Introduction and Review of Literature

Beginning of Era of Immunology

When animals arrived on the Earth immediately surrounded by the pathogenic and non-pathogenic microbes that contained numerous varieties of poisonous substances, these pathogens threatened host homeostasis. As a result, animals exposed to an environmental pathogen started developing innate immunity gradually. The word "Immunity" came into existence after an outbreak of plague in Athens during the 5th century BC, as coined by Thucydides. When smallpox was endemic in China during the 10th century, the process of immunization routinely used for treatment was to expose healthy individuals by inoculating the disease lesions into the skin. But it was abandoned due to safety and ethical issues. Later on, variolation practice became widespread in England mainly because of Lady Mary Wortley Montague, who survived from smallpox but lost her brother. In a while, a surgeon Charles Maitland came and variolated six condemned prisoners and fortunately, all survived. The principle of variolation spreaded from England to the American scientific community and provided a new direction in the field of a vaccine. Finally, Edward Jenner validated the process by using cowpox as a safe vaccine for smallpox. Later on, Koch and Pasteur celebrated success in making vaccines through attenuation. Although the success of vaccination against anthrax and rabies took a new shape, the mechanism of immune response remained a mystery for many years. Years later, Metchnikoff came up with an immunological mechanism termed phagocytosis. Metchnikoff established phagocytosis as the first line of defense against infection at Pasteur Institute, Paris. Soon after, he faced violent opposition from the scientific community which believed that phagocytosis was not the sole source to provide immunity rather body fluid was responsible for immunity. As a support to this humoral factor, Paul Ehrlich predicted the existence of antibodies and their receptors. He suggested that antigen would interact with an antibody. Finally, Ehrlich was able to introduce

the concept between self and non-self discrimination. This provided the concept of Innate and Adaptive immune system.

Innate immunity

The immediate, first line of guard against intruding non-specific microbes starts working within an hour. Due to non-specific recognition, no immunological memory develops for combating the same pathogen in future. Innate immune cells are the first to reach the site of infection or inflammation, release numerous cytokines, lipid mediators, glycoproteins and activate complement system through opsonization of the pathogen making them susceptible to phagocytosis. These cells are described below.

Neutrophils

Granular in nature, multilobed nucleus, type of white blood cell (WBC or granulocytes) short life span (6hr) play a role in phagocytosis involved in antigen presentation to the T-cells. Neutrophils are the first cell to arrive at the site of acute infection, migrate through chemotactic movements such as CXCL8 or IL-8 during the stressed conditions. Neutrophil can interact with pathogen through pathogen-associated molecular patterns (PAMPs) directly or by neutrophil pattern recognition receptors (PRRs), or taking help indirectly by complement receptors. The Phagosomal maturation process establishes fusion with neutrophil granules along with the delivery of antimicrobial molecules, leading to the formation of reactive oxygen species (ROS).

Macrophage

Macrophages originate from monocytes, a blood cell precursor. After monocytes leave bone marrow and reach tissue sites through circulation, these cells are differentiated into macrophages. Macrophages produce numerous cytokines like IL-1, IL-6, TNF-α depending on the microbes. Macrophages produce reactive oxygen species to generate nitrite to kill phagocytosed bacteria. Macrophages migrate into almost every tissue for surveillance and

elimination of unwanted microbes. Macrophages are named depending on the tissue they reside in: Kupffer cells in the liver, Alveolar macrophages in the lung, Microglial cells in the nervous system, and tissue histiocytes in connective tissue.

Basophil

Basophil (granular mononuclear cells) or myeloid progenitor cells are derived from bone marrow. Cross-linking of antigen with FcεRI receptor-bound IgE leads to the activation of basophils causing degranulation and release of cellular contents. It plays a predominant role in allergy by secreting histamine, lipid mediators, IL-4 and IL-13.

Eosinophil

Eosinophils are major effector cells in the immune system play a crucial role against nematodes and other parasitic infections and co-operate actively in other immune response. Eosinophils recruitment at the site of inflammation from blood to tissues and release a multitude of cytokine, chemokine CCR3 an array of inflammatory mediators, cytotoxic proteins and EPO.

Mast Cell

Mast cells (derived from bone marrow precursor) long-lived tissue-resident cells play a central role during parasitic infection and allergic reaction. A Mast cell is released into the blood as mast cell progenitors and does not fully mature until recruited at the site of tissue where they undergo terminal differentiation.

Dendritic Cell

A dendritic cell is bone marrow-derived leukocytes, tree or dendritic shape like appearance responsible for the initiation of adaptive immune response functions like sentinels of the immune system. Dendritic cells most potent APC, capable of antigen processing and presentation to naive T-cell through Major histocompatibility complex (MHC) and co-stimulatory molecule. DC being heterogeneous in nature, reside in both myeloid and

plasmacytoid DC migrate to the lymphoid compartment for residing into lymph node (subcapsular sinus) or Thymus (T-cell zone). Thus, DC functions as a link between innate and adaptive immunity.

Adaptive immunity

When innate immune cell unable to encounter the infectious agent then adaptive immune cell comes in to picture. Adaptive (antigen-dependent), specific in nature involves a time lag between exposure to the antigen to maximize response. It has a capacity for memory to mount a specific immune response against subsequent exposure to a specific pathogen. Adaptive immune cell encounters the pathogen through T-cell and APC along with B-cell.

T-cell

T-cell originated from a hematopoietic bone marrow stem cell precursor followed by maturation into the thymus. T cell expresses T-cell receptor (TCR) on their membrane taking help with APC especially macrophages and DC for recognition of specific pathogens. MHC was expressed on the surface of APC. MHC is also known as HLA (Human Leukocyte Antigen) categorized into two types: MHC-I & MHC-II. MHC-I (intracellular) is widely expressed on nucleated cells whereas MHC-II (Extracellular) was present on B-cell, macrophages, and DC. An encounter of APC with pathogen leads to activation of T-cell that helps in fragmented peptide to reach the T-cell surface resulting secretion of cytokines to check the immune response. T-cell activation either $CD4^+$ gene (T helper cells) or through $CD8^+$ gene (T cytotoxic cells) respectively. T_H response may be triggered by APC whether it is T_H1 or T_H2 mediated. T_H1 response is initiated by IFN-γ which induce macrophages to secrete cytokines that evoke B cells to make opsonizing and neutralizing antibodies. T_H2 dominant anti-inflammatory cytokines secretion is associated with IL-4, IL-5, IL-13 by recruiting antibody-producing plasma cell and B-cell.

B-cell

B-cell arises from hematopoietic stem cell bone marrow precursor till maturation. B-cell not require MHC (as like T-cell) to interact with foreign peptide. B-cell takes action against foreign antigens by producing antibodies. When an encounter with the foreign pathogen B-cell undergo proliferation and differentiation into an antibody-forming plasma cell and memory B-cell. Memory B-cell has a longer life span, in contrast, plasma B-cell cell short half life span and undergoes apoptosis **(Figure 1)**.

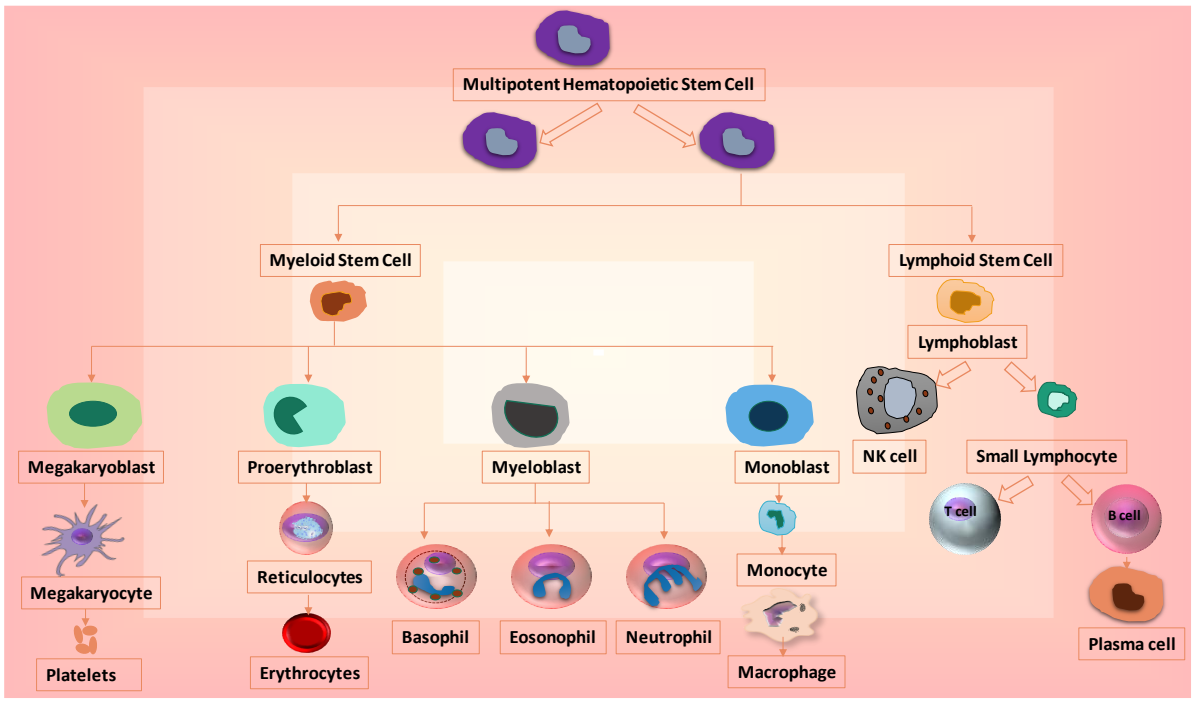

Figure: 1 Overview of the component of Immune System

Leishmaniasis

Leishmaniasis is considered a neglected tropical disease. Leishmania is zoonotic, vector-borne protozoan parasitic diseases transmitted through the bite of female sandfly vector Phlebotomus in old-world or Lutzomya in the new world. On average 20 pathogenic species of *Leishmania* cause a spectrum of diseases associated with infection globally known as leishmaniasis. Leishmaniasis is prevalent in 88 countries especially in the region of tropical

and subtropical of the world. It accounts for 2nd rank after malaria, Annually 0.7-1.5 million new cases of CL are reported whereas 0.2-0.4 million people are at high risk of VL annually (1,2,3). Poor people are the sufferer of this disease mainly in African, Asian, and Latin American continents. It is related with poor housing conditions, dysfunction of the immune response, malnutrition, lack of resources, population migration. Symptoms are associated with a series of episodes like fever, weight loss, cachexia, anemia, lymphadenopathy, splenomegaly, hepatomegaly (4). Treatment options rely on drugs. Most commonly used drugs are: Miltefosine, paromomycin, amphotericin B, Sodium stibogluconate, pentavalent antimony but the risk of resistance always remains a concern. To date, no vaccine is available for human use except for canine-Leishmune and Leishtec.

Epidemiology and geographical distribution

Currently, leishmaniasis is globally distributed among 88 countries that consist of tropical, subtropical, and temperate regions with more than 350 million people are facing risk. 12 million people are victims of leishmaniasis in which 0.2 to 0.4 million people are at risk for VL and 0.7 to 1.2 million cases of CL reported annually (5-8). Globally 90 % chances of occurrence of VL cases in more than 6 countries: Brazil, Ethiopia, Bangladesh, India, South Sudan, and Sudan. CL is more broadly dispersed throughout America, the Mediterranean basin and western Asia. Globally these countries account for 75% incidence of highest no. of CL cases: Afghanistan, Algeria, Brazil, Colombia, Costa Rica, Ethiopia, Iran, Peru, North Sudan and Syria (5,8,9). More than 20 species of leishmania parasite is associated with infection and disseminated through 30 species of phlebotomine sand flies. Transmission of Leishmania through female sand flies of the genus Lutzomyia (new world) in America and Phlebotomus (old world) in the rest of the world. From dusk to dawn or late evening to night time are peak hours for sand fly vectors (8). Transmission of Leishmania through the infected female sand fly vectors to mammalian reservoirs like marsupials, rodents, edentates, monkeys

and wild or domestic canines. Incidentally, humans get infected in prevalent areas (10). *L. tropica* complex (old world) and *L. donovani* complex (especially in the Indian subcontinent) is associated with the anthroponotic transmission (11). India, Bangladesh, Nepal (South Asia) and Sudan, Ethiopia, Kenya, Somalia (East Africa) countries are associated with *L. donovani* infection. In East Africa, *L. donovani* is associated with both anthroponotic and zoonotic disease (12-17). In contrast, *L. infantum* or *L. chagasi* more prevalent in the Mediterranean, the Middle East, Afghanistan, Iran, Pakistan, and Brazil while sporadic cases have been reported in Central Asia, China, Mexico, and Central and Latin America (11,18). The mode of transmission of *L. infantum* infection is mainly zoonotic, in which the dog is the major reservoir (18, 19). Increasing evidence of co-infections of the human immunodeficiency virus (HIV) and Leishmania has been observed during the last 30 years (20). Co-infection cases have been associated with the Mediterranean region, mainly in (Europian countries) France, Italy, Portugal, and Spain. Moreover, VL has emerged as an important opportunistic infection associated with HIV-infected patients living in many Asian (exclusively India) and African countries as well as in Latin America, predominantly in Brazil (21).

Life Cycle of *Leishmania*

When a female sandfly vector of old world genus Phlebotomus or new world genus *Lutzomyia* feeds the blood from an infected individual and injects promastigotes into the skin; in turn, promastigotes phagocytosed by mammalian macrophages becomes round shapes, sessile amastigotes (3-7um in diameter). In macrophages, promastigotes transform into amastigotes. During the blood meal, fly ingested parasitized macrophages release the amastigotes into the stomach of insects (22). Soon amastigotes transform into a flagellated, elongated (10-20 µm), promastigote form. Motile promastigote form reaches the alimentary canal of fly, then comes out and replicates by binary fission (23). Lipophosphoglycan (LPG) a glycoconjugate molecule having dense glycocalyx present on the complete surface of the

Leishmania parasite. Procyclic (immature form, non-infective in nature) have shorter LPG than mature metacyclic form. Metacyclic infective forms move from midgut and move through the proboscis. The Procyclic organism is sensitive towards complement attack through antigen-dependent alternate pathway; however, metacyclic organism activates antibody-dependent classical pathway but resistant towards complement attack. When sandflies attempt to take blood meal another time from the infected host they transfer the metacyclic form of promastigotes in to host together with saliva (24). Within the host, promastigote is engulfed by mammalian macrophages where they immediately transform into amastigote form for their survival and reproduction; ultimately, macrophage gets lysed. Free amastigotes are finally taken over by new macrophages and the fresh cycle starts **(Figure 2)**.

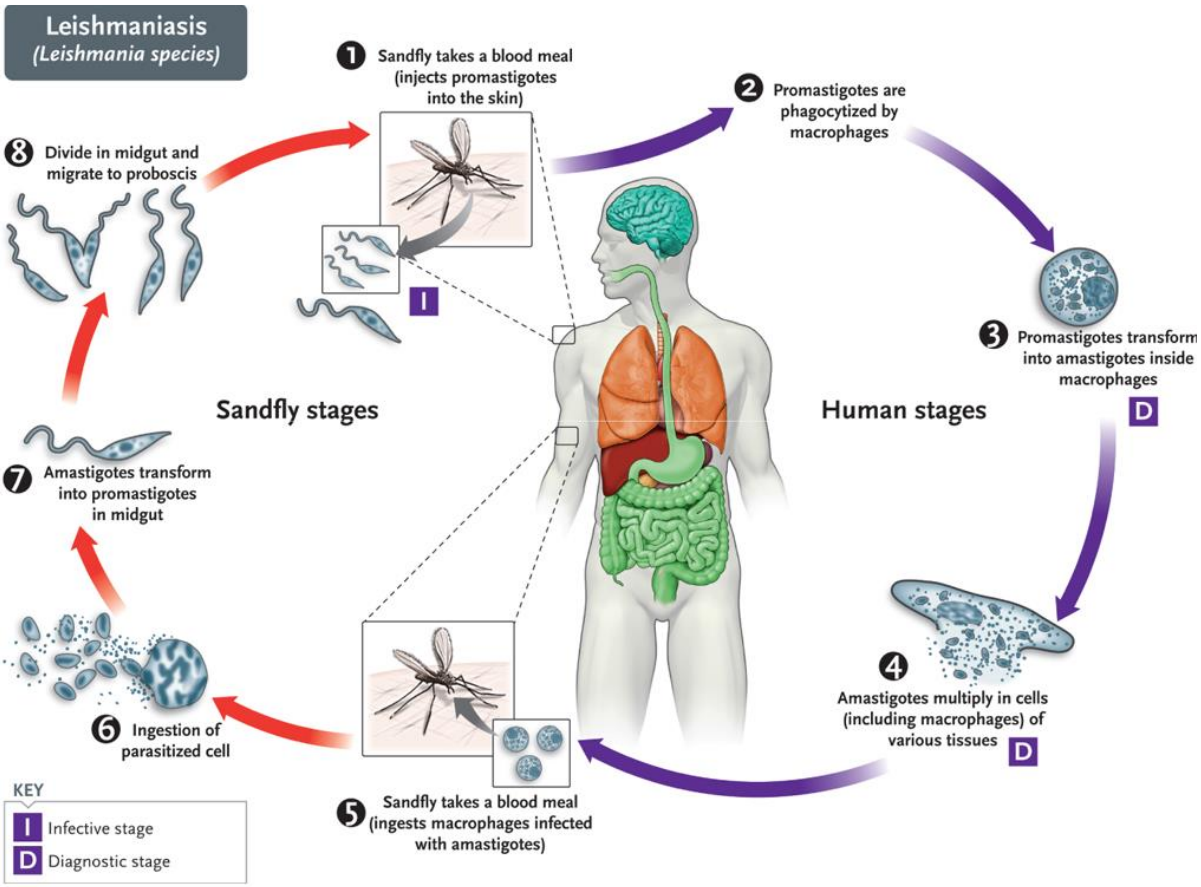

Figure: 2 Life Cycle of Leishmania (Adapted from review of Medical Microbiology and Immunology)

Types of Leishmaniasis

Infection with different species of leishmania accounts for clinical manifestation depends on the nature of species, geographical location, as well as the immune response of the host. It has been categorized into numerous types depending on the disease manifestation of mostly affected body parts. Mainly it has been categorized into three types:

Cutaneous Leishmaniasis (CL):

CL is the most frequent type of leishmaniasis; as the name infers, skin is the most major site of infection. It causes lesions in skin, sores, naked region of the body, leaving everlasting scars and serious infirmity together with stigma. 95% of CL victims were in South America, the Mediterranean Basin, the Middle East, and Central Asia. However, in 2017 more than 95% of new CL sufferer was noticed in six countries: Afghanistan, Algeria, Brazil, Colombia, The Islamic Republic of Iran, Iraq and the Syrian Arab Republic. Very few cases were noticed in other countries, including Southern Europe. It comes to attention that between 600,000 to 1 million new sufferers was reported worldwide annually (25).

Mucocutaneous Leishmaniasis (MCL):

MCL cases occur due to incomplete or complete demolition of the nasal mucous membrane, nasal septum, throat, mouth, and mid-face. 90% of victims of mucocutaneous leishmaniasis sufferers were reported in Ethiopia, Bolivia, and Peru. If left untreated leads to deaths result from secondary infections like pneumonia (25).

Visceral Leishmaniasis (VL)

VL commonly identified as Kala-azar and utmost devastating form of Leishmaniasis if left untreated becomes fatal in 95% sufferer victims. It is categorized by an unbalanced episode of illness, loss in weight, widening morphology in spleen and liver associated with anemia. Majority of striking evidence in East Africa, South-East Asia, Brazil. Annually on an average predicted 50,000 to 90,000 new VL victims were noticed globally from them 25–45%

evidenced in WHO database. In 2017, over 95% of new VL sufferers were found in 10 countries reported to WHO: India, Nepal, China, Bangladesh, Brazil, Ethiopia, Somalia, Kenya, South Sudan, and Sudan. In visceral leishmaniasis (VL), parasite disseminates over skin through blood and lymph fluids to the crucial organs of the body predominantly spleen, liver and bone marrow. This type of infection is less frequent but more life-threatening than CL (25).

Post-Kala-azar dermal leishmaniasis (PKDL)

Post kala-azar dermal leishmaniasis (PKDL) is an outcome of visceral leishmaniasis characterized by nodular, macular or papular rash typically on the face, trunks, upper arms and other body parts in a patient who has recovered from VL and who is otherwise well. The appearance of rashes was usually seen near the mouth from there disseminate to other body parts depending on severity. It occurs mostly in East Africa and on the Indian subcontinent especially in India and Sudan where 5–10% of patients of kala-azar Sufferers are of PKDL. It usually appears after an interval of six months to more than one year after kala-azar symptoms have been cured, but can occur previously. PKDL people are likely considered a selected source of Leishmania infection. A lesion in skins may appear from months to years after healed from VL (25).

Table-1:Species of Leishmania and their clinical outcome:

The Causative agent in the old world	The Causative agent in the New world	Disease manifestations	Clinical outcomes
L. major, L. tropica, L. aethiopica	*L. mexicana, L venezuelensis, L. amazonensis, L. braziliensis, L. panamensis, L. guyanensis*and *L. peruviana*	Cutaneous Leishmaniasis (CL)	Oriental sore, skin ulcers, appearance of necrosis in infected tissues.
L. aethiopica	L. mexicana species complex	Diffuse cutaneous Leishmanaiasis (DCL)	Non-ulcerative skin lesions like lepromatousleprosy.
L. aethiopica (Rare)	*L. braziliensis, L. guyanensis, L. mexicana, L. amazonensis*and *L. panamensis*	Mucocutaneous Leishmaniasis (MCL)	Perforated nasal septum, destruction of mucosal tissue, mutilated face, appearance of metastatic lesion on buccal and nasal mucosa.
L. donovani and *L. infantum*	*L. infantum*	Visceral Leishmaniasis (VL)	Hepatosplenomegaly and substantial weight loss.

Source: Adapted from Awanish Kumar (Leishmania and Leishmaniasis) Springer Briefs in Immunology.

CD40 and Leishmania

CD40, a family of type I transmembrane receptor glycoprotein having an extracellular carboxy-terminal segment consist of 22 cysteine residues, closely resembles other members of the tumour necrosis receptor family (TNRF). CD40 is expressed mostly on monocytes, APC such as macrophages, DCs besides these non-immune cells epithelial cells and platelets may express CD40 (26-28). CD40L a family of Type II transmembrane protein having CD40 receptors exists homotrimer in nature. CD40L is mostly present on the surface of activated mature $CD4^+$ T helper cells, as well as the small population of $CD8^+$ T cells. CD40L is also expressed by other myeloid immune cells like basophils, eosinophils and mast cells but can express under certain circumstances by B cells, NK cells and DCs, monocytes, and macrophages (26).

Interaction with CD40-CD40L plays a major role in the humoral response, particularly with antibody isotype class switching, maturation of memory cell and germinal center formation. CD40 deficient mice infected with *L. major* through footpad route shown protective efficacy that means CD40 have a key role in Leishmania infection. $CD40^{-/-}$ mice show polarity towards T_H2 type by releasing excessive levels of IL-4 (similar to BALB/c mice) and low level of IFN-γ production through draining lymph nodes (29). CD40L deficient mice infected with *L. mexicana* have also show T_H2 phenotype along with low TNF-α, INF- γ, NO production but high IL-4 (30). It indicates that T-cell mediated macrophage activation get impaired result high in parasite burden. Even though IFN- γ is a potent inducer of macrophage activity (31,32). IFN- γ treatment of infected macrophages was unable to clear the parasite. A combination of anti-CD40 antibody along with IFN-γ proves to be very beneficial in terms of lowering the parasite burden as compared to treatment alone. It indicates CD40-CD40L interaction plays a crucial role in macrophage activation and parasite clearance (29). Interaction of CD40 with CD40L leads to activation of T-cell that induces

IFN-γ, a T_H1 type immune response that controls amastigote growth. Treatment of CD40 with anti-CD40 antibody within 6hr after infection leads to a reduction in percent infection followed by a reduction in amastigote number but not in late infected macrophages through INOS dependent pathway. CD40 signaling impairment can be rescued by a p38MAPK activator (Anisomycin) in contrast to an inhibitor (SB203580) accentuate the role of p38MAPK in CD40 signaling in host protective memory T-cell response (33). The low dose of CD40 signaling skews towards Extracellular related kinase (ERK-1/2) dependent promotion of IL-10; paradoxically, high-dose of anti-CD40 induce signaling through p38MAPK dependent IL-12 production. In leishmania infected macrophages, CD40 signals through ERK-1/2 augment IL-10, impairs CD40-induced p38MAPK activation and inducible nitric oxide synthase-2 (iNOS-2) expression as a result IL-12 production get affected. IL-10 neutralization or impairment in ERK-1/2 signaling restores CD40-mediated p38MAPK activation. It indicates dual reciprocity in CD40 signaling either through p38MAPK or ERK-1/2 mediated parasite elimination or parasite survival (34).

Vaccine Development against *Leishmania* so far

Leishmaniasis is among the major but neglected, parasitic diseases. An anti-leishmanial vaccine for human use remains unaccomplished. During the times of Jenner and Pasteur, infection with a pathogen could be prevented by priming individuals with an attenuated or killed pathogen, although the mechanism of attenuation or loss of virulence remained unknown. A century later, DNA was discovered as the genetic material, wherein alterations can change the characteristics including the loss of virulence or attenuation of the pathogen. At the same time, Immunology was being developed as a discipline that is directly associated with resistance to pathogens. So, despite knowledge about the mechanism of attenuation and immunological mechanisms of host-protection, vaccine development for many pathogens has met with little success. The last seven decades witnessed the failure of three major

approaches: live, attenuated or killed *Leishmania*, DNA, RNA or recombinant multi-epitope peptides, and single gene-deleted, attenuated live *Leishmania*. The basic dogma-antigenic priming with an appropriate adjuvant elicits host-protective anti-pathogen immune response has repeatedly failed. Here, we are going to focus on generation wise *Leishmania* vaccine:

First Generation vaccine

Earlier if an individual gets to recover from the abrasion become resistant for further subsequent infection with the same pathogen. During the era of Jenner and Pasteur, they used to isolate active ingredients from the site of infection and then inoculated in to cover part of a normal person's body to achieve protection against lesion. Earlier mothers used to expose their baby's arms to bite through sandflies to protect them from the subsequent severe encounter. Later this technique was used in practice known as Leishmanization whereby individuals were inoculated with a low number of live virulent parasites. Uzbekistan employs the technique of Leishmanization. In practice, 100% efficiency was found in Israel and the Soviet union. The individuals used to develop scars but protection as well, albeit not in all cases. However, the practice was discontinued due to difficulty in standardization, persistent lesion, ethical & safety issues, repeated subculturing leads to a dramatic loss of infectivity and show hypersensitivity reaction as a substantial number of vaccines developed the infection (35). Later on, Autoclaved Leishmania major (ALM) along with BCG adjuvant was used in a trial for the treatment of visceral leishmaniasis in Sudan (36). During practice, this was replaced by the killed but metabolically active (inoculation through live attenuated pathogenic organism by treating with amotosalen, (S-59) an artificial psoralen compound and a low dose of UVA radiation make replication-deficient) but retain the metabolic activity and eliciting the immune response against *L. infantum* and *L. chagasi* (37). Different methods were employed for attenuation like repeated subculturing, temp. sensitivity, γ-attenuation, chemical-based mutation, and culture of the parasite under drug pressure (35). Failure of

killed parasites in eliciting a protective immune response has led to the employment of live but attenuated parasites because it mimics the natural course of infection, help in the representation of antigen to the immune cells, Controlled persistence of parasite evokes memory response.

Second generation vaccine

Due to advancements in leishmania genome and proteomes, various antigenic proteins were identified that are expressed in either of both stages of the parasite promastigote and amastigote. This era includes the integration of specific proteins of Leishmania instead of the whole organism. Initiated with the testing of native Leishmanial proteins extracted by employing through chemical procedures and sonication etc. Although many Leishmania proteins have characterized based on their surface architecture, T-cell based clones, screening of antigen pools or through the screening expression libraries from infected animals and humans sera (38). FML (Fucose mannose ligand) a glycoprotein in which GP36 is a major immunogen component present on the surface of parasite shown immunogenicity in both mice and rabbit models. FML Vaccination against *L. donovani* infection shown promise in mice and hamster models. It reaches up to phase-III trial in the endemic region of brazil in both Humans and canine against kala-azar (39). QuilA saponin (adjuvant) is a mixture of saponins with carbohydrate moiety attach through glycosidic linkage to triterpenoid quillaic acid. Vaccination with FML-QuilA formulation provides long-lasting robust protective efficacy against canine visceral Leishmaniasis during phase-III trial in terms of Kala-azar in an endemic area of Brazil (40). This victory leads the researcher to registered a vaccine under the trademark of Leishmune® in Brazil (41). As we know adjuvant serves as a depot to boost the response of vaccine if the release of antigen in small amount (weak immunogen) but some vaccine has not requirement of adjuvant like *L. major* culture-derived soluble exogenous antigen (SEG) show consistent protection by inhibiting the pathogenesis of *L.*

major in murine model (42). As an alternative to the problem of adjuvanticity, cationic liposomes were used to deliver the antigens. The cell surface is negatively charged, the cationic liposomes were expected to deliver the antigens to antigen-presenting cells more efficiently. Isolation of antigens from *Leishmania donovani* promastigote membrane antigens (LAg) encapsulated with +ve charged liposome shown protective efficacy in the murine model against *L. donovani* infection (43). But simultaneously non-phosphatidylcholine (non-pc) liposomes entrapped promastigote soluble antigen also confer protection in the hamster model (44). Recently, purified soluble L. major antigen (SLA) with adjuvant nuclease-resistant phosphorothioate CpG oligodeoxynucleotides (PS CpG) or nuclease-sensitive phosphodiester CpG ODNs (PO CpG) shown promise in experimental murine CL (45). Gp63 (Leishmanolysin) a cell surface protein help in attachment of the parasite to macrophage. A native and recombinant form of gp63 have tested in various models. Recombinant gp63 have expressed in salmonella & E. coli. Recombinant gp63 with adjuvant liposome have tested in a mouse model and it is considered safe, good response during clinical trial finally approved by the FDA. TLR9 has ligand for CpG to help in the immune cascade mechanism by presenting the antigen to the cell surface. CpG-ODN is used as an adjuvant to boost the immune response. rGP63 co-administered with CpG-ODN in cationic liposome protects against CL in mice model (46). Vaccination with gp63 in cationic distearoyl phosphatidylcholine (DPSC) liposomes also held promise in VL (47). Moreover, glycoprotein gp63 along with CpG-ODN through heterologous prime-boost approach show durability and protective efficacy against visceral leishmaniasis (48). Recombinant histone H1 antigen, recombinant hydrophilic acylated surface protein B1(HASPB1) either alone or combination of H1+ HASPB1 with adjuvant Montanide Tm shown promise during the trial in monkey and canine model (49,50). Maxadilan a salivary component present in sandfly saliva responsible for exacerbating the infection altogether, vaccination against maxadilan protects the mice from *L. major* infection

(51). The possible reasons for the failure of the peptides and recombinant proteins are many folds. 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 humans were a few of them.

Polyprotein, Fusion or Hybrid Vaccine

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 very limited success. Although the mouse experiments were successful, the clinical trials failed again. Leish-111f is multiantigenic leishmania antigens, include TSA (Thiol specific antioxidant), LmSTI1 (Leishmania major stress-inducible protein1) and LEIF (Leishmania elongation initiation factor) having ORF linked in a tandem manner. rLeish-111f with adjuvant MPL (monophosphoryl lipid A) plus squalene (MPL-SE) shown protective efficacy against *L. major* challenge in a murine model (52) but failed to protect dogs against *L. infantum* infection (53). Leish-111f + MPL-SE are the first successful vaccine reached up to phase-I & II human clinical trials in normal healthy subjects by inducing $CD4^+$ T-cell mediated response (54). Later on, Leish-110f +MPL-SE in combination with N-methyl meglumine antimoniate (Glucantime) have assessed in canine visceral leishmaniasis (CVL) by using immunochemotherapeutic approach (55). Finally, the LEISH-F1+MPL-SE vaccine is considered safe and immunogenic in healthy volunteers against visceral leishmaniasis during the clinical trial (56).

Genetically attenuated

Further advancement in genetic manipulation and completion of leishmania genome sequenced think researcher to move towards novel approaches like selectively targeted

disruption with the help of gene knock-out, antibiotic selection marker, homologous recombination, antisense RNA interference technique. Pathogenic stages are the most affordable stages for attenuation due to limited antigenic diversity although it showed the promise but associated with risk of reversion into normal wild type form (57). Previously attenuation of Leishmania parasite DHFR-TS null mutant has generated by targeted deletion of dihydrofolate reductase - thymidylate synthase considered to be a safe and protective response in *L. major*, cross-protection against *L. amazonensis* (58,59). LdCen1($^{-}/^{-}$) Centrin gene knockout also shown promise in Balb/c, SCID, hamster against *L. donovani* infection as well as cross-protection against *L. braziliensis* (60). Silent information regulatory2 single knockout genes (LiSIR2($^{+}/^{-}$)) also confer complete protection against *L. infantum* (61). The deletion of HSP70-II mutant also shown promise in murine and hamster model against *L. major* (62). Later on non-pathogenic species of Leishmania like tarentolae shown protective response in mice against *L. donovani* (63). A2-recombinant *L. tarentolae* strain also shown promise in mice against *L. infantum* (64).

Dendritic cells

DC is the key player of the innate immune system because leishmania primarily infects the dendritic cell and DC was used as a delivery vehicle for leishmania antigen due to highly potent APC help in presenting antigen to the cell surface by activating $CD4^{+}$ and $CD8^{+}$ cells. Interaction of pathogen with 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. DC has shown some promising effect by allocating as immunomodulatory role in vivo and used as an adjuvant for vaccination against visceral Leishmaniasis. DC Vaccination with defined peptide could be a promising strategy against cutaneous leishmaniasis (65). Soluble *Leishmania donovani* Ags (SLDA) Pulsed DC through Adoptive transfer have been

engineered by retroviral gene transfer technique to augment IL-12 antigen-specific immune response (66). Antimony based chemotherapy along with SLDA-pulsed syngeneic bone marrow-derived DC immunotherapy has successfully eliminated the parasite along with parasite burden from the visceral organ (67). KMP-11 (Kinetoplastid membrane protein) protein is highly antigenic in murine, canine, and human T-cells and its expression are dual in terms of the form of the parasite. Bone-marrow derived dendritic cells (BM-DCs) pulsed with a defined peptide of *L. infantum* KMP-11in combination with CpG-ODN as an adjuvant evokes cellular immunity by inducing T_H1 type immune response (68). Finally, Hybrid Cell Vaccination therapy (HCV) induces $CD8^+$ CTL response by suppressing IL-10 not IL-4 and IL-13 against *L. donovani* infection (69). 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 that affect the efficacy of this vaccination method such as antigen loading, maturation state of DC, route, and frequency of administration need to be taken care of in a precise manner. Furthermore, heterogeneity in the DC population adds to the complexity in the selection of DC to be used for antigen pulsing. 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. Therefore, to take care of these problems, the third generation vaccines took several routes.

3rd Generation Vaccine

To develop an immune response against the antigenic protein, the characterization of antigenic protein is essential to inject it into the model system (70). In case of genetic immunization gene of interest is cloned into a bacterial plasmid, under the control of a eukaryotic promoter by injecting directly into muscle or skin into an organism then plasmid enters to the host cell machinery where it remains in the form of episome nor integrated with

the host cells DNA machinery. Genetic immunization fulfills the current perspectives by imitating the effect of the live attenuated vaccine, safety concern, worthwhile, stable, no need to store in the refrigerator. DNA Vaccination satisfies the criteria to present scenario by generating cellular and humoral response and also show cross-priming reaction (71). Earlier DNA vaccine has been assessed in both genetically susceptible BALB/c mice and resistant C3H/He mice (72). P36 LACK Leishmania homolog of receptors for activated C kinase (LACK) DNA vaccination fails to protect mice against *L. chagasi* through the I.M route (73). Whereas pClneo-LACK vaccination through intranasal route (needle-free) gain attention by providing a protective effect against *L. chagasi* infection (74). Besides, the intranasal route of immunization can also show cross-protection against *L. braziliensis* infection (75). Due to emerging resistance against Leishmania, KMP-11 (kinetoplastid membrane protein) and ORFF were used for DNA vaccination and shown sterile protection in hamsters and mice against antimony-susceptible and -resistant strains of *L. donovani* (76,77). Besides, KMP-11 vaccination also shows cross-protection against *L. major* but requires IL-12 as an adjuvant (78). The next approach comes is multiantigenic plasmid DNA vaccine (KMPII, TRYP, LACK & GP63) but failed to show protection in a canine model (79). Paradoxically, multicomponent (10 antigens) DNA vaccine was able to restrict the parasite growth in dogs through intramuscular immunization (80). Leishmania requires heme for exogenous growth for its survival and host dependency. HBR was conserved in various Leishmania strains and antibody was detected in Kala-azar patient's sera. Vaccination with HBR DNA generates multifunctional T-cell response along with sterile protection in rodent and hamster models (81). Recently, a DNA vaccine comprising Minimalistic Immunogenically Defined Gene Expression (MIDGE) vector was developed by Mologen AG called LEISHDNAVAX was constructed from immunogenic epitopes from the pan-species proteins of Leishmania were encoded in the vector. Immunogenicity was assessed from the cured leishmaniasis T-cells

patient. Finally, T-cell epitope-based vaccine against visceral leishmaniasis meets the criteria for the clinical trial (82) **(Figure 3)**. Failure of DNA vaccine focuses on a new approach known as prime-boost.

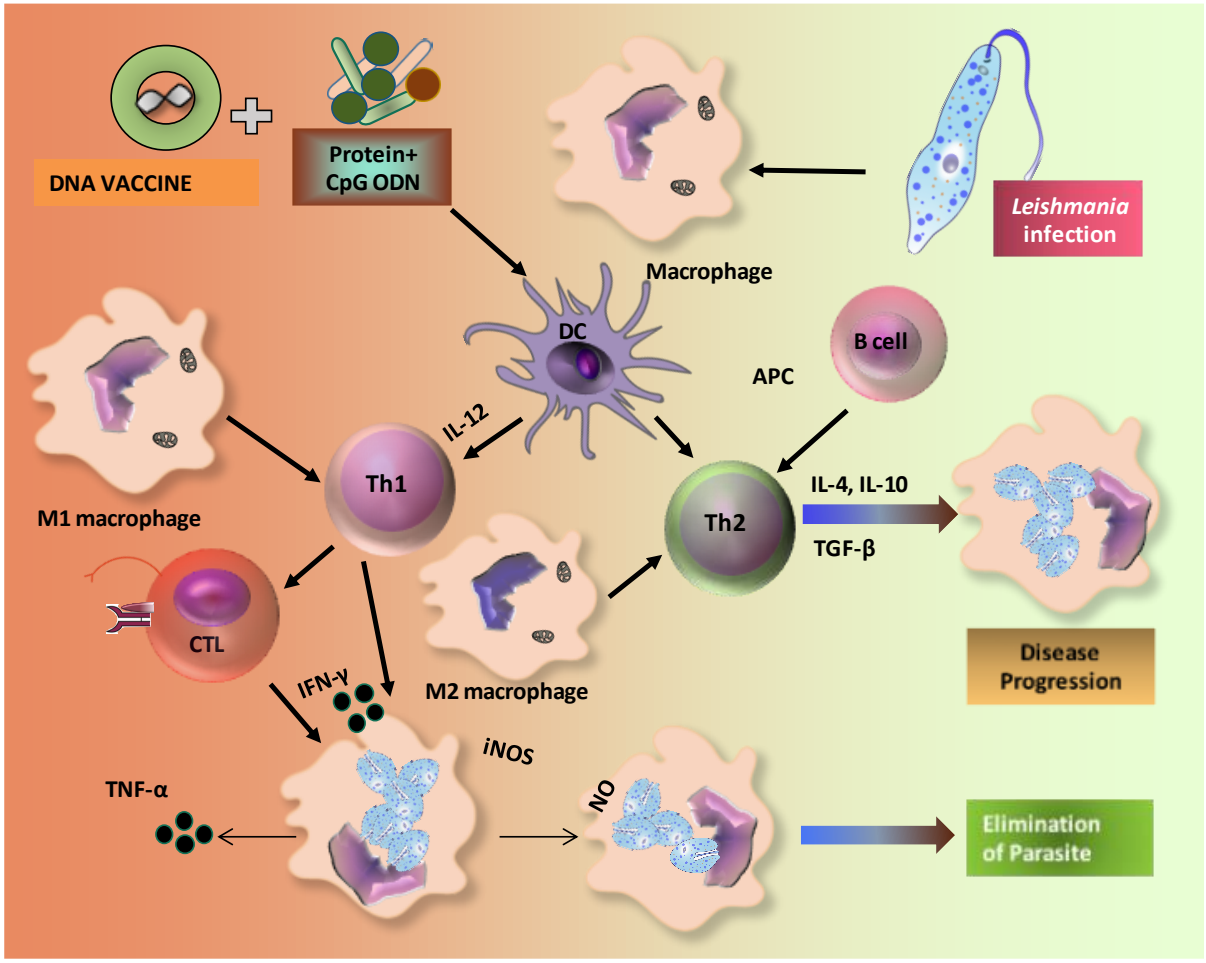

Figure: 3 Mechanism of DNA Based Vaccination

Heterologous Prime-boost (HPB) DNA Vaccine

Usually, priming is done by plasmid DNA followed by a booster with recombinant protein. Heterologous prime-boost is more effective than homologous prime-boost (83). DNA vectors followed by vaccinia virus recombinants (VVr) expressing the *L. infantum* P36/LACK antigen show significant promise in mice (84) and canine model (85) as well as LACK

antigen also show protective efficacy against *L. infantum* (86). Previously, the HPB approach consists of an antibiotic resistance gene due to prophylactic so not recommended. Thus, Antibiotic resistance-free gene pORT-LACK/MVA-LACK were used and shown promise against *L. infantum* in the canine model (87). Cocktail of cysteine proteinases plasmids DNA type I (CPB) and II (CPA) followed by a booster with rCPA/rCPB along with CpG ODN and Montanide720 as adjuvant show protection against *L. infantum* (88). A2-CPA-CPB(-CTE)-recombinant *L. tarentolae* in combination with cationic solid lipid nanoparticles (cSLN) as an adjuvant showed promise in murine VL against *L. infantum* (89). This is a novel tri-gene fusion product considered safe as a live vaccine candidate against visceral leishmaniasis **(Figure 4)**.

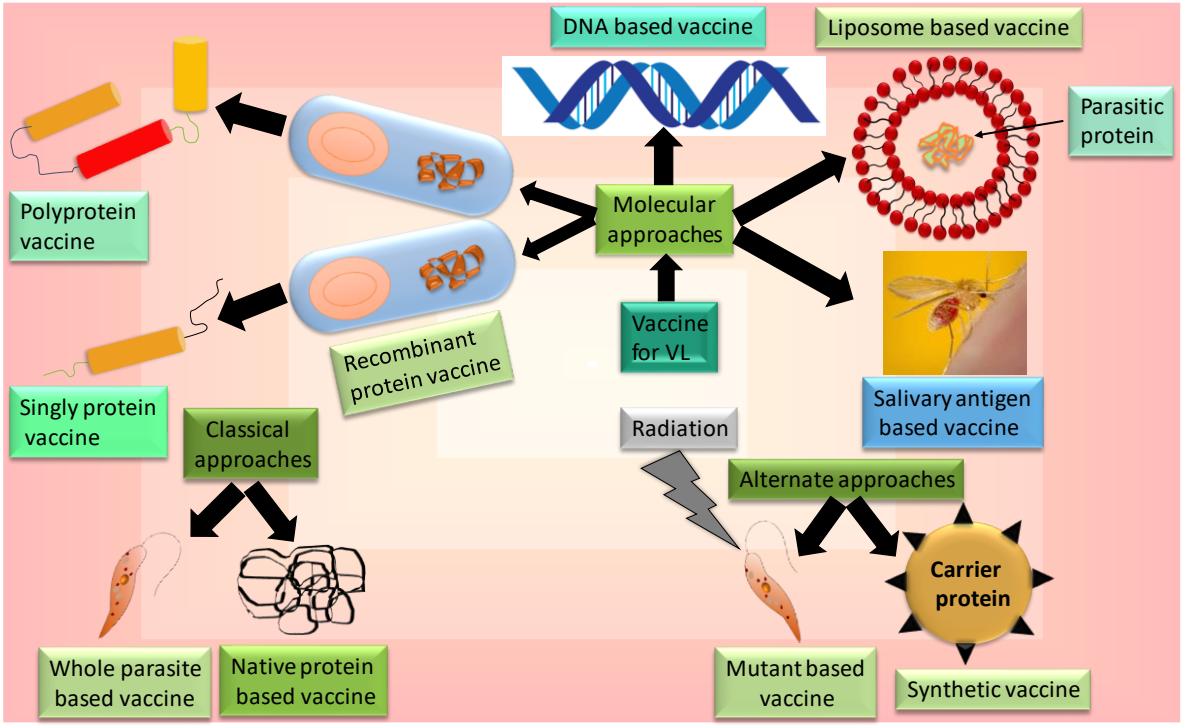

Figure: 4 Compile approaches of Leishmania Vaccine

MAP Kinase pathway and signaling

MAP kinases are mitogen-activated protein kinases that auto phosphorylate both serine and threonine residues after the recognition of extracellular stimuli. MAP kinase is an

evolutionarily conserved group of proteins ubiquitously expressed in most eukaryotic cells. MAP kinase is an ancient signaling intermediate pathway broadly used in the various physiological cellular processes by regulating gene function, metabolism, apoptosis, stress response, cell proliferation, mitosis, motility, survival, differentiation and immune response (90). After receiving stimuli from extracellular milieu and by phosphorylating serine and threonine moiety MAPK module get activated through the cascade of consecutive phosphorylations from the previous stimulus; each MAPK gets phosphorylated by upstream MAPK by phosphorylation through interaction with Ras/Rho GTP binding protein. A MAPK module includes a series of phosphorylation events initially MAP3K activates a MAP2K, further MAP2K activates a MAPK through conserved Thr-X-Tyr motif. MAPK cascade of phosphorylation may be inhibited by specific phosphatase associated with serine and threonine moiety (91). In mammalian cells widely established MAPK pathways and associated signaling cascade are: p38MAPK α, β, δ and γ pathways, ERK, JNK, and p38 isoforms related pathway. ERK1/2 activation depends on hormones, growth factors or pro-inflammatory stimuli, whereas p38MAPK α, β, δ and γ, and JNK1/2/3 activation depends on cellular and environmental stresses (92) **(Figure 5)**.

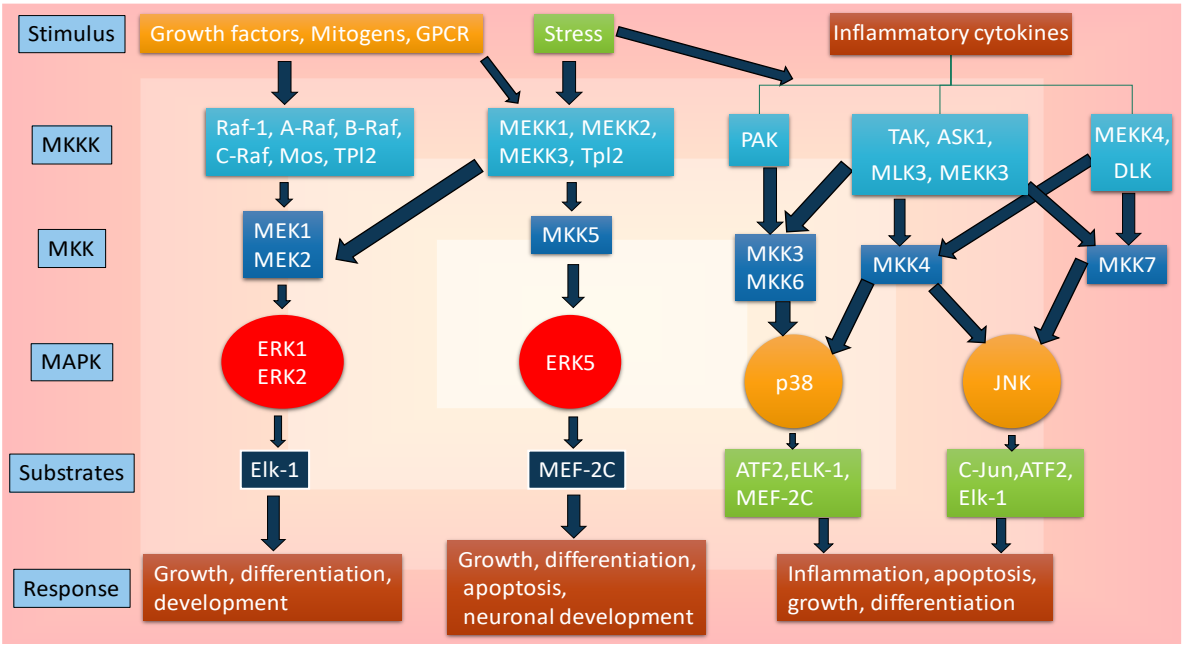

Figure: 5 MAPK Pathway and associated signaling cascade events

Structural classification of MAP Kinases:

There are 14 different kinds of MAP kinases found in mammals finally categorized into seven groups. Generally, these MAP Kinases can be classified into two types: Conventional and Atypical MAP Kinase. ERK1/2 (extracellular signal-regulated kinases), p38 isoforms (α, β, δ) JNK1/2/ 3 (c-Jun amino N-terminal kinases), and ERK5 come under conventional MAP Kinase (93) whereas ERK3, ERK4, ERK7 and NLK (Nemo-like kinase) comes under atypical MAP Kinase respectively. Conventional MAPKs evolutionarily conserved groups are associated with three sequentially acting kinases: a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK) along with Thr-X-Tyr residues in their activation loop of kinase domain whereas Atypical MAP Kinase is not associated with three-tiered kinase cascade pathway. In ERK3/4 and NLK Thr-X-Tyr motif is absent; meanwhile, Tyr residues can be substituted by Gly or Glu. While in ERK7 Thr-Glu-Tyr residues were present in its activation loop, ERK7 itself phosphorylates the residues rather by an upstream MAPKK (94).

MAPK and Immune system

MAP kinase plays crucial roles in innate and adaptive immunity because both components effectively regulate the effector function through signal transduction. Innate cells recognize the intruding microbes with the help of pattern recognition receptors through TLR (Toll-like receptor) family. Each TLR has specific architecture, binding of TLR with specific ligand activates a series of signaling cascade result lead to activation of numerous MAP kinase-like p38, ERK1/2, JNK, and transcription factor NFkB along with inflammatory cytokine IL-1, IL-6, IL-12, and TNF-α. The ultimate goal of signaling cascade is to eradicate the infectious microbes through effector function. In one study, by using p38-specific inhibitors (SB 203580, SB 202190, and SB 202474) or by disruption of one of its activator, MKK3

(MAPKK), leads to defective production of LPS-induced IL-12 at both RNA and protein stage (95). Adaptive immune cells work in co-ordinance with B-cell and T-cell to encounter the foreign invaders. To such an encounter against antigen T cells need to proliferate and differentiate in to mature effector $CD4^+$ and $CD8^+$ cells from their naïve (immature) counterpart. T-cell development originates from bone marrow to till maturation in the thymus where they undergo a series of developmental processes like growth, proliferation, differentiation and thymic selection in cortex and medulla region linked with +ve and -ve selection for higher affinity T-cells. During the whole complex process, MAP Kinase plays an important role. In a transgenic mice model by using dominant-negative mutation of both MKK3 and MKK6, as well as partial inhibition of p38 MAPK impaired the double negative $CD4^-CD8^-$ thymus development and proliferation process but not +ve selection. Hindrance with +ve selection leads to inhibition of p38MAPK, it gives a clue that p38MAPK is a crucial molecule in +ve selection (96). Antigen recognition through TCR (T-cell receptor) bound MHC complex along with costimulatory molecule (B7 molecules) present on APC that bind to CD28, whereas CTLA-4 is present on T_H-cell, leads to differentiation into effector cells. T_H1 and T_H2 are classified based on the type of cytokine they secrete. p38MAP Kinase is necessary for IFN-γ and T_H1 differentiation, not T_H2 (97). In one study, by using activator of p38MAP Kinase GADD45β (retrovirally overexpressed), IFN-γ production linked with p38MAPK activation gets enhanced in T_H1 stimulated cells (98). Imidazole, a p38MAP Kinase inhibitor blocks the production of IFN- γ and T_H1 cells in a dose-dependent manner not IL-4 or T_H2 cells. p38 regulation in both CD4 & CD8 T-cells occurs in similar way inhibition of p38 inhibition inhibits IFN-γ production, whereas p38 activation allows IFN-γ production. In contrast, activation of p38 kinase alone has a different outcome in both $CD4^+$ and $CD8^+$cells (99).

Leishmanial MAP Kinase and their role inside the parasite

MAP Kinase is a crucial molecule in cellular signaling pathways from a single cell to a complex conserved eukaryote cell. The Role of MAP Kinase is widely studied in cell proliferation, apoptosis, motility, and stress response. Leishmania, a protozoan parasite undergo morphological and biochemical changes during their two alternative life stage. In both sandfly gut and mammalian infected macrophages stage, there is a clue that reversible phosphorylation plays a pivotal role in parasite adaptation (100). According to the genome project, 179 eukaryotic protein kinases are present in *L. major* that encode 30% of the human kinome and 2% of the *Leishmania* genome. The major difference between human and leishmania kinome is the absence of receptor-tyrosine kinases and tyrosine kinase-like kinases (101). Earlier 9 MAP Kinase homologues of *L. mexicana* were identified that encodes two motifs of MAP Kinase by using degenerate oligonucleotides (102). Later on, the genome database of *L. major* was also available and 15 MAP Kinase homologues were screened by their amino acid pattern (TXYXXXRXYRXPE) in motif search (103). This motif has two peculiar features: dual phosphorylation in their TXY motif and (P + 1)-specificity pocket associated with site-directed substrate phosphorylation at kinase residues (104). 15 MAP Kinase homologue of *L. major* was reported that could also be present in other *Leishmania* species like *L. Mexicana, L. braziliensis, L. infantum* as well as in trypanosome species. Leishmanial MAP Kinase classically possesses a TXY motif with a slight variation in central amino acid. Out of 15 MAP Kinase, 8 homologue possess a TDY motif, 2TEY, 2TQY, and rest have TNY, THY or TIY in their phosphorylation lip. Table-2 represents the complete list of MAP Kinase in different *Leishmania* species by using the algorithm-based motif search (103).

Table-2 List of MAP Kinase in Leishmania

MAPK	***L. mexicana***	***L. major***	***L. infantum***	***L. brazieliensis***
MPK1	LmxMPK1 (LMPK) Z95887	LmjMPK1 LmjF36.6470	1 copy of a gene	1 copy of a gene
MPK2	LmxMPK2 AJ293280	LmjMPK2 LmjF36.0720	1 copy of a gene	1 copy of a gene
MPK3	LmxMPK3 AJ293281	LmjMPK3 LmjF10.0490	1 copy of a gene	1 copy of a gene
MPK4	LmxMPK4 AJ293282	LmjMPK4 LmjF19.1440	1 copy of a gene	1 copy of a gene
MPK5	LmxMPK5 AJ293283	LmjMPK5 LmjF30.2910	1 copy of a gene	1 copy of a gene
MPK6	LmxMPK6 AJ293284	LmjMPK6 LmjF32.3250	1 copy of a gene	1 copy of a gene
MPK7	LmxMPK7 AJ293285	LmjMPK7 LmjF13.1640	1 copy of a gene	1 copy of a gene
MPK8	LmxMPK8 AJ293286	LmjMPK8 LmjF28.0580	1 copy of a gene	1 copy of a gene
MPK9	LmxMPK9 (MAK) AJ293287	LmjMPK9 LmjF19.0180	1 copy of a gene	1 copy of a gene
MPK10	LmxMPK10 DQ308411	LmjMPK10 LmjF10.0200	1 copy of a gene	1 copy of a gene
MPK11	LmxMPK11 DQ026027	LmjMPK11 LmjF33.1380	1 copy of a gene	1 copy of a gene
MPK12	LmxMPK12 DQ026026	LmjMPK12 LmjF30.0370	1 copy of a gene	1 copy of a gene
MPK13	LmxMPK13 (LF4; MOK) DQ812905	LmjMPK13- LF4 LmjF35.5010	1 copy of a gene	1 copy of a gene
MPK14	LmxMPK14 (MAK) DQ812906	LmjMPK14 LmjF27.0100	1 copy of a gene	1 copy of a gene
MPK15	LmxMPK15 DQ812907	LmjMPK15 LmjF33.2070	1 copy of a gene	1 copy of a gene

IL-7 (Interleukin-7) and Signaling

IL-7 a non-hematopoietic stromal cell-derived cytokine secreted in thymic and bone marrow environment produced by fibroblast reticular T-cell zone of lymph node hence responsible for T-cell development & homeostasis (105,106). IL-7 is not produced by T-cell, B-cell and NK cell rather produced little bit by DC (107). IL-7 has two receptors explicitly IL-7Rα (CD127) common with (TSLP) thymic stromal lymphopoietin and common γ chain (CD132). Expression of IL-7Rα is exclusively present on lymphoid cells whereas γc is expressed mostly by hematopoietic cells. Binding of IL-7 with its receptor initiates signaling through JAK-STAT and PI3K/AKT for survival and differentiation respectively (108). Binding of IL-7 with IL7Rα recruit γc (γ-chain) leads to assembly of both intracellular domain JAK1 and JAK3 by trans phosphorylating the intrinsic kinase and phosphorylating the Y449 site on IL-7Rα (109). Accordingly, the receptor gets phosphorylated and docking sites created for STAT5. STAT5 binds with the SH2-phosphotyrosine domain by dimerizing reciprocal phosphotyrosine/SH2 interactions. Then, the STAT5 dimer reaches into the nucleus to bind with DNA (110). Activation of IL-7 leads to phosphorylation of Y449 residue on IL-7Rα. Phosphorylated Y449 residue via an SH2 domain, where p85α subunit of PI3Kinase directly binds. The PI3K is recruited to the membrane where PIP3 phosphorylates PIP2. Activated PIP3 induce downstream genes such as PDK1 and AKT (111). AKT, in turn, phosphorylates genes such as GSK3β, P27, and Bad protein. After GSK3β phosphorylation, IL-7 is unable to phosphorylate β-catenin (cytoplasmic signaling molecule), which hinders its degradation making a way to reach nucleus where it binds with transcription factors, e.g., TCF/LEF-1, to induce the expression of cyclin D1 gene which helps in progression of cell cycle by regulating RB hyperphosphorylation and inactivation (112) **(Figure 6)**.

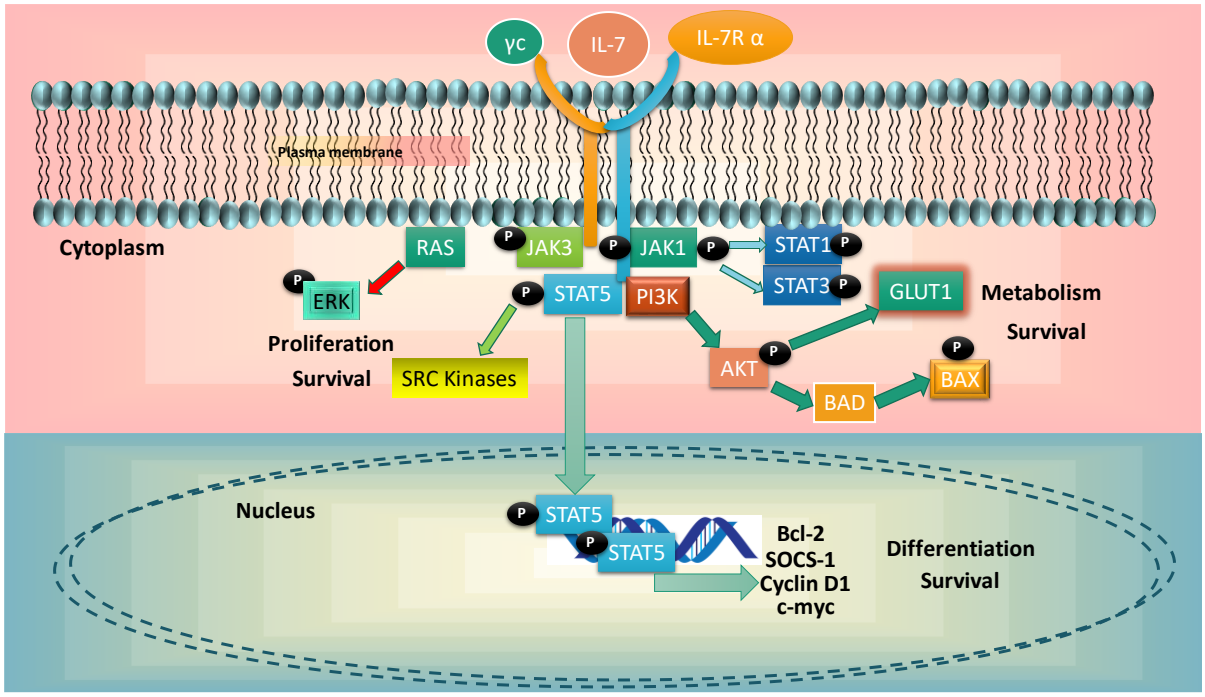

Figure: 6 IL-7 and Signaling Pathway

Role of IL-7 in Leishmania infection

IL-7 treatment in murine macrophage followed by infection with *L. major* reduce the parasite burden per cell along with percent infected cells in a dose-dependent manner. Combinatorial treatment of IFN-γ with IL-7 leads to >99% elimination of amastigotes in infected macrophages. In contrast, anti-TNF-α or N"-monomethyl-L-arginine acetate favors the pro-parasitic effect of IL-7. As it is well-studied IL-7 promotes pro-inflammatory cytokine-like IL-6, IL-1α, IL-1β, TNF- α, MIP-1 β in human peripheral blood monocytes (113). Paradoxically, in vivo IL-7 treatment to Balb/c mice following challenge with *L. major* leads to the aggravation of disease subsequently the death of animals. In vitro IL-7 treatment comparably induces the amount of T_H2 cytokine IL-4, IL-10 but not IFN- γ accordingly, a 40-fold increase in parasite burden was observed in spleen and lymph node. Despite that total cell numbers were also increased in draining lymph node and spleen at the site of infection locally (114). Central memory T-cells generated during chronic *L. major* infection have a high level of IL-7R expression. Moreover, large no. of T_H1 based effector cells also have

increased IFN-γ and T-BET expression. T_H1 based effector cells can also express a high level of IL-7R expression during chronic *L. major* infection. However, blocking the IL-7R signaling reduces the number of T-bet$^+$CD4$^+$ T cells, decreased production of IFN- γ and DTH response indicates maintenance of T_H1 effector cells require IL-7 signaling during *L. major* infected mice (115). For a T-cell to develop into T_H1 cell IL-12 signal is needed to produce leishmania-specific central memory CD4$^+$T-cell however, effector T-cell does not require IL-12 signal to produce IFN-γ both in vitro and in vivo. Moreover, adoptive transferred central memory CD4$^+$ T-cell in an IL-12 deficient host, most of the cells were IL-4 producers. It indicates the development of T-cell into T_H1 or T_H2 effectors cells require a population of central memory CD4$^+$ T cell produced during *L. major* infection (116).