# The role of metabolic enzymes in virulence and immunopathogenesis of *Leishmania* infection

By

**Somtochukwu Stella Onwah**

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment to the requirements of the degree of

Doctor of Philosophy

Department of Immunology

University of Manitoba

Winnipeg

# ABSTRACT

Leishmaniasis is a neglected tropical disease affecting approximately a million people yearly. The disease is caused by intracellular protozoan parasites that belong to the genus *Leishmania*. Different *Leishmania* species cause different clinical forms of the disease, which can present as self-healing cutaneous lesions or often fatal visceral disease that affects the liver, spleen and bone marrow. Unfortunately, there is yet no approved vaccine against human leishmaniasis. Although some drugs are effective, treatment often results in serious drug-mediated toxicity and side effects, and there is increasing evidence of drug resistance. However, vaccination is possible because people who recover from the disease are immune to reinfection. Knowledge of the antigens that induce protective immunity in infected hosts is critical for designing effective vaccines and therapeutic strategies against the disease. Dihydrolipoyl dehydrogenase (DLD) and Phosphoenolpyruvate carboxykinase (PEPCK) are key metabolic enzymes in numerous eukaryotic cells, including *Leishmania* and play critical roles in metabolism. This present study aims to assess the impact of DLD and PEPCK deficiency on parasite virulence and host immune response. Using the CRISPR-Cas 9 gene editing tool *DLD* and *PEPCK* gene in *L. major* and *L. donovani*, respectively, were deleted. The impact of deficiency of these enzymes on parasite survival, infectivity and host immune response were assessed. The gene-deficient parasites demonstrated reduced proliferation in axenic cultures and inside infected macrophages *in vitro*.

Mice infected with DLD-deficient parasites had no observable lesions and harboured significantly lower parasite burden compared to their WT or complementary add-back control-infected animals. Interestingly, DLD deficient *L. major* infected mice mounted blunted immune responses compared to their WT and complementary addback infected control counterpart mice.

Vaccination with DLD deficient parasites induced strong protective responses upon virulent *L. major* rechallenge, and this was associated with strong IFN-γ response.

The ability for PEPCK-deficient *L. donovani* to differentiate into their infective forms was not compromised *in vitro*. Although their infectivity in macrophages were compromised *in vitro*, these parasites maintained their virulence and pathology *in vivo*. In mice infected with PEPCK deficient *L. donovani*, immune responses in the liver and spleens were relatively unaltered apart from increased TNF production in the liver. Interestingly, the infectivity of PEPCK KO parasites in Kupffer cells *in vitro* was compromised compared to their WT or AB parasite controls, suggesting that the *in vivo* microenvironments may predispose PEPCK-deficient parasites to a compensatory mechanism that enables their proliferation.

Collectively, these findings show that DLD is a critical metabolic enzyme for intracellular survival of *L. major* and targeting this molecule could be a viable option for controlling the disease. Although PEPCK-deficient *L. donovani* is attenuated *in vitro* but not *in vivo*, they may not be a suitable live attenuated vaccine candidate against visceral l*eishmania*sis.

# DEDICATION

This Thesis is dedicated to my God Almighty for giving me the strength and tenacity to keep going despite all the odds. I also dedicate it to my daughter, Liora, and her future siblings, proving that if your mind can conceive it, you can achieve it.

# ACKNOWLEDGEMENTS

This thesis would not have been possible without the support of the many people mentioned below.

I would sincerely like to thank my Supervisor, Dr. Jude Uzonna, for accepting me into his laboratory. I am grateful for his mentorship, direction for this project and wise counsel. Over the years, he has nurtured in me the capacity to think critically, and this has been a major confidence boost throughout my graduate program. My committee members, Dr. Abdelliah Soussi Gounni, Dr. Thomas Murooka, and Dr. Kangmin Duan deserve special thanks for their great faith in me and commitment in ensuring my thesis completion. Current and Past Uzonna lab members, Zhirong, Gaurav, Nnamdi, Eni, Gloria, Arnold, Ping, Nonso, Aida, Muna, Anita and Chidimma are the real MVPs for their support with experiments and critiques of my work, especially Dr Mou and Dr Gupta for taking the time to mentor me in experimental designs and result output. Special thanks to Fola, Nnamdi, Roma, Jamie, Marina, Diane, and Fatemeh for the great conversations and laughs. To Bill Stefura, Dr. Christine Zhang, Silvia Panameno, and Angela Peloquin, thank you for always helping with my million questions. Thanks to the entire staff and students of the Department of Immunology, especially previous administrative assistants Karen and Susan, for their immense support during my program. To my collaborators, Dr. Ayesha Saleem, Patience Obi, and Dr. Saeid Ghavami, thank you for helping me study the parasite's mitochondrial metabolism. Also, I thank Dr. Wen Wei Zhang for providing useful troubleshooting guides to generate my KO and AB parasites.

I am grateful for the financial support from the Canadian Institute of Health Research, HSC Foundation, Tom and Mindel Olenik, University of Manitoba Faculty of Graduate Studies,

University of Manitoba Graduate Student Association, Deer Lodge Hospital Staff Association, Canadian Society of Immunology, and the American Society of Tropical Medicine and Hygiene, which have made the progress of my research possible.

To my amazing friends who have now become family: the Anyanwus, the Ndulues, the Chukwukas, Grandpa Murray and Grandma Josse, the Amakoms, the Oyebolas, Tosin and Femo, the Ezenwatas, Itee bebe and Nitesh. You are all beautiful souls who have vibrantly supported me on this journey.

My siblings Nelo, Kene, Buzo, Maero, Yuxie, and Loxie and my parents, Engr and Dr Mrs Mezue and Engr and Dr Mrs Onwah, have supported me massively and unwaveringly. I am so blessed to be a member of this family.

Finally, the love and emotional support from my husband, Maj, and Daughter, Liora, have been the bedrock of my persistence and consistency in showing up every day in this program. I love you both dearly; you are my light.

# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# ABBREVIATIONS

| Acronym | Descriptions |
|---|---|
| AB | Add-back |
| ADP | Adenosine diphosphate |
| AFLP | Amplified fragment length polymorphism |
| APC | Antigen presenting cell |
| ATP | Adenosine triphosphate |
| CDC | Center for disease Control |
| CL | Cutaneous leishmaniasis |
| CR | Complement receptor |
| CRISPR | Clustered Regularly Interspaced Short Palindromic Repeats |
| CRP | C-reactive proteins |
| DAMP | Danger-Associated Molecular Patterns |
| DAT | Direct agglutination tests |
| DC | Dendritic cell |
| DCL | Diffused Cutaneous leishmaniasis |
| DLD | Dihydrolipoyl dehydrogenase |
| DNA | Deoxynucleic acid |
| ECM | Extracellular matric |
| ELISA | Enzyme linked immunosorbent assay |
| FADH2 | Flavin adenine dinucleotide |
| FH | Factor H |
| GCC | Glycine cleavage classification |
| GCVL | Glycine cleavage L protein class |
| GDP | Guanosine diphosphate |
| GIPL | glycoinositol phospholipids |
| GK | Glycerol kinase |
| HDR | Homology directed repair |
| HDT | Host directed therapies |
| HIV | Human Immunodeficiency Virus |
| ICT | Immune chromatographic test |
| IFN | Interferon |
| IL | Interleukin |
| KO | Knock out |
| LAMP | Loop-mediated isothermal amplification |
| LPG | lipophosphoglycan |
| MAC | Membrane Attack Complex |

| | |
|---|---|
| MCL | Mucocutaneous Leishmaniasis |
| MHC | Major histocompatibility Class |
| MMEJ | microhomology-mediated end joining |
| mTOR | mammalian Target Of Rapamycin |
| NAD | Nicotinamide adenine dinucleotide |
| NADH | Nicotinamide adenine dinucleotide Hydrdide |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NASBA | Nucleic acid sequence-based amplification |
| NH36, | Nucleoside hydrolase 36 |
| NK | Natural Killer cells |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NP | Nano particle |
| NRAMP | Natural Resistance Associated Macrophage Protein- |
| PAM | protospacer-adjacent motif |
| PCR | Polymerase Chain Reaction |
| PEPCK | Phosphoenolpyruvate carboxykinase |
| PKDL | Post-kala-azar dermal Leishmaniasis |
| PPDK | pyruvate phosphate dikinase |
| PRR | Pathogen Recognition Receptor |
| PTX3 | Pentraxin 3 |
| PV | Parasitophorous vacoule |
| RAPD, | Randomly amplified polymorphic DNA |
| RFLP | Restriction Fragment Length Polymorphism |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| SAP | Serum Amyloid P |
| SSA | single-stranded annealing |
| TB | Tuberculosis |
| TCA | Tricarboxylic Acid |
| TGF | Tumor growth factor |
| TLR | Toll like receptor |
| TNF | Tumor necrosis factor |
| TNFR1 | Tumor necrosis factor receptor |
| VL | Visceral Leishmaniasis |
| WHO | World Health Organization |
| WT | Wild type |

# 1. CHAPTER 1: Introduction

## 1.1 Leishmaniasis and Global Epidemiology Status

Leishmaniasis is a disease caused by the flagellated intracellular protozoan belonging to the genus *Leishmania* of the order Kinetoplastida and family Trypanosomatidae (1). Transmission can be through the bite of a *Leishmania*-infected sandfly and can be anthroponotic or zoonotic (2). There are about 93 known *Leishmania* sandfly vectors (3). *Leishmania* is known to have a complex lifecycle because there are two life forms of the parasites: promastigotes and amastigotes. The promastigotes and amastigotes are established in their respective hosts: invertebrate (sandfly) or vertebrate (human or animal) host, respectively (4). About 54 identified *Leishmania* species present varying clinical manifestations, with 31 species parasitic to mammals and 20 species uniquely pathogenic to humans (3).

*Leishmania* causes four primary clinical diseases: Cutaneous Leishmaniasis (CL), Visceral Leishmaniasis (VL), Mucocutaneous Leishmaniasis (ML) and diffuse cutaneous Leishmaniasis (DCL). CL causes skin ulcers, while VL is usually fatal if left untreated and affects the liver, spleen, and bone marrow. The mucosal membranes of the mouth, nose, and throat are the target of ML. Another form of Leishmaniasis, Post-kala-azar dermal Leishmaniasis (PKDL), develops in about 5-15% of patients treated for VL (5). *Leishmania* infects over 70 animal species, including humans (6). The heterogenicity of *Leishmania* species and the resulting ability to infect multiple hosts leading to different forms of clinical symptoms, impact the mode of disease control because strategies will be targeted at both the host and vector levels to reduce disease transmission.

According to the World Health Organization (WHO), Leishmaniasis has been classified as a neglected tropical disease of significant public health importance. The main reason for the

negligence of the illness is that less attention is given to ensuring the speedy development of a permanent cure and elimination of the disease, even when millions of people are affected every year (5). Recent reports from the 14th meeting of the WHO's Strategic and Technical Advisory Group for Neglected Tropical Diseases provided a framework to ensure the prompt management of Leishmaniasis and diagnostic outcomes (7). Leishmaniasis is known to affect the world's poorest populations, where there are limited elimination programs, less interest from participating pharmaceutical industries, and poorly maintained healthcare facilities (8).

*Leishmania* is classified into the *Leishmania*, *SauroLeishmania*, *Mundinia* and *Viannia* subgenera. Each species has its preferred host and characteristic symptoms obtainable during infection (9). *Leishmania* is also classified into New and Old World groups because it helps public health professionals effectively map the epidemiological trends and diversity of the parasites. Old World *Leishmania*, for instance, is primarily found in regions like Asia, Europe, and Africa, as these regions have had a long history of Leishmaniasis and have been impacted by the disease since 2,500 B.C (3). Some Old World *Leishmania* species are subdivided into complexes such as the *Leishmania donovani* complex (*L. donovani*, *L. infantum* and *L. chagasi*) and the *L. major* complex (*L. major*, *L. tropica*).

Meanwhile, a New World classification of *Leishmania* refers to species of *Leishmania* identified in the New World, such as in the Americas after the Europeans first migrated there, thus establishing leishmaniasis. Some of the new world *Leishmania* species include *Leishmania braziliensis* complex (*Leishmania braziliensis*, *Leishmania panamensis*, and *Leishmania guyanensis*), and *Leishmania mexicana* complex (*Leishmania mexicana*, *Leishmania pifanoi*, *Leishmania amazonensis*). Interestingly, the sandfly vectors that transmit the new or old world

*Leishmania* species can be distinguished into different groups. For instance, New World sandflies include the genera *Lutzomyia*, *Warileya*, and *Brumptomyia*, found commonly in the Nearctic and neotropical regions, including countries in the Americas, the US and Canada. In contrast, the Old World sandflies belong to the genera *Phlebotomus*, *Sergentomyia*, and *Chinius*, found mostly in the Palaearctic, Afrotropical, Malagasy, Oriental, and Australian regions.

With over 1 billion people estimated to be at risk of being infected with *Leishmania*, there is a great need for increased public health research efforts necessary to curb these risks. An estimated 50,000 to 90,000 new cases of VL occur annually, mainly in Brazil, East Africa, and India (10,11). However, only 25 to 45% of these cases get reported to the WHO. Although the milder form of Leishmaniasis, CL, causes skin lesions that can heal spontaneously, it can leave permanent scars and can cause degenerating stigma and disability. It is estimated that 600,000 to 1 million new cases of CL occur every year, mainly in the Americas, the Mediterranean basin, the Middle East and Central Asia. Only 200,000 of these cases are reported to the WHO (7,12). ML leads to the destruction of the nasal tissues, and over 90% of ML cases occur in Bolivia, Brazil, Ethiopia, and Peru (World Health Organization, 2022).

Leishmaniasis is more commonly presented as CL, ML and VL. According to the WHO, in 2020, out of all the cases of leishmaniasis that occurred in 7 countries; Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan, VL accounted for 90% of them (11). In contrast, it was reported in 2016 that amongst the incidences of leishmaniasis in 10 countries: Afghanistan, Algeria, Brazil, Colombia, Iraq, Pakistan, Peru, the Syrian Arab Republic, Tunisia, and Yemen, 84% were associated with CL (11). Interestingly, according to the WHO repository from 2020, CL and VL

were highly prevalent in the same regions and areas (Figure 1). Although the exposure rate may be high, only a small number of infected individuals develop the disease, and approximately 30,000 eventually die (13). Leishmaniasis is projected to be the ninth-largest disease burden than other infectious diseases (14). Coincidentally, it is also considered in the top 20 list of neglected tropical diseases by the CDC because it grossly affects people with low incomes and historically has not received as much attention as other diseases due to several factors ranging from the complexities associated with the parasites' biology to the underreported incidences in endemic areas (15).

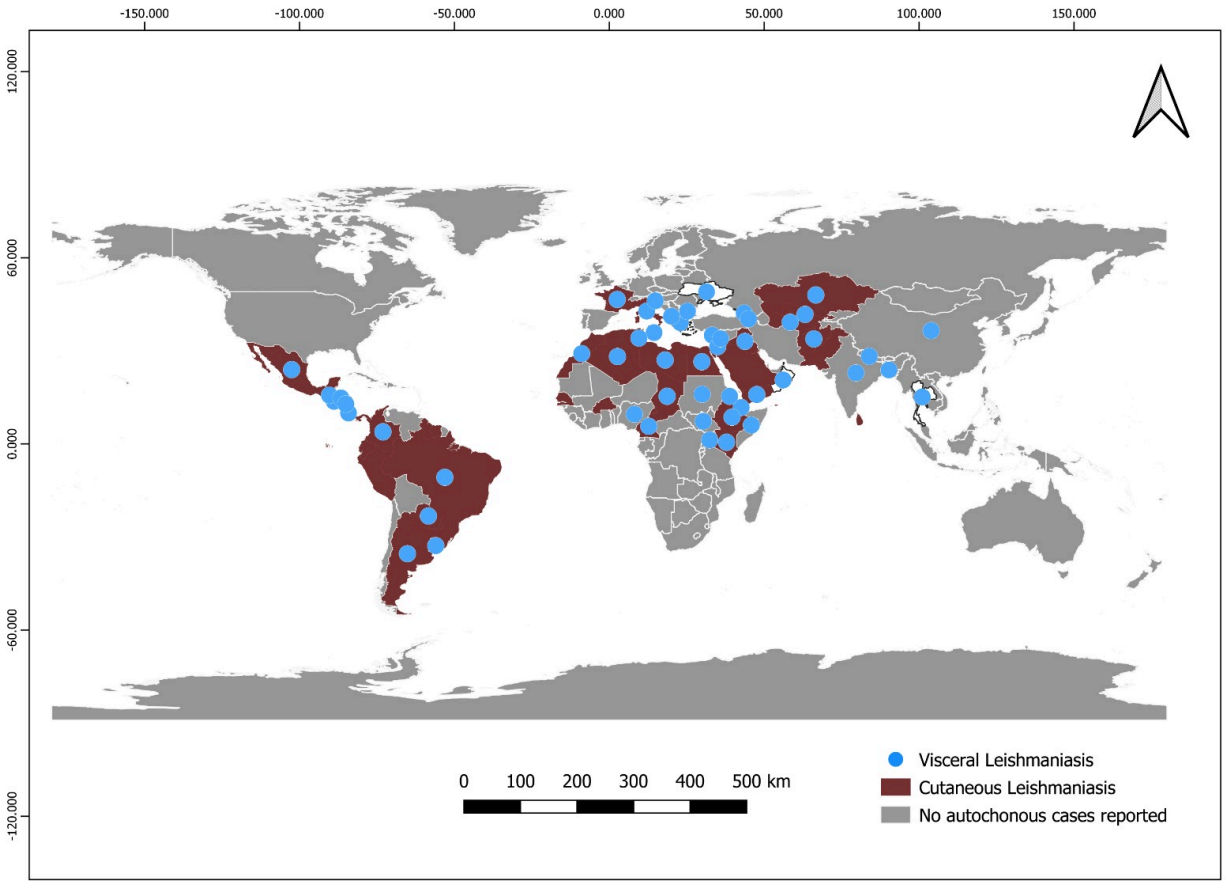



***Figure 1. Global epidemiology status of leishmaniasis.***

Created with qGIS using information from the WHO repository on Leishmaniasis 2020 (https://www.who.int/data/gho/)

## 1.2 Vector and transmission

The main vectors of *Leishmania* are the Sandfly insects of the genus *Phlebotomus* or *Lutzomyia*, which are the most studied genera because they bear great importance for human infection. Other Sandfly genera includes *Warileya*, *Sergentomyia*, *Brumptomyia* and *Chinius*. *Leishmania* parasites are transmitted from sandfly to the human or other mammalian hosts by the bite of an infected sandfly. *Leishmania* parasites undergo morphological and physiological changes in the sandfly hosts to be 'fit' to infect their human or mammalian hosts. Between the subgenera *Leishmania* and *Viannia*, the exact location by which the parasites enter the sandfly differs. The parasites in the *Viannia* genera, for instance, *Leishmania braziliensis,* would first enter the hindgut of the sandfly before settling in their midgut. They are referred to as the peripylarian parasites (16). The *Leishmania* parasites in the *Leishmania* genera are called suprapylarians because they mostly first migrate and develop in the midgut (16). Usually, most studies on vector-parasite interactions focus on the mode of development of the suprapylarians in the sandfly.

The early stages of parasites' development in sandfly begin when amastigotes from a blood meal containing macrophages start their journey to the midgut. Once they are taken up by sandfly, these parasites are immotile and rounded. Still, as the midgut of the sandfly's physiology changes, such as a decrease in temperature and increased pH, these parasites are transformed into a procyclic promastigote that is weakly motile and has a slightly protruding flagella beating at their exterior bodies. This form of the parasite replicates extensively in the peritrophic matric that is distal to the midgut. In approximately 72 hours, their replication slows down as time

passes, and the parasites are differentiated into robust and motile nectomonad (17). They move towards the midgut lumen and develop into short nectomonads, also called leptomonads, where they enter another proliferation cycle (18). Eventually, they are detached from midgut and begin an onward journey into the stomodeal valve, and their location here is critical for transmission (17). In this stage, the parasites become metacyclic promastigotes and can be injected into skin of vertebrate host in the sandfly's next blood meal (17).

With the help of extensive molecular studies on sandfly such as *Lutzomyia longipalpis*, *Phlebotomus papatasi* and *Phlebotomus perniciosus*, it has been shown that specific molecules expressed in the sandfly host enable parasites to survive in the fly midgut (19,20). There are natural barriers to successful establishment and survival of *Leishmania* in sandfly, including proteolytic enzymes, formation of peritrophic matrix, and sandfly immune responses (9). Despite these defences, parasites are able to overcome them and become established in the sandfly (9). Indeed, reports have indicated that there is usually an increasing activity of proteolytic enzymes the moment amastigotes enter the sandfly, which was detected as early as 6 hours post blood meal. The activity of the enzymes peaks around 48 hours after blood meal (21,22). Some of these enzymes are trypsin and trypsin-like enzymes, and they have been known to affect the development of *Leishmania* in sandflies. Parasite numbers are reported to be reduced in the early phase of the parasite colonization in the sandfly (23). Studies demonstrated that over 50% of the parasites taken up by the sandfly after a blood feeding were killed within the first day (17,24). Even though half of the parasites are lost by the degradative action of the digestive enzymes, the parasites that escape the enzymatic pressure develop an enhanced ability to inhibit the proteolytic attack. One identified mechanism is that *Leishmania* parasites express an array of

glycoconjugate molecules, including phospohogylcans (PG) which have repeating units (Gal-ManPO4) attached to lipid anchors, lipophosphoglycans and the proteophosphoglycans. These Glycoconjugate molecules are also found in the secreted forms (secreted phosphoglycans) or secreted acid phosphatase (9). These molecules are generally resistant to the action of proteolytic and digestive enzymes (25). Evidence suggests that *L. major* lacking either secreted or anchored forms of the PGs were more susceptible to killing when exposed to blood-fed sandfly midgut lysates (26). This killing was reversible when exogenous PG or trypsin inhibitor was added (26). Thus, targeting proteolytic enzyme in the sandfly, especially in endemic regions, may be an attractive strategy for controlling vector-based transmission of *Leishmania*.

The peritrophic matrix (PM) is another structure in sandfly that impedes successful colonization of *Leishmania* parasites in the midgut. This matrix separates the gut lumen from midgut epithelium. It comprises of an extracellular chitin envelope whose complex structures become rearranged when the sandfly blood meal digestion begins. The PM is thinly formed around the blood bolus a few hours after a blood meal; however, after about two days, the matrix thickens and breaks down (27,28). The PM plays dual functions regarding the establishment of *Leishmania*; it can protect the parasite against proteolytic damage and prevent it from escaping when mature. Pimenta et al. suggested that thickened PM is a barrier to the influx of digestive enzymes and also prevents exposure of parasites to these enzymes (24).

On the other hand, the parasites are expected to leave the PM so that they are not passed along with the rest of digested blood meal. Walter et al. reported that *Leishmania panamensis* was trapped within the endo peritrophic space of *Phlebotomus papatasi* (28). Another study showed that thickening of the PM reinforces the continued entrapment of *L. major* within the peritrophic

space in *Phlebotomus papatasi* (24). However, the eventual release of the parasites from the PM to the midgut occurs in about five days post blood meal, where the parasites take advantage of the host chitinolytic activities on the PM (9,29,30).

Like other insect vectors, sandfly has immune responses that help to control the survival of either bacterial or parasitic infection in the midgut (31). *Leishmania* infection in *P. duboscqi* induced the expression of an insect defensin. The recombinant defensin peptide inhibited *Leishmania major* in *in vitro* cultures (32). It has been described that plasmodium parasites were cleared in mosquitoes when immune genes such as the immune deficiency pathway, toll pathway and the JAK/STAT pathway were induced (33), Meanwhile the presence of *Leishmania* parasites in the sandfly vector does not significantly modulate the immune genes of the sandfly (30,34). However, the modulation of the immune genes in the sandfly is strongly associated with hemoglobin from the blood meal and the bacterial species present in the sandfly's gut (35).

Interestingly, when an essential immune component of the sandfly, *Caspar*, was deleted, there was a concomitant reduction in the survival of *L. mexicana* and *L. infantum* (36). Also, when the immune component, TGF-beta, is removed in *Lu*. *Longipalpis* there was a concomitant reduction in the number of *L. infantum* found in the midgut (37). All of these suggest that activating immune responses in the sandfly contributes to parasite development (38). There is speculation that a direct regulation of the sandfly's host immune response by *Leishmania* may be non-existent. Yet, there is a link between the sandfly immune response and the gut microbiota (38), which will need further investigation.

Parasites escape from the PM to the midgut, which occurs as the blood digestion progresses. Still, the parasites firmly attach to the midgut epithelium with their flagella enwrapped around the

epithelial microvilli (39). This prevents the parasites from being eliminated by defecation depending on the stage-specific development of the parasites in the gut, restricted to the leptomonads and nectomonads. For instance, the procyclic and metacyclic forms of the parasites do not attach to the sandfly's midgut (40). Also, the expression of the abundant surface glycolipid, lipophosphoglycan (LPG) covering the entire surface of the parasite enables attachment in the midgut epithelium (9). It is interesting to note that some sandfly vectors are permissive to the attachment of the parasites to the midgut epithelia, while others are not. Most sandflies generally support the development of multiple *Leishmania* species and are called 'permissive' vectors. There are instances where specific sandfly vectors have been identified to allow for better survival of *Leishmania* parasites. For example, *L. major* will attach better to *P. papatasi* and *P. duboscqi* than other *Leishmania* species. Also, *L. tropica* appears to survive better in *P. sergenti* only (41).

The transmissible form of *Leishmania* (metacyclic) will no longer be able to attach to the gut epithelium as their LPG are constantly modified due to increasing PG motifs, side chain galactose residues and arabinose capping the LPG molecule (42,43). The development of metacyclic parasites, which are small and rapidly motile forms with more elongated flagella, was observed *in vitro* at low pH or nutrient limitation (39). Although there is little knowledge on what cues drive metacyclic differentiation in sandflies, V-ATPase was identified to be responsible for gut acidification, which was probably a cue for Leptomonads to detach from midgut and begin differentiation into metacyclic forms (20). Although metacyclics were found in salivary gland of sandfly just before a blood meal (44), they were also found excreted in urine of the insects (45). Two mechanisms for transmission of metacyclics have been described. Metacyclics would either

be deposited in the host skin or that the sandfly takes up a blood meal through its proboscis and regurgitates metacyclics that reside in the stomodeal valve through a backflow of ingested blood (46). Whatever the mechanism may be, these parasites, having undergone numerous developmental cycles in sandfly, and become deposited on the skin of vertebrate host during blood feeding.

### 1.3 *Leishmania* lifecycle

The lifecycle of *Leishmania* is digenetic because different life forms alternate between the sandfly and mammalian host. The sandfly vector that transmits Old World *Leishmania* species is mainly of *Phlebotomus (P)* genera, while the New World *Leishmania* species is of the genus, *Lutzomyia* (Lu). These vectors feed on carbohydrates from plant juices. However, female sandflies mainly bite mammals to get a blood meal, which is required to complete their egg development (47). Sandflies obtain blood meals from vertebrates such as canids or rodents. However, they mainly feed on humans, but human transmission of leishmaniasis can be animal or human-induced (48). The non-mammalian hosts usually serve as 'reservoirs' of parasites and do not directly transmit parasites to humans (49). *Leishmania* undergoes morphological changes within their respective hosts into two forms: amastigotes (in mammalian host) and promastigotes (in sandfly host). Once sandfly takes up a blood meal from a *Leishmania*-infected host, amastigotes first differentiate into procyclic promastigotes, which are highly motile, possessing a flagellum about 15-28 μm long at their exterior body. They have a slender body of 15-20 μm in length and 1.5-3.5 μm in width. The parasite differentiates in the sandfly's gut to eventually develop into the metacyclic promastigotes, which move to the sandfly's proboscis in preparation for transmission to their

mammalian host. The transition to becoming metacyclic promastigotes occurs in 7-10 days, and this can be transmitted to their mammalian host through a bite by the sandfly (50) (51). Furthermore, the parasites stealthily enter several phagocytic cells in their mammalian hosts, especially macrophages, to become encapsulated within the parasitophorous vacuole (PV). At this point, promastigotes morph into amastigotes, which are oval, about 2-4 μm wide and replicate extensively. At some point, the phagocytic cell ruptures to release the amastigotes which enters other bystander cells thus spreading the infection leading to either the CL, VL or ML manifestations (49). However, suppose a sandfly happens to be within the vicinity of the infected mammalian host, the insect can take up the amastigote in blood feeding, and the parasites' life continues in the sandfly (Figure 2).

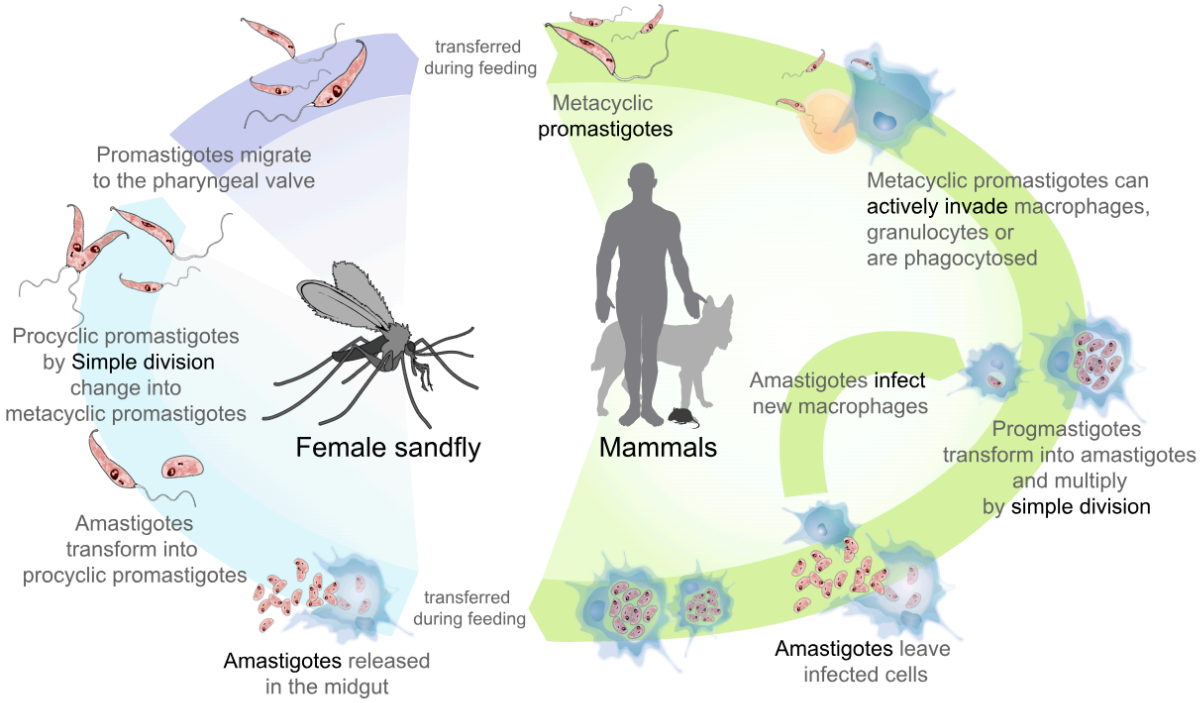



***Figure 2 Life cycle of Leishmania.***

Leishmania parasites alternates between two life forms; Amastigotes in the mammalian host and promastigotes in the sandfly hosts.

## 1.4 Forms of Leishmaniasis

Due to the numerous species of *Leishmania*, leishmaniasis presents in 3 forms previously mentioned: Cutaneous, Mucocutaneous, and Visceral. Cutaneous Leishmaniasis (CL) is the more common and mild form affecting the skin, resulting in sores or ulcers. The skin lesions vary in appearance from simple, painless sores to more severe forms with raised edges and a central crater. Some *Leishmania* species that cause cutaneous leishmaniasis include *L. major*, *L. tropica*, *L. braziliensis*, and *L. donovani* (in some cases). Mucocutaneous Leishmaniasis (MCL) is a more severe form involving the skin and mucous membranes of the mouth, nose, and throat. It is known to have started as a cutaneous sore before progressing to affect the mucous membranes. Some organisms that cause MCL are *L. braziliensis*, *L. panamensis*, and *L. guyanensis*. Visceral Leishmaniasis (VL) is the most fatal form of leishmaniasis, especially if left untreated. It affects internal organs such as the liver, spleen, and bone marrow. VL is characterized majorly by the enlargement of the liver and spleen. *L. infantum* and *L. donovani,* or their hybrids*,* are the main *Leishmania* species causing VL.

## 1.5 Cutaneous Leishmaniasis CL

The WHO reports 1 million new CL cases annually, which can be attributed to increased migration of people into endemic areas (52). As previously described, the organisms that cause Old World (*L. major*, *L. tropica* and *L. aethiopica*) and New World Leishmaniasis (*L. mexicana*, *L. braziliensis*, *L. amazonensis* and *L. guyanensis*) present as CL. CL is characterized first as a minor rash developing at an exposed body site such as the head, hands, or skin. This progresses to a rash

that ulcerates, forming a nodule. The nodular ulcer formed in CL is characteristic of the disease, and it self-heals in 3 to 18 months, depending on the *Leishmania* species. The variability in the clinical outcomes of CL is such that in the New World, solitary lesions are formed, but multiple lesions characterize the Old World disease (53). However, up to 10% of CL cases progress to more chronic and debilitating forms (10,54). CL is called the 'great imitator' because it presents in forms associated with other skin-related diseases like leprosy or dermatitis. It often goes misdiagnosed, especially in non-endemic areas (55). One of the rare manifestations of CL is leishmaniasis recidivans, which is characterized by the appearance of a new lesion around the area of a healed lesion (Figure 3). The more unique nodular lesions formed are usually around the borders of the old lesions. The newer lesions may persist and become enlarged as the years progresses. The postulated mechanism is thought to be the resuscitation of dormant strains due to changes in immune status or the coincidental reinfection of exogenous parasites (56).

On the other hand, some patients with CL have lesions that do not follow the typical timeline for healing. Usually resistant to treatment, these lesions are considered chronic and remain for several years following the initial onset—chronic lupoid Leishmaniasis (Figure 3) (55). Chronic Lupoid Leishmaniasis resembles lupus vulgaris or cutaneous tuberculosis, which often results in misdiagnosis in different settings (57).

Although CL is not fatal, the outcome of the disease usually results in facial disfiguration and stigmatization with imposed psychological effects (58,59). Generally, patients with CL are systematically well and occasionally feel an itch around the lesions, but the lesions do not result in any body pain (10). Lesions caused by *L. major* and *L. tropica* heal in a year but leave permanent scars. Meanwhile, lesions by *L. aethiopica* take years to heal and progresses to severe

mucocutaneous leishmaniasis and diffused forms of CL. Interestingly, the new world *Leishmania* species, such as *L. mexicana*, cause lesions that heal very quickly, unlike the new world *Viannia* species, which can result in more severe ulcerating lesions and progress to MCL forms (10,60).

Diffuse and disseminated CL are other rare variants of CL that are hinged on the difference in the spectrum of the immune responses (61). Diffused CL is characterized as having poor T cell-mediated immunity, poor reaction to the Leishman skin test, wide-spreading nodules, and lesions that resemble leprosy with massive parasites (62). On the other hand, disseminated CL has a strong T cell sensitivity and response to the Leishman skin test; the lesions are usually mixed and present in two or more parts of the body with fewer parasites in them (62). They are known to be caused by the *Viannia* species of the new world leishmaniasis, *L. braziliensis*. At the same time, Diffused CL is caused by *L. amazonensis L. aethiopica*, or *L. mexicana* (10). For both diffused and disseminated CL, immunosuppression is a risk factor (63). The presentation of CL in diffuse and disseminated forms indicates that different immune responses can depict various clinical manifestations.

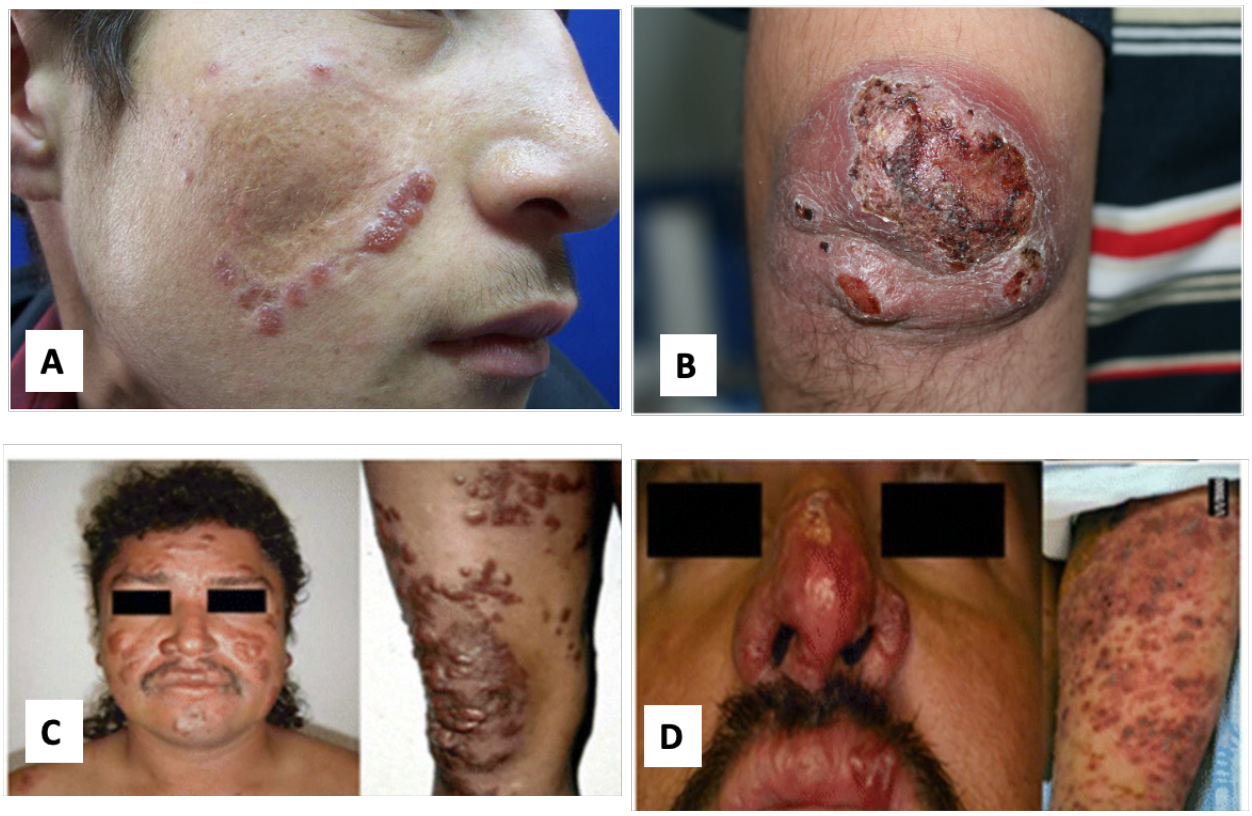



*Figure 3 Clinical variations in the manifestation of cutaneous leishmaniasis*

(A) *Leishmania* recidivans (B) Chronic Lupus Leishmaniasis (C) Diffuse Leishmaniasis (D) Disseminated Leishmaniasis

### 1.6 Visceral Leishmaniasis VL

Unlike CL, VL is a more systemic and lethal form of leishmaniasis. It is usually characterized by non-dousing fever, hypergammaglobulinemia, unintended weight loss, enlargement of the spleen and liver, and anemia (10). Upon the transmission of the parasite from a sandfly, the amastigotes transverse through multiple internal organs like the bone marrow, spleen, and liver, where they settle down to cause clinical manifestations (5). The Old World visceralizing *Leishmania* species are *L. donovani* and *L. infantum*. *L. chagasi* (a variant of L. infantum) causes the new world VL. Some CL-causing species, such as L. amazonensis and L. tropica, have been

implicated in causing VL (64,65). Humans are the main reservoir of VL due to L. donovani, while domestic dogs are the primary reservoir for L. infantum, especially in Latin America. Usually, an outbreak of VL occurs in rural areas where houses have earthen floors that are housed with livestock. Whereas, urban outbreaks associated with a wild hare reservoir have also been reported (66).

The incubation period for the development of VL is two weeks to 8 months, and if two years lapse without treatment, it is often fatal (67). Patients who do not become treated for VL experience extensive bleeding, resulting in leukocytopenia, thus degenerating to immunosuppressive or immunocompromised states (5). Immunocompromised patients are predisposed to other infections, such as HIV and bacteria. While there appears to be a global decrease in the incidence of VL, from about 200,000 to 400,000 new cases in 2012 to about 50,000 and 90,000 in 2017 (15), there is still the co-infection risk with other diseases. In the late 1990s, HIV was responsible for the re-emergence of VL in some parts of Europe, and currently, there is about 6% of HIV-VL co-infection cases reported in Brazil and India (68,69). In Ethiopia, about 18% of VL patients are co-infected with HIV (70). Although the risk of co-infection with malaria is low, there is still potential for a re-emergence of VL in malaria-infected patients, particularly in endemic areas (71). Some cases of VL-pulmonary tuberculosis (TB) coinfection have been reported (72–74). Although minimal information on the prevalence of TB-VL coinfection has been provided, studies show that TB contributes to up to 25% of mortality in VL-HIV coinfected cases (75).

Post-kala-azar dermal Leishmaniasis (PKDL) occurs after treatment for VL due to *L. donovani*. It only occurs in patients previously infected with *L. infantum* in immunocompromised settings (10). The symptoms of patients with PKDL include a hyperpigmented rash or an erythematous

maculopapular rash around the head that spreads throughout the body (Figure 4). In Asia, PKDL appears after about two years of VL treatment in about 5-10% of VL cases. In contrast, in East Africa, it appears only after one year of treatment in about 50% of VL cases (76).

Moreover, in East Africa, PKDL lesions would heal within a year in about 80% of cases but may pose facial disfiguration and stigmatization (76). Although PKDL appears in patients that previously had VL, it is not clear why approximately 5% of patients in India with post-kala-azar dermal leishmaniasis never had VL in their lifetime (76). In these patients, the lesions were observed to contain a pool of parasites that can be quickly taken up by a sandfly in a blood meal and could become a reservoir of infection for the duration of the disease (77). Thus, becoming a source of transmission during interepidemic periods. The onset of PKDL is an ongoing battle of the host's immune response to generate a systemic interferon-gamma against the onslaught of an existing pool of dermal parasites, inducing a T-helper 2 cell response with a robust production of anti-inflammatory cytokine, interleukin, IL-10 (10). Incomplete VL treatment and exposure to ultraviolet light necessitate PKDL, but specific treatments can also increase PKDL cases (78).

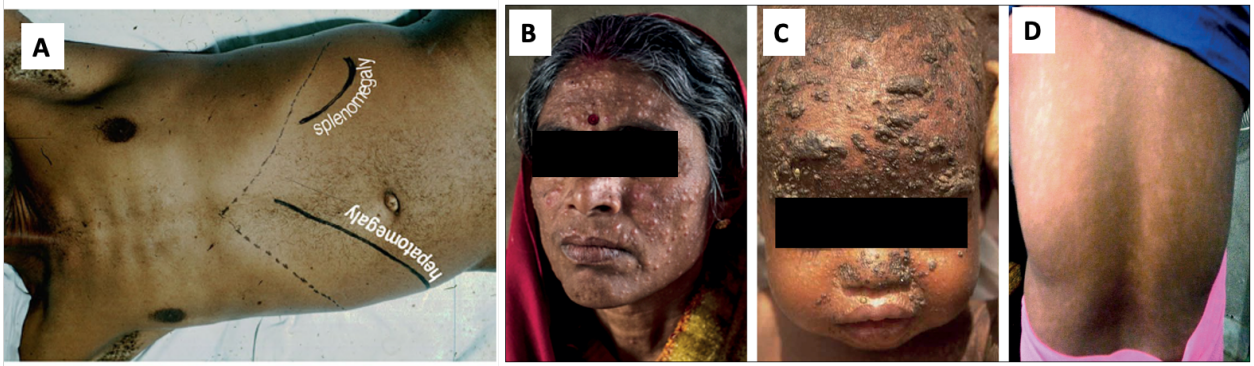



***Figure 4 Clinical presentation of Visceral leishmaniasis***

(A) Characteristic VL presentation: enlargement of the spleen and the liver, (B &C) PKDL in a woman and a child, respectively, with a maculopapular rash, (D) PKDL in a patient with a hypopigmented macula.

## 1.7 Mucocutaneous Leishmaniasis (ML)

Mucocutaneous Leishmaniasis is another severe form of the leishmaniasis caused by *L. braziliensis*, *L. panamensis*, and *L. guyanensis* majorly affecting mucosal membrane. ML is known to first develop as a cutaneous sore before progressing to ulcerous lesions in mucosal regions such as the mouth, nose, or tracheal mucosa. People suffering from ML are at risk of a disability and a life-long scar. Also 600,000 to 1,000,000 new incidences of ML globally occur every year (13).

## 1.8 Diagnosis of Leishmaniasis (all the different methods of diagnosis)

The availability of a rapid and accurate tool in diagnosing leishmaniasis will greatly help global intervention strategies to reduce mortality and morbidity rates of the disease. An excellent method for detecting leishmaniasis should be capable of identifying the exact form of the disease, asymptomatic or co-infective cases, and clearly distinguished from those with similar parasitic diseases (10,79). Some conventional methods for detecting leishmaniasis are parasitological, molecular, and immunological. However, the traditional methods of detecting Leishmaniasis are marred by numerous challenges, such as the existence of different clinical manifestations of the disease, the existence of multiple species even within the same genera for the same or different disease symptoms, increasing incidences of co-infection cases, conflict with other infections with similar leishmaniasis symptoms (Kumari et al., 2021). Hence, in recent years, there has been a wealth of vested interest in developing specific methods to address some

of the challenges in diagnosing leishmaniasis. Some of these advances in diagnosing leishmaniasis involve nano diagnostic tools, advanced molecular methods, Fluorescent-activated cell sorting and proteomics (Table 1). A thorough understanding of the advantages and disadvantages of the available diagnostic tools will enable researchers to develop more targeted alternatives, especially in detecting the early onset of leishmaniasis. Below are some of the conventional and advanced diagnostic tools available to date.

### *1.8.1 Parasitological methods*

These methods refer to procedures for visibly detecting the *Leishmania* parasites through direct microscopy, histopathology, or culturing. Direct microscopy in CL and MCL patients involves taking a scrape of the dermal lesion ulcers and visualizing the amastigotes, which is very common (80). In VL patients, aspirates from the lymph node, spleen or bone marrow are smeared onto a glass slide (81) , and further staining using Giemsa, wright or panoptic is made to enable visualization of the amastigotes under a microscope (79). Extracting aspirate samples from patients for parasitological diagnosis is invasive; a more straightforward approach for sampling is to use a press-imprint-smear or tape-strip disc method which is non-invasive. Press-imprint-smear involves stamping tissues on the clean side of a slide; by doing so, some cells become stuck to the slides, and further analysis is carried out on the cells (82). The tape strip disc approach involves sticking a disc with an adhesive side to the dermal site of the ulcers, and components of the lesion are attached to the disc once it is removed. Also, the print-imprint-smear method demonstrated positivity in more than 80% of CL cases compared to the conventional histopathology methods of sampling, which resulted in over 44% positivity—suggesting that the

print-imprint smear is a more sensitive detection method. In addition, sampling using filter paper cards is less invasive for CL and MCL diagnosis (83).

In VL, even though invasive, aspirate sampling is known to be 100% accurate depending on what organ the aspirate is coming from (84). Splenic aspiration is the gold standard for VL cases (85) , but bleeding can occur when non-specialized persons perform this procedure (86). Collecting bone marrow aspirates will be a preferred option because there is no risk of hemorrhage, although patients must endure the painful process of extracting the samples (87). Aspirates from lymph nodes would have been the best sampling choice, but sensitivity with this method is very low (about 50%) compared to those obtained from bone marrow or spleen (80,84). Circulating blood in VL may be another less invasive sampling method than aspirates, but the sensitivity is very low, especially for people with a lower parasite burden (81).

Samples from the respective tissues and sites of dermal ulcers can be further cultured in an enriched medium like Schneider's insect media or a diphasic medium containing 10 -20% Fetal bovine serum (FBS) (88). Here, the goal is to convert the amastigotes in tissues to promastigotes in a controlled laboratory environment to depict what is obtainable when parasites live inside the Sandfly vector. Further identification of parasites can be made molecularly for species identification, or the parasites can be used for determining a target antigen, animal injection, or screening for potential drug candidates. The downside to parasite culture is that it takes time (days to months), is monotonous, and there is always a risk of parasite contamination. It is also not feasible for field diagnosis because of the level of sophistication and need for a state-of-the-art facility, which may not be readily available in endemic and rural areas. Recent studies showed that culturing parasites using non-invasive sampling methods such as tape strips have more

sensitivity (100%) than those from biopsies, about 51% (89). Thus, non-invasive sampling methods in diagnosing leishmaniasis are a more advantageous tool due to higher sensitivity rate, reduced need for needles used, reduced need for a highly trained professional and efficient monitoring in treatment.

### *1.8.2 Immunological methods:*

These methods refer to tools used to detect current or past *Leishmania* infection by assessing numerous immune parameters. It is a rapid and non-invasive method to detect *Leishmania* in humans. The *Leishmania* skin test or Montenegro test measures type IV hypersensitivity or delayed hypersensitivity responses when suspensions of attenuated parasites are injected into the skin layers (90). This is more commonly used for MCL and CL cases, and the outcome is used to determine the patients' pre-exposure to *Leishmania* by the cellular reaction on the skin. This reaction is in the form of induration, and if it is greater than 5 mm for 48 hours post-injection, then that patient has either been infected with the parasite or has healed from a previous infection. This method is known to have a high sensitivity (86-100%) with a > 90% specificity (79). VL patients usually test negative for *Leishmania* skin test at the early onset of infection, but after treatment, they become positive (91). It suggests that the sensitivity or specificity of this method for diagnosis is limited to the dermal forms of Leishmaniasis but that they can also be used as an indication of successful treatment. The major disadvantage of this method is that it cannot differentiate a previous exposure from an active one, which is critical to reinforce a suitable treatment strategy.

Direct agglutination tests (DATs) are another immunological method of detecting *Leishmania* antibody titres in patients' serums. This method involves assessing the antigen-antibody

reactions by mixing trypsinized *Leishmania* promastigotes with patients' serum/blood/plasma, and specific agglutination of the antibodies with the antigen in the microtitre plates is assessed for exposure to the parasite (92). A positive result will indicate that the patient has a high antibody titre above the stated threshold (92). DATs are rapid, cost-effective, semi-quantitative, and highly sensitive for VL, which makes them valuable tools for diagnosis in fieldwork and laboratory settings (87). It was reported that the sensitivity and specificity rates were 96% and 95%, respectively (93). Although the previous heat-sensitive antigen used for DAT was known to have a shorter life span, mainly when used in tropical regions lacking access to adequate storage facilities, the use of resistant and freeze-dried forms that are stable at room temperatures for up to two years has been developed (81). One questionable problem with DAT is that patients still are positive for DAT even up to 1 year after treatment. While this may be an excellent thing to ascertain the efficacy of the treatment, it is unable to ascertain whether this occurrence of positivity is due to a disease relapse (94). One disadvantage of this method is that it requires multiple rounds of pipetting in serial dilutions and a long incubation period (12-18h). However, this was overcome by a fast agglutination procedure that involved a single serial dilution with a reduced incubation time of 3 hours (95). This upgraded agglutination test had a sensitivity and specificity of 91-95% and 70-88%, respectively, and it is recommended to detect the disease in multiple patient samples at once (96).

Another form of the agglutination test is the indirect hemagglutination assay, wherein the host or patient erythrocytes' reaction to heat-stable *Leishmania* antigen is assessed (91). The sensitivity and specificity are reported to be 96-100% and 86%, respectively, but vary amongst human host immune responses (88). This assay was very useful in detecting VL in non-endemic

areas (97). However, it is suggested that they should not be the only diagnostic tool used because people who recovered years later came out with higher titres (98).

The fluorescent antibody or immuno-fluorescence assay is a precise and rapid method that depends on using a fluorescent probe bound to an antibody specific to *Leishmania*. A signal is detected once it binds to its target (usually in the patient's serum). The sensitivity and specificity of this method were reported to be 91% and 81%, respectively, especially in CL (99). In VL, this method helps assess low antibody titres in the treatment relapse (91). This method can allow the probe to bind to the antigen or antibody target directly or indirectly where the secondary labelled probe binds to the target antigen or antibody (100). One major drawback of using this method, especially in resource-deprived settings, is that you need a fluorescence microscope to detect the signals, usually unavailable in low-income or developing countries.

Enzyme-linked immunosorbent assay (ELISA) is used to diagnose various pathogenic diseases, and this involves detecting a *Leishmania*-specific antibody from a patient's serum in the presence of a known *Leishmania* antigen. The *Leishmania* antigen commonly used is usually freeze-thawed in phosphate-buffered saline. The sensitivity and specificity are relatively high, 80-100% and 82-95%, respectively (98). The advantage of ELISA is that one can screen for multiple antigens in a single patient, which can help to direct specific drug treatments (101). However, it poses a problem when the *Leishmania* antigen used is similar to those from other closely related parasites or bacteria species as it generates similar antibody specificities. This cross-reactivity of the antibody detected in leishmaniasis by ELISA has been observed in other infections like toxoplasmosis, trypanosomiasis, and tuberculosis (81,98). Despite its high sensitivity, ELISA as a diagnostic tool requires expensive equipment for detection, such as a mass spectrometer in a

laboratory with highly trained professionals. There is also the fact that it does not help distinguish a present from a past infection.

Immune chromatographic test (ICT) makes use of the recombinant Kinesin-39 antigen (rK39) from *Leishmania chagasi* is embedded into a strip, and antibody from a patient's serum or blood will react with it and give a colour change in minutes (102). Just like other serodiagnostic tools, it is very rapid and non-invasive. This is very useful for mainly detecting VL. Although there are several schools of thought regarding the sensitivity of ICT, in one Mediterranean region, the sensitivity was around 78%, but in endemic areas of Colombia, it was 91.5%. However, when another study compared ICT with Florescence antibody or ELISA, ICT appeared to have the highest sensitivity of 95.5% (103). A rKE16 antigen from *L. donovani* was used for VL detection by ICT in an Indian community with a sensitivity of 92-100% but around a wide range of 36-90% sensitivity in the East African and Brazilian communities (104). Other antigens from *L. donovani* or *L. infantum* have been proposed for VL diagnosis with ICT. However, WHO recommends rK39 as the most effective (98). Although there is a wide variability in the sensitivity of ICT in detecting VL in different regions, it can also not distinguish between active and resolved cases.

Latex agglutination test is a test that detects VL in patients' urine by measuring the thermal resistance and molecular weight of a specific *Leishmania* molecule after the urine is boiled. It is also non-invasive and can be used as an alternative diagnostic tool for patients with low or undetectable antibody titres, especially in HIV and *Leishmania* co-infection settings (105). There is a wide range of sensitivity with this test, 47 -95%, but immunocompromised individuals had a sensitivity of 85-100%. This method monitors the progress in VL treatments as antigens can be detected from 1 to 6 months after treatment and can also detect preclinical stages of infection

(106). This method is cheap, does not have cross-reactivity issues, and distinguishes present infection and early detection. However, its sensitivity could be higher and false detection is achievable when urine is not boiled. (81,106)

### *1.8.3 Molecular methods*

The introduction of molecular biology revolutionized the diagnosis of leishmaniasis as there appeared to be numerous challenges with the immunological detection of leishmaniasis, such as the wide range of sensitivities, inability to detect active infection from a relapsing one, low antibody titres, etc. Clinicians and scientists recommend polymerase chain reaction (PCR) as the gold standard in molecular detection of antigens (107). The blood samples were more sensitive to molecular methods than invasive bone marrow samples (108). There are numerous molecular methods of detecting a pathogen, but the polymerase chain reaction (PCR) is usually the mother of all. The Conventional PCR method involves identifying the target antigen in a patient's sample by amplifying numerous copies of a genetic fragment of that antigen. The availability of most *Leishmania* species' whole genome sequencing data in an open domain has made it easier to utilize molecular technologies to identify them (86). A comparison of PCR with other parasitological methods suggests that PCR has a higher sensitivity (84.6%) than when observing parasites in culture or under a microscope (109). Despite its high sensitivity, the conventional PCR methods cannot distinguish an acute *Leishmania* infection from a relapsing one, especially in VL. There is also a risk of contamination and the expertise of a skilled professional, which may limit the sensitivity of this method (88).

Semi-nested and nested PCR is an improvement from the conventional PCR methods, where multiple regions of the target genes are amplified using multiple primer sets. This is critical to

identify parasites in a sample in low amounts (101). The nested PCR was determined to have a sensitivity and specificity of 94% and 100%, respectively, primarily for VL diagnosis (110). Unfortunately, there is still a high contamination risk with this method (87).

Real-time PCR (RT-PCR) would amplify the target genetic fragment and help quantify it on the go. It uses a fluorescent probe or dye which binds to the target fragment and then gives off a signal. Another type of reporter probe called Taqman will bind to a specific sequence, and when it becomes degraded, a report is released to give a signal (111). In the PKDL endemic region of Bangladesh, the sensitivity of this method for diagnosis was significantly higher (91.2%) compared to the parasitology methods, which had a sensitivity of 50.6% (112). Even though the overall sensitivity and specificity of the RT-PCR methods are 98 and 100%, respectively, (113) it still does not help identify *Leishmania* species (114). However, this method is an improvement from the nested PCR and conventional PCR methods because of its high sensitivity and reduced contamination (114).

The multiplex PCR method assesses multiple genetic fragments analyzed on gel electrophoreses in a given sample using multiple primer pairs at a go (115). This method is known to have a high sensitivity but may not be recommended for routine diagnosis of leishmaniasis because of the high cost (86).

Nucleic acid sequence-based amplification (NASBA) is used to identify a genetic fragment using RNA polymerase and reverse transcriptase to generate RNA and DNA, respectively. The products are assessed calorimetrically using fluorochrome probes and gel electrophoresis (116). Although there are two forms of NASBA, quantitative NASBA and oligo-chromatography-NASBA, quantitative NASBA is known to have a higher sensitivity than conventional PCR and a higher

specificity (95%) than oligo-chromatography NASBA (117,118). Generally, NASBA cannot identify the *Leishmania* species in the infected host (87).

Randomly amplified polymorphic DNA (RAPD) relies on the amplification of random segments of genomic DNA using short primers by PCR. Although this method can distinguish *Leishmania* species as it was able to identify *L. major* in the case of CL, it is considered unsuitable for diagnosis in the field studies due to difficulties with reproducibility (107). On the other hand, a technique closely similar to RAPD, restriction fragment length polymorphism (RFLP), is reproducible and can distinguish between *Leishmania* species effectively (119,120). The RFLP method digests the genetic fragments using restriction endonucleases, and the broken fragments are put together in a gel electrophoresis system to identify the *Leishmania* species in the sample (121). Amongst all the parasitological methods for detecting *Leishmania* infection, this technique is known to be very sensitive, especially in patients with low parasitemia (122). Another form of RFLP is the Amplified fragment length polymorphism (AFLP), which is reproducible and helps distinguish between *Leishmania* species (123,124). AFLP will amplify the fragments that have been digested using a specific primer set (100). These amplified products will be assessed on gel electrophoresis to check for polymorphisms.

### *1.8.4 Flow cytometry:*

This is one of the recent advances in diagnosing VL. Flow cytometry is a technique that helps assess protein expression in cells, cell viability, and cell-to-cell interactions (125). This method analyses single cells in solution using a combination of their light scattering and fluorescent signals which can be detected by photodiodes or photomultiplier tubes. The signals are further analysed on the computer and stored as a data file (.fcs). A study compared flow cytometry with

ELISA methods to assess Immunoglobulin G (IgG) in a CL case and found that the flow cytometry method has a higher sensitivity but lower specificity than the ELISA method (126).

### *1.8.5 Proteomics:*

Proteomics helps analyze the whole protein composition expressed in an organism, cell, or tissue in the presence of the defined conditions. It measures protein-protein interactions, post-translational modification, and protein abundance by extracting, separating, quantifying, and identifying. A sample specimen will undergo numerous steps of tissue disassociation. After separation of the proteins, the ions in the sample are trapped through a laser beam, which is then desorbed onto a matrix to pass through a vacuum flight tube whose mechanistic levels of detecting the ions by measuring the time of flight compared alongside an existing mass spectra database. The ionization of the samples to detect specific *Leishmania* proteins is called matric-assisted laser desorption ionization. Time-of-flight mass spectrometry has been extensively used to detect different clinical cases of Leishmaniasis (127,128). Liquid chromatography allowed the detection of plasminogen and vitronectin, key biomarkers for PKDL diagnosis using liquid chromatography-mass spectrometry (129).

Immuno-proteomics is a type of proteomics that detects specific immune responses to a particular infection. The process involves protein separation, western blotting, and mass spectrometry (MS) detection. Here, proteins separated from a cell are embedded on a nitrocellulose membrane in a western blotting technique; the patient serum, which is the primary antibody, is applied to the blot, and if there is a specific reaction of the patient serum with the protein on the blot, a secondary antibody that is enzyme labelled will be able to detect the interaction. Specific proteases cut the bound spots to enable the detection of the peptide

fragments by MS for immunogenic fingerprints (130). This method was used to identify specific biomarkers for VL (131). Generally, proteomics methods are highly sophisticated techniques and may require well-trained professionals and established laboratories for analysis. Thus, it is not feasible for use in fieldwork settings.

### *1.8.6 Advanced molecular methods:*

One of the advanced methods for the molecular diagnosis of *Leishmania* infection is Loop-mediated isothermal amplification (LAMP), which makes use of about 4-6 primers to amplify six distinct sequences on the target genetic fragment to form products that look like a Loop of nucleotide sequences (132). Amplified fragments can be detected visually by colour change, viscosity and fluorescence (133). Unlike the conventional PCR methods that use thermocyclers to regulate the temperature of cycles, LAMP uses isothermal conditions like water baths and heat blocks, making them a cheaper option for Leishmaniasis diagnosis. Indeed, LAMP is considered the best tool for a point of care in the fight against Leishmaniasis. Compared to parasitological and conventional methods of detecting Leishmaniasis, LAMPS has the highest sensitivity (>90%) in blood samples in CL and VL cases (134). In an endemic area in India, LAMP was seen to have a 96.9% sensitivity for VL (135). With LAMP, immune-compromised individuals reported a relatively high sensitivity (60-100%) and specificity (85-100%) for LAMP (136). Challenges that limit the use of LAMP are inefficient temperature control associated with isothermal systems and the formation of secondary nucleotide structures (107).

The multilocus enzyme electrophoresis is a non-DNA method of assessing species variants by measuring the electrostatic mobilities of intracellular enzymes during electrophoresis. The mobility pattern is usually strain and species-specific, and their profiles can be used to identify

the particular variant under investigation (137). Although the WHO recommends this method for *Leishmania* species typing (138), it is limited for use, especially in resource-deprived settings, because of the requirement for a highly skilled professional, high cost and need for a large amount of isoenzymes (139).

The multilocus sequence typing will enable the identification of a specific species or subgenera in a target sample by amplifying about 6-8 housekeeping genes, and these genes are further sequenced (107). This method could differentiate between the genetic diversity of *L. braziliensis* and *L. panamensis* (140) and showed the variability in *L. major* and *L. tropica* that was identified in the lesions of patients with CL in Iran (141). The drawback of using this is the high cost and need for a skilled professional in an established laboratory. Further advances in molecular methods will enable researchers to develop alternative diagnostics and treatments for Leishmaniasis readily.

### *1.8.7 Nano-diagnosis:*

The use of nanoscience has revolutionized the diagnostic outcomes of Leishmaniasis. The nano diagnostic methods involve using nanoparticles (NP), nanodiscs, quantum dots, stable lipid NPs, and polymer NPs to identify promastigotes *in vivo* and *in vitro*. A DNA, RNA or protein-based nano-diagnosis has been developed to diagnose leishmaniasis. In the DNA-based nano diagnostic approach, a specific kinetoplast DNA in the target sample was amplified, and nucleic acid biosensor probes were infused with an NP detector such that the hybridization of the amplified product in the target sample with the oligos in the NP gives off a colour that confirms positivity (142). The RNA approach is a method where a subunit of ribosomal RNA from *L. donovani* was packaged in an NP salt aggregated probe; once the isolated target RNA binds with the NP

packaged RNA probe, it triggers a colour change indicating positivity in the target sample (143). In the protein nano diagnostic tool, the crude antigen of *Leishmania infantum* is embedded in the gold NP screen-printed carbon electrode probe. On contact with the sera from a patient, the antibody-antigen interaction is detected by signals from an indirect ELISA assessment (144). This method has been reported to be used in diagnosing canine leishmaniasis (145). Depending on the type of nano-diagnostic tool, the sensitivity and specificity are high and within the range of 98-100% compared with other parasitological or serological methods (146,147). However, the challenge with nano diagnostic measures is that there have been reports of false positivity, as some of the NPs are known to interact chemically with the test samples (148). Nonetheless, with the increased advancement in Nanotechnology, nano-based diagnostic measures have great potential to become the gold standard for detecting Leishmaniasis.

***Table 1 Pros and Cons of the Diagnostic tools used in Leishmaniasis***

| | **Diagnostic method** | **Pros** | **Cons** |
|---|---|---|---|
| | Parasitological | Visualizing amastigote | 1. Invasive sampling<br>2. Time intensive<br>3. Risk of parasite contamination<br>4. Not suitable for field diagnosis |
| | Immunological (Type IV hypersensitivity) | 1. Non-invasive<br>2. High sensitivity | Unable to distinguish prior exposure from active infection |
| | Immunological (Direct Agglutination Tests, DAT) | 1. High sensitivity and specificity<br>2. Easily detected in multiple patients | Error prone due to multiple pipetting |

| | | | |
|---|---|---|---|
| Traditional | Immunological (Flourescent antibody or immunoflorescent | High sensitivity | Sophistication required |
| | Immunological (ELISA) | Allows for multiple screen of samples at a time | 1. Expensive<br>2. Does not distinguish current from past infection |
| | Immunological (Immune chromatographic test, ICT) | 1. Rapid and non-invasive<br>2. Useful for VL<br>3. More sensitive than ELISA | Does not distinguish current from past infection |
| | Immunological (Latex agglutination) | 1. Non-invasive<br>2. Alternative for patients with low antibody titres<br>3. Wide sensitivity range<br>4. distinguish current from past infection | False detection is possible |
| | Molecular (conventional PCR) | Increased sensitivity when blood samples are used | 1. Contamination risk<br>2. Professional expertise required<br>3. Does not distinguish current from past infection |
| | Molecular (semi nested/nested PCR) | Higher sensitivity and specificity | High contamination risk |
| | Molecular (RT-PCR) | 1. Sensitivity higher than parasitological<br>2. Reduced contamination | Does not identify *Leishmania* species |
| | Molecular (multiplex PCR) | High sensitivity | High cost |

| | | | |
|---|---|---|---|
| | Molecular (Nucleic acid sequence-based amplification, NASBA) | Higher sensitivity than conventional PCR | Does not identify *Leishmania* species |
| | Molecular (Randomly amplified polymorphic DNA, RAPD) | Distinguishes between *Leishmania* species | Poor reproducibility |
| Advanced | Flow cytometry | Higher sensitivity than ELISA | Lower specificity |
| | Proteomics | Used to identify specific biomarker in disease | Sophisticated trained professionals required |
| | Loop-mediated isothermal amplification (LAMP) | Higher sensitivity | 1. Formation of secondary nucleotide structures<br>2. Inefficient temperature control |
| | Multilocus enzyme electrophoresis | Recommended for parasite typing | 1. Requires a large amount of isoenzyme<br>2. Sophisticated trained professionals required |
| | Nano diagnosis | High specificity and sensitivity | False positivity |

## 1.9 Treatment and control of VL and CL

The current treatment measures for leishmaniasis have employed chemotherapeutic strategies such as pentavalent antimonial, liposomal amphotericin B, miltefosine, and paromomycin (Table 2). However, these treatments need to be improved due to their toxicities, the rise in co-infection cases, and the emergence of drug resistance. Some strategies to overcome the current challenges

with chemotherapy against leishmaniasis have been combination therapy, host-directed therapy, drug repurposing, anti-microbial peptides, and nano-based treatments. Some of these are discussed briefly below.

### *1.9.1 Pentavalent antimonial:*

Pentavalent antimonials such as meglumine antimonate (Glucantime) or sodium stibogluconate (Pentostam) have been the oldest drugs used to treat leishmaniasis (since 1945) (149). These drugs are inactive before ingestion, but after they are absorbed, they become reduced to an active trivalent form in their host (150). There have been several schools of thought on the mechanism of action for this drug: a) A reduction to the trivalent form could occur when the pentavalent form reacts with a known virulence factor in the parasite (Trypanothione) to form thiols that create oxidative stress and continuous efflux of thiols into the cells is orchestrated by an ABC transporter (151) b) trivalent forms are formed when pentavalent compounds undergo reductions associated with ADP and GDP (152) c) Pentavalent forms directly inhibits metabolism pathways such as glycolysis, fatty acid $\beta$ oxidation, glycolysis, and ADP phosphorylation d) it can also damage DNA and cause cell death (153). It has been regarded as the gold standard for treatment in both old and new world countries, and people diagnosed with VL were given the drug either intravenously or intramuscularly. In contrast, for those with CL, the drug is injected within the lesions (154). In a clinical trial in Brazil, CL patients given the meglumine antimoniate had a cure rate of 87% (155). In comparison, another study showed a cure rate of >90 % among the participants (156). However, in India, there is minimal use of antimonials due to toxicity and resistance issues. Its resistance could be due to inactivation of the trivalent forms, reduced

uptake due to the overexpression of aquaglyceroporin, and downregulation of calcineurin critical for cellular functions (150).

### *1.9.2 Liposomal amphotericin B (amp B):*

Amp B deoxycholate is an antifungal drug that is efficacious in anti-leishmanial treatment and was introduced in the early 1960s (157). Since the emergence of antimonial resistance, Amp B has been used as an alternative drug, and it has been reported to cure VL. The mechanism of action is that they interact with sterols to create a pore within the cells, leading to cell death and inducing cells to go into oxidative stress, releasing reactive oxygen species, which makes parasites unable to survive (150). Amp B deoxycholate appeared to show improvements in patients resistant to antimonials, but there were other side effects, such as nephrotoxicity and hypokalemia. To improve this, amp B was packaged in liposomes (AmBisome, Amp B lipid complex and AmB cholesterol) and delivered to the lipid bilayers (158). This helped to reduce toxicities with improved pharmacokinetics and dynamics (159). Patients in Ethiopia who took liposomal Amp B had a cure rate of >85% for VL (160) , and in another study in India, an increased cure rate of 96% was observed when amp B was combined with amBisome (161). Resistance with ampB has been associated with faulty membranes, increased expression of a known MDR1 gene for drug mutation, and increased trypanothione biosynthetic pathways. However, combining either doxorubicin (an anti-parasitic drug) or liposomal amp B appears to induce better efficacies (162).

### *1.9.3 Miltefosine:*

Miltefosine (hexadecyl-phosphocholine) was given for breast cancer treatments as far back as the 1980s and was also seen to be efficacious in anti-*Leishmania*l treatments. It is administered

orally and has been demonstrated efficacious for both CL and VL (163). Miltefosine is known to a) inhibit phospholipid and alkyl lipid metabolism, thereby disrupting cell membrane (164), p b) affect mitochondria by obstructing cytochrome c oxidase, leading to cell death (151), c) Upregulates expression of inducible nitric oxide synthase, a critical enzyme that produces nitric oxide critical for killing *Leishmania* (165). With just 28 days of administration of miltefosine, >90% of patients were effectively cured of VL (159,166). The cure rates of miltefosine on CL were reported to be between 87 and 100% (163). Despite the improved efficacy, there has been resistance to miltefosine, which was due to inadequate use and extended shelf life and has led to relapsing cases in Bangladesh, India and Nepal (163). Another reason for the resistance may be that miltefosine intercepts the transporter protein LdRos3, which may have affected the uptake of the drug (167,168). In females, miltefosine is known to affect the developing fetus, and its use is limited (169). Combined with other drugs, such as Lopinavir, reduced the parasite burden in *Leishmania*-infected mice compared with miltefosine alone, suggesting that combination therapy will enhance treatment outcomes.

### *1.9.4 Paromomycin:*

Paromomycin is a broad-spectrum antibiotic used to treat both eukaryotic and prokaryotic pathogens, but in the 1960s, it was shown to be effective against CL and VL (170). Its action is to intercept protein machinery by destabilizing the 30S ribosomal subunit. It also affects cell membrane potential by affecting lipid metabolism (152). Paromomycin was approved for VL treatments in India and administered intramuscularly with a cure rate of 94% (166). The application is topical for CL, and the cure rate is about 80% (171). Paromomycin is preferred for VL treatment in resource-limited settings and in India because it is cheaper (172). Although

resistance has been reported in Paromomycin treatments for VL, combination therapy is recommended in these cases (173).

### *1.9.5 Combination therapy:*

Due to the increasing cases of resistance and toxicity associated with the conventional therapy against Leishmaniasis, generally, multiple drug administration was the proposed alternative. It was necessary to do this to reduce the dosage duration with single drug usage and thus reduce the time frame for drug toxicity to become established. It was also vital to reduce relapses and resistance issues (154). The reason for exploring this in leishmaniasis was because combination therapy has been reported successful in treating HIV, malaria, and TB (174–176). Combining an antifungal drug such as itraconazole alongside a hypercholesteremic drug, ezetimibe, with miltefosine in VL model mice drastically reduced the parasite burdens in the liver and spleen of the animals (177). The efficacy of tamoxifen with antimonials also showed anti-*Leishmania* efficacies in vivo and in vitro models of *L. amazonensis* infection (177). In another study, a single therapy drug, liposomal Amp B, was used for skin abrasions associated with *L. major* in mice, which took 64 days to clear. However, when Liposomal Amp B was combined with tretazicar, it cured in 18 days (178). All these mice studies suggested the potential use of a combinatorial approach to anti-*Leishmania*l treatments.

When antimonial resistance was developed, combining two drugs to treat infected individuals was already getting attention in India and the African Continent (150,179). The cure rate of miltefosine and liposomal amp B was >95% in India, while in Sudan or Kenya, this combinatorial drug had a cure rate of 87%. They suggest that the effective use of a multiple-drug combination may depend on the existing resistant strain in the endemic region. The administration of

amBisome and miltefosine in HIV patients co-infected with VL was reported to be safe, tolerable and efficacious in reducing the VL relapse rate (180). In another study, a combination of miltefosine with liposomal amp B over three months in patients suffering from PKDL had a cure rate of 100%. However, relapses were observed 18 months after recovery, whereas in a miltefosine monotherapy, relapses occurred 3 to 4 weeks post-recovery (181). All of these suggest the expansive outcomes from multiple drug administrations in anti-*Leishmania* treatment.

### *1.9.6 Drug repurposing:*

This refers to an alternative drug used to treat an ailment originally manufactured for another medical cure. This approach was established to save costs and time associated with developing new drugs, mainly because these drugs have gone through the required regulatory checks and preclinical/clinical trials (182). When a drug is to be repurposed, in silico approaches are used to assess the potential interactions of the drug with the *Leishmania* parasite of interest to be sure that the drug will be directly targeting the parasite (183). Most conventional anti-*Leishmania* treatment drugs, like paromomycin, amp B, and azoles (itraconazole, fluconazole, and Posaconazole), were repurposed. An anti-depressant like sertraline was recommended to be valuable for anti-*Leishmania* treatment. It was reported to inhibit *Leishmania* infantum parasites directly by decreasing ATP synthesis and mitochondrial membrane potential, suggesting they may be suitable drug candidates against VL (184). Multiple studies have estimated the efficacy of repurposed drugs in anti-*Leishmania* treatments (183,185–189). Repurposing drugs is an innovative way to develop valuable therapy against Leishmaniasis in the future.

### *1.9.7 Nanoscience therapy:*

Nanomedicine allows one to optimize the use of nanoscale to design suitable drugs and toxic-free delivery outcomes. One of the primary reasons for resistance and toxicity with conventional drugs against *Leishmania* is that these drugs damage cellular compartments when they are administered (87). NPs are suitable for drug delivery options because they are usually biologically compatible, deliver to the organ of interest, improve drug solubility, and can be exploited to provide multiple administration routes (190). NPs used for drug delivery are usually made from metal, carbon, liposomes, organic and inorganic polymers, dendrimers and micelles; however, because the significant target of *Leishmania* is usually in the phagocytic cell, most of the drugs for anti-*Leishmania* treatments are packaged in liposomes and polymers (191,192). Drugs administered with NPs produce practical leishmanicidal effects such as enhanced immune responses, increased ROS production, damaged DNA and mitochondrial functions, and inhibited synthetic pathways (193). Even though numerous studies outline the efficacy of Nanomedicine in anti-*Leishmania* therapy, there is a considerable systemic toxicity that has been reported (194) and preparing drugs in NP packaging is expensive and requires a technical skill set (195). With the advancement of science in this era, these challenges will soon be overcome.

### *1.9.8 Host-directed therapy:*

Host-directed therapy (HDT) targets the immune responses rather than the pathogen for a clinical cure. HDT involves using either a drug or molecule that can modulate inflammatory responses or the body's immune system to induce a cure and reduce relapses (196). One such molecule is the Oleuropein from an olive plant that stimulates ROS and NO production, elevated

IL-12, IFN- $\gamma$ and transcription factors responsible for generating Th1 cellular immunity against *Leishmania* donovani infection in mice (197).

***Table 2 Modes of action and challenges in the Drugs used to treat Leishmaniasis***

| Drug | Mode of action | Downside | Reference |
|---|---|---|---|
| Pentavalent antimonial | Forms an active trivalent form that interacts with parasite to create oxidative stress | Toxicity, Resistance | (151), (150) |
| Liposomal amphotericin B (ampB) | Interacts with sterols to create a pore in the cells resulting in oxidative stress | Resistance | (150) |
| Miltefosine | 1. Distupts cell membrane<br>2. Inhibits cytochrome c oxidase<br>3. Upregulate iNOS to kill parasites | Resistance | (151), (164) |
| Paromomycin | Destabilizes 30s ribosomal subunit and affects lipid metabolism | Resistance | (152) |

## 1.10 Vaccination strategies against VL and CL

Due to the increasing challenges associated with chemotherapy, such as drug toxicity and resistance, there is an increasing need for an alternative therapy that will prevent the initial onset

of leishmaniasis through prior exposure to some form or components of the parasite, thus reducing morbidity and mortality associated with the disease. This strategy is referred to as vaccination. Interestingly, an old vaccination rule postulates that a vaccine must induce protection similar to what is obtainable following recovery from the natural disease (198). Vaccination is adoptable in leishmaniasis because recovering patients develop immunity against re-infection (199). While human vaccination against leishmaniasis has been increasingly disappointing, some vaccines have been approved to treat the disease in animals (200). Leishmanization has been the only effective vaccination strategy against cutaneous leishmaniasis but has been discontinued due to safety concerns. *Leishmania* subunit vaccine is a popular vaccination strategy that has received numerous accolades for its inexpensive cost and reproducibility, such that a couple of these vaccines are approved for animal use (201). However, the immune responses from the subunit vaccines do not produce long-lasting protection and require occasional boosts. Thus the need to generate a vaccine candidate that will induce long-lasting protection against leishmaniasis is critical. Some of the previous and recent vaccination strategies are outlined below.

### *1.10.1 Leishmanization:*

Leishmanization refers to conferring immunity on an individual against leishmaniasis by intradermal injection of live virulent *Leishmania* in an unexposed body part to prevent development of a natural disease. It was noticed in those days that individuals living in CL-endemic areas of the Middle East, only got CL lesions once in their lifetime, indicating that immunity was conferred to these individuals (202,203). A sharp object would be used to scrap

out exudates in lesion and inject them into a covered body part of a CL naïve individual. Once the person develops lesions and is healed, they are immune for life against natural disease (204). This practice has been the norm since the 19th century when promastigotes were harvested from culture media and inoculated in humans (204,205). As a result of this, mass immunization of military recruits was enforced in Israel, Uzbekistan and Turkmenistan between 1959 and 1967 and by 1977, leishmanization program was in full effect in Iran (206). The risk assessment of leishmanization reduced the incidence rate of disease to about 69% (207) , and at the time, it was a last resort in the control of CL as other measures were failing. Attempts at reducing the risk associated with leishmanization began with developing an *L. major* stabilates generated by good manufacturing practices (GMP). Participants were vaccinated, and lesions developed in some individuals, but natural CL lesions did not develop even after ten years, suggesting this method induced protective responses. Using these stabilates, there were cases of vaccinated individuals developing non-healing lesions, but it has been known that natural CL can induce non-healing lesions as well. However, this has been the major drawback using these *L. major* stabilates (208).

Since VL is very fatal, vaccination is highly needed. However, recovery after timely treatment is possible, but individuals are at risk of severe complications from either the treatment or developing relapsing conditions. In Sri Lanka, it was reported that a naturally attenuated *L. donovani* isolate protected against VL, which was associated with a Th1/Th2 response (209). This isolate was obtained from an individual who presented cutaneous lesions but did not develop visceralizing disease. Other studies have shown that vaccinating mice with either a low dose of *L. infantum* or sustained levels of *L. infantum* in the spleen protects against virulent challenges

(210,211). Notwithstanding, leishmanization is a cheaper tool to control leishmaniasis. Aside from the risk of reverting to virulence and developing non-healing lesions or severe consequences, the rate at which lesions develop after vaccination with these live parasites is not uniform even when using the same protocols and media growth conditions because individuals respond differently to the immunization (207). Thus, generating non-pathogenic variants of *Leishmania* species became an alternative vaccination strategy to explore.

### *1.10.2 Genetically modified and live attenuated vaccine:*

Genetically modified and live attenuated vaccine strategies have been regarded as second-generation vaccines, where a gene (s) in the parasite responsible for survival and virulence is deleted (212). These parasites do not pose any danger and are preferred because they induce immune responses similar to what can be obtained in a natural infection (213). Non-pathogenic or genetically engineered parasites are the most promising vaccine against the more fatal forms, such as VL. Numerous live attenuated forms of the *Leishmania* parasites have been generated by removing essential genes. These genes include the biopterin transporter and A2-Rel in *L. donovani*, SIR2, heat shock protein Hsp70-II, and KHI in *L. infantum*—the deletion of these genes in the parasites induced protective responses in mice (214–217). We have also shown that the absence of a metabolic enzyme critical for gluconeogenesis in *Leishmania major* induced moderate protective responses (218). The protective responses obtained from centrin-deficient parasites were due to the increased influx of MHCII-expressing macrophages, which resulted in increased production of IFN-$\gamma$ from $CD4^+$ Th1 cells and reduced the levels of IL-10 and IL-4

cytokine-producing $CD4^+$ Th2 cells (219,220). The absence of the abundant surface molecule in *Leishmania* parasites, LPG, in *L. major* and *L. infantum* was attenuated in mice and induced long-term protection against virulent *Leishmania* challenge (221,222). However, LPG deficiency in *L. mexicana* was not attenuated inside macrophages or mice and perhaps not a valuable vaccine target against *L. mexicana* infections (223). Essentially, the most favourable vaccine candidate that is genetically modified but live will be one that will be protective against all forms of leishmaniasis, including VL and CL.

### *1.10.3 Subunit vaccine:*

Subunit vaccines refer to a subunit or recombinant form of the parasites that upon injection, could induce protective responses in preventing a wild-type *Leishmania* challenge. Some of the components in the parasite that have been used as a vaccine in *Leishmania* include LeiF, gp63, p36/LACK, A-2, PSA-2/gp46/M-2, FMK, LCR1, OEFF, KMP11, LmSTI1, TSA, HASPB1, protein Q, cysteine protease B (CPB), and A (CPA) (12). When *Leishmania*'s surface glycoprotein Gp63 was packaged in a cationic liposome, or the DNA forms were used as a vaccine, they were shown to increase the frequency of IFN-$\gamma$-producing $CD4^+$ T cells and confer protection (224). Also, promastigote surface protein PSA-2/gp46/M-2 that was packaged in virus induced protective responses against *L. amazonensis* (225) The more notable subunit vaccines are the LEISH-F1, LEISH-F2, LEISH-F3 which show promising potentials as a vaccine candidate as they have advance to Phase II clinical trials (226–228). LEISH-F1 is a subunit derived form the kinetoplastid membrane protein-11 (KMP-11) conserved in all *Leishmania* species, LEISH-F2 is derived from Leishmania stress inducible protein 1 (LmSTI1) and LEISH-F3 is a combination of multiple immunogenic proteins including LmST1. LEISH-F1 and LEISH-F2 are packaged in a structure that

stimulates toll-like receptors, while LEISH-F3 is contained in a stable oil-in-water emulsion, all showing promising results to protect against *Leishmania* infection (226–228). One advantage of using subunit vaccines is that there is no risk of reverting to natural infection in their host because only a parasite component is used to induce protection. The challenge, however, is that these vaccines could not generate long-term protective immunity because the presence of parasites is vital for lifelong immunity (229).

### *1.10.4 Third-generation vaccines and recent advances:*

The third generation vaccines refer to vaccines that involve naked DNA (230). The DNA of one or multiple specific components in *Leishmania* is usually packaged in a virus and injected intradermally or intramuscularly. They are used because they induce robust IFN-$\gamma$ production and $CD4^+$/$CD8^+$ T cells and activate dendritic cells, which are crucial for protection against *Leishmania* infection (231,232). DNA vaccines comprise DNA from one or multiple antigens in *Leishmania*, such as gp63, LACK, PSA-2 TSA, KMP11, A-2, NH36, LmST11, cysteine proteases and histones (230). Current studies are underway to understand the clinical impact of a DNA vaccine comprised of 2 antigens, *Leishmania* KMP11 and HASPB, in patients with PKDL (233). The advantage of DNA vaccines is that there is no potential to revert to virulence in the vaccinated individual, and they are stable and do not require adjuvants. However, the downside is the risk of these vaccines becoming reintegrated into the host genes, resulting in autoimmune disease and/or cancer (219).

Aside from DNA vaccines, there have been recent advances in developing more efficacious vaccines. It was important to improve the vaccine delivery to control antigen release, prevent the degradation, and ensure that the antigen is delivered to the target organ.

The use of nanoparticle carriers has enabled the progression of studies demonstrating the efficacy of these vaccines (234). For instance, when cationic solid nanoparticles delivered a fusion gene from *Leishmania*, there was a protective cellular immune response (235). In addition, when the poly D, L-lactide-co-glycolide nanoparticle delivered soluble *Leishmania* antigen or monophosphoryl lipid A, protection against *L. infantum* infection was obtained (236). Another exciting advancement is chimeric vaccines, where two or more gene fragments in different chromosomes or loci from *Leishmania* are linked and expressed together to generate a more robust and improved immune response. ChimeraT comprises antigens from *Leishmania* prohibitin, elongation initiation factor 5a, and hypothetical LiHyp1 and LiHyp2, induced a Type1 immune response upon vaccination against *L. infantum* (237). Vaccination with the F1F3 chimeric protein gotten from *Leishmania*'s nucleoside hydrolase NH36, cause reduced lesion developments following challenge with *L. braziliensis*, which was associated with higher IL-2, TNF-$\alpha$, and IFN-$\gamma$-producing $CD4^+$ and $CD8^+$ T cells (238). *L. tarentolae* is a non-pathogenic *Leishmania* species that infects humans. The idea of using them as a vaccine is that they activate DCs, increasing IFN-$\gamma$ Th1 type responses, and they are known to mimic the immune response one would find in a natural infection setting (234,239,240). It has been established that *L. tarentolae* injected intraperitoneally induced protective immune responses against *L. donovani* infections in BALB/c mice (241). The use of virus-like particles is another strategy in vaccine developments that has the potential to generate cellular and humoral responses similar to native viruses but is unable to replicate (242). *L. tarentolae* are known to be a vehicle or antigen that produces viral-like particles or proteins (243). Viral-like particles derived from *L. tarentolae*, were seen to induce neutralizing antibodies during a virulent *Leishmania* infection (242).

RNA vaccines direct the synthesis of the antigen in the host for that can elicit an immune response. The F2-RNA, RNA vaccine administered alongside LEISH-F2 in a VL model significantly reduced the liver parasite burden upon challenge (244). RNA vaccines have the potential to revolutionize vaccine developments against leishmaniasis.

***Table 3 Common vaccination strategies used against Leishmaniasis***

| Vaccine strategy | Pros | Cons | Reference |
|---|---|---|---|
| Leishmanization eg low dose parasite, *L. major* stabilates | 1. Cheaper<br>2. Protection and long term immunity guaranteed | 1. High risk to reverse to virulence<br>2. Risk to developing non-healing lesions | (208) |
| Genetically modified or Live attenuated eg LPG2 KO parasites, centrin deficient parasites | 1. Protection and long term immunity may or may not be guaranteed | 1. Low risk to reversal to virulence (at least not as high as leishmanization) | (219,220). |
| Subunit eg LeishF1, Leish F3 | 1. No risk to revert to virulence or natural infection | 1. Long term immunity not guaranteed<br>2. Adjuvants required | (226–228) |
| DNA vaccines eg KMP+HASPB | 1. No risk to revert to virulence or natural infection<br>2. Stable and requires an adjuvant | 1. Risk of reintegration into host genome | (219) |

## 1.11 Experimental models of CL and VL

To develop drugs for CL and VL, specific models for the disease are required so that the efficacy of the therapeutics can be assessed. There are available *in vitro* studies that examine the efficacy of drugs using techniques such as reporter gene assays and high content/high throughput screens with either promastigote and amastigotes or their components (245,246). In the recent systemic review by Vander Ende and Scahalling, it was noted that hamsters and mice are the more frequently used animal models, and *L. major* and *L. amazonensis* were the species mostly used to induce infection in these animals (247). However, *in vivo* models are popularly known to recapitulate what is closely obtainable in humans. So animal models of CL and VL are preferred and will be explained below.

### *1.11.1 Mouse model:*

Mice are often used because they are inbred with similar genetic makeup within each strain, transferring phenotypic characteristics to offspring consistently (248). Generally, the BALB/c strain of mice has been associated with susceptibility, and the C57BL/6 strain is associated with resistance to *Leishmania* infection (249). Susceptibility is related to the development of a non-healing lesion in *L. major* infection. It correlates with a Th2 immune response that produces IL-10 and IL-4, which promotes upregulation production of arginase that enables parasite survival in macrophages. On the other hand, resistance is associated with the resolution of lesions in *L. major* infected in C57BL/6 and C3H mice. This is due to induction of IFN-$\gamma$ and TNF-$\alpha$ production in $CD4^+$ T cells, which stimulate the upregulation of inducible nitric oxide synthase (iNOS) and production of nitric oxide to initiate the clearance of the parasites in macrophages (250–252).

Studies attempt to recapitulate natural infection in these mice models by inoculating $10^5$-$10^6$ parasites in the footpad, ear, or tail without including the salivary gland components from the sandfly. Natural infection however is described as one where low-dose parasites (100-1000) are deposited by the sandfly with accompanying salivary gland components (253). Studies by Belkaid et al, showed that intradermal low-dose infections rather than subcutaneous infections with high dose parasites ($10^5$-$10^6$) leads to development of lesions slowly in mice that is less extensive compared to what is observed in natural settings (254). Some mice models show distinctions in their responses to different *Leishmania* species. For instance, the C57BL/6 and C3H mice, which are resistant to *L. major*, are susceptible to *L. mexicana* and *L. amazonensis* (255). It is thought that this susceptibility of C57BL/6 mice to *L. amazonensis* infection is not dependent on a Th2 response or IL-10 expression but on the predominance of M2 macrophages (256,257). Still, the control of the infection was dependent on IFN-$\gamma$ and STAT-4 (258,259). Even when exogenous IL-12 was administered to these mice, there was a delayed resolution to the lesion compared to *L. major* infection (260). If using only the *L. major* mouse infection mice model, one is bound to miss exciting dynamics in immune responses associated with other species and thus may limit vaccine discovery or therapeutic outcomes.

For VL caused by either *L. infantum* or *L. donovani*, the BALB/c, DBA/1, C57BL/6, and NMRI mice models are used to determine pathogenesis and test therapeutic agents (261,262). BALB/c depicts an organ-specific immune response to visceralizing disease. After the first few weeks, the parasites will multiply rapidly in the liver. Afterwards, the onset of a Th1 response at 4 weeks post-infection ensures the clearance of the parasites resulting in the development of an organ-resistant phenotype. On the other hand, parasites in the spleen replicate extensively but are

eventually controlled and maintained for a long time as they never get cleared. The persistence of the parasites in the spleen of mice is due to unsuccessful granuloma formation leading to splenomegaly (263). It has also been noted the Natural Resistance Associated Macrophage Protein-1 (NRAMP-1) locus has been identified to play a role in delayed expansion of parasites in the liver and spleen in BALB/c mice compared to the DBA mice (264,265). It is important to note that in BALB/c mice, VL is chronic but not fatal making it a good model for subclinical or self-healing studies. Although the use of BALB/c mice for VL does not quite recapitulate what is observed in humans, it is beneficial for underpinning the effect of specific parasite genes on host immune responses (266).

### *1.11.2 Hamster model:*

The Syrian golden hamster completely reproduces the clinical pathologies of VL disease as seen in humans and thus is the best animal to study *L. donovani* and *L. infantum* infections. However, there are limitations to using this animal model because the reagents required to investigate the specific immune responses are not readily available (245,267,268). Hamsters infected with *L. donovani* develop increased Th1 type cytokine expressions, basal IL-4 and TNF-$\alpha$ expressions as infection progresses, and a potent increase in IL-10 expression within the spleen. In this model, there is uncontrolled parasite replication in the spleen, bone marrow and liver, even in the presence of a strong Th1 response, which was thought to be due to increased proliferation of immune cells producing suppressive cytokines associated with the active disease. This suppressive environment was due to the fact that APCs could not present antigens to T cells; so tolerogenic cells began producing TgF$\beta$ resulting in the apoptosis of lymphocytes and decreased expression of protein kinase C, which is critical for proliferative cellular responses (267).

Interestingly, in the hamster model, fatal and progressive disease has been associated with an unresponsive macrophage which is incapable of upregulating inducible nitric oxide synthase (iNOS), the main component that ensured killing of parasites despite having a strong Th1 presence (267). It was noted that the undetectable level of iNOS in these animals was due to significantly lowered (basal) *NOS2* gene promoter activity compared to that obtained in infected mice (269). Although a good progressive disease model, the disease in hamsters is achieved by intracardial or intraperitoneal injection, which does not quite depict the natural infection dynamics where the parasites are deposited in the mammalian skin by the sandfly vectors. Interestingly, hamsters are also valuable for maintaining the virulence of *Leishmania* species that cause VL (270). Once the parasites are isolated from the hamsters, they can only be grown for a few weeks before they lose virulence, so using cryopreserved parasites will decrease the constant need to maintain virulence in the animals (270).

### *1.11.3 Dog model:*

Domestic and wild dogs are the main zoonotic reservoirs of VL caused by *L infantum* in the Middle East, Latin America and Asia (271). In China, dogs infected with *L. chagasi* are predominant (272). The fact that dogs were reservoirs for VL led to intensive research to understand the cellular mechanisms that promote protective responses in order to develop new therapeutics and control measures that can apply to the human forms of the disease (15,273). Interestingly, dogs are not known to be reservoirs for parasites causing CL (274,275). However, natural infection of dogs with *L.* (*V*.) *braziliensis*, *L*. (*V*.) *peruviana*, *L*. (*V*.) *panamensis*, *L*. (*V*.) *colombiensis* and *L.* (*L*.) *mexicana* has been observed (276). Dogs infected with VL-causing parasites bear similar presentation of symptoms observed in humans, such as long periods of asymptomatic infections,

anemia, enlarged spleen, weight loss and fever. The dogs mostly used for cannie VL studies are German shepherds, beagles or mixed breeds (277–279).

### *1.11.4 Non-human primate model:*

Non-human primate models are valuable because they closely resemble human anatomy, immunology, and physiology. However, using non-human primates for research is expensive and difficult to handle. One non-human primate, the Asian rhesus macaques, is susceptible to *Leishmania* infection, develops human-like disease, produces *Leishmania*-specific antibodies and T cell-mediated responses and is also seen to be protected by numerous vaccination strategies (280). Macaques were also seen to build resistance against a re-infection similar to what is obtainable in humans, indicating the development of infection-induced immunity (281). New world primates such as owl monkeys, squirrel monkeys and marmosets are potential hosts for VL. Owl monkeys will develop fatal VL disease and are usually unable to recover (282) , but squirrel monkeys will develop deadly disease, recover and become resistant to reinfection (283). The occurrence of different phenotypes in these monkey species makes the development of vaccines against VL a possibility. Due to cost limitation in using non-primates for leishmaniasis, they are only an option when trying to resolve a hypothesis that cannot be easily solved using other animal models. If this is the case, most times, monkeys will be preferred (245).

### *1.11.5 Wild Rodent model:*

Although inbred mice have been helpful so far for immunology and oncology research studies, they are limited in their polymorphic diversity. Hence, using wild rodents became an alternative approach to studying host-parasite relationships in rodents since they are genetically polymorphic, just like humans (248). Several rodents have been identified as reservoirs of

*Leishmania* species, such as *Sigmodon hispidus*, *Thrichomys Laurentius*, and P*eromyscu*s *yucatanicus*, demonstrating unique manifestations of both CL and VL (284–287). However, few studies have followed up on the type of host-parasite relationships in these animals due to the difficulties encountered when managing wild animals.

## 1.12 Host immune response against CL and VL

The differences in clinical presentations and outcomes of leishmaniasis represent the divergent immune responses following *Leishmania* infection. Patients who mount a Th1 immune response are believed to effectively clear the infection and eliminate the parasites, which is characteristics of mild and localized CL. Hence, the inability to mount an efficient immune response is associated with severe and chronic infections such as those described in DCL and VL. It is assumed that a certain proportion of the parasites can be cleared in characteristic chronic infections. Still, a growing pathology allows for the persistence of some parasites, leading to an exaggerated inflammatory response. This is seen in MCL pathologies (288). These differences in clinical outcomes and immune responses have led to the classification of leishmaniasis as a spectral disease similar to Tuberculosis and Leprosy (289). The immune responses are, therefore, critical in determining the clinical manifestations of the disease. The innate and adaptive immune systems are the major players in the outcome of the disease. The innate immune system is the first responder to any resulting insult or injury on the host, and it usually alerts other components of the immune system, such as the adaptative immune system. The adaptive immune system would usually step up to resolve the ongoing inflammation and tissue damage, especially where the innate defence mechanisms fail to do so. The chronicity in leishmaniasis arises because of the

inability of both innate and adaptive immune responses to eliminate the inflammation and the disease effectively.

### *1.12.1 Innate immune response (DCs, Macrophages Neutrophils, NK cells, Complement, pentraxin)*

The innate defences include dendritic cells, Macrophages, Neutrophils, and Natural Killer (NK) cells. Other host molecules involved in innate host defences include complements and pentraxins. The significant cells that first internalize the *Leishmania* promastigotes the minute they penetrate the dermis of the host are the phagocytic cells (DCs, Macrophages, Neutrophils). These cells have a specific sensory pattern, such as the pattern recognition receptors (PRRs) and the complement receptors (CRs), that detects the presence of these parasites. Some PRRs include toll-like receptors like TLR 2, TLR3, TLR4, TLR7 and TLR9 (290–293) and CR3 (294), which contributes to sensing *Leishmania*-specific molecules during infection by the innate immune cells. The activation of these receptors initiates downstream signalling activities that set the tone for the initiation of inflammatory responses and parasite proliferation in these cells (294,295). Some key players in the induction of innate immune responses are depicted in Figure 5.

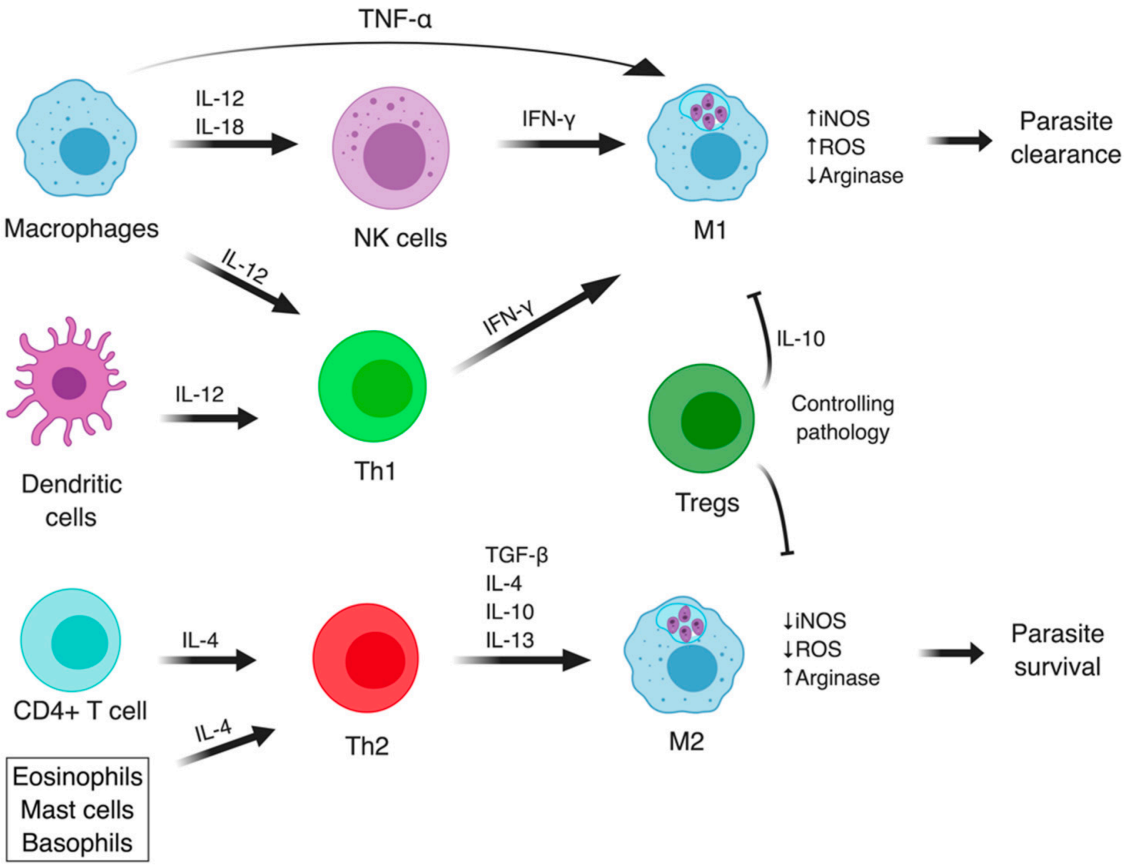



***Figure 5: Key players in the induction of innate and adaptive immune response during Leishmania infection.***

Parasitized macrophages assume either an M1 or M2 phenotype depending on the signals received. IL-12 from DCs or macrophages favours the induction of IFN-$\gamma$ from either Th1 or NK cells, leading to the upregulation of iNOS, which is critical for releasing nitric oxide to clear the parasites in the M1 macrophages. IL-4 from different cell types induces Th2 cell development, producing anti-inflammatory cytokines to promote Arginase upregulation in infected macrophages. Arginases inhibit iNOS to assume an M2 phenotype, metabolizing arginine to polyamines that promote parasite survival.

**Dendritic cells:**

DCs develop from the hematopoietic stem cells in the bone marrow and are evenly distributed throughout the body. They are critical for activating the adaptive immune system and specialize

in the uptake, processing and presentation of antigens to T cells, which has earned them the title 'professional antigen-presenting cells' (296,297). Different types of DCs induce different immune responses during the activation of the adaptive immune response. For instance, the dermal DCs promote the induction of Th1 immune response, but the epidermal DCs induce the activation of parasite-specific regulatory T cells (298). DCs produce IL-12, which is critical for activating naïve $CD4^+$ T cells to differentiate to a Th1 subset and that produces IFN-$\gamma$ essential for parasite killing (296,299). However, different DC subsets drive varying levels of IL-12 production. These differences are believed to be the reason for the distinct dominating T-helper responses found in the BALB/c and C57BL/6 mice infected with *Leishmania* (300). Failure to produce IL-12 in DCs results in the expansion of Th2 cells that produce IL-4 and IL-10 to upregulate arginase expression, which promotes parasite proliferation in macrophages (301,302). The expression of co-stimulatory molecules, especially CD40 in DCs, is critical to influence the quality and robustness of IL-12 production. Inhibiting CD40 expression in DCs using an anti-CD40 antibody reduced IL-12 and IFN-$\gamma$ production, increasing susceptibility to *L. major* infection (303). DCs express other co-stimulatory molecules like CD80 (B7-1) and CD86 (B7-2), which act as the second signal required to activate naïve $CD4^+$ T cells to differentiate into various $CD4^+$ T cell subsets. These costimulatory molecules play vital roles in the outcome of *Leishmania* infection. It has been reported that the Th2 response responsible for susceptibility to *Leishmania* infection in BALB/c mice is dependent on CD86 expression and that the early Th1 response in C57BL/6 mice is also dependent on CD86. Similarly, CD80 expression on DCs was upregulated in mice infected with *L. major*, and it influenced the production of IL-4 and IFN-$\gamma$ from Th2 and Th1 cells, respectively (304).

**Macrophages:**

Macrophages are best described as monocytes from blood circulation but resident in tissues and organs. Although it was shown that monocytes originated from the bone marrow, they are now known to be derived from the yolk sac, which is the leading site for hematopoiesis during embryonic development (305). Unlike DCs, macrophages and neutrophils have better phagocytosing and engulfing capabilities (306). However, macrophages are the definitive hosts for *Leishmania* parasite replication. Neutrophils and macrophages are usually the first cells to migrate to where the Sandfly deposited *Leishmania* promastigotes. After engulfment and eventual establishment, the phagolysosome formation encloses the parasites, which become converted to non-flagellated amastigotes. However, the establishment of amastigotes in neutrophils is usually short-lived, making macrophages the host cell where continuous parasite replication occurs before they are eventually released to infect other cells or taken up by a sandfly during another blood meal (61). Macrophages are the host cells that ensure the killing of the parasites, and for this to happen, the macrophages need to receive specific signals that activate their macrophage-killing abilities. There are two types of states macrophages assume when activated: classically (M1) and alternatively (M2) activated macrophages. IFN-$\gamma$ produced by $CD4^+$ T cells, $CD8^+$ T cells and NK cells, activates macrophages classically to induce the upregulation of inducible nitric oxide synthase (iNOS) that act on L-arginine to produce nitric oxide, critical for parasite killing (307). Interestingly, C57BL/6 mice that could not induce iNOS became susceptible to *L. major* despite having a robust IFN$-\gamma$ response (308). In addition to IFN-$\gamma$, TNF-$\alpha$, IL-1, and IFN$\beta$ also activate M1 macrophages (309).

On the other hand, alternatively activated macrophages receive signals such as anti-inflammatory cytokines, IL-4 and IL-10, which downregulate iNOS to enhance arginase expression. Arginase converts L. arginine to polyamines. *Leishmania* parasites manipulate macrophages to maintain the M2 phenotype because it favours their continued survival. Since IL-12 production is critical for the induction of IFN-γ-producing $CD4^+$ T cells, *Leishmania* suppresses IL-12 production in macrophages. For instance, bone marrow-derived macrophages from BALB/c and C57BL/6 mice infected with *L. major* do not produce IL-12, whereas other pro-inflammatory cytokines produced in the cultures remained unaffected (310). *Leishmania* induces an immunosuppressive state to evade host immune responses, producing anti-inflammatory cytokines such as IL-10 and TGFβ (311).

**Neutrophils:**

Neutrophils are one of the foremost cells that traffic towards the site where the sandfly vector deposits the promastigotes before the infiltration of macrophages (312). They phagocytose parasites like macrophages, but neutrophils can directly kill them by releasing toxic granules and releasing extracellular traps called neutrophil extracellular traps (NETs). NETS contain DNA materials and other neutrophilic granular proteins such as elastase and myeloperoxidase, which immobilize and kill pathogenic organisms (313). *Leishmania* is known to induce NET formation in neutrophils (314). However, some parasite strains resist NETs, while others do not. For instance, *L. amazonensis* would be killed in the presence of NETs, but *L. donovani*, *L. mexicana* and *L. infantum* have mechanisms to evade being killed by NETs (314). *L. donovani* can resist neutrophil killing by inhibiting oxidative burst, escaping confinement around the neutrophil's lytic organelle,

and speedy induction of NETs to ease the entry into macrophages (315). The process of macrophages taking up parasites from neutrophil debris is called a 'trojan horse' infection (316). Macrophages that engulf parasites in this way have low levels of IL-12 and robust IL-10 production, which is favourable for parasite proliferation and survival (317). Indeed, it has been shown in real time using intravital microscopy that neutrophils are usually the first to arrive at the scene of either a sandfly or needle *Leishmania* infection and that the absence of neutrophils significantly reduces parasite burden (318). This indicates that neutrophils are critical drivers of disease severity in leishmaniasis. However, whether neutrophils contribute to disease resolution or progression in leishmaniasis has been debated. Interestingly, increased parasite burden was observed in *L. braziliensis* infected in mice whose neutrophil was depleted (319). Reintroducing exogenous neutrophils reduced parasite burden at the site of infection and draining lymph nodes (319). In a model of VL, the absence of neutrophils was seen to reduce parasite burden in the spleen early on but not the liver (320). It is believed that the absence of neutrophils allowed for the expansion of Th2 responses, which was marked by the increase in IL-10 and IL-4 as well as in *L. donovani*-specific immunoglobulin IgG1/IgG2 levels (320). In canine leishmaniasis, severe disease is associated with reduced neutrophilic infiltrations, increased apoptosis and decreased oxidative bursts. However, in a mild or moderate disease, the neutrophils had no loss of viability (321). Thus, whether neutrophils are protective or harmful during *Leishmania* infection may depend on the *Leishmania* species, host, and stage of disease progression.

**Natural killer (NK) cells:**

NK cells are a component of innate-like lymphocytes and they make up about 10% of the peripheral blood mononuclear cells and 0.4% of the secondary lymphoid organs (322). They

produce proinflammatory cytokines and perform cytolytic activities. Exposure of purified NK cells to *Leishmania* antigen induced a robust IFN-γ production (323,324). The absence of NK cells in a mice model of cutaneous Leishmaniasis resulted in reduced IFN-γ production. This leads to increased parasite burden, suggesting that NK cells are critical for anti-*Leishmania* immunity (323,324). It was reported that in the resistant C3H mice, NK cells were responsible for driving resistance toward *L. major* infection. However, in the susceptible BALB/c mice, there was a reduced NK activity. This was because the production of necessary stimulants for NK activity, such as CXCL10 and CCL3, were diminished (325). NK cells exercise their cytolytic functions by directly lysing *Leishmania*-infected macrophages. This cytolytic function was thought to be due to the release of perforin and granzyme B from NK cells (326). Recent studies indicate *Leishmania*'s LPG suppresses NK cell activation by inhibiting IL-12 synthesis in infected macrophages (327), revealing dimensional approaches through which *Leishmania* parasite molecules hijack host immune responses.

**Complement:**

Complement proteins in the serum play a sequential role in eliminating invading pathogens. Different Complement proteins play specific roles in phagocytosis (C1q, C3b and iC3b), immune cell sequestration, Induction of inflammatory responses (C3a, C5a), the formation of membrane attack complex (MAC) orchestrated by C6-C9 proteins which ultimately ensures the lysis of the pathogen (328). *Leishmania* evades MAC lysis by LPG molecule inhibition of the complex (329,329). The original aim of the breakdown of C3 to C3a and C3b is to aid opsonization and facilitate phagocytosis (by C3b). However, *Leishmania* parasites bind to C3b and penetrate host cells such as macrophages and neutrophils with the complement receptors CR1 and CR3.

Interestingly, *Leishmania*'s metalloprotease GP63 cleaves C3b to an inactivated form iC3b, preventing the completion of the complement activation process (330). Similarly, another *Leishmania* molecule, GP46, from *L. infantum*, *L. braziliensis* and *L. amazonensis*, was seen to interact with the Factor H (FH) proteins (which is a regulator of complement proteins) to inactivate C3b (331). The interaction of *Leishmania* with CR3 on macrophages resulted in reduced IL-12 production, which is critical for the induction of IFN-$\gamma$ -producing $CD4^+$ T cells (331).

**Pentraxin:**

It is becoming clear that host molecules contribute to either susceptibility or resistance during *Leishmania* infection (332,333). One of these host molecules is Pentraxin, a group of evolutionarily conserved proteins with known polymorphisms in their genetic loci within introns, suggesting their critical role in complex organisms (334). There are different classes of pentraxin, including the long pentraxin 3 (PTX3) and the short pentraxins, such as C-reactive proteins (CRP) and Serum Amyloid Protein (SAP). PTX3 has been shown to bind strongly to pathogenic ligands, and is expressed primarily by myeloid cells such as neutrophils, DCs, and macrophages upon TLR activation (335,336). The role of PTX3 in cutaneous leishmaniasis was not previously known. However, we have shown that PTX3 expression was elevated in skin biopsies from humans with active skin lesions and in cutaneous lesions during mouse models of experimental CL (332). Mice lacking PTX3 displayed enhanced resistant to *L. major* infection, which was associated with enhanced differentiation of naïve $CD4^+$ T cells to Th17 subsets (332). This suggests that PTX3 expressed in myeloid cells, particularly DCs during *Leishmania* infections, downregulates the Th17 cell differentiation, resulting in disease progression during leishmaniasis. Interestingly, it was shown that the short pentraxins, CRP and SAP bind to either promastigotes secretory gel or

LPGs and induce complement activation (337). However, more studies must evaluate the implications of this during leishmaniasis.

### *1.12.2 Adaptive immune response (Subsets of $CD4^+$ T cells [Th1, Th2, Th17], $CD8^+$ cells, Tregs, B cells)*

The adaptive immune response becomes necessary when the innate immune system fails to eliminate the invading pathogen or clear the impending inflammatory response. T and B cells are the major cell types that make up the adaptative immune response. Unlike innate immune responses, adaptive immune responses particularly target specific antigens. They also generate immunological memory, which is vital for faster and more precise responses when the host encounters the same antigen. Some of the cells that play a role in the induction of the adaptive immune response during leishmaniasis are depicted in Figure 5.

**T cells**

T cells are immune cells produced in the bone marrow but undergo maturation and development in the thymus. There are two main subsets of T cells, $CD4^+$ and $CD8^+$ T cells, and they are characterized because in addition to the expression of the CD3 ligand, they express the CD4 and the CD8 ligands, respectively. They perform numerous functions: inducing signals that permit the sequestration of other immune cells, producing signals that initiate specific effector function and cell activation, and helping B cells to make antibodies. T cells recognize short sequences (peptides) derived from pathogen using their T cell receptors. The peptides from pathogens are processed and presented to T cells by antigen-presenting cells (APCs) such as macrophages, DCs and B cells. The major histocompatibility class I and II (MHC I &II) molecules are expressed on somatic cells or APCs, respectively, and present peptides from various pathogens to T cells. While

MHC I is expressed solely in somatic cells, MHC I and II are expressed in APCs (338). MHC I-containing peptides would be present in cytotoxic T cells, for instance, $CD8^+$ T cells, while MHC II presents peptides to TCR from $CD4^+$ T cells. T cells make up cellular immunity, which is invaluable for anti-*Leishmania* immunity. It has been reported that the absence of T cells in a mouse resistant to *Leishmania* made it susceptible, and the re-introduction of adoptively transferred T cells abolished the susceptibilities (339).

**CD4 + T-helper 1 and T-helper 2 cells**

Naïve $CD4^+$ T cells receive signals to differentiate into various T cell subsets, and these signals are dependent on the quality of TCR and MHC II-peptide interaction. IFN-$\gamma$ and IL-12 are the signals required for the induction of differentiation of naïve $CD4^+$ T cells to a T helper 1 (Th1) subsets, which produce IFN-$\gamma$ and TNF-$\alpha$ critical for the control of invading intracellular pathogens (340). IL-12 made from the APCs, such as DCs and macrophages, will induce the expansion of Th1 cells through STAT-4 and the expression of T-bet. The induction of expression of Tbet, which is the master Th1 signature transcription factor in Th1 cells, is mediated via IFN-$\gamma$ -induced STAT-1 expression (341). On the other hand, CD4 T helper 2(Th2) cells subsets are the product of differentiation of naïve $CD4^+$ T cells when cytokines such as IL-4 and IL-10 are received. GATA-3, the signature transcription factor of Th2 cells, is usually expressed in response to these signals, and these cells produce anti-inflammatory cytokines such as IL-4 and IL-13 (342). In addition to cytokine signals received by naïve $CD4^+$ T cells, differentiation would not occur unless a strong interaction is achieved between the costimulatory molecules expressed on APCs, such as CD86, CD80 and CD40 and their ligands on the naïve T cells, CD28 and CD40L (343). Macrophages are the most parasitized cells during *Leishmania* infection. Thus, they are the target for Th2 and Th1

cells, which determines the activation status of these macrophages to an M2 or M1 phenotype, respectively (344). Thus, it is critical for either parasite's survival or elimination. The C57BL/6 mice are resistant to *Leishmania* major due to a solid IFN $\gamma$ -producing Th1 cell, while BALB/c mice are susceptible due to a robust IL-4-producing Th2 cell composition (250–252). It has been reported that IL-1 was critical in inducing an enhanced Th1 response in Leishmania infected BALB/c mice (345). When exogenous IL-1a was administered to *L. major* infected mice, there was a marked increase in IFN $\gamma$-producing Th1 cells and a reduced lesion development (345). IL-12 was also valuable in initiating this response (345).

IFN- $\gamma$ produced from Th1 cells binds to the IFN-$\gamma$ receptor on *Leishmania*-infected macrophages, resulting in the upregulation of iNOS, which converts L-arginine to nitric oxide. Nitric oxide is toxic to the parasites, and ends up killing them (302,339,346). IL-4 production from mast cells, basophils, eosinophils and NKT cells induces the differentiation of Th2 subsets (347). IL-4 produced within the vicinity of *Leishmania*-infected macrophages induces the expression of arginases, which compete with iNOS for L-arginine. If arginases successfully catabolize L-arginine, they produce polyamines that are favourable for parasite proliferation (302,339,346). Neutrophils may be the source for the enhanced production of IL-4 in BALB/c mice because the depletion of neutrophils in mice infected with *Leishmania* resulted in a significant reduction in IL-4 and in Th2 cell developments (348). Another indication that Th2 cells drive susceptibility during *Leishmania* infection is that in SCID mice lacking T cell response are generally susceptible to *Leishmania*, and exogenous administration of Th1 or Th2 cells rendered the mice resistant or promoted gross disease progression, respectively (349). Interestingly, the inoculating doses of *Leishmania* determine the development of the Th1 and Th2 cells. It has been noted that when

C57BL/6 mice were infected with low doses of *Leishmania*, Th2 responses were initiated early on. Meanwhile, in the high doses, Th1 responses were favoured (350). At both doses (high and low), healing lesions still developed. The Th2 response was only transient because, later, the induction of IFN- $\gamma$ produced from $CD8^+$ T cells drove protection against the disease. Uzonna et al suggested that $CD8^+$ T cells regulate the Th2 and Th1 responses (350). Further studies need to establish dynamics and origin of Th2 responses in *Leishmania* infection, as targeting this will result in better disease resolution and outcomes.

**$CD4^+$ T helper 17**

$CD4^+$ T helper 17 (Th17) cells are subsets of $CD4^+$ T cells generated when naïve $CD4^+$ T cells are differentiated in the presence of IL-6, IL-23, IL-1$\beta$ and TGF-$\beta$ (351). These cells produce IL-17, which affects macrophages and neutrophils, increasing their production of proinflammatory cytokines and chemotaxis (352). The signature Th17 transcription factor, ROR$\gamma$–T, is noted to cause an inhibitory effect on the Foxp3, the transcription factor for T regulatory cells (another subset of $CD4^+$ T cells) and vice versa (353). In *L. panamensis* infection in mice, anti-inflammatory cytokines suppressed Th1 and Th17 cell responses. However, when these cytokines were neutralized, it led to an enhanced Th1 and Th17 response, resulting in protection and disease resolution (354). This suggests that anti-inflammatory cytokines regulate the induction of Th17 responses, so the presence of IL-10 would dampen Th17 immune responses during *Leishmania* infection. Whether Th17 generally contributes to protection or susceptibility during *Leishmania* infection is debatable. In the absence of IL-10 regulation, *L. major* infection in mice resulted in an expansive induction of Th17 and Th1 cells, leading to disease progression (355). It was reported that the expansive induction of Th17 cells correlated with an increase in the infiltration

of neutrophils, while Th1 cells correlated with an increase in the infiltration of monocytes (355). This suggests that susceptibility during *Leishmania* infection can be mediated by Th17 cells when the measures for controlling the inflammatory responses are defective.

On the other hand, Th17 was associated with protection against *L. donovani* (356) or *L. infantum* (357) infections. It was reported that low levels of IL-10 and reduced frequency of $Foxp3^+$ $CD4^+$ T cells allowed IL-17 from Th17 cells to synergize with IFN-γ, resulting in enhanced nitric oxide production from the infected macrophages, thereby leading to disease resistance (357). In another study, we showed that the absence of a host molecule Pentraxin3, *Leishmania* infected mice induced strong Th17 and IL-17 response with IFN-γ remaining unchanged resulting in resistance to CL (332). From all indications, *Leishmania* species may regulate the induction of Th17 cells differently leading either pathogenic or protective response.

**$CD8^+$ T cells**

$CD8^+$ T cells develop in the thymus and are also known as cytotoxic lymphocytes because they demonstrate effector functions such as releasing granzyme, perforin, and IFN-γ, which are critical for eliminating invasive pathogens (358). They become activated in the presence of MHC I presenting peptides expressed on the surface of either somatic cells or APCs. In the case of viruses, $CD8^+$ T cells are protective; however, in *Leishmania* infections, there are conflicting reports on whether they contribute to a protective response even when they produce IFN-γ. Although *Leishmania*-specific $CD8^+$ T cells were identified (359), the absence of CD8+ T cells or MHC I in mice infected with *Leishmania* resulted in disease control (360). However, $CD8^+$ T cells may only be valuable for protective responses in low-dose inoculum challenges because the absence of $CD8^+$ T cells correlated with an enhanced Th2 response, and the introduction of IL-12

or neutralization with IL-4 resulted in the induction of Th1 responses (350,359). The role of $CD8^+$ T cells in *Leishmania* infections became more complicated when it was reported that $CD8^+$ T cells contribute to disease pathogenesis because MHC I deficient mice infected with L. amazonensis developed reduced lesions compared to the WT controls (361). Without T or B cells, RAG KO mice produce small lesions with uncontrollable parasite burden following *L. major* infection but increased lesions were observed when $CD8^+$ T cells were introduced in these animals (362). This observation suggest that $CD8^+$ T cells may contribute to disease progression in the absence of CD4+ T cells; thus, $CD8^+$ T cells may be a regulator of Th1 responses. Despite the controversies surrounding $CD8^+$ T cells in primary infections, $CD8^+$ T cells have been implicated in playing a role in protective responses during infection-induced immunity (363,364).

**Regulatory T cells**

Regulatory T cells (Tregs) are known to restore homeostasis upon the induction of an inflammatory response and ensure that tolerance amongst immune cells is sustained. There are broadly two classes of Tregs: naturally occurring T regs (nTregs) and inducible T regs. The onset of any infection produces an outburst of inflammatory cytokines and immune cell infiltration, leading to tissue damage. Tregs assume a different T cell subtype; they can either become a Th1 or Th17 (365,366). This plasticity observed with the Tregs makes them able to respond to invasive pathogens as the need arises. In layman's terms, Tregs put a break in the exaggeration of the inflammatory microenvironment to resolve a growing disease pathology and repair damaged tissues. They can do this through multiple mechanisms: by releasing anti-inflammatory cytokines such as IL-10 and TGF$\beta$, through cell-to-cell contact, expression of inhibitory receptors such as CTLA4 and LAG-3 and by competition for IL-2 with other T cells, as increased CD25 expression will

result in reduced $CD4^+$ T cell effector activities (367). The chemokine receptor, CCR5, permits the infiltration of nTregs, and they are maintained around the site of *L. major* infection due to the expression of CD103 (368). The maintenance of nTregs around the site of infection necessitates the induction of a growing population of parasites through IL-10 dependent or independent mechanisms, which were not killed during active inflammatory responses but remain non-proliferative (369). The persistence of parasites allows the induction of lifelong immunity, representing a beneficial host-parasite relationship (370). In *L. infantum* infection, increased levels of Tregs producing TGFβ in the spleen and dLN reduced Th1 and Th2 responses (371). CD40 was seen to regulate Treg expansion in *L. donovani* infection. Enhanced CD40 expression in DCs induced Th1 effector function, whereas poor expression of CD40 in DCs leads to enhanced Treg expansion (372). We previously showed that inactivating the P110 isoform of the P13kinase in mice impaired the expansion and effector function of Tregs during Leishmania infection, resulting in enhanced resistance to susceptible animals (373). In VL patients, increased antigen-driven expansion of Tregs was observed and even present after successful treatment (374).

**B cells**

B cells develop in the bursa of Fabricius in chicken and the bone marrow of other mammals. B cells induce humoral immunity by producing antibodies that neutralize extracellular pathogens. Like DCs and macrophages, B cells are APCs and present antigens to naïve $CD4^+$ T cells to differentiate them into different T cell subsets. Activation of B cells makes them become antibody-producing plasma cells (375). In *Leishmania*, B cells were initially not seen to play a role in protection against *leishmaniasis* (376), and this is because there is already a preconceived notion that antibodies cannot neutralize intracellular pathogens. Indeed, $CD4^+$ T cells but not B

cells transferred to an irradiated mice model of leishmaniasis-induced protective responses (377). There was no protection when serum from a healed mice was transferred (378). Later on, it was discovered that high *Leishmania*-specific antibodies were observed in infected susceptlbe BALB/c model of CL (379) , and parasite-specific IgG antibodies enhanced the secretion of IL-10 to result in disease susceptibility (380). Indications that B cells may induce protective responses were shown in *L. panamensis* infected individuals, where co-cultures of T and B cells from these individuals resulted in increased IFN-$\gamma$ and TNF-$\alpha$ production (381). In addition, B cells infected with *L. panamensis* had higher CD86 expression than those from DC-activated B cells, which enhanced antigen presentation abilities of DCs. *Leishmania* infected mice that lacked B cells had significantly reduced IFN-$\gamma$ production which was restored when IgG-coated parasites were administered (382). In this study, the B-cell deficiency resulted in enhanced parasite burdens and lesion sizes (382).

### *1.12.3 Cytokines and Chemokines (IL-4, IL-10, IL-12, IL-17, IFN-$\gamma$, TNF-$\alpha$)*

Cytokine secretory signals determine the effector responses from the innate and adaptive immune cells. They are the driving forces in determining the outcome of *Leishmania* infections. Numerous cytokines have been proposed to play a role in disease progression or resistance.

Interleukin-12 (IL-12) is one cytokine known to induce protective responses during leishmaniasis. When IL-12 was depleted, it led to a deficit in the induction of Th1 responses during *L. major* infection in resistant C57BL/6 mice, resulting in susceptibility (383). In line with this, treatment of susceptible BALB/c mice with recombinant IL-12 during infection with *L. major* leads to a resistant phenotype which was associated with the induction of a Th1 immune response (383). IL-12 was seen to be the driver of this resistance because the administration of only recombinant

IFN-γ in the BALB/c mice did not induce protective responses during the infection (384). This suggests that IL-12 is the signal needed to induce Th1 responses. IL-12 (IL-12p70) comprises two subunits, p35 and p40; a defect in any unit abolishes resistance during *Leishmania* infection (385), supporting the idea that IL-12 is critical for pathogen control. Although IL-12 is produced by macrophages and DCs, *Leishmania* suppresses the production of IL-12 in macrophages (386), suggesting that DC-derived IL-12 may be the critical pathway for resistance. Infected DCs, especially from resistant mice, produce IL-12, which activates naïve $CD4^+$ T cells to differentiate into IFN-γ -producing cells that influence anti-*Leishmania* immunity (296).

Interferon γ (IFN-γ) is the cytokine believed to be primary in mediating parasite killing in macrophages by the induction of iNOS, whose by-product is nitric oxide (250). IFN-γ can be secreted by the $CD4^+$ T cells (Boyton & Altmann, 2002), NK cells (323,324), and $CD8^+$ T cells (358). Indeed, neutralizing IFN-γ in resistant mice enhanced Th2 response and thus enhanced their susceptibility (387). Similarly, the administration of recombinant IFN-γ in BALB/c mice infected with *L. major* reversed susceptibility as these mice had reduced lesion developments (388). Interestingly, resistant mice that could not produce IFN-γ were susceptible. Still, when they lacked the IFN-γ receptor, they could not induce NO killing of the parasitized macrophages despite the secretion of IFN-γ (389). Thus, disease resolution is contingent on an intact IFN-γ signalling.

Tumour necrosis factor -alpha (TNF-α) is a vital cytokine that macrophages and T cells produce. TNF-α and IFN-γ synergize to induce nitric oxide production for killing the parasites inside macrophages (250). Indeed, the administration of recombinant TNF-α in a mice model of

cutaneous leishmaniasis resulted in a reduced lesion and parasite burden. The neutralization of TNF-α in these mice reversed this (390). Although TNF-α contributes to protective immune responses, the receptors TNFR1 and TNFR2 may not directly regulate Th1 and, by extension, nitric oxide production. Mice that lacked both TNF -α receptors did not have impaired nitric oxide production and successfully killed the parasites upon infection (391). This suggests that there is a mechanism for parasite killing that does not require TNF-α receptor signalling, and thus, TNF-α may be acting independently of their receptor to initiate parasite killing (392). Interestingly, infected TNFR1 KO mice successfully eliminated the parasites, but they had developing lesions suggesting that the TNF-α receptors may play a role in the inflammatory responses (393). Furthermore, it was reported that TNFR1 plays a role in the apoptosis of cells at the lesion sites (394). The dynamics in the interplay of TNF-α and their receptor during *Leishmania* infection is fascinating and may hold promise for future therapeutic anti-*Leishmania* application.

Interleukin 4 (IL-4) is an anti-inflammatory cytokine that majorly comes from $CD4^+$ Th2 cells, but mast cells, basophils, eosinophils, and NKT cells may produce some amounts of the cytokine (347). Interestingly, IL-4 inhibits IFN-γ production from Th1 cells, a classical balancing act between the Th1 and Th2 subsets to sustain inflammation or restore homeostasis, respectively (395). One of the explanations for the susceptibility of BALB/c to *Leishmania* infections is that they produce a robust amount of IL-4, which inhibits IFN-γ expression. Consequently, Th1-mediated infection resolution process is thwarted (396). As expected, suppressing IL-4 secretion using a neutralizing antibody in these mice enhanced the resistance (397). Surprisingly, deleting the *IL-4* gene in mice did not affect their susceptibility to *L. major* (sub strain LV39) as the animals still displayed

comparable lesion developments to the WT controls (398). In contrast, IL-4 receptor a (IL-4Ra) deficient BALB/c mice developed resistance to *L. major* substrain IR173 (399). This indicated that aside from IL-4, other cytokines that signal through IL-4Ra may contribute to susceptibilities during *Leishmania* infection.

Interleukin 13 (IL-13) is an anti-inflammatory cytokine produced by $CD4^+$ Th2 cells, mast cells and eosinophils, and play a role in the elimination of intracellular pathogens, expelling worms and regulating airway inflammatory responses (400). Elevated levels of IL-13 mRNA were observed in patients with VL and CL (401). C57BL/6 mice manipulated to express IL-13 were seen to develop non-healing lesions and uncontrolled parasite burdens during primary *Leishmania* infection, which correlated positively to the secretion of IL-4 (402); suggesting that IL-4 and IL-13 synergistically affect disease susceptibilities. How IL-13 was seen to induce gross disease in acute infection (403), but protection during chronic disease (404) is not very clear. However, this suggests that IL-13 may regulate stage-specific disease outcomes. Indeed, IL-13 inhibits several functions in parasitized macrophages, including IL-12 (405), TNF-$\alpha$ (406) and iNOS expression (407).

Interleukin 10 (IL-10) was previously referred to as a cytokine synthesis inhibitory factor because it was initially thought to be produced by Th1 cells to inhibit cytokine production by Th2 cells (408). It is now known to inhibit both Th2 and Th1 cells and have anti-inflammatory effects on DCs and macrophages, suppressing the release of proinflammatory mediators like TNF-$\alpha$ and IL-12 (409). In addition, IL-10 is made from multiple cells like Tregs, mast cells, B cells, macrophages,

neutrophils, NK cells and eosinophils (410). BALB/c mice deficient in IL-10 were protected against *Leishmania* major infection, suggesting that IL-10 modulates disease susceptibility (311). Interestingly, while wound healing is associated with persistence of the parasites in CL (411), IL-10 deficient mice lost their ability to harbour persistent parasites due in part to absence of IL-10-producing Tregs (369). This suggested that IL-10 may be the critical driver of parasite persistence that maintains infection-induced immunity. Interestingly, IL-10 regulates immune response differently among *Leishmania* species and in different hosts. For instance, BALB/c mice lacking the ability to secrete IL-10 were still susceptible to *L. amazonensis* and *L. mexicana* infection (311).

$CD4^+$ Th17, $CD8^+$ T cells, neutrophils and mast cells produce interleukin 17 (IL-17), a cytokine that is critical for regulating pro- and anti-inflammatory responses and inducing recruitment of neutrophils during *Leishmania* infection (412). Patients with VL (413) and CL had elevated levels of IL-17 (412). The most widely studied member of the IL-17 cytokine family is IL-17A, which functions by inducing neutrophil chemotaxis, regulating IFN-γ secretion from $CD4^+$ Th1 cells (357) and enhancing the production of antimicrobial peptides (414). In the case of VL in humans, it was reported that IL-17 was protective because it enhanced the secretion of chemokines, which are vital for the sequestration of neutrophils and Th1 cells (356). Infection of BALB/c mice with *L. donovani* leads to increased IL-17, IFN-γ and IL-23 production these cytokines mediate parasite killing. Neutralization of IL-17 and IL-23 resulted in uncontrollable parasite replication in the spleen and liver. In line with this, administration of recombinant IL-17 to CL, ML and VL patients led to a drastic increase in IFN-γ and nitric oxide, which enhanced parasite clearance (415–417).

In contrast, it has been reported that increased secretion of IFN-γ and IL-17 in infected IL-10 deficient mice leads to exacerbating disease pathologies. Interestingly, when IFN-γ was inhibited, there was an increase in IL-17 levels (355), suggesting the existence of a regulatory network between IL-17, IL-10 and IFN-γ.

Chemokines determine immune cells' exact flavours and composition at an inflammatory site. They are chemotactic cytokines that play a role in migrating leucocytes to maintain homeostasis and regulate the innate and adaptive immune responses during pathogenesis of leishmaniasis (418,419). The expression of chemokines during an active *Leishmania* infection has been well documented (420–422). The surface glycolipid, LPG, in *Leishmania* promastigotes inhibited the induction of CCL2 in endothelial cells, which limited the expansion and recruitment of monocytes (423). Infection of human macrophage with *L. infantum* led to the downregulation of the chemokine receptor CCR1, which was associated with restriction of the macrophage's sequestration to affected organs (424). Thus, *Leishmania* can modulate both the secretion of host chemokines and the expression of their cognate receptor to limit recruitment of effector cells capable of inducing parasite elimination. In a resistant model of cutaneous disease, a Th1 profile is associated with a strong expression of chemokines like CCL2, CXCL9 and CXCL10 (422), while in a susceptible model, Th2 profile is enriched with CCL3 and CCL7 expression (422,425). CCL2 is also known as monocyte chemoattractant protein-1 or MCP-1. In *Leishmania*, MCP-1 plays a protective role in the early onset of cutaneous leishmaniasis by recruiting macrophages, monocytes, NK cells and other CCR2-expressing leukocytes critical for anti-*Leishmania* responses (426,427). CCL2 was reported to induce protective responses during CL by mediating the oxidative burst (428,429) and synergizing with IFN-γ to induce macrophage activation, an effect

that was abolished in the presence of IL-4 (427). Conversely, it was reported that CCL2-deficient mice were resistant to *L. major* (430). This is because CCL2 also has a Th2 polarizing effect, and other cytokines, such as CCL7 and CCL12, compensate for the absence of CCL2 (431,432). Although evidence suggests that *Leishmania* induces chemokines associated with anti-*Leishmania* immunity and exacerbates disease conditions, it is an oversimplification to pin the control of the disease solely on the chemokines. However, these are exciting fields for future exploration.

### *1.12.4 Tissues and Vasculature*

In ML and CL, the skin undergoes complex damage due to lesion developments, which require a structured repair and regeneration process. In VL, due to the gross pathology in the spleen, extensive remodelling of the splenic architecture occurs in a compartmentalized manner, even in the growing presence of proinflammatory cytokines (433). The extracellular matrix (ECM) proteins, which are usually secreted and regulated by immune cells, are critical for initiating tissue repair processes, and they comprise cytokines, protease growth factors, and ECM components (434). One significant component of ECM in the skin is collagen I, which is produced by fibroblasts. Other extracellular matrix (ECM) proteins include collagen IV and laminin. The initial onset of *Leishmania* infection in the dermis triggers the induction and expression of ECM proteins and components, leading to a colossal inflammatory milieu with areas undergoing necrosis (435,436). For instance, Laminin expressions are recognized as danger-associated molecular patterns (DAMPs), collagen induces the secretion of IL-1$\beta$ in monocytes, elastin is seen to recruit immune cells, and fibronectin induces TLR signalling (437). There are three stages in the tissue repair processes: acute, proliferative, and remodelling. The acute stage is usually

characterized as having a high influx of immune cells and secretion of inflammatory cytokines with no pathological damage on the epidermis, lasting from a few days to a month (435). However, once the parasites proliferate extensively, the proliferative stages commence with massive recruitment of immune cells, which invoke inflammatory reactions. This results in visible necrotizing tissues comprising layers of dead keratinocytes, apoptosed cell debris and live and dead amastigotes (253). As healing progresses, remodelling of the epithelium occurs by forming new epithelia to replace the necrotizing ones (438). This leads to the final stages, where IL-10 and TGFβ are vital. This stage comprises the resolution of inflammation, degradation of the invading pathogen, angiogenesis (new blood vessels), and new collagen formation from fibroblasts with improved tensile strength (439).

During *L. amazonensis* infection, collagen I was highly expressed in early lesions (440) and the parasites extravasated within the collagen structures (441). Parasite-specific proteinases were indicated to break down about 20% of the collagen scaffolds to improve migration and increase recruitment of target host cells like macrophages, DCs and neutrophils. Interestingly, as the expression of collagen I decreased, type III collagen increased in the BALB/c model of *L. amazonensis* infection. This collagen III was found deposited around macrophages infected with the parasites (440). Increased deposition of collagen III was associated with a softer tissue matrix and is believed to help facilitate the ease of parasite migration (441). Deposition of collagen III was also observed in the livers of mice infected with *L. donovani* and was thought to restrict access to T cells (442). The ability of animals infected with *L. major* to develop resistance is associated with wound healing in the lesions (443).

## 1.13 Metabolism in *Leishmania*

The sole purpose of metabolism is to provide energy from numerous available nutrient sources to undertake multiple cellular processes. These cellular processes are vital for growth, structural adaptations, development, and replication. The metabolic process is orchestrated in sequential chemical reactionary pathways where complex nutrients are broken down into simpler metabolites, releasing molecules critical for maintaining homeostasis (444). These pathways are catabolic (energy generating) or anabolic (biosynthetic). Strategies made by *Leishmania* parasites have evolved numerous strategies to modulate host functions in order to increase their survival in their vector, mammalian host or reservoir host (445). The parasites' energy is obtained by sequestration of nutrients found within their surrounding environment (446), followed by the catabolism or anabolism of these nutrients in multiple pathways to generate metabolites capable of performing essential functions within their respective niches. These pathways include carbon dioxide metabolism, B oxidation of fatty acid, ether phospholipid synthesis, carbon dioxide fixation, purine salvage, and pyrimidine synthesis (447). The breakdown and synthesis of essential nutrients require specific and specialized enzymes, and in *Leishmania*, there are multiple metabolic enzymes conserved in glycosomes and the mitochondrion. The glycosomes are unique organelles in *Leishmania* that harbour peroxisomal and glycolytic enzymes (448). The mitochondrion is an organelle representing the parasite's major powerhouse and key for determining whether the parasites will be viable or dead (449).

Interestingly, over 400 genes in *Leishmania major* were seen to code for metabolic enzymes critical for the parasites' major metabolic pathways (450). The focus of numerous research activities today is to understand how the different metabolic pathways could enable parasite

adaptation in their respective niches (444). Thus, targeting the various metabolic enzymes in these niches could be a viable alternative therapeutic approach to controlling the disease (451). *Leishmania* parasites have a digenetic life cycle, with promastigotes capable of flourishing in the sandfly gut and amastigotes in the parasitophorous vacuole of the mammalian host cells. Whole genome sequencing in *Leishmania* has provided a valuable understanding of the exact genes driving the selective adaptation of the parasite throughout its life stages (452). While many of the genes do not have a prescribed function in both the amastigote and promastigote life forms, next-generation transcription and proteomic profiles are critical to gaining insights into the functions of some of these genes (453,454). However, more of the answers into the exact functions of these enzymes, especially those required for parasite metabolism, will take a while to fully understand. One way to gain knowledge of metabolism in these parasite forms is to study the environments in which they reside. The parasitophorous vacuoles (PVs) where the amastigotes live have a very low pH, whereas the promastigotes reside within the gut of sandflies with a neutral pH. In addition to the pH requirements, amastigotes and promastigotes respond differently to the availability of carbon dioxide and oxygen (455,456). It was reported that differentiating amastigotes had a low glycolytic capacity and elevated gluconeogenesis (457) compared to the promastigotes with a high glycolytic flux (458). This suggests that the metabolism of these different stage forms of the parasites will differ based on their environments.

The PVs are characterized as refurbished phagolysosomes with characteristic features like low pH, lysosome-associated proteins, proteases, lipases and glycosidases containing endosomes, autophagosomes and endoplasmic reticulum markers (459,460). The exact nutrient composition

of the PV is yet to be completely elucidated. However, the composition is expected to be similar to what is observed in other lysosomal compartments containing sugars, amino acids, nucleotides and lipids generated from the catabolism of complex molecules like glycolipids, DNA, lipoproteins and proteins (461). Host nutrient transporters are critical to mediating the nutrients' two-way extravasation within and outside the PVs (461). It has been reported that the levels of nutrients in the PVs are regulated by the host signaling molecule, mTOR, which has been suggested to play a vital role in the parasite's intracellular proliferation (461,462). Sugars and fatty acids are the primary carbon sources of amastigotes, which are critical for metabolic pathways such as glycolysis, pentose-phosphate pathways, glycosomal succinate fermentation, fatty acid β oxidation, tricarboxylic acid (TCA) cycle and the mannogen cycle (463).

Analysis of amastigote in lesions demonstrated that despite the nutrient abundance within the PVs, amastigotes grew slowly (doubled in 12 days) compared to the metabolically active logarithmic and stationary phased promastigotes (doubled every 8-12hours) (464,465). The transition of promastigote to amastigote results in the parasite's acquisition of this metabolically stringent response in PVs, which is associated with (1) decreased macromolecule synthesis, (2) decreased expression of sugar or amino acid transporters heavily impacting glycolysis, oxidative phosphorylation and PPP, (3) switch to TCA as a source of amino acid, and increased fatty acid β oxidation, and (4) increased formation of mannogen reserves and lipid bodies (463,465,466). It should be noted that this stage of parasite metabolism is not a shutdown but a physiological phenomenon undertaken to encourage continuous parasite survival. Indeed, the expression of transporters that take up carbon and non-essential nutrients is decreased in amastigotes (467). Still, those that take up essential nutrients like arginine and iron were elevated (467). Some of

the speculated reasons why amastigotes assume this supposedly quiescent metabolic role is to focus on the synthesis of stress response proteins, minimize dependence on pathways (such as TCA and electron transport chains) that are prone to anti-microbial targeting by the host and conserve nutrients in the PV (463). Amastigotes aim to ensure prolonged survival by inducing these stringent metabolic responses. At the same time, they activate host immune and inflammatory responses, leading to disease pathologies in the host. Given the strategic mechanisms by which *Leishmania* stealthily survives within the PVs, increased focus should be geared towards understanding the metabolism of the parasites in this environment, as this holds great promise for developing anti-*Leishmania* targets.

### *1.13.1 Energy Metabolism in Leishmania*

Studies on metabolism in *Leishmania* focus on the promastigotes because they can be easily cultured *in vitro* (450). Thus, many metabolic changes in the amastigote form may be missed if one focuses solely on the promastigote forms. Trypanosomatids, including *Leishmania*, depend on carbon sources to produce energy by releasing ATP, which is critical for proliferation and growth. The major metabolic pathways for energy metabolism in *Leishmania* are glycolysis and mitochondrial respiration (468). While hexoses are the primary substrates for glycolysis and are readily taken up by promastigotes, nutrient starvation makes the parasites dependent on amino acids, which are converted to glucose in a *de novo* synthesis. This process is called gluconeogenesis. Gluconeogenesis is a critical metabolic pathway for energy metabolism in *Leishmania*; it is beneficial for generating metabolites that are valuable for synthesizing macromolecules like DNA and RNA and accumulating mannogen reserves (469). Enzymes that mediate gluconeogenesis, such as phosphoenolpyruvate carboxykinase (PEPCK), glycerol kinase

(GK) and pyruvate phosphate dikinase (PPDK), were found highly expressed in the glycosomes of amastigotes (470).

Moreover, it was reported that the Fatty acid B oxidation pathway was more frequently utilized than glycolysis for amastigotes in lesions, suggesting that they have reduced dependence on the uptake of hexoses (471). This further implies that gluconeogenesis pathway would be preferred if energy metabolism may be required. Mitochondrial respiration involves energy generation in the presence of oxygen in oxidative phosphorylation, which often terminates in the electron transport chain. The main metabolic pathway that initiates mitochondrial respiration is the Tricarboxylic acid cycle (TCA), and TCA-type enzymes have been found to be expressed in *Leishmania* promastigotes. Still, evidence suggests that the cycle is not vital in amastigotes (472). Yet, inhibition of oxidative phosphorylation in *Leishmania* halted metabolism similar to the metabolic quiescence previously described (473). In contrast, inhibition of oxidative phosphorylation in African trypanosomes results in rapid death because of inability to generate energy other than those from oxidative processes (474). The ability of *Leishmania* parasites to undergo metabolic quiescence may have been a survival hack they developed in the sandfly (473). All these metabolic processes involved in energy generation are further discussed in detail below.

### *1.13.2 Glycolysis*

Glycolysis is a process whereby glucose is broken down into substantial metabolites such as ATP and pyruvate. In the presence of oxygen, pyruvate become substrate for the TCA occurring in the mitochondria. However, in the absence of oxygen, pyruvate undergoes anaerobic glycolysis to produce lactate (475). The glycosome is the central organelle where glycolysis occur in

*Leishmania*. It comprises a double membrane with transporters that permit the regular inflow of nutrients (448). Glycolytic enzymes are mainly concentrated in the glycosomes, although gluconeogenetic enzymes and enzymes involved in fatty acid β oxidation are expressed in small amounts (476). The closed compartmentalization of glycolytic enzymes in the glycosomes is thought to sustain a balance of the ATP/ADP and NAD+/NADH and acts as a metabolite reserve (477). Interestingly, glycolytic enzymes are poorly expressed in amastigotes than in promastigotes, suggesting that intracellular proliferation of amastigotes is not highly dependent on energy generation from glycolysis or glucose uptake (457). This reduced dependence on glucose uptake may be associated with metabolic quiescence of differentiated amastigotes, where metabolic programming significantly differs from what is obtainable in promastigotes (450).

Central carbon metabolism is a significant component of parasite metabolism that enables the parasite to escape host oxidative and microbicidal responses (478). Glucose uptake is indispensable for amastigote and promastigote proliferation, although amastigotes have reduced glucose uptake compared to promastigotes (479,480). Inhibition of glucose transporters in *L. mexicana* significantly impaired parasite proliferation in axenic culture, and proliferation of the mutant parasites was compromised in macrophages (480). Similarly, inhibiting the uptake of critical amino acids, glutamine and glutamate, led to impaired parasite growth in macrophages, indicating that amastigotes depend on hexose or amino acid metabolism for their survival in macrophages (481). It is known that *Leishmania* has well-developed mitochondria (482), but they do not always fully metabolize glucose to produce $CO_2$ (a by-product of oxidative phosphorylation). Instead, glucose metabolism is majorly associated with metabolites such as

succinate, L-alanine, pyruvate, ethanol, L. lactate, or D-lactate (482). When these metabolites are produced, they proceed into anaerobic metabolic biosynthetic pathways. For instance, when *L. mexicana* took up glucose, it did not go through the glycolytic pathway but synthesized glutamine via a-ketoglutarate in the TCA pathway (Saunders et al., 2014; McConville et al., 2015). An offshoot of the glycolytic pathway produces mannogen through glucose-6 phosphate flux (Figure 6), which is used as a carbohydrate storage reserve just like starch or glycogen in other eukaryotes. In addition, accumulation of mannogen helps to regulate increased temperatures and limit glucose toxicity in *Leishmania* by lowering sugar uptake and its subsequent utilization in other pathways, such as PPP and nucleotide biosynthesis, which is critical to enable amastigotes to assume the metabolic quiescence in the PVs (463).

### *1.13.3 Gluconeogenesis*

A critical pathway in central carbon metabolism is gluconeogenesis, which represents the reversal of glycolysis, where glucose is synthesized *de novo* from non-sugar sources, such as lactate, amino acid and glycerol (483). Although not a perfect reversal, three specific gluconeogenetic enzymes (PEPCK, PPDK, fructose, 1, 6 bisphosphatase) are not shared with the glycolytic pathway (483). However, just like glycolysis, gluconeogenesis occurs in the glycosomes (Figure 6). The primary gluconeogenesis substrates in *Leishmania* are amino acids and glycerol (469,484). *Leishmania* can take up available glucose and break it down via the glycolytic pathway (485,486). However, in starvation or a limited glucose environment, the parasites heavily depend on gluconeogenesis to synthesize glucose to gain their energy requirement and perform cellular functions (484,487). *Leishmania* parasites utilize gluconeogenesis despite being present in a glucose-rich environment (470), and it has also been implicated as critical for amastigotes

replication in the PVs (469). Indeed, there was an enhanced production of key gluconeogenesis enzymes such as PEPCK, PPDK and fructose I,6 bisphosphate in differentiating promastigotes (477,488), suggesting the importance of this pathway for amastigotes adaptation in the PVs. Gluconeogenesis in *Leishmania* has facilitated the synthesis of structural surface molecules (such as LPG and GIPL), DNA/RNA, and mannan (468). Levels of mannan reserves were severely hampered in the absence of crucial gluconeogenesis enzyme in *Leishmania mexicana* (470). Gluconeogenesis stabilizes the redox potential (NADPH/NADP+) in parasites when metabolites, such as glucose-6-phosphate, are produced and go into the pentose phosphate pathway. An imbalance in redox potential makes parasites vulnerable to attack by the host cells' oxidative burst (482,489), re-emphasizing the importance of this pathway to parasite's continued survival inside macrophages.

### *1.13.4 Oxidative Phosphorylation*

Another critical metabolic pathway in the mitochondria is the TCA cycle, which comprises a chain of reactions producing $CO_2$ through acetyl CoA. The TCA cycle is the central pathway to initiate oxidative phosphorylation processes. More energy (ATP) is produced from oxidative phosphorylation than from the TCA cycle and glycolysis combined (490). However, oxidative phosphorylation is highly dependent on oxygen, and the environment of *Leishmania* PVs is microaerophilic, suggesting that amastigotes will have a reduced dependency on oxidative phosphorylation as their primary energy source. Oxidative phosphorylation involves a series of steps whereby electrons from electron carriers like NADH and FADH2 produced through different metabolic pathways are carried to the electron transport chain to synthesize ATP and ADP (490).

Enzymes involved in the induction of the TCA metabolic pathway are expressed in promastigotes (491) , and the TCA metabolism is active (492). However, in amastigotes, only the pentose phosphate pathway (PPP), glycolysis and glutamate biosynthesis were activated, which appears to yield reduced fluxes and ATP compared to those in the promastigotes (492). However, earlier studies demonstrated the expression of TCA cycle enzymes in amastigotes (493), and the inactive TCA may be due to the stringent metabolic responses that differentiating promastigotes develop (463)—indicating that the activation of the TCA cycle in amastigotes is not entirely inactive but that oxidative phosphorylation may not occur at the same level as the promastigotes. However, more studies are needed to investigate oxidative phosphorylation in amastigotes.

The mitochondria are the organelles that host the induction of both TCA pathways and electron transport chain. Aside from being the powerhouse of the cell, oxidative phosphorylation process in mitochondria produces reactive oxidation species (ROS) (490). Since ROS production can be harmful, Trypanosmatids are known to develop defences against this toxicity (494). However, the capacity of *Leishmania* parasites to produce ROS is yet to be studied compared to mammalian cells or other trypanosomatids. Studies have identified sites on electron transport chains critical for generating ROS in trypanosomatids; the defence mechanisms against ROS have also been explained (494). Accumulation of Mitochondrial Ca+ in trypanosomatids, including *Leishmania*, leads to ROS production within the mitochondrial membrane, which is critical for regulating oxidative phosphorylation (495). Indeed, a mitochondrial Ca+ uniporter was identified in several *Leishmania* species, suggesting that a functional mitochondrial Ca+ deposition occurs in the parasites (496). Indeed, mitochondrial accumulation in *L. mexicana* was associated with virulence

of the parasites (496) , suggesting that increased deposition of the Ca+ harms not only parasites but also the host.

To protect against toxicity associated with ROS production and, by extension, Ca+ accumulation, *Leishmania* parasites express Superoxide dismutases (SOD), ascorbate peroxidases (APx) and peroxiredoxins (TRyXPx), which neutralize the oxidative species produced. Overexpression of a form of SOD, SODA, in *L. chagasi* made them resistant to anti-herbicidal drugs (497). The expression of SODA in the mitochondria of *L. amazonensis* protected the parasites from oxidative stress, and a deletion of only an allele of *SODA* gene resulted in significantly reduced lesions compared to the WT control (498). Expression of APx in *L. major* protected the parasites from oxidative stress (499). Interestingly, overexpression of Apx in *L. braziliensis* made the parasite resistant to routine antimonial drugs (500), suggesting that these enzymes protect against ROS toxicity and prevent being killed by anti-*Leishmania* drugs. Also, Xiang et al. showed that mice infected with APx-deficient *L. amazonensis* parasites developed reduced lesions and parasite burden (501). Finally, TryXPs are vital as chaperones against oxidative stress in both *L. donovani* (502) and *L. infantum* (503).

Furthermore, in *L. donovani*, this TryXP molecule, which was more highly expressed in amastigotes than promastigotes, protected the parasites from ROS-induced death (502). Taken together, oxidative phosphorylation is an indispensable metabolic process the parasites require for survival. Although amastigotes do not highly depend on the TCA cycle for their energy demands (463,492), the parasites have developed a mechanism to prevent being killed by the mitochondrial ROS-producing outcomes during oxidative phosphorylation. With increasing differences between the mammalian cell and *Leishmania* parasites, more studies are needed to

better explain the oxidative phosphorylation pathway in *Leishmania* for effective targeting against disease progression.

## 1.14 Metabolic enzymes in *Leishmania*

*Leishmania* parasites adapt to host PVs because they utilize available nutrients in multiple biosynthetic or catabolic pathways to gain nourishment or energy that promotes survival. The energy metabolism pathways in *Leishmania*, such as glycolysis, gluconeogenesis, and oxidative phosphorylation, are critical for the parasites to escape being killed within the toxic environment of the PVs (476,504). These pathways require enzymes that help replenish depleted nutrients and allow continuous energy production to ensure structural component production or de novo synthesis. Indeed, it has been shown that metabolic enzymes in *Leishmania* are upregulated without an effective transcriptional and translational control in *Leishmania*, unlike their other trypanosomatid counterpart like *T. brucei*, which is capable of strongly regulating the enzyme expression (505). This deficiency in regulation allows the parasites to exploit available and fluctuating nutrients in their host, contributing to parasites' persistence and exacerbating disease despite a solid immune defence. Several metabolic mutants in *Leishmania* strains, such as those lacking enzymes critical for gluconeogenesis (469), iron uptake (506), purine metabolism (507) and glycoconjugate synthesis, (508) were shown to characteristically survive within their macrophage hosts, establishing low-level chronic infection without an overblown pathology. However, it is assumed that low persistence, even in a metabolic deficiency, is an opportunity to prevent parasites from rapidly expanding and proliferating (509). Thus, targeting the parasite’s

metabolic enzymes will be essential for anti-*Leishmania* therapy to limit the parasite's continuous replication.

An approved vaccine against the human forms of *Leishmania* infection is challenging because the antigens that drive protective responses in *Leishmania* are not entirely understood. Interestingly, in an attempt to search for these antigens, high throughput proteomic analysis identified peptides derived from *Leishmania*'s Dihydrolipoyl dehydrogenase ($DLD_{63-79}$) (510,511) and Phosphoenolpyruvate carboxykinase ($PEPCK_{335-351}$) that induced the production of polyfunctional $DLD_{63-79}$ and $PEPCK_{335-351}$ specific $CD4^+$ T cells, respectively during *L. major* infection. During infection, these antigen-specific $CD4^+$ T cells expanded and contracted following parasite clearance, developing into memory T cells that secreted IFN-$\gamma$ and TNF-$\alpha$ to protect mice against secondary challenges (510,511). Interestingly, dihydrolipoyl dehydrogenase (DLD) and Phosphoenolpyruvate carboxykinase (PEPCK) are critical enzymes that play a role in *Leishmania*'s energy metabolism (512,513). These enzymes are discussed in detail below.

### *1.14.1 Phosphoenolpyruvate carboxykinase (PEPCK)*

PEPCK is an enzyme in the gluconeogenesis pathway that reversibly catalyzes oxaloacetate and ADP/ATP to $CO_2$ and phosphoenolpyruvate (Figure 6). In *Leishmania*, PEPCK is expressed in the glycosome and appears to promote parasite differentiation and survival in a limited glucose environment through the de novo synthesis of sugars from non-glucose substrates (470).

The gluconeogenic enzyme, PEPCK, is expressed in both promastigotes and amastigotes (510), and appears to have a 2-fold upregulation in amastigotes than in promastigotes (457). In *Leishmania*, PEPCK is critical for aspartate accumulation (470,513) and ferments succinate to

maintain glycosome ATP pools (514). PEPCK exhibits the highest gluconeogenetic activity (477) and maintains NADP/NADPH+ pools through the pentose phosphate pathway (489).

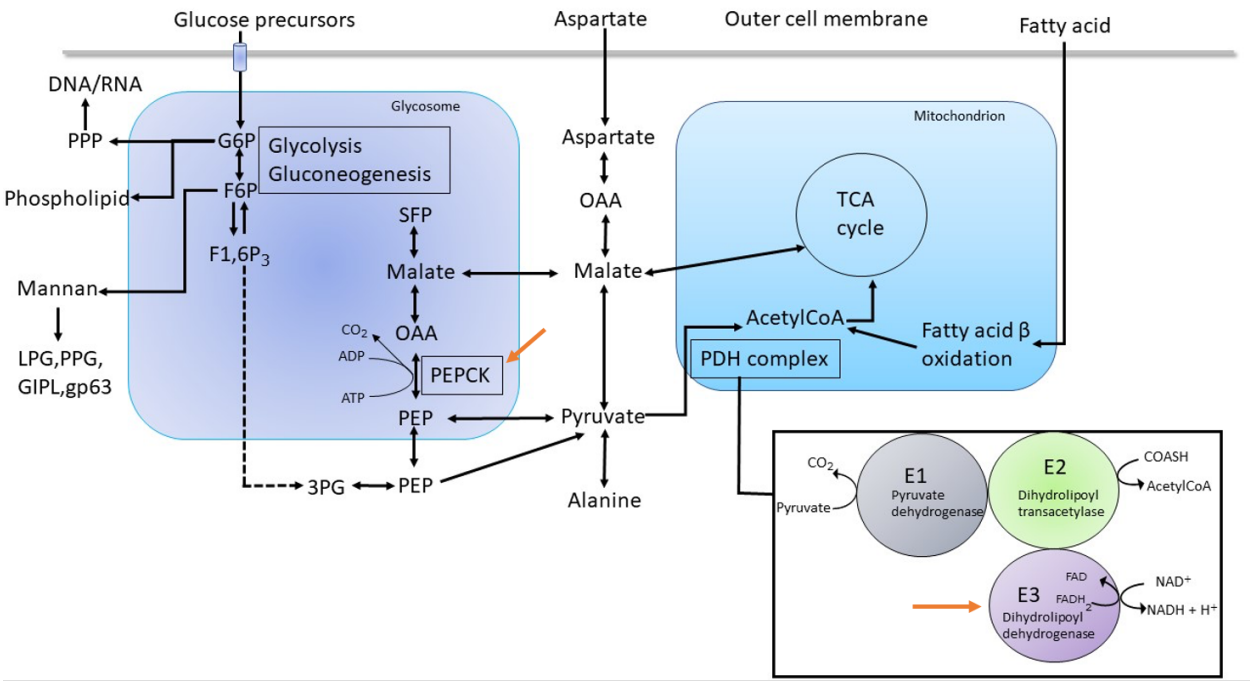



***Figure 6 Organization of the core metabolic pathways in Leishmania***

The energy metabolism pathways in *Leishmania* occur in the glycosomes and mitochondria. Glycolysis and Gluconeogenesis are mostly confined to the glycosomes, while the TCA cycle and oxidative phosphorylation occur in the mitochondria. Most of these pathways produce metabolites that shunt into multiple biosynthetic pathways for macromolecule synthesis, such as mannogen, *Leishmania* surface coats (LPG, PPG, GIPL and gp63), Phospholipid, and nucleic acid (DNA and RNA). PEPCK and DLD (orange arrows) are critical components of the gluconeogenetic pathway and the oxidative phosphorylation process in *Leishmania*. TCA, Tricarboxylic acid cycle; OAA, Oxaloacetate; PDH, pyruvate dehydrogenase; PEP, Phosphoenolpyruvate, ATP, Adenosine triphosphate; ADP, Adenosine diphosphate; CO2, Carbondioxide; 3PG, Glyceraldehyde-3-phosphate, SFP, Succinate fermentative pathway; $F1,6P_3$ Fructose 1,6 bisphosphate; F6P, Fructose, 6 Phosphate; PPP, Pentose phosphate pathway. Adapted from McConville et al., 2015; Opperdoes and Coombs, 2007.

The absence of PEPCK in *Leishmania* resulted in poor *de novo* glucose synthesis and susceptibility to oxidative stress (477). Reduced ROS production in PEPCK-deficient *L. donovani* parasites made

them vulnerable to killing by macrophage antioxidants (477). It has been demonstrated in *L. donovani* that glucose starvation resulted in the upregulation of PEPCK and virulence, which was reversed when the parasite media was supplemented with amino acid (477). This suggests that PEPCK not only helps the parasites survive and proliferate but also that they may be associated with virulence. PEPCK deficient *L. major* infected mice did not produce any lesions and harbour significantly reduced parasite burdens at the site of infection (218). In other pathogenic organisms, such as *Mycobacterium bovis* (515) and *Schistosoma mansoni* (516), PEPCK was associated with virulence. The *PEPCK* gene shares approximately 80% similarity across all pathogenic *Leishmania*, suggesting that targeting PEPCK in anti-*Leishmania* approaches is possible for all forms of leishmaniasis. We identified peptides derived from *Leishmania* Phosphoenolpyruvate carboxykinase ($PEPCK_{335-351}$) (510) that induced $PEPCK_{335-351}$ specific $CD4^+$ T cells during *L. major* infection. During infection, these antigen-specific $CD4^+$ T cells expanded and contracted, and after parasite clearance, memory T-cells that secreted IFN-$\gamma$ and TNF-$\alpha$-protected mice against secondary challenges were developed (510). This indicates that PEPCK might be a plausible vaccine and drug target for anti-*Leishmania* approaches.

### *1.14.2 Dihydrolipoyl dehydrogenase (DLD)*

In *Leishmania*, Dihydrolipoyl Dehydrogenase, DLD is a flavoprotein and usually the third component of multiple enzyme complexes such as the pyruvate, $\alpha$-ketoglutarate, branched-chain amino acid–dehydrogenase complexes, and the glycine cleavage system (GCC). It has diaphorase activity and helps to oxidize NADH/FADH2, which is critical for carrying electrons for energy generation via oxidative phosphorylation (512). When DLD is part of the pyruvate dehydrogenase complex in *Leishmania*, it is associated with the generation of Lipoic acid that E1

and E2 components require to produce acetyl-CoA (Figure 6). Although acetyl CoA can be produced from fatty acid β oxidation, a significant proportion can be produced from the catabolism of pyruvate through the pyruvate dehydrogenase complex (517). An association of purified fractions of DLD with drugs originating from nitrofuran derivatives generated free radicals that could potentially damage the parasite's nucleic acid (512). This suggests that DLD enzyme complex are promising targets for drug discovery. In *Leishmania*, DLD was expressed by both the amastigotes and promastigotes (511); however, during starvation or in nutrient-limiting environments, acetyl-CoA is highly utilized for mitochondrial-mediated ATP production through oxidative phosphorylation in the TCA cycle and the electron transport chain (517). This suggests that the pyruvate dehydrogenase complex and, by extension, DLD is one of the components that maintain acetyl-CoA levels in *Leishmania* amastigotes to promote parasite survival in the limiting nutrient environment of the PVs.

According to the Glycine cleavage (GCV) enzyme classification, DLD belongs to the L protein unit of GCV system (GCVL, 1.8.1.2). Interestingly, there are 2 GCVL genes in *Leishmania*, with GCVL-1 found in chromosome 29 and GCVL-2 found in chromosome 32 (512). GCVL-1 shares a 25% amino acid identity with GCVL-2, which is thought to be less catalytically active than GCVL-2 (512). Moreover, GCVL-1 and GCVL-2 are known to share the same substrate (512), suggesting that GCVL-1 and GCVL-2 may be homologs. Interestingly, signal sequences were detected in GCVL-2, but not GCVL-1 and were found expressed in subcellular mitochondrial fractions of *L. infantum* (518). GCVL-2 product DLD, was present in secreted exogenous vesicles in *Leishmania*, and exosomes are critical for inducing IL-8 secretion in parasitized macrophages (519). IL-8 is a critical pro-inflammatory cytokine produced by *Leishmania*-infected macrophages to recruit neutrophils

to the site of infection (520). This indicates that DLD in *Leishmania* modulates host immune responses during infection, which may be associated with the parasite's virulence.

Furthermore, DLD from taxonomically distinct pathogenic organisms was associated with virulence and regulated host immune responses. DLD contributed to the virulence of *Streptococcus pneumonia* (521), induced protection in *Vibro*-infected fishes (522) , prevented phagocytosis, and efficiently killed the fungi *Paracoccidioides braziliensis* (523). Interestingly, DLD shares over 90% similarity with other pathogenic *Leishmania* species (524), suggesting they may be vaccine or drug targets for all forms of Leishmaniasis. Furthermore, just like PEPCK, we identified peptides derived from *Leishmania*'s Dihydrolipoyl dehydrogenase ($DLD_{63-79}$) (511) that induced the production of polyfunctional $DLD_{63-79}$ during *L. major* infection. During infection, these antigen-specific $CD4^+$ T cells expanded, contracted, and, after parasite clearance, developed into memory T cells that secreted IFN-$\gamma$ and TNF-$\alpha$ to protect mice against secondary challenges (511). The DLD peptide sequences ($DLD_{63-79}$) identified in our lab emanated from the GCVL-2 on chromosome 32 and not the one from chromosome 29 because the peptide sequence $DLD_{63-79}$ is absent in the DLD located on chromosome 29 (511). In this thesis, the DLD I will refer to is located on chromosome 32. Although the role of DLD in the virulence and immunopathogenesis of *Leishmania* has not been elucidated, some indications point to the fact that DLD may either be modulating host immune responses or contribute to the virulence of the parasites.

## 1.15 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) genomic edits.

Clustered regularly interspaced short palindromic repeats (CRISPR) is a group of repeat DNA sequences found in bacteria of the eubacteria and archaea phylogenetic lineage that was initially used to degrade DNA of invading bacteriophages (525–527). Hence, they were the anti-phage defence mechanisms in bacteria. Over 50% of eubacteria and 90% of Archaea contain CRISPR sequences (528). CRISPR sequences do not work alone; a group of CRISPR-associated proteins (Cas) is located on the CRISPR loci that guide the sequences to recognize the target genes (529). Multiple Cas systems have been identified; Cas9 is the more straightforward and commonly used. Cas9 is from the Streptococcus pyogenes, and the Cas9 activity depends on two molecules; the CRISPR RNA (crRNA) and the transactivating crRNA (tracrRNA). In 2012, the Doudna and Charpentier lab fused the crRNA and the tracrRNA into a single guide RNA so that Cas9 could conveniently cut the target DNA (530). In 2015, Cas12a, also known as Cpf1 from *Francisella novicida*, was identified and differed from Cas9 in that it introduced an irregular cut in the DNA and required only the crRNA, whereas Cas9 made a blunted cut and required both crRNA and tracrRNA (531). In 2016, Cas13, also known as C2c2 from the bacteria *Leptotrichia shahii*, was identified. Cas13 initiates cleavage on single-stranded RNA, and just like Cas12a, Cas13 depends on the crRNA to create a cut on the target RNA (532). The CRISPR-Cas9 is versatile and more commonly used, and they are discussed further below.

### *1.15.1 CRISPR-Cas9 genomic editing in Leishmania*

Whole genome sequencing in *Leishmania* has enabled the identification and function of putative or hypothetical genes by performing numerous gene editing techniques (452). The more popular

gene editing strategy involves homologous recombination, but it is marked with limitations such as being labour- and time-intensive and not permitting point mutations (533). RNA interference would have been the best alternative option, but this is absent in *Leishmania* (534). The development of the CRISPR gene editing tool made it possible to identify the functions of *Leishmania* genes for various anti-*Leishmania* applications. CRISPR-Cas9 is more widely used in *Leishmania* and uses the Cas9 and the guide RNA, which comprises 20 nucleotides followed by the crRNA and tracrRNA fusion sequences and is complementary to the gene of interest. Once expressed, the guide RNA and Cas9 form a ribonuclear protein complex and cleavage is initiated that is adjacent to the triple nucleotide NGG sequence (also known as a protospacer-adjacent motif, PAM), generating a double-stranded break (DSB) (Figure 1.7) (535). Generation of a DSB activates an internal repair mechanism in *Leishmania*, such as the microhomology-mediated end joining (MMEJ) or the single-stranded annealing (SSA), which is error-prone as it permits the insertion of short homology sequences or random nucleotides within the cleavage site (536,537). However, a homology-directed repair (HDR) mechanism allows a controlled and error-free mutation within the cleavage site. This repair mechanism involves inserting a long stretch of homology sequence of the cleavage site alongside an antibiotic marker or a fluorescent tag into the site where a DSB was created (538,539). This method allows for a precise and controlled mutation to generate mutant parasites (Figure 7).

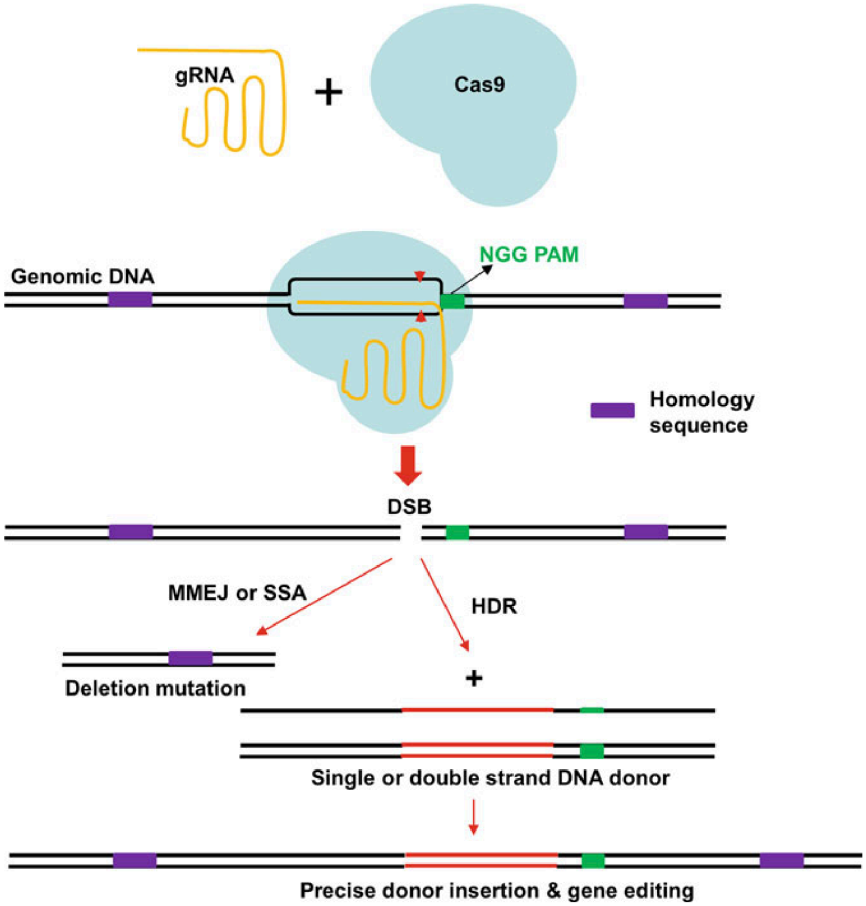



*Figure 7 CRISPR-Cas9 editing in Leishmania*

The versatility and simplicity of the CRISPR-Cas9 gene edits are based on the fact that generating a mutant strain mostly requires altering the 20 nucleotide sequences of the guide RNA. This makes CRISPR-Cas9 tool useful for point mutation deletion of multiple copy genes and gives insights on whether a gene in *Leishmania* is essential (540). In *Leishmania*, Cas9 and guide RNA (gRNA) can be expressed in stable or transient systems. Stable systems allow for the constitutive expression of Cas9 and gRNA using an expression vector and under the control of the U6 or RNA ribosomal promoter that will target the gene of interest (533,541). However, with the transient systems, constitutive expression of Cas9 emanates from the genome under the control of the T7

RNA promoter, and this is followed by a transient expression of the gRNA and donor DNA containing antibiotic-resistant sequences or a fluorescent tag (542,543). Although the transient systems are convenient as they do not involve labour-intensive cloning procedures, multiple gRNA can be introduced simultaneously. However, the process of generating a constitutively expressed Cas9 and gRNA in the parasite's genome with transient system is challenging (542,543). In addition, transient systems have not been used for targeting multicopy families of genes (542,543). The recent advancement with CRISPR-gene editing tool makes it possible to develop loss of function mutant parasite critical to assess the basic biology of *Leishmania*.

### *1.15.2 CRISPR-Cas9 Off targets and mismatches*

CRISPR-Cas systems are unique gene editing systems, but there is often an off-target effect that occasionally occurs (544,545). Off-targets could occur when Cas9 cleaves unintended target sites and are usually dependent on the mismatch of the gRNA on the target gene. Multiple sites within the genome with which the gRNA bears increased similarity can result in Cas9 initiating a cut at multiple regions. Off targets may also be independent of gRNA mismatches, which need further detection and assessment (546,547). *In silico* tools are critical to give insights into the activity of gRNA before using the prescribed CRISPR-Cas9 technique. CRISPRater (https://crispr.cos.uni-heidelberg.de/) is one tool initially created for human and mouse genomes, but it can also give information about the gRNA sequence and its activity (548). The Eukaryotic Pathogen CRISPR guide RNA Design Tool (EuPaGDT) is highly recommended for designing gRNA that potentially has no off-target effect in *Leishmania* (http://grna.ctegd.uga.edu/). This tool gives the designed gRNA a score, determines the potential off-target sites, and identifies the homology sequences that

will flank the predicted cleavage sites (549). Aside from *in silico* methods to detect mismatches of the gRNA, other methods help to detect off-target effects, and they include cell-free methods, *in vitro* (cell base) and *in vivo* methods (550). However, the development of CRISPR-Cas9 derivatives enabled researchers to develop strategies that prevent off-target occurrence. They include (a) improving Cas9 efficiencies (544), (b) assessing gRNA activities through *in silico* approaches and validated by *in vivo* or *in vitro* methods (550), (c) specifically generating single DNA breaks using Adenine and cytosine base editors(551,552) and (d) the use of electroporation and liposomal modes of delivering the Cas9 and gRNA (553).

Furthermore, assessing whether the mutant parasites can restore their WT phenotype by generating a complementary control or add-back strain (AB) is essential. If a restoration in the WT is not observed with the ABs, it will suggest an off-target effect was created in the CRISPR-Cas9 methods used. ABs can be generated by episomally expressing the deleted full-length gene in the mutant parasites or by integrating the gene back into the parasite genome (540).

# 2. CHAPTER 2: Rationale Hypothesis, Aims and Objectives

## 2.1 Rationale

Leishmaniasis, a neglected tropical disease, is a growing public health burden with increasing mortality and morbidities (5,7). Current treatment regimen is plagued with challenges due to drug toxicities and the emergence of drug resistance (12). There is no approved vaccine against human forms of leishmaniasis. However, vaccination is possible because people who recover are protected from developing disease later in life. Understanding the antigens that drive protective responses during *Leishmania* infection will be helpful in generating effective vaccines against leishmaniasis. Recently we identified peptides derived from two key metabolic enzymes in *Leishmania,* Dihydrolipoyl dehydrogenase ($DLD_{63-79}$) (511) and Phosphoenolpyruvate carboxykinase ($PEPCK_{335-351}$) (510) that induced the expansion of polyfunctional $DLD_{63-79}$ and $PEPCK_{335-351}$ specific $CD4^+$ T cells, respectively, during *L. major* infection. During infection, these antigen-specific $CD4^+$ T cells significantly expanded and contracted, and following parasite clearance, developed into memory T cells that secreted IFN-γ and TNF, andprotected mice following secondary challenge (510,511). This suggests that dihydrolipoyl dehydrogenase (DLD) and phosphoenolpyruvate carboxykinase (PEPCK) are potential drug or vaccine targets.

Furthermore, PEPCK and DLD are critical for *Leishmania's* energy metabolism (512,513). Metabolic enzymes play a crucial role in inducing stringent metabolic response that the parasites experience in the PVs of macrophages (463). We have shown previously that *L. major* PEPCK contributed to the parasite's virulence because its deficiency led to impaired progression of cutaneous leishmaniasis. However, the role of PEPCK and DLD in visceral and cutaneous leishmaniasis, respectively, is yet to be elucidated. Here, I aim to uncover the role of PEPCK and

DLD in the immunopathogenesis of visceral leishmaniasis and cutaneous leishmaniasis, respectively.

## 2.2 Global Hypothesis

Deficiency of DLD and PEPCK in *Leishmania major* and *L. donovani,* respectively, will lead to loss of virulence, resulting in reduced disease pathology and altered host immune response during infection.

## 2.3 Aims and Objectives

**Aim 1: Elucidate the role of DLD in Cutaneous Leishmaniasis**

1.1 Generate DLD deficient or knock out (KO) *L. major* and complementary addbacks (AB)

1.2 Assess the impact of DLD deficiency in parasite proliferation in axenic culture.

1.3 Determine whether deleting DLD affected the infective form of *L. major*.

1.4 Examine the proliferation of DLD-deficient *L. major* parasites in the PVs of macrophages.

1.5 Investigate whether DLD-deficient parasites are virulent.

1.6 Assess the impact of DLD-deficient parasites on host immune responses in mice.

1.7 Investigate the potentials of using DLD deficient *L. major* parasite as a vaccine against cutaneous Leishmaniasis.

1.8 Assess the impact of DLD deficiency in *L. major* on mitochondrial respiration and function.

**Aim 2: Elucidate the role of PEPCK in Visceral Leishmaniasis**

2.1 Generate PEPCK deficient or knock out (KO) *L. donovani* and complementary addbacks (AB)

2.2 Assess the impact of PEPCK deficiency in *L. donovani* proliferation in axenic culture.

2.3 Determine whether deleting PEPCK affected the infective form of *L. donovani*.

2.4 Examine the proliferation of PEPCK-deficient *L. donovani* parasites in the PVs of macrophages.

2.5 Investigate whether PEPCK-deficient *L. donovani* induce pathologies.

2.6 Assess the impact of PEPCK-deficient *L. donovani* on host immune responses in mice.

## 2.4 Sex and Gender considerations in leishmaniasis

Like every infectious disease, the susceptibility and or progression of leishmaniasis is dependent on the sex of the individuals, and it is not only due to the environmental influence or inadequate healthcare facilities (554–556). Many factors necessitate the induction of sex-related differences in the onset of leishmaniasis, such as the type of *Leishmania* species, host, and social and gender roles (557). Indeed, sex is now characterized as a biological variable that modulates host immune responses, physiology, drug efficacies and disease progression (556,558). Therefore, research conducted in disease states must consider how the sex and/or gender affects disease outcomes; otherwise, the results become skewed and may not result in efficacious therapies.

Higher incidences of VL and CL were associated with males in both new-world and old-world *Leishmania* species (559–561). In most of these reports, it was not clear whether these differences were due to gender-associated roles or biological factors. In Brazil, it was reported that more males refused to take medications to treat VL caused by *L. infantum*, and this led to higher mortality rates among males than females in that area (562). However, the study did not indicate whether the refusal of male patients to accept treatment was related to gender roles or that biological sex impacted the disease severity (562). However, sex-disparities depend on the mice and the *Leishmania* species in experimental mouse models of leishmaniasis. In the C57BL/6 mice infected with low dose *L. major*, the female mice developed increased parasite burdens compared to the male mice. This enhanced parasite burden in the female mice was associated

with increasing immune cell infiltrations (563). However, in BALB/c and C57BL/6 mice infected with *L. infantum*, the males developed higher parasite burdens in the livers than the females (564). This reduced parasite burdens in the female mice were linked to their enhanced TLR7 expression in the X chromosome, which interacts with Th1-mediated responses, resulting in the control of the parasites by the granuloma formed (564). It has been known that female tissues in mice develop more enhanced granulomas during infection (557). However, whether these sex disparities in inducing granuloma during VL are organ-specific requires further investigations. Generally, the female mice (BALB/c and C57BL/6) predominantly develop more progressive diseases than males during experimental CL. However, in the resistant DBA/2 mice models, females infected with L. mexicana developed slower-growing lesions than the male mice. These mice's immune responses were associated with a stronger Th1 immune response than those from the male mice (565). These stronger Th1 immune responses in the female mice is associated with the differential sex hormones. Testosterone and androgens cause immunosuppression, leading to poor control of parasites in the male mice (566). Considering the sex disparity in the immune responses and disease control during leishmaniasis, this thesis focused on the use of only female mice (C57BL/6 and BALB/c).

# 3. CHAPTER 3: Materials and Methods

## 3.1 Mice

BALB/c and C57BL/6 female mice, six to eight weeks old, were obtained from the Central Animal Care Services (CACS) at the University of Manitoba or the Jackson Laboratory (Maine, US). If C57BL/6 mice are required, the J substrain was used. All mice were kept in the specific-pathogen free of CACS facility at the University of Manitoba, and were used following the instructions outlined by the Canadian Council for Animal Care. Put the protocol number

## 3.2 Parasites preparation and quantification for infection

The parasites used in this study were *Leishmania major* (MHOM/80/Friedlin) and *Leishmania donovan*i (strain LV9). The parasite lines generated were PEPCK KO *L. donovani*, PEPCK AB *L. donovani*, DLD KO *L. major* and DLD AB *L. major*. These parasite lines, including the wild types (WT), were grown in a 25 $cm^3$ or 75 $cm^3$ tissue culture flasks in M199 media containing 20% heat-inactivated fetal bovine serum (FBS) (Cansera, Mississauga, ON, Canada) and 100µg/ml penicillin/streptomycin (Gibco™). When KO or AB parasite lines were grown, 100µg/ml Bleomycin (Invivogen, US) or 100µg/ml Hygromycin (Invivogen, US) were included in the complete M199 media, respectively. All parasite lines were grown in a 28$^oC$ incubator for 3 or 7 days, depending on the experiment. If logarithmic phased promastigotes are required, passaged parasites are grown for three days, and if stationary phased promastigotes are needed, the parasites are grown for seven days. To prepare the parasites for infection *in vitro* or *in vivo,* stationary phase promastigotes (parasites grown in axenic cultures for 7 days) are used. Briefly centrifuge promastigote cultures in a tissue culture flask were transferred to a 50ml Falcon tube and washed twice in sterile PBS at 3000rpm for 15 minutes. Supernatants are discarded and

resuspended in 10ml of sterile PBS. A 1:40 dilution is made to count the parasites, and 20µl of this suspension is transferred to the glass slip in a hemocytometer (Fisher Scientific, Whitby) and counted under an optical microscope. For *L. major* infections in mice, the parasites lines were adjusted as 1-5 X $10^6$ parasites per 50µl of sterile PBS which was administered as a subcutaneous footpad injection, while for *L. donovani* infection in mice, parasite lines were adjusted as 50 X $10^6$ parasites per 10µl of sterile PBS administered in the retro-orbital plexus depending on the mice strain and objective of the experiments. A 1:10 cell-to-parasite ratio was used for *in vitro* infections.

### 3.3 Generation of DLD and PEPCK deficiency in *L. major* and *L. donovani* using CRISPR-Cas9 genome edits

DLD and PEPCK deficient *L. major* and *L. donovani* parasites were generated with the CRISPR-Cas9 gene editing technique. There is only a single copy of the *DLD* gene in *Leishmania* (located on Chromosome 32), and two copies of *PEPCK* gene in *L. donovani* on chromosome 27 (524). The gRNA unique for CRISPR-Cas9 mediated PEPCK and DLD deletion in *L. donovani* and *L. major,* respectively, were designed using the EuPaGDT tool, and they did not have any predicted off-targets. The gRNAs were synthesized and cloned into the BbsI sites of the CRISPR vector pLdCN, which was a gift from Greg Matlashewski (Addgene plasmid #84290) and was used to generate the CRISPR-Cas9 expressing vectors pLdCN-LmPEPCKa&b and pLdCN-LmDLDa&b for specifically deleting PEPCK and DLD, respectively as previously described (567). *L. major* and *L. donovani* logarithmic phase promastigotes were washed twice in ice-cold cytomix electroporation buffer (120 mM KCl, 0.15 mM CaCl2, 9.2 mM K2HPO4, 25 mM HEPES, 2 mM EDTA, 4.75 mM MgCl2, 69 mM sucrose, pH 7.6) and counted so that a concentration of $2 \times 10^8$ cells/ml is actualized and

further incubated for 10 minutes on ice. Four hundred microliter (400 µl) of the parasites were transferred to the electroporation cuvette on ice, and 5-20µg of ice-cold pLdCN-LmPEPCKa&b or pLdCN-LmDLDa&b was introduced and mixed by pipetting up and down. The cuvette was placed in the electroporator (BTX ECM830) to initiate electroporation twice at 25 µF, 1500 V (3.75 kV/cm), pausing 10 s between pulses. Following electroporation, the cells are further incubated on ice for 10 minutes, transferred to fresh complete M199 media, and incubated at 27$^{o}$C overnight. The following day, 50µg/ml of G418 antibiotics was added; once the parasites were stable in 3 to 4 days, the concentration of G418 was increased to 100µg/ml or 150µg/ml. The parasite's growth was monitored under an optical microscope daily until stable cultures are established and parasites survived the antibiotic pressures. Following the establishment of stable cultures, the bleomycin donor templates were generated by PCR using primer sets (PEPCKBleF/R or DLDBleF/R) listed in Table 4 from the pSPBle vector a gift from Greg Matlashewski (Addgene plasmid # 63561). Amplified Bleomycin fragments containing the homology PEPCK or DLD sequences adjacent to the cleaved sites were introduced into the CRISPR-Cas9-containing parasites via electroporation as described above. The flanked arms of the PEPCK or DLD sequences in the donor template allowed for the induction of the homology directed repair (HDR) mechanism. The cultures containing the bleomycin donor template are maintained in increasing concentrations of Hygromycin, starting from 50µg/ml up to 150µg/ml. Following the establishment of stable cultures, limiting dilution was carried out on the parasites to screen for single parasite clones that lack the *PEPCK* or *DLD* genes by PCR using the primer pairs PEPCKF1R1 or DLDF1R1 in Table 4. Confirmation of the bleomycin donor template in the

mutant parasite clones was carried out by PCR using the primer pairs PEPCKF2R2 or DLDF2R2 (Table 4) for the PEPCK and DLD mutants, respectively.

***Table 4 Primer list for PCR***

| Primer pairs | Primer sequence | Amplicon size |
|---|---|---|
| PEPCKBleF/R | F: 5'GCAACTCAGCGACCGTAAATCTAAAATCTTCATCGGATCGGGTAC<br>R: 3' GACTTGTACCAACCTCGTACCGCTATCAGTCCTGCTCCTCGGCCA | 925bp |
| DLDBleF/R | F: 5'AAGCACACACCAGCATGTTCCGCAGATCTTCATCGGATCGGGTAC<br>R: 3'ACACGCTACCAAGCTCCAGCCCAATTCAGTCCTGCTCCTCGGCCA | 584bp |
| DLDF1R1 | F: 5' GTCCTACGACGTGACGGTGA<br>R: 3' TTCTCGTCGAAGGGCAGGAA | 474bp |
| DLDF2R2 | F: 5' AGATCTTCATCGGATCGGGTA<br>R: 3' CTGATGAACAGGGTCACGTC | 313bp |
| PEPCKF1R1 | F: 5' GACGACGTCTGTGTCGCTAA<br>R: 3' AGCTGCTCGCCATAGAATGT | 717bp |
| PEPCKF2R2 | F: 5' CGTGTCTTTCCTCCACGAAT<br>R: 5' CAAACAAGCAGTGAGCCAAA | 925bp |

## 3.4 Generating Complementary control addbacks

DLD or PEPCK genes were episomally expressed into the DLD KO or PEPCK KO parasites to generate complementary controls or add-backs (AB). A *Leishmania*-expressing vector containing the *DLD* gene (pLPHygDLD) was generated by subcloning the *DLD* gene into the *Leishmania* expression plasmid (Figure 8). For the re-introduction of the *PEPCK* gene in PEPCK KO *L. donovani*, pLPHygPEPCK was generated as previously described in our lab (218). pLPHygPEPCK and pLPHygDLD were introduced into the PEPCK KO and DLD KO parasites, respectively, using the electroporation protocol described in section 3.3. Parasites that receive the *Leishmania*

expression vector are maintained in increasing concentrations of hygromycin starting at 50µg/ml up to 150µg/ml over a period. Once stable cultures were achieved, *PEPCK* or *DLD* gene expression confirmation in the parasites was assessed by PCR using PEPCKF1RI or DLDF1R1 primers (Table 4).

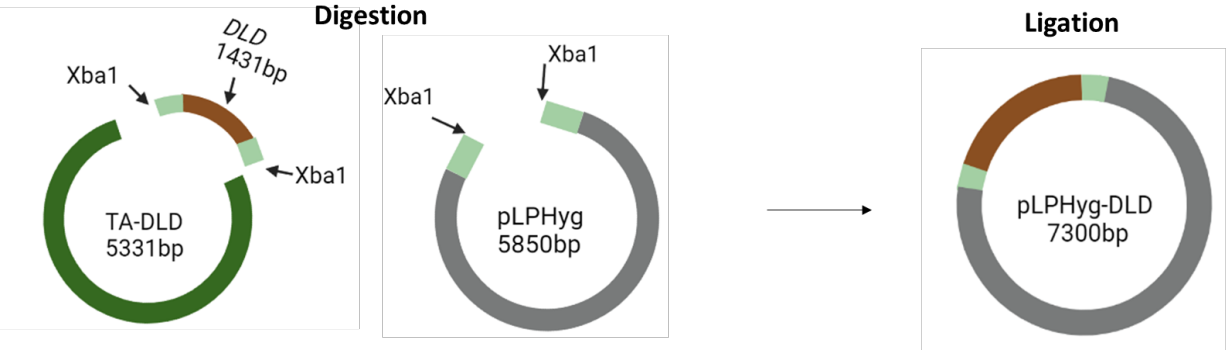



***Figure 8 Subcloning strategy to generate pLPHygDLD***

### 3.5 Western blotting

Logarithmic phase promastigotes were harvested and adjusted to a final concentration of $1\times10^6$/ml and suspended in 2x Laemmli buffer containing 5% 2-mercaptoethanol (Sigma-Aldrich, St Louis MO) in 1.5ml Eppendorf tubes. Following lysis by heating at 95 °C for 5 minutes, the tubes were vortexed vigorously. Afterwards, soluble fractions were recovered in the supernatant following centrifugation at 3000 rpm for 15 minutes. Parasite proteins in these fractions were loaded on a 12% SDS-PAGE gel electrophoresis, and separation of the proteins based on sizes was executed at 100V for 1.5 hour. Separated proteins were transferred to an activated PVDF membrane using a semi-dry western transfer system (Bio-Rad, Mississauga, ON, Canada) at 20V for 1 hour. The PVDF membrane was immersed in tris-buffered saline (TBS) solution containing 0.5% tween and 5% non-fat milk (pH 7.4) and incubated for one hour at room temperature.

Following the incubation of the membrane with a polyclonal rabbit anti-PEPCK antibody (1/1000 dilution) in the blocking solution (TBS supplemented with 3% BSA) overnight at 4$^{o}$C, the membrane was washed thrice in TBS solution supplemented with tween 20% (TBS-T) for 15 minutes per wash. Afterwards, the membrane was incubated with the secondary antibody, mouse anti-rabbit horseradish peroxidase-conjugated (1/10000 dilution) in blocking solution (TBS supplemented with 3% BSA) for 1 hour at room temperature. Following this, the membrane was washed three times in TBS-T for 30 minute/wash, and the ECL blocking agent (GE Healthcare, Mississauga, ON, Canada) was used to visualize the protein bands under a Bio-Rad ChemiDoc™ Imaging System.

### 3.6 Assessment of parasite growth in axenic cultures

To monitor the growth of parasite lines over time, equal numbers of WT, KO, or AB parasites were grown in 10 ml of complete M199 media and incubated at 27$^{o}$C. The parasites were counted daily using a hemocytometer under a microscope and recorded.

### 3.7 Bone marrow-derived macrophages and Kupffer cell preparations.

As previously described, bone marrow-derived macrophages were generated *in vitro* from bone marrow cells (568). Femur and Tibia from BALB/c or C57BL/6 mice were harvested, and the extra fleshy tissues were separated in a petri dish using forceps and surgical scissors to reveal bare bones. The ends of each bone were cut using a sterile razor, and the bone marrow was flushed out using a needle and syringe containing 5 ml RPMI complete medium into 15 ml polypropylene tubes. The bone marrow cells in suspension were spun at 1200 rpm for 5 minutes, and red blood cells in pellets were lysed with ACK buffer (150 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM $Na_2EDTA$, pH 7.2-7.4) for 3 minutes. The addition of complete RPMi media halts the ACK lysis, and the cells are

centrifuged at 1200 rpm for five minutes. The cell pellets were resuspended in complete RPMI medium, and cells were adjusted as 4 X $10^5$/ml cells to be plated in a 100 x 15 $mm^3$ Petri dish (BD Falcon) in 10 ml of complete RPMI media supplemented with 30% L929 cell culture supernatant at 37$^o$C. After 3 days, 10 ml of RPMI complete media containing 30% L929 culture supernatant were added to the petri dish, and on the seventh day when the cells were approximately 80-90% confluent, they were harvested using a cell scraper, washed in complete media, and kept on ice until ready for use in downstream applications.

Isolation of Kupffer cells was carried out using methods previously described (569). Briefly, naïve C57BL/6 mice were euthanized, and the internal organs were revealed through a tear in the peritoneum. The liver was perfused through the right atrium by injecting cold PBS to eliminate circulating blood cells. Following perfusion, the liver was removed and chopped into pieces in 10ml collagenase solution (0.01% DNase, 100mg/ml collagenase in incomplete RPMI media) to break down the collagenous tissues. Following incubation at 37$^o$C for 20 minutes, the minced tissues were homogenized in a cell strainer to release the cells. Cells were washed in cold 1X HBSS twice by spinning at 1200rpm for 5 minutes. Afterwards, the red blood cells were lysed as described above, and the cell pellets were resuspended in 4ml of complete RPMI media. These cells were layered on a 25% and 50% Percoll solution gradient in a 15ml Falcon tube. The cells were centrifuged at 900rpm for 20 minutes with no break. A layer of cells was formed within the middle of the tube and was collected using a transfer pipette and washed twice in complete RPMI media. The cells were kept on ice until further use.

### 3.8 *in vitro* infections

Based on the objectives of the experiments, Kupffer cells and BMDMs were counted using a hemocytometer and were adjusted to cell-to-parasite ratio of 1:10 in a 5 ml polypropylene tube. The tubes containing the cells and parasite were incubated at 37$^{o}$C for 6 hours. After that, uninfected parasites were washed off twice using sterile PBS by centrifuging at 500 rpm for 5 minutes. Afterwards, fresh media was added to the infected cells and incubated at 37$^{o}$C. At the specified time, infected cells were placed on glass slides by centrifugation at 1200 rpm for 5 minutes in a cytospin machine. The infected cells attached to the glass slide are submerged in Giemsa stain and viewed under an optical microscope in a 100X objective lens. Images of the infection were visualized. Parasite quantification and infection rates were assessed.

### 3.9 Isolation of spleen, liver, Lymph node

Depending on the specified time point, mice infected with the parasite lines were euthanized using isoflurane and cervical dislocation and the internal organs were revealed by a tear in the peritoneum. The spleens and draining lymph nodes (dLNs) were dislodged and transferred to 15 ml Falcon tubes containing complete DMEM growth media. Following homogenization of the organs in a cell strainer, cells were washed in complete DMEM media. For the dLN, the pellets were resuspended in complete media, counted and left on ice till required for further use. For the spleens, red blood cells were lysed with ACK buffer for 3 minutes, followed by addition of complete DMEM media to quench lysis reaction. The cells were spun at 1200rpm for 5 minutes, and cell pellets were resuspended in complete DMEM media. Cells are kept on ice till further use. As described in section 3.7, the liver from euthanized mice infected with the parasite lines were perfused by injecting cold PBS with a syringe into the right atrium. The perfused liver was

collected and chopped into small pieces using 10 ml of collagenase solution (see section 3.7 for components). After incubation at 37$^{o}$C for 20 minutes and homogenization in a cell strainer, the cells were washed twice in a cold HBSS buffer. Red blood cells were lysed with ACK buffer for 3 minutes, and the addition of complete DMEM media halted the ACK reaction. The cells were spun at 1200rpm for 5 minute, and the resulting pellets were resuspended in 5 ml of 40% percoll layered on top of a 70% percoll solution and centrifuged at 750 g for 20 minute without brakes. Lymphocytes are formed at the interface and are collected. Following a wash in complete DMEM media, the cells are kept on ice till further use.

## 3.10 Flow cytometry

Flow cytometry is a multi-parametric tool designed to assess the properties of a cell based on its light scattering or fluorescent properties (570). This tool was used to characterize cell populations assessed throughout this thesis. The methods by which flow cytometry was applicable in this thesis are described in detail below.

### *3.10.1 Intracellular cytokine staining*

Single-cell suspensions from the spleen, dLN and liver from infected mice were prepared at the specified time points. Cytokines such as IL-10, IL-4, TNF, and IFN-$\gamma$ were detected through an intracellular staining method. Briefly, single cells were counted and plated at 1-2 X $10^{6}$/ml in 24-well plates in the presence of 2ml stimulation cocktail (Thermo Fischer) containing ionomycin (669.3$\mu$M), PMA (40.5 $\mu$M) and Brefeldin (2.5mg/ml) at 37$^{o}$C. After 4 hours, the cells are harvested, transferred to flow tubes, and spun at 1200 rpm for 5minutes. Cell pellets are resuspended in FACS buffer (PBS containing 0.1% FBS and 0.1% sodium azide) and incubated with 100 µl of TruStain FcX™ Antibody (BioLegend) for 5 minutes on ice. This limits non-specific

interactions of the FC regions of the antibodies with the FCγ receptors on the cells. The cells were surface stained with anti-CD4 and anti-CD90.2 antibodies (see Table 5) for 20 minutes on ice. Thereafter, the cells were fixed by incubating with CytoFast™ Fix/perm solution (BioLegend) at RT for 20 minutes, washed twice in 1 ml of 1x CytoFast™ perm wash by spinning at 350 x g for 5 minutes and stained in the dark for 20 minutes with an optimal concentration of the intracellular antibodies (IL-4, IL10, TNF, IFN--γ) in 1 ml of 1x CytoFast™ perm wash. Afterwards, the cells were washed in 1 ml of 1x CytoFast™ perm wash and the pellets were resuspended in FACS buffer and acquired using FACS Cytoflex flow cytometer (BD Bioscience, Mississauga, ON, Canada). For data analysis, Flowjo software was used.

***Table 5 Antibodies used for flow cytometry***

| Antibody | Clone | Fluorochrome |
|---|---|---|
| CD90.2 | 19E12 | PE |
| CD4 | GK 1.5 | BV421 |
| CD44 | 5035-41.1D | PEcy7 |
| CD11b | M1/70 | PE |
| F480 | BM8 | FITC |
| CD19 | 6D5 | FITC |
| CLEC4f | 3E3F9 | Alexafluor647 |
| IFN-γ | XMG1.2 | APC |

| | | |
|---|---|---|
| TNF | MP6-XT22 | PE |
| IL-10 | JES5-16E3 | PE |
| IL-4 | 11B11 | APC |

### *3.10.2 FITC-Peanut agglutinin staining*

To assess whether differentiation to the infective forms in the mutant parasite lines was compromised, the binding of the parasites to peanut agglutinin conjugated to FITC was executed by flow cytometry as previously described (571). Briefly, logarithmic and stationary phase parasites were washed twice in sterile PBS, adjusted to $1x10^6$ parasites/ml, and transferred into flow tubes. The parasites were stained with FITC-Peanut agglutinin (Sigma) in the dark at RT for 30 minutes and washed in sterile PBS afterwards. Twenty thousand parasites per sample group were acquired using the Cytoflex Flow cytometer (BD Bioscience, Mississauga, ON, Canada). Further analysis was carried out using the FlowJo software.

### *3.10.3 Tetramer staining*

Depending on the objective of the experiment, PEPCK or DLD-specific $CD4^+$ T cells were assessed in the spleens and dLNs of mice infected with the different parasite lines using I-$A^b$-$PEPCK_{335-351}$ PEPCK and I-$A^b$-$DLD_{63–79}$ DLD tetramer, respectively as previously described (572). I-$A^b$-$PEPCK_{335-351}$ and I-$A^b$-$DLD_{63–79}$ DLD tetramer were synthesized by the National Institutes of Health Tetramer core facility (Emory University, Atlanta, GA). Spleen or dLN cell suspensions isolated from infected mice, as described in section 3.9, were stained with 2 μg/ml tetramer I-$A^b$-$PEPCK_{335-351}$ or I-$A^b$-$DLD_{63–79}$ DLD tetramer conjugated to APC and incubated at 37°C for 30minutes. Following

incubation, tetramer-stained cells were enriched for DLD or PEPCK-specific T cells using magnetic beads and the numbers of DLD or PEPCK-specific T cells was assessed by Flow cytometry.

## 3.11 Multiplex cytokine analysis

Liver lymphocytes, spleen and dLN suspension cells prepared in section 3.9 were used for *in vitro* recall response. Briefly, 2-4×$10^6$/ml cells were plated in a 24-well plate, and stimulated with 50 μg/ml freeze-thawed *Leishmania* antigen at 37 °C. After three days, the concentration of cytokines in the culture supernatant fluids was determined using the Luminex™ 200 multiplex system (Luminex, Austin, TX, USA) by Eve Technologies Corp. (Calgary, Alberta). Ten Cytokines were simultaneously measured using Eve Technologies' Mouse Focused 10-Plex Discovery Assay® (MilliporeSigma, Burlington, Massachusetts, USA) according to the manufacturer's protocol. The 10-plex consisted of GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, MCP-1, and TNFα. Assay sensitivities of these markers range from 0.4 – 10.9 pg/mL for the 10-plex.

## 3.12 Metabolism assessment

PEPCK and DLD are critical enzymes for energy metabolism in *Leishmania* (512,513). Thus, assessing the impact metabolic pathways will have in the absence of these enzymes was essential. The methods for evaluating metabolism in these enzyme deficient parasites are described in detail below.

### *3.12.1 Measurement of glycolysis and oxidative phosphorylation*

To measure the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) in the parasite lines, the Mitostress test, which measures overall mitochondrial function and respiration in the parasite lines, was performed using a Seahorse analyzer as previously described

(573,574). Briefly, 1 ml of poly-D-lysine was incubated in the wells of an XF24 analyzer plate for 2 hours at RT. The parasites, 5 X $10^6$ /ml, were immobilized in the treated XF24 pre-hydrated analyzer plate and 100µl of 37°C prewarmed XF DMEM media supplemented with 1 mM Sodium Pyruvate and 11 mM D-glucose. Following centrifugation at 3000 rpm for 10 minutes, the plates were left in the 37°C incubator for 1h. Drugs (oligomycin, carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP), and rotenone/antimycin A) up to a final concentration of 1 mM were added to the wells of the pre-warmed cartridge. Afterwards, the cartridge was put into the XF24 analyzer machine and calibrated. After incubation of the XF plates containing the immobilized parasites, they were topped off with 400 µl of XF complete media. Further analyses were made using the Seahorse analyzer machine, which assessed the oxygen consumption rate (in pmol/min) and the Extracellular acidification rate (in mpH/min). Afterwards, the parasites were lysed, and the total protein concentrations was assessed using the Bradford Assay and used to normalize oxygen consumption rate per protein concentration (pmol/min/µg) or extracellular acidification rate per protein mpH/min/µg.

### *3.12.2 Measurement of mitochondrial permeability and reactive oxygen species*

To assess mitochondrial permeability and the production of reactive oxygen species (ROS) by the mitochondrion, 1x$10^6$ promastigotes of the different parasite lines being tested were stained with Tetramethylrhodamine methyl ester (TMRM) and mitosox dyes (Thermo Fischer Scientific) according to the manufacturer's suggested protocols. Stained parasites were incubated at RT for 20 minutes in the dark and washed twice in PBS afterwards. TMRM- and mitosox-stained

parasites were gated in the PE and PI channels using the Cytoflex flow core after gating 20,000 cells. The mean fluorescent intensity was assessed using Flow jo software (v10).

### 3.13 Lesion size measurement and estimation of parasite burden by limiting dilution and PCR.

A digital vernier calliper was used to measure lesion size, which was recorded as the difference between the size of uninfected and infected footpads. Parasite burdens at the infection site were determined using the limiting dilution assay as previously described (575). Briefly, infected footpads from mice sacrificed by isoflurane and cervical dislocation were kept in 2% pen/streptomycin containing PBS (PBS/PenStrep) on ice. To limit bacteria contamination, the footpad was submerged in a beaker containing 70% EtOH, chlorhexidine disinfectant, and 70% EtOH consecutively for 5 minutes each and then rinsed in PBS/PenStrep. Following chopping of the toes and skin removal, they were cut into smaller pieces and transferred to a sterile tissue homogenizer containing 2 ml of PBS/PenStrep. Once the tissues are fully homogenized, they are resuspended in additional 2 ml PBS/PenStrep and transferred into a 15 ml Falcon tube. The tissue containing the parasites was centrifuged at 600 rpm for 5 minutes. The supernatant containing the parasites was transferred to a new 15 ml tube and centrifuged at 3000 rpm for 15 minutes. The pellets were resuspended in 2 ml of complete Schneider media (Invitrogen, Life Technologies, Burlington, Ontario) supplemented with 20% FBS, 2mM L-glutamine, 1% penicillin/streptomycin (1000X) and 25 mm HEPES. In a 96-well plate containing 180 µl of complete Schneiders media, 20µl of the parasite suspension is transferred to the first row of the plate and mixed gently by pipetting. A 1:10 dilution was performed by transferring 20µl from the first row to the next row, and after mixing, another 20µl was transferred to the next row until the last row, where 20µl of

parasite suspension was discarded. Plates were covered and incubated at 27 °C for 5 to 7 days. Parasite burdens were extrapolated from observing parasite growth in the wells under an optical microscope at x40 magnification.

In some experiments, parasite burden was quantified by absolute quantitative PCR method as previously described (576,577). Briefly, tissues harvested from infected mice were subjected to genomic isolation using the trizol (Invitrogen). The absolute quantification of the parasite burden in the samples was deduced from Cq values of known concentrations of genomic DNA from logarithmic phase promastigotes. Homogenized promastigotes (logarithmic phase) or tissues from infected mice were incubated with 1 ml of Trizol for 5 minutes followed by addition of 0.2ml of chloroform. Thereafter , the tubes were centrifuged at 12000 x g for 15 minutes and the aqueous interface was collected. To precipitate the genomic DNA, 0.3 ml of 100% ethanol was added and further incubated in 1 ml of 0.1 M sodium citrate in 10% ethanol for 30 minutes followed by another centrifugation (2 times) at 2000 x g for 5 minutes. The isolated DNA was washed in 1.5 ml of 75% ethanol by gentle inversion for 10 minutes and centrifugation at 2000 x g for 5 minutes. DNA pellet was air dried and reuspended in 20 µl of DNAse free water. Once the genomic DNA has been extracted, the kinetoplast DNA (kDNA) was identified by real-time PCR in the samples. The primer pair used is 5'-CCTATTTTACACCAACCCCCAGT-3' and 5'-GGGTAGGGGCGTTCTGCGAAA. PCR was carried out in a total reaction mixture of 20 µl. The components of the reaction mixture is as follows; 10 µl of 1X SYBR® green master mix (Strategene, La Jolla, California), followed by 2uM from each primer pair and 2µl of genomic DNA, which was topped off with DNase/RNase free water. The reaction mixture was placed in a thermal cycler (Roche light cycler) and amplification was carried out using the following settings:

1 cycle initial PCR activation for 10 minutes at 95$^{o}$C, 45 cycles of 20 sec at 95$^{o}$C, 20 sec at 60$^{o}$C, and 16 sec at 72$^{o}$C, and a final extension for 10 minutes at 72$^{o}$C. Using the MXPro software (stratagene) to generate a linear standard curve, parasite numbers were assessed from the cycle thresholds of the test samples. Parasite loads are calculated as a percentage of the weight of the organ.

### 3.14 Reverse transcriptase PCR

Confirmation of absence of PEPCK or DLD mRNA expression in mutant parasites was performed by reverse transcriptase PCR. Briefly, mRNA from 1x10$^{6}$ logarithmic promastigotes was isolated using the Trizol (Invitrogen) reagent. Parasites were lysed in 1 ml of trizol by vortexing for 15 seconds and left for 5 minutes at RT. After adding 200 µl of chloroform, the tubes were incubated for 3 minutes and centrifuged at 12000xg at 4°C for 15 minutes. Afterwards, the aqueous layer containing mRNA was carefully collected and transferred to a new 1.5 ml Eppendorf tube. The RNA was precipitated by adding 500 µl isopropanol and incubated further for 10 minutes at RT. The tubes were centrifuged for 10 minutes at 12000xg at 4°C, and the supernatant was discarded. The pellet was washed in 1 ml of 75% alcohol and centrifuged at 7500xg at 4°C for 5 minutes. The supernatant was discarded, the RNA pellet was air dried and further resuspended in 20 µl RNase-free water. The resultant solubilized RNA was stored at -80°C or immediately converted to cDNA using the reverse transcriptase. According to the manufacturer's protocols, parasite cDNA was obtained from isolated RNA by a first-strand cDNA synthesis kit (E6300L – New England Biolabs). Real-time PCR was performed on the cDNA, and PCR was performed using Luna universal qPCR master mix (M3003X - New England Biolabs), according to the manufacturer’s protocols.

### 3.15 Secondary challenge experiments

Secondary challenge experiments were performed to understand whether protection from WT challenge is achievable with the mutant parasites. 5-week-old infected BALB/c mice and their age-matched naïve controls were challenged with 0.1 X $10^6$ WT stationary phased promastigotes in their contralateral footpads. Developing lesions were monitored in the challenged footpad weekly, and after three weeks, mice were sacrificed to assess parasite burden and immune response.

### 3.16 Statistical analysis

All the data presented in this study represent the mean ± SEM from the indicated replicates. To compare parasite burden, immune responses, and sizes of lesions between the multiple groups, a two-tailed student T tes

t and one-way or two-way analysis of variance (One-way or Two-way ANOVA) was used. If the p-value is ≤ 0.05, the results were considered significant.

# 4. CHAPTER 4: Results

## Aim 1 The Role of DLD in Cutaneous Leishmaniasis

### *4.1.1 Background and Rationale*

Leishmaniasis, categorized as a neglected tropical disease, is fast becoming a global health burden of critical importance. Despite the immense strategies designed to reduce the morbidities and mortalities associated with the disease (7), millions of individuals still get infected every year (5). This has been complicated by the fact that *Leishmania,* the primary cause of the disease, is heterogenous and infects multiple hosts, resulting in a plethora of clinical manifestations (15). Cutaneous Leishmaniasis, caused by different *Leishmania* species, including *Leishmania major*, presents as a self-healing lesion that can degenerate to chronic and debilitating forms (10,54). While modern technologies are in the pipeline for an effective treatment regimen, current treatment is plagued with various challenges due to drug toxicities and the emergence of drug resistance (12). Although there is currently no approved vaccine against the human forms of leishmaniasis, vaccination is possible because people who recover from primary infection are protected from developing the disease later on in life (199). This infection-induced immunity is mediated by IFN-γ-producing $CD4^+$ T cells. Understanding the antigens that drive protective responses during *Leishmania* infection will help generate effective vaccines against leishmaniasis. Previously, we found that some peptides derived from Dihydrolipoyl dehydrogenase ($DLD_{63-79}$) of *L. major* induced the production of polyfunctional cytokines during *L. major* infection. Following infection, these antigen (DLD)-specific $CD4^+$ T cells expanded and contracted, and after parasite clearance, memory T-cells that secreted IFN-γ and TNF were developed, and protect mice against secondary virulent *L. major* challenge (511). We also found that mice vaccinated with

recombinant DLD mounted strong protective responses against *L. major* challenge (511), suggesting that DLD may be a good vaccine target.

However, indications that DLD may play a vital role in the virulence of *L. major* are elusive, especially because DLD is a critical enzyme that plays a role in the energy metabolism in *Leishmania* (512). In other pathogenic organisms, DLD has been shown to be critical for either virulence or the induction of host immune responses. For instance, DLD contributed to the virulence of *Streptococcus pneumonia* (521), induced protection in *Vibro*-infected fishes (522) and inhibition of phagocytosis and efficient killing of the fungi *Paracoccidioides braziliensis* (523). Further insights on the possible role of DLD as a virulence factor were assessed from studies that demonstrated the presence of DLD in the *Leishmania* secretory vesicles, exosomes, which is critical for virulence. Thus, given that DLD is a critical enzyme involved in parasite metabolism, **I hypothesized that deficiency of DLD in *Leishmania major* leads to a loss of virulence, resulting in reduced disease pathology and altered host immune response during infection. I further hypothesize that the absence of DLD in *L. major* results in impaired parasite proliferation due to an altered metabolic activity.**

### *4.1.2 Generation and confirmation of DLD KO and addbacks in L. major*

The CRISPR-Cas9 molecular editing tool has been demonstrated efficient in deleting multicopy and essential genes of *Leishmania* (578). Thus, to understand the functions of DLD on *L. major*, I deleted the *DLD* gene using the CRISPR-Cas9 editing tool (Figure 9A&B). To do this, two gRNA were cloned into the *Leishmania* expression vector, PLDCN (Figure 9A) and upon the co-expression of the gRNA and Cas9 in *L. major*, a ribonuclear complex is formed, which initiates a

cleavage in the *DLD* loci (Figure 9B). Following the insertion of the Bleomycin resistance gene cassette within the cleavage site, mutant parasites was confirmed by PCR (Figure 9B). Although off-target effects are usually associated with CRISPR/Cas9 editing processes (544,545), numerous mitigating strategies limit this risk (545,550). One approach to address this off-target effect of the CRISPR/Ca9 system in this study is to generate a complementary control or add-back strain (AB), whose role is to see whether the WT phenotype is restored in order to further confirm that the targeted deletion was not due to errors or off-target from the editing tool. To do this, I first generated a DLD expression vector (Figure 9C), which I episomally introduced into the DLD-deficient parasites. The resulting parasite lines that received the DLD expression vector were referred to as DLD AB parasites (Figure 9C). DLD has been deleted in *L. major* because DLD amplicon (474bp) was absent in the DLD KO parasites by PCR but present in the WT and DLD AB parasites (Figure 9D). This suggests that I successfully generated DLD null mutant *L. major* parasite, and this was restored in the DLD AB parasites. I also confirmed that the homology-directed repair strategy I initiated was successful because the bleomycin resistance gene amplicon (474bp) was observed in the DLD KO parasites but not the WT *L. major* parasites (Figure 9E). Furthermore, DLD KO parasites were confirmed in RT-qPCR by the absence of DLD mRNA expression (Figure 9F). This mRNA expression was restored in the DLD AB parasites to a level comparable to WT parasites (Figure 9F).

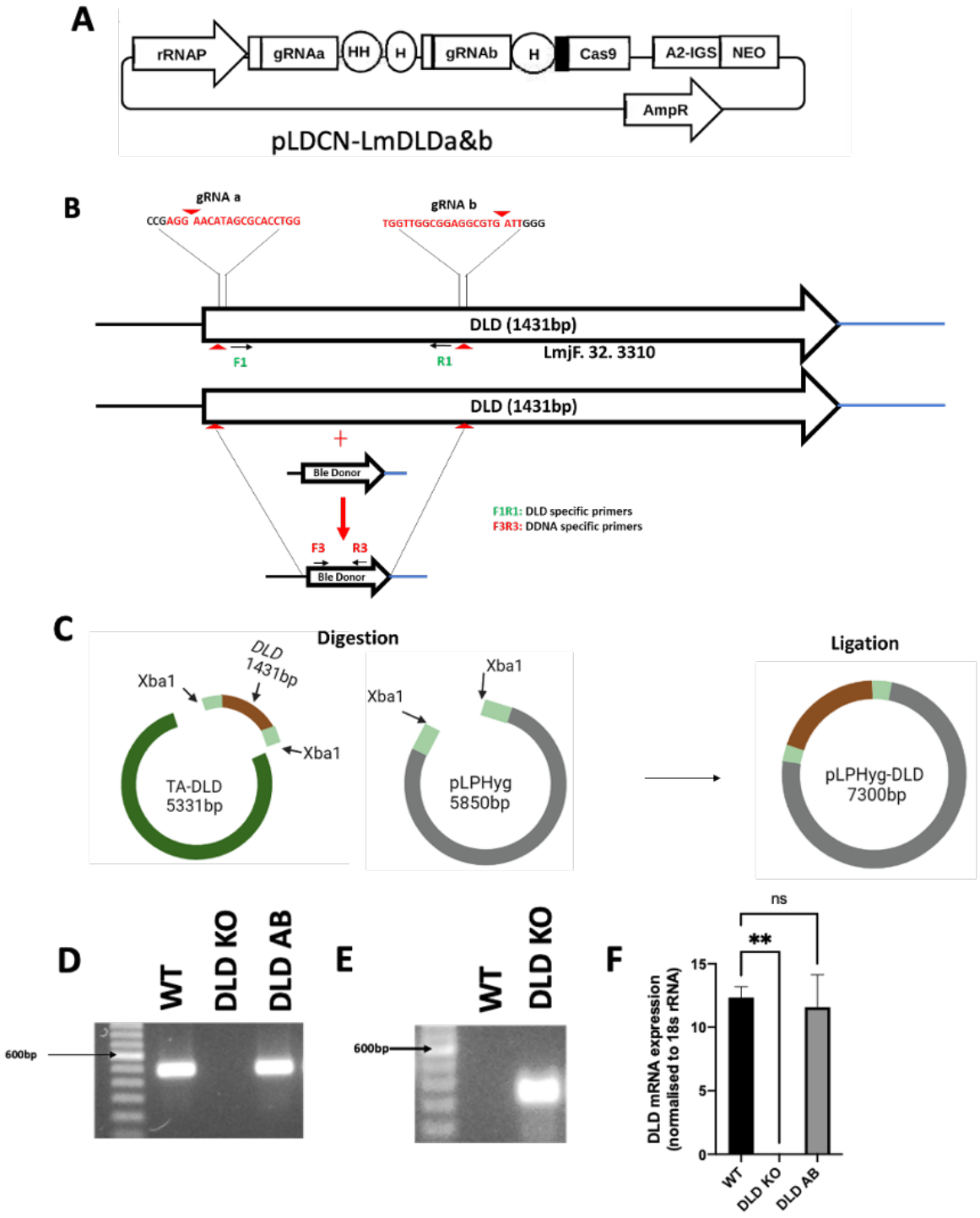



***Figure 9 DLD KO and DLD AB L. major generation and confirmation.***

Plasmid scheme showing the insertion of two-gRNA complementary to the *DLD* gene within the *Leishmania* plasmid, pLDCN (A). DLD deletion strategy using the CRISPR-Cas9, expression of Cas9 and the gRNA initiates a cleavage in the *DLD* loci (red arrows). Introducing the bleomycin resistance gene cassette within the cleaved site generates mutant parasites. Confirmation of DLD

deletion was made by PCR using DLD-specific primers (F1R1), and insertion of the Bleomycin resistance gene was confirmed by PCR using the bleomycin-specific primers (F2R2) (B). DLD AB construct (pLPHyg-DLD) generated by the subclone of the *DLD* gene into the *Leishmania* expression vector, pLPHyg. DLD AB *L. major* was generated by introducing pLPHyg-DLD into DLD KO parasites (C). PCR analysis confirmed DLD deletion by the absence of DLD PCR product (474bp), which was restored in the DLD AB parasite (D). PCR analysis confirms the successful insertion of the bleomycin resistance gene cassette PCR product (313bp) in the DLD KO parasite, which was absent in the WT parasites (E). The absence of DLD mRNA expression in the DLD KO parasites was confirmed by RT-qPCR and restored in the DLD AB parasites (F). **, $p < 0.01$; ns, not significant.

Next, I sought to use another method to confirm deletion of *DLD* gene in the mutant parasites and its complementation (restoration) in the add back strain. Previously, we generated DLD tetramer solution and used it to demonstrate the expansion of DLD-specific $CD4^+$ T cells following *L. major* infection (511). DLD-specific $CD4^+$ T cells produced IFN-$\gamma$ and TNF, cytokines that are critical for anti-*Leishmania* immunity (579). Therefore, I wanted to validate deletion of DLD in *L. major* using an *in vivo* involving assessment of DLD reactivity with the tetramer solution generated in our lab. I infected C57BL/6 mice with WT, DLD KO and DLD AB parasites and at five weeks post-infection, I tracked the expansion of DLD-specific $CD4^+$ T cells using DLD tetramer solution (I-$A^b$-$DLD_{63–79}$) by flow cytometry (Figure 10A). I found that in the spleen and dLN of mice infected with WT or DLD AB parasites, the expansion of DLD-specific $CD4^+$ T cells was statistically different yet almost comparable (Figure 10B&C). However, there was no detectable DLD-specific CD4+ T cells in the spleens and dLNs of mice infected with DLD KO *L. major* parasites (Figure 10B&C), suggesting successful deletion of DLD in *L. major* since infection in mice did not result in the expansion of DLD-specific $CD4^+$ T cells.

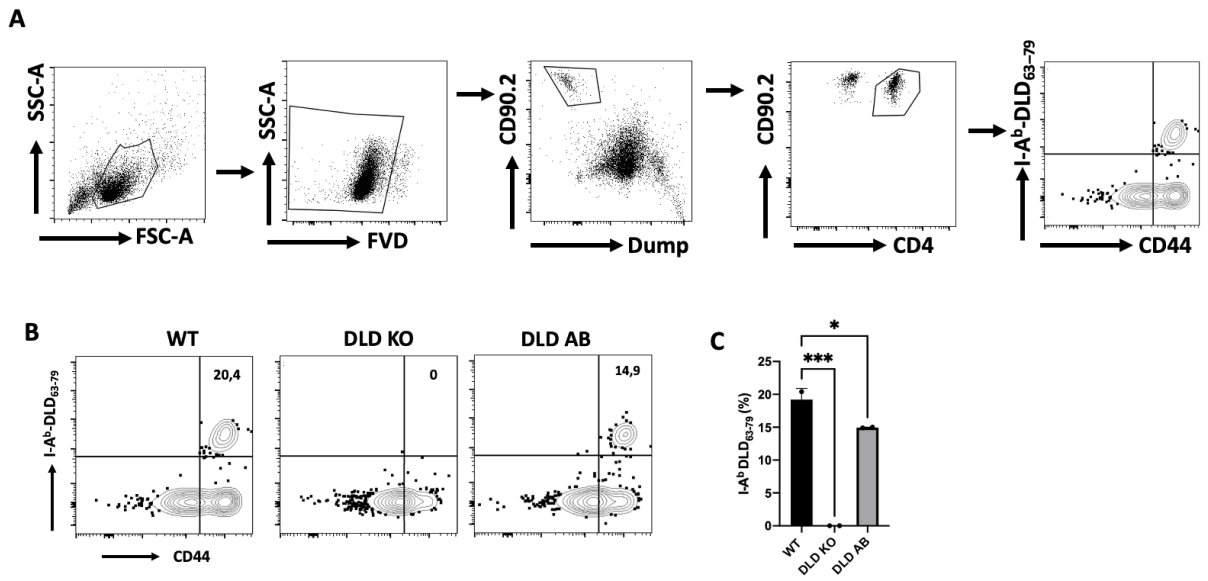



***Figure 10 Validating DLD deletion in vivo.***

C57BL/6 mice were injected subcutaneously with 2 x $10^6$ WT, DLD KO or DLD AB parasites in the foot pad for five weeks. Afterwards, the mice were sacrificed, and the presence of DLD-specific CD4+ T cells was identified from pooled spleen and LN using DLD Tetramer I-Ab-$DLD_{63–79}$ and analyzed by flow cytometry. Gating strategy (A). Flow plots (B) and graphs showing the percentage frequency of DLD-specific T cells (C) in mice infected with WT, DLD KO and DLD AB. *, $p < 0.05$; ***, $p < 0.001$.

### *4.1.3 DLD deficiency impacts parasite growth in axenic culture.*

Impaired growth usually indicates reduced virulence and infectivity and is a feature of null mutants in *Leishmania* (580). For instance, studies in *Leishmania* lacking the translation protein LeishIF4E1, which is critical for the parasite to adapt to their sandfly and mammalian host, were severely impaired in morphology, proliferation, and infectivity (580). To check whether DLD deficiency affects *L. major* proliferation, equal numbers of WT, DLD KO and DLD AB *L. major* promastigotes were grown in axenic cultures. Over time, numerical counts of the parasites were made in a hemocytometer under an optical microscope. In DLD deficiency, parasite numbers were significantly reduced compared to the WT parasites (Figure 11). However,

complementation of the *DLD* gene in the DLD-deficient parasites (DLD AB) restored their proliferation comparable to the WT *L. major* (Figure 11), suggesting that DLD is critical for the proliferation of *L. major* in axenic cultures.

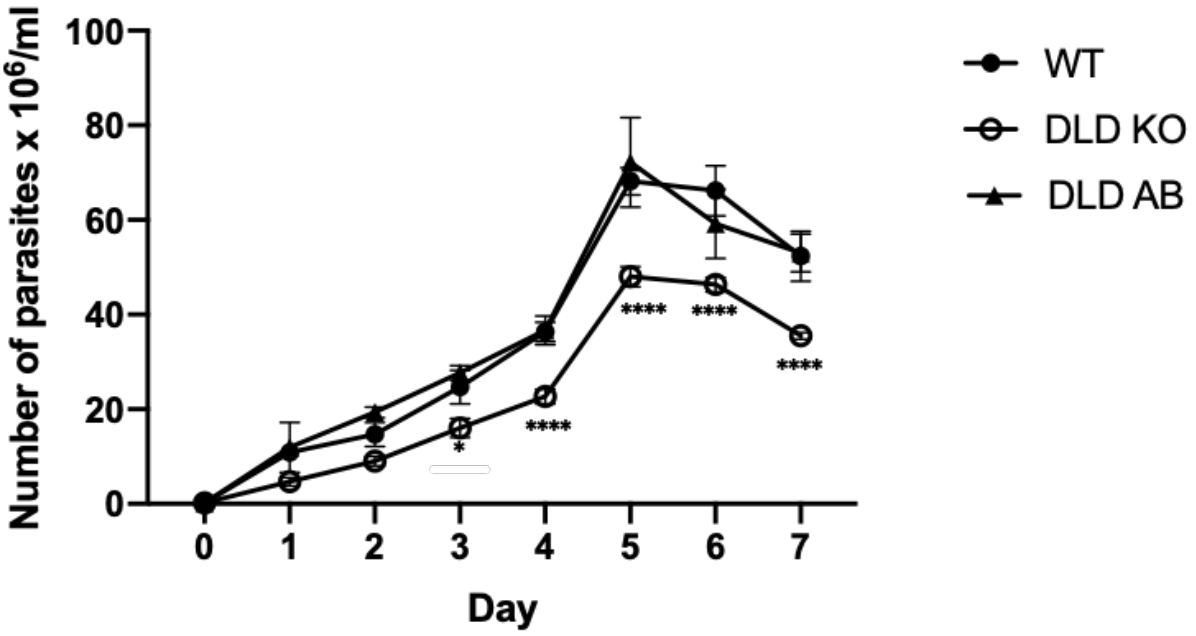



***Figure 11 Impaired proliferation of DLD KO L. major in axenic culture***

Equal numbers of WT, DLD KO or DLD AB parasites were grown in axenic cultures over time. At the indicated time points, parasites were counted under an optical microscope using a hemocytometer. *, $p < 0.05$; ***, $p < 0.001$.

### *4.1.4 DLD deficiency compromises parasites' growth inside macrophages*

Once deposited in the skin, *Leishmania* infects various phagocytic cells. Macrophages are preferable for amastigote replication due to the beneficial nutritional environment (61,302). Since I observed that DLD deficiency in *L. major* impacted promastigote replication in axenic cultures, I wanted to determine whether this will impact amastigote proliferation inside macrophages. To do this, I infected bone marrow-derived macrophages (BMDM) with WT, DLD

KO or DLD AB *L. major* and monitored infection by staining infected cells from cytospin preparations with Giemsa and imaging under an optical microscope at 6, 24, 48 and 72hour post-infection (Figure 12A). At 6 hours post-infection, there was no significant difference in the parasite numbers (Figure 12B) or infection rate (Figure 12B) amongst all the strains. However, as the infection progressed, there was a drastic reduction in the number of amastigotes observed in the cells infected with DLD KO parasites compared to those infected with WT or AB parasites (Figure 12A-C). This suggests that deficiency of DLD in *L. major* did not affect the parasites' ability to enter the cells, but DLD is critical for the proliferation of *L. major* amastigotes inside macrophages.

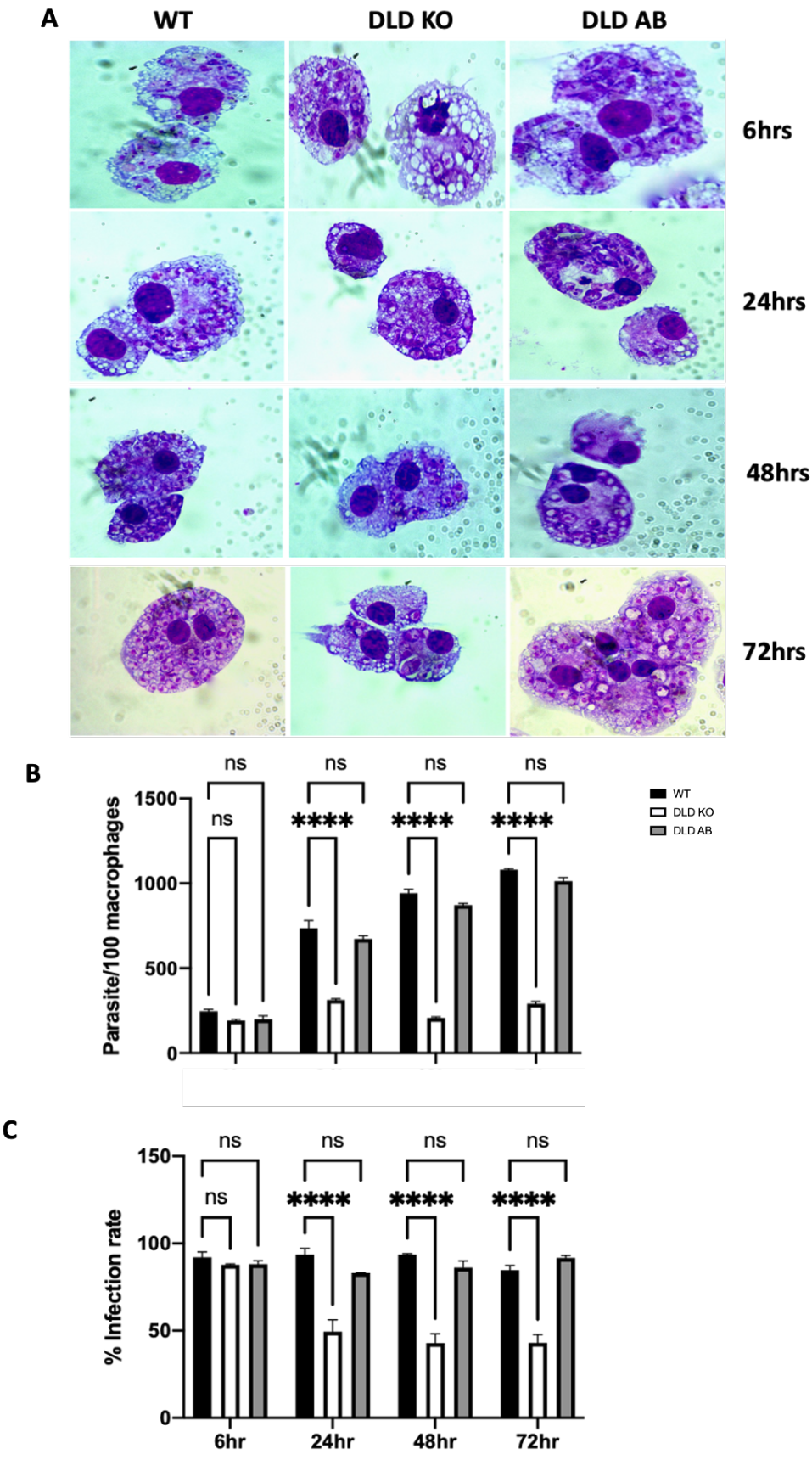

A
WT
DLD KO
DLD AB
6hrs
24hrs
48hrs
72hrs
B
Parasite/100 macrophages
1500
1000
500
0
ns
****
WT
DLD KO
DLD AB
C
% Infection rate
150
100
50
0
6hr
24hr
48hr
72hr

***Figure 12 DLD KO L. major was compromised inside macrophages.***

Bone marrow-derived macrophages (BMDM) were infected with WT, DLD KO or DLD AB parasites in a 10:1 parasite-to-cell ratio. At the indicated times, the giemsa-stained cytospin preparations of the infected cells were prepared, and images were generated (A). Quantification of the parasite/100 macrophages (B) and percentage infection rate (C) during the infection course was analyzed. ****, $p < 0.0001$; ns, not significant.

### *4.1.5 The ability of DLD KO parasites to differentiate into Infective forms was not compromised.*

*Leishmania* promastigotes undergo a translative process termed 'metacyclogenesis,' characterized by the parasite's infectivity and morphology alterations. It is known that infective stationary phase (metacyclic) promastigotes develop from the non-infective logarithmic-phase (procyclic) promastigotes *in vitro* (581). This can be attributed to the structural differences in the major surface glycoconjugate, lipophosphoglycan (LPG), whose repeating units are two-fold longer in the metacyclic forms than the procyclic promastigotes (582). Also, a notable observation is that the LPG of procyclic promastigotes lacks the arabinose caps on the galactose residues, which are present in metacyclic promastigotes (582). This modification forms the basis for which the infective forms can be distinguished using the peanut agglutinin lectin (PNA), which freely binds the galactose residues of the LPG moiety in the procyclic promastigotes causing agglutination but the arabinose capping the galactose residues on the LPG hinders this agglutination with PNA in the metacyclic promastigotes (583). By adopting this characteristic property, I sought to determine whether the ability of DLD KO parasite to differentiate into the infective forms (metacyclic promastigotes) was affected. I stained WT, DLD KO and DLD AB *L. major* logarithmic (day 3) and stationary (day 7) phase parasites with FITC-conjugated PNA and assessed their ability to bind PNA by flow cytometry. On day 3 of axenic growth (procyclic stage),

there was a slight dip in the percentage of metacyclic (PNA-negative) population in the DLD KO parasites compared to the WT and AB parasites. However, as the culture progressed to the stationary growth phase (Day 7), the percentage of PNA-negative(metacyclic) populations in the DLD KO cultures were comparable to those of the WT and AB parasites (Figure 13). Perhaps, DLD deletion in *L. major* may have delayed the development of procyclic promastigotes early during the culture but they matched up in their ability to differentiate into infective forms (metacyclics, PNA negative population) comparable to the WT or DLD AB parasites. This observation is consistent with the finding (shown in Figure 12 above), that DLD KO parasites were not impaired in their ability to enter macrophages as there was no significant difference in parasite numbers at 6 hours post-infection between WT, KO and DLD AB groups. Taken together, these findings indicate that loss of DLD in *L. major* does not impair their ability to either differentiate into the infective form or enter macrophages.

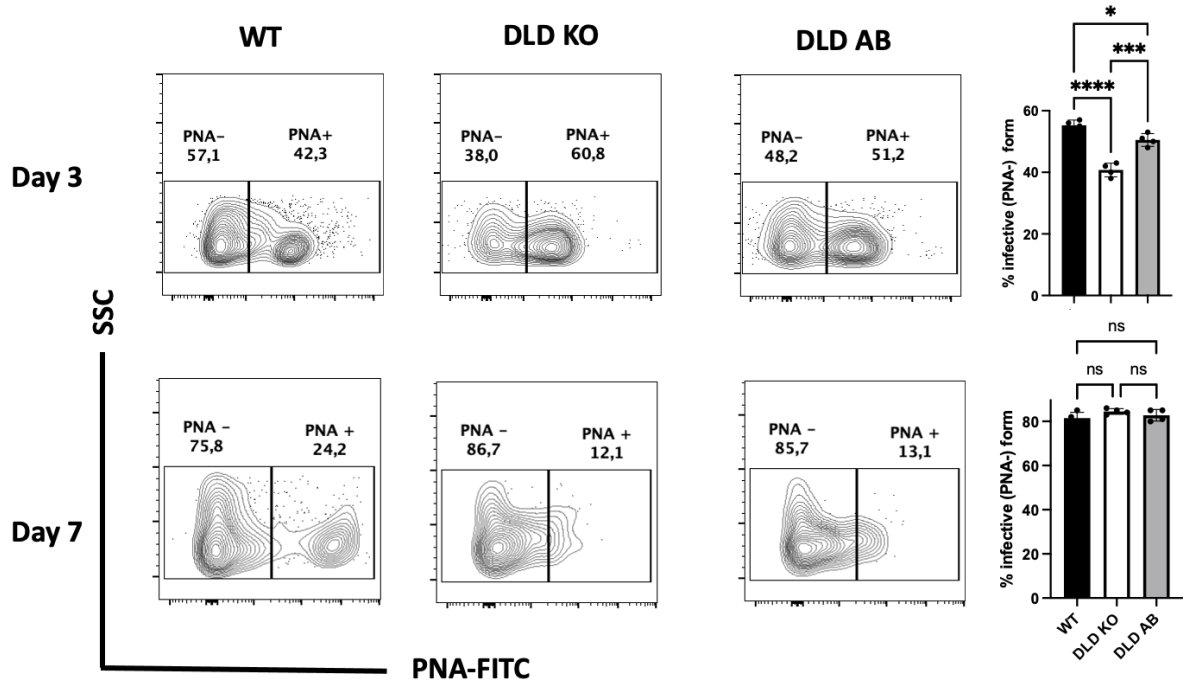



***Figure 13 DLD deficiency in L. major did not impact their ability to differentiate into infective forms.***

WT, DLD KO and DLD AB *L. major* parasites grown in axenic cultures at the logarithmic phase (day 3) and stationary phase (day7) were stained with PNA-FITC, and differentiated infective forms were assessed in the PNA negative gates by Flow cytometry.

### *4.1.6 DLD is a virulence factor.*

Previous studies have demonstrated a critical role of DLD in the virulence of *Streptococcus pneumonia* (584) and *Mycobacterium tuberculosis* (585). Whether DLD may be critical for parasite virulence in vivo is unknown, although the recent finding of DLD in exosomal vesicles (519), which are usually known to contain *Leishmania* molecules known to induce virulence, (586) may be suggestive. Therefore, I sought to determine whether DLD is affects virulence in *L. major* by infecting the highly susceptible (BALB/c) and relatively resistant (C57BL/6) mice with WT, DLD KO, and DL AB parasites in their footpads and monitor lesion progression over time. I observed

that progressive lesions developed in both the C57BL/6 (Figure 14A) and BALB/c (Figure 14B) mice infected with WT or DLD AB parasites, but no lesion developed overtime in both mice models infected with DLD KO parasites (Figure 14A&B). As expected, in infected C57BL/6 mice, lesion development peaked at four weeks post-infection with WT and DLD AB groups and afterward began to resolve (Figure 14A). In contrast, no lesion was observed in the footpad of mice infected with DLDL KO parasites for the duration of the experiment (Figure 14A&B). When infected animals were sacrificed at five weeks post-infection, the footpads from C57BL/6 (Figure 14C) and BALB/c (Figure 14D) mice infected with DLD KO parasites did not have a noticeable swelling compared to those from mice infected with WT or DLD AB parasites (Figure 14C & 14D). Consistent with the impaired development of cutaneous lesions, parasite burdens in the footpad of mice infected with DLD KO parasites were significantly lower compared to the WT and the DLD AB infected mice (Figure 14E&F).

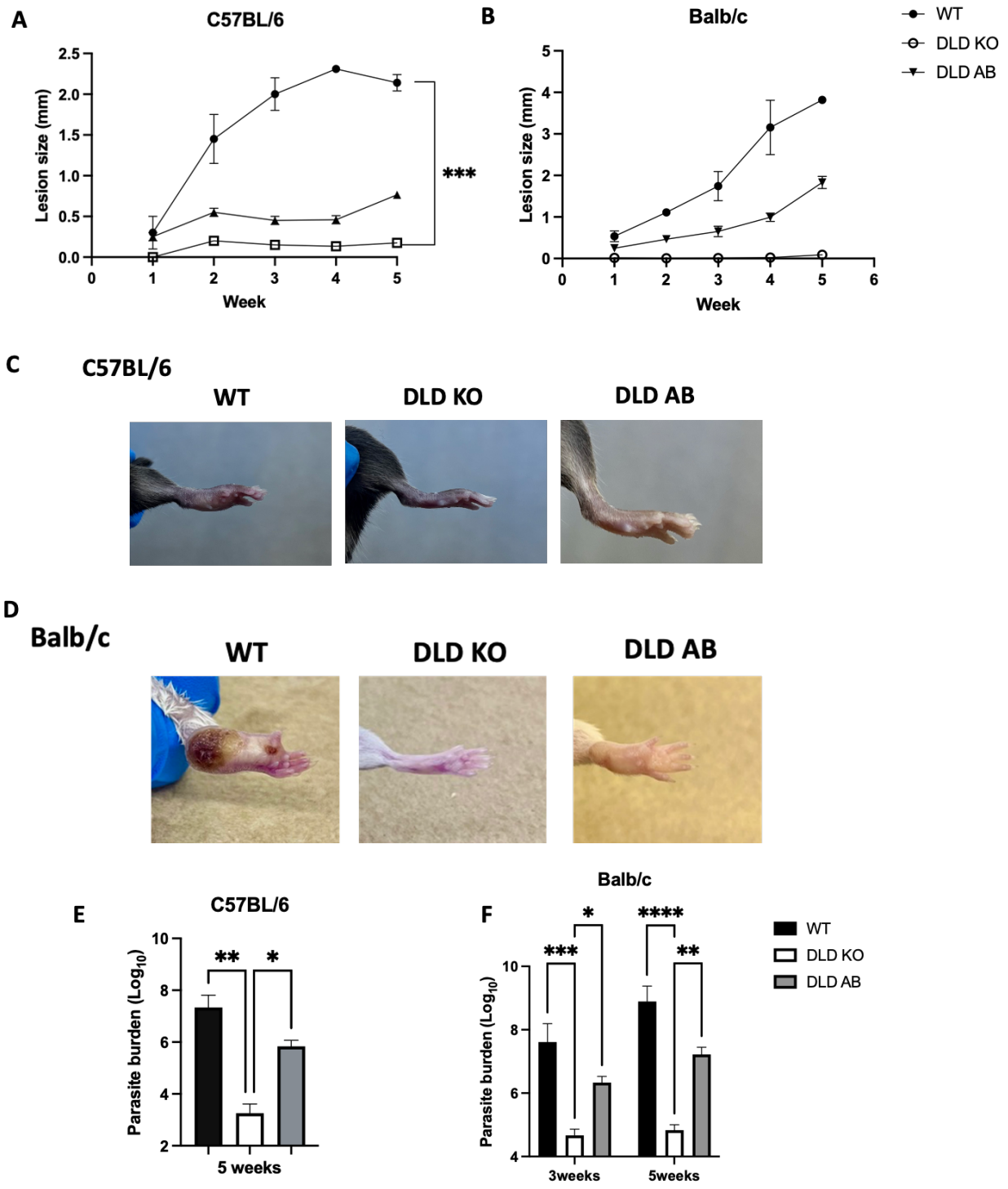



***Figure 14 DLD deficiency in L. major did not induce pathologies in susceptible and resistant mice models.***

*C57BL/6 and BALB/c mice were infected with 1 X10$^6$ WT, DLD KO or DLD AB L. major s.c in the footpad and lesion development was monitored over time using a digital calliper (A & B). A representative image showed the foot pad of the C57BL/6 (C) and BALB/c (D) infected mice at five*

*weeks post-infection. At the indicated time, the mice were sacrificed, and the parasite burden in the foot pads of the C57BL/6 (E) and BALB/c (F) mice was assessed by limiting dilution. This result is representative of 2 independent sets of experiments with similar results.* *, $p < 0.05$; **, $p < 0.01$; ****, p < 0.001; ****, p < 0.0001*

Partial restoration in the lesion developments and parasite burdens was observed in the footpad of mice infected with the DLD AB parasites (Figure 14), which appeared to be progressive at the indicated time points albeit at a reduced level with their WT infected counterpart mice, especially in the BALB/c mice. These observations (partial restoration of virulence phenotype in episomally expressed complementary controls) are consistent with about 30% restoration of WT phenotype in another mutant strains that were complemented episomally (587). Still, episomal gene expression delivery methods in *Leishmania* are chosen due to their versatility in gene delivery for stable expression (588). Taken together, these results demonstrate that DLD is a virulence factor in *L. major* and its absence results in loss of virulence and impaired parasite proliferation *in vivo*.

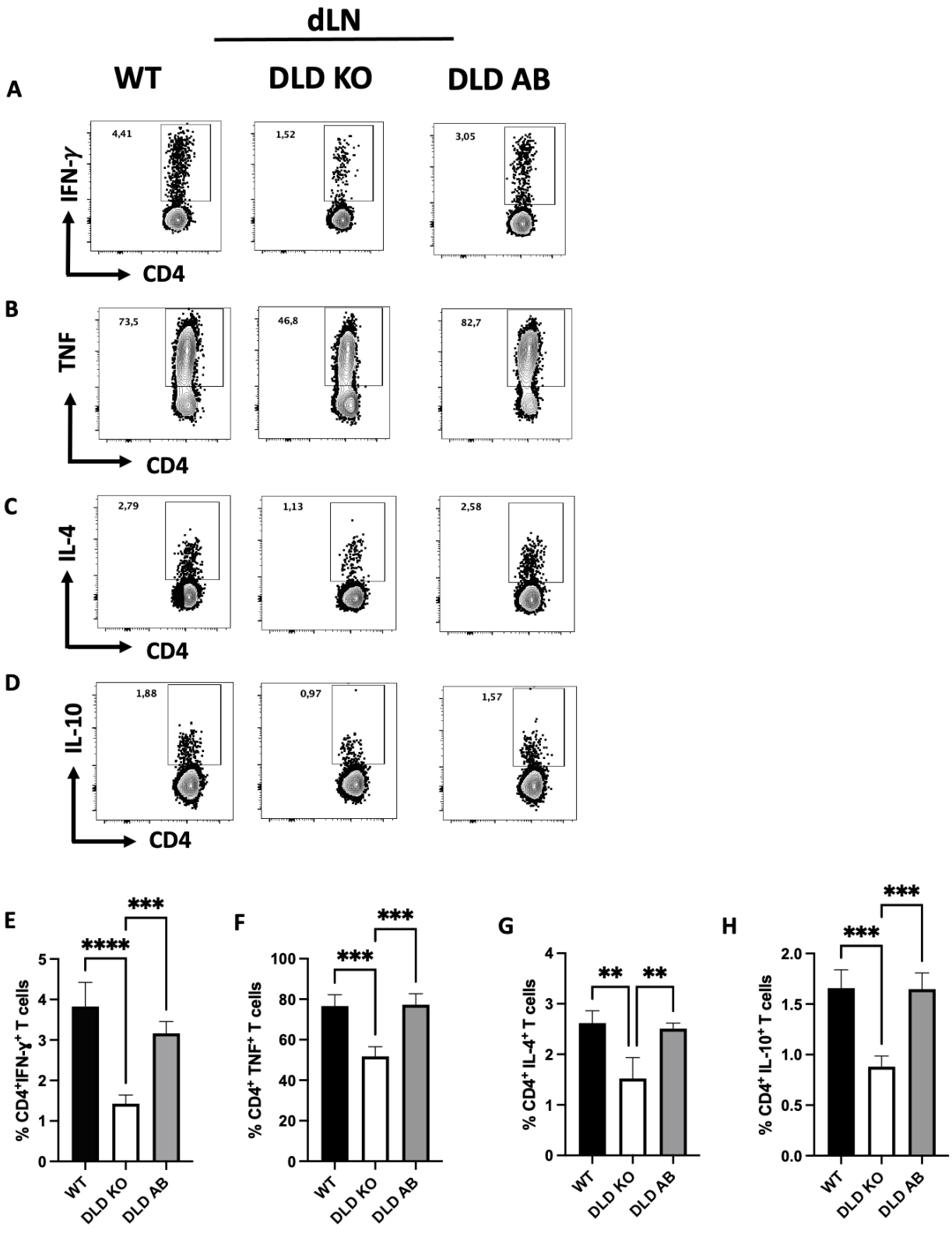
### *4.1.7 Absence of DLD in L. major results in blunted host immune responses following infection.*

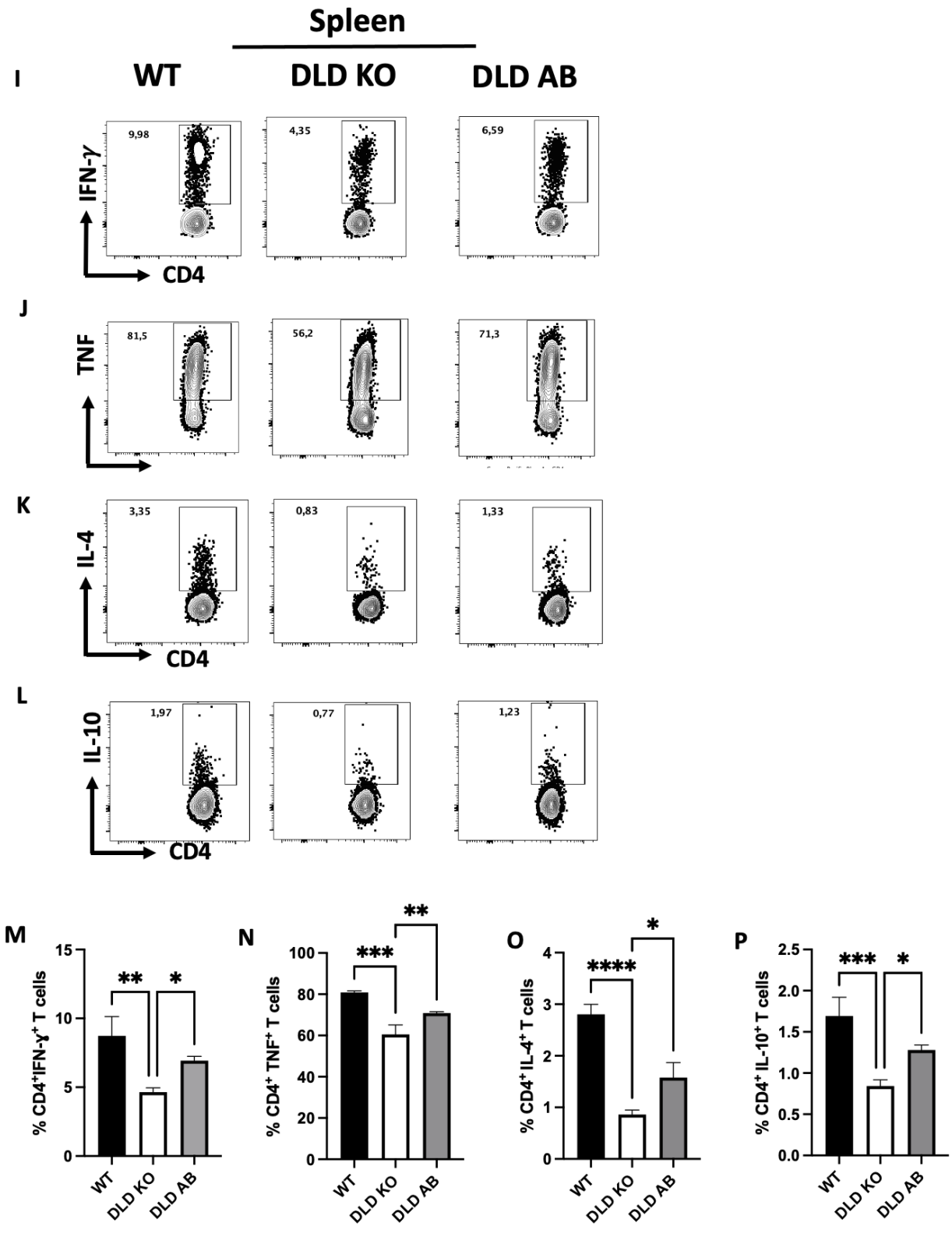
Enhanced resistance to *Leishmania* infection (manifested as reduction in lesion size and parasite burden) is associated with the induction of strong IFN-γ response in infected host (302). In contrast, susceptibility manifested as an uncontrollable lesion progression and high parasite burden is associated with the production of anti-inflammatory cytokines like IL-4, IL-13, and IL-10 (302). The reduced lesion size and parasite burden in the footpad of BALB/c mice infected with DLD KO parasites (Figure 14F) suggest that they may modulate host immune response differently than WT parasites (589). We previously showed that *L. major* DLD peptide ($DLD_{63-79}$) induces

robust anti-*Leishmania* immunity in mice (511). However, whether the absence of the entire *DLD* gene in the parasite will alter the host immune response is uncertain.

To examine the impact of DLD deficiency on host immune response, I infected BALB/c mice with 1x $10^6$ WT, DLD KO or DLD AB promastigotes and assessed the frequency of cytokine-producing $CD4^+$ T cells from spleens and dLNs directly *ex vivo*. Flow cytometry analysis showed that the frequency of cytokine (IL-10, IL-4, IFN-$\gamma$ and TNF) producing $CD4^+$ T cells were significantly lower in the dLN (Figure 15A-H) and spleen (Figure 15I-P) of mice infected with DLD KO parasites compared to those with WT or DLD AB parasites. Next, I assessed the secretion of cytokines produced in the cultures of cells from these tissues following stimulation with Freeze-thawed *Leishmania* antigen using multiplex cytokine profiling platforms (Figure 15Q-T). Consistent with the flow cytometry data, the levels of IL-10, IL-4, IFN-$\gamma$ and TNF in the culture supernatant fluids of mice infected with DLD KO parasites were significantly lower than those infected with WT and DLD AB parasites (Figure 15Q-T). To determine whether the blunted cytokine response was related to infection in the highly susceptible mice, I infected C57BL/6 mice with 1 x$10^6$ WT, DLD KO or DLD AB parasites and assessed the frequency of cytokine (TNF and IFN-$\gamma$) producing $CD4^+$ T cells in the spleen and dLN by flow cytometry. Like BALB/c mice, the frequency of TNF and IFN-$\gamma$ producing $CD4^+$ T cells were significantly higher in the dLN (Figure 16A-D) and spleen (Figure 16E-H) of mice infected with WT of DLD AB parasite than those infected with DLD KO parasites. Similarly, the level of cytokines secreted in the culture supernatant fluid were significantly higher in cells from mice infected with WT and DLD AB parasites compared to those infected with DLD KO parasites (Figure 16I-L), suggesting that the impaired ability of DLD KO parasites to induce

cytokine response was unrelated to the mice strain. Collectively, these results show that the absence of DLD in *L. major* leads to blunted host immune response.


dLN
WT
DLD KO
DLD AB
A
IFN-γ
CD4
4,41
1,52
3,05
B
TNF
CD4
73,5
46,8
82,7
C
IL-4
CD4
2,79
1,13
2,58
D
IL-10
CD4
1,88
0,97
1,57
E
% CD4+IFN-γ+ T cells
F
% CD4+ TNF+ T cells
G
% CD4+ IL-4+ T cells
H
% CD4+ IL-10+ T cells
WT
DLD KO
DLD AB


Spleen
I
WT
DLD KO
DLD AB
9,98
4,35
6,59
IFN-γ
CD4
J
81,5
56,2
71,3
TNF
K
3,35
0,83
1,33
IL-4
CD4
L
1,97
0,77
1,23
IL-10
CD4
M
% CD4+IFN-γ+ T cells
N
% CD4+ TNF+ T cells
O
% CD4+ IL-4+ T cells
P
% CD4+ IL-10+ T cells
WT
DLD KO
DLD AB

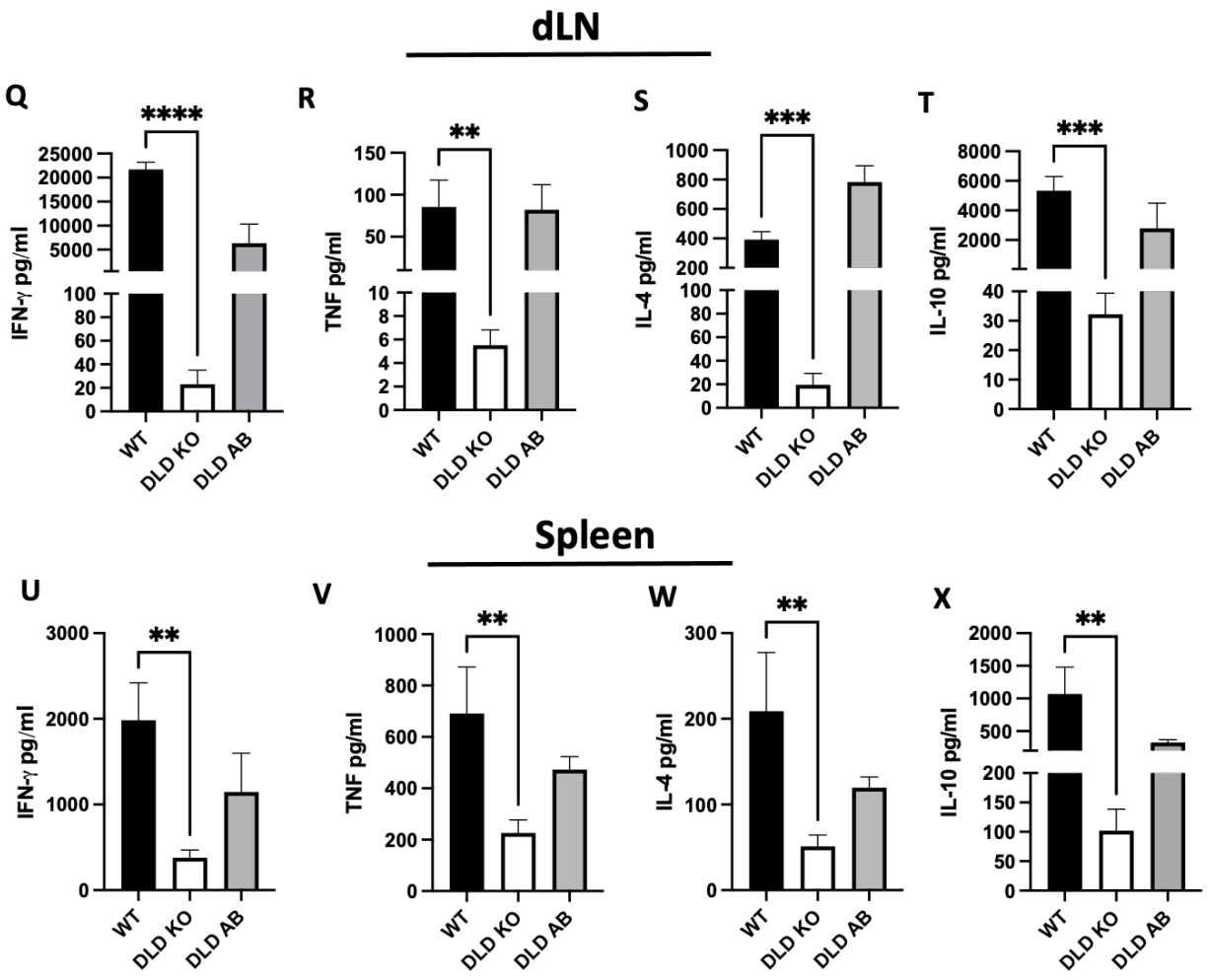
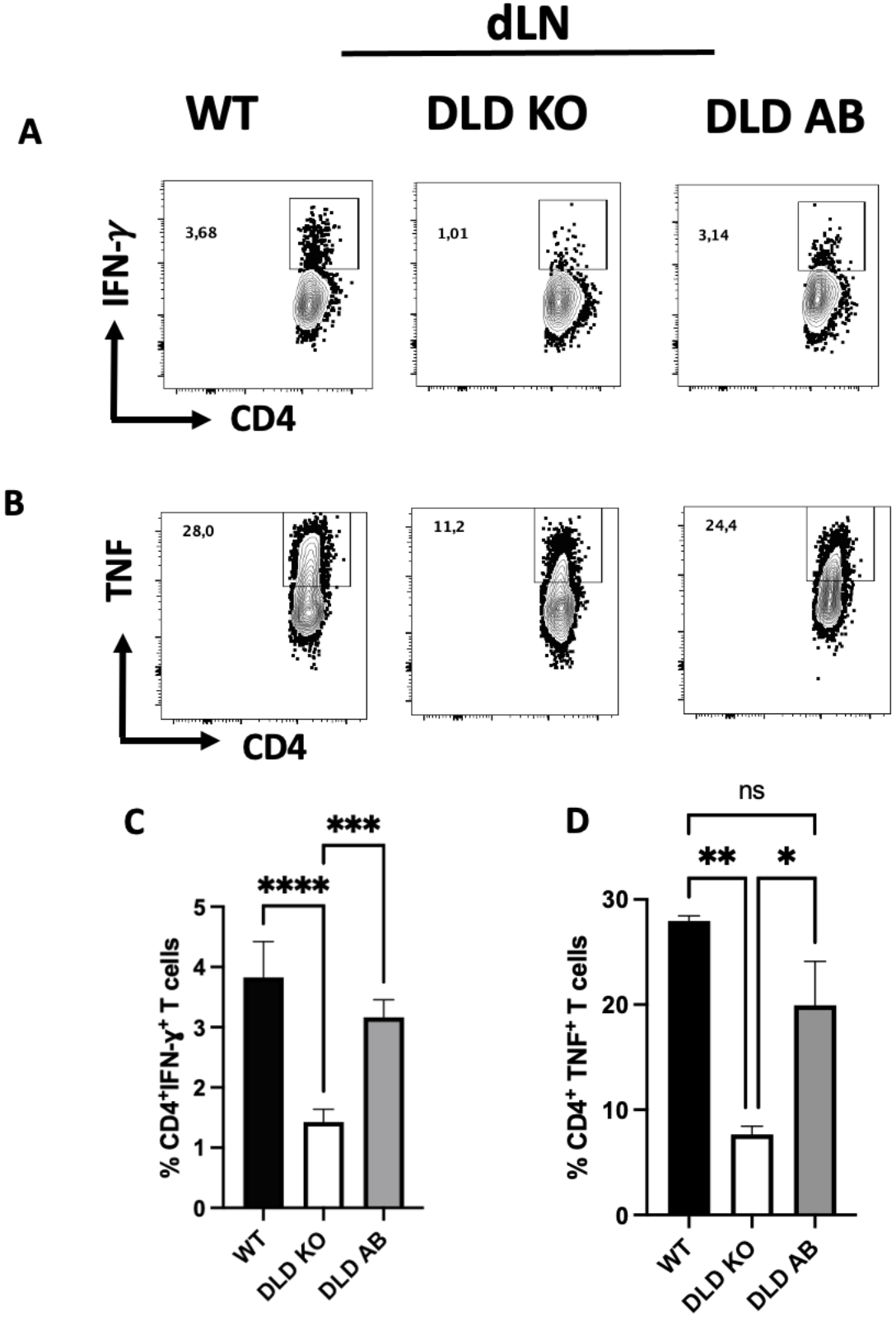
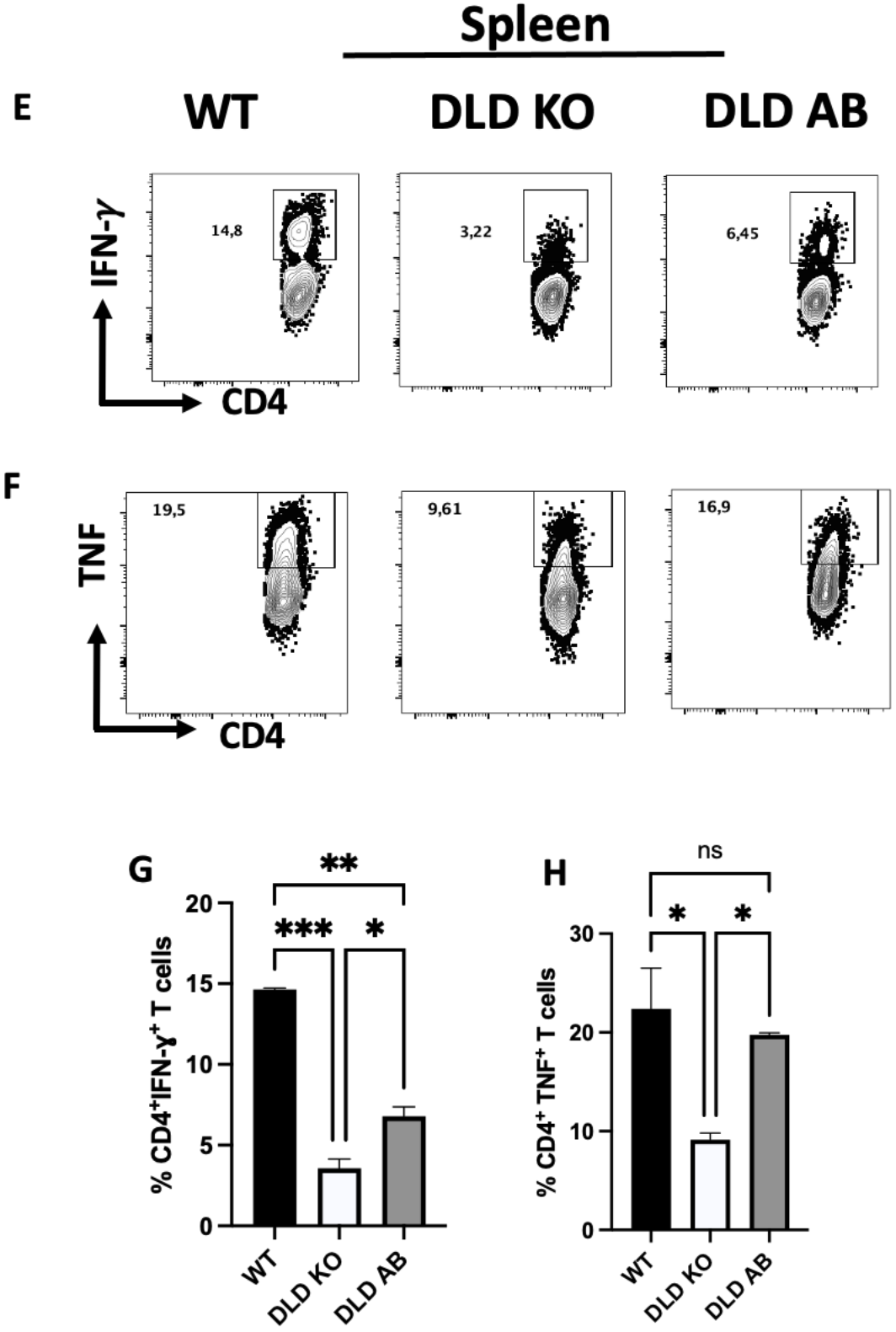



***Figure 15 DLD deficiency results in blunted host immune responses in BALB/c mice.***

BALB/c mice (6-8 mice per group) were infected in the footpad with 1 x$10^6$ WT, DLD KO or DLD AB stationary phased *L. major* parasites, and after 5 weeks, mice were sacrificed, and the spleen and dLN were harvested. The cells from the dLN (A-D) and spleen (I-L) were stimulated with PMA, ionomycon and brefeldin cocktail for 4hours and further stained to assess the frequency of IFN-$\gamma$, TNF, IL-4 and IL-10 producing CD4+ T cells by flow cytometry (E-H, M-P). Some of the dLN (Q-T) and spleen (U-X) cells were restimulated with freeze-thawed *Leishmania* antigen. After three days, multiplex cytokine profiling assessed the cytokines accumulated in the culture supernatant. This result is representative of 2 independent sets of experiments with similar results. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$


dLN
A
WT
DLD KO
DLD AB
IFN-γ
CD4
3,68
1,01
3,14
B
TNF
CD4
28,0
11,2
24,4
C
% CD4+IFN-γ+ T cells
****
***
WT
DLD KO
DLD AB
D
% CD4+ TNF+ T cells
ns
**
*
WT
DLD KO
DLD AB


Spleen
E
WT
DLD KO
DLD AB
IFN-γ
CD4
14,8
3,22
6,45
F
TNF
CD4
19,5
9,61
16,9
G
% CD4+IFN-γ+ T cells
**
***
*
WT
DLD KO
DLD AB
H
% CD4+ TNF+ T cells
ns
*
*
WT
DLD KO
DLD AB

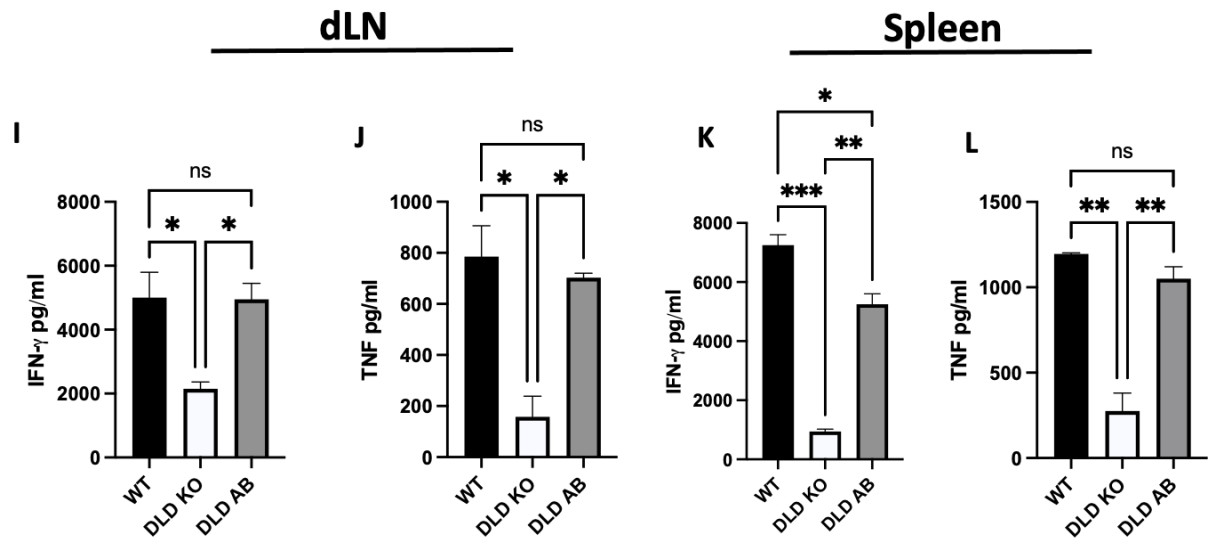



***Figure 16 DLD deficiency blunts host immune responses in C57BL/6 mice.***

C57BL/6 mice (5-6 mice per group) were infected in the footpad with 1 x$10^6$ WT, DLD KO or DLD AB stationary phased *L. major* parasites, and after 5 weeks, mice were sacrificed, and the spleen and dLN were harvested. The cells from the dLN (A-D) and spleen (E-H) were stimulated with PMA, ionomycin and brefeldin cocktail for 4hours and further stained to assess the frequency of IFN-$\gamma$, TNF, IL-4 and IL-10 producing CD4+ T cells by flow cytometry (C&D, G&H). Some of the cells from the dLN (Q-T) and spleen (U-X) were restimulated with freeze-thawed *Leishmania* antigen and after 3 days, the cytokines accumulated in the culture supernatant were assessed by ELISA. This result is representative of 2 independent sets of experiments with similar results. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$

### *4.1.8 Mice infected with DLD KO parasites had a compromised antigen-specific CD4+ T cell response.*

It has been reported that blunting of the host immune responses correlates with poor proliferation of *Leishmania major* parasites during infection (590,591). However, the immunodominance of a parasite molecule in *Leishmania* could result in the blunting of host immune responses when that molecule is removed (592). Immunodominance is a bias toward selecting specific epitopes critical for anti-inflammatory responses (592). Indeed, an epitope derived from *Leishmania* DLD ($DLD_{63–79}$) has been shown to induce strong expansion of DLD-

specific $CD4^+$ T cells that mediate resistance (effective resolution of cutaneous lesions and rapid parasite control) during *Leishmania* infection (511). It is conceivable that the blunting of the host immune response in mice infected with DLD KO parasites is because DLD is an immunodominant antigen. Alternatively, it is also possible that the blunting of the host immune responses is related to lack of availability of antigens due to impaired proliferation of DLD KO parasites in infected mice (Figure 14E&F). Thus, to rule out the possibility that the blunting host immune response is due to DLD immunodominance, I assessed antigen (*Leishmania*)-specific responses in the mice infected with DLD deficient *L. major*. I infected C57BL/6 mice with $2X10^6$ WT or DLD KO *L. major* and tracked the kinetics of PEPCK-specific $CD4^+$ T cell responses over time using PEPCK tetramer that was generated in our lab to track $CD4^+$ T cells that have specificity for $PEPCK_{335-351}$ derived from *Leishmania major* (510). There were no statistical differences in the frequency (Figure 17B &C) or absolute numbers (Figure 17D) of PEPCK-specific $CD4^+$ T cells in the spleen and dLN from mice infected with WT or DLD KO parasites at 1-week post-infection. However, as infection progressed (4 weeks), the frequency (Figure 17B &C) and absolute numbers (Figure 17D) of PEPCK-specific $CD4^+$ T cells in the spleen and dLN of mice infected with WT parasites were significantly higher than those from the DLD KO parasites. This time point coincides with the time where we observed impaired proliferation of the DLD KO parasites in mice (Figure 17C & D). This suggests that the blunting of the host immune response following infection with DLD KO parasites may be due to their reduced proliferation, which leads to reduced antigen availability.

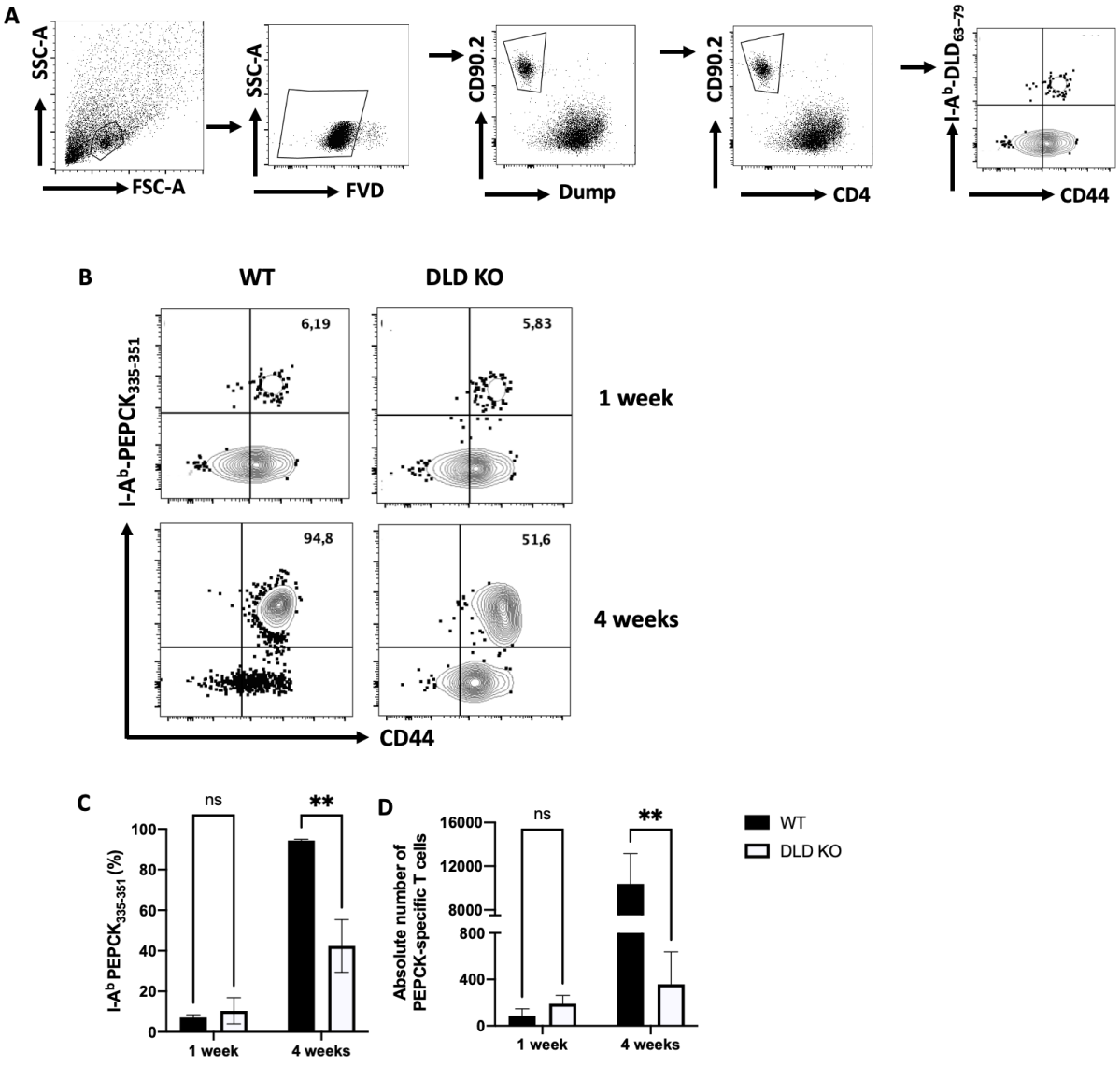



***Figure 17 Antigen specific CD4⁺ T cells were compromised in mice infected with DLD KO L. major.***

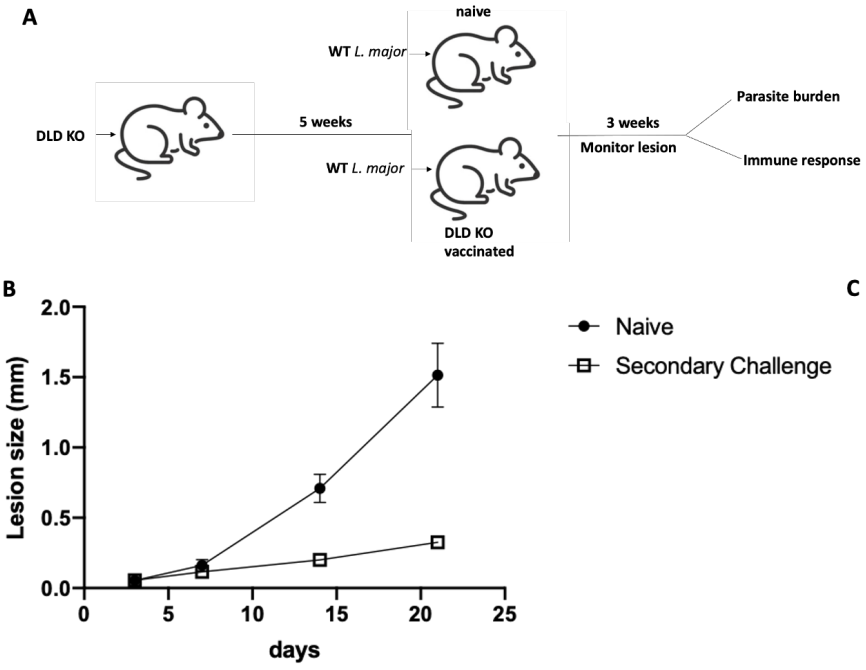
C57B/6 mice were infected in the footpad s.c with $2X10^6$ WT or DLD KO *L. major* stationary phased parasites. At the indicated times, mice were sacrificed, and cells pooled from the spleen and dLN were enriched with PEPCK tetramer (I-$A^b$-$PEPCK_{335-351}$) and analyzed by flow cytometry. The gating strategy (A) and percentage frequency of PEPCK-specific $CD4^+$ T cells in flow plots (B) and graph (C). The absolute number of PEPCK-specific $CD4^+$ T cells was assessed using counting beads (D). This result is representative of 2 independent sets of experiments with similar results. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$

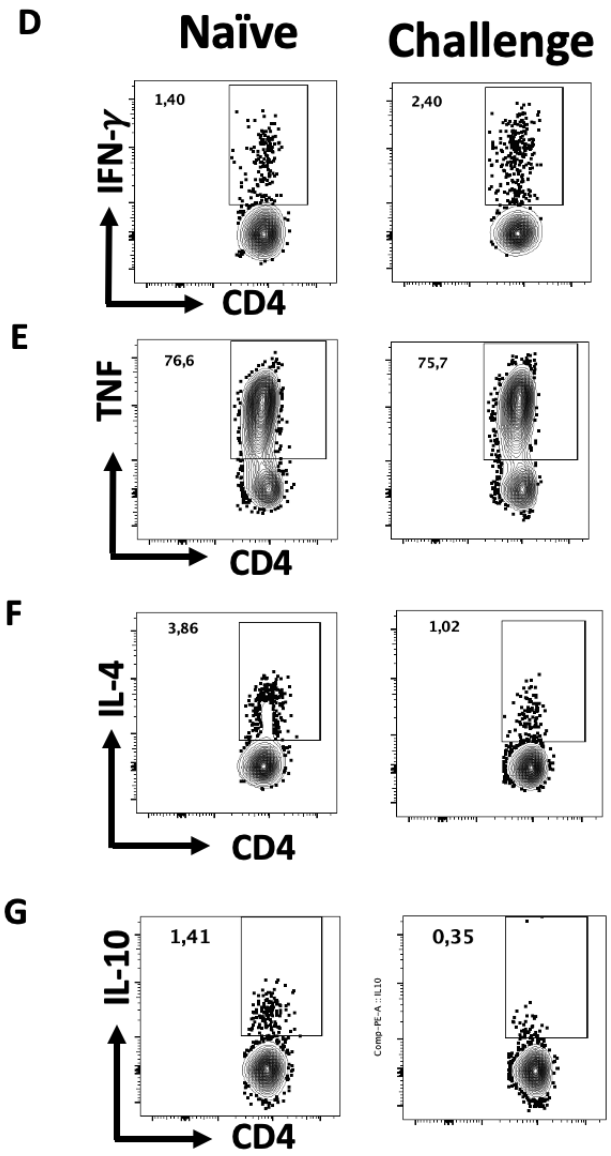
### *4.1.9 DLD-deficient parasites induce protective immune responses.*

During *Leishmania* infection, a good primary immune response must be able to protect against a secondary virulent challenge (339). It has been reported that a suitable vaccine against leishmaniasis in humans should provide an infection-induced or concomitant immunity (593,594). Although I observed a significantly reduced IFN-γ immune response in mice infected with DLD KO parasites (Figure 15&16), studies have shown that even in reduced levels of Th1 immune responses, secondary protection was still achieved if anti-inflammatory cytokines such as IL-10 and IL-4 are reduced (222). Also, since DLD KO parasites persist in mice without causing overt lesions (Figure 14), this suggests they can potentially be a candidate for live-attenuated vaccine. Therefore, despite the blunted immune response observed with the DLD KO parasites in mice, I wanted to know whether this would be associated with protective immunity against secondary virulent WT challenge. To do this, I vaccinated (infected) BALB/c mice s.c in their footpads with DLD KO parasites for five weeks and challenged them with $10^5$ WT *L. major* in their contralateral footpads alongside naïve controls. I monitored lesion development in the footpads and measured parasite burden and immune responses after the animals were sacrificed three weeks post-challenge (Figure 18A). I observed that challenged mice had a reduced lesion development at 2 and 3 weeks post-challenge compared to their infected naïve control (Figure 18B). At three weeks post-challenge, the parasite burdens in mice vaccinated with DLD KO parasites were significantly lower compared to their naïve controls (Figure 18C).

Next, I assessed the frequency of cytokine (IFN-γ, TNF, IL-4 and IL-10) producing $CD4^+$ T cells in the spleen and dLN of the challenged mice (Figure 18D-L). Compared to the naïve controls, there was a significant reduction in the frequency of IL-10 and IL-4-producing $CD4^+$ T cells in the dLN of

challenged mice (Figure 18D-L). Conversely, the frequency of IFN-γ-producing $CD4^+$ T cells were significantly increased in the vaccinated groups compared to their naïve controls (Figure 18D&H). At the same time, no differences were observed in the frequency of TNF between both groups (Figure 18D-K). Interestingly, the ratio of the frequency of IFN-γ to IL-4-producing $CD4^+$ cells was significantly higher in the vaccinated groups compared to the naïve controls (Figure 18L). I also stimulated cells from the dLN of infected animals with freeze-thawed *Leishmania* antigen for three days and assessed the levels of cytokines produced in the culture supernatant fluids by ELISA. Consistent with the flow cytometry data, the levels of IL-10 and IL-4 in the culture supernatant fluids of cells from vaccinated groups were significantly lower compared to those from the naïve groups (Figure 18M-P). In contrast, there was a significant increase in the IFN-γ levels in the culture supernatant fluids from vaccinated groups compared to their naïve controls (Figure 18M-P). Again, the ratio of IFN-γ to IL-4 secreted in this recall responses from the vaccinated groups was significantly higher than those from their naïve controls. These observations suggest that the enhanced resistance (better parasite control) observed in the vaccinated group may be related to this stronger IFN-γ. Taken together, these observations suggest that the attenuation of DLD KO parasites in mice is associated with secondary protective immunity against virulent WT challenge despite the blunted primary immune response.


A
naive
WT L. major
DLD KO
5 weeks
WT L. major
DLD KO
vaccinated
3 weeks
Monitor lesion
Parasite burden
Immune response
B
Lesion size (mm)
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
days
Naive
Secondary Challenge
C
**
Parasite burden (Log 10)
10
8
6
4
Naïve
Challenge


D
Naïve
Challenge
1,40
2,40
IFN-γ
CD4
E
76,6
75,7
TNF
CD4
F
3,86
1,02
IL-4
CD4
G
1,41
0,35
IL-10
CD4

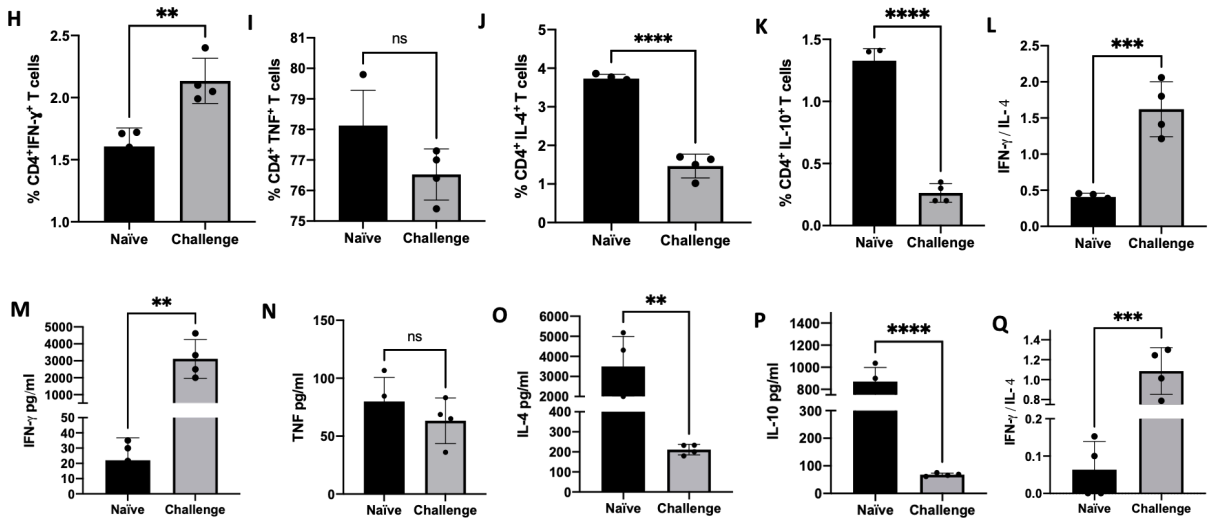



***Figure 18 DLD KO vaccinated mice challenged with WTT parasites develop reduced pathologies, displaying enhance protective immune responses***

BALB/c mice vaccinated mice and naïve control mice (4 mice per group) were challenged with 100 X$10^5$ WT parasites s.c in their contralateral footpad (A), and lesion development was monitored (B). After three weeks, the mice were sacrificed. Parasite burden in the footpad was assessed (C), and IFN-$\gamma$, IL-10, IL-4 and TNF $CD4^+$ producing cells were analyzed from the cells in the dLN by flow cytometry (D-K). The IFN-$\gamma$ to IL-4-producing $CD4^+$ T cells ratio was determined (L). Some of the dLN cells were stimulated with freeze-thawed Leishmania antigen. After three days, the cytokines accumulated in the culture supernatant were assessed by multiplex cytokine profiling (M-P). The ratio of IFN-$\gamma$ to IL-4 cytokine produced was assessed (Q). This result is representative of 2 independent sets of experiments with similar results. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$

The protective response I observed upon WT challenge in mice vaccinated with DLD KO parasites were short-term, i.e., mice were only challenged after 5 weeks of vaccination with DLD KO. Therefore, I wanted to know whether protection can be sustained long-term by intending to challenge vaccinated mice after 12 weeks. Surprisingly, significant lesions began developing in the mice infected with the DLD KO parasites after 8 weeks and they seem to be progressive by the $10^{th}$ week (Figure 19A). Rather than challenge the vaccinated mice with WT parasites, I wanted to confirm that the sudden lesion developments in these animals was not due to the

restoration of the *DLD* gene in the DLD KO parasite. Therefore, I isolated the parasites from the developing lesions and screened for the *DLD* gene expression by PCR analysis. The *DLD* gene amplicon (474bp) was absent in the DLD KO parasite but present in the WT parasite (Figure 19B). To further confirm this, I checked for the presence of bleomycin resistance gene cassette that was used to replace the *DLD gene* in the KOs by PCR analysis. The bleomycin gene amplicon (313bp) was still present in the parasites isolated from mice vaccinated with DLD KO parasites but not in those infected with WT parasites (Figure 19C). Collectively, these observations suggest that long-term vaccination with DLD KO parasites would not be possible, mainly because reactivation of disease (virulence), which is not due to the reappearance/reacquisition of the deleted *DLD* gene.

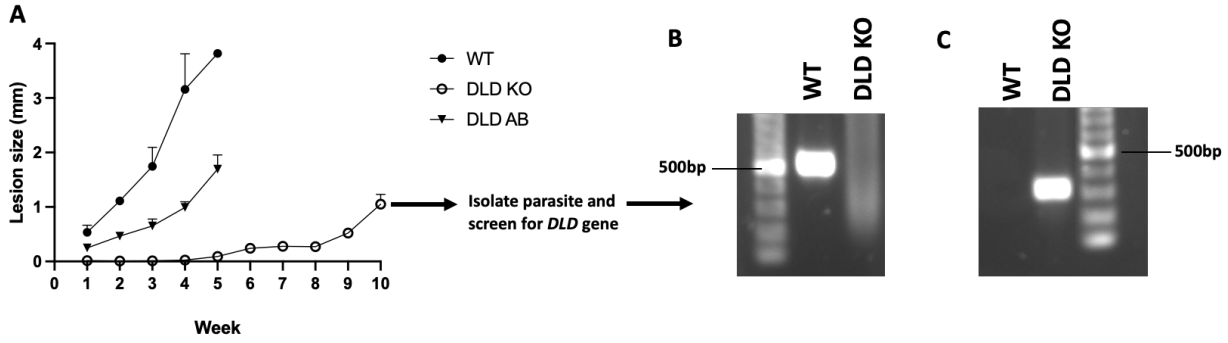



***Figure 19 Appearance of lesion developments in mice vaccinated with DLD KO L. major long term.***

BALB/c mice vaccinated with $1X10^6$ DLD KO promastigotes for 10 weeks started developing lesions long-term (A). At 10 weeks, Parasites in these mice were isolated from the footpad, and the absence of the DLD Amplicon (474bp) was assessed using DLD-specific primers by PCR (B). The Bleomycin resistance gene cassette (313bp) was analyzed in the parasites by PCR (C).

### *4.1.10 DLD deficiency compromises L. major metabolism and mitochondrial functions*

DLD is a critical mitochondrial enzyme that plays a role in energy generation (512,595,596). Thus, deleting DLD in *L. major* could severely hamper its mitochondrial functions resulting in the parasite's impaired proliferation *in vitro* and *in vivo*. To check whether deleting DLD in *L. major* impacted mitochondrial function, I assessed whether the permeability of the mitochondria in these parasites were compromised. To do this, I checked whether the ability of DLD KO parasites to uptake Tetramethylrhodamine methyl ester (TMRM) dye into their mitochondria was hampered because tmrm dye will penetrate healthy and functional mitochondria easily (597). I observed that DLD KO parasites grown in axenic cultures had a significantly reduced Mean Fluorescent Intensity (MFI) when stained with TMRM dye compared to their WT and DLD AB controls (Figure 20A). This suggests that the mitochondrial membrane of DLD KO parasites is leaky and unable to retain TMRM dyes (Figure 20B).

Subsequently, I checked whether the impaired mitochondrial membrane permeability in DLD KO parasites may have impacted their ability to produce mitochondrial reactive oxygen. Thus, I assessed the production of reactive oxygen species in mitochondria using the Mitosox reagent by flow cytometry. In the absence of DLD in *L. major*, there was a significant reduction in Mitosox signals compared to their WT and DLD AB controls (Figure 20B), suggesting that DLD KO parasites produce reduced reactive oxygen species due in part to impaired mitochondrial membrane permeability.

A faulty mitochondrial permeability and reduced production of reactive oxygen species indicate compromised mitochondrial respiration, which can be assessed by the amount of oxygen consumed (598). Thus, to assess whether mitochondrial respiration was impacted in DLD KO *L.*

*major*, I subjected axenic growing promastigotes to a Mito stress test. In these tests, WT, DLD KO or DLD AB parasites were exposed to drugs that inhibit one or two oxidative metabolic pathways. These drugs, which include oligomycin (inhibitor of ATP synthase), FCCP (proton gradient uncoupler and disruptor of the mitochondrial membrane potential) and Rotenone/Antimycin (which shuts down mitochondrial respiration by inhibiting ubiquinone oxidation), were administered to the parasites at different times and assessed by the seahorse assay using an XF24 analyzer. The results show that DLD KO parasites were consuming more oxygen than their DLD AB or WT counterpart parasites (Figure 20C). Indeed, there was a significant increase in the basal oxygen consumed by the DLD KO parasites compared to their WT or AB counterpart parasites (Figure 20D), suggesting that the absence of DLD in *L. major* makes them develop a higher demand for oxygen to possibly meet their energy needs for survival.

Furthermore, the extracellular acidification rate (ECAR), which is a measure of glycolytic flux in cells (599), can be assessed from a mitostress test. Throughout the period of the drug administration to the parasite lines, DLD KO parasites had higher ECAR compared to their WT or DLD AB counterpart controls (Figure 20E). Indeed, before the drugs were added, culture media containing DLD KO parasites had significantly higher acidification rate basally than their WT and AB controls (Figure 20E). This suggests that the absence of DLD in *L. major* has taken a toll on the parasites respiration such that they are tenaciously looking to increase their energy demands through other means such as the glycolytic pathway. Taken together, these observations suggest that the absence of DLD in *L. major* results in impaired mitochondrial functions, making the parasites develop a need for alternative energy-generating pathways.

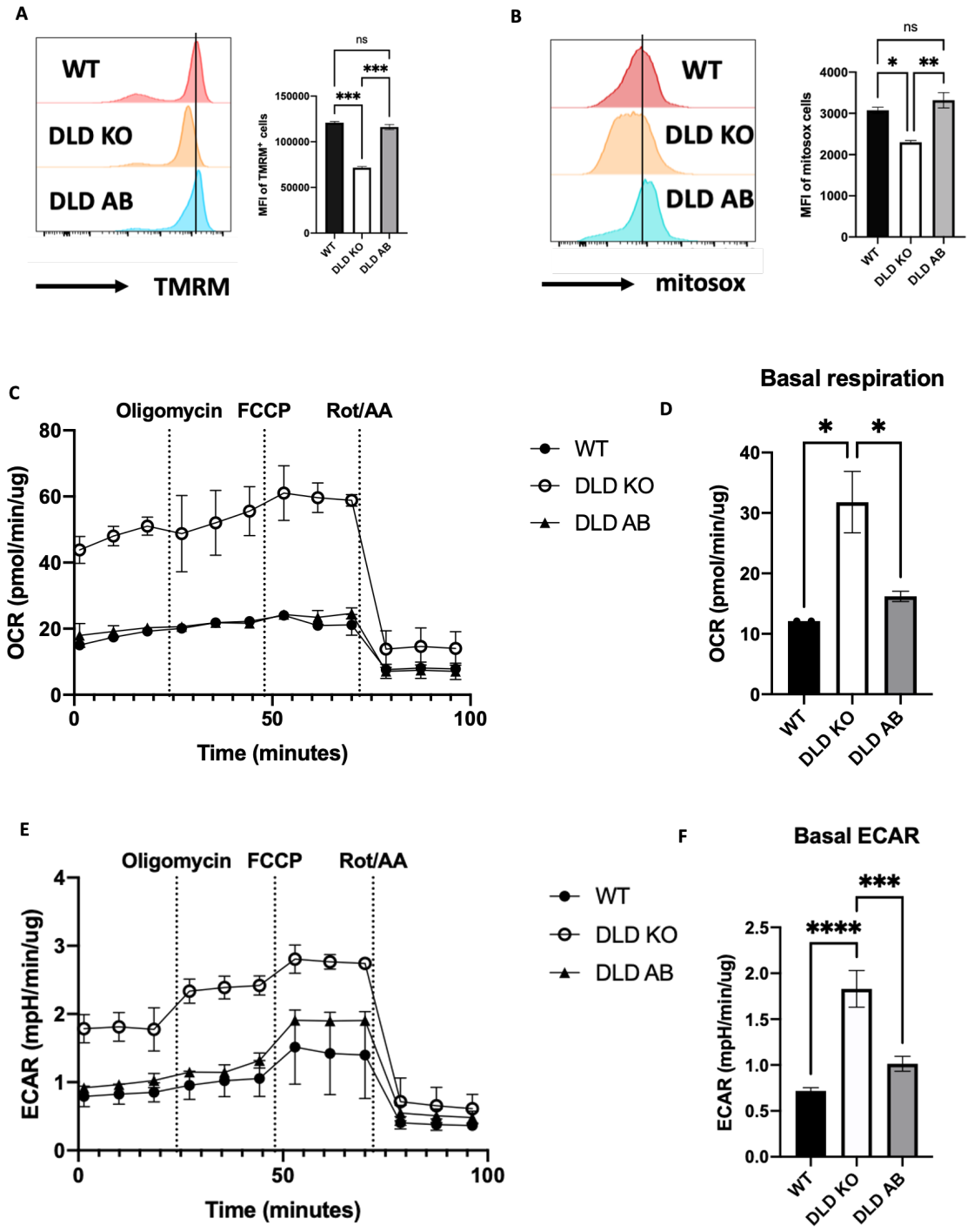



*Figure 20 DLD deficiency in L. major compromises parasite metabolism.*

Histogram plots and mean fluorescent intensity of WT, DLD KO and DLD AB *L. major* promastigotes stained with TMRM (A) and mitosox dyes (B) and signals detected by Flow cytometry (B). 5 X $10^6$ WT, DLD KO and DLD AB *L. major* promastigotes were immobilized using poly-D-lysine and subjected to different drug treatments such as Oligomycin, FCCP, Rotenone/antimycin in a Mito stress test using the XF24 analyzer in a XF supplemented media.

Following normalization with parasite protein concentration, oxygen consumption rates over time were assessed (C) and Basal respiration calculated (D). The acidification of the media is assessed as ECAR over time (E) and the basal levels determined (F). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant.

***Summary 1:***

Cutaneous leishmaniasis is still a public health burden with complications and challenges surrounding the successful eradication and elimination of the disease. However, a clear understanding of the antigens in the disease-causing parasites, *Leishmania major*, which could induce a protective response during infection, is critical for vaccine developments. Peptides derived from Leishmania's Dihydrolipoyl dehydrogenase ($DLD_{63–79}$) induced protective responses (511) during infection. However the exact role of DLD in the virulence and immunopathogenesis of *Leishmania major* is unknown. By successfully deleting the entire *DLD* gene in *Leishmania major* using the CRISPR/Cas9 tool, I assessed the impact of this deletion on parasite virulence and host immune response during infection. DLD-deficient parasites had impaired proliferation in axenic culture and inside macrophages. The ability of these parasites to differentiate into their infective forms was unhindered. However, gene-deficient parasites infected in mice did not cause disease pathology but led to the blunting of the host immune response. Mice vaccinated with the DLD deficient parasites induced protective responses after exposure to virulent *Leishmania* parasites. Deleting DLD, which is a critical mitochondrial enzyme in *L. major*, compromised the parasite's mitochondrial metabolism and function.

## Aim 2 The role of PEPCK in Visceral Leishmaniasis

### *4.2.1 Background and Rationale*

Unlike CL, visceral leishmaniasis (VL) is a systemic and more severe form of leishmaniasis that affects *major* organs like the liver, spleen, and bone marrow. It becomes fatal and life-threatening if patients are left untreated (10). Current strategies for the control and elimination of VL from 2012 to 2017 resulted in a decrease in incidence rate. However, it is becoming difficult to control VL due to the risk of co-infection with other diseases. Reports indicate that patient with VL are at risk of HIV infections, malaria and even TB. These complications with VL make treatment against the disease challenging. With the current toxicities and emergence of drug resistance in the current treatment regimen, vaccination is believed to be the best alternative prophylactic route for the control of VL. Developing a safe and efficacious vaccine against VL requires identification of protective antigens, efficient vaccine delivery approach and excellent vaccine composition that can induce sustained effector and memory T cell responses. More importantly, identifying a cross-species protective antigen that will induce protective responses against multiple *Leishmania* species will be highly advantageous. Recently, our lab identified a peptide derived from *Leishmania* Phosphoenolpyruvate carboxykinase ($PEPCK_{335\text{-}351}$) (510) that induced the expansion of $PEPCK_{335\text{-}351}$ specific $CD4^+$ T cells in mice infected *L. major* infection. During infection, these PEPCK-specific $CD4^+$ T cells expanded, contracted and after parasite clearance led to the development of memory T-cells that secrete IFN-$\gamma$ and TNF to protect mice against secondary challenge (510). We also found that vaccination with PEPCK-expressing vector induced protection in C57BL/6 infected with L. *donovani* as evidenced by significant reduction in parasite burden in the spleen and liver (510). This is not surprising given that PEPCK has more than 90% homology

across multiple *Leishmania* species, suggesting that PEPCK may be a viable drug or vaccine target against different forms of leishmaniaisis including VL. PEPCK is a critical enzyme in trypanosomatids (514) and was shown to play vital roles in virulence in *Leishmania major* (218), *Mycobacterium tuberculosis* (515) and *Schistosoma mansoni* (516). If PEPCK will be a cross species vaccine target against all forms of leishmaniasis, it is crucial to know whether it is a virulence factor `in VL and understand how they modulate host immune responses. Although, PEPCK is a virulence factor in *L. major*, whether is also virulence factor in *L. donovani* is unknown. **I hypothesized that deficiency PEPCK in *L. donovani* will lead to a loss of virulence resulting in reduced disease pathology and altered host immune response during infection.**

### *4.2.2 Generation and confirmation of PEPCK KO and addbacks in L. donovani*

The CRISPR-Cas9 molecular editing tool has been demonstrated efficient in deleting multicopy and essential genes of *Leishmania* (578). We have previously used this gene manipulation tool in deleting PEPCK in *L. major* (218). Similar CRISPR/Cas9 system described in section 4.1.2 was used to delete PEPCK in *L. donovani.* To do this, two gRNA was cloned into the *Leishmania* expression vector, PLDCN (Figure 21A) containing Cas9 and introduced into logarithmic phase *L. donovani* promastigotes by electroporation. This results in deletion of the two copies of *PEPCK* gene that is within a 7kb region (Figure 21B). Introduction of the bleomycin resistance gene cassettes into the cleaved sites generates mutant parasites that can be confirmed by PCR. PCR analysis confirmed the presence of the PEPCK amplicon (717bp) in WT parasites (Figure 21C) which was absent in the PEPCK KO parasites. Complementary control or addback (PEPCK AB) parasites were generated by episomally introducing *PEPCK* gene into the PEPCK KO L. *donovani*. The *PEPCK*

amplicon (717bp) was restored in the PEPCK AB parasites (Figure 21C). The successful homology directed repair was confirmed by the presence of bleomycin resistance gene amplicon (925bp) in the PEPCK KO parasites which was absent in the WT parasites (Figure 21D). Furthermore, the absence of PEPCK mRNA expression (Figure 20E) as well as protein expression (Figure 21F) by RTqPCR and western blot, respectively, in the PEPCK KO parasites confirms the deletion of both copies of the *PEPCK* gene.

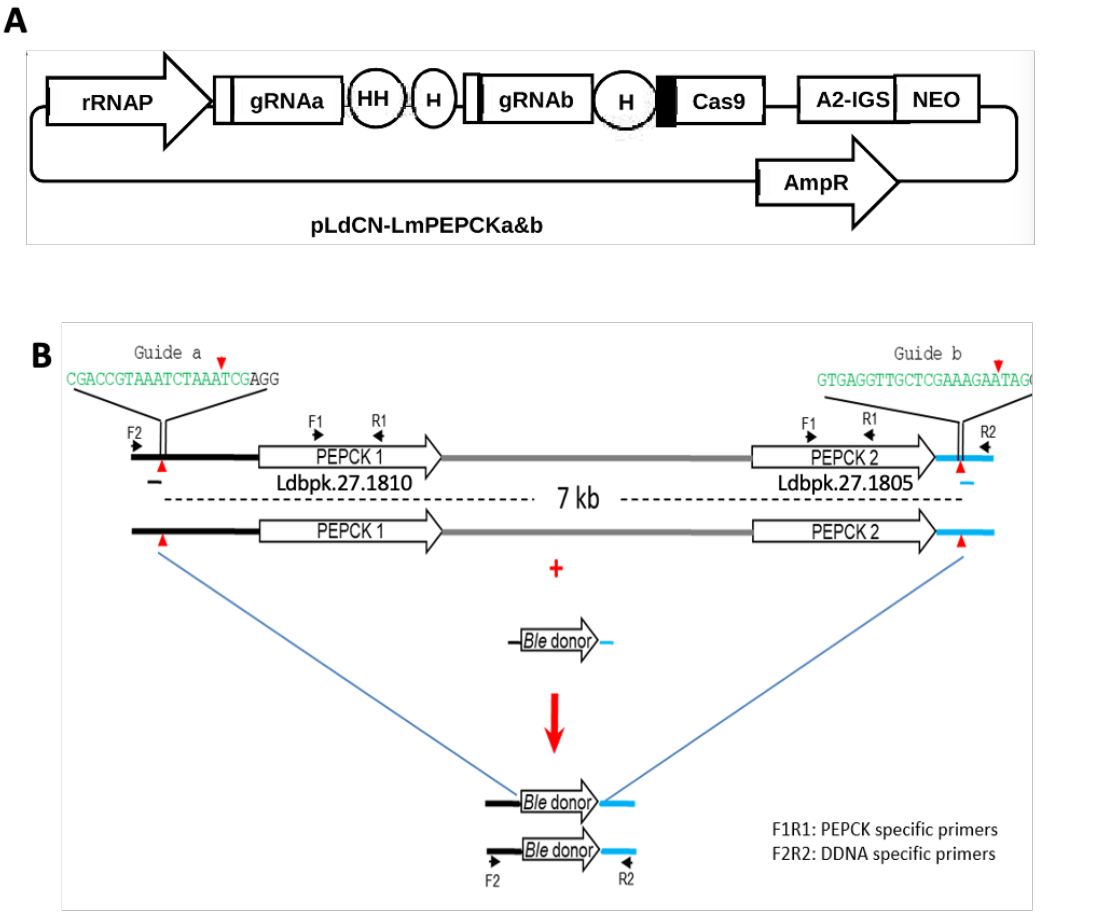

A
rRNAP
gRNAa
HH
H
gRNAb
H
Cas9
A2-IGS
NEO
AmpR
pLdCN-LmPEPCKa&b
B
Guide a
CGACCGTAAATCTAAATCGAGG
Guide b
GTGAGGTTGCTCGAAAGAATAG
F2
F1
R1
PEPCK 1
Ldbpk.27.1810
7 kb
F1
R1
PEPCK 2
Ldbpk.27.1805
R2
PEPCK 1
PEPCK 2
+
Ble donor
Ble donor
Ble donor
F2
R2
F1R1: PEPCK specific primers
F2R2: DDNA specific primers
C
WT
PEPCK KO
PEPCK AB
1000bp
717bp
D
WT
PEPCK KO
1500bp
925bp
E
ns
**
150
100
50
0
PEPCK mRNAexpression
(normalised to 18srRNA)
WT
PEPCK KO
PEPCK AB
F
WT
PEPCK KO
PEPCK AB
58kDa

***Figure 21 Generation and confirmation of PEPCK KO and PEPCK AB L. donovani.***

Plasmid scheme showing the insertion of two-gRNA complementary to the *PEPCK* gene within the *Leishmania* plasmid, pLDCN (A). PEPCK deletion strategy using the CRISPR-Cas9, expression of Cas9 and the gRNA initiates a cleavage towards the end of both copies of the PEPCK loci (red arrows). Introduction of the bleomycin resistance gene cassette within the cleaved site generates mutant parasites. Confirmation of PEPCK deletion was made by PCR using PEPCK specific primers (F1R1) and insertion of the Bleomycin resistance gene confirmed by PCR the bleomycin specific primers (F2R2) (B). PCR analysis confirmed PEPCK deletion by the absence of PEPCK PCR product (717bp) which was restored in the PEPCK AB parasite (C). PCR analysis confirms the successful insertion of the bleomycin resistance gene cassette PCR product (925bp) in the PEPCK KO parasite which was absent in the WT parasites (D). Absence of DLD mRNA expression in the DLD KO parasites was confirmed by RT-qPCR and restored in the DLD AB parasites (E). Western blots confirm the absence of PEPCK protein expression in the PEPCK KO parasites (F) **, $p < 0.01$; ns, not significant.

### *4.2.3 PEPCK impacts L. donovani growth in axenic culture.*

Impaired growth is usually an indication for reduced virulence and infectivity in null mutants in *Leishmania*. We previously demonstrated that PEPCK is an important gluconeogenic enzyme that is critical for axenic culture proliferation of *L. major*. Absence of PEPCK in *L. major* resulted in the significantly reduced proliferation of the parasites that was completely rescued by addition of exogenous glucose into the cultures (218). To determine whether PEPCK deficiency also impairs *L. donovani* proliferation, equal numbers of WT, PEPCK KO and PEPCK AB promastigotes were grown in axenic cultures and numerical counts were performed over time using a hemocytometer. There was a significant reduction in numbers of PEPCK KO parasites compared to their WT counterpart controls (Figure 22). However, complementation of the *PEPCK* gene in the PEPCK KO parasites restored their proliferation to WT levels. This observation suggest that PEPCK is critical for proliferation of *L. donovani* in axenic cultures.

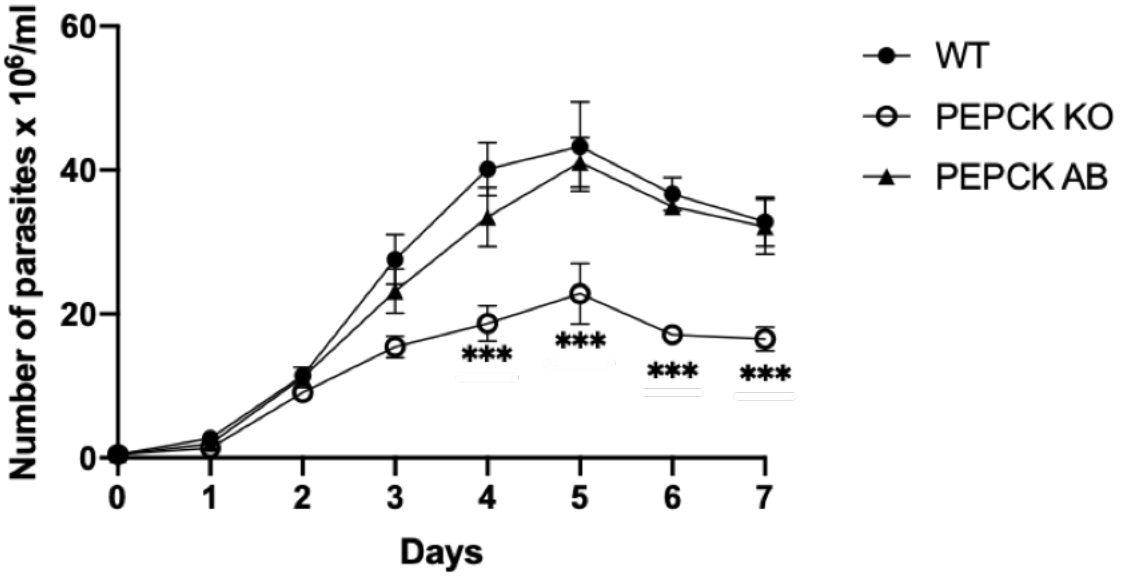



*Figure 22 Impaired proliferation of PEPCK KO L. donovani in axenic cultures.*

Equal numbers of WT, PEPCK KO or PEPCK AB *L. donovani* were grown in axenic cultures over time. At the indicated time points, parasites were counted under an optical microscope with a hemocytometer. *, $p < 0.05$; ***, $p < 0.001$.

### *4.2.4 PEPCK deficiency in L. donovani impairs parasite growth inside macrophages.*

Once, deposited in the skin, *Leishmania* infects various phagocytic cells including macrophages which are the definitive host for amastigote replication (61,302). We previously demonstrated that PEPCK deficiency in *L. major* impaired amastigote proliferation in macrophages (218). However, whether PEPCK deficiency would impair infectivity and proliferation of *L. donovani* in macrophages is unknown. Since I observed that the PEPCK deficiency in *L. donovani* impacted promastigote replication in axenic cultures, I wanted to know whether this will correspondingly impact amastigote proliferation inside macrophages. To do this, I infected bone marrow-derived macrophages (BMDM) with WT, PEPCK KO or PEPCK AB *L. donovani* and at different times,

monitored infection by staining infected cells after cytospin preparations followed by visualizations under an optical microscope (Figure 23A). The parasite numbers in cells infected with WT or PEPCK AB *L. donovani* parasites steadily increased over time demonstrating proliferation (replication) of the parasites in infected cells. In contrast, a significant reduction in the numbers of amastigotes were observed in the cells infected with PEPCK KO promastigotes compared to those infected with WT or AB counterpart controls (Figure 23B). This shows that deficiency of PEPCK in *L. donovani* impairs the ability of the parasite to proliferate inside macrophages, suggesting that it is a critical molecule for parasite virulence.

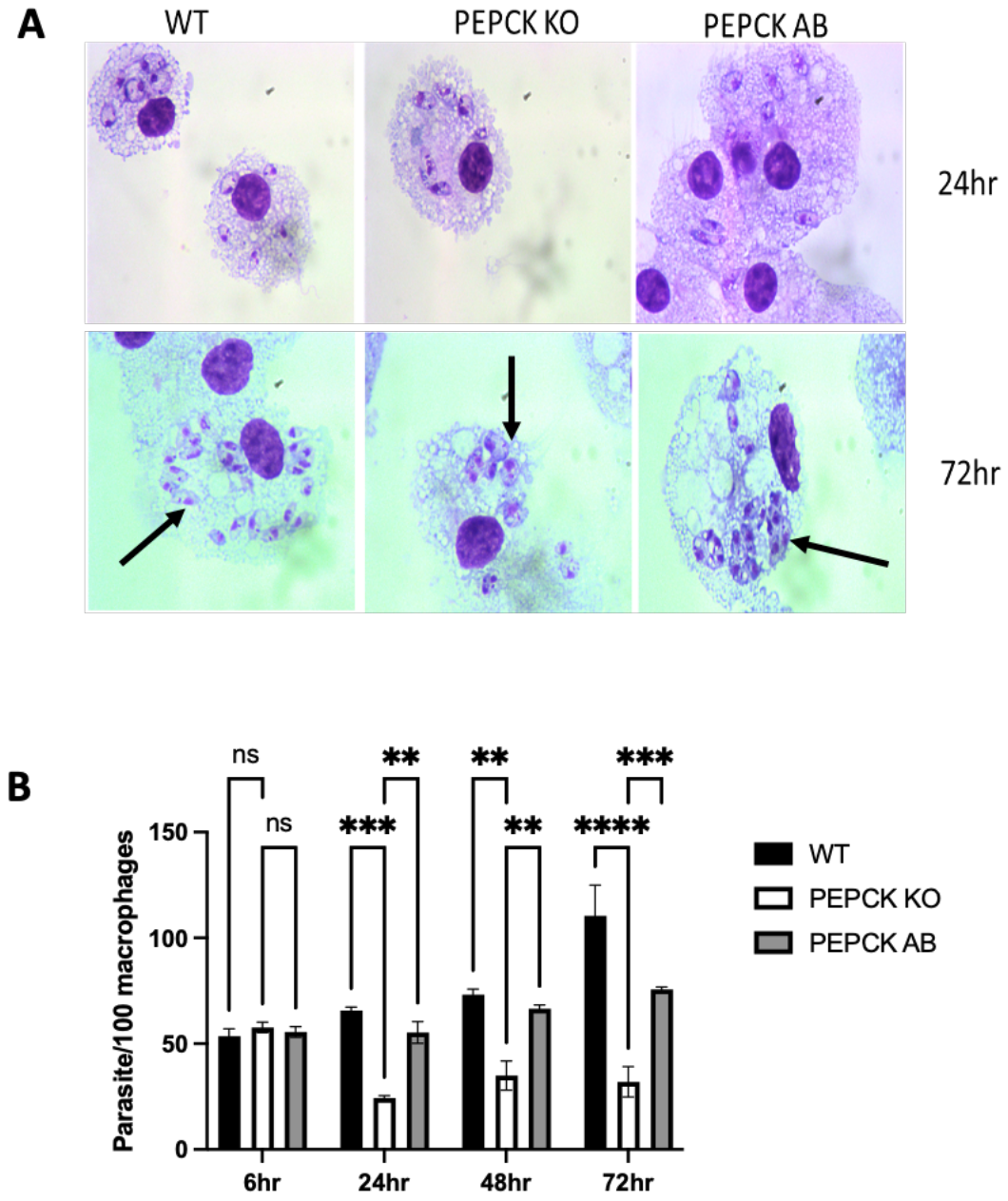


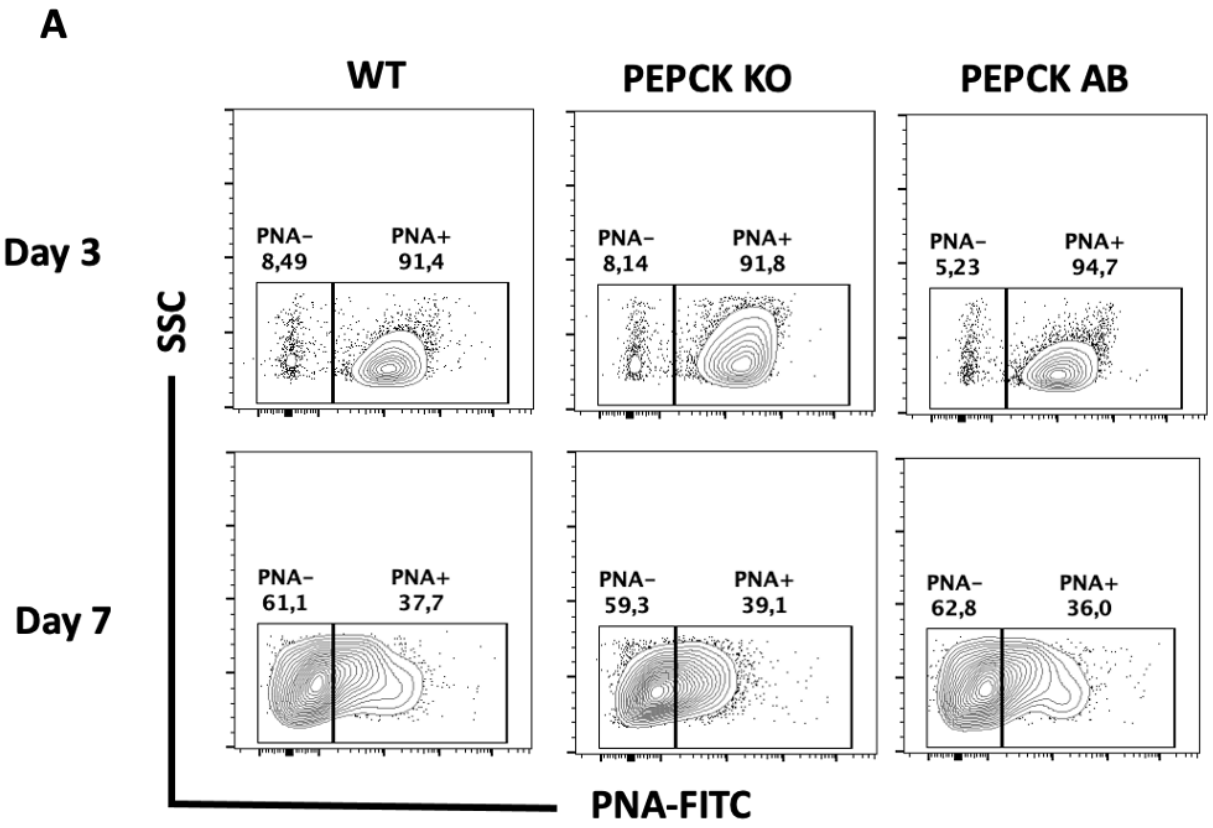

*Figure 23 PEPCK KO L. donovani was compromised inside macrophages.*

Bone marrow derived macrophages (BMDM) were infected with WT, PEPCK KO or PEPCK AB parasites in a 10:1 parasite to cell ratio. At the indicated times the giemsa stained cytospin preparations were prepared and images generated (A). Quantification of the parasite/100 macrophages (B) during the infection course were analyzed. **, $p < 0.01$; ****, $p < 0.0001$; ns, not significant.

### *4.2.5 Metacyclogenesis in PEPCK KO L. donovani was not compromised.*

*Leishmania* promastigotes undergoes a transformative process termed 'metacyclogenesis' which is characterized by alterations in the parasite's infectivity and morphology. Parasites become infective when they differentiate from procyclic parasite into metacyclic promastigotes (581). This transformation in the parasites is attributed to differences in the *major* surface glycoconjugate, lipophosphoglycan (LPG), whose repeating units are 2 folds longer in the metacyclic forms than in the procyclic parasites (582). Also, a notable observation is that the LPG of procyclic promastigotes lacks the arabinose caps on the galactose residues, which are present in metacyclic promastigotes (582). This modification forms the basis for which the infective forms can be distinguished using the peanut agglutinin lectin (PNA), which freely binds the galactose residues of the LPG moiety in the procyclic promastigotes causing agglutination. The arabinose capping the glactose residues on the LPG hinders this agglutination with PNA in the metacyclic promastigotes (583). Although, metacyclogenesis is a phenomenon occurring in the sandfly, this can be replicated in *in vitro* cultures (600). Logarithmic-phase promastigotes are parasites grown typically at day 3 of axenic cultures and under a microscope they move speedily, have rounder body that resemble the procyclic parasites in the sandfly midgut. As parasites progress to stationary phase of growth typically on day 7 of axenic culture, their movement is reduced and under a microscope, they have a narrow-slender body with a long flagella (longer than those from procyclic parasites), which resembles the metacyclic promastigotes found in the sandfly (571). Hence, I wanted to know whether the deficiency of PEPCK in L. *donovani* impacted their ability to differentiate into their infective forms. To do this, I stained WT, PEPCK KO and PEPCK AB L.

*donovani* logarithmic (day 3) and stationary (day 7) phase promastigotes with FITC-conjugated PNA reagent and assessed the PNA binding capacity of the parasites from signals detected by flow cytometry. Increase in the PNA-negative populations from day 3 to day 7 of axenic growth were observed with all the parasite lines stained with PNA-FITC (Figure 24A). However, there was no difference in the PNA-negative populations of all the parasite lines grown for 7 days (Figure 24A). Examination of the parasite lines stained with giemsa under the microscope at day 3 and 7 of axenic culture growth showed similar structural morphologies (Figure 24B), with the logarithmic phase WT, PEPCK KO or PEPCK AB parasites having rounded bodies which became slender with an extended flagella in their stationary phase of growth (Figure 24B). This suggests that PEPCK deficiency in *L. donovani* did not impact metacyclogenesis or the parasites' ability to differentiate into infective forms.


A
WT
PEPCK KO
PEPCK AB
Day 3
Day 7
SSC
PNA-FITC
PNA–
8,49
PNA+
91,4
PNA–
8,14
PNA+
91,8
PNA–
5,23
PNA+
94,7
PNA–
61,1
PNA+
37,7
PNA–
59,3
PNA+
39,1
PNA–
62,8
PNA+
36,0

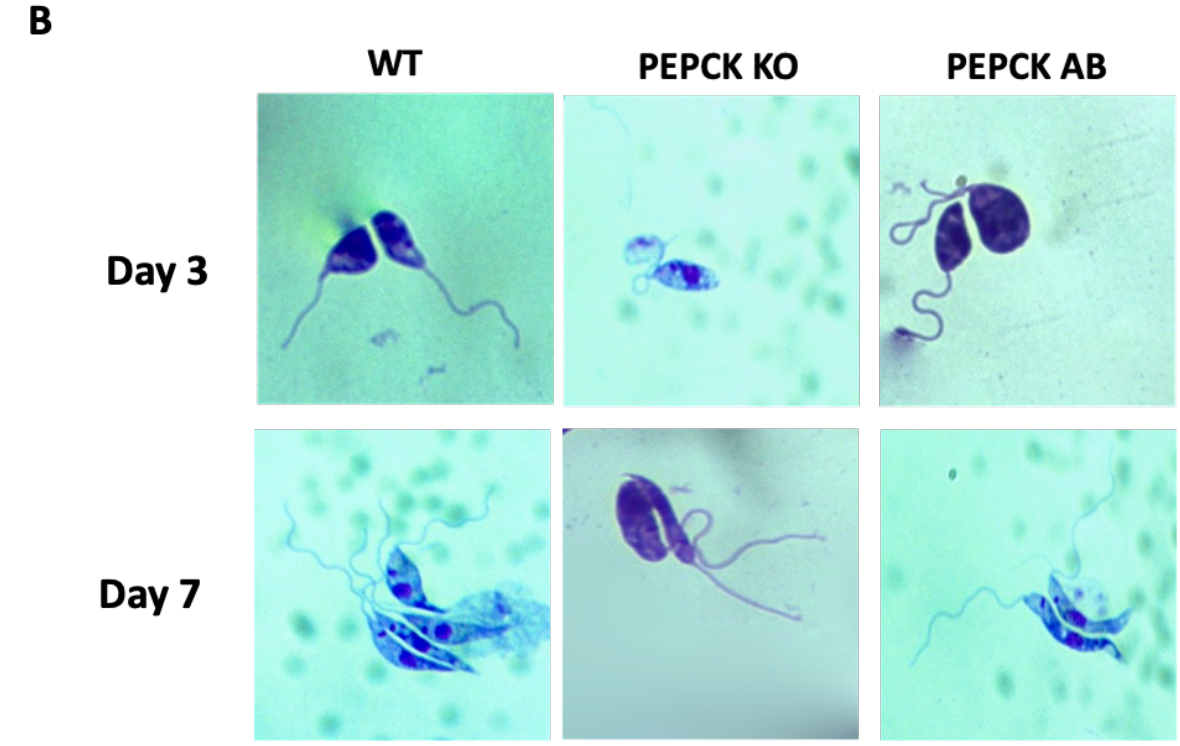

B
WT
PEPCK KO
PEPCK AB
Day 3
Day 7

***Figure 24 PEPCK deficiency in L. donovani did not affect their ability to differentiate to their infective forms.***

WT, PEPCK KO and PEPCK AB L. *donovani* parasites grown in axenic cultures at the logarithmic phase (day 3) and stationary phase (day7) were stained with PNA-FITC and differentiated infective forms were assessed in the PNA- gates by Flow cytometry (A). The morphology of the giemsa-stained parasites grown on day 3 and day 7 was observed under an optical microscope (B).

### *4.2.6 Spleen and liver weights from mice infected with PEPCK KO L. donovani were not different.*

VL is an organ-specific systemic disease affecting the bone marrow, liver, and spleen. The mechanisms by which the spleen and bone marrow becomes chronically infected during VL is still not understood. It has been reported that in experimental VL in mice, the spleen becomes enlarged taking up about 15% of the animal's body weight (601). Although, the disease in liver is known to be self-resolving, the enlargement of the liver (hepatomegaly) is the most common finding associated with VL (602) because it is characterized in over 30-100% of parasitized individuals (263). Since PEPCK deficient *L. donovani* was impaired in proliferation inside macrophages, I wanted to know whether PEPCK affects splenomegaly and hepatomegaly in mice. I infected BALB/c mice with 5 x $10^7$ WT, PEPCK KO or PEPCK AB stationary phase promastigotes intravenously through the retroorbital plexus. At the indicated time points, the infected mice were sacrificed, the organs were harvested and weighed on a scale. Over time, the spleen and liver weights slightly increased in infected mice (Figure 25A). However, it was surprising that there were insignificant differences in liver (Figure 25A) and spleen (Figure 25B) weights amongst

all the groups at the indicated time points. This suggests that PEPCK may not be critical for inducing splenomegaly and hepatomegaly during *L. donovani* infection.

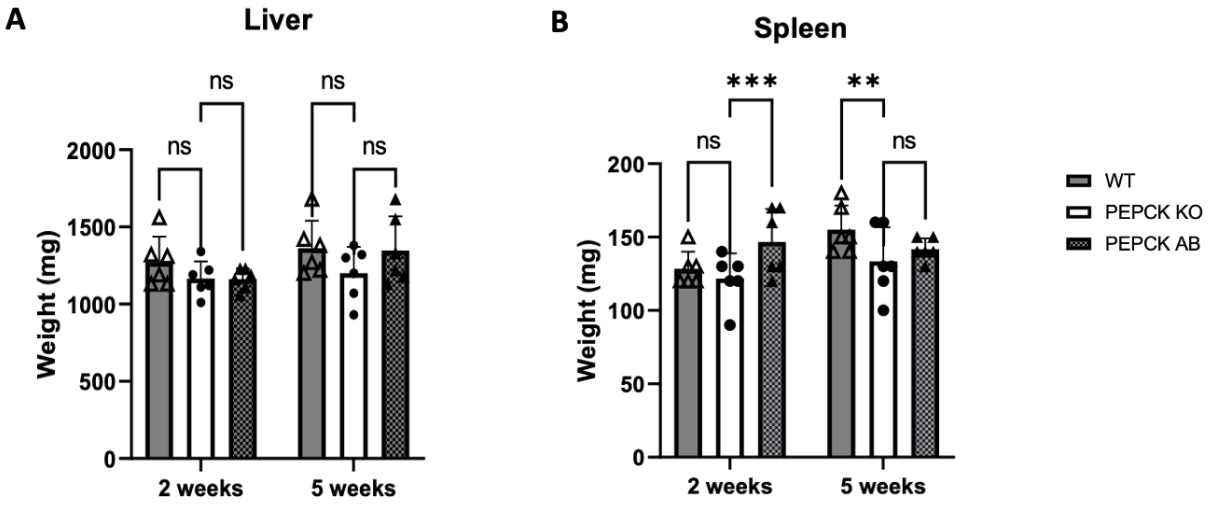



***Figure 25 PEPCK deficiency did not dramatically impact splenomegaly and hepatomegaly.***

BALB/c (6-8 mice per group) was retro-orbitally infected intravenously with 50 X $10^6$ WT, PEPCK KO or PEPCK AB stationary phased L. *donovani* promastigotes. At the indicated times, the mice were sacrificed, and liver (A) and spleen (B) were weighed. This result is representative of 3 independent sets of experiments with similar results. **, $p < 0.01$; ***, $p < 0.001$; ns, not significant

### *4.2.7 Parasite burdens in the spleen and liver of mice infected with PEPCK KO L. donovani were not different from those infected with WT or AB parasites.*

Increased parasitemia results in enhanced cellular recruitment which is associated with the enlargement of the liver and spleen during VL (263). Although PEPCK deficiency was observed to impair proliferation of *L. donovani* promastigotes and amastigotes *in vitro*, I wanted to know whether the proliferation of *L. donovani* will be impacted in their respective organs *in vivo*. I infected BALB/c mice with 5 x $10^7$ WT, PEPCK KO or PEPCK AB stationary phase promastigotes intravenously through the retroorbital plexus and at the indicated time points, sacrificed the

infected mice and assessed parasite burden in the liver and spleen by limiting dilution. Generally, the parasite burdens in the liver from all the mice groups were decreased over time which is an indication of a resolving infection (Figure 26A). However, there was no significant difference in parasite burden in liver from mice infected with PEPCK KO compared to those infected with their WT or PEPCK AB counterpart controls (Figure 26A). As expected, in the spleen parasite burden from all mice groups increased overtime which is an indication of chronicity of the disease. However, akin to the liver, there was insignificant differences in the parasite burden from mice infected with PEPCK KO parasites compared to their WT or PEPCK AB infected counterpart controls. Consistent with the parasite burden in the liver and spleen, the number of cells in liver and spleen tissues was not significantly different amongst all mice groups (Figure 26 C&D), suggesting similar inflammatory responses. To confirm that the parasite load obtained by limiting dilution were not misleading, I also assessed parasite burden in the homogenized spleen and liver tissues in infected mice by real time PCR. At 5 weeks post infection, there was insignificant differences in the amount of *Leishmania* kinetoplast DNA in the liver and spleen from mice infected with PEPCK KO L. *donovani* comparable to their WT or PEPCK AB infected counterpart controls (Figure 26E &F). Taken together, these findings indicated that deficiency of PEPCK in *L. donovani* does not impair its ability to cause disease *in vivo*.

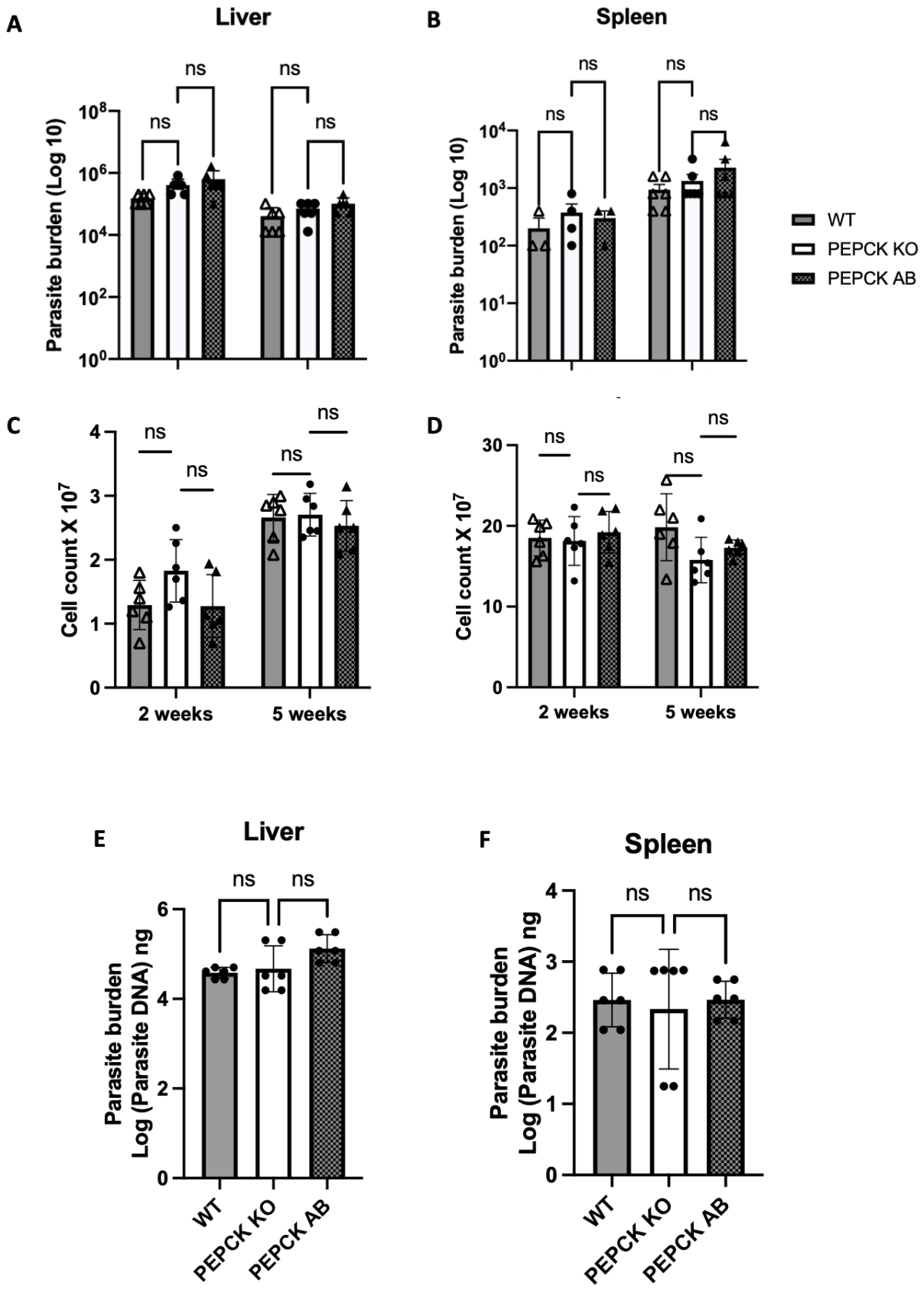

A
Liver
Parasite burden (Log 10)
ns
B
Spleen
WT
PEPCK KO
PEPCK AB
C
Cell count X 10^7
2 weeks
5 weeks
D
E
Liver
Parasite burden Log (Parasite DNA) ng
F
Spleen

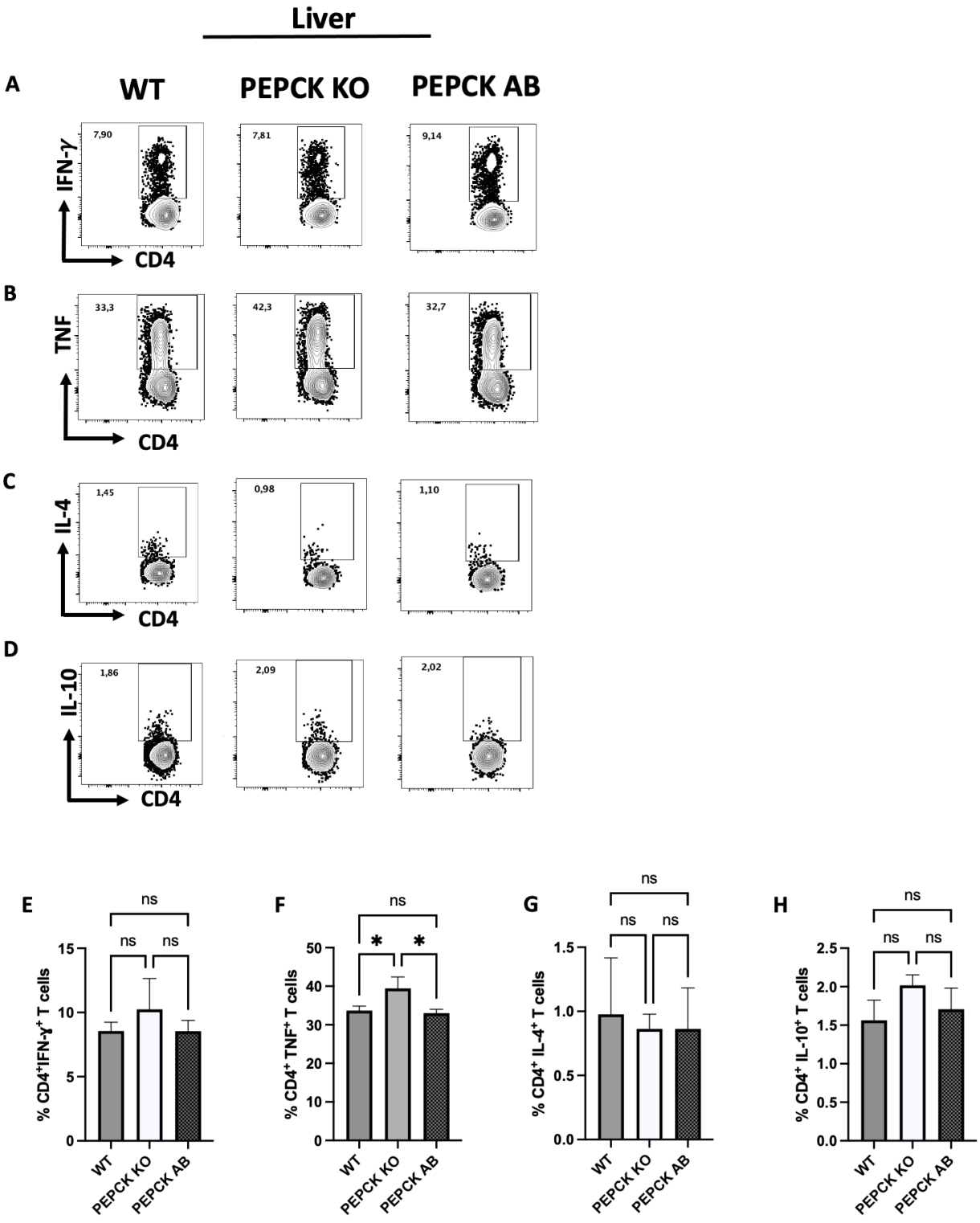
***Figure 26 Parasite burden kinetics in the spleen and liver from mice infected with the L. donovani parasite lines.***

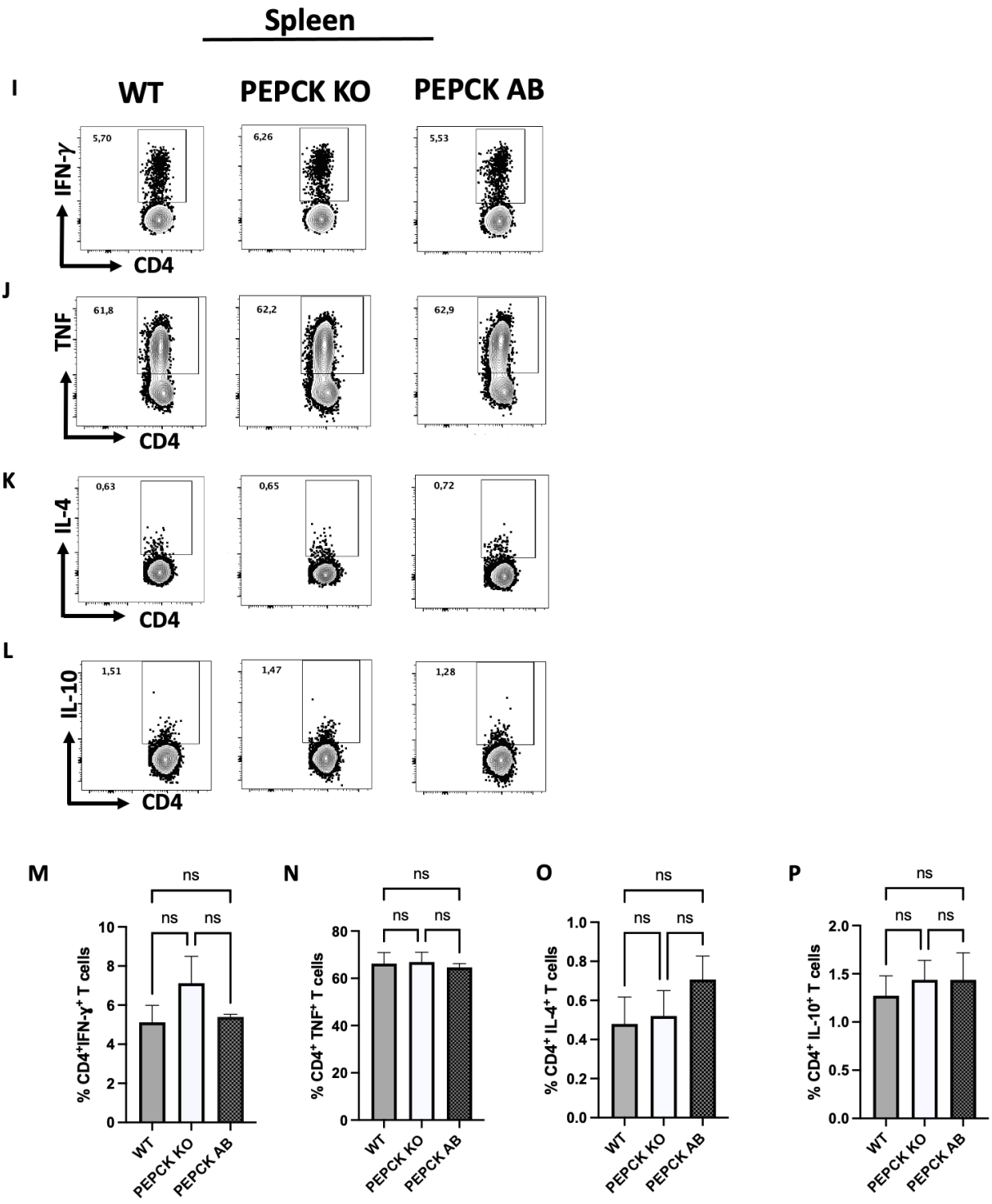
BALB/c (6-8 mice per group) was retro-orbitally infected with 50 X $10^6$ WT, PEPCK KO or PEPCK AB stationary phased L. *donovani* promastigotes. At the indicated times, the mice were sacrificed and parasite burdens in the liver (A) and spleen (B) were assessed by limiting dilution. At the indicated times, the total cell counts in the liver (C) and spleen (D) were made. At 5 weeks post infection, parasite burdens in the liver (E) and spleen (F) were assessed by absolute quantitative real time PCR. This result is representative of 3 independent sets of experiments with similar results. ns, not significant

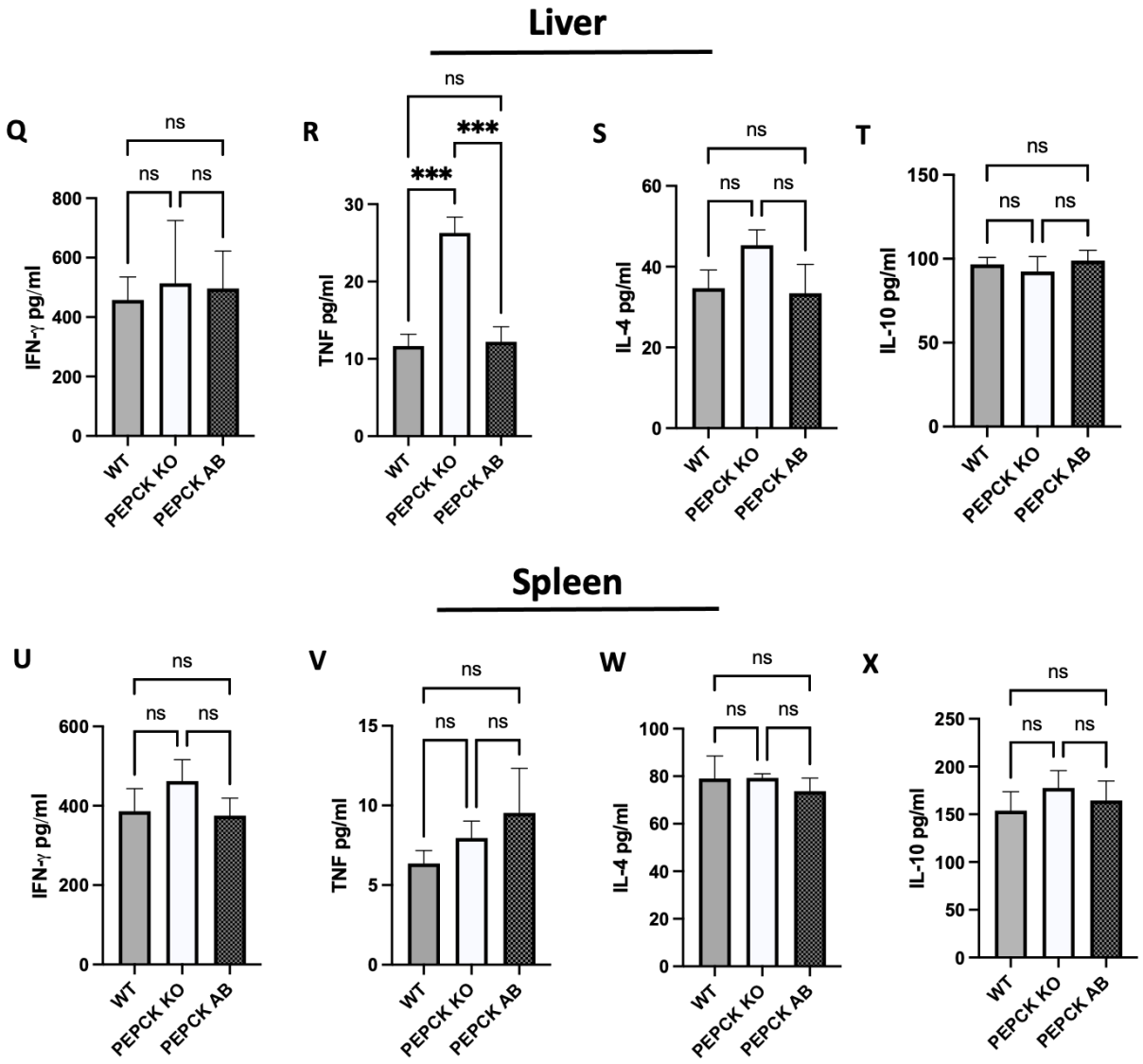
### *4.2.8 Immune responses in the liver and spleen from mice infected with PEPCK KO L. donovani were not affected.*

Susceptibility and resistance to VL is dependent on varying factors including the quality of the immune responses which can be associated with the IFN-γ and TNF-mediated macrophage activating or IL-10 and IL-4-mediated macrophage deactivating cytokines (603). The *major* producers of these cytokines are T cells. Since deficiency of PEPCK in *L. donovani* was not associated with higher parasite loads in the liver and spleen, I wanted to assess whether the immune responses in these organs remained unaffected. I infected BALB/c mice with 5 x $10^7$ WT, PEPCK KO or PEPCK AB stationary phase promastigotes intravenously through the retroorbital plexus and at 5 weeks post-infection, I assessed the frequency of cytokine (IFN-γ, TNF, IL-10 and IL-4) producing $CD4^+$ T cells in the liver and spleen of infected mice. The frequency of IFN-γ, IL-10 and IL-4 producing $CD4^+$ T cells in the liver of mice infected with PEPCK KO parasites was comparable to those infected with WT or PEPCK AB controls (Figure 27A-H). In contrast, the frequency of TNF-producing $CD4^+$ T cells was significantly higher in the liver of the mice infected with PEPCK KO parasites compared to those infected with WT or PEPCK AB controls (Figure 27B

& F). However, in the spleen, the frequency of IFN-γ, TNF, IL-10 and IL-4-producing $CD4^+$ T cells from mice infected with PEPCK KO parasites remained comparable to those infected with WT and PEPCK AB controls (Figure 27I-P). Next, I stimulated cells from spleen and liver of infected mice with freeze-thawed *Leishmania* antigen for 3 days and assessed the levels of cytokines secreted in the culture supernatant fluids by multiplex cytokine ELISA. Except for TNF, there was no significant difference in IL-10, IL-4 or IFN-γ secreted from the isolated liver lymphocytes of mice infected with PEPCK KO parasites in comparison to those infected with WT or PEPCK AB controls, consistent with flow cytometric data (Figure 27Q-T). TNF secreted from the liver lymphocytes in PEPCK KO infected animals were significantly higher than those from the WT or PEPCK AB infected controls (Figure 26R). There was no significant difference in the level of cytokines secreted by splenocytes in mice infected with PEPCK KO compared to those from WT or PEPCK AB infected controls (Figure 27U-X). I also confirmed these observations by assessing mRNA levels of these cytokines in homogenized liver and spleen tissues by RT-qPCR. Consistent with the flow cytometry results, the mRNA expression of IFN-γ, IL-10 and IL-4 from the liver of mice infected with PEPCK KO parasites remained comparable to those from mice infected with WT or PEPCK AB parasites (Figure 27Y). Again, the fold change in the TNF mRNA expression from the liver of PEPCK KO infected animals was significantly higher than those from WT or PEPCK AB infected controls (Figure 27Y). As with flow cytometry and ELISA, there was no significant difference in the mRNA expression of all the cytokines tested in spleen tissues amongst all the groups (Figure 27Z). Taken together, these results suggest that deficiency of PEPCK does not affect immune responses to *L. donovani* infection in both the spleens and liver.


Liver
A
WT
PEPCK KO
PEPCK AB
IFN-γ
CD4
7,90
7,81
9,14
B
TNF
CD4
33,3
42,3
32,7
C
IL-4
CD4
1,45
0,98
1,10
D
IL-10
CD4
1,86
2,09
2,02
E
% CD4+IFN-γ+ T cells
ns
ns
ns
WT
PEPCK KO
PEPCK AB
F
% CD4+ TNF+ T cells
ns
*
*
G
% CD4+ IL-4+ T cells
ns
ns
ns
H
% CD4+ IL-10+ T cells
ns
ns
ns


Spleen
I
WT
PEPCK KO
PEPCK AB
IFN-γ
CD4
5,70
6,26
5,53
J
TNF
CD4
61,8
62,2
62,9
K
IL-4
CD4
0,63
0,65
0,72
L
IL-10
CD4
1,51
1,47
1,28
M
% CD4+IFN-γ+ T cells
N
% CD4+ TNF+ T cells
O
% CD4+ IL-4+ T cells
P
% CD4+ IL-10+ T cells
ns
WT
PEPCK KO
PEPCK AB


Liver
Q
IFN-γ pg/ml
800
600
400
200
0
ns
ns
ns
WT
PEPCK KO
PEPCK AB
R
TNF pg/ml
30
20
10
0
ns
***
***
S
IL-4 pg/ml
60
40
20
0
T
IL-10 pg/ml
150
100
50
Spleen
U
V
W
X

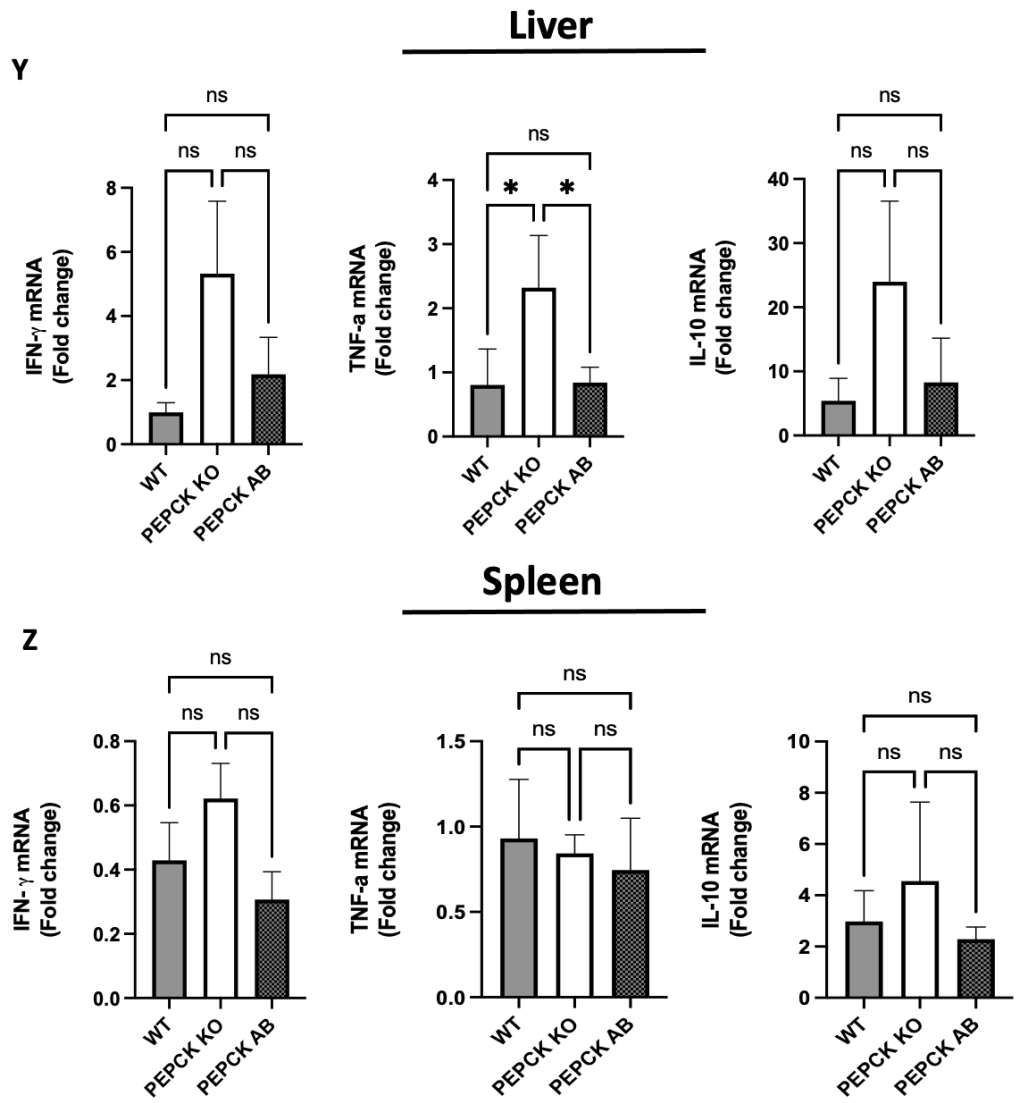



***Figure 27 PEPCK deficiency in L. donovani does not blunt the host immune responses.***

BALB/c mice (6-8 mice per group) were infected in the footpad with stationary phased 50 x$10^6$ WT, PEPCK KO or PEPCK AB L. *donovani* parasites and after 5 weeks, mice were sacrificed and the liver and spleen were harvested. The cells from the Liver (A-H) and spleen (I-P) were stimulated with PMA, ionomycon and brefeldin cocktail for 4hrs and further stained to assess the frequency of IFN-$\gamma$, TNF, IL-4 and IL-10 producing $CD4^+$ T cells by flow cytometry (E-H, M-P). Some of the cells from the liver (Q-T) and spleen (U-X) were restimulated with freeze-thawed *Leishmania* antigen and after 3 days, the cytokines accumulated in the culture supernatant were assessed by multiplex cytokine profiling. The fold change in the mRNA of the cytokines from homogenized liver (Y) and spleen (Z) in the infected mice were assessed by RTPCR. This result is representative of 3 independent sets of experiments with similar results. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$

### *4.2.9 PEPCK KO L. donovani infected in Kupffer cells poorly proliferated in vitro.*

We previously found that PEPCK is critical for virulence for *L. major* because its deficiency leads to attenuation of disease and blunted immune response in mice (218). Therefore, it was surprising that deficiency of PEPCK in *L. donovani* did not affect disease outcome and host immune responses in mice (Figure 26 & 27). It is widely established that *in vitro* systems differ remarkably from *in vivo* systems, and in some cases scientific findings are preferably confirmed by combined *in vivo* and *in vitro* data (604). These contrasting observations with PEPCK KO L. *donovani in vitro* and *in vivo* suggest that the *in vivo* microenvironments in mice may contribute to the lack of difference between WT and PEPCK KO parasites. Interestingly, the liver-spleen axis is important for coordinating numerous processes such as immune regulation, metabolism, and blood filtration especially in diseased states (605,606). It has been reported that the Liver-spleen axis share similar metabolic activities (605,606). The spleen is the main organ that supplies IL-16 critical for maintaining the hepatic growth factor levels and basal metabolic rates (607). Indeed, glucose metabolic rates in the spleen and liver are similar which is mediated by insulin (608). Thus, I hypothesized that a unique Liver-spleen axis microenvironment spleen may have rescued the proliferative potential of PEPCK KO parasites in infected mice. Therefore, I decided to check the proliferation of PEPCK-deficient parasites in isolated liver macrophages following *in vitro* infection. I isolated liver macrophages (Kupffer cells) *in vitro*, infected them with WT, PEPCK KO or PEPCK AB parasites in a 10: 1 parasite to cell ratio and monitored infection after 48 hours by Giemsa staining of cytospin preparations. The quality of the isolated Kupffer cells (KCs) was confirmed by staining and assessing for specific KC markers like Clec4F by Flow cytometry (Figure

28A). At 48 hours post infection, parasite numbers in the KCs infected with PEPCK KO parasites was significantly reduced compared to those infected WT and PEPCK AB controls. This observation suggests that the microenvironment of the liver may have altered the macrophages, which could account for the differences observed in the proliferation of PEPCK KO parasites *in vitro* and *in vivo*.

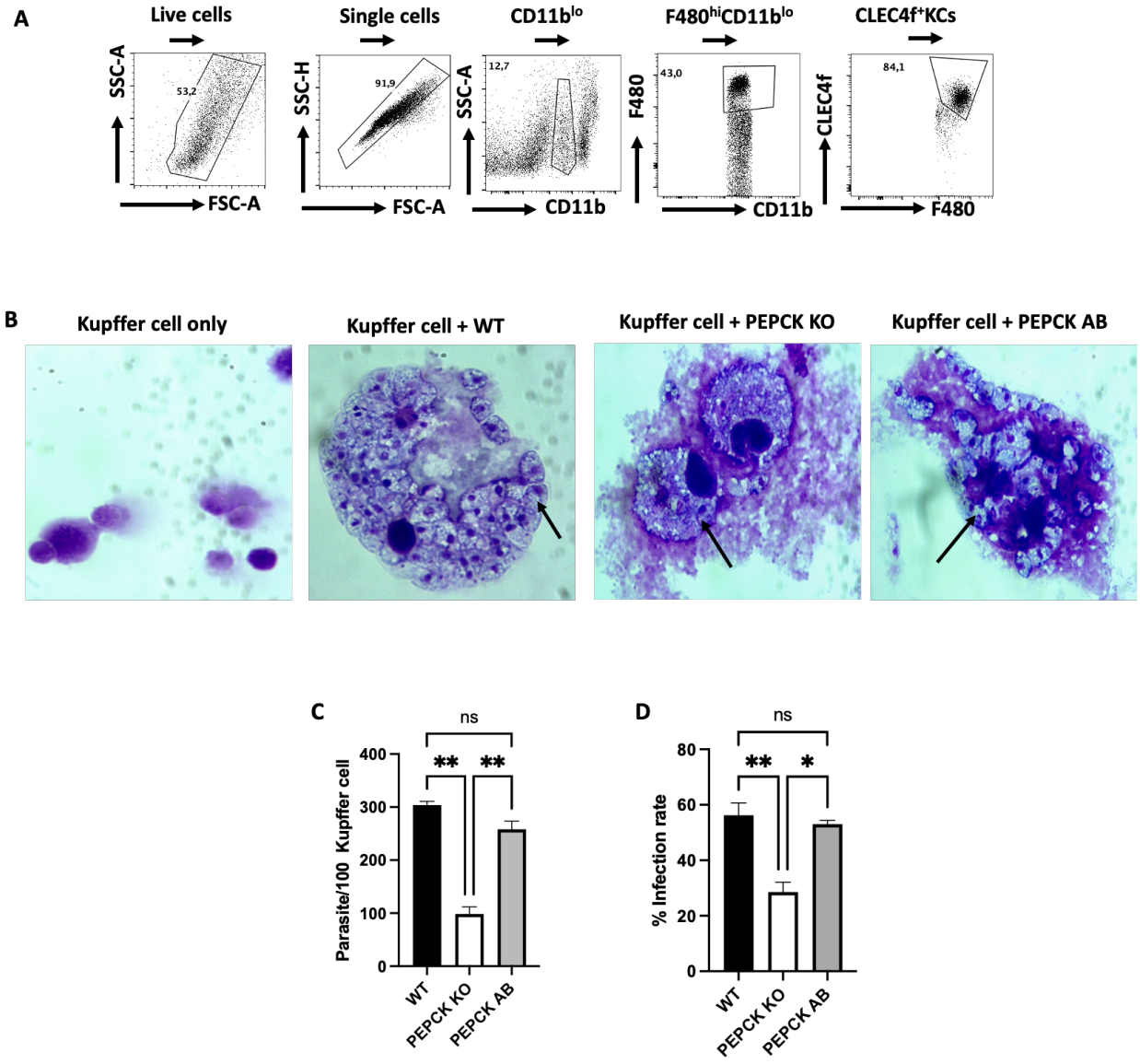



***Figure 28 PEPCK deficient L donovani was compromised inside Kupffer cells isolated in vitro.***

Kupffer cells were isolated from C57BL/6 mice and infected with stationary phased WT, PEPCK KO or PEPCK AB L. *donovani* promastigotes in a 10: 1 parasite to cell ratio. Gating strategy showing the phenotypic characterization of the isolated KCs by flow cytometry (A). After 6hours post infection, uninfected parasites were washed off and incubated for 48hours and giemsa stained cytospin preparations of infected infected cells were made to produce images observed in an optical microscope (B). Quantification of the parasite/100 macrophages (C) and percentage infection rate (D) during the infection course were analyzed. *, $p < 0.05$; **, $p < 0.01$; ns, not significant.

***Summary 2:***

Visceral Leishmaniasis is the most fatal and life-threatening form of Leishmaniasis and urgent therapeutic and vaccination strategies will help reduce the global transmission of the disease. Understanding the antigen responsible for protective responses during *Leishmania* infection is critical for a vaccine design. Peptides derived from *Leishmania*'s Phosphoenol pyruvate carboxylase kinase ($PEPCK_{335-351}$) induced protective responses during Leishmania infection (510). Although the role of PEPCK to cause cutaneous leishmaniasis has been previously elucidated (218), yet the role of PEPCK during visceral leishmaniasis is currently unknown. I successfully performed and confirmed the deletion of the *PEPCK* gene in *Leishmania donovani* (causative parasite for visceral leishmaniasis) and assessed the impact of the deletion on the parasite virulence and host immune response. PEPCK deficient *L. donovani* parasites had impaired proliferation in axenic culture, inside Bone marrow derived macrophages and Kupffer cells in vitro. The ability for the gene-deficient parasites to differentiate into their infective forms was not hampered. However, inside mice, PEPCK deficient *L. donovani* parasites still caused pathology because spleen and liver weights and parasite burden were comparable to the WT and complementary control counterparts. In mice infected with PEPCK deficient *L. donovani*, the immune response remain unchanged compared to the WT and complementary control groups.

# 5. CHAPTER 5: Discussion

## 5.1 The Role of DLD in CL

Although leishmaniasis is a neglected tropical disease, it is still a growing public health concern with increasing incidences and mortalities which needs heightened attention. It is vital to implement strategies that will control disease progression and transmission. Understanding the mechanisms of resistance and susceptibility to *Leishmania* infection is critical for developing novel therapeutic approaches including development of new drugs and vaccines. Protection during cutaneous leishmaniasis is primarily associated with the induction of IFN-γ-producing $CD4^+$ T cells that activate parasitized macrophages leading to destruction of parasites. On the contrary, susceptibility is associated with the production of anti-inflammatory cytokines like IL-4 and IL-10, which deactivates infected macrophages and limit their ability to clear parasites. Although there is currently no approved vaccine against human forms of the disease, identifying the antigens that drive protective immune responses during *Leishmania* infection is critical for identifying new vaccine targets. One of such antigens is Dihydrolipoyl dehydrogenase (DLD), which is a key metabolic enzyme in *Leishmania*. During *Leishmania major* infection, a peptide derived from DLD, $DLD_{63–79}$, induced the expansion of DLD-specific polyfunctional $CD4^+$ T cells that produce protective cytokines including IFN-γ and TNF. The expansion of these cells was followed by classical contraction and stable maintenance of memory T cell that induce protective immunity following secondary infection (511). However, the role of DLD in *Leishmania major* was not known.

One of the aims of this thesis was to understand the impact of DLD on virulence and immunopathology of *Leishmania major* following infection in mice. Using the powerful gene

editing tool, CRISPR/Cas9, I successfully deleted *DLD* gene in *L. major* and confirmed this deletion by the absence of the DLD amplicon in PCR analysis. Further validation of DLD deficiency in *L. major* was made in mice by demonstrating the lack of expansion of DLD specific $CD4^+$ T cell in infected. I found that DLD deficiency resulted in impaired proliferation of *L. major* promastigotes and amastigotes *in vitro*. The ability of these parasites to differentiate into their infective forms were unhindered. Furthermore, I showed that DLD is a virulence factor in *L. major* because its absence leads to attenuated disease phenotype as manifested by lack of lesion development and significantly reduced parasite burden at the infection sites. In addition, deficiency of DLD resulted in blunted host immune responses in infected mice. I found that the blunting of the host immune responses was most likely related to impaired proliferation of the DLD deficient parasites in mice and may not be due to the immunodominance of the antigen. Short-term vaccination with DLD deficient parasites protected vaccinated mice against virulent WT parasites challenge as evidenced by a reduced parasite burden, and this was associated with the induction of strong protective immune responses. Finally, I found that the DLD deficient parasites had impaired mitochondrial metabolism and glycolytic capacity, which may be associated with the parasites impaired proliferation (Figure 29).

Whole genome sequencing in *Leishmania* has made it possible to explore the numerous gene editing platforms available to dissect the functions of putative or hypothetical genes (452). The use of homologous recombination in gene editing in *Leishmania* was limited due to the random homologous insertions that occur and the fact that point mutations were impossible with this method (609). RNA inference using small regulatory RNAs is critical for downregulating gene expressions to assess and validate gene functionality (534). However, mechanisms for RNA

interference (RNAi) are evolutionarily lost with most eukaryotes including *Leishmania* species (534). Interestingly, the development and optimization of RNAi in the *Vianna* subspecies of *Leishmania* has been described (610). CRISPR/Cas9 is one of foremost gene editing platforms available today. Multiple studies have demonstrated the effectiveness of this tool in directing point mutations or deleting multiple or single genes in leishmania (119,212,533,540,542). In my studies, I used this tool to delete the single copy of *DLD* gene located on chromosome 32 in *L. major*. I introduced the CRISPR system containing complementary gRNA and Cas9 proteins into the logarithmic phase *L. major* promastigotes by electroporation which induced a cleavage between both ends of the *DLD* loci (Figure 9B). Absence of DLD amplicon in DLD KO *L. major* (Figure 9D) or DLD mRNA expression (Figure 9F) was confirmed by PCR or RT-qPCR analysis, respectively. A cleavage break within the DNA in any cell sets the stage for innate DNA repair mechanisms. In *Leishmania*, the microhomology-mediated end joining (MMEJ) or the single-stranded annealing (SSA) is normally initiated as a response to DNA damage but these repair mechanisms are error-prone, introducing short homology sequences or random nucleotides within the cleavage site (536,537). However, to limit the induction of an uncontrolled mutation that could arise with the DLD CRISPR/Cas9 -edited parasites, I introduced the Bleomycin resistance gene cassette that shares homology arms of the cleavage sites. This homology directed repair (HDR) mechanism allowed for the generation of a controlled DLD deficient mutant because PCR analysis confirmed the presence of the Bleomycin amplicon (Figure 9E). In *Leishmania*, HDR is a valuable repair process that increases the efficiency and specificity of the CRISPR/Cas9 editing systems (533,542).

One of the downsides in using CRISPR/Cas9 editing technology is the tendency to create off target effect or mismatches (544,545). Sometimes, these off target effect could be dependent on gRNA bearing similarities to other regions of the genome. In some situations, off-target effects are independent of gRNA mismatches (546,547). To eliminate the idea that the CRISPR/Cas9 targeting system resulted in a phenotype associated with an off-target activity, it was necessary to add back the WT *DLD* gene to complement the DLD KO mutant and see whether restoration of wild type phenotype in the complementary control add back is achieved (578,611). In this study, I generated the complementary add-back by episomally expressing WT *DLD* gene contained in a *Leishmania* expression vector (Figure 9C) into the DLD KO parasites. Episomal expression is considered to be efficient because the episomes contain antibiotic resistance markers such that when the concentration of the drug is raised, the copy number of the gene increases to enhance protein expression (609). I confirmed the presence of the *DLD* gene amplicon in the DLD complementary add backs (DLD AB) by traditional PCR analysis (Figure 9D), and mRNA expression was confirmed by RT-PCR (Figure 9F). The restoration of the DLD gene amplicon and the DLD mRNA expression in the DLD AB parasites suggest that the WT phenotype will be restored with these parasites.

I validated DLD deletion *in vivo* by infecting the C57BL/6 mice with the parasite lines (WT, DLD KO and DLD AB *L. major*) and assessed the expansion of DLD specific $CD4^+$ T cells using DLD tetramer reagent by flow cytometry (Figure 10A). This DLD tetramer I-$A^b$-$DLD_{63–79}$ has been previously shown to identify the expansion of DLD specific $CD4^+$ T cells at clonal levels (511) during *Leishmania* infection. The spleens and dLN of mice infected with WT or DLD AB *L. major* parasites contained similar numbers of DLD specific $CD4^+$ T cells at 4 weeks post-infection (Figure

10B &C). In contrast, spleens and dLN of mice infected with the DLD KO parasites had no detectable numbers of DLD-specific $CD4^+$ T cells, indicating that the inability of the DLD-specific $CD4^+$ T cells to expand in these mice was due to loss of DLD gene product in the knock out strain, thereby confirming the deletion of *DLD* gene in this strain.

*Leishmania* promastigotes growth in axenic cultures is targeted to depict parasite development in the insect's gut. To become procyclic promastigote, the parasite must rapidly divide in axenic cultures containing M199 media supplemented with serum. This stage of parasite growth is regarded as the logarithmic phase of growth. At day 6 or 7 of axenic culture growth, a higher population of parasites morph into a more infective form referred to as metacyclic promastigotes in a developmental process called metacyclogenesis. This stage of parasite growth is also referred to as stationary phase promastigotes (612,613). In this study, the growth progression of WT, DLD KO or DLD AB parasites in axenic cultures were monitored over time starting with similar parasite numbers. As expected, DLD AB and WT parasite growths were comparable at all times tested. In contrast, DLD KO parasites proliferated significantly lower than those of their control counterparts (Figure 11), suggesting that the targeted loss of DLD, (which is a key enzyme for energy metabolism in *Leishmania,* (512)) slowed the parasites proliferative capacity in axenic culture. The proliferation of the DLD KO parasites was impaired at their logarithmic phase (day 3) as well as in their stationary phase (day 6-7) of growth.

Sandfly vector control is a critical strategy to reduce disease transmission. Since, both metacyclic and procyclic DLD KO promastigotes were impaired in axenic cultures, this observation suggests injection of these parasites by the sandfly vector may result in proliferative deficiencies and development into infective metacyclic promastigotes. However, this remains to be tested in

transmission studies. Because my studies did not assess the impact of DLD deficiency in *L. major* inside the sandfly vector, how this deficiency affects parasite development inside the gut of the insect calls for further investigation. Water labelling studies showed that axenically grown amastigotes *in vitro* grew slower than their promastigote counterpart (614) (615). However, since amastigotes and not promastigotes are the culprits for cutaneous disease manifestations more efforts are targeted at controlling amastigote proliferation in the mammalian host instead (616). Generally, it has been established that impaired promastigote proliferation is an indication of reduced infectivity (580). Amastigote proliferation albeit at a reduced level compared to promastigotes is critical for overriding the host cell defense mechanisms such as altering the hosts ROS and reactive nitrogen species (RNS) levels, distorting the Th1/Th2 cytokine balance and modulating signaling pathways like the MAPK (617). Thus, both amastigotes and promastigote life forms should equally be considered in the control of leishmaniasis.

Interestingly, macrophages are the host cells that are mainly targeted to halt amastigote proliferation through nitric oxide mediated killing but these cells are also the incubation ground for the amastigotes to rapidly proliferate (302,339). Thus, with an impaired promastigote proliferation, it automatically indicates that the amastigote proliferation will be affected inside the macrophages. Indeed, I observed that DLD KO promastigotes were not only poorly proliferating in axenic cultures (Figure 11), they were also impaired in proliferation inside infected bone marrow derived macrophages (BMDM) in *in vitro* compared to their WT and DLD AB counterparts (Figure 12 A-C). This suggests that DLD plays a critical role not only in promastigote growth in axenic cultures, but also in establishment and proliferation of amastigotes in the mammalian host.

Amastigote metabolism is a crucial factor in parasite proliferation inside the PVs of macrophages because these parasites assume a stringent metabolic requirement needed to conserve the limited nutrient availability to prevent being killed by host cell activation or host cell apoptosis (463). One of the features of these stringent metabolic responses are the fact that the expression of amino acid or sugar transporters are significantly downregulated, which negatively impacts glycolysis, gluconeogenesis, and PPP inside the PV. Once this happens, the parasites are left with increased dependence on metabolic pathways like TCA and fatty acid $\beta$ oxidation (463). These pathways are critical for the generation of non-glucose molecules like amino acids or acetyl CoA. It was observed that when axenically grown amastigotes or lesion- derived amastigotes were inhibited with mitochondrial biogenesis inhibitors, the parasites were severely impacted, indicating that mitochondrial metabolism is an indelible requirement for the parasite survival and/or proliferation in the PV of macrophages (481).

In this thesis, DLD was seen to be critical for mitochondrial metabolism. The absence of DLD resulted in an impaired mitochondrial membrane permeability (Figure 20A). This indicates a reduction in the mitochondrial membrane potential ($\Delta\Psi m$), which combines forces with proton gradient ($\Delta pH$), to create a hydrogen ion critical for the production of ATP (618), and reactive oxygen species (ROS) which is a by-product of oxidative phosphorylation and critical for producing ATP in the electron transport chain (495). DLD KO parasites produced significantly lower ROS compared to their WT or AB parasites (Figure 20B). Interestingly, DLD is a key player in electron transfer processes via the NADH and FADH by oxidizing lipoamides. These electron carrying molecules NAD+, NADP+ and FAD+, can be generated during multiple metabolic pathways such as glycolysis, TCA cycle and oxidative phosphorylation but are extremely

important for ATP generation in the electron transport chain (619,620). Although, in this thesis I did not measure ATP production in the DLD KO parasites, these parasites were consuming more oxygen basally compared to their WT and DLD AB counterparts (Figure 20D). Despite the administration of oligomycin (inhibitor of ATP synthase), FCCP (which uncouples proton gradient and disrupts the mitochondrial membrane potential) or rotenone/antimycin A (which shuts down mitochondrial respiration by inhibiting ubiquinone oxidation) all of which contributes to impede mitochondrial respiration, DLD KO parasites demand for oxygen was increased more than those from the controls (Figure 20C). DLD is linked to ATP production and the absence of DLD in *L. major* drove the parasites to usurp other metabolic pathways where they can increase their ATP generation. One of such pathway is the anaerobic glycolytic pathway where lactate is oxidized contributing to the acidification of the extracellular milieu (599). I found that the absence of DLD in *L. major* made the parasites resort to other ATP yielding metabolic pathways because DLD deficient parasites in axenic cultures had increased acidification rates compared to their WT and AB controls (Figure 20F). Indeed, I observe that generally DLD deficient *L. major* had impaired mitochondrial membrane permeability (Figure 20A & B) using the TMRM dye. This impairment of mitochondrial permeability was confirmed by the mitostress test and seemed to have driven the DLD deficient parasites to consume more oxygen. Taken together, these observations suggest that DLD is critical for mitochondrial respiration and metabolism in *Leishmania*.

Targeting parasite metabolism is a critical therapeutic strategy that has gained significant traction in recent years. The idea behind this strategy is hinged on the fact that *Leishmania* parasites have developed an overt strategy of surviving within the toxic parasitophorous vacuoles and escaping killing mediated by the host immune responses through their stringent metabolic responses

(463,621). Metabolic enzymes are critical for establishing these stringent metabolic responses. It was established that a metabolic enzyme, transketolase in *Leishmania mexicana*, critical in the pentose phosphate pathway (Figure 6), played a significant role in this stringent metabolic response. The absence of transketolase in *L. mexicana* resulted in reduced glycolytic flux, poor glucose uptake, disruptions in central carbon metabolism and the PPP (621). Essentially, despite these deficiencies in their metabolism, the promastigotes growth in axenic cultures was unaffected and amastigotes did not cause virulence in mice (621). Indeed low level chronic infection without an overblown pathology were observed in *Leishmania* parasites where metabolic enzymes critical for gluconeogenesis (218,469), iron uptake (15), purine metabolism (16) and glycoconjugate synthesis (17) were absent. We previously showed that PEPCK, a critical enzyme for gluconeogenesis, is a virulence factor in *L. major* (218). Similarly, my study demonstrates that the absence of DLD, which is a key enzyme for mitochondrial metabolism in *Leishmania,* caused impaired lesion developments in both susceptible (BALB/c) and resistant (C57BL/6) mice models (Figure 6), suggesting that metabolic enzymes may indeed be a virulence determinant during *Leishmania* infections.

Most trypanosomatids are covered in a dense glycocalyx containing glycosylphosphatidylinositol (GPI) anchors. However, *Leishmania*'s glycocalyx comprises the GPI anchors, glycan and a linear peptidoglycan that is capped with an oligosaccharide (622). LPG is the most common and abundantly expressed glycoconjugate on the surface of *Leishmania* parasites (622,623). LPG performs many functions in parasite survival in the sandfly vector and mammalian host. It is one of the foremost virulence factors to be described in *Leishmania* (622). Interestingly, the synthesis of LPG is complex, however some of the components in this molecule are derived from mannan

synthesis (Figure 6), which are offshoots of the core metabolic programs in *Leishmania* (624). This suggests that the grassroots origin of LPG synthesis is linked to metabolic pathways, further underpinning the importance of parasite metabolism in inducing virulence in mammalian hosts. One striking feature of the LPG expression in *Leishmania* is in their notable structural distinctions in the promastigote and amastigote forms of the parasites. Although LPG expression in amastigote is highly downregulated about a thousand fold more than in amastigotes (625), there are distinct modifications in the LPG structures amongst both life forms of *Leishmania*. Procylic promastigotes in the sandfly midgut will express LPG molecules that are capped with arabinose on their oligosaccharides moiety but as the parasites move towards the stomodeal valve for transmission, the LPG of the metacyclic promastigotes and amastigotes (in the mammalian host) lose the arabinose capping (42,43). This transition of LPG structural composition is part of a process in *Leishmania* where the parasites transform to a more infective form (metacylic promastigote), and is regarded as metacyclogenesis. It is based on this distinction that separation of the infective forms of *Leishmania* parasites can be made as peanut agglutinin binds to procyclic but not able to attach to the LPG of metacyclic parasites due to the limitation of the arabinose caps. In my studies, one argument for the impaired proliferation of DLD-deficient parasites inside macrophages (Figure 12) is that the infective forms of these parasites were compromised. However, it is very unlikely because parasites can establish within their host cells in 4-6hours post-infection (302,626,627) and in my studies at this time, there were no differences in the parasite numbers inside the cells, suggesting that the infection rates of the DLD deficient parasites were comparable to their WT and DLD AB controls (Figure 12B & C). Although, there is currently no established link of DLD in LPG synthesis, nevertheless, it is possible that the process

of deleting DLD in *L. major* may have impacted the parasite's infective forms. I found that DLD deficient *L. major* grown to become stationary phase promastigotes (which would normally have higher numbers of metacyclic parasites) had comparable infective forms to their WT and AB counterparts because there was no difference in the fractions that were unable to bind to PNA (Figure 13). This observation is consistent with our previous findings that PEPCK deletion in *L. major* did not affect the parasite's morphological structures in their infective forms and that PNA-negative populations in the PEPCK deficient parasites were comparable to their WT counterparts (218). Collectively, these observations suggest that metabolic enzymes may not affect LPG structural modifications, but this does not completely rule out the fact that the core metabolic pathways are not associated with LPG synthesis.

Resistance to cutaneous leishmaniasis is associated with the induction of IFN-$\gamma$ from $CD4^+$ T cells or NK cells, which mediates nitric oxide-mediated killing inside parasitized macrophages. On the other hand susceptibility to CL is related to the induction of anti-inflammatory cytokines like IL-4 and IL-10, which favour the continuous proliferation of the parasites in infected macrophages (302,339,383). Multiple factors (including host and parasite) trigger the establishment of Th1/Th2 responses during *Leishmania* infection. In my studies, I found that infection of both susceptible (BALB/c) and resistant (C57BL/6) mice with DLD deficient *L. major* resulted in blunting of the host immune responses as evidenced by reduction in IL-10, IL-4, IFN-$\gamma$ and TNF production by cells from spleens and lymph nodes draining the infection site compared to their WT or DLD AB counterparts (Figure 15). Since, we found that a peptide ($DLD_{63–79}$) derived from DLD induced strong and dominant IFN- $\gamma$ from $CD4^+$ T cells in infected animals, it was expected that absence of DLD (and by implication absence of the $DLD_{63–79}$), would result in impaired induction IFN-$\gamma$.

Thus, the observation indicates that DLD may not be an immunodominant antigen as we previously proposed (Mou et al). The blunting of the IFN-γ in animals infected with DLD deficient parasites raised some concern that the attenuated parasites I generated may not be able to induce protective responses. However, studies have demonstrated that strong IFN-γ responses were not always needed to control infection as long as the induction of anti-inflammatory/inhibitory cytokines such as IL-10 and IL-4 were non-existent or reduced (222). In my studies, all the cytokines produced from the mice infected with DLD deficient parasites were reduced. Despite this global blunting of immune response, mice infected with DLD-deficient parasites appeared to control their infection because they did not develop any observable lesion and had significantly reduced parasite burden (Figure 14). Similarly, our lab observed that when PEPCK was deleted in *L. major*, there was no observable lesion in infected mice (218) but yet were able to control a secondary virulent WT infection (218). This suggests that my DLD-deficient parasite, which is also attenuated, may also be able to induce protection during secondary infections despite the blunted immune response observed in the primary infection.

Furthermore, I wanted to understand why the immune responses were blunted when mice were infected with DLD deficient parasites. This could have been due to the immunodominance of DLD considering that the $DLD_{63–79}$ epitopes in *Leishmania* induced stronger IFN- γ than PEPCK epitope ($PEPCK_{335-351}$) (511). However, the blunting of immune responses could also be associated with limited antigen availability related to low parasite proliferation (590,591). My findings suggest that mice infected with DLD deficient *L. major* had blunted immune responses because the parasites have reduced proliferation resulting in limited antigen availability. During early infection (1week) (usually a time where 90% of infecting parasites are dead and hence most like

equal amount of antigens released by the dead parasites from the different strains), there was comparable expansion of PEPCK-specific $CD4^+$ T cells from mice infected with WT and DLD deficient parasites (Figure 17). With time, as parasite proliferation progressed, the WT parasites proliferated more than DLD-deficient parasites in mice (Figure 14E) creating more PEPCK antigen availability to necessitate the enhanced expansion of PEPCK-specific $CD4^+$ T cell. In contrast, impaired proliferation of DLD deficient parasites would have resulted in limited antigen (including PEPCK) availability thus resulting in the significantly reduced expansion of the PEPCK-specific $CD4^+$ T cells in the mice infected with DLD KO *L. major.*

Despite the reduced expansion of antigen-specific $CD4^+$ T cells and the blunted cytokine responses, I observed that the mice vaccinated with DLD deficient parasites still produced strong IFN-$\gamma$ response during secondary WT challenge and the production of anti-inflammatory and regulatory cytokines were significantly reduced (Figure 18D-Q). Since the IFN-$\gamma$:IL-4 was significantly increased in the challenged animals, IFN-$\gamma$ must have been involved in the killing of parasites inside macrophages in the contralateral footpad that resulted in the significant reduction in parasite burden and lesion developments compared to the infected naïve counterparts (Figure 18 B&C). This indicates that the blunted immune response induced in the primary infection with DLD-deficient parasites primed the host to develop an effective secondary recall response. In contrast to the findings reported here, it had been previously reported that PEPCK-deficient parasites produced a reduced Th1 response during primary infection. Still, this response could only produce marginal protection during secondary challenge infections (218). However, like my studies, primary infection with LPG2 deficient parasites led to minimal Th1 and Th2 responses while inducing strong protection against secondary WT infections. Also, mice

vaccinated with the LPG2 deficient parasites produced significant increase in IFN-γ during secondary WT infection that was followed by a concomitant reduction in anti-inflammatory/regulatory cytokines like IL-4 and IL-10 (222). This correlate of protection observed in my studies is similar to what was previously described where susceptible BALB/c mice given low dose parasites ($3.3 \times 10^2$) became resistant (628) upon virulent high dose *L. major* challenge. These observations underscore the possibility that individuals who are susceptible to leishmaniasis have the intrinsic potential to mount protective immune responses if the dose of antigen is properly regulated.

Delayed-type hypersensitivity (DTH) is a diagnostic method to determine the cellular response of the host to prior exposure of an antigen or pathogen. To confirm sensitization to *Leishmania*, Leishmanin, a soluble leishmania or other purified protein fractions, are routinely used to assess T cell recruitment to the skin by measuring the thickness of the swelling formed (629). However, Bretscher et al, demonstrated the potential for animals vaccinated with low dose parasites to mount prolonged DTH responses critical for protection against a high or low dose challenge (628). Meanwhile, in my studies and from Uzonna et al, 2004, no DTH response was observed from vaccination with DLD deficient or LPG2 deficient parasites (222). While it is unclear why the attenuated parasites could not generate DTH responses, previous reports suggest that protective immune responses were induced during *Leishmania* infections due to parasite persistence (630), which is critical for generating effector memory T cell responses (594). It has been established that persistent parasites are maintained by a pool of T regulatory cells during healing, which limits the induction of Th1 responses (369,631,632). From this thesis (Figure 14E&F) and Uzonna et al (222), DLD and LPG2 deficient parasites had low proliferation during primary infection in mice

such that due to limited antigen availabilities, the recruitment of T cell in the contralateral footpad exposed to WT parasites may have delayed the induction of the DTH responses. However, this low parasite proliferation in the DLD or LPG2 deficiencies may have also delayed the induction of regulatory T cells (Treg) so that there is no hindrance to the Th1 immune responses observed (Figure 18 L&Q) to control the infection during secondary WT exposures (Figure 18 B &C). Taken together, whether DLD-deficient parasites are maintained by a pool of Tregs like what is obtainable in healed animals during primary infection remains to be examined. Also, uncovering the molecular mechanisms for the induction of a strong IFN-γ response during WT re-challenge in the contralateral footpad of mice vaccinated with DLD deficient parasite will give more insight into quality of protection with these parasites.

I have already established that DLD is a critical mitochondrial enzyme in *L. major* and the absence of the *DLD* gene product resulted in the low proliferation of the parasites as well as the blunting of the host immune response in mice due to a significant disruption in the parasites mitochondrial functions and respiration. Sometimes, DLD is interchangeably referred to as dihydrolipoamide dehydrogenase. DLD is part of several enzyme complexes in the pyruvate dehydrogenase complex and is critical for the generation of acetyl-CoA (517). Interestingly, according to the Glycine cleavage (GCV) enzyme classification, DLD belongs to the L protein unit of the GCV system (GCVL, 1.8.1.2). There are 2 copies of *GCVL* gene in *Leishmania: GCVL-1 and GCVL-2,* which are found on chromosomes 29 and 32, respectively (512). GCVL-2, the target of my study, has 25% similarity with GCVL-1, which is thought to be less catalytically active than the GCVL-2 (512). Moreover, GCVL-1 and GCVL-2 are known to share the same substrate (512), suggesting that GCVL-1 and GCVL-2 may be distant homologs. $DLD_{63-79}$, which we previously identified that

induces strong protective immune response in mice infected with *Leishmania major*, is completely absent in GCVL-1 (511). Therefore, the observations reported in this study strongly suggest that the deletion of *DLD* gene on chromosome 32 is most likely responsible for the phenotype we observed in the DLD deficient parasites *in vitro* and *in vivo*. Furthermore, while GCVL-1 have been shown to lack signal sequences in their N-terminus and is not certain to localize in the mitochondria (512,596) in *L. infantum*, its location and activity has not be studied in *L. major*. GCVL-2 DLD was present in the secreted exogenous vesicles (exosomes) in *Leishmania* and are critical for inducing IL-8 secretion in parasitized macrophages (519). IL-8 is a critical pro-inflammatory cytokine produced by *Leishmania*-infected macrophages to recruit neutrophils to the site of infection (520). This indicates that DLD in *Leishmania* modulates host immune responses during infection, which may be associated with the parasite's virulence.

The question on whether the protection observed in the mice vaccinated with DLD deficient parasites can be long-lasting was not assessed in my studies, because around 9 weeks post infection, observable swellings in the mice infected with DLD deficient parasites were recorded (Figure 19A). This sudden lesion development was not due to the restoration of *DLD* gene in these parasites because *DLD* gene was absent in the parasites isolated from the footpad of the infected mice (Figure 19B). The presence of the Bleomycin resistance gene cassette in these parasites were also intact suggesting that my CRISPR/Cas9 gene editing strategy remained untarnished in the parasites (Figure 19C). The use of attenuated live parasites for vaccination will always be an issue due to the safety concerns. However, reversion to virulence is not uncommon in parasites attenuated through genetic manipulation. LPG2 deficient *L. major* parasites were originally suggested to induce long-term protective responses without inducing lesions after about 12

weeks (222). However, about 40% of these infected animals began developing lesion in mice after about 17 weeks and this progressed rapidly until 49 weeks when the animals were sacrificed (633). These revertant LPG2 deficient strains were unable to synthesize phosphoglycans but they induced lesions in these mice and amastigotes maintained proliferation in mice and inside the macrophages (633). In my studies, the restoration of lesion development after about 8 weeks post-infection with the DLD deficient parasites may be due to a yet-to-be-identified compensatory mechanism. I speculate that the presence of intact GCVL-1 DLD in my DLD-deficient *L. major* parasites may be driving the sudden development of this lesion. Although the sequences of GCVL-1 DLD and GCVL-2 DLD are only similar by 25%, the GCVL-1 DLD has a stronger diaphorase activity that they are able to transfer hydrogen ions to their respective acceptors (NADPH, NAD, FAD) effectively (512,596). Whether this ability was compensated for in the DLD deficient parasite I generated (GCVL 2 DLD KO) to cause reversal to lesion development in mice is uncertain.

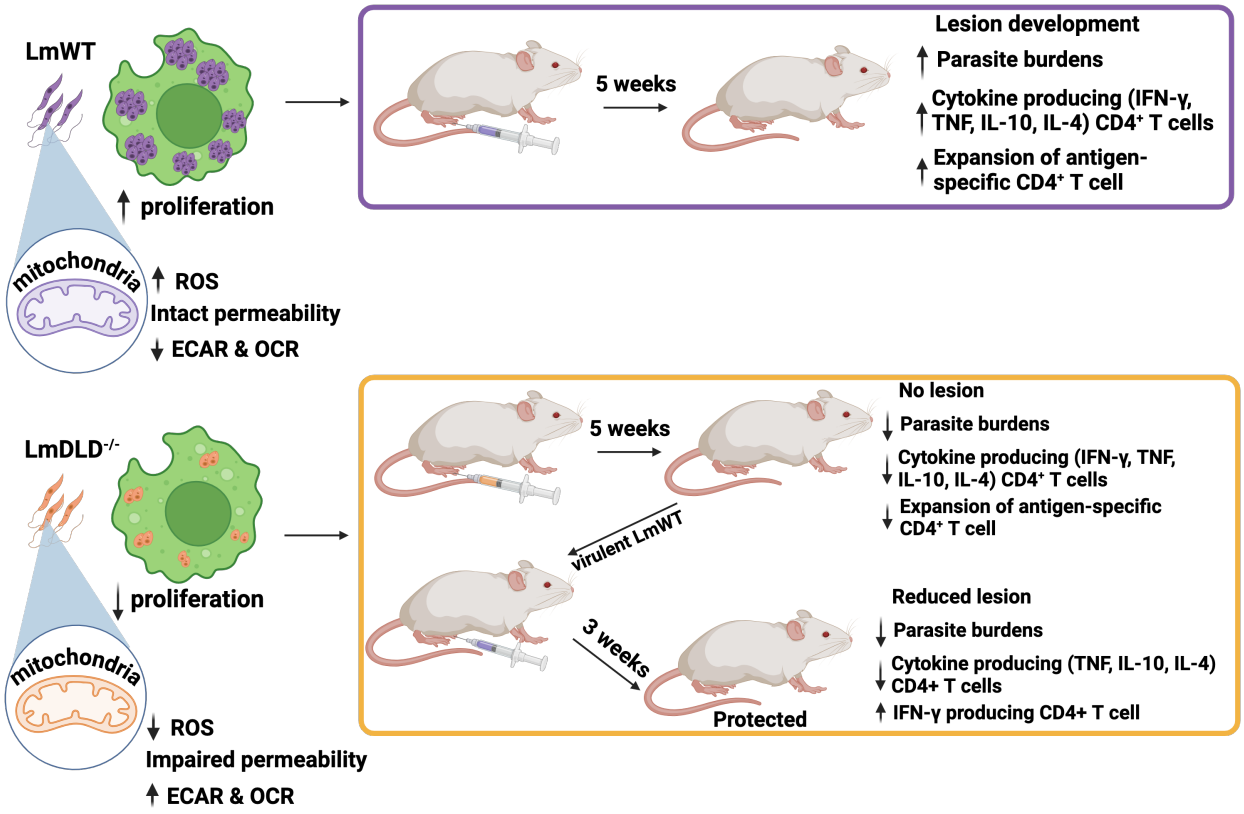



*Figure 29 Graphical summary for the role of DLD in CL.*

The absence of DLD in *L. major* led to reduced proliferation of the parasites both in axenic cultures and inside macrophages compared to the WT *L.major* parasites. Meanwhile, in mice after 5 weeks post-infection with the gene-deficient parasites, no lesion was observed, with reduced parasite burdens and blunting of the host immune responses compared to the WT counterparts. Mice vaccinated with the DLD deficient parasites were able to control a WT virulent challenge by evidence of a reduced parasite burden and lesion development which was associated with an elevated IFN-γ production. The impaired proliferation of the DLD deficient parasites may be due to an impaired mitochondrial function.

## 5.2 The role of PEPCK in VL

Visceral Leishmaniasis is still a growing public health burden with complications of co-morbidity risks from HIV, TB and malaria (75,634,635). Like cutaneous leishmaniasis, vaccination is possible because people who recover from the disease are immune to reinfection (601). Yet, there is

currently no approved vaccine against human disease. Knowing the antigens that drive protective response during leishmaniasis is critical for establishing a vaccine candidate against the disease. We previously identified a peptide ($PEPCK_{335-351}$) derived from phosphoenol pyruvate carboxykinase (PEPCK) in *Leishmania major*, a critical gluconeogenic enzyme, that induces strong $CD4^+$ T cell response in a model of experimental cutaneous leishmaniasis (510). I believe PEPCK would be a cross-protective antigen because this epitope is 100% conserved in all species of *Leishmania* and vaccination in mice using PEPCK-expressing vector protected them against virulent *L. donovani* challenge as evidenced by reduction in parasite burdens in the spleen and liver (510). Furthermore, it was confirmed that PEPCK is a virulence factor in *L. major* in mice, and vaccination with PEPCK-deficient *L. major* parasites induces moderate protective responses (218). Since PEPCK exhibits more than 90% similarity across all *Leishmania* species (636), I wanted to know whether the expression of PEPCK by *L. donovani* impacts its virulence and immune responses in mice.

To fully understand the impact of PEPCK during visceral leishmaniasis, I generated a deletion mutant of PEPCK in *L. donovani* using the CRISPR/Cas9 technology, which is a highly efficient method of gene editing than the previous cumbersome homologous recombination methods (637). I also generated the PEPCK AB complementary control parasites to eliminate the possibility that the effects seen with the mutants were due to errors from the CRISPR/Cas9 system. Consistent with the previous findings in *L. major* (218), I found that PEPCK-deficient *L. donovani* parasites had impaired proliferation in axenic culture and inside macrophages. Deleting PEPCK in *L. donovani* did not impact the parasite's ability to differentiate into their infective forms. However, in mice, PEPCK deficiency still induced hepatomegaly and splenomegaly, causing

pathology with comparable parasite burden in the liver and spleens as their WT or PEPCK AB counterparts, an observation that was completely different from that seen in *L. major* (218). Similarly, mice infected with PEPCK deficient *L. donovani* did show altered host immune responses compared to those infected with WT or PEPCK AB controls. I observed an increased TNF production in the liver of animals infected with PEPCK KO *L. donovani*. The unique microenvironment *in vivo* may have contributed to the continued proliferation of these deficient parasites in mice because the proliferation of these parasites was compromised in isolated Kupffer cells cultured *in vitro*. Although attenuated *in vitro*, PEPCK-deficient *L. donovani* may not be recommended as a live attenuated vaccine candidate against visceral leishmaniasis due to safety concerns.

*Leishmania* parasites can survive the limited nutrient environments within the parasitophorous vacuoles (638) in phagocytic cells due to the induction of a stringent metabolic response (463,481). This response is a survival mechanism by the parasites to conserve resources and escape being detected or killed by the host's immune responses (463). The parasitophorous vacuole is usually poor in glucose, and the pathway utilized in this stringent nutrient deprivation is gluconeogenesis (639,640). Gluconeogenesis is majorly the generation of glucose from non-glucose substrates such as aspartate, alanine and glycerol (483). In *Leishmania*, these substrates are critical for synthesizing carbohydrate storage reserves referred to as mannogen (484). Mannogen, despite being a backup of glucose reserves for *Leishmania*, especially the amastigotes, mediates virulence *in vivo* (641,642). The gluconeogenesis pathway can be activated through glycerol kinase (GK) from the glycerol substrate (457) or through pyruvate phosphate dikinase (PPDK) and PEPCK from alanine and aspartate (470,484). GK catalyzes

glycerol to glycerol-3-phosphate, PEPCK reversibly catalyzes oxaloacetate into Phosphoenolpyruvate (PEP), while PPDK catalyzes pyruvate to PEP. These enzymes are located in the glycosomes and are critical for energy metabolism and have been extensively described (643–645). However, aside from their catabolic roles, they are known to play other specific functions. PEPCK maintains the redox potential ($NADPH/NADP^+$) critical for ATP production in the glycosomes (514), GK regulates oxygen tension (457) while PPDK contributes to glycolysis and maintain ATP flux in glycosomes (646,647).

PEPCK is an important enzyme for carbohydrate metabolism in *Leishmania* (514). Although PEPCK is expressed in both amastigote and promastigote stages of the parasites (510); it exhibits a two-fold expression in amastigotes compared to the promastigotes (457). Out of all the gluconeogenesis enzymes, PEPCK is known to have the highest activity (477). We previously identified PEPCK as an immunodominant antigen in *L. major* (510), whose absence greatly impacted the proliferation of the promastigote and amastigote forms (218). Consistent with this, my thesis demonstrates that PEPCK deficiency in *L. donovani* affected proliferation of promastigote and amastigote forms *in vitro* (Figures 22, 23 and 28). Saini et al. also demonstrated that PEPCK-deficient *L. donovani* was significantly impaired *in vitro* (477,648), but this deficiency was not assessed *in vivo* in mice. From my literature search, no study has assessed PEPCK deficiency in visceralizing *Leishmania* species like *L. donovani* or *L. infantum* in mice. My thesis is the first to demonstrate the impact of PEPCK deficiency in *L. donovani in vivo*. Given that these parasites were compromised inside Kupffer cells cultured *in vitro,* I speculate that the microenvironment of the liver and spleen *in vivo* might contribute to their continued proliferation in these organs in infected mice (Figure 26) (Figure 28). However, it is usually anticipated that *in*

*vitro* data should tally with *in vivo* results. Still, in some cases, it does not and a successful hypothesis is expected to be backed up with both *in vitro* and *in vivo* evidence (604).

I wondered if the comparable proliferation of PEPCK KO *L. donovani* to their WT controls in the spleen and liver of the infected mice is because they had reverted to virulence by acquiring the *PEPCK* gene *in vivo*. I isolated parasites from the livers and spleens of animals infected with PEPCK-deficient parasites and assessed their expression of *PEPCK* gene. There was no expression of the *PEPCK* gene in these isolates by PCR analysis (Data not shown), suggesting that the maintenance of their proliferative capacity in infected animals was not due to reacquisition of *PEPCK* gene, but must be related to the in vivo microenvironmental milieu of the tissues. Indeed, studies have demonstrated that parasites deficient in the gluconeogenesis enzyme, Fructose, 1,6- bisphosphatase in *Leishmania major*, which competes with phosphofructokinase for the same substrate, were severely attenuated *in vitro* (469). Yet, the administration of glucose restored the deficient parasites' proliferation to WT status (469). Our lab have previously demonstrated that the impaired proliferation of PEPCK KO *L. major* promastigotes was abolished when additional glucose was introduced into the axenic cultures (218). Indeed, *Leishmania* parasites utilize gluconeogenesis during periods of glucose limitation (469). PEPCK was highly upregulated during periods of glucose starvation in *L. donovani* (477). While the role of PEPCK in promastigotes has been established, in amastigotes, it is associated with redundancy because of an existing competition for aspartate (470). Although *in vitro* models are less complicated and controlled (649), I observed that PEPCK deficiency in *L. donovani* amastigotes was compromised in an *in vitro* setting (Figures 23 and 28). Whether the complexity of *in vivo* systems may have

contributed to the enhanced proliferation of PEPCK-deficient *L. donovani* is yet to be investigated.

The compartmentalized organ-specific immune responses during visceral leishmaniasis are due to parasite growth in those organs, resulting in gross tissue damage. Chronicity occurring in the spleen is associated with increased parasite burden (601,650). A reduced parasite burden linked to disease resolution occurs in the liver by the formation of a modest granuloma (263) compared to those from *Mycobacterium tuberculosis* or *Schistosoma mansoni* (651). Granuloma formation is marked by increased recruitment of Kupffer cells and subsequent interactions with invariant natural killer cells result in further amplification, which is followed by influx of other immune cells, leading to the maturation of the granuloma (652). Parasite clearance is initiated in the granulomas due to the surge of the inflammatory responses around 4 weeks post-infection (652). Granuloma clears around 4 weeks later and the recruited cells move out, restoring the liver to the original state before infection (653). Aside from playing a role in parasitized macrophage activation, TNF, one of the inflammatory mediators, contributes to early control of granuloma in the liver because TNF KO in C57BL/6 mice develop abrupt and accelerated granuloma during *L. donovani* infection (654). In contrast, blocking TNF production in BALB/c mice infected with *L. donovani* did not affect hepatic granuloma formation (655). These differences may be associated with the mice backgrounds used as the resistant C57BL/6 mice produce robust pro-inflammatory cytokines while the highly susceptible BALB/c mice produce more anti-inflammatory mediators (266). Although in this present study granuloma formation in infected mice was not assessed, I consistently found increased amounts of TNF produced from the liver in mice infected with PEPCK deficient *L donovani* (Figure 27B, F,R and Y). However, this enhanced TNF production was not

associated with a concomitant reduction in the parasite burden in the liver. Whether the enhanced TNF production observed in mice infected with PEPCK deficient parasites could result in early granuloma formation and produce a faster resolution of liver pathogenesis, is yet to be elucidated. However, it has been reported that over-expression of PEPCK in *S. mansoni* eggs resulted in a solid Type 1 immune response linked to granuloma formation during infection (516). It is, however, not clear whether PEPCK deficiency may only regulate Th1 responses (TNF) in the liver of *L. donovani*-infected animals. To date, no study has demonstrated the regulation of liver granuloma during visceral leishmaniasis by PEPCK in *Leishmania*. More studies defining the role of PEPCK in *L. donovani* in infected animals need to be investigated.

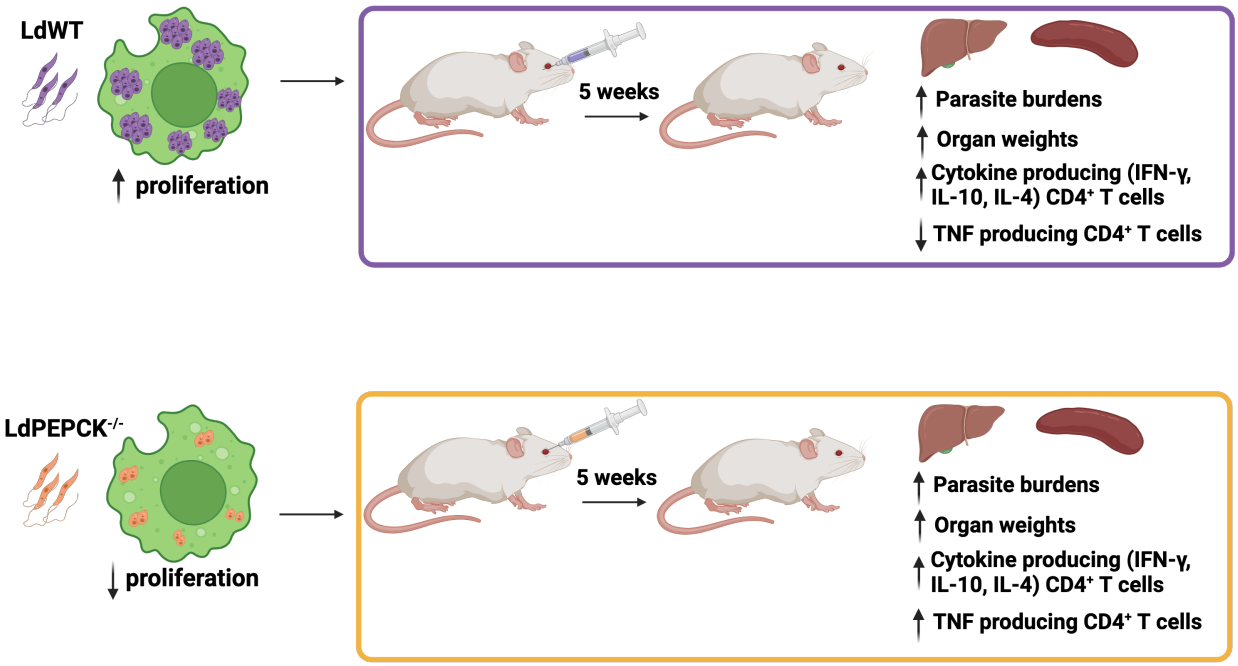



*Figure 30 Graphical summary on the role of PEPCK in VL.*

PEPCK deficient *L. donovani* parasites had reduced proliferation *in vitro* (axenic culture, bone marrow derived macrophages and Kupffer cells). However, these parasites proliferated inside infected ani in the liver and spleen, comparable to the WT counterparts. There were no changes in the weights and spleen of mice infected with the PEPCK deficient *L. donovani* parasites compared to the WT controls. Also, cytokine production (IFNg, IL-4, IL-10) from CD4+ T cells from the liver and spleen of the animals infected with PEPCK deficient *L. donovani* was comparable to those of the WT groups except TNF.

## 5.3 Exploring DLD KO *L. major* as a live attenuated vaccine candidate against CL

The current toxicity associated with the available drugs for treating leishmaniasis drives the need for alternative therapeutic approaches against the disease. Since recovery from prior exposure to *Leishmania* induces protection against reinfection, this suggests that vaccination against the disease is possible. Further investigation into the nature of the protective responses during secondary responses revealed that the presence of persistent parasites was critical to drive this protection (656). This suggests that complete clearance of the parasites would result in loss of protective immunity (229,593). This so called infection-induced protection is mediated by $CD4^+$ T cells which are also critical cells in the control of the infection. Our lab found that exposure to non-persisting parasites, dihydrofolate reductase thymidylate synthase (dhfr-ts) *L. major*, does not induce protection against virulent challenge in mice, and this was associated with a loss of *Leishmania* PEPCK specific $CD4^+$ T cells (Mou et al, submitted). Although infection with dhfr-ts *L. major* parasites in mice induced protection when mice were challenged around 5 weeks post-infection, but around 25 weeks, when the parasites were completely cleared, the effector responses were significantly reduced compared to the effector responses gotten from persistent parasites leading to loss of protection (593). It has been noted that the little protection observed with the sterile immunity was due to central memory T cells ($CD62^{high}$). Meanwhile, the generation of effector memory T cells is guaranteed to keep the supply of IFN-$\gamma$ constant as long as there is a persisting parasite population (229). Thus, the generation of live or attenuated parasites (through genetic methods), which are not completely eliminated, is critical for the induction of protective responses and hence necessary for developing an effective vaccine.

In my studies, vaccination with DLD deficient mice followed by challenge with virulent WT parasites after a short time induced a strong IFN-γ response from $CD4^+$ T cells and this was associated with suppression of anti-inflammatory cytokines. This elevated IFN-γ response was associated with parasite control because parasite burdens in the challenged mice were significantly reduced. Whether the IFN-γ responses in the challenge mice were from effector memory T cells are uncertain. The primary infection with DLD deficient parasites must have primed the mice to induce this protective response against a virulent WT challenge.

The fact that vaccination with DLD deficient parasites protected mice against virulent WT challenge indicates the generation of memory cells in vaccinated mice. Whether these memory responses can be long-lived needs to be thoroughly evaluated. Our lab recently demonstrated the requirement for continuous presence of the cognate antigen for maintenance of *Leishmania*-specific memory T cells (Mou et al. 2024, Submitted). Despite the absence of $DLD_{63–79}$ in my DLD deficient *L. major* parasites, which appeared to induce stronger IFN-γ than the $PEPCK_{335-351}$ (511), my study demonstrated the expansion of PEPCK-specific $CD4^+$T cells in primary infection with DLD deficient parasites (Figure 17). This indicates that the antigen-specific $CD4^+$ T cell responses may be blunted in the mice infected with the DLD deficient parasites (Figure 17, Figure 16 ), but they may have been the culprits that must have controlled the virulent WT challenge in the secondary infection (Figure 18). We found that PEPCK-specific $CD4^+$ T cells eventually undergo clonal expansion during infection to produce effector memory responses (510). However, it is important to investigate the longevity of these antigen-specific $CD4^+$ T cells in sustaining protective responses in the long term.

Furthermore, in my mice I infected with the DLD deficient *L. major* parasites, the longevity of these antigen-specific memory CD4+ T cells may be primarily questionable because of the presence of intact GCVL-1 DLD, which may have caused lesion developments in the primary infection after 9 weeks (Figure 19). Whether the recrudescence of lesion in the primary infection site after several weeks affects the overall quality of the antigen-specific $CD4^+$ T cells to induce protective responses during secondary challenge infection is uncertain. Notwithstanding, DLD-deficient parasites I generated in this study may not be suitable live attenuated vaccine candidate against cutaneous leishmaniasis owing to the sudden reoccurrence of lesions at the primary infection site which raises safety concerns. The development of these lesions at the primary infection site is potentially due to developing a compensatory mechanism for the lipoamide and electron transfer activities carried out by the DLD. Although, GCVL-1 DLD and GCVL-2 DLD are considered structurally distinct owing to their low sequence similarities (512), yet they act on the same substrates and even perform similar diaphorase activities (512). Interestingly, the exact localization of GCVL-1 DLD in *L. donovani* has not been elucidated, and reports show that the GCVL-2 DLD in *L. donovani* is found in the inner leaflet of the plasma membrane (596). Multiple experiments from Chiranjivi et al, showed that the GCVL-1 DLD is located in multiple compartments such as the nucleus, plasma membrane, kinetoplast and mitochondria (596). Indications that the GCVL-1 DLD in *Leishmania* cause disease is uncertain, however this thesis have demonstrated for the first time that GCVL-2 DLD is indeed a virulence determinant in *L. major*; it may be critical for lesion developments early on while GCVL-1 DLD may be a crucial driver for virulence later. Thus, further investigation is required to assess whether GCVL-1 DLD plays a role in the virulence of primary *Leishmania major* infection, perhaps, by double deletion

of both *GCVL-1* and *GCVL-2* genes. This will bring insights on whether total deficiency of DLD (GCVL-1 and GCVL-2) in *L. major* may be a suitable live attenuated vaccine against cutaneous leishmaniasis.

## 5.4 General Discussion and Conclusion

Leishmaniasis is a growing global health concern of clinical relevance, yet it is classified as a neglected tropical disease affecting millions of people every year. With increasing challenges in the current treatment modalities, alternative therapeutics strategies are necessary. Although there is no approved vaccine for human use, vaccination is considered possible because infection-induced immunity is acquired after recovery from a prior infection, resulting in protection upon a re-exposure to a virulent parasite. This infection-induced immunity, which is mediated by effector memory $CD4^+$ T cells, is lost in the absence of persistent parasites. So far, the only strategy known to maintain the longevity of the effector memory $CD4^+$ T cell response is through maintenance of stable population of persisting parasites. Thus, developing live-attenuated parasites (through genetic manipulation) is one way to consistently build parasite persistence required to generate long-lived infection-induced resistance.

Although cutaneous leishmaniasis produces self-healing lesions, disfiguration and long-term complications may arise. Visceral leishmaniasis is even the most fatal form of leishmaniasis, with a significantly growing public health burden. Vaccinating humans with either attenuated visceralizing or cutaneous *Leishmania* species needs to undergo a series of checks to confirm their viability or potential to revert to virulence. In this thesis, I generated a DLD deficient *L. major*

parasite that did not induce pathology in experimental mice model short term and has the potential of becoming a live-attenuated vaccine candidate because it induces strong IFN-γ response in vaccinated mice following secondary infections. On the other hand, PEPCK-deficient *L. donovani* promastigote and amastigote forms appeared attenuated *in vitro*, but they may be unsafe for use as a vaccine candidate in humans because *in vivo* data from this thesis suggest they induce virulence comparable to their WT counterpart controls. Thus, further studies are needed to investigate why the PEPCK-deficient parasites were not attenuated *in vivo* especially when we showed that the PEPCK deficiency in *L. major* was attenuated in *in vitro* and *in vivo* settings (Barazandeh et al., 2021a).

# 6. CHAPTER 6: Significance, Limitations and Future Directions

## 6.1 Significance

An indication of protective immunity in leishmaniasis is the induction of Th1 cellular immunity. Since we have shown that naturally processed peptides derived from metabolic enzymes (PEPCK and DLD) play a role in protective immunity to leishmaniasis, this study is the first to have demonstrated the importance of these enzymes (DLD and PEPCK) in causing cutaneous or visceral leishmaniasis, respectively. Additionally, results from this study have provided insights into the potential use of gene-deficient parasites (genetic manipulation) as a live attenuated vaccine candidate to protect against cutaneous and visceral leishmaniasis. These gene-deficient parasites generated in this thesis might be useful in the future to assess the quality and longevity of cellular memory responses produced during *Leishmania* infections.

## 6.2 Limitations

1. As with many studies carried out in animal models, the major caveat in my thesis is the uncertainty that the results generated from the gene deficient parasites can be ultimately recapitulated in humans. The BALB/c mice, which is the mice model that is more susceptible to cutaneous leishmaniasis, is widely used because of the cost and general acceptance that the symptoms of the disease in mice mirrors what is obtained in clinical settings. The infection of C57BL/6 mice with my mutant parasites, which is usually resistant to experimental cutaneous leishmaniasis, produced similar results obtained in the susceptible mice, further underscoring a potential reproducibility in other mice or animal models. The findings in the C57BL/6 mice model of infection further provides

credibility and validity to the phenotypes observed in my DLD deficient parasites. However, I did not assess my gene deficient parasites in multiple mice models such as in immune compromised models to confirm their attenuation and increase their potential to be valuable for therapeutic application in humans.

2. This study did not factor in sex or gender considerations with the mice models infected with the gene deficient parasites. Males are known to be mostly affected by both CL and VL (559–561). However, these sex disparities are not very clear in mice models. In cutaneous leishmaniasis, female mice appear to be more susceptible than male mice (563) but in visceral leishmaniasis the male mice developed higher parasite burdens in livers than in the female mice (563). In my study, I did not factor this discrepancy as I used female mice in all my animal experiments. However, it is vital to include sex variables in these studies to be able to assess the need for sex targeted therapies.
3. Leishmaniasis, is a disease caused by *Leishmania* which is transmitted through the bite of the sandfly. However, it is not entirely possible to mimmick the natural transmission of my gene deficient parasites in my animal models in a laboratory setting. The sandfly saliva plays a major role in amplifying the severity of *Leishmania* infection (657,658). In my studies, the impact of the sandfly saliva delivered alongside my gene-deficient parasites in mice was excluded. Multiple methods have been developed to mimic *Leishmania* infection in a natural setting. It is popularly known that inoculation of $10^5$-$10^6$ metacyclic parasites in footpad, ear or tail is sufficient to mimic infection in the natural setting. However, low dose parasite (100-1000) inoculated intradermally rather than subcutaneously is now accepted as the gold standard because lesion formation would be

gradual and recapitulates what is observed in a natural sandfly transmission (254). Whether inoculating low doses of my gene-deficient parasites in mice will change the disease manifestation stated in my thesis was not explored. Also, the size of the inoculum is essential during infection (659); I ensured large numbers of stationary phased culture-derived promastigotes (containing a higher percentage of metacyclic promastigotes) were used during infection. Since I did not use a specified amount of purified metacyclic promastigote, there is variability in the in the amount of metacyclics injected into mice with every experiment. My data, however, did not show many variabilities between repeated experiments. Finally, the gene deficient parasite may be a valuable tool for vector control in endemic regions, but this study did not access the impact this deficiency will have on sandfly host.

4. In my mitostress studies, the assessment of proton leak, respiration linked to ATP production, total and spare respiratory capacity, and the oxygen consumption due to non-mitochondrial processes was omitted intentionally. This is because, the administration of mitochondrial inhibitors such as oligomycin, which is supposed to restrict electron flow by inhibiting ATP synthase, ideally should drastically reduce the OCR in the kinetics study of the DLD deficient parasites. However, after oligomycin treatment, the OCR of the WT, and AB parasites remained unchanged and those of the DLD KO parasites were reduced. This suggested to me that maybe *Leishmania* parasites may be unresponsive to oligomycin and that deleting DLD somehow made them responsive. Also, I observed that FCCP administration, which is supposed to uncouple electrons which in turn increases the OCR, appeared to minimally do so in the DLD KO parasites but in the AB or WT parasite

this drug did not impact their OCR levels much (Figure 20). Attempts at troubleshooting this several times by titrating parasite numbers or drug concentrations yielded similar results. Thus, it is possible that these drugs were resistant to the WT or AB parasites and somehow *DLD* gene deletion in *L. major* made the drugs permeable. Thus, to prevent complications in the interpretation of the results in the mitostress test, only Basal ECAR and OCR values were displayed.

## 6.3 Future directions

### *1. Investigating whether the impaired proliferation in the gene-deficient parasites is linked to autophagy*

Autophagy is an 'in-house' cleaning system every cell adopts to maintain cellular homeostasis by engagement in protein and organelle turnover (660–662). Worn-out cellular organelles are degraded through numerous autophagosomal machinery (663). These processes have evolved to eliminate invading intracellular pathogens. The initiation of autophagosomes arises from damaged cellular organelles. Given that DLD deletion in *L. major* resulted in functionally impaired mitochondria and was associated with low proliferation of the parasites in macrophages and mice, however, whether this is due to alteration in autophagy is unknown. In mammalian cells, autophagy is well studied, but in *Leishmania* parasites, there are evidence that these processes exist and *Leishmania*-specific autophagic proteins have been identified in *Leishmania* to play a role in parasite survival and virulence. However, the autophagic processes in *Leishmania* are not well understood. Whether DLD is a critical regulator of this process in *Leishmania* is elusive. Using the recently described methods identified to measure autophagy in *Leishmania* (664), I hope to

understand whether the metabolic deficiency in my gene-deficient parasites is linked to autophagic processes. To do this, I will assess the presence of autophagic organelles such as damaged mitochondria, double membrane autophagosomes or mitochondria undergoing fusion/ fission using transmission electron microscopy. Since there are no available autophagosomal antibodies for western blots in *Leishmania* parasites, I will assess the accumulation of acidic vacuolar organelles in these gene-deficient parasites by measuring the fluorescence of acridine orange in those compartments by Flow cytometry, which have been previously described (664).

***2. Long-term vaccination experiments in GCVL-1 DLD and GCVL-2 DLD double KO parasites.***

This thesis assessed short-term vaccination potential of DLD deficient parasites (GCVL-2 KO). However, mice vaccinated with DLD deficient parasites developed lesions later (9 weeks post vaccination), and this may have been due to compensation due to the presence of *GCVL-1* gene in my DLD KO parasites. This speculation arose because of the observation that although GCVL-1 and GCVL-2 have low sequence similarities, yet they act on similar substrates (512,596). No study had demonstrated whether GCVL-1 DLD variant plays a role in virulence of *Leishmania major*. Thus, future studies will need to delete *GCVL-1* gene DLD in *Leishmania* to characterize whether it is necessary for the parasite to cause disease. Afterwards, generating double KO of both DLD variants (GCVL-1 and GCVL- 2) will be made by using the CRISPR/Cas9 tool to remove *GCVL-1* gene in my DLD (GCVL-2 DLD) deficient parasites. This double KO parasites will be critical to assess whether long term vaccination is possible in these gene deficient strains.

### *3. Understanding the cellular mechanisms behind the generation of memory responses during vaccination studies*

My study has identified DLD deficient *L. major* as a potential vaccine candidate against cutaneous leishmaniasis. During *Leishmania* infections, cellular memory response is a critical component for infection-induced resistance to re-exposure in secondary infections (229,593). These gene-deficient parasites will be valuable in understanding the quality and longevity of cellular memory responses in vaccinated mice, especially since we had previously observed that DLD and PEPCK-specific $CD4^+$ T cells undergo normal clonal expansion following activation and produce memory cells upon disease resolution (510,511). Therefore, these attenuated parasites can contribute to assessing the durability of these memory cells using our PEPCK and DLD TCR transgenic mice.

### *4. Assess humoral immune response in serum and granuloma formation from histological stains of liver and spleen tissue sections in infected mice*

Humoral immunity which comprises of antibodies that promotes neutralization, opsonization and activation of the complement system during visceral leishmaniasis are critical to the disease progression (376,665). Yet, B lymphocytes, the main cell that produces these antibodies are often neglected players during infection because the parasites infect and reside inside macrophages. As a result, it is frequently thought that an extracellular protein (such as antibody) cannot impact an intracellular pathogen such as *Leishmania*. Studies have demonstrated that the absence of the B cell activating factor (BAFF) suppressed splenomegaly and not hepatomegaly (666), and BAFF expression in VL patient is about 4 folds higher than in controls, suggesting a crucial role of B lymphocytes during VL (667). Formation of immune complexes is associated with high antibody

titres often prevalent in VL (263). Although, my studies demonstrate no change in the parasite burden and immune responses in the livers and spleen of mice infected with PEPCK deficient *L. donovani*, whether PEPCK deficiency affects antibody responses (IgG, IgM and IgE) without contributing to VL disease progression is unknown. However, since my *in vitro* and *in vivo* studies were not in tandem in my PEPCK study, it is important to assess the serum antibody levels in my animals infected with PEPCK deficient *L. donovani* to validate this discrepancy.

### *5. PEPCK deficient L. donovani in nutritionally enriched or starved mice.*

We have previously shown that PEPCK-deficient *L. major* promastigotes proliferated like wild-type parasites when exogeneous glucose was administered *in vitro* (218), thus indicating that the impaired proliferation of the deficient parasites is due to unmet nutritional need. This thesis, however, did not elucidate whether this nutritional need in PEPCK-deficient *L. donovani* parasites was met inside the infected mice, especially given that I found the deficient parasites maintained their ability to induce pathology comparable to WT parasites *in vivo*. Thus, since PEPCK is a critical metabolic enzyme important for parasite metabolism, assessing the immunopathologies from PEPCK-deficient *L. donovani* in mice with nutrient deficiencies, sufficiency, or overexpression will be essential to understand whether PEPCK deficiency in *L. donovani* causes VL due to the unique nutritional milieu in the liver and/or spleen.

### *6. TNF-mediated regulation of liver granuloma during PEPCK KO L. donovani infections.*

My thesis showed that PEPCK deficiency in *L. donovani* only enhanced TNF production in the liver. It has been established that TNF contributes to liver granuloma formation which is critical for

enhanced parasite clearance. My study did not assess the formation of liver granuloma in the infected mice since I did not observe a significant change in parasite burdens in infected animals. However, understanding whether PEPCK in *L. donovani* regulates TNF-mediated granuloma formation during visceral leishmaniasis will provide further insights into the hepatic tissue pathologies during infection.

# 7. CHAPTER 7: References and Appendices


1. Kaufer A, Ellis J, Stark D, Barratt J. The evolution of trypanosomatid taxonomy. Parasit Vectors. 2017 Jun 8;10(1):287.

2. Montaner-Angoiti E, Llobat L. Is leishmaniasis the new emerging zoonosis in the world? Vet Res Commun. 2023 Jul 12;

3. Akhoundi M, Kuhls K, Cannet A, Votýpka J, Marty P, Delaunay P, et al. A Historical Overview of the Classification, Evolution, and Dispersion of Leishmania Parasites and Sandflies. Vol. 10, PLoS Neglected Tropical Diseases. 2016.

4. Pace D. Leishmaniasis. J Infect. 2014 Nov;69 Suppl 1:S10-18.

5. Yadav P, Azam M, Ramesh V, Singh R. Unusual Observations in Leishmaniasis—An Overview. Pathogens. 2023 Feb 10;12(2):297.

6. World Health Organization. Leishmaniasis Fact sheet N 375. Geneva, Switzerland; 2015.

7. World Health Organization. Fifteenth meeting of the Strategic and technical advisory group for neglected tropical diseases. Geneva; 2022 Feb. Report No.: 15.

8. Knight CA, Harris DR, Alshammari SO, Gugssa A, Young T, Lee CM. Leishmaniasis: Recent epidemiological studies in the Middle East. Front Microbiol. 2023 Feb 2;13:1052478.

9. Dostálová A, Volf P. Leishmania development in sand flies: parasite-vector interactions overview. Parasit Vectors. 2012 Dec 3;5:276.

10. Burza S, Croft SL, Boelaert M. Leishmaniasis. The Lancet. 2018;392(10151):951–70.

11. Scarpini S, Dondi A, Totaro C, Biagi C, Melchionda F, Zama D, et al. Visceral Leishmaniasis: Epidemiology, Diagnosis, and Treatment Regimens in Different Geographical Areas with a Focus on Pediatrics. Microorganisms. 2022 Sep 21;10(10):1887.

12. Mathison BA, Bradley BT. Review of the Clinical Presentation, Pathology, Diagnosis, and Treatment of Leishmaniasis. Lab Med. 2023 Jul 5;54(4):363–71.

13. World Health Organization. Endemicity of Leishmaniasis 2018.pdf [Internet]. 2018. Available from: https://www.who.int/leishmaniasis/burden/GHO_VL_2018.pdf?ua=1

14. Hotez PJ, Molyneux DH, Fenwick A, Ottesen E, Sachs SE, Sachs JD. Incorporating a rapid-impact package for neglected tropical diseases with programs for HIV/AIDS, tuberculosis, and malaria: A comprehensive pro-poor health policy and strategy for the developing world. PLoS Medicine. 2006;3(5):576–84.

15. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS ONE. 2012;7(5).

16. Lainson R, Ward RD, Shaw JJ. Leishmania in phlebotomid sandflies: VI. Importance of hindgut development in distinguishing between parasites of the Leishmania mexicana and L. braziliensis complexes. Proc R Soc Lond B Biol Sci. 1977 Nov 14;199(1135):309–20.

17. Rogers ME, Chance ML, Bates PA. The role of promastigote secretory gel in the origin and transmission of the infective stage of Leishmania mexicana by the sandfly Lutzomyia longipalpis. Parasitology. 2002 May;124(Pt 5):495–507.

18. Rogers ME, Bates PA. Leishmania manipulation of sand fly feeding behavior results in enhanced transmission. PLoS Pathog. 2007 Jun;3(6):e91.

19. Dostálová A, Votýpka J, Favreau AJ, Barbian KD, Volf P, Valenzuela JG, et al. The midgut transcriptome of Phlebotomus (Larroussius) perniciosus, a vector of Leishmania infantum: comparison of sugar fed and blood fed sand flies. BMC Genomics. 2011 May 10;12:223.

20. Ramalho-Ortigão M, Jochim RC, Anderson JM, Lawyer PG, Pham VM, Kamhawi S, et al. Exploring the midgut transcriptome of Phlebotomus papatasi: comparative analysis of expression profiles of sugar-fed, blood-fed and Leishmania-major-infected sandflies. BMC Genomics. 2007 Aug 30;8:300.

21. Dillon RJ, Lane RP. Influence ofLeishmania infection on blood-meal digestion in the sandfliesPhlebotomus papatasi andP. langeroni. Parasitol Res. 1993;79(6):492–6.

22. Telleria EL, de Araújo APO, Secundino NF, d'Avila-Levy CM, Traub-Csekö YM. Trypsin-like serine proteases in Lutzomyia longipalpis--expression, activity and possible modulation by Leishmania infantum chagasi. PLoS One. 2010 May 18;5(5):e10697.

23. Schlein Y, Jacobson RL. Resistance of Phlebotomus papatasi to infection with Leishmania donovani is modulated by components of the infective bloodmeal. Parasitology. 1998 Nov;117 ( Pt 5):467–73.

24. Pimenta PF, Modi GB, Pereira ST, Shahabuddin M, Sacks DL. A novel role for the peritrophic matrix in protecting Leishmania from the hydrolytic activities of the sand fly midgut. Parasitology. 1997 Oct;115 ( Pt 4):359–69.

25. Sacks D, Kamhawi S. Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis. Annu Rev Microbiol. 2001;55:453–83.

26. Secundino N, Kimblin N, Peters NC, Lawyer P, Capul AA, Beverley SM, et al. Proteophosphoglycan confers resistance of Leishmania major to midgut digestive enzymes induced by blood feeding in vector sand flies. Cell Microbiol. 2010 Jul;12(7):906–18.

27. Secundino NFC, Eger-Mangrich I, Braga EM, Santoro MM, Pimenta PFP. Lutzomyia longipalpis peritrophic matrix: formation, structure, and chemical composition. J Med Entomol. 2005 Nov;42(6):928–38.

28. Walters LL, Irons KP, Guzman H, Tesh RB. Formation and composition of the peritrophic membrane in the sand fly, Phlebotomus perniciosus (Diptera: Psychodidae). J Med Entomol. 1993 Jan;30(1):179–98.

29. Coutinho-Abreu IV, Sharma NK, Robles-Murguia M, Ramalho-Ortigao M. Targeting the midgut secreted PpChit1 reduces Leishmania major development in its natural vector, the sand fly Phlebotomus papatasi. PLoS Negl Trop Dis. 2010 Nov 30;4(11):e901.

30. Coutinho-Abreu IV, Serafim TD, Meneses C, Kamhawi S, Oliveira F, Valenzuela JG. Leishmania infection induces a limited differential gene expression in the sand fly midgut. BMC Genomics. 2020 Sep 4;21(1):608.

31. Feldhaar H, Gross R. Immune reactions of insects on bacterial pathogens and mutualists. Microbes Infect. 2008 Jul;10(9):1082–8.

32. Boulanger N, Lowenberger C, Volf P, Ursic R, Sigutova L, Sabatier L, et al. Characterization of a defensin from the sand fly Phlebotomus duboscqi induced by challenge with bacteria or the protozoan parasite Leishmania major. Infect Immun. 2004 Dec;72(12):7140–6.

33. Meister S, Kanzok SM, Zheng XL, Luna C, Li TR, Hoa NT, et al. Immune signaling pathways regulating bacterial and malaria parasite infection of the mosquito Anopheles gambiae. Proc Natl Acad Sci U S A. 2005 Aug 9;102(32):11420–5.

34. Sloan MA, Sadlova J, Lestinova T, Sanders MJ, Cotton JA, Volf P, et al. The Phlebotomus papatasi systemic transcriptional response to trypanosomatid-contaminated blood does not differ from the non-infected blood meal. Parasit Vectors. 2021 Jan 6;14(1):15.

35. Kelly PH, Bahr SM, Serafim TD, Ajami NJ, Petrosino JF, Meneses C, et al. The Gut Microbiome of the Vector Lutzomyia longipalpis Is Essential for Survival of Leishmania infantum. mBio. 2017 Jan 17;8(1):e01121-16.

36. Telleria EL, Sant'Anna MRV, Ortigão-Farias JR, Pitaluga AN, Dillon VM, Bates PA, et al. Caspar-like gene depletion reduces Leishmania infection in sand fly host Lutzomyia longipalpis. J Biol Chem. 2012 Apr 13;287(16):12985–93.

37. Di-Blasi T, Telleria EL, Marques C, Couto R de M, da Silva-Neves M, Jancarova M, et al. Lutzomyia longipalpis TGF-β Has a Role in Leishmania infantum chagasi Survival in the Vector. Front Cell Infect Microbiol. 2019;9:71.

38. Serafim TD, Coutinho-Abreu IV, Dey R, Kissinger R, Valenzuela JG, Oliveira F, et al. Leishmaniasis: the act of transmission. Trends Parasitol. 2021 Aug 10;S1471-4922(21)00168-9.

39. Bates PA. Leishmania sand fly interaction: progress and challenges. Curr Opin Microbiol. 2008 Aug;11(4):340–4.

40. Wilson R, Bates MD, Dostalova A, Jecna L, Dillon RJ, Volf P, et al. Stage-specific adhesion of Leishmania promastigotes to sand fly midguts assessed using an improved comparative binding assay. PLoS Negl Trop Dis. 2010 Sep 7;4(9):e816.

41. Volf P, Myskova J. Sand flies and Leishmania: specific versus permissive vectors. Trends Parasitol. 2007 Mar;23(3):91–2.

42. McConville MJ, Blackwell JM. Developmental changes in the glycosylated phosphatidylinositols of Leishmania donovani. Characterization of the promastigote and amastigote glycolipids. J Biol Chem. 1991 Aug 15;266(23):15170–9.

43. Pimenta PF, Turco SJ, McConville MJ, Lawyer PG, Perkins PV, Sacks DL. Stage-specific adhesion of Leishmania promastigotes to the sandfly midgut. Science. 1992 Jun 26;256(5065):1812–5.

44. Killick-Kendrick R, Killick-Kendrick M, Tang Y, Bastien P. Metacyclic promastigotes of Leishmania in the salivary glands of experimentally infected phlebotomine sandflies. Parasite. 1996 Mar 1;3(1):55–60.

45. Sádlová J, Volf P. Occurrence of Leishmania major in sandfly urine. Parasitology. 1999 May;118 ( Pt 5):455–60.

46. Rogers ME, Ilg T, Nikolaev AV, Ferguson MAJ, Bates PA. Transmission of cutaneous leishmaniasis by sand flies is enhanced by regurgitation of fPPG. Nature. 2004 Jul 22;430(6998):463–7.

47. Claborn D. The biology and control of leishmaniasis vectors. Journal of Global Infectious Diseases. 2010;2(2):127.

48. Steverding D. The history of leishmaniasis. Parasites and Vectors. 2017;10(1):1–10.

49. Sasidharan S, Saudagar P. Leishmaniasis: where are we and where are we heading? Parasitol Res. 2021 May;120(5):1541–54.

50. Gossage SM, Rogers ME, Bates PA. Two separate growth phases during the development of Leishmania in sand flies: implications for understanding the life cycle. Int J Parasitol. 2003 Sep 15;33(10):1027–34.

51. Lodge R, Descoteaux A. Leishmania invasion and phagosome biogenesis. Subcell Biochem. 2008;47:174–81.

52. Pavli A, Maltezou HC. Leishmaniasis, an emerging infection in travelers. International Journal of Infectious Diseases. 2010;14(12):e1032–9.

53. Grevelink SA, Lerner EA. Leishmaniasis. Journal of the American Academy of Dermatology. 1996;34(2 I):257–72.

54. Uzun S, Gürel MS, Durdu M, Akyol M, Fettahlıoğlu Karaman B, Aksoy M, et al. Clinical practice guidelines for the diagnosis and treatment of cutaneous leishmaniasis in Turkey. Int J Dermatol. 2018 Aug;57(8):973–82.

55. Gurel MS, Tekin B, Uzun S. Cutaneous leishmaniasis: A great imitator. Clinics in Dermatology. 2020 Mar 1;38(2):140–51.

56. Saravia NG, Segura I, Labrada LA, Weigle K, Giannini SH, Pacheco R, et al. Recurrent lesions in human Leishmania braziliensis infection-reactivation or reinfection? The Lancet. 1990;336(8712):398–402.

57. Handler MZ, Patel PA, Kapila R, Al-Qubati Y, Schwartz RA. Cutaneous and mucocutaneous leishmaniasis: Differential diagnosis, diagnosis, histopathology, and management. J Am Acad Dermatol. 2015 Dec;73(6):911–26; 927–8.

58. Bennis I, Thys S, Filali H, De Brouwere V, Sahibi H, Boelaert M. Psychosocial impact of scars due to cutaneous leishmaniasis on high school students in Errachidia province, Morocco. Infectious Diseases of Poverty. 2017;6(1):1–8.

59. Yanik M, Gurel MS, Simsek Z, Kati M. The psychological impact of cutaneous leishmaniasis. Clin Exp Dermatol. 2004 Sep;29(5):464–7.

60. Fikre H, Mohammed R, Atinafu S, van Griensven J, Diro E. Clinical features and treatment response of cutaneous leishmaniasis in North-West Ethiopia. Trop Med Int Health. 2017 Oct;22(10):1293–301.

61. Scott P, Novais FO. Cutaneous leishmaniasis: Immune responses in protection and pathogenesis. Nature Reviews Immunology. 2016;

62. Hashiguchi Y, Gomez EL, Kato H, Martini LR, Velez LN, Uezato H. Diffuse and disseminated cutaneous leishmaniasis: clinical cases experienced in Ecuador and a brief review. Trop Med Health. 2016;44:2.

63. van Griensven J, Carrillo E, López-Vélez R, Lynen L, Moreno J. Leishmaniasis in immunosuppressed individuals. Clin Microbiol Infect. 2014 Apr;20(4):286–99.

64. Centers for Disease Control (CDC). Viscerotropic leishmaniasis in persons returning from Operation Desert Storm--1990-1991. MMWR Morb Mortal Wkly Rep. 1992 Feb 28;41(8):131–4.

65. Herwaldt BL. Leishmaniasis. Lancet. 1999 Oct 2;354(9185):1191–9.

66. Arce A, Estirado A, Ordobas M, Sevilla S, García N, Moratilla L, et al. Re-emergence of leishmaniasis in Spain: community outbreak in Madrid, Spain, 2009 to 2012. Euro Surveill. 2013 Jul 25;18(30):20546.

67. Ready PD. Epidemiology of visceral leishmaniasis. Clin Epidemiol. 2014;6:147–54.

68. Desjeux P, Alvar J. Leishmania/HIV co-infections: Epidemiology in Europe. Annals of Tropical Medicine and Parasitology. 2003;97(SUPPL. 1):3–15.

69. Burza S, Mahajan R, Sanz MG, Sunyoto T, Kumar R, Mitra G, et al. HIV and visceral leishmaniasis coinfection in Bihar, India: An underrecognized and underdiagnosed threat against elimination. Clinical Infectious Diseases. 2014;59(4):552–5.

70. Yimer M. Proportion of Visceral Leishmaniasis and Human Immune Deficiency Virus Co-Infection among Clinically Confirmed Visceral Leishmaniasis Patients at the Endemic Foci of the Amhara National Regional State, North-West Ethiopia. American Journal of Biomedical and Life Sciences. 2014;2(1):1.

71. Aschale Y, Ayehu A, Worku L, Tesfa H, Birhanie M, Lemma W. Malaria-visceral leishmaniasis co-infection and associated factors among migrant laborers in West Armachiho district, North West Ethiopia: Community based cross-sectional study. BMC Infectious Diseases. 2019;19(1):1–7.

72. Li XX, Zhou XN. Co-infection of tuberculosis and parasitic diseases in humans: a systematic review. Parasites & Vectors. 2013 Mar 22;6(1):79.

73. Mahajan R, Kazmi S, Pandey K, Harshana A, Alexander N, Moretó-Planas L, et al. TUBERCULOSIS IN PATIENTS CO-INFECTED WITH VISCERAL LEISHMANIASIS AND HIV – A NEW DIAGNOSTIC AND MANAGEMENT CHALLENGE. International Journal of Infectious Diseases. 2023 May 1;130:S7–8.

74. Shweta, Bhatnagar S, Gupta AK, Murti K, Pandey K. Co-infection of visceral leishmaniasis and pulmonary tuberculosis: a case study. Asian Pac J Trop Dis. 2014 Feb;4(1):57–60.

75. van Griensven J, Mohammed R, Ritmeijer K, Burza S, Diro E. Tuberculosis in Visceral Leishmaniasis-Human Immunodeficiency Virus Coinfection: An Evidence Gap in Improving Patient Outcomes? Open Forum Infect Dis. 2018 Mar 16;5(4):ofy059.

76. Zijlstra EE. The immunology of post-kala-azar dermal leishmaniasis (PKDL). Parasites and Vectors. 2016;9(1):1–9.

77. Addy M, Nandy A. Ten years of kala-azar in west Bengal, Part I. Did post-kala-azar dermal leishmaniasis initiate the outbreak in 24-Parganas? Bull World Health Organ. 1992;70(3):341–6.

78. Uranw S, Ostyn B, Rijal A, Devkota S, Khanal B, Menten J, et al. Post-kala-azar dermal leishmaniasis in Nepal: a retrospective cohort study (2000-2010). PLoS Negl Trop Dis. 2011 Dec;5(12):e1433.

79. De Brito RCF, Aguiar-Soares RD de O, Cardoso JM de O, Coura-Vital W, Roatt BM, Reis AB. Recent advances and new strategies in Leishmaniasis diagnosis. Appl Microbiol Biotechnol. 2020 Oct;104(19):8105–16.

80. de Vries HJC, Reedijk SH, Schallig HDFH. Cutaneous leishmaniasis: recent developments in diagnosis and management. Am J Clin Dermatol. 2015 Apr;16(2):99–109.

81. Elmahallawy EK, Sampedro Martinez A, Rodriguez-Granger J, Hoyos-Mallecot Y, Agil A, Navarro Mari JM, et al. Diagnosis of leishmaniasis. J Infect Dev Ctries. 2014 Aug 13;8(8):961–72.

82. Sousa MC, Varandas R, Santos RC, Santos-Rosa M, Alves V, Salvador JAR. Antileishmanial activity of semisynthetic lupane triterpenoids betulin and betulinic acid derivatives: synergistic effects with miltefosine. PLoS One. 2014;9(3):e89939.

83. Miranda ADC, González KA, Samudio F, Pineda VJ, Calzada JE, Capitan-Barrios Z, et al. Molecular Identification of Parasites Causing Cutaneous Leishmaniasis in Panama. Am J Trop Med Hyg. 2021 Jan 11;

84. Singh OP, Sundar S. Developments in Diagnosis of Visceral Leishmaniasis in the Elimination Era. J Parasitol Res. 2015;2015:239469.

85. Mantur BG, Mulimani MS, Bidari LH, Akki AS, Tikare NV. Splenic puncture: diagnostic accuracy and safety in infectious diseases. International Journal of Infectious Diseases. 2008 Jul 1;12(4):446–7.

86. Sundar S, Singh OP. Molecular Diagnosis of Visceral Leishmaniasis. Mol Diagn Ther. 2018 Aug;22(4):443–57.

87. Kumari D, Perveen S, Sharma R, Singh K. Advancement in leishmaniasis diagnosis and therapeutics: An update. Eur J Pharmacol. 2021 Aug 21;174436.

88. Sakkas H, Gartzonika C, Levidiotou S. Laboratory diagnosis of human visceral leishmaniasis. J Vector Borne Dis. 2016 Mar;53(1):8–16.

89. Taslimi Y, Sadeghipour P, Habibzadeh S, Mashayekhi V, Mortazavi H, Müller I, et al. A novel non-invasive diagnostic sampling technique for cutaneous leishmaniasis. PLoS Negl Trop Dis. 2017 Jul;11(7):e0005750.

90. Bettaieb J, Toumi A, Ghawar W, Chlif S, Nouira M, Belhaj-Hamida N, et al. A prospective cohort study of Cutaneous Leishmaniasis due to Leishmania major: Dynamics of the

Leishmanin skin test and its predictive value for protection against infection and disease. PLoS Negl Trop Dis. 2020 Aug;14(8):e0008550.

91. Reimão JQ, Coser EM, Lee MR, Coelho AC. Laboratory Diagnosis of Cutaneous and Visceral Leishmaniasis: Current and Future Methods. Microorganisms. 2020 Oct 22;8(11):1632.

92. do Vale INPC, Saliba JW, Fonseca GSF, Peruhype-Magalhães V, de Araújo FF, Pascoal-Xavier MA, et al. Laboratorial algorithm for serological diagnosis of visceral leishmaniasis using rK39-ICT, DAT-LPC and FC-Simplex IgG1. J Immunol Methods. 2020 May;480:112765.

93. Cota GF, de Sousa MR, Demarqui FN, Rabello A. The diagnostic accuracy of serologic and molecular methods for detecting visceral leishmaniasis in HIV infected patients: meta-analysis. PLoS Negl Trop Dis. 2012;6(5):e1665.

94. Srivastava P, Dayama A, Mehrotra S, Sundar S. Diagnosis of visceral leishmaniasis. Trans R Soc Trop Med Hyg. 2011 Jan;105(1):1–6.

95. Silva ES, Schoone GJ, Gontijo CMF, Brazil RP, Pacheco RS, Schallig HDFH. Application of direct agglutination test (DAT) and fast agglutination screening test (FAST) for sero-diagnosis of visceral leishmaniasis in endemic area of Minas Gerais, Brazil. Kinetoplastid Biol Dis. 2005 Jun 14;4:4.

96. Hailu A, Schoone GJ, Diro E, Tesfaye A, Techane Y, Tefera T, et al. Field evaluation of a fast anti-Leishmania antibody detection assay in Ethiopia. Trans R Soc Trop Med Hyg. 2006 Jan;100(1):48–52.

97. Iqbal J, Hira PR, Saroj G, Philip R, Al-Ali F, Madda PJ, et al. Imported visceral leishmaniasis: diagnostic dilemmas and comparative analysis of three assays. J Clin Microbiol. 2002 Feb;40(2):475–9.

98. Kumar A, Pandey SC, Samant M. A spotlight on the diagnostic methods of a fatal disease Visceral Leishmaniasis. Parasite Immunol. 2020 Oct;42(10):e12727.

99. Sarkari B, Ashrafmansouri M, Hatam G, Habibi P, Abdolahi Khabisi S. Performance of an ELISA and indirect immunofluorescence assay in serological diagnosis of zoonotic cutaneous leishmaniasis in iran. Interdiscip Perspect Infect Dis. 2014;2014:505134.

100. Thakur S, Joshi J, Kaur S. Leishmaniasis diagnosis: an update on the use of parasitological, immunological and molecular methods. Journal of Parasitic Diseases [Internet]. 2020;(Dhiman 2014). Available from: https://doi.org/10.1007/s12639-020-01212-w

101. Deepachandi B, Weerasinghe S, Soysa P, Karunaweera N, Siriwardana Y. A highly sensitive modified nested PCR to enhance case detection in leishmaniasis. BMC Infect Dis. 2019 Jul 15;19(1):623.

102. Karimi Kakh M, Golchin M, Kazemi Arababadi M, Daneshvar H. Application of the Leishmania infantum 21-kDa recombinant protein for the development of an immunochromatographic test. Parasite Immunol. 2020 Oct;42(10):e12770.

103. Mniouil M, Fellah H, Amarir F, Sadak A, Et-Touys A, Bakri Y, et al. Comparative evaluation of immunochromatographic dipstick test (ICT) rk39, soluble antigen ELISA and IFAT for the sero-diagnosis of visceral leishmaniasis in Morocco. Acta Trop. 2018 Jun;182:185–9.

104. Abass E, Bollig N, Reinhard K, Camara B, Mansour D, Visekruna A, et al. rKLO8, a novel Leishmania donovani - derived recombinant immunodominant protein for sensitive detection of visceral leishmaniasis in Sudan. PLoS Negl Trop Dis. 2013;7(7):e2322.

105. Rezvan H, Nourian A reza, Hamoon Navard S. An Overview on Leishmania Diagnosis. Journal of Medical Microbiology and Infectious Diseases. 2017 Jan 10;5(1):1–11.

106. Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, et al. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat Rev Microbiol. 2007 Nov;5(11):873–82.

107. Akhoundi M, Downing T, Votýpka J, Kuhls K, Lukeš J, Cannet A, et al. Leishmania infections: Molecular targets and diagnosis. Mol Aspects Med. 2017 Oct;57:1–29.

108. de Ruiter CM, van der Veer C, Leeflang MMG, Deborggraeve S, Lucas C, Adams ER. Molecular tools for diagnosis of visceral leishmaniasis: systematic review and meta-analysis of diagnostic test accuracy. J Clin Microbiol. 2014 Sep;52(9):3147–55.

109. Lemrani M, Hamdi S, Laamrani A, Hassar M. PCR detection of Leishmania in skin biopsies. J Infect Dev Ctries. 2009 Sep 15;3(2):115–22.

110. Salam MA, Huda MM, Khan MGM, Shomik MS, Mondal D. Evidence-based diagnostic algorithm for visceral leishmaniasis in Bangladesh. Parasitol Int. 2021 Feb;80:102230.

111. Paiva-Cavalcanti M, Regis-da-Silva CG, Gomes YM. Comparison of real-time PCR and conventional PCR for detection of Leishmania (Leishmania) infantum infection: a mini-review. J Venom Anim Toxins incl Trop Dis. 2010;16:537–42.

112. Ghosh P, Hasnain MG, Hossain F, Khan MAA, Chowdhury R, Faisal K, et al. Evaluation of Real-time PCR for Diagnosis of Post-Kala-azar Dermal Leishmaniasis in Endemic Foci of Bangladesh. Open Forum Infect Dis. 2018 Oct;5(10):ofy234.

113. Filgueira CPB, Moreira OC, Cantanhêde LM, de Farias HMT, Porrozzi R, Britto C, et al. Comparison and clinical validation of qPCR assays targeting Leishmania 18S rDNA and HSP70 genes in patients with American Tegumentary Leishmaniasis. PLoS Negl Trop Dis. 2020 Oct;14(10):e0008750.

114. Galluzzi L, Ceccarelli M, Diotallevi A, Menotta M, Magnani M. Real-time PCR applications for diagnosis of leishmaniasis. Parasit Vectors. 2018 May 2;11(1):273.

115. Giantsis IA, Chaskopoulou A, Claude Bon M. Direct Multiplex PCR (dmPCR) for the Identification of Six Phlebotomine Sand Fly Species (Diptera: Psychodidae), Including Major Leishmania Vectors of the Mediterranean. J Econ Entomol. 2017 Feb 1;110(1):245–9.

116. Gill P, Ghaemi A. Nucleic acid isothermal amplification technologies: a review. Nucleosides Nucleotides Nucleic Acids. 2008 Mar;27(3):224–43.

117. Saad AA, Ahmed NG, Osman OS, Al-Basheer AA, Hamad A, Deborggraeve S, et al. Diagnostic accuracy of the Leishmania OligoC-TesT and NASBA-Oligochromatography for diagnosis of leishmaniasis in Sudan. PLoS Negl Trop Dis. 2010 Aug 3;4(8):e776.

118. van der Meide WF, Schoone GJ, Faber WR, Zeegelaar JE, de Vries HJC, Ozbel Y, et al. Quantitative nucleic acid sequence-based assay as a new molecular tool for detection and quantification of Leishmania parasites in skin biopsy samples. J Clin Microbiol. 2005 Nov;43(11):5560–6.

119. Espada CR, Ortiz PA, Shaw JJ, Barral AMP, Costa JML, Uliana SRB, et al. Identification of Leishmania (Viannia) species and clinical isolates of Leishmania (Leishmania) amazonensis from Brazil using PCR-RFLP of the heat-shock protein 70 gene reveals some unexpected observations. Diagn Microbiol Infect Dis. 2018 Aug;91(4):312–8.

120. Mohammadi MA, Bamorovat M, Fasihi Harandi M, Karimi T, Sharifi I, Aflatoonian MR. Comparison of Three PCR-based Methods for Simplicity and Cost Effectiveness Identification of Cutaneous Leishmaniasis Due to Leishmania tropica. Iran J Parasitol. 2017;12(2):215–23.

121. Montalvo AM, Fraga J, Monzote L, Montano I, De Doncker S, Dujardin JC, et al. Heat-shock protein 70 PCR-RFLP: a universal simple tool for Leishmania species discrimination in the New and Old World. Parasitology. 2010 Jul;137(8):1159–68.

122. Blaizot R, Simon S, Ginouves M, Prévot G, Blanchet D, Ravel C, et al. Validation of Swab Sampling and SYBR Green-Based Real-Time PCR for the Diagnosis of Cutaneous Leishmaniasis in French Guiana. J Clin Microbiol. 2021 Jan 21;59(2):e02218-20.

123. Kumar A, Boggula VR, Misra P, Sundar S, Shasany AK, Dube A. Amplified fragment length polymorphism (AFLP) analysis is useful for distinguishing Leishmania species of visceral and cutaneous forms. Acta Trop. 2010 Feb;113(2):202–6.

124. Restrepo CM, De La Guardia C, Sousa OE, Calzada JE, Fernández PL, Lleonart R. AFLP polymorphisms allow high resolution genetic analysis of American Tegumentary Leishmaniasis agents circulating in Panama and other members of the Leishmania genus. PLoS One. 2013;8(9):e73177.

125. Abraham RS, Aubert G. Flow Cytometry, a Versatile Tool for Diagnosis and Monitoring of Primary Immunodeficiencies. Clin Vaccine Immunol. 2016 Apr;23(4):254–71.

126. Pedral-Sampaio G, Alves JS, Schriefer A, Magalhães A, Meyer R, Glesby MJ, et al. Detection of IgG Anti-Leishmania Antigen by Flow Cytometry as a Diagnostic Test for Cutaneous Leishmaniasis. PLoS One. 2016;11(9):e0162793.

127. Caraballo-Guzmán AJ, Ospina-Villa JD, Cuesta-Caicedo AP, Sánchez-Jiménez MM. Immunoproteomics characterization of Leishmania panamensis proteins for potential clinical diagnosis of mucosal Leishmaniasis. Parasite Immunol. 2021 Jun;43(6):e12824.

128. Culha G, Akyar I, Yildiz Zeyrek F, Kurt Ö, Gündüz C, Özensoy Töz S, et al. Leishmaniasis in Turkey: Determination of Leishmania Species by Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS). Iran J Parasitol. 2014;9(2):239–48.

129. Jaiswal P, Ghosh M, Patra G, Saha B, Mukhopadhyay S. Clinical Proteomics Profiling for Biomarker Identification Among Patients Suffering With Indian Post Kala Azar Dermal Leishmaniasis. Front Cell Infect Microbiol. 2020;10:251.

130. Ohyama K, Kuroda N. Immune complexome analysis. Adv Clin Chem. 2013;60:129–41.

131. Machado AS, Ramos FF, Oliveira-da-Silva JA, Santos TTO, Tavares GSV, Costa LE, et al. An immunoproteomics approach to identify Leishmania infantum proteins to be applied for the diagnosis of visceral leishmaniasis and human immunodeficiency virus co-infection. Parasitology. 2020 Aug;147(9):932–9.

132. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 2000 Jun 15;28(12):e63.

133. Sriworarat C, Phumee A, Mungthin M, Leelayoova S, Siriyasatien P. Development of loop-mediated isothermal amplification (LAMP) for simple detection of Leishmania infection. Parasit Vectors. 2015 Nov 14;8:591.

134. Erber AC, Sandler PJ, de Avelar DM, Swoboda I, Cota G, Walochnik J. Diagnosis of visceral and cutaneous leishmaniasis using loop-mediated isothermal amplification (LAMP) protocols: a systematic review and meta-analysis. Parasit Vectors. 2022 Jan 24;15(1):34.

135. Dixit KK, Verma S, Singh OP, Singh D, Singh AP, Gupta R, et al. Validation of SYBR green I based closed tube loop mediated isothermal amplification (LAMP) assay and simplified direct-blood-lysis (DBL)-LAMP assay for diagnosis of visceral leishmaniasis (VL). PLoS Negl Trop Dis. 2018 Nov;12(11):e0006922.

136. Nzelu CO, Kato H, Peters NC. Loop-mediated isothermal amplification (LAMP): An advanced molecular point-of-care technique for the detection of Leishmania infection. PLoS Neglected Tropical Diseases. 2019;13(11):1–21.

137. Caierão J, Paiva JACD, Sampaio JLM, Silva MG da, Santos DR de S, Coelho FS, et al. Multilocus enzyme electrophoresis analysis of rapidly-growing mycobacteria: an alternative tool for identification and typing. Int J Infect Dis. 2016 Jan;42:11–6.

138. Van der Auwera G, Dujardin JC. Species typing in dermal leishmaniasis. Clin Microbiol Rev. 2015 Apr;28(2):265–94.

139. Sundar S, Singh B. Understanding Leishmania parasites through proteomics and implications for the clinic. Expert Rev Proteomics. 2018 May;15(5):371–90.

140. Herrera G, Hernández C, Ayala MS, Flórez C, Teherán AA, Ramírez JD. Evaluation of a Multilocus Sequence Typing (MLST) scheme for Leishmania (Viannia) braziliensis and Leishmania (Viannia) panamensis in Colombia. Parasit Vectors. 2017 May 12;10(1):236.

141. Hosseini M, Nateghi Rostami M, Hosseini Doust R, Khamesipour A. Multilocus sequence typing analysis of Leishmania clinical isolates from cutaneous leishmaniasis patients of Iran. Infect Genet Evol. 2020 Nov;85:104533.

142. Toubanaki DK, Athanasiou E, Karagouni E. Gold nanoparticle-based lateral flow biosensor for rapid visual detection of Leishmania-specific DNA amplification products. J Microbiol Methods. 2016 Aug;127:51–8.

143. Bose PP, Kumar P. Visual assessment of parasitic burden in infected macrophage by plasmonic detection of leishmania specific marker RNA. Biochem Biophys Res Commun. 2016 Nov 4;480(1):81–6.

144. Martins BR, Barbosa YO, Andrade CMR, Pereira LQ, Simão GF, de Oliveira CJ, et al. Development of an Electrochemical Immunosensor for Specific Detection of Visceral Leishmaniasis Using Gold-Modified Screen-Printed Carbon Electrodes. Biosensors (Basel). 2020 Jul 23;10(8):81.

145. Maciel MODS, Soares MF, Costa SF, Bragato JP, de Freitas JH, Venturin GL, et al. Development of plasmonic ELISA for the detection of anti-Leishmania sp. IgG antibodies. J Immunol Methods. 2019 Nov;474:112664.

146. Gedda MR, Madhukar P, Shukla A, Mudavath SL, Srivastava ON, Singh OP, et al. Nanodiagnostics in leishmaniasis: A new frontiers for early elimination. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2021 Mar;13(2):e1675.

147. Maza Vega D, Di Meglio M, Alonso S del V, Alvira F, Montanari J. Nanomaterials for diagnosis, treatment, and prevention of human cutaneous leishmaniasis: A review. OpenNano. 2023 Jul 1;12:100158.

148. Bailly AL, Correard F, Popov A, Tselikov G, Chaspoul F, Appay R, et al. In vivo evaluation of safety, biodistribution and pharmacokinetics of laser-synthesized gold nanoparticles. Sci Rep. 2019 Sep 9;9(1):12890.

149. Pospíšil J, Konrádová D, Strnad M. Antileishmanial Activity of Lignans, Neolignans, and Other Plant Phenols. Prog Chem Org Nat Prod. 2021;115:115–76.

150. Singh K, Garg G, Ali V. Current Therapeutics, Their Problems and Thiol Metabolism as Potential Drug Targets in Leishmaniasis. Curr Drug Metab. 2016;17(9):897–919.

151. Ponte-Sucre A, Gamarro F, Dujardin JC, Barrett MP, López-Vélez R, García-Hernández R, et al. Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. PLoS Negl Trop Dis. 2017 Dec;11(12):e0006052.

152. No JH. Visceral leishmaniasis: Revisiting current treatments and approaches for future discoveries. Acta Trop. 2016 Mar;155:113–23.

153. Roatt BM, de Oliveira Cardoso JM, De Brito RCF, Coura-Vital W, de Oliveira Aguiar-Soares RD, Reis AB. Recent advances and new strategies on leishmaniasis treatment. Appl Microbiol Biotechnol. 2020 Nov;104(21):8965–77.

154. Sundar S, Singh B. Emerging therapeutic targets for treatment of leishmaniasis. Expert Opin Ther Targets. 2018 Jun;22(6):467–86.

155. Ramalho DB, Silva RE da, Senna MCR de, Moreira HSA, Pedras MJ, Avelar DM de, et al. Meglumine antimoniate intralesional infiltration for localised cutaneous leishmaniasis: a single arm, open label, phase II clinical trial. Mem Inst Oswaldo Cruz. 2018 Jun 21;113(9):e180200.

156. Arboleda M, Barrantes S, Úsuga LY, Robledo SM. Successful treatment of cutaneous leishmaniasis with intralesional meglumine antimoniate: A case series. Rev Soc Bras Med Trop. 2019 May 16;52:e20180211.

157. Nagle AS, Khare S, Kumar AB, Supek F, Buchynskyy A, Mathison CJN, et al. Recent developments in drug discovery for leishmaniasis and human African trypanosomiasis. Chem Rev. 2014 Nov 26;114(22):11305–47.

158. Sundar S, Mehta H, Suresh AV, Singh SP, Rai M, Murray HW. Amphotericin B treatment for Indian visceral leishmaniasis: conventional versus lipid formulations. Clin Infect Dis. 2004 Feb 1;38(3):377–83.

159. Freitas-Junior LH, Chatelain E, Kim HA, Siqueira-Neto JL. Visceral leishmaniasis treatment: What do we have, what do we need and how to deliver it? Int J Parasitol Drugs Drug Resist. 2012 Dec;2:11–9.

160. Tamiru A, Tigabu B, Yifru S, Diro E, Hailu A. Safety and efficacy of liposomal amphotericin B for treatment of complicated visceral leishmaniasis in patients without HIV, North-West Ethiopia. BMC Infect Dis. 2016 Oct 10;16(1):548.

161. Sundar S, Chakravarty J, Agarwal D, Rai M, Murray HW. Single-dose liposomal amphotericin B for visceral leishmaniasis in India. N Engl J Med. 2010 Feb 11;362(6):504–12.

162. Wei P, Ye Z, Cao S, Bai S, Seeberger PH, Yin J, et al. Combination therapy with amphotericin B and doxorubicin encapsulated in mannosylated nanomicelles for visceral leishmaniasis. Colloids and Surfaces A: Physicochemical and Engineering Aspects. 2020 Aug 5;598:124804.

163. Monge-Maillo B, López-Vélez R. Miltefosine for Visceral and Cutaneous Leishmaniasis: Drug Characteristics and Evidence-Based Treatment Recommendations. Clinical Infectious Diseases. 2015 May 1;60(9):1398–404.

164. Pinto-Martinez AK, Rodriguez-Durán J, Serrano-Martin X, Hernandez-Rodriguez V, Benaim G. Mechanism of Action of Miltefosine on Leishmania donovani Involves the Impairment of Acidocalcisome Function and the Activation of the Sphingosine-Dependent Plasma Membrane Ca2+ Channel. Antimicrob Agents Chemother. 2018 Jan;62(1):e01614-17.

165. Ortega V, Giorgio S, de Paula E. Liposomal formulations in the pharmacological treatment of leishmaniasis: a review. J Liposome Res. 2017 Sep;27(3):234–48.

166. Sinha PK, Jha TK, Thakur CP, Nath D, Mukherjee S, Aditya AK, et al. Phase 4 pharmacovigilance trial of paromomycin injection for the treatment of visceral leishmaniasis in India. J Trop Med. 2011;2011:645203.

167. Dorlo TPC, Balasegaram M, Beijnen JH, de Vries PJ. Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. J Antimicrob Chemother. 2012 Nov;67(11):2576–97.

168. Sánchez-Cañete MP, Carvalho L, Pérez-Victoria FJ, Gamarro F, Castanys S. Low plasma membrane expression of the miltefosine transport complex renders Leishmania braziliensis refractory to the drug. Antimicrob Agents Chemother. 2009 Apr;53(4):1305–13.

169. Ware JM, O'Connell EM, Brown T, Wetzler L, Talaat KR, Nutman TB, et al. Efficacy and Tolerability of Miltefosine in the Treatment of Cutaneous Leishmaniasis. Clinical Infectious Diseases. 2021 Oct 1;73(7):e2457–562.

170. Goto H, Lindoso JAL. Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis. Expert Rev Anti Infect Ther. 2010 Apr;8(4):419–33.

171. Sosa N, Pascale JM, Jiménez AI, Norwood JA, Kreishman-Detrick M, Weina PJ, et al. Topical paromomycin for New World cutaneous leishmaniasis. PLoS Negl Trop Dis. 2019 May;13(5):e0007253.

172. Pokharel P, Ghimire R, Lamichhane P. Efficacy and Safety of Paromomycin for Visceral Leishmaniasis: A Systematic Review. J Trop Med. 2021 Jul 24;2021:8629039.

173. Jhingran A, Chawla B, Saxena S, Barrett MP, Madhubala R. Paromomycin: uptake and resistance in Leishmania donovani. Mol Biochem Parasitol. 2009 Apr;164(2):111–7.

174. Larkins-Ford J, Greenstein T, Van N, Degefu YN, Olson MC, Sokolov A, et al. Systematic measurement of combination-drug landscapes to predict in vivo treatment outcomes for tuberculosis. Cell Syst. 2021 Nov 17;12(11):1046-1063.e7.

175. Stellbrink HJ, Lazzarin A, Woolley I, Llibre JM. The potential role of bictegravir/emtricitabine/tenofovir alafenamide (BIC/FTC/TAF) single-tablet regimen in the expanding spectrum of fixed-dose combination therapy for HIV. HIV Med. 2020 Mar;21 Suppl 1:3–16.

176. Tindana P, de Haan F, Amaratunga C, Dhorda M, van der Pluijm RW, Dondorp AM, et al. Deploying triple artemisinin-based combination therapy (TACT) for malaria treatment in Africa: ethical and practical considerations. Malar J. 2021 Feb 27;20(1):119.

177. Trinconi CT, Reimão JQ, Bonano VI, Espada CR, Miguel DC, Yokoyama-Yasunaka JKU, et al. Topical tamoxifen in the therapy of cutaneous leishmaniasis. Parasitology. 2018 Apr;145(4):490–6.

178. Caridha D, Sciotti RJ, Sousa J, Vesely B, Teshome T, Bonkoungou G, et al. Combination of Subtherapeutic Doses of Tretazicar and Liposomal Amphotericin B Suppresses and Cures Leishmania major-Induced Cutaneous Lesions in Murine Models. ACS Infect Dis. 2021 Feb 12;7(2):506–17.

179. van Griensven J, Balasegaram M, Meheus F, Alvar J, Lynen L, Boelaert M. Combination therapy for visceral leishmaniasis. Lancet Infect Dis. 2010 Mar;10(3):184–94.

180. Mahajan R, Das P, Isaakidis P, Sunyoto T, Sagili KD, Lima MA, et al. Combination Treatment for Visceral Leishmaniasis Patients Coinfected with Human Immunodeficiency Virus in India. Clin Infect Dis. 2015 Oct 15;61(8):1255–62.

181. Ramesh V, Dixit KK, Sharma N, Singh R, Salotra P. Assessing the Efficacy and Safety of Liposomal Amphotericin B and Miltefosine in Combination for Treatment of Post Kala-Azar Dermal Leishmaniasis. J Infect Dis. 2020 Feb 3;221(4):608–17.

182. Breckenridge A, Jacob R. Overcoming the legal and regulatory barriers to drug repurposing. Nat Rev Drug Discov. 2019 Jan;18(1):1–2.

183. Bustamante C, Ochoa R, Asela C, Muskus C. Repurposing of known drugs for leishmaniasis treatment using bioinformatic predictions, in vitro validations and pharmacokinetic simulations. J Comput Aided Mol Des. 2019 Sep;33(9):845–54.

184. Lima ML, Abengózar MA, Nácher-Vázquez M, Martínez-Alcázar MP, Barbas C, Tempone AG, et al. Molecular Basis of the Leishmanicidal Activity of the Antidepressant Sertraline as a Drug Repurposing Candidate. Antimicrob Agents Chemother. 2018 Dec;62(12):e01928-18.

185. Domínguez-Asenjo B, Gutiérrez-Corbo C, Álvarez-Bardón M, Pérez-Pertejo Y, Balaña-Fouce R, Reguera RM. Ex Vivo Phenotypic Screening of Two Small Repurposing Drug Collections Identifies Nifuratel as a Potential New Treatment against Visceral and Cutaneous Leishmaniasis. ACS Infect Dis. 2021 Jun 11;

186. Khadir F, Shaler CR, Oryan A, Rudak PT, Mazzuca DM, Taheri T, et al. Therapeutic control of leishmaniasis by inhibitors of the mammalian target of rapamycin. PLoS Negl Trop Dis. 2018 Aug;12(8):e0006701.

187. Mesquita JT, Romanelli MM, de Melo Trinconi Trinconi Cm C, Guerra JM, Taniwaki NN, Uliana SRB, et al. Repurposing topical triclosan for cutaneous leishmaniasis: Preclinical efficacy in a murine Leishmania (L.) amazonensis model. Drug Dev Res. 2022 Apr;83(2):285–95.

188. Tabrez S, Rahman F, Ali R, Muhammad F, Alshehri BM, Alaidarous MA, et al. Repurposing of FDA-approved drugs as inhibitors of sterol C-24 methyltransferase of Leishmania donovani to fight against leishmaniasis. Drug Dev Res. 2021 Apr 30;

189. Tunes LG, Morato RE, Garcia A, Schmitz V, Steindel M, Corrêa-Junior JD, et al. Preclinical Gold Complexes as Oral Drug Candidates to Treat Leishmaniasis Are Potent Trypanothione Reductase Inhibitors. ACS Infect Dis. 2020 May 8;6(5):1121–39.

190. Karuppusamy C, Palanivel V. Role of nanoparticles in drug delivery system: A comprehensive review. 2017 Mar 1;9:318–25.

191. Nafari A, Cheraghipour K, Sepahvand M, Shahrokhi G, Gabal E, Mahmoudvand H. Nanoparticles: New agents toward treatment of leishmaniasis. Parasite Epidemiol Control. 2020 Aug;10:e00156.

192. Ahmad MZ, Rizwanullah Md, Ahmad J, Alasmary MY, Akhter MdH, Abdel-Wahab BA, et al. Progress in nanomedicine-based drug delivery in designing of chitosan nanoparticles for cancer therapy. International Journal of Polymeric Materials and Polymeric Biomaterials. 2022 May 24;71(8):602–23.

193. Kumar P, Shivam P, Mandal S, Prasanna P, Kumar S, Prasad SR, et al. Synthesis, characterization, and mechanistic studies of a gold nanoparticle-amphotericin B covalent conjugate with enhanced antileishmanial efficacy and reduced cytotoxicity. Int J Nanomedicine. 2019;14:6073–101.

194. Kumar A, Choudhary A, Kaur H, Guha S, Mehta S, Husen A. Potential Applications of Engineered Nanoparticles in Plant Disease Management: A Critical Update. Chemosphere. 2022 May 1;295:133798.

195. Sahu T, Ratre YK, Chauhan S, Bhaskar LVKS, Nair MP, Verma HK. Nanotechnology based drug delivery system: Current strategies and emerging therapeutic potential for medical science. Journal of Drug Delivery Science and Technology. 2021 Jun 1;63:102487.

196. Novais FO, Amorim CF, Scott P. Host-Directed Therapies for Cutaneous Leishmaniasis. Front Immunol. 2021;12:660183.

197. Kyriazis ID, Koutsoni OS, Aligiannis N, Karampetsou K, Skaltsounis AL, Dotsika E. The leishmanicidal activity of oleuropein is selectively regulated through inflammation- and oxidative stress-related genes. Parasit Vectors. 2016 Aug 9;9(1):441.

198. Solana JC, Moreno J, Iborra S, Soto M, Requena JM. Live attenuated vaccines, a favorable strategy to provide long-term immunity against protozoan diseases. Trends Parasitol. 2022 Apr;38(4):316–34.

199. Sacks DL. Vaccines against tropical parasitic diseases: a persisting answer to a persisting problem. Nat Immunol. 2014 May;15(5):403–5.

200. Wylie CE, Carbonell-Antoñanzas M, Aiassa E, Dhollander S, Zagmutt FJ, Brodbelt DC, et al. A systematic review of the efficacy of prophylactic control measures for naturally-occurring canine leishmaniosis, part I: vaccinations. Prev Vet Med. 2014 Nov 1;117(1):7–18.

201. Velez R, Gállego M. Commercially approved vaccines for canine leishmaniosis: a review of available data on their safety and efficacy. Trop Med Int Health. 2020 May;25(5):540–57.

202. Sergiev V, Kondrashin A, Litvinov S, Morozova L, Turbabina N, Stepanova E, et al. Epidemiology and Control of Leishmaniasis in the Former USSR: A Review Article. Iranian Journal of Parasitology. 2018 Sep 23;13(3):342–50.

203. Wenyon, C. M. Protozoology: a manual for medical men, veterinarians and zoologists. Vol. 1. Baillière, Tindall and Cox.; 1926.

204. Senekji HA, Beattie CP. Artificial infection and immunization of man with cultures of Leishmania tropica. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1941 May 27;34(6):415–9.

205. Rioux JA, Albaret JL, Houin R, Dedet JP, Lanotte G. Ecologie des Leishmanioses dans le sud de la France. 2. - Les réservoirs selvatiques: Infestation spontanée du Renard *(Vulpes vulpes* L.). Ann Parasitol Hum Comp. 1968;43(4):421–8.

206. A.Nadim, Javadian E, Mohebali M. The experience of leishmanization in the Islamic Republic of Iran. Eastern Mediterranean Health Journal [Internet]. 1997 [cited 2023 Oct 13]; Available from: https://www.semanticscholar.org/paper/The-experience-of-leishmanization-in-the-Islamic-of-A.Nadim-Javadian/79b4b1734dcba7d37828f876a50dc983938f2c76

207. Mohebali M, Nadim A, Khamesipour A. An overview of leishmanization experience: A successful control measure and a tool to evaluate candidate vaccines. Acta Tropica. 2019;200(September):105173.

208. Nadim A, Javadian E, Mohebali M. The experience of leishmanization in the Islamic Republic of Iran. EMHJ - Eastern Mediterranean Health Journal, 3 (2), 284-289, 1997 [Internet]. 1997 [cited 2023 Oct 19]; Available from: https://iris.who.int/handle/10665/117339

209. McCall LI, Zhang WW, Ranasinghe S, Matlashewski G. Leishmanization revisited: immunization with a naturally attenuated cutaneous Leishmania donovani isolate from Sri Lanka protects against visceral leishmaniasis. Vaccine. 2013 Feb 27;31(10):1420–5.

210. Ferrua B, Luci C, Le Fichoux Y, Paul A, Marty P. Imprinting of BALB/c mice with low Leishmania infantum parasite dose markedly protects spleen against high-dose challenge. Vaccine. 2006 Jan 30;24(5):589–96.

211. Rousseau D, Demartino S, Anjuère F, Ferrua B, Fragaki K, Le Fichoux Y, et al. Sustained parasite burden in the spleen of Leishmania infantum-infected BALB/c mice is accompanied by expression of MCP-1 transcripts and lack of protection against challenge. Eur Cytokine Netw. 2001;12(2):340–7.

212. Zhang WW, Karmakar S, Gannavaram S, Dey R, Lypaczewski P, Ismail N, et al. A second generation leishmanization vaccine with a markerless attenuated Leishmania major strain using CRISPR gene editing. Nature Communications. 2020 Dec;11(1).

213. Gannavaram S, Dey R, Avishek K, Selvapandiyan A, Salotra P, Nakhasi HL. Biomarkers of safety and immune protection for genetically modified live attenuated Leishmania vaccines against visceral leishmaniasis - discovery and implications. Frontiers in Immunology. 2014;5(MAY):1–9.

214. Carrión J, Folgueira C, Soto M, Fresno M, Requena JM. Leishmania infantum HSP70-II null mutant as candidate vaccine against leishmaniasis: a preliminary evaluation. Parasit Vectors. 2011 Jul 27;4:150.

215. Ghosh A, Labrecque S, Matlashewski G. Protection against Leishmania donovani infection by DNA vaccination: increased DNA vaccination efficiency through inhibiting the cellular p53 response. Vaccine. 2001 Apr 30;19(23–24):3169–78.

216. Papadopoulou B, Roy G, Breton M, Kündig C, Dumas C, Fillion I, et al. Reduced infectivity of a Leishmania donovani biopterin transporter genetic mutant and its use as an attenuated strain for vaccination. Infect Immun. 2002 Jan;70(1):62–8.

217. Silvestre R, Cordeiro-Da-Silva A, Santarém N, Vergnes B, Sereno D, Ouaissi A. SIR2-deficient Leishmania infantum induces a defined IFN-gamma/IL-10 pattern that correlates with protection. J Immunol. 2007 Sep 1;179(5):3161–70.

218. Barazandeh AF, Mou Z, Ikeogu N, Mejia EM, Edechi CA, Zhang WW, et al. The Phosphoenolpyruvate Carboxykinase Is a Key Metabolic Enzyme and Critical Virulence Factor of Leishmania major. J Immunol. 2021 Mar 1;206(5):1013–26.

219. Volpedo G, Pacheco-Fernandez T, Bhattacharya P, Oljuskin T, Dey R, Gannavaram S, et al. Determinants of Innate Immunity in Visceral Leishmaniasis and Their Implication in Vaccine Development. Front Immunol. 2021;12:748325.

220. Volpedo G, Pacheco-Fernandez T, Holcomb EA, Cipriano N, Cox B, Satoskar AR. Mechanisms of Immunopathogenesis in Cutaneous Leishmaniasis And Post Kala-azar Dermal Leishmaniasis (PKDL). Front Cell Infect Microbiol. 2021;11:685296.

221. Lázaro-Souza M, Matte C, Lima JB, Duque GA, Quintela-Carvalho G, Vivarini Á de C, et al. Leishmania infantum lipophosphoglycan-deficient mutants: A tool to study host cell-parasite interplay. Frontiers in Microbiology. 2018;9(APR):1–10.

222. Uzonna JE, Späth GF, Beverley SM, Scott P. Vaccination with Phosphoglycan-Deficient Leishmania major Protects Highly Susceptible Mice from Virulent Challenge without Inducing a Strong Th1 Response. The Journal of Immunology. 2004;172(6):3793–7.

223. Ilg T. Lipophosphoglycan is not required for infection of macrophages or mice by Leishmania mexicana. The EMBO Journal. 2000 May 2;19(9):1953–62.

224. Olobo JO, Anjili CO, Gicheru MM, Mbati PA, Kariuki TM, Githure JI, et al. Vaccination of vervet monkeys against cutaneous leishmaniosis using recombinant Leishmania “major surface glycoprotein” (gp63). Vet Parasitol. 1995 Dec;60(3–4):199–212.

225. McMahon-Pratt D, Rodriguez D, Rodriguez JR, Zhang Y, Manson K, Bergman C, et al. Recombinant vaccinia viruses expressing GP46/M-2 protect against Leishmania infection. Infect Immun. 1993 Aug;61(8):3351–9.

226. Chakravarty J, Kumar S, Trivedi S, Rai VK, Singh A, Ashman JA, et al. A clinical trial to evaluate the safety and immunogenicity of the LEISH-F1+MPL-SE vaccine for use in the prevention of visceral leishmaniasis. Vaccine. 2011 Apr 27;29(19):3531–7.

227. Coler RN, Duthie MS, Hofmeyer KA, Guderian J, Jayashankar L, Vergara J, et al. From mouse to man: safety, immunogenicity and efficacy of a candidate leishmaniasis vaccine LEISH-F3+GLA-SE. Clin Transl Immunology. 2015 Apr;4(4):e35.

228. Gillespie PM, Beaumier CM, Strych U, Hayward T, Hotez PJ, Bottazzi ME. Status of vaccine research and development of vaccines for leishmaniasis. Vaccine. 2016;

229. Scott P, Artis D, Uzonna J, Zaph C. The development of effector and memory T cells in cutaneous leishmaniasis: The implications for vaccine development. Immunological Reviews. 2004;201:318–38.

230. Moafi M, Rezvan H, Sherkat R, Taleban R. Leishmania Vaccines Entered in Clinical Trials: A Review of Literature. Int J Prev Med. 2019;10:95.

231. Aunguldee T, Gerdprasert O, Tangteerawatana P, Jariyapongskul A, Leelayoova S, Wongsatayanon BT. Immunogenicity and potential protection of DNA vaccine of Leishmania martiniquensis against Leishmania infection in mice. J Infect Dev Ctries. 2021 Sep 30;15(9):1328–38.

232. Prasanna P, Kumar P, Kumar S, Rajana VK, Kant V, Prasad SR, et al. Current status of nanoscale drug delivery and the future of nano-vaccine development for leishmaniasis - A review. Biomed Pharmacother. 2021 Jul 20;141:111920.

233. Kaye P. A Phase IIb Study to Assess the Safety, Efficacy and Immunogenicity of the Leishmania Vaccine ChAd63-KH in Post-kala Azar Dermal Leishmaniasis [Internet]. clinicaltrials.gov; 2023 May [cited 2022 Dec 31]. Report No.: NCT03969134. Available from: https://clinicaltrials.gov/study/NCT03969134

234. Dinc R. Leishmania Vaccines: the Current Situation with Its Promising Aspect for the Future. Korean J Parasitol. 2022 Dec;60(6):379–91.

235. Taslimi Y, Zahedifard F, Taheri T, Doroud D, Latif Dizaji S, Saljoughian N, et al. Comparison of Protective Potency of DNA and Live Vaccines Expressing A2-CPA-CPB-CTE Antigens against Visceral Leishmaniasis in Syrian Hamster as Preliminary Study. Iran J Parasitol. 2020;15(3):383–92.

236. Margaroni M, Agallou M, Athanasiou E, Kammona O, Kiparissides C, Gaitanaki C, et al. Vaccination with poly(D,L-lactide-co-glycolide) nanoparticles loaded with soluble Leishmania antigens and modified with a TNFα-mimicking peptide or monophosphoryl lipid A confers protection against experimental visceral leishmaniasis. Int J Nanomedicine. 2017;12:6169–84.

237. Lage DP, Ribeiro PAF, Dias DS, Mendonça DVC, Ramos FF, Carvalho LM, et al. A candidate vaccine for human visceral leishmaniasis based on a specific T cell epitope-containing chimeric protein protects mice against Leishmania infantum infection. NPJ Vaccines. 2020;5:75.

238. Alves-Silva MV, Nico D, De Luca PM, Palatnik De-Sousa CB. The F1F3 Recombinant Chimera of Leishmania donovani-Nucleoside Hydrolase (NH36) and Its Epitopes Induce Cross-Protection against Leishmania (V.) braziliensis Infection in Mice. Frontiers in Immunology. 2019;10(APR):1–21.

239. Klatt S, Simpson L, Maslov DA, Konthur Z. Leishmania tarentolae: Taxonomic classification and its application as a promising biotechnological expression host. PLoS Neglected Tropical Diseases. 2019;13(7):1–29.

240. Mendoza-Roldan JA, Mendoza-Roldan MA, Otranto D. Reptile vector-borne diseases of zoonotic concern. Int J Parasitol Parasites Wildl. 2021 Aug;15:132–42.

241. Breton M, Tremblay MJ, Ouellette M, Papadopoulou B. Live nonpathogenic parasitic vector as a candidate vaccine against visceral leishmaniasis. Infect Immun. 2005 Oct;73(10):6372–82.

242. Panasiuk M, Zimmer K, Czarnota A, Grzyb K, Narajczyk M, Peszyńska-Sularz G, et al. Immunization with Leishmania tarentolae-derived norovirus virus-like particles elicits high humoral response and stimulates the production of neutralizing antibodies. Microb Cell Fact. 2021 Sep 24;20(1):186.

243. Bandi C, Mendoza-Roldan JA, Otranto D, Alvaro A, Louzada-Flores VN, Pajoro M, et al. Leishmania tarentolae: a vaccine platform to target dendritic cells and a surrogate pathogen for next generation vaccine research in leishmaniases and viral infections. Parasites & Vectors. 2023 Jan 26;16(1):35.

244. Duthie MS, Van Hoeven N, MacMillen Z, Picone A, Mohamath R, Erasmus J, et al. Heterologous Immunization With Defined RNA and Subunit Vaccines Enhances T Cell Responses That Protect Against Leishmania donovani. Front Immunol. 2018;9:2420.

245. Gupta S, Nishi. Visceral leishmaniasis: Experimental models for drug discovery. Indian J Med Res. 2011 Jan;133(1):27–39.

246. Gopu B, Kour P, Pandian R, Singh K. Insights into the drug screening approaches in leishmaniasis. Int Immunopharmacol. 2023 Jan;114:109591.

247. van der Ende J, Schallig HDFH. Leishmania Animal Models Used in Drug Discovery: A Systematic Review. Animals (Basel). 2023 May 16;13(10):1650.

248. Guénet JL, Bonhomme F. Wild mice: an ever-increasing contribution to a popular mammalian model. Trends Genet. 2003 Jan;19(1):24–31.

249. Aguilar-Torrentera F, Carlier Y. Immunological factors governing resistance and susceptibility of mice to Leishmania major infection. Rev Latinoam Microbiol. 2001;43(3):135–42.

250. Liew FY, Wei XQ, Proudfoot L. Cytokines and nitric oxide as effector molecules against parasitic infections. Philos Trans R Soc Lond B Biol Sci. 1997 Sep 29;352(1359):1311–5.

251. Mauel J. Macrophage-parasite interactions in Leishmania infections. Journal of Leukocyte Biology. 1990;47(2):187–93.

252. Murray HW, Delph-Etienne S. Roles of endogenous gamma interferon and macrophage microbicidal mechanisms in host response to chemotherapy in experimental visceral leishmaniasis. Infect Immun. 2000 Jan;68(1):288–93.

253. Belkaid Y, Mendez S, Lira R, Kadambi N, Milon G, Sacks D. A natural model of Leishmania major infection reveals a prolonged “silent” phase of parasite amplification in the skin before the onset of lesion formation and immunity. J Immunol. 2000 Jul 15;165(2):969–77.

254. Belkaid Y, Kamhawi S, Modi G, Valenzuela J, Noben-Trauth N, Rowton E, et al. Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of Leishmania major infection in the mouse ear dermis. J Exp Med. 1998 Nov 16;188(10):1941–53.

255. Loría-Cervera EN, Andrade-Narváez FJ. Review: Animal models for the study of leishmaniasis immunology. Revista do Instituto de Medicina Tropical de Sao Paulo. 2014;56(1):1–11.

256. Afonso LC, Scott P. Immune responses associated with susceptibility of C57BL/10 mice to Leishmania amazonensis. Infect Immun. 1993 Jul;61(7):2952–9.

257. Jones DE, Buxbaum LU, Scott P. IL-4-independent inhibition of IL-12 responsiveness during Leishmania amazonensis infection. J Immunol. 2000 Jul 1;165(1):364–72.

258. Buxbaum LU, Uzonna JE, Goldschmidt MH, Scott P. Control of New World cutaneous leishmaniasis is IL-12 independent but STAT4 dependent. Eur J Immunol. :10.

259. Satoskar A, Bluethmann H, Alexander J. Disruption of the murine interleukin-4 gene inhibits disease progression during Leishmania mexicana infection but does not increase control of Leishmania donovani infection. Infect Immun. 1995 Dec;63(12):4894–9.

260. Torrentera FA, Laman JD, Van Meurs M, Adorini L, Muraille E, Carlier Y. Endogenous interleukin-12 is critical for controlling the late but not the early stage of Leishmania mexicana infection in C57BL/6 mice. Infection and Immunity. 2002;70(9):5075–80.

261. Garg R, Dube A. Animal models for vaccine studies for visceral leishmaniasis. Indian J Med Res. 2006 Mar;123(3):439–54.

262. Hommel M, Jaffe CL, Travi B, Milon G. Experimental models for leishmaniasis and for testing anti-leishmanial vaccines. Ann Trop Med Parasitol. 1995 Dec;89 Suppl 1:55–73.

263. Kumar R, Nylén S. Immunobiology of visceral leishmaniasis. Frontiers in Immunology. 2012;3(AUG):1–10.

264. Blackwell JM. Genetic susceptibility to leishmanial infections: studies in mice and man. Parasitology. 1996;112 Suppl:S67-74.

265. Bradley DJ. Letter: Genetic control of natural resistance to Leishmania donovani. Nature. 1974 Jul 26;250(464):353–4.

266. Loría-Cervera EN, Andrade-Narváez FJ. Review: Animal models for the study of leishmaniasis immunology. Revista do Instituto de Medicina Tropical de Sao Paulo. 2014;56(1):1–11.

267. Melby DLS, C P. Animal Models for the Analysis of Immune Responses to Leishmaniasis. Vol. 176, Physiology & behavior. 2017. 139–148 p.

268. Wilson ME, Jeronimo SMB, Pearson RD. Immunopathogenesis of infection with the visceralizing Leishmania species. Microb Pathog. 2005 Apr;38(4):147–60.

269. Perez LE, Chandrasekar B, Saldarriaga OA, Zhao W, Arteaga LT, Travi BL, et al. Reduced nitric oxide synthase 2 (NOS2) promoter activity in the Syrian hamster renders the animal functionally deficient in NOS2 activity and unable to control an intracellular pathogen. J Immunol. 2006 May 1;176(9):5519–28.

270. Lei SM, Ramer-Tait AE, Dahlin-Laborde RR, Mullin K, Beetham JK. Reduced hamster usage and stress in propagating Leishmania chagasi promastigotes using cryopreservation and saphenous vein inoculation. J Parasitol. 2010 Feb;96(1):103–8.

271. Kushwaha AK, Scorza BM, Singh OP, Rowton E, Lawyer P, Sundar S, et al. Domestic mammals as reservoirs for Leishmania donovani on the Indian subcontinent: Possibility and consequences on elimination. Transbound Emerg Dis. 2021 Mar 8;

272. Liu G, Wu Y, Wang L, Liu Y, Huang W, Li Y, et al. Re-emergence of canine Leishmania infantum infection in mountain areas of Beijing. One Health Advances. 2023 Mar 30;1(1):11.

273. Baneth G, Koutinas AF, Solano-Gallego L, Bourdeau P, Ferrer L. Canine leishmaniosis - new concepts and insights on an expanding zoonosis: part one. Trends Parasitol. 2008 Jul;24(7):324–30.

274. Reithinger R, Davies CR. Is the domestic dog (Canis familiaris) a reservoir host of American cutaneous leishmaniasis? A critical review of the current evidence. Am J Trop Med Hyg. 1999 Oct;61(4):530–41.

275. Santaella J, Ocampo CB, Saravia NG, Méndez F, Góngora R, Gomez MA, et al. Leishmania (Viannia) infection in the domestic dog in Chaparral, Colombia. Am J Trop Med Hyg. 2011 May;84(5):674–80.

276. Dantas-Torres F. Canine leishmaniosis in South America. Parasit Vectors. 2009 Mar 26;2 Suppl 1(Suppl 1):S1.

277. Abranches P, Santos-Gomes G, Rachamim N, Campino L, Schnur LF, Jaffe CL. An experimental model for canine visceral leishmaniasis. Parasite Immunol. 1991 Sep;13(5):537–50.

278. Campino L, Santos-Gomes G, Riça Capela MJ, Cortes S, Abranches P. Infectivity of promastigotes and amastigotes of Leishmania infantum in a canine model for leishmaniosis. Vet Parasitol. 2000 Oct 20;92(4):269–75.

279. Lasri S, Sahibi H, Sadak A, Jaffe CL, Rhalem A. Immune responses in vaccinated dogs with autoclaved Leishmania major promastigotes. Vet Res. 1999;30(5):441–9.

280. Grimaldi G. The utility of rhesus monkey (Macaca mulatta) and other non-human primate models for preclinical testing of Leishmania candidate vaccines. Mem Inst Oswaldo Cruz. 2008 Nov;103(7):629–44.

281. Amaral VF, Teva A, Porrozzi R, Silva AJ, Pereira MS, Oliveira-Neto MP, et al. Leishmania (Leishmania) major-infected rhesus macaques (Macaca mulatta) develop varying levels of resistance against homologous re-infections. Mem Inst Oswaldo Cruz. 2001 Aug;96(6):795–804.

282. Broderson JR, Chapman WL, Hanson WL. Experimental visceral leishmaniasis in the owl monkey. Vet Pathol. 1986 May;23(3):293–302.

283. Dennis VA, Lujan R, Chapman WL, Hanson WL. Leishmania donovani: cellular and humoral immune responses after primary and challenge infections in squirrel monkeys, Saimiri sciureus. Exp Parasitol. 1986 Jun;61(3):319–34.

284. Chable-Santos JB, Van Wynsberghe NR, Canto-Lara SB, Andrade-Narvaez FJ. Isolation of Leishmania (L.) mexicana from wild rodents and their possible role in the transmission of localized cutaneous leishmaniasis in the state of Campeche, Mexico. Am J Trop Med Hyg. 1995 Aug;53(2):141–5.

285. De Lima H, De Guglielmo Z, Rodríguez A, Convit J, Rodriguez N. Cotton rats (Sigmodon hispidus) and black rats (Rattus rattus) as possible reservoirs of Leishmania spp. in Lara State, Venezuela. Mem Inst Oswaldo Cruz. 2002 Mar;97(2):169–74.

286. Roque ALR, Cupolillo E, Marchevsky RS, Jansen AM. Thrichomys laurentius (Rodentia; Echimyidae) as a putative reservoir of Leishmania infantum and L. braziliensis: patterns of experimental infection. PLoS Negl Trop Dis. 2010 Feb 2;4(2):e589.

287. Van Wynsberghe NR, Canto-Lara SB, Sosa-Bibiano EI, Rivero-Cárdenas NA, Andrade-Narváez FJ. Comparison of small mammal prevalence of Leishmania (Leishmania) mexicana in five foci of cutaneous leishmaniasis in the State of Campeche, Mexico. Rev Inst Med Trop Sao Paulo. 2009;51(2):87–94.

288. Marsden PD. Mucosal leishmaniasis (“espundia” escomel, 1911). Transactions of the Royal Society of Tropical Medicine and Hygiene. 1986;80(6):859–76.

289. Turk JL, Bryceson ADM. Immunological Phenomena in Leprosy and Related Diseases. Advances in Immunology. 1971;13(C):209–66.

290. Flandin JF, Chano F, Descoteaux A. RNA interference reveals a role for TLR2 and TLR3 in the recognition of Leishmania donovani promastigotes by interferon-gamma-primed macrophages. Eur J Immunol. 2006 Feb;36(2):411–20.

291. Murray HW, Zhang Y, Zhang Y, Raman VS, Reed SG, Ma X. Regulatory actions of Toll-like receptor 2 (TLR2) and TLR4 in Leishmania donovani infection in the liver. Infect Immun. 2013 Jul;81(7):2318–26.

292. Pratti JES, da Fonseca Martins AM, da Silva JP, Ramos TD, Pereira JC, Firmino-Cruz L, et al. The role of TLR9 on Leishmania amazonensis infection and its influence on intranasal LaAg vaccine efficacy. PLoS Negl Trop Dis. 2019 Feb 25;13(2):e0007146.

293. Regli IB, Passelli K, Martínez-Salazar B, Amore J, Hurrell BP, Müller AJ, et al. TLR7 Sensing by Neutrophils Is Critical for the Control of Cutaneous Leishmaniasis. Cell Rep. 2020 Jun 9;31(10):107746.

294. Polando R, Dixit UG, Carter CR, Jones B, Whitcomb JP, Ballhorn W, et al. The roles of complement receptor 3 and Fcγ receptors during Leishmania phagosome maturation. J Leukoc Biol. 2013 Jun;93(6):921–32.

295. Faria MS, Reis FCG, Lima APCA. Toll-like receptors in Leishmania infections: Guardians or promoters? Journal of Parasitology Research. 2012;2012.

296. Marovich MA, McDowell MA, Thomas EK, Nutman TB. IL-12p70 Production by Leishmania major -Harboring Human Dendritic Cells Is a CD40/CD40 Ligand-Dependent Process. The Journal of Immunology. 2000;164(11):5858–65.

297. von Stebut E, Belkaid Y, Jakob T, Sacks DL, Udey MC. Uptake of Leishmania major amastigotes results in activation and interleukin 12 release from murine skin-derived dendritic cells: implications for the initiation of anti-Leishmania immunity. J Exp Med. 1998 Oct 19;188(8):1547–52.

298. Ashok D, Acha-Orbea H. Timing is everything: dendritic cell subsets in murine Leishmania infection. Trends Parasitol. 2014 Oct;30(10):499–507.

299. Kelsall BL, Biron CA, Sharma O, Kaye PM. Dendritic cells at the host-pathogen interface. Nat Immunol. 2002 Aug;3(8):699–702.

300. von Stebut E, Tenzer S. Cutaneous leishmaniasis: Distinct functions of dendritic cells and macrophages in the interaction of the host immune system with Leishmania major. International Journal of Medical Microbiology. 2018 Jan 1;308(1):206–14.

301. dos Santos Meira C, Gedamu L. Protective or Detrimental? Understanding the Role of Host Immunity in Leishmaniasis. Microorganisms. 2019 Dec 13;7(12):695.

302. Liu D, Uzonna JE. The early interaction of Leishmania with macrophages and dendritic cells and its influence on the host immune response. Frontiers in cellular and infection microbiology. 2012;2(June 2012):83.

303. Okwor I, Jia P, Uzonna JE. Interaction of Macrophage Antigen 1 and CD40 Ligand Leads to IL-12 Production and Resistance in CD40-Deficient Mice Infected with *Leishmania major*. JI. 2015 Oct 1;195(7):3218–26.

304. Hathcock KS, Laszlo G, Pucillo C, Linsley P, Hodes RJ. Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. J Exp Med. 1994 Aug 1;180(2):631–40.

305. McGrath KE, Frame JM, Palis J. Early hematopoiesis and macrophage development. Semin Immunol. 2015 Dec;27(6):379–87.

306. Lim JJ, Grinstein S, Roth Z. Diversity and Versatility of Phagocytosis: Roles in Innate Immunity, Tissue Remodeling, and Homeostasis. Frontiers in Cellular and Infection Microbiology [Internet]. 2017 [cited 2023 Nov 1];7. Available from: https://www.frontiersin.org/articles/10.3389/fcimb.2017.00191

307. Liew FY, Li Y, Millott S. Tumour necrosis factor (TNF-α) in leishmaniasis. II. TNF-α-induced macrophage leishmanicidal activity is mediated by nitric oxide from L-arginine. Immunology. 1990;71(4):556–9.

308. Wei XQ, Charles IG, Smith A, Ure J, Feng GJ, Huang FP, et al. Altered immune responses in mice lacking inducible nitric oxide synthase. Nature. 1995 Jun 1;375(6530):408–11.

309. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. Nature Reviews Immunology. 2005;5(8):641–54.

310. Reiner NE. Altered cell signaling and mononuclear phagocyte deactivation during intracellular infection. Immunol Today. 1994 Aug;15(8):374–81.

311. Kane MM, Mosser DM. The role of IL-10 in promoting disease progression in leishmaniasis. J Immunol. 2001 Jan 15;166(2):1141–7.

312. Sunderkötter C, Kunz M, Steinbrink K, Meinardus-Hager G, Goebeler M, Bildau H, et al. Resistance of mice to experimental leishmaniasis is associated with more rapid appearance of mature macrophages in vitro and in vivo. J Immunol. 1993 Nov 1;151(9):4891–901.

313. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science. 2004 Mar 5;303(5663):1532–5.

314. Hurrell BP, Regli IB, Tacchini-Cottier F. Different Leishmania Species Drive Distinct Neutrophil Functions. Trends Parasitol. 2016 May;32(5):392–401.

315. Gabriel C, McMaster WR, Girard D, Descoteaux A. Leishmania donovani promastigotes evade the antimicrobial activity of neutrophil extracellular traps. J Immunol. 2010 Oct 1;185(7):4319–27.

316. Laskay T, van Zandbergen G, Solbach W. Neutrophil granulocytes as host cells and transport vehicles for intracellular pathogens: apoptosis as infection-promoting factor. Immunobiology. 2008;213(3–4):183–91.

317. Ribeiro-Gomes FL, Sacks D. The influence of early neutrophil-Leishmania interactions on the host immune response to infection. Front Cell Infect Microbiol. 2012;2:59.

318. Peters NC, Egen JG, Secundino N, Debrabant A, Kimblin N, Kamhawi S, et al. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. Science. 2008;321(5891):970–4.

319. Novais FO, Santiago RC, Báfica A, Khouri R, Afonso L, Borges VM, et al. Neutrophils and macrophages cooperate in host resistance against Leishmania braziliensis infection. J Immunol. 2009 Dec 15;183(12):8088–98.

320. McFarlane E, Perez C, Charmoy M, Allenbach C, Carter KC, Alexander J, et al. Neutrophils Contribute to Development of a Protective Immune Response during Onset of Infection with Leishmania donovani. Infection and Immunity. 2008 Feb 1;76(2):532–41.

321. Almeida BFM, Narciso LG, Bosco AM, Pereira PP, Braga ET, Avanço SV, et al. Neutrophil dysfunction varies with the stage of canine visceral leishmaniosis. Vet Parasitol. 2013 Sep 1;196(1–2):6–12.

322. Korbel DS, Finney OC, Riley EM. Natural killer cells and innate immunity to protozoan pathogens. Int J Parasitol. 2004 Dec;34(13–14):1517–28.

323. Maasho K, Sanchez F, Schurr E, Hailu A, Akuffo H. Indications of the protective role of natural killer cells in human cutaneous leishmaniasis in an area of endemicity. Infect Immun. 1998 Jun;66(6):2698–704.

324. Nylén S, Maasho K, Söderstrom K, Ilg T, Akuffo H. Live Leishmania promastigotes can directly activate primary human natural killer cells to produce interferon-gamma. Clin Exp Immunol. 2003 Mar;131(3):457–67.

325. Scharton TM, Scott P. Natural killer cells are a source of interferon gamma that drives differentiation of CD4+ T cell subsets and induces early resistance to Leishmania major in mice. J Exp Med. 1993 Aug 1;178(2):567–77.

326. Cavalcante MK de A, de Freitas E Silva R, Pereira VRA, Brelaz-de-Castro MCA. Opinion Article: NK Cells in Cutaneous Leishmaniasis: Protection or Damage? Front Immunol. 2022;13:933490.

327. Alizadeh Z, Omidnia P, Altalbawy FMA, Gabr GA, Obaid RF, Rostami N, et al. Unraveling the role of natural killer cells in leishmaniasis. Int Immunopharmacol. 2023 Jan;114:109596.

328. Rashidi S, Mansouri R, Ali-Hassanzadeh M, Muro A, Nguewa P, Manzano-Román R. The Defensive Interactions of Prominent Infectious Protozoan Parasites: The Host's Complement System. Biomolecules. 2022 Oct 26;12(11):1564.

329. Späth GF, Garraway LA, Turco SJ, Beverley SM. The role(s) of lipophosphoglycan (LPG) in the establishment of Leishmania major infections in mammalian hosts. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(16):9536–41.

330. Brittingham A, Morrison CJ, McMaster WR, McGwire BS, Chang KP, Mosser DM. Role of the Leishmania surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. J Immunol. 1995 Sep 15;155(6):3102–11.

331. Marth T, Kelsall BL. Regulation of interleukin-12 by complement receptor 3 signaling. J Exp Med. 1997 Jun 2;185(11):1987–95.

332. Gupta G, Mou Z, Jia P, Sharma R, Zayats R, Viana SM, et al. The Long Pentraxin 3 (PTX3) Suppresses Immunity to Cutaneous Leishmaniasis by Regulating CD4+ T Helper Cell Response. Cell Reports. 2020 Dec;33(11):108513.

333. Ikeogu NM, Edechi CA, Akaluka GN, Feiz-Barazandeh A, Zayats RR, Salako ES, et al. Semaphorin 3E Promotes Susceptibility to *Leishmania major* Infection in Mice by Suppressing CD4 $^{+}$ Th1 Cell Response. JI. 2021 Feb 1;206(3):588–98.

334. Volanakis JE. Human C-reactive protein: expression, structure, and function. Mol Immunol. 2001 Aug;38(2–3):189–97.

335. Hajishengallis G, Russell MW. Innate Humoral Defense Factors. Mucosal Immunology. 2015;251–70.

336. Roy N, Ohtani K, Hidaka Y, Amano Y, Matsuda Y, Mori K, et al. Three pentraxins C-reactive protein, serum amyloid p component and pentraxin 3 mediate complement activation using Collectin CL-P1. Biochim Biophys Acta Gen Subj. 2017 Feb;1861(2):1–14.

337. Seow ES, Doran EC, Schroeder JH, Rogers ME, Raynes JG. C-reactive protein binds to short phosphoglycan repeats of Leishmania secreted proteophosphoglycans and activates complement. Frontiers in Immunology [Internet]. 2023 [cited 2023 Nov 3];14. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10500826/

338. Trombetta ES, Mellman I. Cell biology of antigen processing in vitro and in vivo. Annu Rev Immunol. 2005;23:975–1028.

339. Scott P, Novais FO. Cutaneous leishmaniasis: Immune responses in protection and pathogenesis. Nature Reviews Immunology. 2016;

340. Boyton RJ, Altmann DM. Is selection for TCR affinity a factor in cytokine polarization? Trends Immunol. 2002 Nov;23(11):526–9.

341. Barbi J, Snider HM, Bhardwaj N, Lezama-Dávila CM, Durbin JE, Satoskar AR. Signal transducer and activator of transcription 1 in T cells plays an indispensable role in immunity to Leishmania major by mediating Th1 cell homing to the site of infection. FASEB J. 2009 Nov;23(11):3990–9.

342. Ouyang W, Löhning M, Gao Z, Assenmacher M, Ranganath S, Radbruch A, et al. Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. Immunity. 2000 Jan;12(1):27–37.

343. Bogdan C, Röllinghoff M. The immune response to Leishmania: mechanisms of parasite control and evasion. Int J Parasitol. 1998 Jan;28(1):121–34.

344. Tomiotto-Pellissier F, Bortoleti BT da S, Assolini JP, Gonçalves MD, Carloto ACM, Miranda-Sapla MM, et al. Macrophage Polarization in Leishmaniasis: Broadening Horizons. Frontiers in immunology. 2018;9(October):2529.

345. Filippi C, Hugues S, Cazareth J, Julia V, Glaichenhaus N, Ugolini S. CD4+ T cell polarization in mice is modulated by strain-specific major histocompatibility complex-independent differences within dendritic cells. J Exp Med. 2003 Jul 21;198(2):201–9.

346. Meira C dos S, Gedamu L. Protective or detrimental? Understanding the role of host immunity in leishmaniasis. Microorganisms. 2019;7(12).

347. Gadani SP, Cronk JC, Norris GT, Kipnis J. Interleukin-4: A Cytokine to Remember. J Immunol. 2012 Nov 1;189(9):4213–9.

348. Tacchini-Cottier F, Zweifel C, Belkaid Y, Mukankundiye C, Vasei M, Launois P, et al. An immunomodulatory function for neutrophils during the induction of a CD4+ Th2 response in BALB/c mice infected with Leishmania major. J Immunol. 2000 Sep 1;165(5):2628–36.

349. Holaday BJ, Sadick MD, Wang ZE, Reiner SL, Heinzel FP, Parslow TG, et al. Reconstitution of Leishmania immunity in severe combined immunodeficient mice using Th1- and Th2-like cell lines. J Immunol. 1991 Sep 1;147(5):1653–8.

350. Uzonna JE, Joyce KL, Scott P. Low Dose Leishmania major Promotes a Transient T Helper Cell Type 2 Response That Is Down-regulated by Interferon γ–producing CD8+ T Cells. Journal of Experimental Medicine. 2004 Jun 7;199(11):1559–66.

351. Zúñiga LA, Jain R, Haines C, Cua DJ. Th17 cell development: from the cradle to the grave. Immunol Rev. 2013 Mar;252(1):78–88.

352. Tesmer LA, Lundy SK, Sarkar S, Fox DA. Th17 cells in human disease. Immunol Rev. 2008 Jun;223:87–113.

353. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. Annu Rev Immunol. 2009;27:485–517.

354. Castilho TM, Goldsmith-Pestana K, Lozano C, Valderrama L, Saravia NG, McMahon-Pratt D. Murine model of chronic L. (Viannia) panamensis infection: role of IL-13 in disease. Eur J Immunol. 2010 Oct;40(10):2816–29.

355. Gonzalez-Lombana C, Gimblet C, Bacellar O, Oliveira WW, Passos S, Carvalho LP, et al. IL-17 mediates immunopathology in the absence of IL-10 following Leishmania major infection. PLoS Pathog. 2013 Mar;9(3):e1003243.

356. Pitta MGR, Romano A, Cabantous S, Henri S, Hammad A, Kouriba B, et al. IL-17 and IL-22 are associated with protection against human kala azar caused by Leishmania donovani. J Clin Invest. 2009 Aug;119(8):2379–87.

357. Nascimento MSL, Carregaro V, Lima-Júnior DS, Costa DL, Ryffel B, Duthie MS, et al. Interleukin 17A acts synergistically with interferon γ to promote protection against leishmania infantum infection. Journal of Infectious Diseases. 2015;

358. Kägi D, Ledermann B, Bürki K, Seiler P, Odermatt B, Olsen KJ, et al. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature. 1994 May 5;369(6475):31–7.

359. Belkaid Y, Von Stebut E, Mendez S, Lira R, Caler E, Bertholet S, et al. CD8+ T cells are required for primary immunity in C57BL/6 mice following low-dose, intradermal challenge with Leishmania major. J Immunol. 2002 Apr 15;168(8):3992–4000.

360. Huber M, Timms E, Mak TW, Röllinghoff M, Lohoff M. Effective and long-lasting immunity against the parasite Leishmania major in CD8-deficient mice. Infect Immun. 1998 Aug;66(8):3968–70.

361. Soong L, Chang CH, Sun J, Longley BJ, Ruddle NH, Flavell RA, et al. Role of CD4+ T cells in pathogenesis associated with Leishmania amazonensis infection. J Immunol. 1997 Jun 1;158(11):5374–83.

362. Novais FO, Carvalho LP, Graff JW, Beiting DP, Ruthel G, Roos DS, et al. Cytotoxic T cells mediate pathology and metastasis in cutaneous leishmaniasis. PLoS Pathog. 2013;9(7):e1003504.

363. Gurunathan S, Sacks DL, Brown DR, Reiner SL, Charest H, Glaichenhaus N, et al. Vaccination with DNA encoding the immunodominant LACK parasite antigen confers protective immunity to mice infected with Leishmania major. J Exp Med. 1997 Oct 6;186(7):1137–47.

364. Jayakumar A, Castilho TM, Park E, Goldsmith-Pestana K, Blackwell JM, McMahon-Pratt D. TLR1/2 activation during heterologous prime-boost vaccination (DNA-MVA) enhances CD8+ T Cell responses providing protection against Leishmania (Viannia). PLoS Negl Trop Dis. 2011 Jun;5(6):e1204.

365. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006 May 11;441(7090):235–8.

366. Zeng W ping, Chang C, Lai J jyh. Immune suppressive activity and lack of T helper differentiation are differentially regulated in natural regulatory T cells. J Immunol. 2009 Sep 15;183(6):3583–90.

367. Sojka DK, Huang YH, Fowell DJ. Mechanisms of regulatory T-cell suppression – a diverse arsenal for a moving target. Immunology. 2008 May;124(1):13–22.

368. Suffia I, Reckling SK, Salay G, Belkaid Y. A role for CD103 in the retention of CD4+CD25+ Treg and control of Leishmania major infection. J Immunol. 2005 May 1;174(9):5444–55.

369. Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL. CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. Nature. 2002 Dec 5;420(6915):502–7.

370. Belkaid Y, Sun CM, Bouladoux N. Parasites and immunoregulatory T cells. Curr Opin Immunol. 2006 Aug;18(4):406–12.

371. Rodrigues OR, Marques C, Soares-Clemente M, Ferronha MH, Santos-Gomes GM. Identification of regulatory T cells during experimental Leishmania infantum infection. Immunobiology. 2009;214(2):101–11.

372. Martin S, Agarwal R, Murugaiyan G, Saha B. CD40 expression levels modulate regulatory T cells in Leishmania donovani infection. J Immunol. 2010 Jul 1;185(1):551–9.

373. Liu D, Uzonna JE. The p110 delta isoform of phosphatidylinositol 3-kinase controls the quality of secondary anti-Leishmania immunity by regulating expansion and effector function of memory T cell subsets. J Immunol. 2010 Mar 15;184(6):3098–105.

374. Rai AK, Thakur CP, Singh A, Seth T, Srivastava SK, Singh P, et al. Regulatory T cells suppress T cell activation at the pathologic site of human visceral leishmaniasis. PLoS One. 2012;7(2):e31551.

375. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. B Cells and Antibodies. In: Molecular Biology of the Cell 4th edition [Internet]. Garland Science; 2002 [cited 2023 Nov 4]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK26884/

376. Deak E, Jayakumar A, Cho KW, Goldsmith-Pestana K, Dondji B, Lambris JD, et al. Murine visceral leishmaniasis: IgM and polyclonal B-cell activation lead to disease exacerbation. Eur J Immunol. 2010 May;40(5):1355–68.

377. Howard JG, Hale C, Liew FY. Immunological regulation of experimental cutaneous leishmaniasis. IV. Prophylactic effect of sublethal irradiation as a result of abrogation of

suppressor T cell generation in mice genetically susceptible to Leishmania tropica. J Exp Med. 1981 Mar 1;153(3):557–68.

378. Mitchell GF, Handman E. Leishmania tropica major in mice: vaccination against cutaneous leishmaniasis in mice of high genetic susceptibility. Aust J Exp Biol Med Sci. 1983 Feb;61(Pt 1):11–25.

379. Kima PE, Constant SL, Hannum L, Colmenares M, Lee KS, Haberman AM, et al. Internalization of Leishmania mexicana complex amastigotes via the Fc receptor is required to sustain infection in murine cutaneous leishmaniasis. J Exp Med. 2000 Mar 20;191(6):1063–8.

380. Miles SA, Conrad SM, Alves RG, Jeronimo SMB, Mosser DM. A role for IgG immune complexes during infection with the intracellular pathogen Leishmania. J Exp Med. 2005 Mar 7;201(5):747–54.

381. Rodriguez-Pinto D, Saravia NG, McMahon-Pratt D. CD4 T cell activation by B cells in human Leishmania (Viannia) infection. BMC Infect Dis. 2014 Feb 25;14:108.

382. Woelbing F, Kostka SL, Moelle K, Belkaid Y, Sunderkoetter C, Verbeek S, et al. Uptake of Leishmania major by dendritic cells is mediated by Fcgamma receptors and facilitates acquisition of protective immunity. J Exp Med. 2006 Jan 23;203(1):177–88.

383. Sacks D, Noben-Trauth N. The immunology of susceptibility and resistance to Leishmania major in mice. Nat Rev Immunol. 2002 Nov;2(11):845–58.

384. Heinzel FP, Schoenhaut DS, Rerko RM, Rosser LE, Gately MK. Recombinant interleukin 12 cures mice infected with Leishmania major. J Exp Med. 1993 May 1;177(5):1505–9.

385. Mattner F, Di Padova K, Alber G. Interleukin-12 is indispensable for protective immunity against Leishmania major. Infect Immun. 1997 Nov;65(11):4378–83.

386. Carrera L, Gazzinelli RT, Badolato R, Hieny S, Muller W, Kuhn R, et al. Leishmania promastigotes selectively inhibit interleukin 12 induction in bone marrow-derived macrophages from susceptible and resistant mice. J Exp Med. 1996 Feb 1;183(2):515–26.

387. Singh N, Sundar S. Combined neutralization of interferon gamma and tumor necrosis factor alpha induces IL-4 production but has no direct additive impact on parasite burden in splenic cultures of human visceral leishmaniasis. PLOS ONE. 2018 Jun 28;13(6):e0199817.

388. Scott P. IFN-gamma modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. The Journal of Immunology. 1991 Nov 1;147(9):3149–55.

389. Swihart K, Fruth U, Messmer N, Hug K, Behin R, Huang S, et al. Mice from a genetically resistant background lacking the interferon gamma receptor are susceptible to infection

with Leishmania major but mount a polarized T helper cell 1-type CD4+ T cell response. J Exp Med. 1995 Mar 1;181(3):961–71.

390. de Kossodo S, Grau GE, Louis JA, Müller I. Tumor necrosis factor alpha (TNF-alpha) and TNF-beta and their receptors in experimental cutaneous leishmaniasis. Infect Immun. 1994 Apr;62(4):1414–20.

391. Derouich-Guergour D, Brenier-Pinchart MP, Ambroise-Thomas P, Pelloux H. Tumour necrosis factor alpha receptors: role in the physiopathology of protozoan parasite infections. Int J Parasitol. 2001 Jun;31(8):763–9.

392. Nashleanas M, Kanaly S, Scott P. Control of Leishmania major infection in mice lacking TNF receptors. J Immunol. 1998 Jun 1;160(11):5506–13.

393. Vieira LQ, Goldschmidt M, Nashleanas M, Pfeffer K, Mak T, Scott P. Mice lacking the TNF receptor p55 fail to resolve lesions caused by infection with Leishmania major, but control parasite replication. J Immunol. 1996 Jul 15;157(2):827–35.

394. Scott P. Differentiation, regulation, and death of T helper cell subsets during infection with Leishmania major. Immunol Res. 1998;17(1–2):229–38.

395. Chatelain R, Varkila K, Coffman RL. IL-4 induces a Th2 response in Leishmania major-infected mice. J Immunol. 1992 Feb 15;148(4):1182–7.

396. Lazarski CA, Ford J, Katzman SD, Rosenberg AF, Fowell DJ. IL-4 attenuates Th1-associated chemokine expression and Th1 trafficking to inflamed tissues and limits pathogen clearance. PLoS One. 2013;8(8):e71949.

397. Gurung P, Karki R, Vogel P, Watanabe M, Bix M, Lamkanfi M, et al. An NLRP3 inflammasome-triggered Th2-biased adaptive immune response promotes leishmaniasis. J Clin Invest. 2015 Mar 2;125(3):1329–38.

398. Noben-Trauth N, Kropf P, Müller I. Susceptibility to Leishmania major infection in interleukin-4-deficient mice. Science. 1996 Feb 16;271(5251):987–90.

399. Noben-Trauth N, Paul WE, Sacks DL. IL-4- and IL-4 receptor-deficient BALB/c mice reveal differences in susceptibility to Leishmania major parasite substrains. J Immunol. 1999 May 15;162(10):6132–40.

400. Wynn TA. IL-13 effector functions. Annu Rev Immunol. 2003;21:425–56.

401. Babaloo Z, Kaye PM, Eslami MB. Interleukin-13 in Iranian patients with visceral leishmaniasis: relationship to other Th2 and Th1 cytokines. Trans R Soc Trop Med Hyg. 2001 Feb;95(1):85–8.

402. Hurdayal R, Brombacher F. The role of IL-4 and IL-13 in cutaneous Leishmaniasis. Immunol Lett. 2014 Oct;161(2):179–83.

403. Alexander J, Brombacher F, McGachy HA, McKenzie ANJ, Walker W, Carter KC. An essential role for IL-13 in maintaining a non-healing response following Leishmania mexicana infection. Eur J Immunol. 2002 Oct;32(10):2923–33.

404. Mohrs M, Ledermann B, Köhler G, Dorfmüller A, Gessner A, Brombacher F. Differences between IL-4- and IL-4 receptor alpha-deficient mice in chronic leishmaniasis reveal a protective role for IL-13 receptor signaling. J Immunol. 1999 Jun 15;162(12):7302–8.

405. Skeen MJ, Miller MA, Shinnick TM, Ziegler HK. Regulation of murine macrophage IL-12 production. Activation of macrophages in vivo, restimulation in vitro, and modulation by other cytokines. J Immunol. 1996 Feb 1;156(3):1196–206.

406. Doyle AG, Herbein G, Montaner LJ, Minty AJ, Caput D, Ferrara P, et al. Interleukin-13 alters the activation state of murine macrophages in vitro: comparison with interleukin-4 and interferon-gamma. Eur J Immunol. 1994 Jun;24(6):1441–5.

407. Rutschman R, Lang R, Hesse M, Ihle JN, Wynn TA, Murray PJ. Cutting edge: Stat6-dependent substrate depletion regulates nitric oxide production. J Immunol. 2001 Feb 15;166(4):2173–7.

408. Moore KW, Vieira P, Fiorentino DF, Trounstine ML, Khan TA, Mosmann TR. Pillars article: homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. Science. 1990. 248: 1230-1234. J Immunol. 2012 Sep 1;189(5):2072–6.

409. Couper KN, Blount DG, Riley EM. IL-10: The Master Regulator of Immunity to Infection. The Journal of Immunology. 2008 May 1;180(9):5771–7.

410. Saraiva M, Vieira P, O'Garra A. Biology and therapeutic potential of interleukin-10. J Exp Med. 2020 Jan 6;217(1):e20190418.

411. Aebischer T, Moody SF, Handman E. Persistence of virulent Leishmania major in murine cutaneous leishmaniasis: a possible hazard for the host. Infect Immun. 1993 Jan;61(1):220–6.

412. Gonçalves-de-Albuquerque S da C, Pessoa-e-Silva R, Trajano-Silva LAM, de Goes TC, de Morais RCS, Oliveira CN da C, et al. The equivocal role of Th17 cells and neutrophils on immunopathogenesis of leishmaniasis. Frontiers in Immunology. 2017;

413. Babaloo Z, Oskoei MR, Kohansal MH, Barac A, Ahmadpour E. Serum profile of IL-1β and IL-17 cytokines in patients with visceral leishmaniasis. Comparative Immunology, Microbiology and Infectious Diseases. 2020 Apr 1;69:101431.

414. Kawaguchi M, Takahashi D, Hizawa N, Suzuki S, Matsukura S, Kokubu F, et al. IL-17F sequence variant (His161Arg) is associated with protection against asthma and antagonizes wild-type IL-17F activity. J Allergy Clin Immunol. 2006 Apr;117(4):795–801.

415. Kumar P, Misra P, Yadav NK, Joshi S, Sahasrabuddhe AA, Dube A, et al. Prophylactic interferon-γ and interleukin-17 facilitate parasite clearance in experimental visceral leishmaniasis. Tropical Parasitology. 2019 Jun;9(1):30.

416. Ghosh K, Sharma G, Saha A, Kar S, Das PK, Ukil A. Successful therapy of visceral leishmaniasis with curdlan involves T-helper 17 cytokines. J Infect Dis. 2013 Mar 15;207(6):1016–25.

417. Faria DR, Gollob KJ, Barbosa J, Schriefer A, Machado PRL, Lessa H, et al. Decreased in situ expression of interleukin-10 receptor is correlated with the exacerbated inflammatory and cytotoxic responses observed in mucosal leishmaniasis. Infect Immun. 2005 Dec;73(12):7853–9.

418. de Araújo FF, Costa-Silva MF, Pereira AAS, Rêgo FD, Pereira VHS, de Souza JP, et al. Chemokines in Leishmaniasis: Map of cell movements highlights the landscape of infection and pathogenesis. Cytokine. 2021 Nov 1;147:155339.

419. Oghumu SO, Lezama-Dávila CM, Isaac-Márquez AP, Satoskar AR. Role of Chemokines in Regulation of Immunity against Leishmaniasis. Exp Parasitol. 2010 Nov;126(3):389–96.

420. Antoniazi S, Price HP, Kropf P, Freudenberg MA, Galanos C, Smith DF, et al. Chemokine gene expression in toll-like receptor-competent and -deficient mice infected with Leishmania major. Infect Immun. 2004 Sep;72(9):5168–74.

421. Racoosin EL, Beverley SM. Leishmania major: promastigotes induce expression of a subset of chemokine genes in murine macrophages. Exp Parasitol. 1997 Mar;85(3):283–95.

422. Ritter U, Körner H. Divergent expression of inflammatory dermal chemokines in cutaneous leishmaniasis. Parasite Immunol. 2002 Jun;24(6):295–301.

423. Lo SK, Bovis L, Matura R, Zhu B, He S, Lum H, et al. Leishmania lipophosphoglycan reduces monocyte transendothelial migration: modulation of cell adhesion molecules, intercellular junctional proteins, and chemoattractants. J Immunol. 1998 Feb 15;160(4):1857–65.

424. Panaro MA, Spinelli R, Lisi S, Sisto M, Acquafredda A, Fumarola L, et al. Reduced expression of the chemokine receptor CCR1 in human macrophages and U-937 cells in vitro infected with Leishmania infantum. Clin Exp Med. 2004 Apr;3(4):225–30.

425. Katzman SD, Fowell DJ. Pathogen-imposed skewing of mouse chemokine and cytokine expression at the infected tissue site. J Clin Invest. 2008 Feb;118(2):801–11.

426. Allavena P, Bianchi G, Zhou D, van Damme J, Jílek P, Sozzani S, et al. Induction of natural killer cell migration by monocyte chemotactic protein-1, -2 and -3. Eur J Immunol. 1994 Dec;24(12):3233–6.

427. Ritter U, Moll H. Monocyte chemotactic protein-1 stimulates the killing of leishmania major by human monocytes, acts synergistically with IFN-gamma and is antagonized by IL-4. Eur J Immunol. 2000 Nov;30(11):3111–20.

428. Moll H. The role of chemokines and accessory cells in the immunoregulation of cutaneous leishmaniasis. Behring Inst Mitt. 1997 Mar;(99):73–8.

429. Rollins BJ, Walz A, Baggiolini M. Recombinant human MCP-1/JE induces chemotaxis, calcium flux, and the respiratory burst in human monocytes. Blood. 1991 Aug 15;78(4):1112–6.

430. Gu L, Tseng S, Horner RM, Tam C, Loda M, Rollins BJ. Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. Nature. 2000 Mar 23;404(6776):407–11.

431. Quinones MP, Estrada CA, Jimenez F, Martinez H, Willmon O, Kuziel WA, et al. CCL2-independent role of CCR2 in immune responses against Leishmania major. Parasite Immunol. 2007 Apr;29(4):211–7.

432. Teixeira MJ, Teixeira CR, Andrade BB, Barral-Netto M, Barral A. Chemokines in host-parasite interactions in leishmaniasis. Trends Parasitol. 2006 Jan;22(1):32–40.

433. Montes de Oca M, Engwerda CR, Kaye PM. Cytokines and splenic remodelling during Leishmania donovani infection. Cytokine X. 2020 Dec;2(4):100036.

434. Bhattacharjee O, Ayyangar U, Kurbet AS, Ashok D, Raghavan S. Unraveling the ECM-Immune Cell Crosstalk in Skin Diseases. Front Cell Dev Biol. 2019;7:68.

435. de Almeida MC, Vilhena V, Barral A, Barral-Netto M. Leishmanial infection: analysis of its first steps. A review. Mem Inst Oswaldo Cruz. 2003 Oct;98(7):861–70.

436. Watt FM, Fujiwara H. Cell-extracellular matrix interactions in normal and diseased skin. Cold Spring Harb Perspect Biol. 2011 Apr 1;3(4):a005124.

437. Pfisterer K, Shaw LE, Symmank D, Weninger W. The Extracellular Matrix in Skin Inflammation and Infection. Front Cell Dev Biol. 2021;9:682414.

438. Abdoli A, Maspi N, Ghaffarifar F. Wound healing in cutaneous leishmaniasis: A double edged sword of IL-10 and TGF-β. Comp Immunol Microbiol Infect Dis. 2017 Apr;51:15–26.

439. Wang PH, Huang BS, Horng HC, Yeh CC, Chen YJ. Wound healing. J Chin Med Assoc. 2018 Feb;81(2):94–101.

440. Silva-Almeida M, Carvalho LO, Abreu-Silva AL, Souza CS, Hardoim DJ, Calabrese KS. Extracellular matrix alterations in experimental Leishmania amazonensis infection in susceptible and resistant mice. Vet Res. 2012 Feb 8;43(1):10.

441. Petropolis DB, Rodrigues JCF, Viana NB, Pontes B, Pereira CFA, Silva-Filho FC. Leishmania amazonensis promastigotes in 3D Collagen I culture: an in vitro physiological environment for the study of extracellular matrix and host cell interactions. PeerJ. 2014;2:e317.

442. LEITE VHR, CROFT SL. Hepatic extracellular matrix in BALB/c mice infected with Leishmania donovani. Int J Exp Pathol. 1996 Aug;77(4):181–90.

443. Sakthianandeswaren A, Elso CM, Simpson K, Curtis JM, Kumar B, Speed TP, et al. The wound repair response controls outcome to cutaneous leishmaniasis. Proc Natl Acad Sci U S A. 2005 Oct 25;102(43):15551–6.

444. Ginger ML. Niche metabolism in parasitic protozoa. Philos Trans R Soc Lond B Biol Sci. 2006 Jan 29;361(1465):101–18.

445. Camus D, Zalis MG, Vannier-Santos MA, Banic DM. The art of parasite survival. Braz J Med Biol Res. 1995 Apr;28(4):399–413.

446. Burchmore RJ, Barrett MP. Life in vacuoles--nutrient acquisition by Leishmania amastigotes. Int J Parasitol. 2001 Oct;31(12):1311–20.

447. de Souza W. Special organelles of some pathogenic protozoa. Parasitol Res. 2002 Dec;88(12):1013–25.

448. Jamdhade MD, Pawar H, Chavan S, Sathe G, Umasankar PK, Mahale KN, et al. Comprehensive proteomics analysis of glycosomes from Leishmania donovani. OMICS. 2015 Mar;19(3):157–70.

449. Tasbihi M, Shekari F, Hajjaran H, Khanmohammadi M, Hadighi R. Comparative mitochondrial proteomics of Leishmania tropica clinical isolates resistant and sensitive to meglumine antimoniate. Parasitology Research. 2020;

450. Opperdoes FR, Coombs GH. Metabolism of Leishmania: proven and predicted. Trends in Parasitology. 2007;23(4):149–58.

451. Jain S, Sahu U, Kumar A, Khare P. Metabolic Pathways of Leishmania Parasite: Source of Pertinent Drug Targets and Potent Drug Candidates. Pharmaceutics. 2022 Jul 30;14(8):1590.

452. Downing T, Imamura H, Decuypere S, Clark TG, Coombs GH, Cotton JA, et al. Whole genome sequencing of multiple Leishmania donovani clinical isolates provides insights into population structure and mechanisms of drug resistance. Genome Res. 2011 Dec;21(12):2143–56.

453. Cortazzo da Silva L, Aoki JI, Floeter-Winter LM. Finding Correlations Between mRNA and Protein Levels in Leishmania Development: Is There a Discrepancy? Front Cell Infect Microbiol. 2022;12:852902.

454. McNicoll F, Drummelsmith J, Müller M, Madore E, Boilard N, Ouellette M, et al. A combined proteomic and transcriptomic approach to the study of stage differentiation in Leishmania infantum. Proteomics. 2006 Jun;6(12):3567–81.

455. Darling TN, Davis DG, London RE, Blum JJ. Carbon dioxide abolishes the reverse Pasteur effect in Leishmania major promastigotes. Mol Biochem Parasitol. 1989 Mar 1;33(2):191–202.

456. Hart DT, Coombs GH. The effects of carbon dioxide and oxygen upon the growth and in vitro transformation of Leishmania mexicana mexicana. Mol Biochem Parasitol. 1981 Nov;4(1–2):117–27.

457. Rosenzweig D, Smith D, Opperdoes F, Stern S, Olafson RW, Zilberstein D. Retooling Leishmania metabolism: from sand fly gut to human macrophage . The FASEB Journal. 2008;22(2):590–602.

458. Lemos-Silva T, Telleria EL, Traub-Csekö YM. The gene expression of Leishmania infantum chagasi inside Lutzomyia longipalpis, the main vector of visceral leishmaniasis in Brazil. Mem Inst Oswaldo Cruz. 2021;116:e200571.

459. Veras PST, de Menezes JPB, Dias BRS. Deciphering the Role Played by Autophagy in Leishmania Infection. Front Immunol. 2019;10:2523.

460. Young J, Kima PE. The Leishmania Parasitophorous Vacuole Membrane at the Parasite-Host Interface. Yale J Biol Med. 2019 Sep;92(3):511–21.

461. Chaparro V, Leroux LP, Masvidal L, Lorent J, Graber TE, Zimmermann A, et al. Translational profiling of macrophages infected with Leishmania donovani identifies mTOR- and eIF4A-sensitive immune-related transcripts. PLoS Pathog. 2020 Jun;16(6):e1008291.

462. Abu-Remaileh M, Wyant GA, Kim C, Laqtom NN, Abbasi M, Chan SH, et al. Lysosomal metabolomics reveals V-ATPase- and mTOR-dependent regulation of amino acid efflux from lysosomes. Science. 2017 Nov 10;358(6364):807–13.

463. Saunders EC, Sernee MF, Ralton JE, McConville MJ. Metabolic stringent response in intracellular stages of Leishmania. Curr Opin Microbiol. 2021 Jul 30;63:126–32.

464. Jara M, Maes I, Imamura H, Domagalska MA, Dujardin JC, Arevalo J. Tracking of quiescence in Leishmania by quantifying the expression of GFP in the ribosomal DNA locus. Sci Rep. 2019 Dec 12;9(1):18951.

465. Kloehn J, Boughton BA, Saunders EC, O'Callaghan S, Binger KJ, McConville MJ. Identification of Metabolically Quiescent Leishmania mexicana Parasites in Peripheral and Cured Dermal Granulomas Using Stable Isotope Tracing Imaging Mass Spectrometry. mBio. 2021 Apr 6;12(2):e00129-21.

466. Sernee MF, Ralton JE, Dinev Z, Khairallah GN, O'Hair RA, Williams SJ, et al. Leishmania beta-1,2-mannan is assembled on a mannose-cyclic phosphate primer. Proc Natl Acad Sci U S A. 2006 Jun 20;103(25):9458–63.

467. Goldman-Pinkovich A, Kannan S, Nitzan-Koren R, Puri M, Pawar H, Bar-Avraham Y, et al. Sensing Host Arginine Is Essential for Leishmania Parasites' Intracellular Development. mBio. 2020 Oct 13;11(5):e02023-20.

468. Mcconville MJ, Saunders EC, Kloehn J, Dagley MJ. Leishmania carbon metabolism in the macrophage phagolysosome- feast or famine ? [ version 1 ; referees : 3 approved ] Referee Status : 2015;4:1–11.

469. Naderer T, Ellis MA, Sernee MF, De Souza DP, Curtis J, Handman E, et al. Virulence of Leishmania major in macrophages and mice requires the gluconeogenic enzyme fructose-1,6-bisphosphatase. Proc Natl Acad Sci U S A. 2006 Apr 4;103(14):5502–7.

470. Rodriguez-Contreras D, Hamilton N. Gluconeogenesis in Leishmania mexicana: contribution of glycerol kinase, phosphoenolpyruvate carboxykinase, and pyruvate phosphate dikinase. J Biol Chem. 2014 Nov 21;289(47):32989–3000.

471. Semini G, Paape D, Blume M, Sernee MF, Peres-Alonso D, Calvignac-Spencer S, et al. Leishmania Encodes a Bacterium-like 2,4-Dienoyl-Coenzyme A Reductase That Is Required for Fatty Acid β-Oxidation and Intracellular Parasite Survival. mBio. 2020 Jun 2;11(3).

472. Hellemond JJV, Meer PVD, Tielens AGM. Leishmania infantum promastigotes have a poor capacity for anaerobic functioning and depend mainly on respiration for their energy generation. Parasitology. 1997 Apr;114(4):351–60.

473. Van Hellemond JJ, Tielens AG. Inhibition of the respiratory chain results in a reversible metabolic arrest in Leishmania promastigotes. Mol Biochem Parasitol. 1997 Mar;85(1):135–8.

474. Chaudhuri M, Ott RD, Hill GC. Trypanosome alternative oxidase: from molecule to function. Trends in Parasitology. 2006 Oct 1;22(10):484–91.

475. Chaudhry R, Varacallo M. Biochemistry, Glycolysis. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 [cited 2023 Nov 7]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK482303/

476. Hannaert V, Bringaud F, Opperdoes FR, Michels PA. Evolution of energy metabolism and its compartmentation in Kinetoplastida. Kinetoplastid Biol Dis. 2003 Oct 28;2(1):11.

477. Saini S, Kumar Ghosh A, Singh R, Das S, Abhishek K, Kumar A, et al. Glucose deprivation induced upregulation of phosphoenolpyruvate carboxykinase modulates virulence in Leishmania donovani. Molecular Microbiology. 2016;102(6):1020–42.

478. Saunders EC, De Souza DP, Naderer T, Sernee MF, Ralton JE, Doyle MA, et al. Central carbon metabolism of Leishmania parasites. Parasitology. 2010;137(9):1303–13.

479. Burchmore RJS, Rodriguez-Contreras D, McBride K, Merkel P, Barrett MP, Modi G, et al. Genetic characterization of glucose transporter function in Leishmania mexicana. Proc Natl Acad Sci U S A. 2003 Apr 1;100(7):3901–6.

480. Feng X, Rodriguez-Contreras D, Buffalo C, Bouwer HGA, Kruvand E, Beverley SM, et al. Amplification of an alternate transporter gene suppresses the avirulent phenotype of glucose transporter null mutants in Leishmania mexicana. Mol Microbiol. 2009 Jan;71(2):369–81.

481. Saunders EC, Ng WW, Kloehn J, Chambers JM, Ng M, McConville MJ. Induction of a stringent metabolic response in intracellular stages of Leishmania mexicana leads to increased dependence on mitochondrial metabolism. PLoS Pathog. 2014 Jan;10(1):e1003888.

482. Michels PAM, Villafraz O, Pineda E, Alencar MB, Cáceres AJ, Silber AM, et al. Carbohydrate metabolism in trypanosomatids: New insights revealing novel complexity, diversity and species-unique features. Experimental Parasitology. 2021 May 1;224:108102.

483. Melkonian EA, Asuka E, Schury MP. Physiology, Gluconeogenesis. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 [cited 2023 Nov 8]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK541119/

484. Rodríguez-Contreras D, Landfear SM. Metabolic changes in glucose transporter-deficient Leishmania mexicana and parasite virulence. J Biol Chem. 2006 Jul 21;281(29):20068–76.

485. Burchmore RJ, Hart DT. Glucose transport in amastigotes and promastigotes of Leishmania mexicana mexicana. Mol Biochem Parasitol. 1995 Oct;74(1):77–86.

486. Rainey PM, MacKenzie NE. A carbon-13 nuclear magnetic resonance analysis of the products of glucose metabolism in Leishmania pifanoi amastigotes and promastigotes. Mol Biochem Parasitol. 1991 Apr;45(2):307–15.

487. Simon MW, Martin E, Mukkada AJ. Evidence for a functional glyoxylate cycle in the leishmaniae. J Bacteriol. 1978 Sep;135(3):895–9.

488. Rosenzweig D, Smith D, Opperdoes F, Stern S, Olafson RW, Zilberstein D. Retooling Leishmania metabolism: from sand fly gut to human macrophage. The FASEB Journal. 2008;22(2):590–602.

489. Jin ES, Sherry AD, Malloy CR. Interaction between the pentose phosphate pathway and gluconeogenesis from glycerol in the liver. J Biol Chem. 2014 Nov 21;289(47):32593–603.

490. Cooper GM. The Mechanism of Oxidative Phosphorylation. In: The Cell: A Molecular Approach 2nd edition [Internet]. Sinauer Associates; 2000 [cited 2023 Nov 8]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK9885/

491. Louassini M, Foulquié M, Benítez R, Adroher J. Citric-acid cycle key enzyme activities during in vitro growth and metacyclogenesis of Leishmania infantum promastigotes. J Parasitol. 1999 Aug;85(4):595–602.

492. Subramanian A, Jhawar J, Sarkar RR. Dissecting leishmania infantum energy metabolism - A systems perspective. PLoS ONE. 2015;10(9):1–34.

493. Meade JC, Glaser TA, Bonventre PF, Mukkada AJ. Enzymes of Carbohydrate Metabolism in. 1984;31(1):156–61.

494. Docampo R, Vercesi AE. Mitochondrial Ca2+ and Reactive Oxygen Species in Trypanosomatids. Antioxid Redox Signal. 2022 May 1;36(13–15):969–83.

495. Vercesi AE, Docampo R, Moreno SN. Energization-dependent Ca2+ accumulation in Trypanosoma brucei bloodstream and procyclic trypomastigotes mitochondria. Mol Biochem Parasitol. 1992 Dec;56(2):251–7.

496. Lu HG, Zhong L, Chang KP, Docampo R. Intracellular Ca2+ pool content and signaling and expression of a calcium pump are linked to virulence in Leishmania mexicana amazonesis amastigotes. J Biol Chem. 1997 Apr 4;272(14):9464–73.

497. Paramchuk WJ, Ismail SO, Bhatia A, Gedamu L. Cloning, characterization and overexpression of two iron superoxide dismutase cDNAs from Leishmania chagasi: role in pathogenesis. Mol Biochem Parasitol. 1997 Dec 1;90(1):203–21.

498. Mittra B, Laranjeira-Silva MF, Miguel DC, Perrone Bezerra de Menezes J, Andrews NW. The iron-dependent mitochondrial superoxide dismutase SODA promotes Leishmania virulence. J Biol Chem. 2017 Jul 21;292(29):12324–38.

499. Dolai S, Yadav RK, Pal S, Adak S. Overexpression of mitochondrial Leishmania major ascorbate peroxidase enhances tolerance to oxidative stress-induced programmed cell death and protein damage. Eukaryot Cell. 2009 Nov;8(11):1721–31.

500. Moreira D de S, Xavier MV, Murta/ SMF. Ascorbate peroxidase overexpression protects Leishmania braziliensis against trivalent antimony effects. Mem Inst Oswaldo Cruz. 2018 Nov 14;113(12):e180377.

501. Xiang L, Laranjeira-Silva MF, Maeda FY, Hauzel J, Andrews NW, Mittra B. Ascorbate-Dependent Peroxidase (APX) from Leishmania amazonensis Is a Reactive Oxygen Species-Induced Essential Enzyme That Regulates Virulence. Infect Immun. 2019 Dec;87(12).

502. Harder S, Bente M, Isermann K, Bruchhaus I. Expression of a Mitochondrial Peroxiredoxin Prevents Programmed Cell Death in Leishmania donovani. Eukaryot Cell. 2006 May;5(5):861–70.

503. Teixeira F, Castro H, Cruz T, Tse E, Koldewey P, Southworth DR, et al. Mitochondrial peroxiredoxin functions as crucial chaperone reservoir in Leishmania infantum. Proc Natl Acad Sci U S A. 2015 Feb 17;112(7):E616-624.

504. Subramanian A, Jhawar J, Sarkar RR. Dissecting leishmania infantum energy metabolism - A systems perspective. PLoS ONE. 2015;10(9):1–34.

505. Saunders EC, De Souza DP, Naderer T, Sernee MF, Ralton JE, Doyle MA, et al. Central carbon metabolism of Leishmania parasites. Parasitology. 2010;137(9):1303–13.

506. Huynh C, Sacks DL, Andrews NW. A Leishmania amazonensis ZIP family iron transporter is essential for parasite replication within macrophage phagolysosomes. Journal of Experimental Medicine. 2006;203(10):2363–75.

507. Boitz JM, Ullman B. Amplification of adenine phosphoribosyltransferase suppresses the conditionally lethal growth and virulence phenotype of Leishmania donovani mutants lacking both hypoxanthine-guanine and xanthine phosphoribosyltransferases. Journal of Biological Chemistry. 2010;285(24):18555–64.

508. Gaur U, Showalter M, Hickerson S, Dalvi R, Turco SJ, Wilson ME, et al. Leishmania donovani lacking the Golgi GDP-Man transporter LPG2 exhibit attenuated virulence in mammalian hosts. Experimental Parasitology. 2009;122(3):182–91.

509. McConville MJ, Naderer T. Metabolic Pathways Required for the Intracellular Survival of Leishmania . Annual Review of Microbiology. 2011;65(1):543–61.

510. Mou Z, Li J, Boussoffara T, Kishi H, Hamana H, Ezzati P, et al. Identification of broadly conserved cross-species protective Leishmania antigen and its responding CD4+ T cells. Science Translational Medicine. 2015;7(310):1–12.

511. Mou Z, Barazandeh AF, Hamana H, Kishi H, Zhang X, Jia P, et al. Identification of a Protective Leishmania Antigen Dihydrolipoyl Dehydrogenase and Its Responding CD4+ T Cells at Clonal Level. J Immunol. 2020 Sep 1;205(5):1355–64.

512. Chiranjivi AK, Dubey VK. Dihydrolipoamide dehydrogenase from Leishmania donovani: New insights through biochemical characterization. International Journal of Biological Macromolecules. 2018;112:1241–7.

513. Sosa MH, Giordana L, Nowicki C. Exploring biochemical and functional features of Leishmania major phosphoenolpyruvate carboxykinase. Archives of Biochemistry and Biophysics. 2015 Oct 1;583:120–9.

514. Saunders EC, Ng WW, Chambers JM, Ng M, Naderer T, Krömer JO, et al. Isotopomer profiling of Leishmania mexicana promastigotes reveals important roles for succinate fermentation and aspartate uptake in tricarboxylic acid cycle (TCA) anaplerosis, glutamate synthesis, and growth. J Biol Chem. 2011 Aug 5;286(31):27706–17.

515. Liu K, Ba X, Yu J, Li J, Wei Q, Han G, et al. The phosphoenolpyruvate carboxykinase of Mycobacterium tuberculosis induces strong cell-mediated immune responses in mice. Molecular and Cellular Biochemistry. 2006;288(1–2):65–71.

516. Asahi H, Hernandez HJ, Stadecker MJ. A novel 62-kilodalton egg antigen from Schistosoma mansoni induces a potent CD4+ T helper cell response in the C57BL/6 mouse. Infection and Immunity. 1999;67(4):1729–35.

517. Shi L, Tu BP. Acetyl-CoA and the Regulation of Metabolism: Mechanisms and Consequences. Curr Opin Cell Biol. 2015 Apr;33:125–31.

518. Vincent IM, Racine G, Légaré D, Ouellette M. Mitochondrial Proteomics of Antimony and Miltefosine Resistant Leishmania infantum. Proteomes. 2015 Oct 21;3(4):328–46.

519. Silverman JM, Clos J, de'Oliveira CC, Shirvani O, Fang Y, Wang C, et al. An exosome-based secretion pathway is responsible for protein export from Leishmania and communication with macrophages. J Cell Sci. 2010 Mar 15;123(Pt 6):842–52.

520. Maspi N, Abdoli A, Ghaffarifar F. Pro- and anti-inflammatory cytokines in cutaneous leishmaniasis: a review. Pathogens and Global Health. 2016;

521. Smith AW, Roche H, Trombe MC, Briles DE, H\aakansson A. Characterization of the dihydrolipoamide dehydrogenase from Streptococcus pneumoniae and its role in pneumococcal infection. Molecular Microbiology. 2002;44(2):431–48.

522. Pang H, Chen L, Hoare R, Huang Y, ZaoheWu, Jian J. Identification of DLD, by immunoproteomic analysis and evaluation as a potential vaccine antigen against three Vibrio species in Epinephelus coioides. Vaccine. 2016;34(9):1225–31.

523. Landgraf TN, Costa MV, Oliveira AF, Ribeiro WC, Panunto-Castelo A, Fernandes FF. Involvement of dihydrolipoyl dehydrogenase in the phagocytosis and killing of Paracoccidioides brasiliensis by macrophages. Frontiers in Microbiology. 2017;8(SEP):1–10.

524. The UniProt Consortium. UniProt: the Universal Protein Knowledgebase in 2023. Nucleic Acids Research. 2023;51:D523–31.

525. Barrangou R. The roles of CRISPR-Cas systems in adaptive immunity and beyond. Curr Opin Immunol. 2015 Feb;32:36–41.

526. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, et al. CRISPR provides acquired resistance against viruses in prokaryotes. Science. 2007 Mar 23;315(5819):1709–12.

527. Marraffini LA, Sontheimer EJ. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. Science. 2008 Dec 19;322(5909):1843–5.

528. Hille F, Richter H, Wong SP, Bratovič M, Ressel S, Charpentier E. The Biology of CRISPR-Cas: Backward and Forward. Cell. 2018 Mar 8;172(6):1239–59.

529. Jansen R, Embden JDA van, Gaastra W, Schouls LM. Identification of genes that are associated with DNA repeats in prokaryotes. Mol Microbiol. 2002 Mar;43(6):1565–75.

530. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012 Aug 17;337(6096):816–21.

531. Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, et al. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell. 2015 Oct 22;163(3):759–71.

532. Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, Cox DBT, et al. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. Science. 2016 Aug 5;353(6299):aaf5573.

533. Zhang WW, Lypaczewski P, Matlashewski G. Optimized CRISPR-Cas9 Genome Editing for Leishmania and Its Use To Target a Multigene Family, Induce Chromosomal Translocation, and Study DNA Break Repair Mechanisms. mSphere. 2017;2(1):e00340-16.

534. Kolev NG, Tschudi C, Ullu E. RNA Interference in Protozoan Parasites: Achievements and Challenges ▿ . Eukaryot Cell. 2011 Sep;10(9):1156–63.

535. Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N, et al. Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell. 2014 Feb 27;156(5):935–49.

536. Her J, Bunting SF. How cells ensure correct repair of DNA double-strand breaks. J Biol Chem. 2018 Jul 6;293(27):10502–11.

537. Sallmyr A, Tomkinson AE. Repair of DNA double-strand breaks by mammalian alternative end-joining pathways. J Biol Chem. 2018 Jul 6;293(27):10536–46.

538. Lin S, Staahl BT, Alla RK, Doudna JA. Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. Elife. 2014 Dec 15;3:e04766.

539. Morrical SW. DNA-pairing and annealing processes in homologous recombination and homology-directed repair. Cold Spring Harb Perspect Biol. 2015 Feb 2;7(2):a016444.

540. Zhang WW, Lypaczewski P, Matlashewski G. Application of CRISPR/Cas9-Mediated Genome Editing in Leishmania. In: Michels PAM, Ginger ML, Zilberstein D, editors. Trypanosomatids [Internet]. New York, NY: Springer US; 2020 [cited 2021 Feb 21]. p. 199–224. (Methods in Molecular Biology; vol. 2116). Available from: http://link.springer.com/10.1007/978-1-0716-0294-2_14

541. Zhang WW, Matlashewski G. CRISPR-Cas9-mediated genome editing in Leishmania donovani. mBio. 2015;6(4):1–14.

542. Beneke T, Madden R, Makin L, Valli J, Sunter J, Gluenz E. A CRISPR Cas9 high-throughput genome editing toolkit for kinetoplastids. R Soc Open Sci. 2017 May;4(5):170095.

543. Beneke T, Gluenz E. LeishGEdit: A Method for Rapid Gene Knockout and Tagging Using CRISPR-Cas9. Methods Mol Biol. 2019;1971:189–210.

544. Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, et al. DNA targeting specificity of RNA-guided Cas9 nucleases. Nat Biotechnol. 2013 Sep;31(9):827–32.

545. Pacesa M, Lin CH, Cléry A, Saha A, Arantes PR, Bargsten K, et al. Structural basis for Cas9 off-target activity. Cell. 2022 Oct 27;185(22):4067-4081.e21.

546. O'Geen H, Henry IM, Bhakta MS, Meckler JF, Segal DJ. A genome-wide analysis of Cas9 binding specificity using ChIP-seq and targeted sequence capture. Nucleic Acids Res. 2015 Mar 31;43(6):3389–404.

547. Richter MF, Zhao KT, Eton E, Lapinaite A, Newby GA, Thuronyi BW, et al. Phage-assisted evolution of an adenine base editor with improved Cas domain compatibility and activity. Nat Biotechnol. 2020 Jul;38(7):883–91.

548. Labuhn M, Adams FF, Ng M, Knoess S, Schambach A, Charpentier EM, et al. Refined sgRNA efficacy prediction improves large- and small-scale CRISPR-Cas9 applications. Nucleic Acids Res. 2018 Feb 16;46(3):1375–85.

549. Peng D, Tarleton R. EuPaGDT: a web tool tailored to design CRISPR guide RNAs for eukaryotic pathogens. Microb Genom. 2015 Oct;1(4):e000033.

550. Guo C, Ma X, Gao F, Guo Y. Off-target effects in CRISPR/Cas9 gene editing. Front Bioeng Biotechnol. 2023 Mar 9;11:1143157.

551. Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI, et al. Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. Nature. 2017 Nov 23;551(7681):464–71.

552. Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature. 2016 May 19;533(7603):420–4.

553. Taha EA, Lee J, Hotta A. Delivery of CRISPR-Cas tools for in vivo genome editing therapy: Trends and challenges. J Control Release. 2022 Feb;342:345–61.

554. Klein SL, Flanagan KL. Sex differences in immune responses. Nat Rev Immunol. 2016 Oct;16(10):626–38.

555. Markle JG, Fish EN. SeXX matters in immunity. Trends Immunol. 2014 Mar;35(3):97–104.

556. vom Steeg LG, Klein SL. SeXX Matters in Infectious Disease Pathogenesis. PLoS Pathog. 2016 Feb;12(2):e1005374.

557. Lockard RD, Wilson ME, Rodríguez NE. Sex-related differences in immune response and symptomatic manifestations to infection with Leishmania species. Journal of Immunology Research. 2019;2019.

558. Pinzan CF, Ruas LP, Casabona-Fortunato AS, Carvalho FC, Roque-Barreira MC. Immunological basis for the gender differences in murine Paracoccidioides brasiliensis infection. PLoS One. 2010 May 21;5(5):e10757.

559. Diniz LFB, Souza CDF de, Carmo RF do. Epidemiology of human visceral leishmaniasis in the urban centers of the lower-middle São Francisco Valley, Brazilian semiarid region. Rev Soc Bras Med Trop. 2018;51(4):461–6.

560. Harhay MO, Olliaro PL, Vaillant M, Chappuis F, Lima MA, Ritmeijer K, et al. Who is a typical patient with visceral leishmaniasis? Characterizing the demographic and nutritional profile of patients in Brazil, East Africa, and South Asia. Am J Trop Med Hyg. 2011 Apr;84(4):543–50.

561. Medina-Morales DA, Machado-Duque ME, Machado-Alba JE. Epidemiology of Cutaneous Leishmaniasis in a Colombian Municipality. Am J Trop Med Hyg. 2017 Nov;97(5):1503–7.

562. Reis LLD, Balieiro AA da S, Fonseca FR, Gonçalves MJF. Changes in the epidemiology of visceral leishmaniasis in Brazil from 2001 to 2014. Rev Soc Bras Med Trop. 2017;50(5):638–45.

563. Rodríguez NE, Lima ID, Gaur Dixit U, Turcotte EA, Lockard RD, Batra-Sharma H, et al. Epidemiological and Experimental Evidence for Sex-Dependent Differences in the Outcome of Leishmania infantum Infection. Am J Trop Med Hyg. 2018 Jan;98(1):142–5.

564. de Freitas EO, Leoratti FM de S, Freire-de-Lima CG, Morrot A, Feijó DF. The Contribution of Immune Evasive Mechanisms to Parasite Persistence in Visceral Leishmaniasis. Front Immunol. 2016;7:153.

565. Satoskar A, Al-Quassi HH, Alexander J. Sex-determined resistance against Leishmania mexicana is associated with the preferential induction of a Th1-like response and IFN-gamma production by female but not male DBA/2 mice. Immunol Cell Biol. 1998 Apr;76(2):159–66.

566. de Araújo Albuquerque LP, da Silva AM, de Araújo Batista FM, de Souza Sene I, Costa DL, Costa CHN. Influence of sex hormones on the immune response to leishmaniasis. Parasite Immunol. 2021 Oct;43(10–11):e12874.

567. Li Y, Sun Y, Hines JC, Ray DS. Identification of new kinetoplast DNA replication proteins in trypanosomatids based on predicted S-phase expression and mitochondrial targeting. Eukaryot Cell. 2007 Dec;6(12):2303–10.

568. Ikeogu NM, Edechi CA, Akaluka GN, Feiz-Barazandeh A, Uzonna JE. Isolation and Preparation of Bone Marrow-Derived Immune Cells for Metabolic Analysis. In: Methods in Molecular Biology. Humana Press Inc.; 2020. p. 273–80.

569. Andreata F, Blériot C, Di Lucia P, De Simone G, Fumagalli V, Ficht X, et al. Isolation of mouse Kupffer cells for phenotypic and functional studies. STAR Protoc. 2021 Sep 14;2(4):100831.

570. McKinnon KM. Flow Cytometry: An Overview. Curr Protoc Immunol. 2018 Feb 21;120:5.1.1-5.1.11.

571. Saraiva EM, Pinto-da-Silva LH, Wanderley JLM, Bonomo AC, Barcinski MA, Moreira MEC. Flow cytometric assessment of Leishmania spp metacyclic differentiation: Validation by morphological features and specific markers. Experimental Parasitology. 2005 May 1;110(1):39–47.

572. Moon JJ, Chu HH, Hataye J, Pagán AJ, Pepper M, McLachlan JB, et al. Tracking epitope-specific T cells. Nature Protocols. 2009;4(4):565–81.

573. Gu X, Ma Y, Liu Y, Wan Q. Measurement of mitochondrial respiration in adherent cells by Seahorse XF96 Cell Mito Stress Test. STAR Protocols. 2021 Mar 19;2(1):100245.

574. Ohms M, Ferreira C, Busch H, Wohlers I, Guerra de Souza AC, Silvestre R, et al. Enhanced Glycolysis Is Required for Antileishmanial Functions of Neutrophils Upon Infection With Leishmania donovani. Front Immunol. 2021;12:632512.

575. TITUS RG, MARCHAND M, BOON T, LOUIS JA. A limiting dilution assay for quantifying Leishmania major in tissues of infected mice. Parasite Immunology. 1985;7(5):545–55.

576. Antonia AL, Wang1 L, Ko DC. A Real Time PCR Assay for Quantification of Parasite Burden in Murine Models of Leishmaniasis. 2018;44(3):320–5.

577. Srivastava A, Sweat JM, Azizan A, Vesely B, Kyle DE. Real-time PCR to quantify Leishmania donovani in hamsters. J Parasitol. 2013 Feb;99(1):145–50.

578. Wen-Wei Zhang, Patrick Lypaczewski and GM. Trypanosomatids - Methods and Protocols. 2020;2116:463–83.

579. Darrah PA, Patel DT, De Luca PM, Lindsay RWB, Davey DF, Flynn BJ, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat Med. 2007 Jul;13(7):843–50.

580. Tupperwar N, Shrivastava R, Shapira M. LeishIF4E1 Deletion Affects the Promastigote Proteome, Morphology, and Infectivity. mSphere. 2019;4(6):1–20.

581. Sacks DL. MINIREVIEW Metacyclogenesis in Leishmania Promastigotes. Vol. 69, EXPERIMENTAL PARASITOLOGY. 1989.

582. Descoteaux A, Turco SJ. Glycoconjugates in Leishmania infectivity. Biochimica et Biophysica Acta - Molecular Basis of Disease. 1999;1455(2–3):341–52.

583. Alcolea PJ, Alonso A, Degayón MA, Moreno-Paz M, Jiménez M, Molina R, et al. In vitro infectivity and differential gene expression of Leishmania infantum metacyclic promastigotes: Negative selection with peanut agglutinin in culture versus isolation from the stomodeal valve of Phlebotomus perniciosus. BMC Genomics. 2016;17(1):1–14.

584. Smith AW, Roche H, Trombe MC, Briles DE, Håkansson A. Characterization of the dihydrolipoamide dehydrogenase from Streptococcus pneumoniae and its role in pneumococcal infection. Molecular Microbiology. 2002;44(2):431–48.

585. Venugopal A, Bryk R, Shi S, Rhee K, Rath P, Schnappinger D, et al. Virulence of mycobacterium tuberculosis depends on lipoamide dehydrogenase, a member of three multienzyme complexes. Cell Host and Microbe. 2011;9(1):21–31.

586. Atayde VD, Hassani K, da Silva Lira Filho A, Borges AR, Adhikari A, Martel C, et al. Leishmania exosomes and other virulence factors: Impact on innate immune response and macrophage functions. Cellular Immunology. 2016;309:7–18.

587. Beneke T, Demay F, Hookway E, Ashman N, Jeffery H, Smith J, et al. Genetic dissection of a Leishmania flagellar proteome demonstrates requirement for directional motility in sand fly infections. PLoS Pathog. 2019 Jun 26;15(6):e1007828.

588. Roy G, Dumas C, Sereno D, Wu Y, Singh AK, Tremblay MJ, et al. Episomal and stable expression of the luciferase reporter gene for quantifying Leishmania spp. infections in macrophages and in animal models. Mol Biochem Parasitol. 2000 Oct;110(2):195–206.

589. Costa-da-Silva AC, Nascimento D de O, Ferreira JRM, Guimarães-Pinto K, Freire-de-Lima L, Morrot A, et al. Immune Responses in Leishmaniasis: An Overview. Trop Med Infect Dis. 2022 Mar 31;7(4):54.

590. Formaglio P, Alabdullah M, Siokis A, Handschuh J, Sauerland I, Fu Y, et al. Nitric oxide controls proliferation of Leishmania major by inhibiting the recruitment of permissive host cells. Immunity. 2021 Dec 14;54(12):2724-2739.e10.

591. Postat J, Olekhnovitch R, Lemaître F, Bousso P. A Metabolism-Based Quorum Sensing Mechanism Contributes to Termination of Inflammatory Responses. Immunity. 2018 Oct 16;49(4):654-665.e5.

592. Zutshi S, Kumar S, Chauhan P, Bansode Y, Nair A, Roy S, et al. Anti-leishmanial vaccines: Assumptions, approaches, and annulments. Vaccines. 2019;7(4):1–33.

593. Zaph C, Uzonna J, Beverley SM, Scott P. Central memory T cells mediate long-term immunity to Leishmania major in the absence of persistent parasites. Nat Med. 2004 Oct;10(10):1104–10.

594. Scott P, Artis D, Uzonna J, Zaph C. The development of effector and memory T cells in cutaneous leishmaniasis: the implications for vaccine development. Immunol Rev. 2004 Oct;201:318–38.

595. Babady NE, Pang YP, Elpeleg O, Isaya G. Cryptic proteolytic activity of dihydrolipoamide dehydrogenase. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(15):6158–63.

596. Chiranjivi AK, Prakash J, Saha G, Chandra P, Dubey VK. Mutational studies on Leishmania donovani dihydrolipoamide dehydrogenase (LdBPK291950.1) indicates that the enzyme may not be classical class-I pyridine nucleotide-disulfide oxidoreductase. International Journal of Biological Macromolecules. 2020;164:2141–50.

597. Creed S, McKenzie M. Measurement of Mitochondrial Membrane Potential with the Fluorescent Dye Tetramethylrhodamine Methyl Ester (TMRM). Methods Mol Biol. 2019;1928:69–76.

598. Nath S, Villadsen J. Oxidative phosphorylation revisited. Biotechnol Bioeng. 2015 Mar;112(3):429–37.

599. Mookerjee SA, Brand MD. Measurement and Analysis of Extracellular Acid Production to Determine Glycolytic Rate. J Vis Exp. 2015 Dec 12;(106):53464.

600. Giraud E, Martin O, Yakob L, Rogers M. Quantifying Leishmania Metacyclic Promastigotes from Individual Sandfly Bites Reveals the Efficiency of Vector Transmission. Commun Biol. 2019;2:84.

601. Stanley AC, Engwerda CR. Balancing immunity and pathology in visceral leishmaniasis. Immunol Cell Biol. 2007;85(2):138–47.

602. Maeda K, Sadoughi S, Morimoto A, Uchida K, Chambers JK, Sanjoba C, et al. Hepatomegaly Associated with Non-Obstructive Sinusoidal Dilation in Experimental Visceral Leishmaniasis. Pathogens. 2021 Oct 20;10(11):1356.

603. van Griensven J, Diro E. Visceral leishmaniasis. Infect Dis Clin North Am. 2012 Jun;26(2):309–22.

604. Graudejus O, Wong R, Varghese N, Wagner S, Morrison B. Bridging the gap between in vivo and in vitro research: Reproducing in vitro the mechanical and electrical environment of cells in vivo. Frontiers in Cellular Neuroscience. 2018 Jan 1;12.

605. Tarantino G, Scalera A, Finelli C. Liver-spleen axis: Intersection between immunity, infections and metabolism. World J Gastroenterol. 2013 Jun 21;19(23):3534–42.

606. Tarantino G, Citro V, Balsano C. Liver-spleen axis in nonalcoholic fatty liver disease. Expert Rev Gastroenterol Hepatol. 2021 Jul;15(7):759–69.

607. Tarantino G, Citro V, Conforti P, Balsano C, Capone D. Is There a Link between Basal Metabolic Rate, Spleen Volume and Hepatic Growth Factor Levels in Patients with Obesity-Related NAFLD? J Clin Med. 2019 Sep 20;8(10):1510.

608. Keramida G, Dunford A, Kaya G, Anagnostopoulos CD, Peters AM. Hepato-splenic axis: hepatic and splenic metabolic activities are linked. Am J Nucl Med Mol Imaging. 2018;8(3):228–38.

609. Roberts SC. The genetic toolbox for Leishmania parasites. Bioengineered Bugs. 2011;2(6):320–6.

610. Lye LF, Owens KL, Jang S, Marcus JE, Brettmann EA, Beverley SM. An RNA Interference (RNAi) Toolkit and Its Utility for Functional Genetic Analysis of Leishmania (Viannia). Genes. 2023 Jan;14(1):93.

611. Zhang WW, Matlashewski G. Single-Strand Annealing Plays a Major Role in Double-Strand DNA Break Repair following CRISPR-Cas9 Cleavage in Leishmania. mSphere. 2019;4(4):1–26.

612. Santarém N, Cunha J, Silvestre R, Silva C, Moreira D, Ouellette M, et al. The impact of distinct culture media in Leishmania infantum biology and infectivity. Parasitology. 2014 Feb;141(2):192–205.

613. Siripattanapipong S, Boontanom P, Leelayoova S, Mungthin M, Tan-ariya P. In vitro growth characteristics and morphological differentiation of Leishmania martiniquensis promastigotes in different culture media. Acta Tropica. 2019 Sep 1;197:105039.

614. Kloehn J, Saunders EC, O'Callaghan S, Dagley MJ, McConville MJ. Characterization of metabolically quiescent Leishmania parasites in murine lesions using heavy water labeling. PLoS Pathog. 2015 Feb;11(2):e1004683.

615. Sunter J, Gull K. Shape, form, function and Leishmania pathogenicity: from textbook descriptions to biological understanding. Vol. 7, Open biology. 2017.

616. Davies CR, Kaye P, Croft SL, Sundar S. Leishmaniasis: new approaches to disease control. BMJ. 2003 Feb 15;326(7385):377–82.

617. Gupta AK, Das S, Kamran M, Ejazi SA, Ali N. The pathogenicity and virulence of Leishmania - interplay of virulence factors with host defenses. Virulence. 2022 Dec 31;13(1):903–35.

618. Zorova LD, Popkov VA, Plotnikov EY, Silachev DN, Pevzner IB, Jankauskas SS, et al. Mitochondrial membrane potential. Anal Biochem. 2018 Jul 1;552:50–9.

619. Møller IM, Rasmusson AG. The role of NADP in the mitochondrial matrix. Trends in Plant Science. 1998 Jan 1;3(1):21–7.

620. Walker MA, Tian R. NAD(H) in mitochondrial energy transduction: implications for health and disease. Curr Opin Physiol. 2018 Jun;3:101–9.

621. Kovářová J, Pountain AW, Wildridge D, Weidt S, Bringaud F, Burchmore RJS, et al. Deletion of transketolase triggers a stringent metabolic response in promastigotes and loss of virulence in amastigotes of Leishmania mexicana. PLoS Pathog. 2018 Mar;14(3):e1006953.

622. Turco SJ, Descoteaux A. The lipophosphoglycan of Leishmania parasites. Annu Rev Microbiol. 1992;46:65–94.

623. Forestier CL, Gao Q, Boons GJ. Leishmania lipophosphoglycan: how to establish structure-activity relationships for this highly complex and multifunctional glycoconjugate? Front Cell Infect Microbiol. 2014;4:193.

624. Saunders EC, De Souza DP, Naderer T, Sernee MF, Ralton JE, Doyle MA, et al. Central carbon metabolism of Leishmania parasites. Parasitology. 2010;137(9):1303–13.

625. Turco SJ, Sacks DL. Expression of a stage-specific lipophosphoglycan in Leishmania major amastigotes. Mol Biochem Parasitol. 1991 Mar;45(1):91–9.

626. Aikawa M, Road A. Interactions Between Macrophagelike Leishmania braziliensis in Vitro Cells and. 1982;

627. Uezato H, Kato H, Kayo S, Hagiwara K, Bhutto AM, Katakura K, et al. The attachment and entry of Leishmania (Leishmania) major into macrophages: Observation by scanning electron microscope. Journal of Dermatology. 2005;32(7):534–40.

628. Bretscher PA, Wei G, Menon JN, Bielefeldt-Ohmann H. Establishment of Stable, Cell-Mediated Immunity that Makes “Susceptible” Mice Resistant to Leishmania major. Science. 1992;257(5069):539–42.

629. Khabiri AR, Bagheri F, Assmar M. *Leishmania major*: Species specific delayed hypersensitivity reaction induced by exogenous secreted antigen in the guinea pig. Experimental Parasitology. 2006 Mar 1;112(3):184–6.

630. Okwor I, Uzonna J. Persistent parasites and immunologic memory in cutaneous leishmaniasis: implications for vaccine designs and vaccination strategies. Immunol Res. 2008 Jun;41(2):123–36.

631. Okwor I, Liu D, Beverley SM, Uzonna JE. Inoculation of killed Leishmania major into immune mice rapidly disrupts immunity to a secondary challenge via IL-10-mediated process. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(33):13951–6.

632. Zayats R, Mou Z, Yazdanpanah A, Gupta G, Lopez P, Nayar D, et al. Antigen recognition reinforces regulatory T cell mediated Leishmania major persistence. Nat Commun. 2023 Dec 19;14(1):8449.

633. Späth GF, Lye LF, Segawa H, Turco SJ, Beverley SM. Identification of a Compensatory Mutant (lpg2−REV) of Leishmania major Able To Survive as Amastigotes within Macrophages without LPG2-Dependent Glycoconjugates and Its Significance to Virulence and Immunization Strategies. Infect Immun. 2004 Jun;72(6):3622–7.

634. Aschale Y, Ayehu A, Worku L, Tesfa H, Birhanie M, Lemma W. Malaria-visceral leishmaniasis co-infection and associated factors among migrant laborers in West Armachiho district, North West Ethiopia: Community based cross-sectional study. BMC Infectious Diseases. 2019;19(1):1–7.

635. Desjeux P, Alvar J. Leishmania/HIV co-infections: Epidemiology in Europe. Annals of Tropical Medicine and Parasitology. 2003;97(SUPPL. 1):3–15.

636. Bateman A. UniProt: A worldwide hub of protein knowledge. Nucleic Acids Research. 2019;47(D1):D506–15.

637. Zhang WW, Matlashewski G. CRISPR-Cas9-Mediated Genome Editing in Leishmania donovani. mBio. 2015 Jul 21;6(4):e00861.

638. Batista MF, Nájera CA, Meneghelli I, Bahia D. The Parasitic Intracellular Lifestyle of Trypanosomatids: Parasitophorous Vacuole Development and Survival. Front Cell Dev Biol. 2020;8:396.

639. McConville MJ, Naderer T. Metabolic pathways required for the intracellular survival of Leishmania. Annu Rev Microbiol. 2011;65:543–61.

640. Courret N, Fréhel C, Gouhier N, Pouchelet M, Prina E, Roux P, et al. Biogenesis of Leishmania-harbouring parasitophorous vacuoles following phagocytosis of the metacyclic

promastigote or amastigote stages of the parasites. J Cell Sci. 2002 Jun 1;115(Pt 11):2303–16.

641. Sernee MF, Ralton JE, Nero TL, Sobala LF, Kloehn J, Vieira-Lara MA, et al. A Family of Dual-Activity Glycosyltransferase-Phosphorylases Mediates Mannogen Turnover and Virulence in Leishmania Parasites. Cell Host Microbe. 2019 Sep 11;26(3):385-399.e9.

642. Zhang K, Beverley SM. Mannogen-ing Central Carbon Metabolism by Leishmania. Trends Parasitol. 2019 Dec;35(12):947–9.

643. Bringaud F, Baltz D, Baltz T. Functional and molecular characterization of a glycosomal PPi-dependent enzyme in trypanosomatids: pyruvate, phosphate dikinase. Proc Natl Acad Sci U S A. 1998 Jul 7;95(14):7963–8.

644. Cymeryng C, Cazzulo JJ, Cannata JJ. Phosphoenolpyruvate carboxykinase from Trypanosoma cruzi. Purification and physicochemical and kinetic properties. Mol Biochem Parasitol. 1995 Jul;73(1–2):91–101.

645. Steinborn K, Szallies A, Mecke D, Duszenko M. Cloning, heterologous expression and kinetic analysis of glycerol kinase (TbGLK1) from Trypanosoma brucei. Biol Chem. 2000 Nov;381(11):1071–7.

646. Besteiro S, Biran M, Biteau N, Coustou V, Baltz T, Canioni P, et al. Succinate secreted by Trypanosoma brucei is produced by a novel and unique glycosomal enzyme, NADH-dependent fumarate reductase. J Biol Chem. 2002 Oct 11;277(41):38001–12.

647. Michels PA, Hannaert V, Bringaud F. Metabolic aspects of glycosomes in trypanosomatidae - new data and views. Parasitol Today. 2000 Nov;16(11):482–9.

648. Saini S, Kumar Ghosh A, Singh R, Das S, Abhishek K, Kumar A, et al. Glucose deprivation induced upregulation of phosphoenolpyruvate carboxykinase modulates virulence in Leishmania donovani. Molecular Microbiology. 2016;102(6):1020–42.

649. Oliveira JM. Complex in vitro models: do not complicate it. In vitro models. 2023 Aug 1;2(3):67–8.

650. Engwerda CR, Kaye PM. Organ-specific immune responses associated with infectious disease. Immunol Today. 2000 Feb;21(2):73–8.

651. Chensue SW, Warmington K, Ruth JH, Lukacs N, Kunkel SL. Mycobacterial and schistosomal antigen-elicited granuloma formation in IFN-gamma and IL-4 knockout mice: analysis of local and regional cytokine and chemokine networks. J Immunol. 1997 Oct 1;159(7):3565–73.

652. Murray HW. Tissue granuloma structure-function in experimental visceral leishmaniasis. International Journal of Experimental Pathology. 2001;82(5):249–67.

653. Moore JWJ, Moyo D, Beattie L, Andrews PS, Timmis J, Kaye PM. Functional complexity of the Leishmania granuloma and the potential of in silico modeling. Front Immunol. 2013;4:35.

654. Murray HW, Jungbluth A, Ritter E, Montelibano C, Marino MW. Visceral Leishmaniasis in Mice Devoid of Tumor Necrosis Factor and Response to Treatment. Infect Immun. 2000 Nov;68(11):6289–93.

655. Tumang MC, Keogh C, Moldawer LL, Helfgott DC, Teitelbaum R, Hariprashad J, et al. Role and effect of TNF-alpha in experimental visceral leishmaniasis. J Immunol. 1994 Jul 15;153(2):768–75.

656. Uzonna JE, Wei G, Yurkowski D, Bretscher P. Immune Elimination of Leishmania major in Mice: Implications for Immune Memory, Vaccination, and Reactivation Disease. The Journal of Immunology. 2001;167(12):6967–74.

657. Kaye P, Scott P. Leishmaniasis: Complexity at the host-pathogen interface. Nature Reviews Microbiology. 2011;9(8):604–15.

658. Norsworthy NB, Sun J, Elnaiem D, Lanzaro G, Soong L. Sand fly saliva enhances Leishmania amazonensis infection by modulating interleukin-10 production. Infect Immun. 2004 Mar;72(3):1240–7.

659. Stamper LW, Patrick RL, Fay MP, Lawyer PG, Elnaiem DEA, Secundino N, et al. Infection parameters in the sand fly vector that predict transmission of Leishmania major. PLoS Negl Trop Dis. 2011 Aug;5(8):e1288.

660. Guhe V, Soni B, Ingale P, Singh S. Autophagy proteins and its homeostasis in cellular environment. Adv Protein Chem Struct Biol. 2021;123:73–93.

661. Yeganeh B, Ghavami S, Kroeker AL, Mahood TH, Stelmack GL, Klonisch T, et al. Suppression of influenza A virus replication in human lung epithelial cells by noncytotoxic concentrations bafilomycin A1. Am J Physiol Lung Cell Mol Physiol. 2015 Feb 1;308(3):L270-286.

662. Yeganeh B, Rezaei Moghadam A, Alizadeh J, Wiechec E, Alavian SM, Hashemi M, et al. Hepatitis B and C virus-induced hepatitis: Apoptosis, autophagy, and unfolded protein response. World J Gastroenterol. 2015 Dec 21;21(47):13225–39.

663. Nakatogawa H. Mechanisms governing autophagosome biogenesis. Nat Rev Mol Cell Biol. 2020 Aug;21(8):439–58.

664. Onwah SS, Uzonna JE, Ghavami S. Assessment of Autophagy in Leishmania Parasites. In: Methods in Molecular Biology (Clifton, NJ). 2024.

665. Conde L, Maciel G, de Assis GM, Freire-de-Lima L, Nico D, Vale A, et al. Humoral response in Leishmaniasis. Front Cell Infect Microbiol. 2022 Dec 12;12:1063291.

666. Omachi S, Fujii W, Azuma N, Morimoto A, Sanjoba C, Matsumoto Y, et al. B-cell activating factor deficiency suppresses splenomegaly during Leishmania donovani infection. Biochem Biophys Res Commun. 2017 Aug 5;489(4):528–33.

667. Goto Y, Omachi S, Sanjoba C, Matsumoto Y. Elevation of serum B-cell activating factor levels during visceral leishmaniasis. Am J Trop Med Hyg. 2014 Nov;91(5):912–4.