Review

Host–Pathogen Interaction in Leishmaniasis: Immune Response and Vaccination Strategies

Hadida Yasmin [1], Anureeta Adhikary [1], Mohammed N. Al-Ahdal [2], Syamal Roy [3] and Uday Kishore [4,*]

1 Immunology and Cell Biology Laboratory, Department of Zoology, Cooch Behar Panchanan Barma University, Cooch Behar 736101, West Bengal, India; hadiday77@gmail.com (H.Y.); anureeta.adhikary@gmail.com (A.A.)
2 Department of Infection and Immunity, King Faisal Specialist Hospital and Research Centre, Riyadh 11211, Saudi Arabia; profahdal@gmail.com
3 Council for Scientific and Industrial Research-Indian Institute of Chemical Biology, Kolkata 700032, West Bengal, India; drsyamalroy@yahoo.com
4 Biosciences, College of Health, Medicine and Life Sciences, Brunel University London, Uxbridge UB8 3PH, UK
* Correspondence: uday.kishore@brunel.ac.uk or ukishore@hotmail.com

Abstract: Leishmaniasis is a zoonotic and vector-borne infectious disease that is caused by the genus *Leishmania* belonging to the trypanosomatid family. The protozoan parasite has a digenetic life cycle involving a mammalian host and an insect vector. Leishmaniasisis is a worldwide public health problem falling under the neglected tropical disease category, with over 90 endemic countries, and approximately 1 million new cases and 20,000 deaths annually. *Leishmania* infection can progress toward the development of species–specific pathologic disorders, ranging in severity from self-healing cutaneous lesions to disseminating muco-cutaneous and fatal visceral manifestations. The severity and the outcome of leishmaniasis is determined by the parasite's antigenic epitope characteristics, the vector physiology, and most importantly, the immune response and immune status of the host. This review examines the nature of host–pathogen interaction in leishmaniasis, innate and adaptive immune responses, and various strategies that have been employed for vaccine development.

Keywords: *Leishmania*; sand fly; macrophage; innate immunity; Th1/Th2; vaccine

1. Introduction

Leishmaniasis is a protozoan parasitic disease caused by over 20 *Leishmania* species; it is among the major neglected vector-borne tropical diseases with outbreak and considerable mortality. *Leishmania* is a dimorphic obligate intracellular protozoan parasite with two distinct and alternating developmental stages: the promastigote form, which is the flagellated and motile stage residing in the midgut of Sandfly vectors, and the amastigote, non-motile form that resides within the mononuclear phagocytes in the mammalian host. All species of *Leishmania* are transmitted by the female *Phlebotomine* Sandflies, which infect a range of mammals including humans, rodents and canids [1]. There are over 90 sandfly species that are known to transmit *Leishmania* parasites.

Leishmaniasis has three main forms: visceral, cutaneous and mucocutaneous. Nearly 95% cases of visceral leishmaniasis (VL), also known as kala-azar, if left untreated, become fatal. Visceral, cutaneous (CL) or mucocutaneous leishmaniasis (MCL) are endemic in Algeria and countries in East Africa, where outbreaks of VL occur frequently [2]. In addition, Post-kala-azar dermal leishmaniasis (PKDL) also exists, mainly in East Africa and the Indian subcontinent. PKDL is a dermal manifestation of VL, developing in 5–10% of kala-azar patients after 2–3 years of treatment who are also considered a potential source of *Leishmania* infection. As estimated by the WHO, between 50,000 and 90,000 new cases of VL and between 600,000 and 1 million new cases of CL occur worldwide annually, where different species of *Leishmania* cause varied clinical manifestations, from self-healing cutaneous lesions to

life-threatening VL (https://www.who.int/news-room/fact-sheets/detail/leishmaniasis (accessed on 30 November 2021)). VL has a broad range of clinical manifestations, primarily affecting the lymphoreticular system, leading to lymphadenopathy, hepatomegaly and splenomegaly, pancytopenia, hypergammaglobulinaemia, and renal disease [3]. CL represents a small localised erythematous nodule developing at the site of the sandfly bite, which gradually develops into an ulcerative condition, rarely penetrating into the subcutaneous tissue [4]. MCL is a highly disfiguring and life-threatening condition occurring in patients with a history of CL with mucosal involvement after 1–5 years of healing [5,6]. Hematogenous or lymphatic dissemination of the parasite in MCL affects the mucosal surfaces of the mouth, nose, and pharynx.

Leishmaniasis can be subclinical (inapparent), localized (skin lesions), and/or disseminated (cutaneous, mucosal, or visceral). There are several factors that determine the clinical presentation and outcome of the disease, such as the parasite and host factors, inflammatory responses, parasite-escape mechanisms as well as the host immunocompetence [7,8]. This review will focus on the mechanisms of leishmanial pathogenesis, host immune responses against the parasite, and the current status of the vaccine development.

2. Life Cycle of *Leishmania*

Leishmania transmission occurs primarily via the bite of infected female sandflies belonging to the genus *Phlebotomous* (Old World) or *Lutzomyia* (New World). *Leishmania* species have their individual characteristics but they share a similar digenetic life involving a mammalian host and an insect vector. However, in the case of VL in India, and CL caused by *Leishmania tropica*, instead of sandflies, human beings are incidental hosts of infection (human anthroponosis), and other mammals act as reservoir hosts. Through blood transfusion, sharing needles and due to transplacental spread or organ transplantation, VL can be initiated by amastigotes [9,10]. The chances of transmitting infection by sub-clinically infected humans are feeble [11]. Canids, especially dogs, act as a reservoir of the parasite in most cases; cats, rats, hares, opossums, foxes and other wild animals may also serve as sylvatic reservoirs [12–16]. Environmental factors such as climate change involving alterations in temperature, deforestation, natural disasters and poor socio-economic factors, also contribute to the spread of this disease. Due to geographical overlap, malaria–VL co-infections occur in large populations; it is common in East African countries where malaria and VL are co-endemic [17,18]. Drug resistance, organ transplantation and immunosuppression by HIV-1 contribute to the spread of Leishmaniasis [19]. VL patients co-infected with HIV-1 may also be important reservoirs for sustained *Leishmania* transmission [20].

Leishmania parasite exists in two forms: Promastigote, a flagellated form that is found in sandflies, and Amastigote, a non-flagellated tissue form that can survive within the phagosomes of macrophage in mammalian hosts. An infected female sandfly (*Phlebotomus* and *Lutzomyia* sp.) injects the promastigotes while biting a mammalian host which then enter into the phagocytic cells. Sandflies are relatively weak, prefer moist and dark areas to rest and become active during evening and thus, prefer the bloodmeal at or after dusk. Within the phagocytic cells, promastigotes transform into round non-flagellated amastigotes. The replicative amastigotes then enter into the sandfly while feeding on an infected host.

2.1. Sandfly-Specific Stages of the Parasite

Amastigotes transform into infective metacyclic promastigotes inside the sandfly gut [21], which at a subsequent bloodmeal, are regurgitated [22] and injected back into the mammalian skin to complete the life cycle (Figure 1). This is the only established route of Leishmanial infection [23]; the amastigote forms are present in the skin and are not usually found in the peripheral circulation.
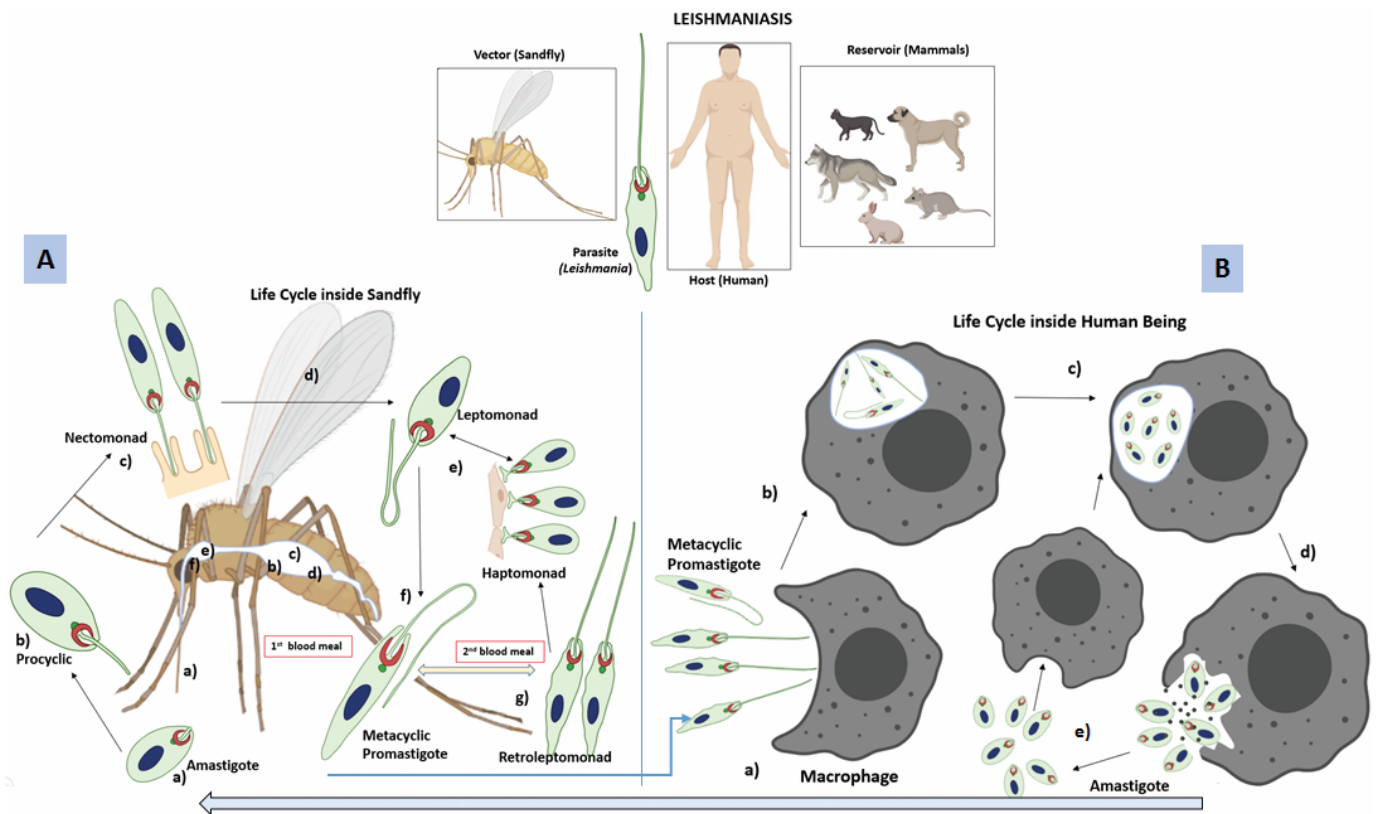
Figure 1. Digenetic life cycle of *Leishmania*: (**A**) Inside the sandfly, amastigotes undergo several non-infective stages before they finally differentiate into metacyclic promastigotes; (**a**) amastigotes enter into the sandfly (vector), while it bites an infected mammal (host), (**b**) amastigotes transform into replicative procyclic promastigotes (flagellated form) inside the fly abdominal midgut, (**c**) Procyclic stage transforms into an elongated nectomonad promastigote that attaches to the microvilli of the abdominal midgut through its flagella, (**d**) nectomonad then differentiates into a replicative form (leptomonad promastigote), and migrates towards the thoracic midgut, (**e**) leptomonad promastigotes can differentiate into either heptomonad promastigotes, or (**f**) metacyclic promastigotes. The haptomonad promastigotes can attach to the stomodeal valve, (**g**) during the second blood meal, a new replicative stage, known as retroleptomonad promastigote, exists due to reverse metacyclogenesis, where they multiply rapidly and differentiate into metacyclic promastogotes enhancing sandfly infectivity. (**B**) The metacyclic promastigote belongs to the infective stage that is transmitted to the mammalian host when an infected sandfly bites; (**a**) metacyclic promastigotes are taken up by phagocytic cells such as macrophages and neutrophils present in the skin, (**b**) promastigotes are internalised into phagosomes (later, it transforms into a parasitophorus vacuole), (**c**) promastigotes change into amastigotes inside the parasitophorus vacuole and proliferate, (**d**) amastigotes burst out from the phagocytes, (**e**) amastigotes can enter into another life cycle inside the sandfly when taken with the blood meal, or can re-infect fresh phagocytes.

The amastigotes, while inside the sandfly, undergoe transformation via several non-infective stages; first, it becomes a procyclic promastigote, which after few days, differentiates into a strong, elongated motile nectomonad promastigote; this is followed by its transformation into shorter replicative forms, known as leptomonad promastigotes [24], allowing flies to regurgitate parasites into the skin before it takes the second blood meal. The infection is amplified in the vector's gut [24]; sometimes, a small number of the nectomonad/leptomonad promastigotes also differentiate into haptomonad promastigotes [25]. Finally, they develop into infective metacyclic promastigotes (metacyclogenesis). These promastigotes, while inside the sandfly gut, secrete filamentous proteophosphoglycan (fPPG), which condenses into the promastigote secretory gel (PSG), helping in its trans-

mission [26]. Usually, it takes about 7 to 14 days for a transmissible infection to develop within the vector [27]. Up to 76% of infected bites contain enriched doses of metacyclic promastigotes (80 to 100%). *L. mexicana* and *L. infantum* bites contain less than 75% metacyclic promastigotes [26]. PSG production is vital in creating a blocked fly as it compels the infected sandfly to go for successive blood meals [27] because metacyclic promastigotes are capable of de-differentiation inside the sandfly into retroleptomonad promasigotes (via reverse metacyclogenesis), increasing parasite load in its next blood meal (Figure 1). Rogers et al. have shown that a single sandfly can ingest around 3200 amastigotes in a 1.6 mL bloodmeal, and within 5 days, the parasite population grows to approximately 35,000 per fly [28]. By the 10th day, the parasite number falls to 1000, probably due to exhaustion of the nutrient, or the immune response mounted by the sandfly [29].

2.2. Stages Inside the Mammalian Phagocytes

Metacyclic promastigotes of *Leishmania* are highly motile cells, capable of migrating through a collagen matrix [30]. They can invade beyond the bite site either directly or through infected phagocytic cells, fostering the ability of the parasite to establish the disease from CL to VL. The metacyclic promastigotes interact with phagocytic cells such as macrophages through its active motile flagellum [31]. Inside the macrophage, the elongated promastigote transforms into a spherical body with a shorter flagellum. The growth rate of the amastigote is slower than the promastigote, possibly to reduce metabolic load and proliferate and survive for a longer time, thus enhancing the infectivity. The amastigote protects itself from the surrounding acidic medium containing proteases by keeping the flagellar pocket neck closed [29]. The amastigotes proliferate inside the phagosome and then get out through cell rupture to infect other phagocytic cells.

3. Host Immune Response

There are several important factors that contribute to a successful transmissible infection, such as the species of the parasite, the vector combination, PSG production, and the host immune response against the parasite. The first crucial event is the initial contact and stable interaction of highly polarized and motile promastigotes with mammalian host cells for their efficient phagocytosis. Macrophages act as the primary host cells for *Leishmania*; however, monocytes, dendritic cells (DCs), and neutrophils are also infected and have important roles in the immunopathology of Leishmaniasis.

3.1. Innate Immune Response

Promastigote uptake is a receptor-mediated process where it binds avidly to macrophages and other phagocytic cells, but poorly to lymphocytes. The cell surface components of *Leishmania*, which change in various parasitic forms (promastigote to amastigote), are important determinants of parasite virulence. Promastigotes are covered in a thick glycocalyx composed of a variety of extensively branched, glycosylphosphatidylinositol (GPI)-anchored glycoconjugates, including lipophosphoglycan (LPG), metaloprotease glycoprotein (gp) 63, and members of a secreted family of heavily glycosylated proteins [32]. Amastigotes are covered by a densely packed glycocalyx mainly composed of glycoinositolphospholipids and glycosphingolipids [32], LPG, gp63 and Proteophoglycan (PPG) are the major determinants in initiating phagocytosis and for intracellular survival of *L. major* [33,34]. LPG of *L. donovani* is also capable of blocking LPS-mediated expression of E-selectin, intercellular adhesion molecule–1, and vascular cell adhesion molecule–1 on endothelial cells [35]. Thus, *Leishmania* LPG exerts its inhibitory effect possibly by affecting the endothelial cell functions and gene expression that are required for inflammation.

3.1.1. Role of Neutrophils and Salivary Components of Sandfly during Early Immune Response

During the early phase of CL, neutrophils and eosinophils increase in number, which is followed by an increasing phagocytic response by macrophages. Neutrophils predomi-

nate at the inoculation site within first few hours of infection, they take up promastigotes, and serve as a vehicle for subsequent infection of monocyte-derived or tissue-resident macrophages [36]. Neutrophils and eosinophils possess leishmanicidal activity that restrains parasite progression [37]. Neutrophils could enhance killing of *L. braziliensis* in infected macrophages through upregulation of TNF-α and ROS [38]. Activated neutrophils are capable of killing promastigotes via the neutrophil trap (NET) [39] and also through oxygen metabolites generated during phagocytosis-induced respiratory burst [40]. In NET associated with promastigotes, DNA, elastase, and histones are detected; histones likely exhibit the leishmanicidal activity [41].

While injecting the promastigotes into the host skin, the sandfly also salivates and delivers certain salivary proteins that are chemotactic and inducer of inflammatory response facilitating *Leishmania* infection [42]. The saliva also contains vector gut microbiota that activates inflammasome-derived IL-1β production and aids in neutrophil recruitment [43]. The saliva of *Lutzomyia longipalpis* contains a female-specific secreted endonuclease called Lundep capable of hydrolysing DNA, thus negating the effect of neutrophil NETosis, in order to protect the promastigotes [44]. A 45 kDa neutrophil chemotactic salivary protein acts through a G-protein-coupled receptor, which enhances lesion pathology and increases the parasite burden in mice upon co-infection with *Leishmania* parasites [45]. Co-infection with helminths such as Filaria may modulate the immune response to *Phlebotomus duboscqi* (Pd) saliva; repeated exposure to Pd saliva polarizes human monocyte function towards a tolerized phenotype while co-infection with filaria favors a *Leishmania*-promoting Th2/regulatory immune response [46].

Neutrophils appear to restrain parasite progression [36] by secreting cytokines and chemokines, releasing its granular contents, triggering pattern recognition signals, and by interacting directly with other inflammatory and resident cells [47–49]. *Leishmania* promastigotes release a soluble chemotactic factor (LCF), as confirmed in the supernatants of *L. major*, *L. aethiopica*, and *L. donovani* parasites cultures, which aids in neutrophil recruitment. This induces IL-8 secretion by neutrophils, thus, amplifying neutrophil recruitment. Often, gut microbes present in the saliva of the sandfly are egested into host skin along with *Leishmania* parasites. The egested microbes trigger the inflammasome to produce significantly higher levels of IL-1β compared to levels induced by parasites alone. Removing gut microbiota, or blocking IL-1β before transmission, abolishes neutrophil recruitment and impairs *Leishmania* dissemination [43]. IL-17A-producing Innate Lymphoid Cells (ILCs), which are RORγt$^+$, are involved in the microbiota-driven immunopathology in CL. Pateints infected with *L. braziliensis* were found often with dominant *Staphylococcus* dysbiosis, and this probably can influence IL-17 synthesis. The lesion size in mice infected with *L. major* was also found to be increased due to *Staphylococcus* colonization [50]. Thus, investigating the mechanistic aspects of immunomodulation due to sandfly microbiota can offer insight into the immunopathology of early infection in Leishmaniasis.

There is also evidence to suggest the survival of *Leishmania* promastigotes following their rapid uptake by neutrophils. Under an experimental condition, inhibiting neutrophilic infiltration increased host resistance to infection [49]. *L. major* and *L. donovani* promastigotes were observed to be surviving in human neutrophils which correlated with a lack of respiratory burst. Inhibition of IP-10 (gamma interferon-inducible protein 10) production by neutrophils in presence of *Leishmania* can inhibit recruitment and activation of NK cells and Th1 cells (Figure 2). Thus, by infecting neutrophils, promastigotes find an escape from neutrophil-mediated defense mechanisms [51]. IL-17 seems important for neutrophil recruitment during the development of *Leishmania*-induced human and murine lesions. CD4$^+$ T cells and neutrophils produce increased amounts of IL-17 in *L. major* infected BALB/c mice [52,53]. However, promastigotes also somewhat contribute to programmed cell death of neutrophils [50], which are cleared via phagocytosis by macrophages and DCs. Infected neutrophils often deliver viable promastigotes to macrophages during their phagocytosis [54,55].
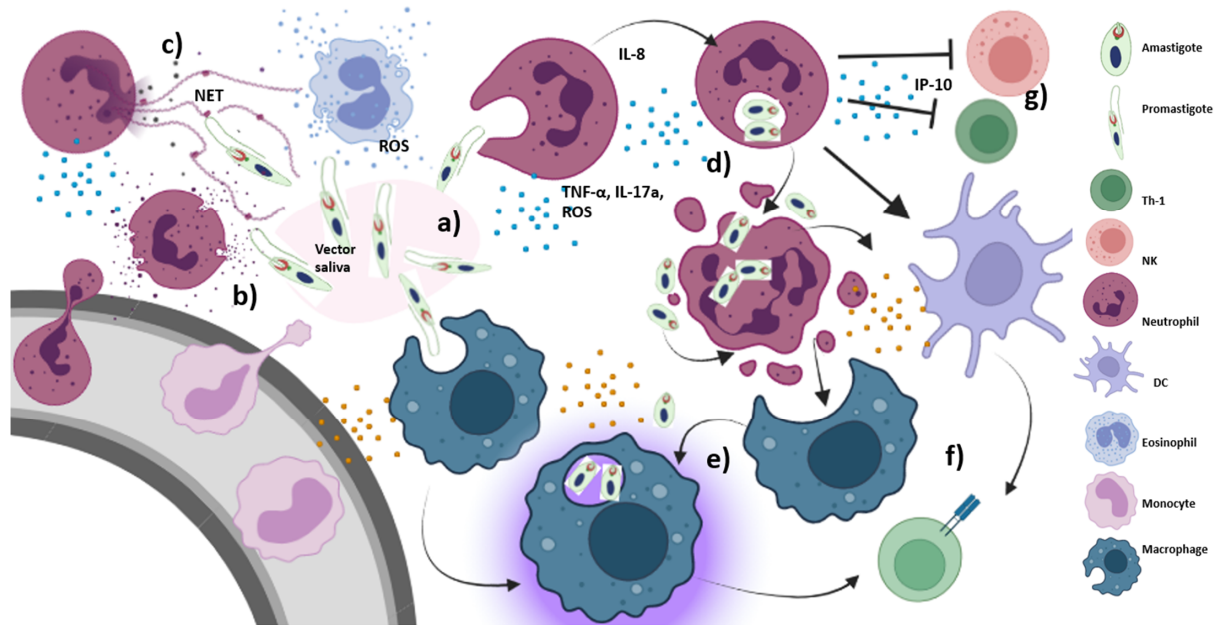
Figure 2. Early immune response triggered by *Leishmania*: (**a**) within minutes of infection, promastigotes are rapidly taken up by phagocytic cells, including neutrophils and macrophages, (**b**) promastigotes are injected into the host skin along with certain vector-derived salivary proteins as well as vector salivary microbiota, which exhibit neutrophil chemotactic properties and trigger inflammasome formation. IL-8 secretion by neutrophils also amplifies further neutrophil recruitment. Monocyte and eosinophil infiltration also occur, (**c**) neutrophils and eosinophils possibly show leishmanicidal activity that controls parasite proliferation. Activated neutrophils are capable of killing promastigotes via NET and ROS generation, (**d**) *Leishmania* is capable of accelerating neutrophil apoptosis, which are then phagocytosed by macrophages and dendritic cells along with the amastigotes, (**e**) Amastigotes released from apoptotic neutrophils can infect uninfected macrophages where they are internalized into phagosomes, divide many times by binary fission, eventually getting released by rupturing the macrophages, (**f**) activated macrophages and dendritic cells present the processed parasitic antigens to T cells for adaptive immune response, (**g**) IP-10 secretion by neutrophils inhibits recruitment and activation of NK cells and Th1 cells.

3.1.2. Macrophage as a Cellular Host of *Leishmania*

In leishmaniasis, amongst the myeloid host cells, macrophages play quite a significant role: one, as a replicative niche during the acute phase of infection, and two, as anti-leishmanial effector, immunoregulatory, and permissive host cells for long-term survival of the parasite [36,56]. During CL, infected macrophages remain near the sandfly bite. Other forms of leishmaniasis develop when infected macrophages migrate away from the sandfly bite site; VL involves dissemination throughout the body, especially to the liver and spleen via infected macrophges.

Parasitophorous Vacuoles (PV) as a Safe Haven for *Leishmania*

Uptake of promastigotes by macrophage involves complement receptors and PAMPs such as LPG or gp63 [33,57]. Among the *Leishmania* promastigote surface molecules, glycoinositol phospholipids (GIPLs) are most abundant and are actively expressed on promastigotes as well as amastigotes [58–60]. The promastigotes bind to the complement receptor 1 (CR1), CR3, fibronectin receptor, and the mannose-fucose receptor on the surface of macrophages (Figure 3) [61]. Various opsonins such as C3b/iC3b, mannan-binding lectin (MBL), and galectins can bind LPG, gp63, and PPG present on the surface of *L. major* promastigotes [33,34,62–64]. TLR-2 and TLR-9 play opposite roles in host response to *Leishmania* infection; TLR-2 is involved in parasite survival in macrophages upon activation by LPG, whereas TLR-9 seems to promote a host-protective response [65]. The best studied

Leishmania protease is gp63, which is capable of activating host phosphatases such as PTP1B, PTP-PEST, TCPTP, and SH-1. Upon activation, SHP-1 downregulates the macrophage response to IFN-γ by interacting with JAK-2, which further modulates TLR and lectin-mediated pathways [66]. However, *Leishmania* phosphatases are equally relevant for the development of the parasite and its survival. LmPRL-1, one of the phosphatases that promotes virulence during macrophage infection, is secreted within exosomes and localized in the PV membrane. During metacyclogenesis, LmPRL-1 is constantly expressed by promastigotes that seems to help the intracellular replication of the parasite in primary mouse macrophages [66]. LPG supports survival of *Leishmania* within human peripheral blood macrophages by suppressing oxidative burst [67]. Murine peritoneal macrophages, stimulated with *L. braziliensis* LPG, show higher TNF-α, IL-1β, IL-6 and NO production than *L. infantum*. Furthermore, *L. braziliensis* LPG activates NF-κB [68]. Induction of TGF-β by murine peritoneal macrophages following infection with *L. amazonensis* has been reported. TGF-β treated mice develop large, non-healing lesions, thus overcoming the inherent resistance to leishmaniasis in C57BL/6 (Th1) mice [69].

Once the contact has been made between the promastigote and the macrophage, it is internalized inside the phagosome. The phagosome surrounding the ingested promastigote fuses with lysosomes and endosomes modify both the membrane and the lumen of the phagosome [70]. These changes transform phagosome into a hydrolytic compartment with a privileged environment for *Leishmania*, termed as parasitophorous vacuoles (PV), an endocytic organelle. The actin-based flagellar motility plays an important role in the phagocytic uptake, taking the promastigote smoothly towards the cell centre inside a PV. The flagellum exhibits active beating, reorienting itself towards the plasma membrane and even protruding out of the membrane. At the end of the intracellular flagellar oscillation, they are drawn close to the host cell nucleus as observed, in real time and at high-resolution time-lapse microscopy, during the encounter between hamster-derived virulent metacyclic-enriched *L. donovani* promastigotes and primary bone-marrow-derived macrophages (BMMs) [71]. This continuous flagellar movement leads to plasma membrane damage, favouring lysosomal exocytosis. The flagellum also acts as a sensory organ being often in contact with the PV membrane to sense the longevity of the macrophages by assessing its metabolites [72,73]. This allows the amastigotes to decide whether to proliferate inside the macrophage or not.

Leishmania species not only survive inside the PV but also multiply without being degraded by the lysosomal enzymes [74]. Phagosomes containing promastigotes poorly interact with endosomes as well as lysosomes and delay the recruitment of LAMP-1. Insertion of LPG into the phagosome membrane destabilizes its lipid microdomains hampering the microbicidal activity in J774 macrophages [75]. This event leads to the exclusion of the membrane fusion regulator, synaptotagmin V (Syt V), which dampens recruitment of the V-ATPase and interferes with the phagosome acidification [76]. Hamster peritoneal macrophages infected with *L. donovani* promastigotes show a decrease in the lysosomal enzymes such as p-galactosidase, N-acetyl-β-D-glucosaminidase, and α-mannosidase [77]. The pinocytic rate of macrophages increases following *Leishmania* infection, resulting in the expression of leishmanial antigens on their surfaces [70]. PV of mouse bone marrow derived macrophage infected with *L. amazonensis* WHOM/BR/75 Josefa strain consisted of host plasma membrane, phagosomal organelles, and parasite-derived [78] proteases, phosphatases, and cytosolic expression of Rab7, LAMP1, and LAMP2 under highly acidic pH [79,80]. Promastigote transforms into amastigote inside the PV and its intracellular metabolism requires a neutral pH. An increase in temperature and a decrease in pH is believed to trigger differentiation of promastigote into amastigote [81]. In the case of *L. amazonensis,* iron uptake and generation of hydrogen peroxide are the major inducers of parasite differentiation [82,83]. The densely packed glycocalyx comprising glycoinositolphospholipids and glycosphingolipids protects the amastigote from the acidic pH as low as 4.0 [84]. Autophagosomes have also been shown to fuse with *Leishmania* PVs [85]. Two distinct types of PVs have been observed inside the infected macrophages, one with

multiple amastigotes, as in *L. amazonensis*, whereas in the case of *L. major,* PV can have a single amastigote parasite [86,87].
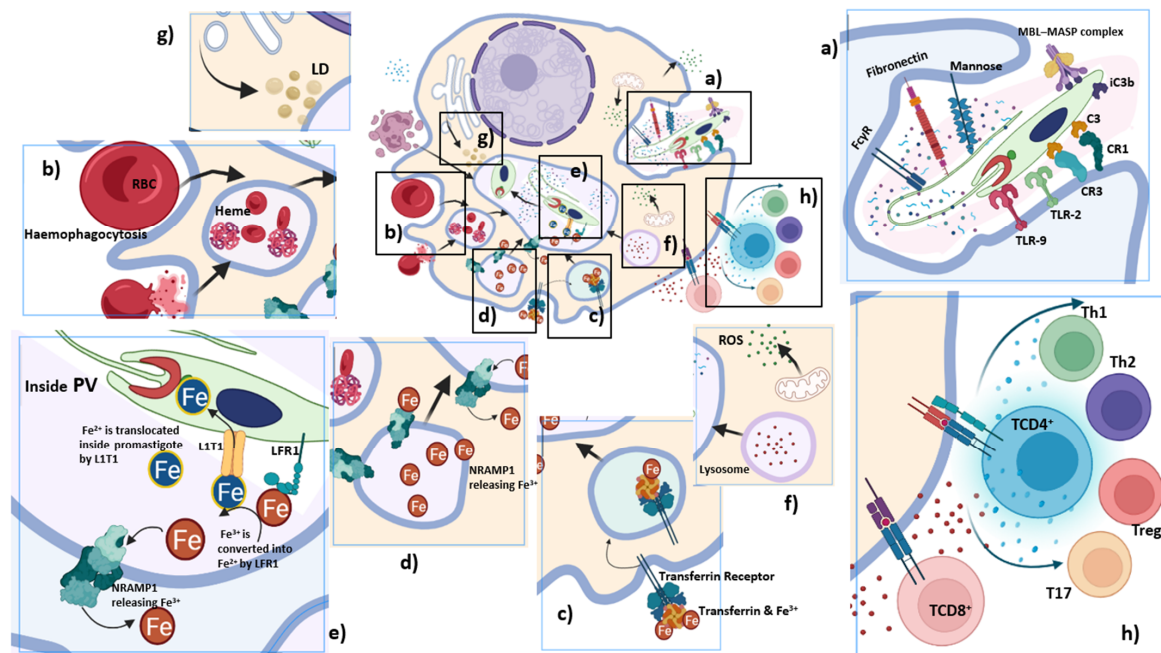

Figure 3. Macrophage as the final destination of *Leishmania*: Once promastigote interacts with the macrophage, it gets internalized inside the phagosome. The phagosome fuses with lysosomes and endosomes; modifications within the membrane and the lumen of the phagosome transforms it into a hydrolytic compartment with privileged environment for *Leishmania*, termed as parasitophorous vacuoles (PV). Inside PV, promastigotes differentiate into amastigotes; (**a**) Uptake of promastigotes by macrophages is a receptor-mediated phagocytosis, where the PAMPs of the *Leishmania* parasite, LPG and gp63, interact with complement receptors (CR1, CR3), Fc receptor, fibronectin receptor, mannose receptor, TLR-2, and TLR-9. *Leishmania* can fix C3, and induce its subsequent cleavage to iC3b. The MBL-MASP complex can also bind to the surface of promastigotes likely via mannose residues, enhancing parasite uptake by macrophages; (**b**) *Leishmania* lack in heme synthesis machinery; hence, it induces infected macrophages to become hyperactive and triggers hemophagocytosis that takes place at a higher rate for the supply of heme; LHR1 helps in the transport of iron; (**c**) *Leishmania* activates iron regulatory proteins to stimulate expression of transferrin receptors for iron uptake. Transferrin, taken up by *Leishmania*-infected macrophages, is delivered to PV. Transferrin endocytosed by amastigotes is degraded within them, since the availability of transferrin promotes amastigote multiplication; (**d**) Nramp1, a membrane protein present on late endosomes and lysosomes of macrophages, fuses with PV and translocates Fe^{2+} from the vesicles into the cytosol. Amastigotes seek continuous iron sources to compete with the host; (**e**) Ferric iron reductase (LFR1) and ferrous iron transporter (LITI) are mediators for iron acquisition and their expression in *Leishmania* is upregulated while inside the PV to compete with the host for iron. LFR1 reduces the insoluble Fe^{3+} to soluble Fe^{2+} and L1T1 transports Fe^{2+} inside the PV; (**f**) Lysosomal vesicles fuse with the PV containing the parasite, but *Leishmania* survives without being degraded by the lysosomal enzymes. The delay in the recruitment of LAMP-1 induced by *Leishmania* also dampens the interactive activity of phagosomes with the lysosomal vesicles inside macrophages. Macrophage activates ROS generation for killing promastigotes and amastigotes. However, during *Leishmania* infection, ROS generation is significantly reduced via upregulation of Heme-oxygenase-1 (HO-1); (**g**) Lipid droplets (LDs) rich in triacylglycerols and sterol esters from ER are released into the cytoplasm and are taken up by PV. Since *Leishmania* cannot synthesize cholesterol, host LDs, low density lipoproteins (LDL), high density lipoproteins (HDL) and lipoprotein lipase compensate for its energy and lipid requirements; (**h**) Th1, Th2, Th17 and Treg cells play an important role in leishmaniasis. Impairment of CD8$^+$ T cell proliferation/function as well as anergy facilitates survival of *Leishmania*.

Upon infection of mouse macrophages with *Leishmania*, ROS generation is significantly reduced, through the upregulation of antioxidant enzyme, Heme-oxygenase-1 (HO-1). An anti-inflammatory condition is created due to the release of carbon monoxide (CO) by HO-1 during heme degradation. CO then inhibits TLR-4 association with MyD88 and TRIF. This further inactivates NF-κB- and IRF3-dependent proinflammatory cytokine production, making the environment conducive for the parasite [88]. Nitric Oxide Synthase (iNOS, NOS2) induced NO production is important for the elimination of *Leishmania* [36]. iNOS gene knock-out mice become more susceptible to *L. major* infection; macrophages derived from iNOS deficient mice are unable to eliminate *L. major* in vitro [89,90].

Leishmania-Induced Hemophagocytosis and Iron Uptake

During erythropoiesis, red pulp macrophages in the spleen, Kupffer cells in the liver and central nurse macrophages in the bone marrow ensure a coordinated metabolism of iron. Macrophages can recycle up to 95% of the iron found in the body while maintaining erythropoiesis through hemophagocytosis, a process where erythrocytes and leukocytes are consumed (phagocytosed) in the bone marrow, liver, or spleen by macrophages or histiocytes. Spleen and liver macrophages phagocytose senescent or injured blood cells, providing continuous delivery of recycled iron under steady-state conditions and during anaemic stress frequency [91]. Trypanosomatid parasites are incapable of producing heme, hence, they infect macrophages for heme acquisition. Following infection (e.g., *Salmonella*, *Mycobacterium*, *Babesia*, and *Leishmania*), hemophagocytosis by macrophages occurs at a high rate [91–95]. Hematophagocytosis has been observed in the bone marrow of infected VL patients recovering from amphotericin B and sodium stibogluconate treatment [96–101].

Hemophagocytosis by heavily infected macrophages in the spleen is the possible cause of anemia in VL. IFN-γ and TNF-α are capable of inducing hemophagocytosis associated with infection by *Salmonella*, Epstein-Barr virus, lymphocytic choriomeningitic virus, cytomegalovirus and *Trypanosoma brucei* [102–106]. *L. donovani* infected macrophages show significant level of hemophagocytosis; uninfected macrophages rarely show hemophagocytosis, suggesting that *Leishmania* is capable of modulating macrophage function directly [107]. Phagocytosis of RBCs also allows nutrient availability for the pathogen, helping in their growth and survival. Down-regulation of Signal regulatory protein α (SIRPα) was observed in *L. donovani*-infected RAW264.7 cells, as well as in MGCs in the spleen of mice infected with *L. donovani* [107]. CD47-SIRPα signaling is the known mechanism for limiting hemophagocytosis, which recognises RBCs and gives the phagocytosis-inhibitory signals. *L. donovani* infection thus induces the downregulation of SIRPα in macrophages and disrupts the CD47-SIRPα interaction resulting in hemophagocytosis.

Slc11a1 (formerly Nramp1; Natural resistance-associated macrophage protein) is a membrane protein of late endosomes and lysosomes of macrophages, which translocates Fe^{2+} from the vesicles into the cytosol [108]. This is immediately recruited to the membrane of microbe-containing phagosomes so that *Leishmania* amastigotes need to compete with the host for iron. This is recognized as a host susceptibility gene for infections with *L. donovani* and *L. infantum* [109]. Nramp1 is downregulated by ubiquitin-proteasome degradation pathway in case of *Leishmania* infection [110]. Hepcidin, the iron-regulatory peptide hormone, induces the signalling for downregulation of Nramp1. Thus, when Nramp1 degradation is blocked using proteasome inhibitor, or by transcriptional agonist of hepcidin, it leads to depletion of phagolysosomal iron pool [110]. *Leishmania* can also induce the host cell to internalize iron. *L. donovani* activates iron regulatory proteins (IRPs), which further stimulate expression of transferrin receptors [111]. PVs of L. amazonensis infected macrophages fuse with transferrin-containing endosomes to maintain a constant supply of iron for the proliferating amastigotes [112]. There are three mediators for iron acquisition that are upregulated in Leishmaniasis; ferric iron reductase (LFR1), ferrous iron transporter (LITI) and heme transporter (LHR1). LFR1 reduces the insoluble Fe^{3+} to soluble Fe^{2+} and L1T1 transports Fe^{2+} inside the PV. *Leishmania* expresses LHR1 and LIT1 to uptake iron within macrophages (Figure 3) [81]. Macrophages infected with *L. donovani*

promasigotes as well as amastigotes show inhibition of NAD(P)H oxidase assembly at the phagosome membrane [113].

Lipid Uptake by *Leishmania*

For an intracellular parasite like *Leishmania,* lipid metabolism is of considerable importance to fulfil its need of lipids. A significant increase in cholesterol content and moderate decrease in Phospholipid (PL) content has been observed during the differentiation of promastigotes into amastigotes. A decline in the membrane and serum cholesterol levels [114] in the VL patient was found to be inversely proportional to the parasitic load in the spleen [115,116]. An increase in Phosphatidylserine (PS) in amastigotes may also suggest its role in the pathogenicity of leishmaniasis [117]. *Leishmania* can degrade CerPCho (sphingomyelin) from host cells possibly to enhance its virulence [118]. Lipid droplets (LD), rich in triacylgylcerols and sterol esters, are derived from the accumulation of newly formed lipids within ER. These LDs bud off from ER and are released into the cytoplasm [119]. *Leishmania* cannot synthesize cholesterol, and thus, host LDs, low density lipoproteins (LDL), high density lipoproteins (HDL) and lipoprotein lipase, compensate for its energy and lipid requirement. *Leishmania* is capable of capturing cholesterol from LDL through its specific LDL binding sites. *L. amazonensis* has both HDL and LDL binding sites and can scavenge cholesterol from plasma. Host cholesterol has been identified on the membrane extensions of *L. amazonensis* [120]. In *L. donovani,* COX-2-mediated PGE_2 release is involved in downregulation of microbiocidal activity of macrophages [121]. *Leishmania* vector saliva can modulate eicosanoids metabolism and LD formation in the host cells [122,123]. The LD formation in macrophages infected with *L. major* has also been documented; LDs are observed inside PV as well as in the parasite cytoplasm. [124].

Cytokine Induced Macrophage Activation

Macrophages are indispensable for parasite survival, replication and differentiation; at the same time, they are also the major effector cells responsible for elimination of the parasites. For *Leishmania,* the macrophage is the final host cell for its proliferation. There are two functionally distinct macrophage phenotypes: M1, a classically activated pro-inflammatory subtype with microbicidal properties, and M2 that is an alternatively activated anti-inflammatory subtype that is associated with the resolution of inflammation. Th1 cell-mediated production of IFN-γ, TNF-α, and GM-CSF polarizes macrophages to M1 phenotype, whereas Th2 cell-mediated production of IL-4, IL-10, IL-13, TGF-β, and M-CSF polarizes macrophages to M2 phenotype [125]. M1 macrophages are characterized by a high production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12, IL-18, IL-23, and Type 1 IFN [126]. Restriction of parasite growth has been associated with the induction of robust Th1 responses and activation of iNOS-expressing M1 macrophage [127]. During *Leishmania* infection, mTOR (mammalian target of rapamycin) pathway plays an important role in regulating M2 polarization. It increases expression of M2 markers such as arginase-1, IL-10, TGF-β, CD206, and CD163, while causing reduction in the expression of M1 macrophage parameters such as ROS, NO, iNOS, NOX-1, IL-12, IL-1β, and TNF-α. This M2 polarization induced by *Leishmania* aids in the survival of parasite inside the host [128]. Reciprocal changes in histone lysine methylation/demethylation of M (LPS + IFN-γ)/M(IL-10) genes is one of the factors that direct macrophage polarization for its successful establishment within the host [129].

L. donovani infection stimulates TNF-α production by macrophages but abolishes IL-1β generation in vitro and in vivo [130–133]; in the case of infection with *L major,* IL-1β generation by macrophages is enhanced [134]. Expression of TNF-α in response to LPS is also inhibited in *L. donovani*-infected macrophages [130]. Cells of the mouse pouch exudate recruited in response to *Leishmania* infection seem to express RANTES, MIP-1α, MIP-1β, MIP-2, IP-10, MCP-1 and TCA-3 [135], suggesting recruitment of a mixed immune cell population including neutrophils, monocytes, macrophages, and eosinophils.

Active leishmaniasis in humans is associated with an increased level of serum IL-10 as well as increased IL-10 mRNA expression in tissue lesions [136,137]. IL-10 production represents a shift from a pro-inflammatory and protective immune response to a regulatory immune response, causing uncontrolled disease progression. Infected macrophages are activated by IFN-γ and TNF-α to kill intracellular amastigotes via the L-arginine nitric oxide pathway [138,139]. *Leishmania*-specific lymphoproliferative unresponsiveness and lower production of IFN-γ upon *Leishmania* antigen stimulation lead to susceptivity towards development of clinical VL [140,141]. Th1 polarization leads to immunosuppressive response [137]; thus, negating IFN-γ can be vital for *Leishmania* survival.

3.1.3. Dual Role of Complement

The complement system acts as a first line of host immune defense that is activated by a proteolytic cascade to eliminate the invading pathogens through the membrane attack complex (MAC) formation and opsonisation. Complement receptors are the primary mediators of parasitic adhesion; in the presence of complement, *Leishmania* can bind efficiently with macrophages. Complement plays a diabolically opposite role: complement-mediated lysis by MAC leads to the elimination of parasites, whereas opsonization by C3b/iC3b enhances phagocytic activity that aids in the internalization and eventual survival of the parasite. Opsonization with C3 leads to enhancement of binding and phagocytosis of *L. major* promastigotes by macrophages. *L. enrietti* and *L. tropica* activate the complement alternative pathway leading to iC3b deposition on its surface, and hence, enhanced uptake of promastigotes via complement receptors [142].

The cutaneous species of *Leishmania* (*L. major, L. mexicana, L. mexicana amazonensis, L. braziliensis guyanens, L. enriettii* and *L. tropica*) are susceptible to lysis by normal serum through activation of the alternative pathway on the promastigote surface [142,143]. For visceral strains such as *L. chagasi* promastigotes, complement seems to be a vehicle to escape from the inoculation site to viscera [144]. Complement plays an important role in controlling the CL lesions caused by *L. amazonensis*. Complement-dependent adhesion of *Leishmania* is mediated by leukocyte integrin Mac-1 and CR1 [145]. Both metacyclic and logarithmic-phase promastigotes of human *L. major* are capable of binding to Mac-1 or CR1. Mac-1 is also the predominant receptor mediating the internalization of complement-opsonized metacyclic *L. major* promastigotes by monocyte-derived macrophages [146]. Promastigotes of *L. donovani* activate the complement classical pathway when opsonized by host natural antibodies and deposit C3 on the parasite surface [146]. C3-coated promastigotes bind to RBC as well as mononuclear phagocytes through CR1 and CR3 [147], leading to enhanced phagocytosis. Promastigotes of *L. braziliensis* can bind to the MBL–MASP complex likely via mannose, activate the complement lectin pathway, and thus, enhance parasite uptake by phagocytes [148]. The binding of MBL, CL-11, ficolin -1, and ficolin-3, but not ficolin-2, was observed on the surface of live metacyclic promastigotes, highlighting the possible role MBL and ficolins play in the initial innate immune response to *L. infantum* [149]. The complement regulatory protein, factor H, from human serum and factor H-like proteins from dog serum, were found to bind to *L. infantum* and inactivate C3b in the presence of factor I [150].

3.1.4. Role of Dendritic Cell

DCs are capable of recognizing PAMPs expressed by *Leishmania* via PRRs leading to their activation, which can greatly influence innate and adaptive immune responses in order to promote parasite eradication. During infection with *L. braziliensis*, TLR-2 deficiency (TLR2$^{-/-}$ mice) resulted in activation of DC with increased IL-12 p40 production and decreased IL-10 production. This induced reduction in lesion size in TLR2$^{-/-}$ mice, with limiting *L. braziliensis* infection. Deficiency of MyD88 inhibited IL-12 p40 by *Leishmania*-infected DCs, which resulted in lower levels of DC activation, impairing the protective immunity [151]. In the case of *L. mexicana*, TLR-2 of monocyte derived DCs interacts with LPG to enhance MHC class II and CD86 expression [152]. DC/TLR-9 interaction enhances

neutrophil recruitment during *L. infantum* infection in mice [153]. Bone marrow-derived DCs via intracellular TLR-9 interaction produce IL-12, which further enhances cytotoxicity of NK cells and IFN-γ expression [154].

Cell surface DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) mediates efficient internalization of *L. mexicana* promastigotes in vitro [155]. DCs stimulated with excreted-secreted antigens (ESA) of *L. major* and *L. donovani* show reduction in the expression of DC-SIGN [155]. Parasitic uptake by DCs leads to their enhanced migration to lymph node for antigen presentation to T cells. *Leishmania* have evolved strategies to interfere with DC migration, by inhibiting expression of CCR7 through IL-10 production [156]. In animal models, CCR2 gene knock-out [157] as well as deficiency in CCL19 and CCL21 [156] caused reduced DC migration to secondary lymphoid organs for antigen presentation.

DCs produce IL-12, and promote Th1 differentiation, characterized by IFN-γ production by Th1 cells, which leads to parasite elimination. Epidermal Langerhans cells (LCs), observed near the inoculation site, internalise *L. major* amastigotes more than promastigotes. The parasite uptake sets off protective Th1 immunity, which is associated with upregulation of MHC-class I and class-II antigens, costimulatory molecules such as CD40, CD54, CD80, and CD86, and IL-12 p40 production [158]. However, infection with *L. amazonensis* can alter DC functions, favouring parasite survival. Amastigote infection is incapable of inducing CD40-dependent IL-12 production [159]. *L. infantum* infected DCs express less costimulatory molecules and higher levels of IL-10 [160]. Thus, *Leishmania* is also capable of downregulating Th1 mediated adaptive immune response as one of its pathogenic mechanisms.

3.2. Adaptive Immune Response

The course of *Leishmania* infection and its resolution greatly depends on efficient establishment of cell mediated immune response. T cell response correlates with recovery from human leishmaniasis as well as resistance to it. CD4$^+$ T cell response seems to correlate with lesion development, while activation of CD4$^+$ and/or CD8$^+$ T cells appears to be essential for the healing process [161]. Th1, Th2, Th17, and Treg subsets play important roles in leishmaniasis [162,163]. There is some evidence of antibody-mediated protection; however, cell-mediated immune response is principally instrumental in providing protection against leishmaniasis [164–166].

3.2.1. Immunomodulation by T Helper Subtypes
Th1 and Th2 Polarization

The Th1 immune response not only plays a critical role in protecting the host against primary infection but also provides lifelong immunity to reinfection [167,168]. A protective immune response against CL due to *L. major, L. mexicana*, or *L. amazonensis*, as well as VL caused by *L. donovani* or *L. infantum*, relies on the establishment of the pro-inflammatory T cell profile [163]. CD4$^+$ Th1 cells induce macrophage-driven proinflammatory response which secrete TNF-α, IL-1β, IL-6, IL-12, IL-18, and IL-23 cytokines, induce ROS generation, and enhance phagocytosis [126,169]; together, they are very effective in generating protective immunity against *L. donovani* [170]. In murine experimental CL, protective immunity appears to be dependent on IFN-γ producing CD4$^+$ Th1 and CD8$^+$ Tc1 cells. Whether CD4$^+$ and CD8$^+$ T-cell responses are involved in resistance and/or cure of VL depends on their efficient memory responses, influenced by IFN-γ, IL-2, and TNF-α. CD4$^+$TNF-α$^+$IFN-γ$^+$, CD4$^+$IL-2$^+$TNF-α$^+$IFN-γ$^+$, CD4$^+$TNF-α$^+$, and CD4$^+$IFN-γ$^+$ T cells increase throughout the treatment. CD8$^+$IL-2$^+$TNF-α$^+$IFN-γ$^+$ and CD8$^+$TNF-α$^+$IFN-γ T cell counts also increase, contributing to the healing process [171]. Th2 response is correlated with an anti-inflammatory phenotype producing IL-4, IL-13, IL-10, and TGF-β cytokines. IL-4, IL-10, and TGF-β modulate Th1 responses by dampening macrophage activation, and hence, aggravate the disease [172,173]. Th2 response can also induce IL-21-mediated downregulation of iNOS, TNF-α, and TLR-4, allowing the *Leishmania* parasite to

proliferate [174,175]. Poudel et al. (2020) have shown that IL-4 promotes CL pathology by promoting Th2 immune response as well as via pathogenic CD8$^+$ T cell responses [176]. IL-10 is an important regulatory cytokine and its sources have been identified as CD4$^+$/CD25$^+$ T cells (Th2), CD4$^+$/CD25$^-$/FoxP3$^+$ regulatory T cells (Tregs) and CD4$^+$/CD25$^-$/FoxP3$^-$ T cells (Th1) [177–179]. IL-10 is capable of inhibiting phagocytosis, and thus, it contributes to the growth and spread of *Leishmania* [180,181]. IL-10 acts as an immunosuppressive factor in VL and facilitates the spread of *Leishmania* parasites. IL-10 secretion by T cells can influence immune activation at the early stage of infection, which renders BALB/c mice susceptible to an uncontrolled *L. major* infection [182]. IL-27 was found to have pleiotropic effects on Th1, Th2, and Th17 cells during *L. major* infection; it exhibits a regulatory role in balancing between protective immunity and pathogenesis during leishmaniasis. Thus, *Leishmania* infection is characterized by a mixed Th1/Th2 response, where a dominant Th1 response provides resistance, whereas a dominant Th2 response confers susceptibility.

T Regulatory and T17 Cells

T regulatory cells (Tregs) play a fundamental role in the infection and persistence of *Leishmania*. In the case of *L. donovani* and *L. major* infection, Tregs lead to disease exacerbation and prevent immune-mediated parasite clearance and disease reactivation [183,184]. Tregs are also beneficial in resolving a hyper-inflammatory state and lead to disease remediation in case of *L. amazonensis* [185]. In the murine model of CL, Tregs act as a major suppressor of T effector cells. There is an accumulation of IL-10 producing Tregs (CD4$^+$CD25$^+$FoxP3$^+$) at the CL lesions as well as in VL patients with persistence of parasite load and reactivation of the pathology [137,184]. Tregs isolated from bone marrow produce IL-10 and inhibit effector T cell activation. Drug unresponsive patients exhibit higher levels of IL-10, indicating its role in immunosuppression in VL patients [186]. Anderson et al. have suggested CD4$^+$CD25$^-$Foxp3$^-$Th1 cells as the dominant player in IL-10–mediated immune suppression in chronic CL induced by *L. major;* in the absence of Foxp3$^+$ cells, expansion of antigen-induced CD4$^+$ T effector cells takes place optimally [179,187]. PBMCs from CL patients, when challenged with *Leishmania* antigens, show upregulation of the activation markers CD25 and CD69 and expression of costimulatory molecule, CD86 [188]. Adoptive transfer of Tregs from naive mice can halt disease progression, reduce the parasite burden, and downregulate production of IL-10, IL-13, IL-17, and IFN-γ in a mouse model of chronic *L. (Viannia) panamensis* infection. Impaired Treg cell response during *L. (Viannia) panamensis* infection has also been reported [189]. However, the Treg-mediated immune suppression among human patients still needs validation.

Th17 cells can play a role in balancing the pro- and anti-inflammatory responses in experimental models as well as in patients with *Leishmania* infection [162,163] Elevated levels of IL-17, suggesting activation of Th17 cells, have been observed in patients with CL and MCL, due to *L. major* [53,190], *L. braziliensis* [191], *L. tropica* [192], *L. panamensis* [191], *L. guyanensis, L. amazonensis*, and *L. naiffi* [193] infections. IL-17 also acts as a crucial modulator of adaptive immunity against *Leishmania* by initiating neutrophil recruitment [162]. At the early phase of VL infection, a strong IL-17 response was observed which progressively reduced to basal level during chronic VL, due to suppression of Th17 cell proliferation by Tregs (CD4$^+$CD25$^+$ T cells). TGF-β and IL-35, derived from CD4$^+$CD25$^+$ T cells, are the key mediators for the downregulation of IL-17 during chronic VL [194]. A recent experiment in BALB/c mice with *L. donovani* infection showed that low levels of Th17 cytokines, IL-17, IL-22 and IL-23, and elevated levels of IL-6, IL-1β and TGF-β were associated with active infection. Amphotericin B treatment restored production of IL-17 and IL-22, suggesting Th17 cytokines are possibly associated with protection against VL infection [195].

3.2.2. CD8 T Cells

In human CL, CD8$^+$ T cells are capable of aggravating disease pathogenesis as well providing protection to the host [196,197]. Joshi et al. have shown impairment of CD8$^+$ T cell function facilitating survival of *L. donovani;* chronic infection in the mouse model leads to

proliferation of defective/anergic CD8$^+$ T cells [198]. The expressions of negative regulators of T cell activation, Cytotoxic T lymphocytes antigen 4 (CTLA-4) and programmed death protein 1 (PD1) are elevated in VL patients, together with upregulated IL-10 mRNA expression, CD94, CD158a, and CD158b [199]. T cells from VL patients stained more positive for Fas and Annexin-V in comparison to post-treatment or healthy controls [200]. CD8$^+$ T cells secreting IL-10 have been noted in PKDL as well as with *L. guanyensis* infection [201,202]. In the lesions from PKDL patients, CTLA-4 mRNA expression was higher in the case of pre-treatment compared to post-treatment or controls [203]. B7-H1 (290-amino-acid type I transmembrane glycoprotein belonging to B7-CD28 family) preferentially co-stimulates IL-10 production in resting T cells; PD-1/B7-H1 interaction mediates the inhibition of activated T cell response [204]. Blockade of B7-H1 thus is possibly a way to enhance T cell response and inhibit *L. donovani* infection [198–206].

3.2.3. Defects in Antigen Presentation to T Cells

Leishmania infection-induced defects in antigen presentation has been observed. It inhibits induction of MHC class I and II, as observed in case of *L. donovani* [131], or brings about defects in peptide loading while expressing normal level of MHC class II in *L. amazonensis* infection [207,208]. Defective antigen loading has also been observed in *L. major* infected macrophages [209]. Reduction of MHC class I-restricted antigen presentation upon infection with *L donovani* parasites has been observed in murine studies. [131,210]

Subsequent to the binding of T-cell receptor (TCR) to the MHC II-peptide complex on the antigen presenting cells (APCs), binding of CD28 or CD40L on T cells to costimulatory molecules such as those of the B7 family or CD40 provides a key element for the exquisite control of T cell activation. *Leishmania* can interfere with macrophage costimulatory signals. *L. donovani* infection blocks LPS-mediated B7-1 expression in infected macrophages [211,212]. In the case of infection with *L. amazonensis*, disruption of CD40/CD40L ligation results in enhanced susceptibility [213] through inhibition of iNOS expression [213,214] and IL-12 production [215] by infected macrophages. Macrophages infected with *L. major* also show defects in CD40 signalling in a p38-dependent manner [216]. Interaction between CD28 on T cells and B7 molecule is a major costimulatory signal for T cell activation on murine peritoneal macrophages; its expression is also decreased on the surface of *L. donovani*-infected BALB/c macrophages [212]. *L. chagasi* infection downregulates CD11b expression in monocytes, diminishes CD54 and HLA-DR expression in infected monocytes, and IFN-γ stimulated HLA-DR and HLA-ABC expression in infected macrophages. There is a negative correlation between CD54 and CD86 expression in both monocytes and macrophages, possibly leading to anergy [210]. Infected macrophages with *L. donovani* demonstrate profound effects on the self-peptide repertoire presented by MHC I molecules, as evident through changes in antigen processing such as in the composition of proteasomes, altered protein expression and turn-over in different cellular compartments [217].

4. Vaccine Strategies

Antimonial drug-based treatment does not confer significant protection as it is toxic to the host and sometimes fail to achieve recovery due to antimicrobial resistance [218,219]. A range of pre-clinical models using murine, canine, and hamsters have been developed to assess the candidate vaccines to prevent VL and CL. Currently, there is no licenced vaccine for human leishmaniasis although several vaccines are undergoing clinical trials. Recently, two new strains of *L. major*- MHOH/IL/2019/MRC-01 (*L. major* MRC-01) and MHOH/IL/2019/MRC-02 (*L. major* MRC-02) were found in Israel; *L. major* MRC-02 strain was selected as a new vaccine candidate for CL [220].

4.1. First-Generation Vaccine

The first-ever vaccine against Leishmaniasis, developed in the early 1940s, called 'Leishmanilization', has been used for over 60 years [221]. The technique involves injecting

live virulent parasites in healthy individuals usually in an inconspicuous area of the body (face or limbs), to protect the recipient from subsequent disfiguring natural infection [222]. This approach was later developed and live virulent *L. major* promastigotes were harvested and used in large-scale vaccination trials. In 1967, it began in Uzbekistan where a mixture of live and killed promastigotes were used. During the 1970s in Iran and in the 1980s in Israel, Leishmanization (LZ) was initiated, but subsequently discontinued [223]. In Iran, in preliminary trials, ~80% protection was achieved; however, the practice of LZ was discontinued due to major complications including non-healing skin lesions, exacerbation of skin diseases, loss of infectivity of the parasites upon repeated subculturing and the potential impact of immunosuppression [224,225]. LZ got replaced by First generation vaccine based on live attenuated, fractionated *Leishmania* antigen and killed parasites, offering a safer vaccine.

4.1.1. Live Attenuated Vaccine

Attenuated parasites that are infectious but not pathogenic have major advantages as live vaccines since they can mimic the natural course of infection [226]. Extensive research has been carried out to test the efficacy of attenuated live vaccines in canine and mice models with *L. mexicana* and *L. major* for CL, and *L. infantum* and *L. donovani* for VL [227]. Razi Vaccine and Serum Institute, Iran conducted phase I and II studies on the safety and immunogenicity of different doses of inactivated *L. major* promastigotes with or without BCG. BCG was used as an adjuvant in a few versions of the Venezuelan, Ecuadorian and Iranian candidate vaccines to enhance the cell-mediated immunity. A trivalent preparation consisting of *L. brazilensis, L. guyanensis* and *L. amazonensis* antigens was evaluated in Ecuador. The autoclaved *L. major* preparation mixed with BCG adjuvant was used in several field trials in Iran and Sudan, which was later replaced by a formulation involving precipitation of the autoclaved *L. major* in aluminium hydroxide (alum-ALM). Alum-ALM mixed with BCG showed significantly higher ability to convert the leishmanin skin test [228].

Live attenuated parasites can be classified into two groups depending on the attenuation techniques. Undefined attenuation can be carried out through irradiation or by chemical mutagenesis with or without overt selection; for example, in vitro selection with the aminoglycoside antibiotic gentamicin was used to attenuate *L. major, L. mexicana* and *L. infantum* [229]. Defined attenuation involves specific mutagenesis to derive mutant parasites through targeted gene knock-out, where the parasites are unlikely to recover the deleted genes. Several gene targets have been selected for defined attenuated *Leishmania* vaccines, such as *dhfr, lpg2, cpa, cpb, Ufm1, p27, SIR2, BT1, HSP70, centrin*, and the paraflagellar rod-2 locus [227,230–232]. However, genetically modified vaccines showed varying degree of stability and protection in animal models [227,233]. *L. major* dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene knock-out was the first to be tested against virulent *L. amazonensis* and *L. major* infections as a potential vaccine [234,235] in susceptible and resistant murine models, but failed to confer protective immunity in rhesus monkey [236]. *L. mexicana* lacking cysteine proteinase genes, *cpa* and *cpb*, was found to be protective in murine models [237]. The cpa/cpb-deficient *L. mexicana* exhibited significantly lower levels of Th2-associated cytokines such as IL-10 and TGF-β than the wild types in the primary lesion of hamsters [238].

Centrin is a calcium-binding cytoskeletal protein required for centrosome duplication and segregation in higher eukaryotes. Attenuated *L. donovani* strain with centrin gene deletion (Ldcen$^{-/-}$) when used in BALB/c mice showed growth arrest in amastigotes but promastigotes were unaffected [239]. Ldcen$^{-/-}$ parasites also induced high antibody titre when compared with commercially available vaccine against Canine VL [240]. A single dose (1×10^7 Ldcen$^{-/-}$) without any adjuvant elicited strong CD4$^+$ and CD8$^+$ T cell activation along with Th1 predominant immune response and considerable reduction in bone marrow *L. infantum* load [241].

Co-cultured CD4$^+$ T cells and macrophages from dogs, immunized with Ldcen$^{-/-}$ and challenged with *L. infantum,* exhibit high microbicidal activity [242]. Ldcen$^{-/-}$ vaccination offered significant protection only in young mice against *L. donovani* which correlated with increased Ig2a antibody, lymphoproliferative response, and significant NO production. However, it failed to induce an adaptive immune response in aged mice [243]. Ldcen$^{-/2W-}$ parasite vaccination also showed efficacy in asymptomatic infection against VL. When C57Bl/6 mice were infected with 10^3 parasites (i.v.) of wild *L. donovani* expressing LL0 epitope (bacterial exotoxin listeriolysin-o of *L. monocytogenes*) and after 3 weeks, they were immunized with Ldcen$^{-/-}$ expressing 2W epitope, the result showed comparable CD4$^+$ T cell proliferation and CD4$^+$ memory cell response (T$_{cm}$) in both asymptomatic and naive animals that received *Ldcen$^{-/-}$* immunization. They also showed reduction in splenic parasite burden [244]. Intradermal immunization with Ldcen$^{-/-}$, combined with an adjuvant salivary protein LJM19 from sand fly vector, conferred long-lasting protection against VL in hamsters in a 9-months study period [245]. Banerjee et al. showed that Ldcen$^{-/-}$ parasites induced IL-23-dependent IL-17 production in a murine model of VL [246]. In various CL endemic areas in Iran, the *centrin* gene in *L. infantum* expressed more highly in exponential growth phase than in stationary phase, highlighting its essential role in parasite proliferation, and hence, be a promising candidate for developing a genetically modified live attenuated vaccine [247].

L. donovani parasites lacking amastigote specific protein p27 (Ldp27$^{-/-}$) are safe as an immunogen. Twenty week following virulent challenge (3 × 10^6 stationary phase Ldp27$^{-/-}$), a significant reduction in parasite burden, induction of pro-inflammatory cytokines responses, and increased leishmanial activity in association with NO were observed. Thus, Ldp27$^{-/-}$ vaccination is capable of offering long-term protection in BALB/c mice [248]. A genetically modified live attenuated *L. major* (MRH0/IR/75/ER) strain lacking the p27 gene has been evaluated for its immunogenicity. BALB/c mice were inoculated subcutaneously (s.c.) with 3 × 10^6 stationary phase Lmp27$^{-/-}$ mutant promastigotes which induced Th1 response, along with no skin lesion and low parasitic burden in liver and spleen [249]. *L. major* p27 gene knock-out (Lmp27$^{-/-}$) strain also elicited its protective immunity against homologous *(L. major)* and heterologous (*L. infantum*) infections [250]. IFN-γ and IgG2a levels were increased in both immunized groups, while the IFN-γ/IL-4 and IgG2a/IgG1 ratios showed a polarization towards a Th1 response [250].

LiΔHSP70-II attenuated vaccine includes *L. infantum* deletion mutant, lacking both *HSP70-II* alleles (ΔHSP70-II). BALB/c VL mice model, vaccinated subcutaneously with 10^7 LiΔHSP70-II stationary promastigotes in the right footpad, showed a reduction in liver damage and rapid parasite-specific IFN-γ production by CD4$^+$ and CD8$^+$ T cells; however, this vaccine failed to control the chronic phase of the disease [251]. LiΔHSP70-II vaccinated BALB/c mice controlled the progression of CL caused by *L. amazonensis* infection, with enhanced IFN-γ and systemic Th2 mediated humoral response, and reduction of IL-10 secretion that favours the IFN-γ to activate leishmanicidal activity by macrophages [252].

4.1.2. Killed Parasite Vaccines

In the late 1930s, the pioneering work of Brazilian scientists demonstrated the efficacy of killed parasites as therapeutic as well as prophylactic vaccines against CL and VL [253]. However, no first-generation killed vaccine has shown sufficient efficacy as a prophylactic vaccine [254]. A whole-cell vaccine approach using *L. infantam* and *L. chagasi* promastigotes, treated with amotosalen (S-59), a synthetic psoralen, and with low UV irradiation, resulted in permanent covalent DNA cross-linking within parasites. These vaccines were called Killed but Metabolically Active (KBMA). Mice immunized with KBMA stationary-phase promastigotes (3 times at 2-week intervals) were capable of activating macrophages producing NO [255]. Whole-cell killed recombinant *L. tropica* stress-inducible protein (LFST-II) conferred better protection from whole-cell killed soluble *L. tropica* antigen (SLA). It showed higher delayed type hypersensitivity (DTH) response and IFN-γ production [256]. *L. tropica*

has also been used to construct DNA vaccine recently based on its ribosomal L5 gene, which exhibited up-regulated Th1 response following leishmanial infection [257].

4.2. 2nd Generation Vaccination

Second-generation vaccines are based on recombinant/synthetic antigens/peptides, recombinant bacteria/virus expressing antigens, or genetically modified *Leishmania* species, and native fractions purified from parasites [230,258]. Several *Leishmania* proteins have been identified as vaciine candidates based on their abundance and surface localizations.

4.2.1. gp63

gp63 is a major immunodominant 63-kDa glycoprotein expressed on *Leishmania* promastigotes that greatly influences host cell signaling mechanisms and aids in survival inside macrophages [259]. Liposomal formulations have been used as a potent adjuvant for gp63-based vaccine. gp63, purified from *L. donovani* and formulated in cationic distearoylphatidylcholine (DSPC) liposomes, induced protective response against VL in BALB/c mice; animals were intraperitoneally immunized with 3 doses at 2 weeks' interval (2.5 mg of gp63 entrapped in liposome). Mice showed IFN-γ augmentation and down-regulation of IL-4 [260]. Monophosphoryl lipid-trehalosedicorynomycolate (MPL-TDM)- based liposomes containing gp63 elicited high IgG2a and IFN-γ at three weeks; early immune response was mixed Th1/Th2 along with IL-12 and increased NO from DCs [261]. HLA-A2 restricted peptide derived from *L. mexican* or *L. major* gp63, and HLA-DR1 restricted peptide derived from *L. major* gp63, showed immunogenicity when tested in HHD-II mice (HLA-A0201 transgenic mice containing human HLA-A0201 gene and mouse H2-K^b gene, for MHC Class I in BALB/c mice) and FVB/N-DR1 mice (transgenic mice having HLA-DR1 gene inserted) for MHC Class II, respectively [262,263]. Another vaccine formulation containing HLA-DR1 or HLA-AA2 peptides derived from *L. major* gp63 with an oily adjuvant (Montanide^{TM}), or both the peptides associated with adjuvant, were able to generate a strong IgG response against *L. infantum* and cellular response for up to 211 days after the last vaccination for VL without presenting any toxicity in immunized animals [264].

BALB/c mice vaccinated with amastin-gp63 (15 μg) showed the best result among three of the epitope-based vaccines (KMP-11-gp63, Amastin-Gp63, and Amastin-KMP-11). At the 12th week post-infection, parasite reduction rates of KMP-11-gp63, Amastin-gp63, and Amastin-KMP-11 groups were 88.42%, 91.01%, and 89.38%, respectively. These epitope-based vaccines are rich with HLA-DR1, HLA-A2, HLA-A24 restricted pepides. Amastin-gp63 contained a largest number of HLA-A2, HLA-A24, HLA-DR1, and H2-d epitopes, and exhibited better protective efficacy. IgG1 and IgG2 isotype levels were also high post immunization, suggesting that the vaccines induced a dominant Th2 response [265]. Another multi-epitope vaccine approach, using gp63 along with LPG3, Thiol-specific antioxidant (TSA), LmSti1 antigens from *L. major,* showed poor antigenicity (0.92%) [266].

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

L. infantum Activated protein kinase A receptor analogue (LACK) antigen has been found to confer protection against *L. major* in BALB/c mice when administered along with IL-12 before infection [267]. Recombinant *Lactobacillus lactis*, secreting IL-12 and expressing LACK antigen, was administered subcutaneously in mice (0.5×10^9 live bacteria) fortnightly. The delay in footpad swelling with significant reduction in parasite burden in immunized mice was seen; immunization induced Ag-specific CD4$^+$ Th1 and CD8$^+$ CTL response [268]. Oral immunization using live *L. lactis* co-expressing LACK and IL-12 elicited protective Th1 response against *L. major* infection in BALB/c mice [269].

L. tarentulae-LACK/KMP-11/EGFP in stationary phase along with CpG oligonucleotide (ODN) adjuvant was tested in mouse left footpad (2×10^7 *L. tarentulae*–LACK/KMP-11/EGFP parasites plus 20 μg CpG ODN), which showed significant reduction in parasite load [270]. When *L. major* soluble antigen (SLA) or recombinant LACK (rLACK) antigen were delivered subcutaneously together with cholera vaccine to BALB/c mice, a Th1 type response

was observed with increased IFN-γ production; however, an exacerbation of infection occurred following challenge with *L. major* [271]. Liposomes consisting of 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) encapsulating SLA with polyethelene glycol (PEG) failed to confer protection in the murine model of *Leishmania* infection [272]. However, immunostimulatory cationic lipids formed from DOTAP, 1,2-dioleoylsn Glycero-3-phosphoethanolamine (DOPE), and cholesterol encapsulating single-gene expression plasmid of pcLACK showed protection against CL [273].

4.2.3. Kinetoplastid Membrane Protein-11 (KMP-11)

KMP-11 is found in both promastigotes and amastigotes of *Leishmania*. KMP-11 transfected macrophages derived from bone marrow of BALB/c mice and allogenic DCs derived from the bone marrow of C57BL/6 mice, elicited significantly strong CD8^{+} CTL response [274]. Ex-vivo, KMP-11 pulsed bone marrow-derived DCs and CpG oligonucleotide also induced protection in a murine model of VL by reducing parasite load in visceral organs. This reflected in restored lymphoproliferative responses and modulation of parasite-specific Th1 and Th17 responses, isotope switching towards IgG2a, and enhanced production of IFN-γ. The vaccinated group also showed downregulation of IL-10 and TGF-β, which were upregulated in control mice group, promoting persistence of infection [275]. KMP-11 delivered through DCs elicited protective cellular immunity through pro-inflammatory cytokines (IFN-γ, IL-12, TNF-α), followed by upregulated P38-MAPK signaling [276]. KMP-11, when encapsulated in polyglycolide-co-L-lactide (PGLA) nanoparticles, also reduced the parasitic burden via induction of macrophage-driven pro-inflammatory immune response [277].

In a recent report, eukaryotic subunit vaccine comprising of recombinant LACK and KMP-11 antigen along with CpG ODN adjuvant showed a more effective immune response in BALB/c mice than prokaryotic subunit vaccine by shifting the immune response towards Th1 response [278].

4.2.4. Fucose Mannose Ligand (FML) and Monophosphoryl Lipid A (MPL-A)

The Fucose-mannose ligand (FML) glycoprotein of *L. donovani* combined with saponin induced 92–97% protection against zoonotic VL [279]. FML pulsed DCs also confer Th1 mediated protection against the lethal infection with *L. infantum* [280].

In recent years, adjuvant compounds derived from plants have been used to improve the immunogenicity of the vaccine. Glycyrrhizin (GL) is a natural triterpenoid saponin having immunomodulatory activities. When LPS stimulated murine peritoneal macrophages were treated with FML along with various GL concentrations and infected with *L. infantum*, the result showed enhanced production of NO, TNF-α and IL-12p70 and reduction of IL-10 levels in comparison with FML treatment alone [281]. Recently, Chimera A formulated with Monophosphoryl lipid A (MPL-A) was also used to vaccinate BALB/c mice, followed by i.v. 1×10^7 promastigotes of *L. infantum* challenge. Brito et al. designed Chimera A by mapping T cell epitopes of known *L. infantum* antigens [282]. This chimera A formulated with MPL-A stimulated Th1 response with IFN-γ, INF-α, IL-2 expression and decreased IL-4 and IL-10 expression along with reduced splenic parasitic load [283].

4.2.5. Hypothetical, In Silico Protein Constructs and Other Proteins

A vaccine combining two *L. braziliensis* protein, hypothetical (LbHyp) and the eukaryotic initiation factor 5a (EiF5a), was able to induce heterologous protection against VL [284]. The immunogenicity of another *Leishmania*-specific hypothetical protein, LiHyT, along with saponin was evaluated in BALB/c mice; the vaccinated mice showed higher levels of IFN-γ, IL-12, and GM-CSF and good titres of LiHyT- and parasite-specific IgG2a antibodies [285]. The recombinant enolase associated with saponin induced high levels of IFN-γ and IL-12 in a murine model along with low levels of IL-4, IL-10, and IgG1 antibody [286]. A peptide-based vaccine has also been constructed based on in silico modelling with 6 HTL, 18 CTL, and 25 B-cell epitopes from three hypothetical membrane proteins of

L. donovani. An adjuvant was added at the N-terminus to enhance its immunogenicity. The vaccine construct was found to be non-toxic, non-allergic, and thermally stable. Molecular docking confirmed its binding with TLR-2 [287]. Recently, another fusion vaccine was constructed using immunoinformatics, targeting KMP-11 gene, and B-type flagellin (Fli-C) as an adjuvant; it was found to be a potent stimulator of the cellular and humoral immune response against VL [288].

4.2.6. Vaccines against Canine Leishmaniasis

Immune regulation of *L. infantum*, the causative agent of most canine leishmaniasis (CanL), requires a balance between the pro-inflammatory CD4+ Th1 cells, which controls parasite replication, and Tregs that induce immunosuppressive/regulatory response that is required to dampen aggravated inflammation. The control of infection by *L. infantum* (syn. *L. chagasi* [289]. Four vaccines are available for the prevention of CanL: Leishmune® and Leish-Tec® in Brazil, and CaniLeish® and LetiFend® in Europe [290].

Leishmune vaccine is the first licensed vaccine against canine visceral leishmaniasis (CVL) registered in Brazil in 2004. It is a FML glycoprotein preparation enriched with *L. donovani* promastigotes, with the adjuvant *Quillaja saponaria* saponin, and has undergone phase I, II, and III filed trials [291]. In phase III field trials, the vaccine demonstrated 92% to 95% protection against CVL in the vaccinated group [292]. However, in 2014, the Leishmune® vaccine production and marketing license were withdrawn by the Brazilian Ministry of Agriculture as the company failed to comply with the technical regulation approved in the Interministerial Normative Instruction 31 (IN-31/2007) [293]. Thus, currently, there are three vaccines available against CanL in the market; (1) Leish-Tec® from Ceva Animal Health, Brazil, (2) CaniLeish® from Virbac Santé Animale, France, and (3) Letifend® from Laboratorios Leti, Spain. Their reported efficacies vary between 68.4% and 80% [290,294].

Leish-Tec® comprises a recombinant protein A2 from *L. donovani* amastigotes and saponin as vaccine adjuvant. It was licensed in Brazil in 2007 and is currently the only authorized CanL vaccine in the country [290]. Leish-Tec® induced protective immunity in beagle dogs against a high dose intravenous infection of *L. chagasi*; however, it provided only partial protection [295]. Vaccination of sub-clinical dogs with LeishTec® was found to be safe with a mild and site-specific adverse event of 3.06% [296,297]. A study conducted in dogs under field conditions suggested that about 43% of Leish-Tec® recipients developed disease over time [298].

CaniLeish (LiESP/QA-21) is the first European CanL vaccine, released in 2011. It is composed of purified excreted–secreted proteins of *L. infantum* (LiESP), together with *Quilaja saponaria* saponin (QA-21) as an adjuvant. LiESP/QA-21 increased significantly IFN-γ secretion and IgG2 production in vaccinated dogs, concomitant with induction of iNOS pathway and production of NO derivatives [299]. Dogs vaccinated with LiESP/QA-21 or CaniLeish® developed Th1 immune response within 3 weeks. In a one-year randomized controlled CaniLeish® vaccine field trial in the CanL Mediterranean endemic area with a heterogeneous canine population, no infection was observed during the first-year [300]; however, the vaccine failed to maintain *L. infantum* specific IFN-γ at 9 months. Thus, the field trial did not support the previously reported efficacy to prevent active *L. infantum* infection in dogs from endemic areas and naturally exposed parasites [301].

In 2016, LetiFend® (Laboratorios LETI, Spain) was licensed for veterinary use in Europe [302]. The active component of LetiFend® is recombinant protein Q formed by five antigenic fragments from four different *L. infantum* proteins (ribosomal proteins LiP2a, LiP2b and LiP0 and the histone H2A), without any adjuvant. Its efficacy was evaluated in different breeds of dogs in the endemic area of Spain and France in randomized, double-blind, placebo-controlled, and field trials. The vaccine reduced the risk of developing CanL following natural infection with *L. infantum* [303,304].

Use of salivary proteins has recently gained much attention for preparing a vaccine against CVL. A vaccine comprising *L. braziliensis* promastigote protein, sand fly gland extract (SGE) and saponin adjuvant was evaluated in a dog model. The vaccine exhibited strong antigenicity and induced SGE-specific humoral immune response. Increase in the circulating CD21^{+} B-cells and CD5^{+} T-cells was observed [305]. Intradermal challenge with *L. infantum* plus salivary gland extract in dogs immunized with a vaccine composed of *L. braziliensis* antigens plus saponin as an adjuvant (LBSap vaccine) was found to elevate total anti-Leishmania IgG as well as IgG1 and IgG2 isotypes in vaccinated dogs. The number of circulating B cells (CD21^{+}) as well as CD4^{+} and CD8^{+} T lymphocytes were also high and exhibited a high level of IFN-γ and low IL-10 and TGF-β1 expression in spleen with reduced parasite load [306].

LiESA-MDP is another second-generation dog vaccine candidate, based on a 54 kDa protein derived from *L. infantum*. Holzmuller et al. showed that a combination of LiESA$_p$ and muramyl dipeptide (MDP) induced total protection against experimental CL in 18 healthy Beagle dogs when challenged with 10^8 metacyclic *L. infantum* promastigotes [307]. The efficacy of LiESA$_P$-MDP was evaluated in a randomized field trial in a large population of dogs in an endemic area of France; dogs were vaccinated with 100 μg lipophilized LiESA$_P$ antigen and 200 μg MDP. After 2 years, the vaccinated dogs showed 92% efficacy, as evident from the detection of Leishmanial DNA in bone marrow aspirates. Enhancement of NO mediated anti-leishmanial activity of canine macrophages were observed with higher IFN-γ production by Th1 cells [308,309].

Another heterologus therapeutic vaccine composed of *L. braziliensis* with MPL adjuvant (LBMPL) was tested in dogs naturally infected by *L. infantum*. Animals developed strong antigen-specific lymphoproliferation mainly of CD4^{+} T cells and CD8^{+} T cells with high TNF-α and low IL-10 levels. It also blocked transmission of parasites to sandflies in LBMPL-vaccinated dogs [310,311].

Recently, phase I and phase II clinical trial in dogs were performed to evaluate the toxicity of LBSap vaccine (comprising *L. braziliansis* antigen and saponin), in comparison to Leishmune[®] and Leish-tech[®]. The results showed an increase in CD21^{+} B lymphocytes in case of the Leishmune[®] group and CD14^{+} monocytes in LBSap and Leishmune[®] groups. In the LBSap group, an increase in both IFN-γ producing subpopulations of T cells was observed; in the Leish-Tec[®] group, an increase in IFN-γ producing CD8^{+} lymphocytes were observed. The three vaccines, Leishmune[®], LiESP/QA-21, and LBSap, did not induce adverse reactions and prevented CL [292].

4.3. Third-Generation Vaccine

Third-generation vaccines are DNA vaccines that provide an extremely flexible method of vaccination for inducing stronger Th1 immune response. gp63, LACK and ORFF are the most extensively studied DNA vaccine candidates against VL [312]. DNA vaccines are advantageous as they are relatively simple to produce and administer, stable, and often very immunogenic than their recombinant protein counterparts [313]. DNA vaccines are able to induce both humoral and cellular immune responses, which can be further tailored through vector modifications, or by incorporation of immunomodulators such as CpG motifs, GM-CSF, Flt3 ligands, and/or cytokine genes [314,315]. The concept of heterologous prime boosting has been employed successfully against *Leishmania* [316]. The first DNA vaccine had the gp63 gene expressed in *S. typhimurium*, which stimulated the CD4^{+} Th1 subset without any detectable DTH response [317]. The level of protection was enhanced by adding gp46 and cysteine proteinase b (CPb) along with gp63 [318]. The use of aluminum phosphate [319] and plasmid encoding CD40L (as adjuvants) further increased the vaccine efficiency [320].

Multi-epitope vaccine comprising gp63, KMP-11, and amastin from *Leishmania* was used as a prime-boost strategy against VL, where immune response was primed with DNA vaccine, then boosted with protein antigen/vector [321]. NH36 DNA, a main component of FML, together with aluminium phosphate, induced a strong Th1 response providing

protection in mice against *L. chagasi*, *L. mexicana* and *L. amazonensis* [319,322,322]. Plasmid DNA encoding TSA and LmSTI1 used alone or in tandem, protected mice against *L. major* infection by inducing specific CD4^{+} Th1 responses without requiring any adjuvant [323]. Plasmid cocktails encoding LmSTI1, TSA, and LACK induced full protection against *L. major* infection [324]. Constructs pCMV3ISS-TSA and pCMV3ISS-LmSTI1 induced a comparable level of protection in mice infected with a highly virulent strain MHOM/TN/95/GLC94 of *L. major* (Zymodeme MON-25; isolated from a Tunisian patient). Administration of combination plasmids containing LACKp24, TSA, LmsTI1 and CPa genes yielded the strongest protective effects [325]. Immunization of dogs with multicomponent DNA vaccine, which contained genes for *L. donovani* H1, H2A, H2B, H3, H4, p36 (LACK), PSA-2, TSA, STI1, and ARP-1, resulted in antigen-induced lymphoproliferative and IFN-γ (but not IL-4) response [326].

Leishmania histones are excellent immunogens for the host immune system. Plasmids containing *L. infantum* nucleosomal histones (H2A, H2B, H3, H4) (dose 25 μg per leg) have been tested in a murine model of *L. major* infection, which developed a strong Th1 response against CL [327,328]. The intradermal vaccination increased IFN-γ production, in addition to CCXC29, CXCL10, and CC12 [329]. Hamsters immunized with recombinant histone proteins of *L. donovani* (rLdh2-4) provided protection against VL (10^7 amastigotes) [330]. BALB/c mice, immunized with plasmids expressing H2A, H2B, H3, and H4 histone proteins, and challenged with *L. braziliensis*, had lesion development prevented [331].

A recombinant lentiviral vaccine expressing a KMP11-Hydrophilic acylated surface protein B (HASPB) fusion protein of *L. infantum* induced IFN-γ and IL-4, and a specific antibody response against *L. infantum*, reducing the parasite burden [332]. A DNA vaccine comprising KMP11-HASPB genes of *L. major* induced a strong immune response in BALB/c mice and reduced the parasitic burden in spleen and lymph [333]. A pEGFP-N1-KMP11-gp96 DNA vaccine also induced a robust Th1 response and protected against *L. major* (MRHO/IR/75/ER) [334].

Leishmania sp. obtains heme from HbR-mediated endocytosis and is an important glycolytic enzyme in the parasite. Thus, HbR-full length-DNA (HbR-FL) and HbR-N-terminal extracellular domain-DNA (HbR-N-DNA) have been tested in BALB/c mice and hamsters for protection against the AG83 strain of *L. donovani*; a total reduction in splenic and hepatic parasite burden matched with full protection against VL [335]. Another vaccine, LEISHDNAVAX, comprising plasmids coding for five leishmania antigenic determinants with T cell restricted epitopes (KMP-11, TSA, CPA, CPB and P74), showed protection in mice against *L. donovani* infection [336].

Recombinant vaccine containing LEISH-F2 and LEISH-F3^{+}, when administered with a TLR4 antagonist, has been shown to induce CD4^{+} mediated T cell immune response in animal models and provide therapeutic efficacy in CL. RNA vaccines expressing F2 and F3^{+} sequences have also been found to enhance CD4^{+} and CD8^{+} T cell responses, protecting mice against *L. donovani* infection [337]. A DNA vaccine using LmxMBA gene lacking the transmembrane regions of *L. mexicana*, when immunized prophylactically, reduced the size of the lesion and parasitic load in the mouse footpad, compared to the control groups [338].

LACK, when administered as a DNA vaccine, is able to protect against *L. major* and *L. infantum* in mice, and against *L. infantum* in Beagle dogs [339–343]. A combination construct containing LACK + Native thiol-specific antioxidant (TSA) increased survival upto 32 weeks following challenge with 2×10^6 *L. major* promastigotes, compared to the control mice that died within 11 weeks [344]. The protective effect correlated well with strong IFN-γ response and reduced levels of IL-4 [345]. A multivalent vaccine containing LeIF, LACK and TSA genes against CL elicited stronger Th1 immune response, and delayed progression of lesion size [346]. Another multivalent vaccine comprising the most immunogenic regions of *L. major* (MRHO/IR/75/ER) including TSA, LmST11, LACK and KMP11 was tested in BALB/c mice with or without vector containing IL-12. Mice immunized with lL-12 + multiple antigens constructs showed Th1 polarisation, and hence, reduced parasite

burden [347]. However, the protective effect of a prime-boost vaccine using LACK construct in Beagle dogs against *L. infantum* was suppressed by IL-12 administration [348].

ChAd63-KH is a replication-defective simian adenoviral vector expressing a novel synthetic gene KH (a self-cleaving polyprotein) comprising KMP-11 and HASPB. In the phase I human trial, it induced IFN-γ production significantly and DC activation, in addition to eliciting CD8⁺ T cells [349]. The phase IIa safety and immunogenicity trial was conducted in Sudanese patients with persistent PKDL. ChAd63-KH vaccine showed minimal adverse reactions and induced potent innate and cell-mediated immune responses [350].

L. infantum pyridoxal kinase (PK) recombinant protein with saponin, or DNA vaccine, significantly reduced the parasite load in spleen, liver, draining lymph nodes and bone marrow of the BALB/c mice challenged with *L. infantum*. The protective Th1 response was characterized by high levels of IFN-γ, IL-12, GM-CSF and specific IgG2a antibodies, whereas IL-4, IL-10 and parasite-specific IgG1 antibodies were lowered [351].

As an innovative strategy, an immunomodulatory drug, sirolimus (SIR), was also used to boost a prophylactic DNA vaccine against leishmaniasis. Syrian hamsters were treated with SIR along a DNA vaccine consisting of four plasmids (LACK, TRYP, PAPLE22 and KMPII). Although the DNA vaccine on its own efficiently reduced the burden of parasites in the skin and lymph nodes, SIR offered enhanced protection [352].

5. Conclusions

Early inflammatory events inducing macrophage dysfunction and T-cell dependent immune response direct the species-specific pathogenesis in Leishmaniasis. The intracellular strategies by the parasite and the subtlities of the host–pathogen stand off that allow the parasite to reside indefinitely inside the macrophage, make the cure challenging. Controlling Leishmaniasis through vaccination is also a challenge. Although a number of vaccine candidates in the form of plasmid coding genes, defined subunits, live or killed or fractionated forms have been evaluated, only a small number of them have proven promising. Several attenuated and protein-based vaccines have shown encouraging results in offering protection against different species of *Leishmania* in animal models, but their potential to revert back to virulent parasite is fraught with risk. Use of nano-carriers for *Leishmania* vaccine containing combination vaccines with adjuvants also seems promising; however, the cure from Leishmaniasis is still a dream.

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