1. Introduction and review of literature:

1.1 History and concepts of vaccination

Vaccination started from the concept of variolation, derived from Latin term *varius* (meaning 'spotted') used for smallpox disease, in which dried pustules from mildly infected individuals were blown into noses of uninfected individuals to protect them from severe forms of disease (1). Evidences indicate that variolation started around 10th century AD and was practiced extensively in China, India, Turkey and African continent (2). This practice is based on the several empirical observations, the first written record of which is contained in the description of plague that affected Athens, Greece in 430 BCE. Thucydides, an Athenian historian and general, describes in *The history of Peloponnesian war* that individual on verge of death found solace in company of those who recovered from disease as they knew from experience that the same person can't be affected twice (3). Also, Persian physician Rhazes, also known as Abu Bakr, in 910 AD reported that cured patients of smallpox are resistant to future infections by same virus (2).

Smallpox was a deadly epidemic all over the world in 18th century. Local observations suggested that cowpox-contracted individuals are resistant to smallpox infection which prompted Edward Jenner, referred as Father of Vaccination, in 1796 AD to try variolation by injecting pustules from Cowpox-infected individuals into skin of healthy milkmaids and proved conclusively that it protected them from smallpox. He termed this procedure *Vacca* - latin word for cow - a widely used term at that time. Although initially looked upon suspiciously, this represented mankind's first experimental attempt for vaccination against a life-threatening disease (1,4). Edward Jenner had no idea about the causal mechanism of

the success of vaccination; it was Louis Pasteur who deciphered the scientific principle of vaccination in 19th century. In 1885, while working on chicken cholera caused by *Pasteurella multocida*, he observed that an injected inocculum from long-left culture of *P. multocida* was able to protect chickens from same disease (5). He hypothesized that micro-organisms can be attenuated by exposing them to stress-conditions such as high temperature, oxygen, dry air, chemical agents or *in vitro* passsaging which are incapable of producing disease symptoms. Later, attenuated rabies and anthrax virus vaccines were prepared employing same principles (6). Thus, it became clear that inactivation of micro-organisms or infectious agents was necessary for generating a vaccine. In 1890, Emil Von Behring and Kitasato reported the mechanism of passive immunity by injecting diphtheria and tetanus toxins that produces 'antitoxins' (now called 'antibodies') which protect the animals from subsequent exposure to same pathogens (7).

Around the time from 1895 to 1905, Élie Metchnikoff and Paul Ehrlich were instrumental in discovering the role of specialized cells in the generation of immunity. In 1897, Paul Ehrlich proposed 'side-chain receptor hypothesis' which was later replaced by Ehrlich himself and proposed the name 'receptors' for side-chains. This work proved to be crucial in deciphering the process of antibody formation (8). Around the same time, Zoologist Metchnikoff observed that defense cells, now known as phagocytes, move towards the site of inflammation and put forward the 'theory of cellular immunity'; suggesting the role of specialized cells in body's defense mechanism (9). These discoveries laid the foundation of subsequent research in cellular and molecular immunology which forms the basis of modern vaccinology till date and Paul Ehrlich received the first Nobel Prize in 1901 while

Elie Metchnikoff and Paul Ehrlich shared the prize in Physiology and Medicine in 1908 for their path-breaking discoveries.

Concept of vaccination revolves around two phenomena: 1) ability of a vaccine preparation to induce a strong and adequate immune response which mimics the natural infection, but without inducing pathological symptoms associated with it 2) ability of a vaccine to induce immune memory i.e. a secondary challenge with same antigen produces a heightened and rapid generation of immune response. The main task of vaccinology is to identify immune correlates of protection against a disease, especially the cellular subsets and effector molecules which are responsible for protective immunity against a pathogen (10). Antigen presenting cell, mainly dendritic cells (DC's) or macrophage, acts as a bridge between antigen and adaptive branch of immune system. An immunogenic antigen should be able to activate the antigen presenting cells (APC's) which can thereby efficiently interact with naïve T- and B-cells. Thus, adjuvants were incorporated in formulation to increase the immunogenicity of vaccine. Originally, adjuvants served this purpose by increasing retention time of antigen and exposure to immune system inside tissues. A greater understanding of vaccinology led to the discovery of specific adjuvants like TLR ligands which aid in activating innate immune cells like APC's and increase the efficiency of anti-pathogenic inflammatory response (11,12). Thus, vaccinology is one the most important concept to emerge out of immunology. It is the most important medical intervention in terms of efficiency in reducing mortality- estimated to reduce nearly 7,000 deaths per day (13). In days to come, it can become one of the most important medical techniques serving mankind and enhancing well-being of disease-prone individuals worldwide.

1.2 Leishmaniasis

1.2.1 Disease and epidemiology

Leishmaniases are group of vector-borne, protozoan parasitic diseases caused by at least 20 different species of genus *Leishmania* with varying clinical manifestations and severity. Clinical outcome and disease pathogenesis depends on plethora of factors: parasite tropism, vector biology and a variety of host factors- majorly host immune response which depends on host genetics (14,15). **C**utaneous **L**eishmaniasis (CL) is characterized by non-healing skin lesions and its closely related forms are **D**iffuse **C**utaneous **L**eishmaniasis (DCL) and **M**uco-**C**utanoeus **L**eishmaniasis (MCL) which causes severe damage to mucous membranes. Life-threatening leishmanial species cause **V**isceral **L**eishmaniasis (VL) which affects spleen, liver and bone marrow, while cured cases of VL can show cutaneous lesions within few years and this syndrome is known as **P**ost **K**ala-azar **D**ermal **L**eishmaniasis (PKDL). Different *Leishmania* sp., clinical pathology and manifestations, endemic regions are listed in **Table 1 and 2**.

Parasite completes its life cycle in two hosts: sand fly and human, although there are other zoonotic reservoirs of *Leishmania* such as dogs, rodents and kangaroos. Leishmaniasis is primarily a zoonotic disease, with the exception of *L. donovani* and *L. tropica*. Leishmaniasis has been categorized as one of the neglected tropical diseases, although it has been ranked as second and fourth on mortality and morbidity causing disease among tropical infections (16,17).

Table 1. Pathological form and associated manifestations of major infecting species of *Leishmania* across the globe (adapted from Burza *et al*. *Lancet*. 2018)

Leishmania sp.	Clinical pathology	Pathological manifestations
L. donovani	VL and PKDL	Persistent fever, splenomegaly, weight loss, and anaemia in VL; multiple macular, papular, or nodular lesions in PKDL
L. tropica	CL and rarely VL	Ulcerating dry lesions, painless, and frequent multiple lesions
L. aethiopica	DL, DCL and oronasal CL	Localised cutaneous nodular lesions; occasionally oronasal; rare ulceration occurs
L. infantum	VL and CL	Persistent fever and splenomegaly in VL; single nodules and minimal inflammation in CL
L. major	CL	Rapid necrosis, multiple wet sores, and chronic inflammation
L. mexicana	CL and DCL	single or multiple ulcerating lesions
L. amazonensis	CL and DCL	single or multiple ulcerating lesions
L. braziliensis	CL, MCL and DCL	Ulcerating lesions can progress to muco-cutaneous form; local lymph nodes are palpable before or in early stage of lesion progression
L. guyanensis	CL and DCL	Ulcerating lesions, single or multiple that can progress to mucocutaneous form; palpable lymph nodes.

Table 2. Animal reservoirs and endemic regions for different forms of leishmaniasis (adapted from Burza *et al*. *Lancet*. 2018).

***Leishmania* sp.**	**Animal Reservoir**	**High-risk associated regions**
L. donovani	Humans	India, Bangladesh, Ethiopia, Sudan and South Sudan
L. tropica	Humans, reports of zoonosis	East Mediterranean, Middle East, Northeastern and Southern Africa
L. aethiopica	Hyraxes	Ethiopia and Kenya
L. infantum	Dogs, hares, and humans	China, Southern Europe, Brazil and South America for VL and CL, Central America for CL
L. major	Rodents	Iran, Saudi Arabia, North Africa, Middle East, Central Asia and West Africa
L. mexicana	Rodents and marsupials	South America
L. amazonensis	Possum and rodents	South America
L. braziliensis	Dogs, human, rodent and horses	South America
L. guyanensis	Possum, sloth, and anteaters	South America

Leishmaniasis is prevalent across Africa, Latin America, Asia, Mediterranean regions and the Middle East (16). It is endemic to regions where the socio-economic conditions are dismal, health care delivery and disease awareness are in poor shape; and thus the disease has huge impact on psychosocial status of individuals, mainly women. Negligence regarding leishmaniasis also stems from the focal distribution and remote locations of disease prevalence which makes extrapolation of epidemiological data a difficult task (18). A 5 year case study analyzing the incidence and epidemiological distribution of leishmaniasis concluded that 0.2-0.4 million CL and 1.5-1.7 VL cases occur annually. While CL is more widespread across the globe, 90% of VL cases are reported from six countries viz. India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil. Around 30,000-40,000 deaths occur every year with a fatality-rate of 10%, virtually all occurs due to VL (19). However, Burza *et al* cited a WHO report which estimated a near 100% increase in VL-related mortality in 2017. Brazil accounts for more than 99% of VL cases reported in Latin America. While dog is the main reservoir of *L. infantum*, human is the primary host for the *L. donovani* in the Indian subcontinent. It usually thrives and propagates in the areas of high humidity and rainfall; typically in alluvial plains of Ganges River at a temperature between 15°C to 25°C in households containing livestock creating a favorable breeding habitat for *Phlebotomous argentipus*. In endemic regions of Africa, *P. orientalis* thrives in acacia and balanite forests containing black cotton soil whereas areas around termite hounds are favorable growing regions for *P. martini* (14).

CL is endemic in 88 countries and 90% of cases are reported from Algeria, Saudi Arabia, Iran, Iraq and Afghanistan in Old world region; Brazil and Peru in New world region (20).

Leishmaniasis cases are on a considerable rise in the travelers which have been to *Leishmania*-endemic regions. A 2010 case-review report by Pavli and Maltezou states that out of all forms of leishmaniasis, nearly 80% of the travel-related leishmaniasis cases are of CL and identified South America as the major spreading region for CL (21). Old world species of CL such as *L. major* generally occurs in rural and wet regions and desert rodents are the main animal reservoirs. It is prevalent in desert regions of Northern Africa and Sudan, Middle East and Central Asia and travelers to endemic areas are acquisition-prone. *L. tropica* is prevalent in urban areas of Mediterranean basin, Central and Middle-east regions of Asia. It is considered as anthroponotic form of CL as human has been considered as primary reservoir, although rock hyraxes have been identified as animal reservoirs of *L. tropica* in Israel and *L. aethiopica* in hilly regions of Ethiopia and Kenya. *L. infantum*, a visceralizing species in infants, causes a mild form of CL in adults and canines (wild or domesticated) acts as animal reservoir. New World CL limited to rural regions of Central and South America and caused by several species of *L. viannia* and *L. mexicana* genus subgroup; reservoirs include foxes, sloths and forest rodents (22). MCL is a chronic, debilitating and life-threatening form of CL which occurs in 3-10% post-healing cases of CL caused by *L. braziliensis*. It is mainly endemic to Latin American countries such as Peru and Bolivia (23).

1.2.2 Pathological manifestations

CL is a topical infection which causes chronic, ulcerative and generally self-healing form of lesion in exposed areas of skin. Depending on the infecting species of *Leishmania*; appearance, extent, severity and persistence of skin lesions vary. In patients infected with

Old World CL, lesions generally appear at or around site of sandfly bite within 6 weeks and starts as a small, pruritic erythematous papule which later enlarges and breaks down into a chronic ulcer or nodular lesion of 2-5 cm with indurated margins. While *L. major* causes multiple lesions and has a quick course of disease period where lesions self-heal usually within 6 months, *L. tropica* and *L. aethiopica* infection are characterized by single and indolent lesions which generally takes 1 to several years to heal. New World CL are chronic, mostly ulcerative, larger (upto 7 cm) in diameter and usually does not heal without medication and lesions take > 1 year to heal (22,23). MCL manifests in some healed cases of *L. braziliensis* where it spreads to mucosal membranes of oral and upper respiratory tract, causes destruction of cartilaginous tissue of nose such as nasal septum and nasal bridge and results in severe disfigurement with symptoms like rhinorrhea and epistaxis (24,25).

VL in different geographical locations differs in clinical manifestations. Although infants and elderly mainly bear the brunt of disease, VL is becoming common in adults in East Africa and India. A cutaneous nodule is formed at the site of sand fly bite which disappears after a while and systemic pathologies start developing after an incubation period which varies from 2-6 months. Initial symptom during acute phase is fever and enlargement of spleen and liver. Other symptoms of VL include anorexia, weight loss, abdominal distension and weakness which may persist for months. Lymphadenopathy commonly occurs in the patients from Mediterranean countries, East Africa and China and has been observed in VL cases from Sudan. Major symptoms of VL arise due to the infiltration and hyperplasia of mononuclear phagocytic and reticulo-endothelial cells in spleen, liver and

bone marrow. Due to the development of grayish cast in patients of chronic VL, term 'Black Fever' (Hindi: Kala Azar) has been given to this disease (26,27). If left untreated for long time, infection becomes chronic and complicated due to occurrence of secondary infections such as pneumoniae, pulmonary tuberculosis, dysentery and often proves fatal. Cured VL cases caused by *L. donovani* in Sudan and Ethiopia (<60%) and India (<10%) may develop into a dermatosis causing macular, maculopopular or nodular rash on skin, trunks and limbs, known as PKDL. It is caused by IFN-γ secreting PBMC's which are skewed towards Th1 from Th2 subtype post-VL treatment and cause inflammatory reaction to the persistent parasite reservoir in the skin (28).

1.3 Life cycle of *Leishmania* sp. and survival strategies in host niche

1.3.1 Basic cellular organization of *Leishmania*

Other than the multiple reservoir host species in different geographical locations, *Leishmania* completes its digenetic life cycle in a mammalian host and an insect vector. Basic cellular structure of *Leishmania* remains constant throughout its life cycle and is characterized by smooth plasma membrane, a pellicular longitudinal array of corset microtubules, flagellar pouch, double membraned nucleus (containing nucleolus) and kinetoplast (performing mitochondrial functions), blepharoblast, vacuoles, basal body and flagellum (29). During the developmental stages, *Leishmania* undergoes many developmental changes in the morphology to adapt, survive and multiply in different niche. During intracellular stage to leptomonad conversion, it has been shown that kinetoplast becomes large and irregular and nucleolus fuses into one and becomes less

compact. These changes are suggestive of metabolic changes to adapt in a markedly different extracellular environment (30). Flagellum is an important structure in both promastigote and amastigote stages of *Leishmania*. Although amastigote is non-motile, it contains a short flagellum whose axoneme has been shown to resemble more with ciliary arrangement pattern and termed as 9υ (9-variable). It has been associated with performing cell-cell interactions inside host macrophage as it interacts closely with parasitophorous vacuole (PV) and also transports parasitic proteins to host cell through endocytic vesicles (31,32), as flagellar pocket (FP) is main site of exocytosis and endocytosis in promastigotes and amastigotes (33). Basic cellular organization is retained as parasite completes its life cycle shuttling between sand fly and human host, but it has been precisely defined of late. Depending on the cell size and shape, length of flagellum, cellular localization of organelles such as kinetoplast and nucleus; *Leishmania* has been differentiated into different cellular forms. However, major cellular forms of the parasite during the course of life cycle remain promastigote and amastigote. Promastigote represent the ovoid, elongated and motile form with a long, elongated flagellum extending from flagellar pocket which helps in motility across the sand fly gut while amastigote is typified by a small, spherical and non-motile cell shape that stays inside the parasitophorous vacuole of mammalian macrophages. Although it contains a small flagellum, it performs sensory functions (34).

1.3.2 Life cycle in sand fly vector: survival in transmission host

Infection in insect vector is initiated when sand fly bites an infected human host. During the course of blood meal, it takes up the *Leishmania*-infected macrophages containing

amastigotes. Macrophages contained in blood meal are ruptured and amastigotes are released in posterior mid-gut which undergoes a series of morphological and physiological changes to adapt, survive and multiply inside insect's digestive tract. **Figure 1** shows the steps in digenetic life cycle of *Leishmania* parasite.

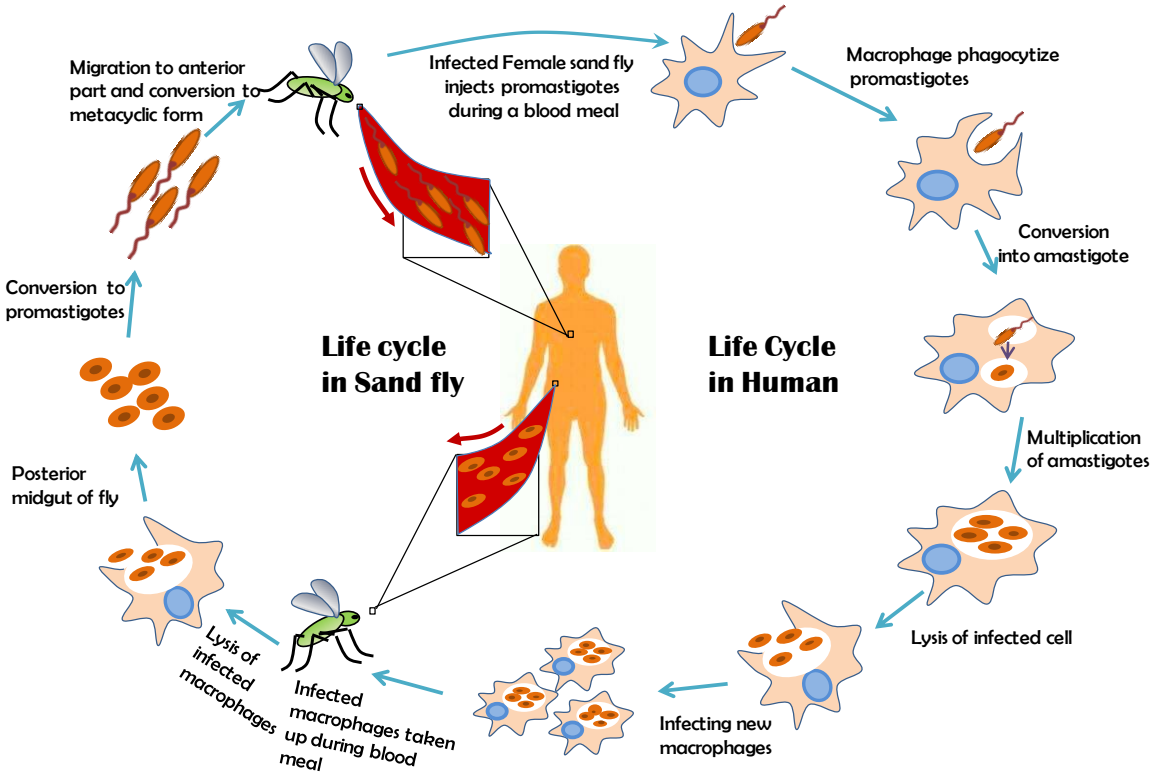

Figure 1. Multiplicative and infectious stages of *Leishmania* sp. life cycle in natural hosts. Sand fly ingests amastigote-containing macrophages which are converted to promastigotes and multiply in sand fly midgut while they undergo several morphological changes. Metacyclic promastigotes are transferred to human by infected sand fly during blood meal, where they transform into amastigotes and replicate inside macrophages.

Initially, amastigotes are converted to short, slow-moving procyclic promastigotes with a short flagellum which starts multiplication cycles in hind-gut (35). These forms are enclosed in type I **P**eritrophic **M**embrane (PM) of insect, which is primarily a chitinous

membrane performing multi-varied role in sand fly immunity and digestive processes. During *Leishmania* life cycle, it was shown to protect susceptible parasite to the proteolytic action of digestive enzymes and aid in their survival (36). Also, timing of PM formation and degradation, defecation of bloodmeal remnants was found to be important in determining the susceptibility of sandfly to *L. donovani* infection. Susceptible sand fly vectors (*P. argentipus* and *P. orientalis*) to *L. donovani* exhibited slower PM formation and longer intermediate time between PM degradation and defecation of waste bloodmeal, suggesting the importance of PM in establishing infection (37). After 48-72 hours, procyclics convert into nectomonads which are long, slender and motile forms and escapes from PM. They are slow-replicating and bind to the epithelial cells lining the wall of abdominal midgut and migrate to the anterior thoracic portion, near the stomodeal valve. Later, these convert into another proliferative form known as leptomonads, which again starts a new proliferative cycle (38). Wilson *et al* showed that gut-binding capability of *Leishmania* is strictly stage-specific and possessed mainly by nectomonad and leptomonad forms. Although it is necessary for establishment of infection, other factors may also play a role in incompatibility of certain parasite-sand fly combinations (39). Finally, two forms of promastigotes may arise from this stage: a haptomonad form, arising either from nectomonad/leptomonads, which are non-motile, having short flagella and attaches to the cuticle lining of stomodeal valve and forms a parasite plug. Attachment of haptomonads at the stomodeal valve is accomplished by converting flagella into hemidesmosome-like structure which binds via hydrophobic interactions (40,41). Haptomonads were observed at a very low number (<10%) post-day 4 infection of *L. donovani* and *L. infantum* infected

Lu. longipalpis (42). Finally, metacyclics arise from leptmonads at the thoracic midgut and cardia. They are the main infective forms that are transmitted during sand fly bite with the help of **P**romastigote **S**ecretory **P**lug (PSG). This gel-like structure acted as reservoir of leptomonad promastigotes and acted as site of metacyclogenesis, by which metacyclics arise from leptomonads. PSG occludes the midgut, causes the stomodeal valve to remain open and affects the feeding ability of flies, thereby making the environment conducive for *Leishmania* transmission (43). **Figure 2** depicts the basic cellular components and different cell forms of *Leishmania* during life cycle in sand fly and humans.

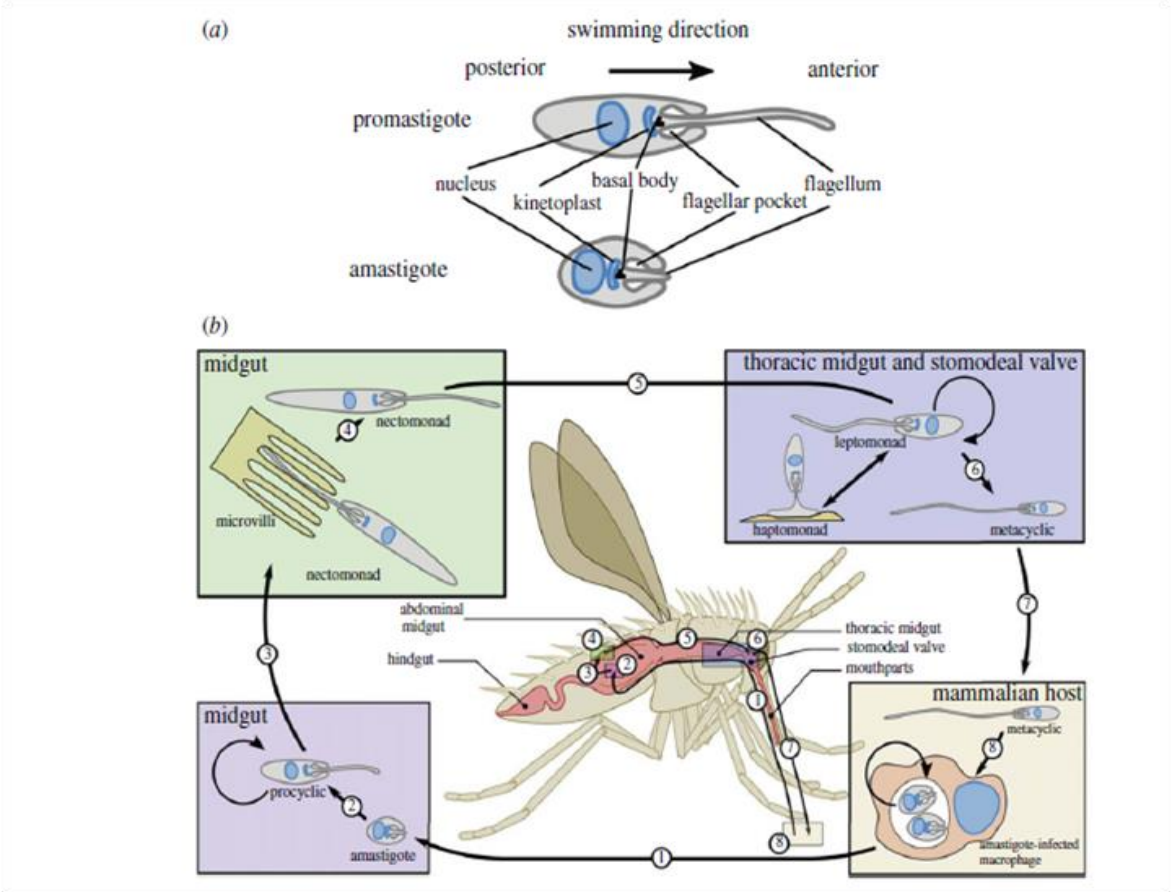

Figure 2. Basic structural organization and different morphological forms of *Leishmania*. (a) *Leishmania* contains anteriorly placed basal body, flagellar pocket,

kinetoplast and flagellum and posterior section contains nucleus. (b) Released amastigotes are converted to procyclic promastigotes with replicative ability. They convert to microvilli-attaching nectomonad form. *Leishmania* moves anteriorly in midgut and converts to leptomonad and haptomonad forms, and finally converting to infectious metacyclic form at the stomodeal valve (adapted from Sunter J., Gull K. *Open Biol*. 2017).

Thus, blood-feeding female sandflies (Order: Diptera; Family: Psychodidae; Subfamily: Phlebotominae) are the major natural vectors for leishmaniasis. 70 species of sand fly have been implicated in transmission of leishmaniasis out of total 900 known species. *Phlebotomus* sp. transmits old world leishmaniasis and *Lutzomyia* sp. is the transmission vector for new world leishmaniasis (44, **Table 3**).

Table 3. Sand fly-parasite combinations in different *Leishmania* sp. (Adapted from Ready PD., *Annu Rev Entomol.* 2013)

***Leishmania* sp.**	**Incriminated vector**
L. donovani	*P. argentipes, P. orientalis, P. martini* s.l.
L. infantum	*P. ariasi, P. perniciosus* (OW), *L. longipalpis* (NW)
L. major	*P. duboscqi, P. papatasi*
L. tropica	*P. sergenti, P. arabicus, P. guggisbergi*
L. aethiopica	*P. longipes, P. pedifer*
L. braziliensis	*L. wellcomei, L. neivai, L. whitmani*
L. amazonensis	*L. flaviscutellata*
L. guyanensis	*L. umbratilis*
L.mexicana	*L. olmeca olmeca*

1.3.3 Life cycle in human host and immune-evasion strategies by *Leishmania*

1.3.3.1 Early cellular and molecular interactions in host niche

Once inside the human host, parasite needs to outcome various hurdles by host's innate and adaptive immune system to establish infection. *Leishmania* sp. defies the general principle that the pathological severity and chances of infection establishment is related to pathogen inoculum. Sandfly bite generally injects a low number (1-1000) metacyclics, still *Leishmania* is able to infect host macrophages (45,46). Resistance of metacyclics to complement-mediated lysis may partially explain this paradox. Surface molecules such as LPG and gp63 contribute to this resistance by either modifying structural features of promastigote membrane and inhibiting C5b-9 complex lytic pore formation or hampering the conversion of C3b to C3bi respectively (47,48). A recent study showed that egested sand fly microbes induces neutrophil recruitment by IL-1β release, inflammasome activation and helps parasite persistence during initial stages of infection (49). Neutrophils are described as 'Trojan horses' for *Leishmania* due to parasite's ability to survive and multiply inside neutrophils, exploiting their presence at bite site to progress infection during initial stages (50,51); however, there are reports of dual roles of neutrophils in *Leishmania* infection (52). *L. donovani* promastigotes have been shown to neutralize the anti-microbial activity of Neutrophil Extracellular Traps (NET) through gp63-dependent mechanism (53). Thus, neutrophils are important players in the initial stages of infection.

Sand fly bite confers significant damage to the skin layers and creates a blood pool containing **E**xtra**C**ellular **M**atrix (ECM) components and tissue cells. This attracts innate immune cells such as macrophages and neutrophils to the wound site. Parasite may need to migrate in a complex extracellular environment of dermal milieu before the establishment of intracellular infection. Studies have found that *Leishmania* promastigotes are able to

bind collagen through their cell-surface receptors and can invade and migrate through a 3D-collagen matrix by the action of metalloproteases and cysteine proteases. This interaction with collagen fibres also explains the skin tissue tropism of *Leishmania* (54,55). Fibronectin (FN) is another major component of ECM which helps in regulation of *Leishmania* infection. Initial studies reported that promastigotes releases extrinsically supplied FN constitutively and infectivity of parasites increases by FN addition to culture medium. This study corroborated with findings by Kulkarni *et al* as they showed that *L. major* and *L. donovani* degrades FN in a gp63-dependent manner and resulting fragments have the capacity to downregulate the production of **R**eactive **O**xygen **I**ntermediates (ROI) (56,57). *Leishmania* promastigotes also interacts with plasminogen; exocytic vesicles secreted by *L. mexicana* were found to contain a plasminogen-binding enzyme known as enolase which helps in immune evasion (58). Moreover, it interacts with Laminin, a component of basement membrane, via its 67-KDa **L**aminin **B**inding **p**rotein (LBP) and was shown to be essential in *L. donovani* virulence and infectivity (59). *Leishmania* was also reported to bind to other ECM molecules such as heparin, heparin sulphate and degrade tropoelastin. Hence, the protozoan parasite interacts, degrades and compromises the structural integrity of many ECM components to facilitate its invasion into host cell, primarily macrophages (60,61). BALB/c mice pre-exposed to salivary gland lysate from *L. intermedia* enhances neutrophil migration, decreases CXCL10 and increases IL-10 secretion in *L. braziliensis*+SGS infected mice; thus creating a pro-parasitic environment. This study suggests that sand fly saliva acts as an immune-modulator during early phase of infection (62).

1.3.3.2 Internalization and delay in phagosomal maturation process

Leishmania entry into macrophages is facilitated by various receptors such as **C**omplement **R**eceptor 3(CR3), **C**omplement **R**eceptor 1(CR1), **M**annose **R**eceptor (MR), depending upon the infecting *Leishmania* sp. and form. Stage-specific internalization receptor utilization by *L. infantum* was demonstrated by Uneo *et al.* Metacyclics entered macrophages via CR3-dependent pathway and were resistant to lysosomal fusion while many procyclics utilized MR-based pathway for internalization (63,64). Real-time host cell imaging revealed that *Leishmania* interacts with macrophage membrane via flagellar tip. Post-recognition, the parasite is internalized via an Endoplasmic reticulum (ER)-membrane derived phagocytic vesicle and phagolysosomes (65). In fact, promastigotes and amastigotes (during successive replication cycles) utilize different modes of macrophage entry. While promastigotes utilizes cholesterol-rich membrane domains like caveolae and delay lysosomal fusion, amastigotes enter via caveolae-independent pathway (66). Upon entry into macrophages, *Leishmania* reorients its flagellum towards cell periphery and oscillatory movement causes cell membrane injury. This cellular injury favors lysosomal exocytosis and alters **P**arasitophorous **V**acuole (PV) composition to promote parasite survival (67). Before promastigotes are converted to amastigotes which can efficiently survive and multiply inside acidic environment of PV's, promastigotes containing PV's show limited interaction and fusion with the endocytic organelles such as lysosomes and shown to be dependent on gp63 abundantly expressed in promastigote form (68). Moreover, *L. donovani* LPG was also found to be responsible for accumulation of F-actin around promastigote-containing phagosome which delays the recruitment of LAMP-1,

PKC-alpha and inhibits phagosomal maturation. *L. donovani* LPG also inhibits phagosomal acidification process by interfering with SytV recruitment to phagosome, impairing the proton pumping- vesicular ATPase and Cathepsin D to be acquired by nascent phagosome (69,70).

1.3.3.3 Modulation of various signaling receptors and pathways

TLR are one of the major components of innate immune system which recognizes various **P**athogen **A**ssociated **M**olecular **P**atterns (PAMP's) and have been associated with protective and exacerbating role in different forms of *Leishmania* infection **(Figure 3)**. *L. donovani* modulates TLR-2 signaling by upregulating host deubiquitination machinery via A20 and hampers the recruitment of TRAF6 in TLR2 signaling, thereby hampering IL-12 and TNF-α production (71). LPG was found to activate MyD88 dependent TLR-2 signaling and two amastigote proteins were found to activate TLR2 signaling and initiate pro-inflammatory, anti-leishmanial immune response (72). Moreover, leishmanial LPG was found to interact with TLR2 to decrease host-protective TLR-9 expression via TGF-β and IL-10 dependent mechanism and exacerbates *L. major* infection (73). TLR2 activation has been shown to augment CL via IFN-β/PKR and IL-12p40 mediated pathways in *L. amazonensis* and *L. braziliensis* respectively (74,75). TLR4 signaling is also exploited by *L. braziliensis* amastigotes to induce TNF-α and IL-10 expression from human PBMC. TLR4 and TLR2 surface expression were found to be higher in healing forms of *L. major* than non-healing ones. *L. donovani* was shown to inhibit ubiquitination-mediated degradation of **T**umor **N**ecrosis **F**actor **R**eceptor-associated **F**actor 3 (TRAF3) to impair TLR4-mediated immune response in macrophages (76–78). Intracellular TLR-3, TLR-7

and TLR-9 signaling was essential for initiating autophagy in *L. major* infected macrophages, a mechanism meant to curb intracellular parasite replication. However, TLR-3 mediated immune response induced by ***L****eishmania* **R**NA **V**irus (LRV1) of *L. viannia* subgenus was shown to exacerbate infection and promotes parasite persistence. Thus, *Leishmania* infection causes modulation of TLR signaling to skew the response to favor its replication inside host cell, which depends on the infecting *Leishmania* sp. (79,80) CD40 is an important co-stimulatory molecule in generation of adaptive immune response. *L. major* infection induces a CD-40 dependent differential regulation of **M**itogen-**A**ctivated **P**rotein **K**inase (MAPK) signaling in macrophages, skews it towards ERK-1/2 which induces IL-10 and inhibits iNOS-2 mediated parasite killing. Later, it was shown that depletion of membrane cholesterol by *Leishmania* was responsible for this immune-evasion mechanism by forming a TRAF-6/Syk containing CD40 signalosome which induces IL-10 and induces anti-inflammatory, pro-parasitic immune response (81,82). Also, TLR-9 and CD40 were shown to be involved in a cross-talk like activation mechanism, increasing each other's expression and reduces the progression of *L. major* infection (83). These findings explained the as to why leishmanial LPG decreases TLR-9 expression as an immune-evasion strategy.

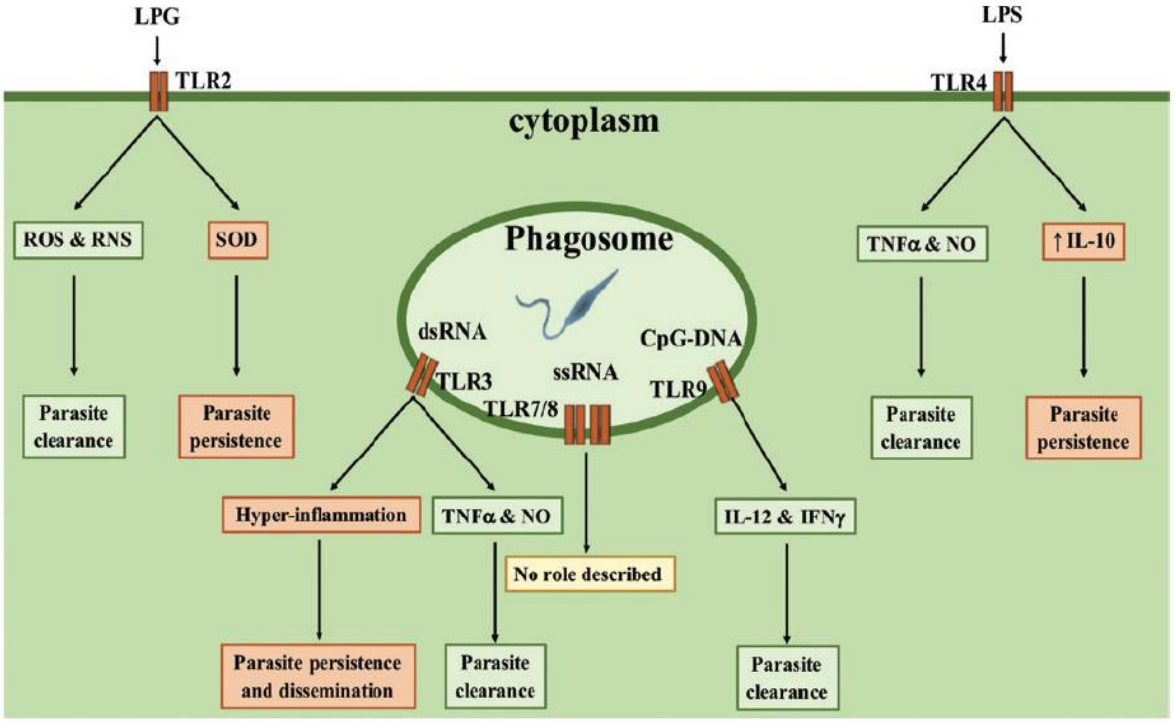

Figure 3. Effect of TLR's on immune signaling during *Leishmania* infection. TLR's have differential effects on the outcome of infection depending on *Leishmania* sp. and stimulation timing. While TLR2 stimulation during *L. major* infection causes ROS-mediated parasite killing, it exaggerates infection in *L. braziliensis* infection through SOD. TLR4 signaling has varied outcomes depending on pre- and post-infection stimulation timing. TLR3 promotes parasite clearance in *L. donovani* infection and helps in L. guyanensis survival. TLR9 mediated host-protection via induction of Th1 response through IL-12 and IFN-γ production (adapted from Rossi M., Fasel N., *Int Immunol*. 2018).

L. donovani infection was shown to impair the activation of PKCβ which is responsible for generation of anti-microbicidal superoxide anion (O2-) in parasitized macrophages. Also, *L. major* selectively utilizes PKC δ and ζ/λ to induce pro-parasitic Th2 immune response and enhance its survival, while subsequently diminishing the activation of anti-parasitic PKC α, βI, βII and γ isoforms (84,85). Moreover, *L. major* infection increases p38-specific MAPK phosphatase-1(MKP-1) and decreasing ERK-1/2 specific MKP-3, thereby skewing immune response towards IL-10 inducing Th2 subtype. Also, *Leishmania* induces **S**rc

Homology-2 domain containing **P**hosphotyrosine phosphatase (SHP-1) activation and increases anti-inflammatory IL-10 based signaling inside infected macrophages (86,87). *Leishmania*-secreted exosomes are also responsible for activating anti-inflammatory molecules like SHP-1, subversion of immune signaling such as PI3K-AKt and delivery of virulence factors inside macrophages (88,89). *Leishmania* sp. induces the proteasome mediated degradation of STAT-1α and halts the anti-parasitic Janus-activated Kinase 2(JAK-2)/STAT-1α signaling for its survival (90).

1.3.3.4 Interference with antigen presentation machinery and co-stimulatory signals

Antigen presentation is the main function of host macrophage for relaying the danger signal to adaptive branch of immune system. *Leishmania* impairs antigen presentation in infected macrophages through various mechanisms. Early studies have suggested decrease in MHC-II presentation capability of *Leishmania*-infected macrophages with the progression of infection (91). *Leishmania* have been shown to either degrade MHC-II molecules by parasitic proteases via uptake into *L. braziliensis* amastigotes (92); they can also decrease the affinity of MHC-II/peptide binding by altering the MHC-II confirmation through depletion of membrane cholesterol (93). PV's containing amastigotes of *L. braziliensis* and *L. mexicana* were also shown to internalize and degrade MHC-II and H-2M molecules (94). Expression of co-stimulatory molecules on the surface of Antigen-presenting cells is essential for proper T-cell activation. *L. donovani* infection decreases the expression of B7-1 on the surface of macrophages. It also hampers the activation of apoptotic signals by decreasing PD-1 levels via NFAT1c inhibition and enhancing AKT

signaling which subsequently inhibits pro-apoptotic **B**cl-2 **A**ssociated **D**eath promoter (BAD) protein (95,96).

Hence, after surviving in the initial moments after internalization, promastigotes are converted to non-motile amastigotes which stays inside PV's. *Leishmania* employs various immune evasion tactics to subvert the host cell's immune signaling, immune receptors and cytokine response, antigen presentation and parasite-killing mechanisms. Meanwhile, it replicates inside the macrophages and ultimately ruptures the cell and goes on to infect a new host cell and the cycle continues inside the human host.

1.4 Animal models for leishmaniasis: pillars of immunological and vaccine research

Use of animal models has been an integral part of vaccination research for many infectious diseases. Use of experimental animals confers lots of advantages such as affordability, ease of utility and formation of large study groups where a larger number of potential vaccine candidates can be tested and immunological parameters can be analyzed. It allows for easy observation and analysis of disease progression. Also, infected tissues and organs can be isolated for experiments or stored for histopathological analysis. Immune response and immunological memory-associated factors can be studied in detail by using various in-bred, transgenic and knockout animal strains (97). *L. enrietti* infection in guinea pigs was the first experimental model for CL. It was a natural host-parasite combination model which showed disease manifestations in 2 weeks and lesions self-healed in about 10 weeks and has now been replaced by rodent models of leishmaniasis (98). Nowadays, a high dose of subcutaneously injected *L. major* signifies the mouse model for CL. BALB/c mice are susceptible to *L. major* infection and develop uncontrolled skin lesions characterized by a

skewed Th2-based response, while C57BL/6 mice are naturally resistant to *L. major* infection, produce Th1-type response and develop self-healing, smaller lesions which resembles the human form of disease (99). To better mimic the dynamics of natural infection, researchers are injecting a small number of promastigotes dermally in ear lobes of mice to develop infection (100). The Syrian Golden Hamster (*Mesocricetus auratus*) has been considered the best animal model till date for studying VL. It was Chang and Dwyer who conclusively demonstrated that *L. donovani* is phagocytosed by hamster macrophages and forms phagolysosmes. It is highly susceptible to visceralizing forms of *Leishmania* such as *L. donovani* and *L. infantum* and manifests progressive form of disease, closely mimicking human form. Administration of VL-causing parasite is mainly achieved by i.v., intracardial and or i.p. route. VL in hamster causes parasite proliferation in spleen, liver and bone marrow in spite of a Th1 response, probably due to hindrance in lymphocyte proliferation (101–103).

Dogs serve as animal models for **C**anine **V**isceral **L**eishmaniasis (CVL) as they are the natural reservoir for *L. infantum* in Mediterranean, Middle-East, Asia and African countries. They serve as important model organism in studying the immunology of CVL and helped in designing new diagnostic aids and vector-transmission blocking methods and vaccines (19,104). Other animals include use of non-human primates such as Asian Rhesus Macaque (*Macaca mulatta*) which is a good experimental model for CL and exhibits a human-like diseases progression which usually self-heals in 3 months and produces *Leishmania*-specific B- and T- cell response. New World primates such as owl monkeys

(*Aotus trivirgatus*), squirrel monkeys (*Saimiri sciureus*), and marmosets (*Callithrix jaccusjaccus*) are animal hosts for studying VL caused by *L. donovani* (103).

1.5 Immune response against Leishmaniasis

1.5.1 Role of innate immune cells

The earliest cells which encounter *Leishmania* parasite after the sand fly bite are **P**oly**M**orpho**N**uclear (PMN) phagocytes known as neutrophils. Their antimicrobial activity was attributed to respiratory-burst induced generation of myeloperoxidase-H2O2-halide microbicidal system and several oxygen metabolites (105,106). A recent innate immune activity associated with neutrophils is **N**eutrophil **E**xtracellular **T**raps (NET) formation, which happens when eu- and heterochromatin of activated, dying neutrophils gets homogenized and released along with granular protein contents. It binds to micro-organisms and prevents dissemination, maintains high concentration of anti-microbial compounds (107,108). It was shown to be effective in killing of *L. amazonensis* promastigotes in an LPG-dependent manner (109). Rochael *et al* showed that *L. amazonensis* promastigotes induces both classical ROS-dependent and vital/early, ROS-independent NETosis occurring as early as 10 minutes post neutrophils-*Leishmania* interaction. In fact, different *Leishmania* sp. induces varying neutrophils functions which indicates role of sand fly components on neutrophils function and NETosis as a function of disease outcome (110,111). Various *Leishmania* sp. induces differential mode of neutrophil activation as *L. infantum* infected neutrophils released higher DNA content, TNF-α and exclusively produced IL-6 as compared to *L. major* infected neutrophils. *L.*

infantum infection suppresses neutrophil activation and ROS generation; inflammatory environment generated by bystander neutrophils is also ineffective in controlling parasite dissemination (112,113). *L. braziliensis* infection activated neutrophils with TNF-α and elastase release and displayed early apoptotic markers. This mechanism could be helpful for *Leishmania* as phagocytosis of apoptotic neutrophils by *L. amazonensis* infected macrophages was shown to aid in parasite survival via TGF-β and PGE2 mediated mechanism (114,115). Thus, neutrophils are important players during early stages of *Leishmania* infection.

Pathological manifestations associated with leishmaniasis prompted researchers to study the role of macrophages, which ultimately proved that macrophages are site of parasite multiplication and also acts as **A**ntigen-**P**resenting **C**ell (APC) for leishmanial proteins (116). Macrophages serve as effector cells for parasite killing through an IFN-γ dependent generation of **N**itric **O**xide (NO) inside macrophages. IL-4, IL-10 and TGF-β induced L-arginase was shown to be essential for intracellular growth of *L. major* and *L. infantum*; the effect was reversed by, N(omega)-hydroxy-l-arginine (LOHA), a physiological inhibitor of L-arginase (117,118). Although NO-based killing is the key mechanism in *L. major* infected mice, parasite clearance in case of *L. braziliensis* requires both NO and superoxide species generation (119,120). Also, IL-12 is the major Th1- inducing cytokine secreted by activated macrophages as it induces IFN-γ from Th1 cells, hence important in NO-based *Leishmania* killing. However, it was shown that TNF-α also mediates NO generation in experimental model of *L. major* (121,122). Also, TNF-α mediated IFN-γ secretion was also observed in the splenic aspirates from VL patients (123).

Dendritic-cell (DC) is an important APC which resides in the dermal layer of skin. Out of many types, dermal DC's are involved in *Leishmania* uptake and antigen presentation during natural infection. Activated DC's are capable of activating T- and NK-cells by migrating to draining lymph node and helps in production of IFN-γ (124,125). While *L. major* and *L. donovani* infection induces DC to produce effector cytokine IL-12 in early stages of infection and later in lesion self-healing (126,127), *L. mexicana* and *L. braziliensis* amastigotes were unable to activate DC's to an inflammatory phenotype . This may indicate differential responses induced by DC's activated during various phases of *Leishmania* infection (128–130). A host-protective role of NK cells was shown in cured cases of *L. aethiopica* and an indirect activation of NKT cells, a novel component of innate branch, was observed via *L. mexicana* LPG-mediated stimulation of TLR2 causing upregulation of MHC II, CD86 and IL-12p70 expression resulting in IFN-γ secretion by NKT cells (131,132).

1.5.2 Role of adaptive immunity: from effector functions to immunological memory

T cells play an important role in the generation of immune responses against intracellular pathogens like *Leishmania*. After being activated by innate immunocytes, T cells undergo differentiation to different effector and memory lineages are helps in pathogen elimination by release of various cytokines. Protection against CL is mainly linked to the production of IFN-γ by Th1 cells and susceptibility to infection is associated with Th2 response initiate by IL-4 secreting Th cells (133). However, accumulating evidences later suggested that dichotomy between resistant and susceptible mice strains is probably due to inhibition of early IL-12 signaling and active secretion of other Th2 cytokines such as TGF-β and IL-10,

rather than just IL-4 (134). Indeed, many studies have pointed to the fact that high levels of IFN-γ do not necessarily correlate with protection induced by vaccination. Higher IFN-γ/IL-10 ratio, resulting from lower levels of IL-10 and other Th2 cytokines like IL-13 may render a host-protective immunity in leishmaniasis. This implies that multifunctional immune profile needs to be assessed for determining immune correlates in disease protection (135–137). Major IL-10 producing cells are CD4+ CD25+ natural Treg cells which have been implicated in curbing the local anti-leishmanial Th1 effector functions at the site of infection through IL-10 dependent and independent mechanisms (138). They are also shown to be involved in establishing a latent reservoir of *L. major* in skin and helps in persistence of parasites. CD4+ CD25+ FOXP3+ CTLA4+ TNFR+ expressing, IL-10 and TGF-β secreting Treg cells were isolated from the skin lesions of CL patients and aim to control local effector T cell functionality (139,140).

Initially, B-cell mediated humoral response was not directly associated with development of skewed Th responses induced in resistant and susceptible mice strains (141). Later, studies showed that antigen presentation function of *Leishmania*-specific B cells, or IL-10 production by a CD1d+ CD105+ regulatory subset of B cells were involved in generation of Th2 responses during *L. major* infection (142,143). However, during *L. mexicana* infection, IgG1 and IgG2a/c were equipotent in eliciting IL-10 from macrophages, yet IgG1 deficient mice induce stronger IgG2a/c, IgG3 and IgM and are more resistant to infection; indicating that only IgG1 is disease-exacerbating IgG isotype (144). Recently, higher IgG2a/IgG1 ratio was correlated with a protective immune response in *L. tropica* infection, indicative of an isotype-specific Th response in *Leishmania* infection (145).

1.5.2.1 Th1-associated cytokines

IL-12 is a monokine which is released from the activated macrophages and serve two functions: activation of IFN-γ from Th1 cells and inhibition of Th2 cytokines like IL-4 (146,147). It is also released by DC's activated by engulfing *L. major* amastigotes in early stages of infection, but the IL-12 burst is very likely suppressed by concomitant production of Th2 type molecules such as IL-4, IL-10 and TGF-β (148,149). CD103+ DC's have been identified as a major producer of IL-12 at lesion site in *L. major* resistant C57BL/6 mice (150). *L. major* infected human DC's were shown to activate IFN-I signaling molecules like IRF2, IRF7 and IFIT5 to induce the production of IL-12, while *L. major* infected mouse DC's activated IRF4 to inhibit IL-12 production (151,152), probably due to opposing disease susceptibility of human and mice to *L. major* infection. Recently, a novel interaction between CD40L and Mac-1 was shown to be involved in production of IL-12 from macrophages and dendritic cells. Furthermore, CD4+ central memory cells require constant IL-12 stimulus for their conversion to IFN-γ secreting effector Th1 population (153,154).

IFN-γ is the major Th1 cytokine which is directly associated with leishmanial killing by virtue of NO generation through inducible-nitric oxide synthase (iNOS-2) and has been used as a systemic injection to cure complicated cases of CL (155,156). IFN-γ may exhibit its NO-mediated killing in synergism with TNF-α signaling in pre-activated macrophages, or in combination with IL-7, through a TNF-α dependent pathway (157,158). However, Carneiro *et al* demonstrated contrasting functions of IFN-γ in *L. amazonensis* infected BALB/c mice (159). IFN-γ was involved in progression of pathological symptoms via

induction of chemokines, in addition to NO-mediated effector mechanisms. Macrophages treated with IFN-γ+IL-12 confers macrophages resistant to *L. major* which signifies the importance of these cytokines during initial stages of infection (160).

Tumor Necrosis Factor-alpha (TNF-α) is an anti-parasitic cytokine secreted by macrophages. A protective role for TNF-α was established in *L. major* infection and TNF-α blocked mice showed disease exacerbation (161). It, along with other Th1 cytokines such as IFN-γ, exerts anti-leishmanial effects and mediates NO-dependent intracellular killing of amastigotes (157,162). Production of TNF-α was higher during active disease and showed significant decreasing trend post-treatment and in healed cases of human CL. Also, a recent report from endemic region showed association between treatment of psoriatic arthritis with TNF-α blockers such as infliximab and susceptibility to CL (163,164). However, in case of CL caused by *L. braziliensis*; similar to IFN-γ, TNF-α was shown to contribute to disease pathology and detrimental inflammation (165). Other Th1 associated cytokines include IL-27 and IL-23 which show structural and functional homology to IL-12. IL-23, termed as p19, associates with p40 subunit of IL-12 to form physiologically active IL-23 cytokine (166,167). IL-23 stimulates IFN-γ production from T cells and mice lacking WSX1, a component of IL-27R, were deficient in IFN-γ production and unable to control *L. major* progression. Also, IL-27 and IL-23 levels were found to be higher in healed forms of CL than the non-healing ones, implying their probable role in resolution of lesions in human CL (168,169).

1.5.2.2 Th2 cytokines

IL-4 is the signature molecule secreted by Th2 cells and is associated with pro-parasitic anti-inflammatory response in leishmaniasis. Transfer of IL-4 and IL-5 secreting cell line exacerbated *L. major* disease progression in BALB/c mice. Therapeutic treatment of susceptible BALB/c mice with IL-4 neutralizing antibody controls disease pathology and makes them resistant to re-infection with *L. major* (170,171). Exacerbation of CL in SOCS3-overexpressing transgenic mice was a result of hyper-production of IL-4 from T cells, indicating importance of IL-4 in leishmaniasis pathology (172). IL-4 was found to be the only Th2 associated factor which is augmented in antimony-resistant *L. braziliensis* isolates than the susceptible ones and it was associated with slower healing and higher parasitic burden in lesions. IL-4 also limits the expression of Th1 associated chemokines such as CCL5 and CCR5 at the lesion site and limits the recruitment of IFN-γ secreting Th1 cells and promotes parasite persistence (173,174).

IL-10 is an immunosuppressive cytokine produced by T cells, macrophages and monocytes. It was shown to directly involved in the suppression of macrophages, even in the presence of IL-12 in lesions of individuals with active human CL, and also known to inhibit the maturation of DC's (175–177). Saliva from *L. intermedia* was shown to skew immune response in *L. braziliensis* exposed patients towards Th2 subtype which is dependent on production of IL-10 by CD25+ FOXP3+ Treg cells (178). In fact, IL-4 deficient mice remain susceptible to *L. major* infection (179), probably due to immunosuppressive role of IL-10 which is independent of IL-4/IL-13 axis, and from sources other than CD4+FOXP3+ T cells (180,181). Chronicity in *L. mexicana* infection was also shown to be contributed by IL-10 secreted by cellular sources other than CD25+

Treg cells (182). Genetic basis of chronic CL was recently identified where IL-10-819 C/T SNP was found to be significantly higher in patients with severe infections (183). Hence, due to multi-varied nature of IL-10 in *Leishmania* pathogenesis, immunotherapeutic intervention via IL-10 blockade showed promising results and decreased the levels of IL-10, IL-4 and TNF-α and showed partial inhibition of CXCL10 (184).

TGF-β is an anti-inflammatory cytokine with multiple effects on wide variety of leukocytes such as macrophages, lymphocytes, dendritic cells and neutrophils (185). TGF-β is produced during *Leishmania* infection and is an important determinant of susceptibility as anti-TGF-β treatment protects mice from *L. major* infection; even with low levels of IFN-γ in treated mice (186,187). Higher population of TGF-β and IL-10 secreting cells were found in the chronic phase of disease as compared to acute or early phase, implying a synergistic role of TGF-β with IL-10 in disease progression by *L. major* (188). Recently, G/G genotype at 25^{th} codon of TGF-β1 was associated with resistance and G/C or C/C genotype was associated with susceptibility against American tegumentary leishmaniasis in South Brazilian population study cohort (189). IL-17 is a pro-parasitic cytokine in CL which exacerbates *L. major* infection and disease progression by influencing neutrophil recruitment (190). WSX1 deficient mice developed a severe form of CL and exhibited an increase in CD4+IL17+ cells which suggest that IL-27 acts antagonistically to IL-17 mediated disease progression mechanism (191). IL-17 was also observed to mediate the immunopathology in IL-10 dysregulated, *L. major* infected C57BL/6 mice, which exhibit larger lesions with fewer parasites, probably via IL-1β induction (192). Higher IL-17-secreting cell population in ML as compared to CL form of

L. braziliensis in patients, suggests its pathogenic role in destructive inflammatory reaction in leishmaniasis (193). Th1 and Th2 cytokines, along with their effector functions in CL are shown in **figure 4.**

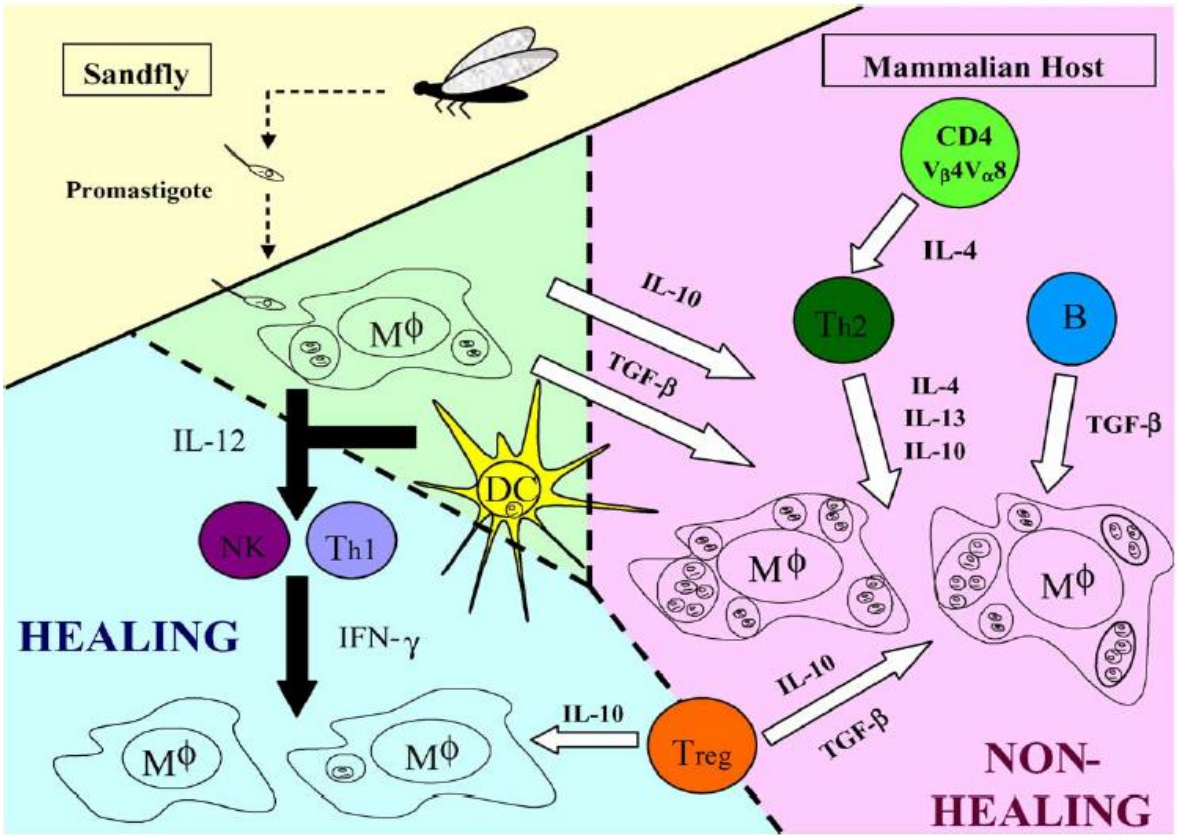

Figure 4. Th1/Th2 phenotype association with disease progression in *L. major* infection. IL-12 production by infected macrophages can induce NK cells and induces Th1-based immune response. IL-12 induces IFN-γ production from Th cells, which in turn activates the infected macrophages for NO-dependent parasite killing. On the other hand, IL-4/IL-13 signaling activation in infected cells, which is further enhanced by IL-10 production by host cells and CD4+ CD25+ Treg cells induces pro-parasitic Th2 response (adapted from Alexander J., Brysan K., *Immunol Lett*. 2005).

1.5.3 T cell memory subsets and importance in leishmaniasis

T cell memory is an important tool in disease clearance upon re-infection with previously encountered pathogen. Memory subsets have enhanced proliferative capacity and effector

functions than naïve T cells. This is particularly important in vaccination because an effective vaccination should generate an adequate pool of disease-clearing Th memory cells upon priming, which can undergo quick activation, proliferation and cytokine release upon encountering the same pathogen and helps in pathogen clearance. Following pathogen clearance or absence of antigenic stimulation, majority of short-lived effector cells die and leave a pool of long-lived memory cells which are classified on the basis of proliferative capacity, effector function and migratory ability into following subsets: 1) **T**-**E**ffector **M**emory (T_{EM}) cells, having low proliferative and high cytokine secretion ability, migrate between spleen, blood and peripheral tissue and express high CD44, low amounts of CD62L (L-selectin) but does not express CCR7 2) **T**-**C**entral **M**emory cells (T_{CM}), having high proliferative capacity, re-circulate between lymphoid organs and blood and does not have tissue-homing capacity **(Figure 5)**. They express high CD44, CD62L and expresses CCR7 3) A new subset known as **T**issue-**R**esident **M**emory cells (T_{RM}) also exist, which is similar to T_{CM}, except that they express CD103, CD69 and CD11a on their surface and reside locally at the site of infection (194–196). Although the functional difference between T_{EM} and T_{RM} is yet to be established, some reports suggests that T_{EM} are actually T_{RM} which are rapidly recruited to tissues following inflammation; while the activation of T_{EM} by successive antigenic signals point towards a separate circulating subset of effector memory cells (196,197).

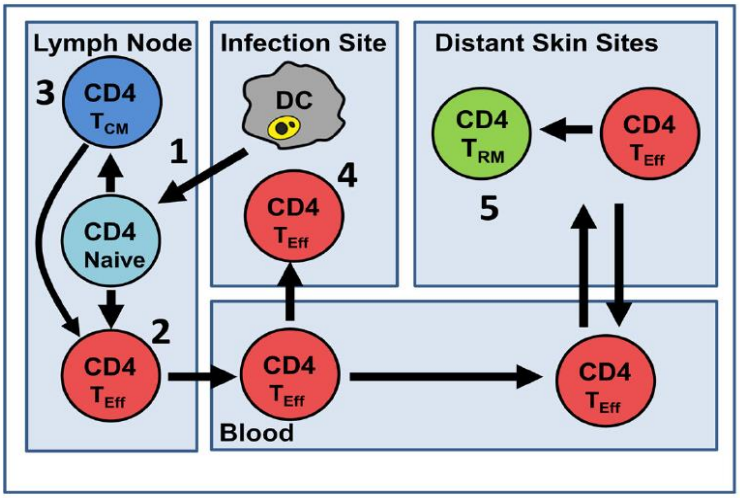

Figure 5. Residence and migration pattern of different T-helper memory subset in cutaneous leishmaniasis. APC's migrate from infection site to draining lymph node and activates naïve CD4 T cells into short-lived effector (T_{Eff}) and T_{CM}. While T_{CM} resides in lymph nodes and maintain long-term antigen-specific pool, T_{Eff} migrates to bite site for parasite clearance and homes to distant skin tissues as T_{RM} cells (Adapted from Glennie N.D., and Scott P., *Cell Immunol*. 2016)

Early studies have debated on the importance of a persistent parasite pool in defining susceptibility and maintenance of long-lived central memory cells (198,199). Recent studies have highlighted the importance to T_{RM} in mediating protection against subsequent *L. major* challenge by recruiting circulatory memory cells, via CXCR3, and inflammatory monocytes via NO-mediated pathway (200,201). These studies, irrespective of Th memory subset, reinforce the importance of memory cells in long-term anti-leishmanial immunity. Also, antigen-restimulated PBMC's from long-term healed patients of CL caused by *L. braziliensis* exhibited effective expansion of effector memory T cell population (202), indicating long-term maintenance of *Leishmania*-specific memory pools in natural infection as well. Colpitts *et al* delineated the importance of IL-7R signaling in

maintaining T_{EM} and T_{CM} pools in *L. major* infection (203). Hence, these studies provide conclusive evidence that generation and maintenance of anti-parasitic Th memory cells are pre-requisite for generating an effective anti-leishmanial vaccine.

1.6 Development of Vaccine modalities against leishmaniasis

Efforts for development of life-long immunity against leishmaniasis started way back at the beginning of 20th Century. Different methods have been tried and tested, numerous modifications have been done in the existing methodologies for the generation of a suitable vaccine against leishmaniasis and it went hand in hand with the advancement in the field of immunology.

1.6.1 First Generation Vaccines: bug as its own nemesis

During the first generation of anti-leishmanial vaccine, it used to be live leishmanization whereby individuals were inoculated with low number of live virulent parasites. At the earliest point of time, inoculation of live virulent promastigotes of *Leishmania* was used to develop intentional cutaneous lesions for subsequent life-long immunity against the parasite. The technique came to known as Leishmanization (LZ). Various tribal societies of the Middle East deliberately expose their babies' buttocks to be bitten by sandflies in order to avert facial lesions (100). The individuals used to develop scars but protection as well, albeit not in all cases. First attempt at vaccine trials using Leishmanization strategy was done at Turkmenistan and subsequently Uzbekistan tried this technique. Thereafter, Iran in 1970's and Israel in 1980's, adapted LZ as a prophylactic treatment against CL. After initial success, LZ was discontinued in these two countries owing to drawbacks such as loss of infectivity after continuous in-vitro passages, inefficacy of other vaccines owing to

immuno-suppression in Israel and few reports of prolonged and non-healing lesions in Iran (204). Still, Uzbekistan is the sole country where the mixture of live and dead *L. major* parasites is licensed in the form of the vaccine preparation. A fresh isolate from human is used every year to maintain the virulence of vaccine preparation. However, standardization and safety issues still pose a challenge (205,206). Use of live, virulent parasites in LZ carries the risk of immuno-suppression due to preferential skewing of immune response towards T_H2 type by *Leishmania* which could lead to failure of vaccination regimens for diseases such as measles, mumps, diphtheria to name a few. Immunocompromised individuals such as HIV patients can become more prone to debilitating diseases by further weakening of the immune system by chance aggravation of parasitic infection.

Drawbacks associated with the use of virulent parasites had shifted focus towards the use of killed *Leishmania* strains by means of autoclaving and irradiation. Interest in this strategy waned due to conflicting results in Middle East and Brazil in 1940's but the importance revived in late 1970's when Mayrink *et al* started field efficacy trials of a preparation containing five killed *Leishmania* isolates from four different species and found positive results. This was later prepared as *L. amazonensis* based vaccine and tested for prophylactic and therapeutic use. In the next decade, Convit and colleagues used *L. mexicana* or *L. braziliensis* promastigotes along with BCG both prophylactically and therapeutically in Venezuela against CL (100,206). In field efficacy trials, it emerged as low risk and cost effective treatment as compared to antimonials (207). Later, Mayrink's vaccine was found to reduce the dose of antimony treatment when used as an adjunct to chemotherapy and was subsequently registered in Brazil (208). In late 1990's, vaccine

containing autoclaved *L. major* (ALM) with BCG as an adjuvant was produced by Razi serum and vaccine institute in Iran. It was tested in various Phase I-III double-blind trials where the vaccinated groups were better Leishmanin skin test (LST) responders than the control groups, but multiple vaccination regimens and better adjuvants were advised to increase its immunogenicity and efficacy (209,210). Similarly, efficacy trials against VL produced similar results with LST conversion higher in vaccinated than control groups (211). A technique in which *L. infantum* chagasi promastigotes were treated with amotosalen (S-59) and subsequently irradiated with low dose of UV-A radiation, thereby making it replication deficient, was also used as a vaccine preparation. This killed but metabolically active parasite provided protection against experimental VL in mice (212). Killed parasite vaccine preparations were tested in different endemic foci containing population susceptible to different *Leishmania* species and it seems that these were more successful rather as an accessory to chemotherapeutic treatment than prophylactically. Although economical and easy to prepare, difficulty in standardizing *Leishmania* cultures and inconsistent results with respect to efficacy have made this an unpopular vaccination strategy.

Failure of killed parasites in eliciting a protective immune response led to employment of live but attenuated parasites. Earlier, scientists used chemical mutagenesis, irradiation, culture under antibiotic pressure to decrease the virulence of *Leishmania*, but the associated risks of virulence reversion and the subsequent advances in the field of genetic manipulation has made these techniques to be replaced by more specific methods such as "genetic attenuation" (100,213). Although attenuation of *Leishmania* by genetic alteration

was conceptualized in the era of third generation vaccines i.e. in late 1990's, it inherits the basic features of live-attenuated first generation vaccines. Incorporating the advantages of inherent adjuvanticity and the maximization of the parasite antigen repertoire availability to the immune system, genetically defined attenuation with targeted gene disruption such as of Centrin, DHFR or A2 in the *Leishmania* parasite was attempted. Although these parasites showed promise in experimental models, little is seen in clinical trials. *L. major* Dihydrofolate reductase - thymidylate synthetase knockout (DHFR-TS$^{-/-}$) was found to be protective against *L. major* and cysteine protease knockout (cpa and cpb) gave protection against *L. major* and *L. amazonensis*. Lpg2$^{-/-}$ mutants conferred protection but regained virulence over time. Centrin knockouts (CEN$^{-/-}$) and P27 knockouts (LdP27$^{-/-}$) in *L. donovani* were designed to inhibit the growth of parasite in amastigote stage but the parasites remained viable i*n vitro* in the form of promastigotes. This may allow the propagation of genetically attenuated cell line. Although single knockouts in the form of SIR2$^{+/-}$ in *L. infantum* and A2-A2rel gene cluster in *L. donovani* afforded protection in mice models, but the risk of reversion to virulent phenotype due to presence of wild type allele hampers their use as vaccine candidates (100,213,214). The main argument advocating the use of live attenuated vaccines has been the persistence of parasites which can mimic the natural course of infection. Life-long immunity against *Leishmania* after a healed infection implies key role of central memory T cells in the maintenance of immunity. Uzonna and colleagues suggested that parasite clearance led to destruction of central and effector pool of memory T cells making mice re-susceptible to challenge infection (198), but later investigations by Zaph *et al* clearly showed that central memory T

cells are maintained even in the absence of parasites (199). While many experiments have delineated the key role of memory T cells in long term anti-leishmanial immunity, the importance of parasite persistence in this regard needs to be reassessed.

Although, attenuated parasite vaccines mimics natural parasitic infection, presents a greater diversity of parasitic antigens leading to a broader immune response, but some antigenic proteins may themselves skew the response towards T_H2 type as demonstrated by Liu *et al* (215). Furthermore, lack of pertinent indicators for a successful attenuation, assessment of genetic and physiological stability of attenuated parasites, standardization of culture systems and possibility of genetic recombination by attenuated strain are the important factors to be taken care of before a live attenuated vaccine for *Leishmania* comes into market (100,216). **Table 4(a)** (shown at the end of text) enlists various vaccine candidates tried under first-generation vaccines.

1.6.2 Second Generation Vaccines: interdisciplinary approach opening new avenues

As the knowledge about the T cells providing antigen-specific protective responses was just developing while the first generation vaccines were being tried, the second generation of vaccines replaced the previous approach and followed the trend of host-protection by T cells, which recognize antigen as peptides. Therefore, many attempts were made using recombinant proteins or T cell peptides. Although the mouse experiments were successful, the clinical trials failed again. **Table 4(b)** (shown at the end of text) presents a brief overview of the status quo of second generation vaccination strategies against leishmaniasis.

With the increase in knowledge about the genomics and proteomics of *Leishmania*, various antigenic proteins were identified which are expressed in either of the two stages of the parasite i.e. promastigote and amastigote. This era included the incorporation of specific proteins of *Leishmania* instead of whole organism. It started with the testing of native leishmanial proteins extracted by employing chemical procedures, sonication etc. Glycoproteins such as gp63, gp36, **F**ucose-**M**annose **L**igand (FML) were purified from *Leishmania* cultures and tested in mice models for protection against CL and VL caused by *L. major* and *L. donovani* respectively. Similarly, *Leishmania infantum* excreted secreted antigens (LiESA) was tested in dogs for protection against CVL caused by *L. infantum* (217). Out of these, FML remains the most studied and promising candidate. Initial trials using FML with various adjuvants in mice and hamster model of *Leishmania* exhibited significant protection in the form of delayed hypersensitivity reactions and skewed response towards T_H1 type. This encouraged scientists to extend trials in dogs which are the key reservoirs of *Leishmania*. FML + QuilA saponin formulation was found to be immunogenic and 95% protection was achieved against VL infection in dogs. Furthermore, predominant T_H1 response and long-term immunological memory was seen in immunized dogs. These results ultimately led to registration of FML + QuilA saponin formulation under the trade name Leishmune® in Brazil (217–220).

At the advent of this vaccination strategy, various leishmanial stage-specific proteins were tested which are preferentially expressed in one of the parasitic stage. gp63, one of the earliest candidates to be tested, is preferentially expressed in promastigotes. Another promastigote specific protein is **P**romastigote **S**urface **A**ntigen (PSA-2) which is a complex

comprising of three antigenically similar polypeptides. While distinct gene products were expressed in promastigotes and amastigotes of *L. major* and *L. donovani*, promastigote specific expression is seen in *L. mexicana*. Later, many amastigote specific antigenic proteins were tried namely A2, P4, P8 against *L. mexicana pifanoi* and *L. donovani* (100,206). Another licensed vaccine under the name Leishtec® was developed by Hertape Calier Saude Animal containing the recombinant A2 antigen expressed by adenovirus. It was licensed for commercial use in 2007 by Brazilian Government. Later, immunization of mice with adenovirus expressing A2 significantly reduced parasite load in spleen and liver with a contrasting low antibody response while producing high numbers of IFN-γ producing CD4+ and CD8+ T-cells. Furthermore, a recent comparative study between Leishmune® and Leishtec® finds them equally efficacious in decreasing parasitism and reducing parasite transmission (221–223). Variable results were obtained with these stage-specific proteins highlighting the need for selection of proteins expressed in both stages of the dimorphic parasite. Later, proteins such as Cysteine proteases (CPA/CPB), LD1, Hydrophilic acylated surface protein B1 (HASPB1), Histone proteins (H2A, H2B, H3, H4), LeIF, TSA, LmSTI1 which are present in both stages of the parasite were tested in VL and CL. In many of these proteins, there is difference in immunogenicity observed when they are administered in native and recombinant forms. This puts light on the importance of conformational structure of protein in antigenic proteins (224). But this difference may be due to other factors as anti-leishmanial immunity is majorly T-cell dependent which recognizes linear epitopes attached to MHC-I/II. Many of these proteins were used in combination in a single preparation to elicit a broader anti-leishmanial

immunity. One such preparation is LEISH-111f which is a single polyprotein incorporating three proteins namely - *L.major* homologue of eukaryotic thiol- specific antioxidant (TSA), *L.major* stress-inducible protein-1(LmSTI1) and *L.braziliensis* elongation and initiation factor(LeIF) fused in tandem (224). Leish-111f provide protection against VL in murine model by enhancing T_H1 response (225), however, it failed to provide protection against *L. infantum* in Phase-II clinical trial in dogs (226). However, it has been tested in Phase I and Phase II clinical trials in healthy volunteers, CL and ML patients in combination with chemotherapy and most recently in an *L. donovani* endemic area in India (227–230). All of these studies in a span of 3 years concluded LEISH-111f + MPL-SE (a TLR4 agonist) to be safe and immunogenic in volunteers. An engineered version of LEISH-111f which doesn't contain His-tag at amino terminus and a proteolytic hot-spot, termed as LEISH-110f exhibited protective effect in mice against *L.major* or *L .infantum* challenge (231). In recent times, LEISH-113f or LEISH-F3 which is a fusion protein incorporating **N**ucleoside **H**ydrolase (NH) and **S**terol 24-c **M**ethyl **T**ransferase (SMT) was manufactured by IDRI and set up for pre-clinical and Phase I clinical trial in United States in 2012. Pre-clinical data of this preparation, along with GLA-SE as an adjuvant, showed protection against *L. donovani* and *L. infantum* in mice, generating a skewed T_H1 response. Futhermore, Phase I trials demonstrated it to be safe and immunogenic in healthy volunteers. Companion Phase I trials are going on in India in collaboration with Banaras Hindu University, Varanasi (232,233).

An antigen contains multiple combination of epitopes which may elicit/activate a T-cell towards a host protective or a pro-pathogen response. Julia *et al* conducted an experiment

in which mice were tolerized to LACK protein of *Leishmania* parasite early in their life. LACK transgenic mice showed resistance to subsequent *L. major* infection characterized by predominant T_H1 response as compared to untolerized mice (234). One possibility suggest that epitopes generated from degradation of LACK antigen during infection generated a predominant T_H2 response which overshadowed T_H1 response generated by other antigenic proteins. Early tolerization deleted the LACK specific T cells resulting in absence of LACK specific immune response at the time of infection. Failure of most subunit vaccines in generating complete protective response against *Leishmania* can be due to predominance of T_H2 inducing epitopes in protein candidates. Moreover, some proteins may be less immunogenic when administered alone and are more successful when administered with other antigenic proteins in a cocktail vaccine. In many cases, combination of different antigenic proteins fared well than their single counterparts. Dose of an antigenic protein may also influence the type of response generated by the cleavage and processing during presentation by MHC molecules. Increasing the amount of antigenic protein beyond a certain limit during the immunization schedule may result in the presentation of cryptic epitopes which are not presented normally during the antigenic processing as suggested by Warnock and Goodacre (235). This may alter the type of immune response generated by that antigen and can shift it towards T_H2 type. In addition, over exposure to an antigenic protein may result in the tolerance and deletion of antigen specific T-cell clones resulting in the failure of immune response.

Skewness of a response towards T_H1 type can be achieved by administration of specific high-affinity and immunogenic epitopes instead of whole-length protein. This has led to

emergence of synthetic peptide based vaccines in 1980's. Peptide portions of gp63, Cysteine protease (CP), histone protein H1 were put to test in animal models. While T_H1 response was observed in case of gp63 and CP's with increased production of IL-2, IFN-γ and increased proliferation of CD8+ T-cells, epitopes of H1 gave only partial protection in animal models (217,236–238). Although later studies with gp63 peptides showed long-term protection against *L. major* (239), initial enthusiasm in synthetic peptides soon faded due to inappropriate memory T-cell response. To address the inadequate presentation of the antigenic repertoire from the pathogen, the recombinant proteins were replaced by a combination of chimeric peptides, wherein immunodominant peptides from different immunogenic proteins were put together. However, this also met with very limited success. Furthermore, the complexity is enhanced by presence of multiple alleles of MHC-II molecules in population of different geographical areas (100). There is a possibility that MHC-II prevalent in Sudanese population may bind an epitope with less avidity or may not interact at all than that of the MHC-II found in the Indian population due to structural constraints associated with each MHC allele and that an MHC allele needs their binding peptides to fulfill particular structural criteria as reviewed by J.C. Howard (240). For addressing the lack of correlation between the mouse and human data, the *Leishmania* antigens dominant over a huge population covering extensive HLA polymorphism were identified and then put together in a construct. However, the results from clinical trials are still awaited**.** Increase in knowledge about MHC polymorphism together with better management of production economics can make synthetic peptides as a plausible vaccination strategy in near future.

1.6.3 Third generation vaccines: Biotechnological era; exploiting the host and parasite genetic machinery

The possible reasons for the failure of the peptides and recombinant proteins are manifold. Their stability, lack of inherent adjuvanticity, lack of peptide-specific memory T cells and inadequate priming of the potentially available T cell repertoire and the lack of successful extrapolation of mouse data to human were a few of them. Therefore, in order to take care of these problems, the third generation vaccines took several routes. DNA vaccine was proposed to overcome the drawbacks related to adjuvanticity and stability of the proteins/peptides. This was because the CpG motif was found to be protective and is now known to be mediated by TLR9 activation. As an alternative to the problem of adjuvanticity, cationic liposomes were used to deliver the antigens. The cell surface being negatively charged, the cationic liposomes were expected to deliver the antigens to antigen-presenting cells more efficiently. As a live alternative for delivering the antigens, the leishmanial antigens used to be cloned and expressed in different bacteria such as *Salmonella*, which being alive would provide danger signal and also activate both TLR4 and TLR9 to boost the immune system with their adjuvant effects.

This generation of vaccine strategy comprise of nucleic acid vaccination in which a plasmid DNA vector encoding the gene of interest under the control of a eukaryotic promoter is injected directly into muscle or skin. Although the efficiency of uptake and translation of the encoded protein is quite low by the cells, it is adequate to evoke cellular immunity against the antigen as proven in various animal models (100,241–243). An added advantage of DNA vaccination is the induction of cytolytic T cell (CD8+ T-cell)

responses possibly due to the cross-presentation of antigen by professional APC which may engulf apoptotic cells, re-present free peptides or peptides complexed with heat shock proteins (243). This is particularly important in case of *Leishmania* as CD8+ cells have been shown to play a protective role in anti-leishmanial immunity (244). Antigenic proteins of *Leishmania* which were tested in purified native and recombinant form were given encoded in plasmid vector. Gp63 was the first candidate delivered as a plasmid preparation. Subsequently, LACK, PSA-2, gp36, A2, LCR, PapLe22 and numerous other candidates were tested in animal models of *Leishmania*. The protective effect of same protein when delivered in the form of DNA vaccine was found to be greater than its recombinant protein preparation, for example in the case of LACK, PSA-2 and Cysteine proteases. Histone proteins such as H2A, H2B, H3, H4 and cathepsin-L like Cysteine proteases CPA, CPB and CPC exhibited better protection in *L. major* infection when given as a mixture of plasmid preparations rather than immunization with single construct (206,224).

A recent technique employing DNA vaccination is heterologous prime boost in which priming is done by plasmid DNA preparation and boosting is done by purified protein. LACK and Cysteine proteases have been used in this form against experimental visceral leishmaniasis in dogs (208,245). Heterologous prime boost provided better results than homologous prime boost and reverse-heterologous prime boost due to unknown reasons as reviewed by Shan Lu (246). More recently, a DNA vaccine comprising **M**inismastically **I**mmunogenically **D**efined **G**ene **E**xpression (MIDGE) vector developed by Mologen AG called LEISHDNAVAX was constructed in which immunogenic epitopes from the pan-

species proteins of *Leishmania* were encoded in the vector. The vaccine provided protection against *L. donovani* in rodent and elicited CD4+ and CD8+ T-cell responses from cured patients of leishmaniasis (247).

DNA vaccination enjoys many advantages. Expression of protein in the eukaryotic cell ensures proper folding and post-translational modifications as *Leishmania* itself is a eukaryotic organism. Longer persistence of plasmid inside the cell and in different tissues ensures the constant encounter of antigenic protein with the immune system. C_PG motifs present in plasmid backbone works as intrinsic adjuvant having TLR9 activating property, although it may effect transgene expression in some cases. Furthermore, plasmid preparations have better stability, longer shelf-life and a cold chain is not a pre-requisite for their transport (100,248). The major concern which surrounds nucleic acid vaccination is the possible integration of foreign sequences into host's genome. Although several studies concluded the possibility as negligible and Ledwith *et al* determined the plasmid integration rate and found it to be three times lower than the spontaneous mutation in mammalian genomes. Better understanding of integration mechanism will help us in limiting the probability of integration or, at least, developing a sensitive method for detecting an integration event in future. This achievement will surely make DNA vaccination more feasible for human trials (249). While third generation strategies were the least one to be tested against this disease, **table 4(c)** (shown at the end of text) briefly overviews the pros and cons of vaccination strategies under this class.

1.6.4 Dendritic-cell based Vaccination: generating a potent immunocyte population

Dendritic **C**ells (DC) are important regulators of innate and adaptive immunity. Being the most potent APC's, they are able to activate CD4+ and CD8+ cells and interaction of Pathogen associated molecular pattern (PAMP) with Toll-like receptor (TLR) present on their surface helps in activating them and increasing the expression of co-stimulatory molecules such as CD80, CD86 which again helps in T cell activation (18,250). DC's have been tested against both cutaneous and visceral forms of leishmaniasis. In separate studies, DC's pulsed with crude extract of *L. major* and *L. donovani* gave persistent protection against respective forms of leishmaniasis (251,252). Although initial studies used *Leishmania* infected DC's for inducing protective immunity against *L. major*, antigen pulsed DC's became a popular strategy later due to ethical reasons associated with administration of live parasite containing DC's in humans (18,148). While IL-12 plays a key role in the induction of protective immunity via DC's (253), paradoxical reports delineating the role of IL-12 suggests that additional signaling cascades may be necessary for mediating protection against leishmaniasis (252,254).

DC's pulsed with leishmanial antigens such as LACK, gp63, PSA, KMP-11, LeIF have afforded protection in rodent models of *L. major*. Soluble *Leishmania donovani* antigen (SLDA) pulsed **B**one **M**arrow DC (BMDC) in combination with antimonials cured VL infected mice. Furthermore, BMDC pulsed with a 20 amino acid long peptide of KMP-11 along with C_PG as an adjuvant exhibited protection against VL. As it is a new strategy in its developing phase, standardized protocols for isolation, maintenance and expansion of DC's from humans needs to be developed. Parameters which affect the efficacy of this vaccination method such as antigen loading, maturation state of DC, route and frequency

of administration needs to be taken care of in a precise manner (18). Furthermore, heterogeneity in the DC population adds to the complexity in selection of DC for antigen pulsing.

In totality, we can say that progress to a successful vaccine against *Leishmania* sp. has seen various vaccination modes being tried and tested, both in pre-clinical and clinical studies, with mixed results and subsequent increase in knowledge about parasite biology and correlates of protection against *Leishmania* has led scientists to formulate new strategies to tackle this disease. Newer approaches like DNA vaccination or a combination of DNA vaccination and recombinant protein priming strategy, known as **H**eterologous **P**rime-**B**oost (HPB), show promise as they are shown to be more immunogenic and efficacious than other vaccination strategies for many infectious disease.

Table 4(a). First-generation vaccines against leishmaniasis

S. No.	Vaccine Modality	Vaccine Candidate/s	Advantages	Limitations	References
1.	Leishmanization (LZ)	Live virulent promastigotes of *Leishmania major*	➢ Subsequent lifelong immunity after first healed infection. ➢ Uzbekistan currently employs this strategy.	➢ Loss of Infectivity, immunosupression, protracted and non-healing lesions.	(204,206)
2.	Live attenuated	Less virulent parasites through repeated culture, radiation.	➢ Closest mimic to natural infection, skewed T_H1 response.	➢ A chance of reversion to virulent state, difficulties in standardization and quality control hinders commercialization.	(100,204,206)
3.	Whole Killed Parasites	Autoclaved or heat killed *Leishmania* sp.(single or multiple)	➢ No reversion to virulence, good safety profile and immunogenicity. ➢ Encouraging results in therapeutic trials.	➢ Lack of reproducible efficacy and long lasting immune response, immunogenicity didn't get converted to protective effect.	(100,213)
4.	Genetically altered	LdCen-/-, DHFR-TS-/-, Lpg1-/-, Lpg2-/-, CP-/-, BT1-/-, ΔPMM, SIR2+/-, LdP27-/-, HSP70-II-/-.	➢ Longer persistence in host and lesser chances of virulence reversion. ➢ Large scale production in well-defined conditions. ➢ Compatible with various adjuvants. ➢ Presence of whole organism during the initial stages elicits a better protective response. ➢ Safe even in immunocompromised individuals.	➢ DHFR-TS-/- didn't protected from challenge in Rhesus monkey. ➢ Lpg2-/- partially reverted to virulence after prolonged periods. ➢ Safety issues such as blocking of vaccine strain transmissibility by sand fly vector are to be addressed.	(18,213,250,255)

Table 4(b). Second-generation vaccine candidates against leishmaniasis

S. No.	Vaccine modality	Vaccine candidate/s	Advantages	Limitations	References
1.	Purified *Leishmania* fractions	FML, LAg, SLA, gp36, dp72, gp63, LD 31/51/72/91, gp70-2/dp72, Hsp70/Hsp83, gp63/gp70, LPG, 78kDa, Leishmune (FML + Saponin adjvant).	➢ Leishmune is the first licensed vaccine for CVL, used as a prophylactic in Brazil since 2004.	➢ Difficulties in large-scale production, in-vitro culture standardization and purification.	(221,250)
2.	Recombinant Proteins (Single Component)	Gp63, PSA-2, LeIF, LmSTI1, LACK, A2, P4, P8, lcr1, TSA, GRP78, H1, HASPB1, gp46/M2, LD1, SP-15, dp72, KMP-11, ORFF, NH36, F14, papLe22, HSP90, Ldp23, PFR-2, rcp5, rLdccys5, LRP, Lip0, LJM17, LJM19, LJL143, Maxidilan, Leishtec.	➢ Some proteins (LeIF) can work itself as an adjuvant. ➢ Adjuvants such as IL-12, TLR agonists increase the protective T_H1 response. ➢ CD8+ T cell response was generated by some proteins (HASPB1).	➢ Immunogenic variability due to non-native conformation. ➢ Complete protection not achieved with few proteins. ➢ Significant T_H2 response, either through IL-10 (KMP-11, papLe22, HSP90 and LACK) or I_gG1 production in some cases. ➢ Poor generator of CD8+ response and no single antigen confers complete protection.	(18,100,213,217,224, 250,256,257)

3.	Recombinant proteins (Multicomponent)	Q protein (Lip2a, Lip2b, P0 and H2A), TSA/LmSTI1/LeIF/Lbhsp83, Leish-111f(LmSTI1+LeIF+TSA), Leish110f, CPA/CPB fusion protein, Leish-F3, KSAC (KMP-11, SMT, A2 and CPB)	➢ Wide range of protection conferred by multiple antigens. ➢ Leish-F3 completed Phase I trial in U.S. and companion Phase I trial is undergoing in India.	➢ LeishF1 failed in Phase III clinical trials in dogs. ➢ Fusion protein of CPA and CPB was recognized weakly by PBMC of recovered patients.	(213,217,221,232,258,259)
4.	Synthetic Peptides	PT3 of gp63, Peptide I of CP, peptides from PSA .	➢ Specific epitopes for potent T-cell induction. ➢ Synthetic vaccine under RAPSODI project funded by EU showed 75% positive immune response and can be used as enhancers in leishmaniasis vaccines.	➢ Lesser T-cell memory response. ➢ Inter-population variability to immune response. ➢ Economic considerations.	(100,217,260,261)

Table 4(c). Third-generation vaccine candidates against leishmaniasis

S.No.	Vaccine modality	Vaccine candidate/s	Advantages	Limitations	References
1.	DNA Vaccines (Single and Multicomponent)	Gp63, LACK, PSA-2, gp46, A2, H1, CP's, p36LACK, PapLe22, KMPII, TRYP, NH36, γGCS, A2/NH, H2A/H2B/H3/H4, H2A/H2B/H3/H4/LACK, gp63/gp46/CPb, KMPII/TRYP/LACK/gp63, KMP-11, PPG N-terminal domain, ORFF, HSP20	➢ Protein expression is still sufficient to induce immune responses. ➢ CD8+ T-cell mediated protection is achieved with candidates such as LACK, H2A/H2B/ H3/H4, ➢ Cost-effective and simple production. ➢ C_PG motifs in DNA vaccine can themselves function as adjuvant.	➢ Partial protection is achieved with some candidates such as H1, CP's, γGCS, NH36, PapLe22. ➢ KMPII, TRYP, LACK and gp63 afforded no protection in dog. ➢ P36LACK has variable results in different studies. ➢ A viral vector based and LEISHDNAVAX is being tested in Europe.	(100,213,217,224,250,260,262)
2.	Heterologous Prime-Boost Strategy	P36 LACK, CPA/CPB, CTE of CPIII, CPC, ORFF, KMP-11, gp63, CTE of CPb, Lip0.	➢ Same candidates showed more amount of protection than their older vaccination strategies. ➢ Higher specific immune response than DNA vaccination or recombinant proteins.		(213,217,263)
4.	Dendritic cell (DC) based vaccines	Ag-pulsed DC (SLDA, KMP-$11_{12\text{-}31\ aa}$, LACK/gp63/PSA/KMP-11/LeIF, H1, HSP20), Plasmacytoid DC, Hybrid cell vaccination therapy,	➢ Integrating DC vaccination with Chemotherapy cured established VL infection. ➢ Protection was dependent on regulation of T_H, T_C and T_{reg} cells.	➢ Procedure is laborious and expensive, limits feasibility in developing countries. ➢ Standardized protocols for production and delivery needs to be developed.	(18,250,263)