sustained scientific efforts and global health initiatives to develop effective, safe, and universally applicable vaccines.

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