THE CRITICAL ROLE OF PHOSPHOENOLPYROVATE CARBOXYKINASE (PEPCK) IN IMMUNOPATHOGENESIS OF CUTANEOUS LEISHMANISIS

BY

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ABSTRACT

Leishmaniasis is a tropical vector-borne infection caused by protozoan *Leishmania* parasites which are transmitted by the bite of various species of infected female phlebotomine sandflies. This neglected disease with a wide range of clinical symptoms affects 350 million individuals in 88 countries and is considered the second leading cause of mortality after malaria. Cutaneous leishmaniasis is the most common form of the disease caused by several species of parasites. Despite tremendous efforts, no effective vaccine against leishmaniasis that induces a robust and long-lasting immunity yet exists. This could be possibly explained by the lack of sufficient knowledge on *Leishmania* immunogenic antigens and immunological correlations of protection. We recently found that *Leishmania* glycosomal phosphoenolpyruvate carboxykinase (PEPCK) is a highly immunogenic antigen that produces a robust T cell-mediated immunity in both mice and human. Remarkably, PEPCK is a gluconeogenic enzyme involved in the conversion of oxaloacetate into phosphoenolpyruvate and is constitutively expressed by both life stages of all pathogenic species of *Leishmania* parasites.

In the current study, I utilized molecular and immunological approaches to investigate the role of PEPCK in virulence and immunopathogenicity of *Leishmania major*. I successfully generated a PEPCK deficient *L. major* and demonstrated that the targeted loss of PEPCK results in a compromised growth in axenic cultures once glucose is depleted from media. Although the selective loss of PEPCK does not influence metacyclogenesis, the PEPCK deficient parasites exhibit a severely impaired proliferation in bone marrow-derived macrophages. Moreover, the absence of PEPCK leads to an attenuated pathology *in vivo*. Indeed, PEPCK null mutants permanently fail to induce cutaneous lesions in highly susceptible BALB/c mice infected with these parasites despite the persistence of a low number of parasites at the site of infection in these mice. Furthermore, the ablation of PEPCK, as an immunogenic antigen, leads to a reduction in the frequency of cytokine (IFN-γ, IL-4, and IL-10) -producing $CD4^+$ T cells, and consequently blunted immune response in susceptible mice. Surprisingly, vaccination with PEPCK deficient *L. major* confers moderate protection (minor DTH response and low level of IFN-γ) against secondary virulent challenge in BALB/c mice which is not associated with a strong *in vitro* recall response. Lastly, I reported here that PEPCK deficient parasites exhibit

markedly higher extracellular acidification rate, enhanced oxygen consumption rate and proton leak, and reduced ATP coupling efficiency compared to wild type and addback counterparts.

Collectively, these findings indicate that PEPCK is a key metabolic enzyme and its targeted loss leads to an attenuated phenotype *in vitro* (axenic culture and bone marrow-derived macrophages) and *in vivo* (susceptible mice), altered metabolic activity and blunted host immune response.

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DEDICATION

I dedicate this work to my parents for their sincere support of my endeavor towards learning. And to my beloved son, Adrián, who made me stronger, better, and more fulfilled than I could have ever imagined.

Table of Contents

List of Tables

List of Figures

ABBREVIATIONS

Acronym	Definition
2-DG	2-Deoxyglucose
AmphB	Amphotericin B
ANOVA	Analysis of Variance
ADP	Adenosine Diphosphate
APC	Antigen-presenting cell
ATP	Adenosine Triphosphate
BMDM	Bone marrow-derived macrophage
BFA	Brefeldin A
BSA	Bovine Serum Albumin
CACS	Central Animal Care Services
Cas9	CRISPR-associated protein 9
CD	Cluster of differentiation molecules
CFSE	Carboxyfluorescein succinimidyl ester
CL	Cutaneous Leishmaniasis
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
CTL	Cytotoxic T cell
DC	Dendritic cell
DCL	Diffuse Cutaneous Leishmaniasis
dLNs	Draining Lymph nodes
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ECAR	Extracellular Acidification Rate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FBP	Fructose 1,6-biphosphatase

FCCP	Carbonyl cyanide-4-phenylhydrazone
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box p3
GK	Glycerol Kinase
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
HDR	Homology-directed repair
HEPES	Hydroxyethyl-1-piperazineethanesulfonic acid
HPAEC	high-pH Anion Exchange Chromatography
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
K_2HPO_4	Dipotassium Phosphate
KO	Knock Out
LACK	Leishmania homologue of receptors for Activated C Kinase
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
M199	Medium 199
MCL	Mucocutaneous Leishmaniasis
mg	Milligram
$MgCl_2$	Magnesium Chloride
MHC I	Major Histocompatibility Complex I
MHC II	Major Histocompatibility Complex II
ml	Millilitre
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
NHEJ	Non-homologous end-joining
ng	Nanogram
NK	Natural Killer
NO	Nitric Oxide

OCR	Oxygen Consumption Rate
PAM	Protospacer Adjacent Motif
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pg	Picogram
PKDL	Post-kala-azar dermal *leishmaniasis*
PMA	Phorbol Myristate Acetate
PMNs	Polymorphonuclear neutrophil
PNA	Peanut Agglutinin
PEPCK	Phosphoenolpyruvate carboxykinase
PPDK	Pyruvate Phosphate Dikinase
rIL-12	Recombinant Interleukin twelve
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute Medium
SD	Standard Deviation
SLA	Soluble Leishmania Antigen
TALEN	Transcription Activator-Like Effectors Nucleases
TCR	T Cell Receptor
TGF-ß	Transforming Growth Factor Beta
Th	T helper cell
TNF-α	Tumor Necrosis Factor Alpha
Tregs	Regulatory T Cells
VL	Visceral Leishmaniasis
WT	Wild Type
µg	Microgram
ZFN	Zinc-Fingers Nucleases

CHAPTER 1

1.0 Introduction

1.1 Background to leishmaniasis

Leishmaniasis is a vector born disease transmitted by the bite of various species of infected female phlebotomine sandflies, and puts the life of approximately 350 million people in 88 countries in danger This neglected with a broad range of protean manifestation is the second parasitic diseases with the most mortality after malariav[1] [2]. An increase in the number of immunosuppressed individuals and international travel accounts for the growing number of new leishmaniasis cases[3]. Leishmaniasis not only affects people in poor countries across South East Asia, East Africa, Latin America and, the Mediterranean, but also is considered a potential threat to those from affluent countries who travel to endemic regions **(Figure I)**[4]. The disease manifestation ranges from a self-healing cutaneous ulcers that leave life-long scars to a deadly disseminated cutaneous or visceral infections depending on the interplay between parasite species and the type of host immune response induced during *Leishmania* infection[2] [5]. Leishmaniasis is often considered a zoonotic disease, however, the transmission between humans has been reported in endemic regions such as Afghanistan, India, and Sudan[6].

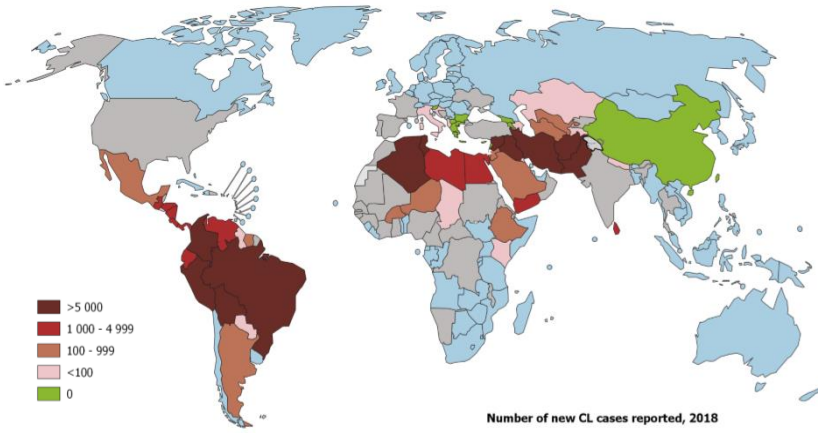


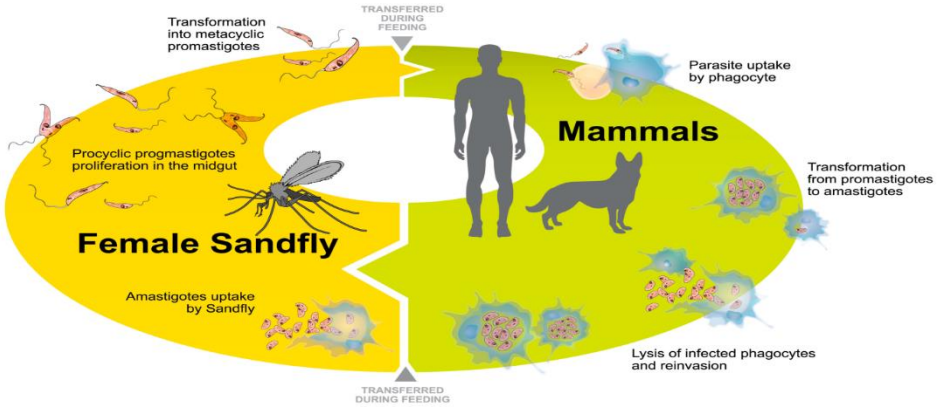
Figure I. The geographical distribution of cutaneous leishmaniasis, 2018

1.2 The Vector

The female sandfly of the genera *Phlebotomus* and *Sergentomyia* in the Old World and *Lutzomyia* in the New World are the only vectors that are capable of transmitting the parasite to the mammalian host[7]. Various sandfly species have different behavioral patterns that enable them to spread the disease. Outdoor and indoor biting, several probing on the same host, and being noiseless while flying are among some of the most notable sandfly feeding and life behaviors that enhance the chances of parasite transmission[2].

1.3 The parasite and life cycle

The complex life cycle of *Leishmania* consists of two divergent stages, the flagellated promastigotes form within the sandfly midgut and non-flagellated amastigote form inside the phagocytic cells of the mammalian host[8]. During a blood meal, the infective metacyclic-stage promastigotes are injected into the skin of the mammalian host along with various components of sandfly saliva[9]. The promastigotes are subsequently engulfed by several phagocytic immune cells recruited to the site including macrophages and monocytes. Within the phagocytic cells, the promastigotes undergo a tremendous morphological change to ultimately convert into a non-flagellated, dividing form called amastigotes. Phagocytic cells that carry multiplying amastigotes eventually rupture to release the amastigotes and facilitating the infection of other local phagocytic cells. The final step to complete the parasite life cycle is the transformation of amastigotes into promastigotes that occurs in the midgut of new sandfly vector following uptake of



amastigotes[10 1] **(Figure II)**[11]**.**

1.4 Metacyclogenesis in *Leishmania*

The infectious cycle [illegible] mammalian and a [illegible]

Figure II. The *Leishmania* digenetic life cycle

internalized by various innate immune cells, mostly macrophages, upon deposition into the host skin via the bite of an infected sandfly. In the mammalian host, promastigotes differentiate into round, non-flagellated amastigotes and remain in this form for the duration of their intracellular life cycle[12]. The developmental transition of promastigotes in the sandfly gut, termed metacyclogenesis, is very poorly understood[13]. This process, which is involved in the transformation of procyclic promastigotes into metacyclic promastigotes, is characterized by substantial alterations in parasites infectivity and morphology[14]. Thus, metacyclic parasites are preferably utilized in both *in vitro* and *in vivo* experimental studies of parasite infectivity[15]. Metacyclogenesis could also take place within the axenic culture as the properties of logarithmic-phase promastigotes and stationary-phase promastigotes often resemble those of procyclic and metacyclic forms, respectively[13]. Additionally, infective metacyclic promastigotes morphologically differ from their non-infective procyclic counterparts. Under the light microscope, procyclic and metacyclic parasites could be simply distinguished, as the former mostly assumes a small round-shape body whereas the latter form exhibits a narrow-slender body and a relatively elongated flagella[15].

1.4.1 Basis for Peanut Agglutinin (PNA) Isolation of Metacyclic Promastigotes

Metacyclogenesis is not only associated with modifications in promastigotes morphology and infectivity but is also accompanied by multiple structural alterations in lipophosphoglycan (LPG)[15 16]. Procyclic promastigotes display a smaller LPG that facilitates their binding to sandfly midgut, whereas metacyclic promastigotes display a larger LPG during metacyclogenesis that limits midgut binding [17]. However, the most notable structural change in LPG is that in

metacyclic promastigotes, arabinose residues are capped by galactosyl side chains instead of the mannose caps found in procyclic promastigotes. This modification is considered the basis of purification methods utilized for the isolation of *Leishmania* metacyclic promastigotes using peanut agglutinin lectin (PNA). Therefore, the metacyclic-stage promastigotes are negatively isolated as unlike procyclic parasites, they fail to be agglutinated by PNA[13]. Despite the limitations involved in the use of PNA or anti-LPS monoclonal antibodies in studies of LPG-deficient parasites[15], these methods remain the most preferred techniques of isolating metacyclic promastigotes used for *Leishmania* infectivity assays in macrophages and mammalian host[15].

1.5 Metabolism in Trypanosomatids

The metabolism of Trypanosomatids considerably varies among various species as well as different life stages of the same species. It is widely believed that variations in the metabolism of distinct Trypanosomatids are dictated by nutrients availability in their habitats[18]. Indeed, most trypanosomatids heavily rely on the available carbon sources in their host to produce ATP for functioning and replication. Strikingly, some species such as *Trypanosoma brucei* and *Trypanosoma cruzi* rather consume glucose as the major carbon source despite the abundance of amino acids in their natural environments[19]. Trypanosomatids possess special energy metabolism as the greater part of the glycolysis pathway takes place in a peroxisome-like organelle called glycosome[20]. In addition, a plant-like alternative oxidase exists in the mitochondrion of some bloodstream trypanosomatids which is involved in the terminal step of mitochondrial respiration[21]. The development of RNA interference (RNAi) tool, as a reverse genetic approach, in *T. brucei* and completion of genome projects in *L. major* have provided novel insights into the analysis of metabolic processes[19]. However, owing to lack of RNA interference pathway in *Leishmania* species and *T. cruzi*[22], key questions regarding maintenance of glycosomal ADP/ATP balance, production of nucleobase precursor, and biosynthesis of glycoconjugates through gluconeogenesis in a glucose-depleted environment in these parasites remain to be addressed[19].

1.5.1 Energy metabolism in *Leishmania*

The genome analysis of *L. major* has identified over 4000 genes involved in central metabolic pathways of this protozoan parasite[23]. This finding indicates that *L. major* has the most extensive metabolic capabilities compared to two major trypanosomatid parasites, *T. brucei* and *T. cruzi*.

The complicated metabolic makeup, as present in *L. major*, could be in part explained by the parasite's life history strategies[23] [19]. Indeed, a wealth of knowledge on the genomic organization of *Leishmania* could provide valuable information about the metabolic profile of parasites for each life cycle. However, due to poor knowledge of the niche that *Leishmania* resides, the precise prediction of adaptions of these parasites during their distinct life stages is not feasible[24]. It is widely believed that the metabolic profile in *Leishmania* is highly influenced by the of type carbon sources available in the parasite's habitat. *Leishmania* initially thrives as promastigote forms inside the nectar-feeding sandfly host where glucose is abundant[19]. Notably, the knowledge of metabolism in *Leishmania* promastigotes is often obtained from the biochemical analysis of promastigotes cultivated in nutrient-rich medium. For that reason, it is not clear whether metabolic routes as naturally occur in sandfly-derived promastigotes are similar to that of axenic promastigotes[25]. Glycolysis and mitochondrial respiration appear to be the major energy-generating metabolic pathways in *Leishmania* promastigotes[26]. Hexoses such as glucose, if available, is the preferred carbon source for *Leishmania* promastigotes. However, under glucose starvation, promastigotes are capable of consuming amino acids to synthesize hexoses *de novo* via gluconeogenesis pathway[27]. The metabolism of *Leishmania* promastigotes, to date, is supported by findings of genomics analyses. However, a better understanding of metabolism seems to require the genetic ablation of key enzymes involved in core metabolic pathways[25].

Promastigotes are internalized by macrophages and differentiate into amastigotes following localization in the mature phagolysosome. The biochemical composition of the phagolysosome compartment is believed to be rich in amino acids and poor in sugars[29]. The genome-based analyses of *Leishmania* metabolism and known nutrient requirements of amastigotes suggest that *Leishmania* amastigotes supply the large part of amino acids and purines required for proliferation and functioning from the macrophage[30]. The uptake of essential metabolites is facilitated by polytopic membrane transporters of acidic phagolysosome[31]. Furthurmore, *Leishmania* amastigotes are capable of scavenging complex lipids from the phagolysosome since the host glycosphingolipids are often delivered to this compartment during parasite phagocytosis[26]. Notably, proteolysis of exogenous proteins that enter into the phagolysosome through autophagy and endocytosis provides amastigotes with a considerable amount of essential amino acids and heme[32]. Semini *et al* reported that unlike *L. mexicana* promastigotes, there is an elevated fatty acid oxidation pathway in lesion-derived amastigotes which significantly reduces

the glucose need of the parasites inside the phagolysosome[33]. Gluconeogenesis seems to be an essential pathway in phagolysosome, as this organelle is a glucose-poor niche. It is believed that gluconeogenesis actively participates in maintaining the pool of precursors required for the biosynthesis of DNA/RNA and reserved oligosaccharide 1,2 β mannan. As compared to promastigotes, glycosomes are present in fewer numbers in amastigotes. However, amastigotes glycosomes seem to sequester a greater amount of gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK)[34], (glycerol kinase) GK and (Pyruvate phosphate dikinase) PPDK[27].

Altogether, the complexity of amastigotes nutrient requirements as well as limited knowledge on the biochemical composition of phagolysosome could be part of the reason why energy metabolism in *Leishmania* amastigotes is comparatively less understood[35]. Moreover, studies on the metabolic capabilities of amastigotes are limited and mainly reliant on lesion-derived amastigotes. However, the cultivation of amastigotes in the absence of host cells has been plausible for some *Leishmania* species with the caveat that *in vitro* forms often fail to completely mimic properties of host-isolated amastigotes[36 37 38].

1.5.2 Glycolysis in *Leishmania*

Glycolysis is a series of reactions in which, under aerobic conditions, glucose is broken down into pyruvate and ATP. In *Leishmania* parasites, glycolysis is known to be a central metabolic pathway that mostly takes place in peroxisome-like organelles called glycosomes[19]. These organelles are confined by a phospholipid bilayer membrane with specific transporters to facilitate metabolite trafficking. Although glycolytic enzymes constitute the vast majority of proteins sequestered in glycosomes (up to 90%), other enzymes controbute to gluconeogenesis and fatty acid oxidation pathways are also localized in these organelles[24]. The notion of closed compartment suggests a possible link between substrates and products of pathways that concurrently take place inside glycosomes. This implies that during metabolic processes, the glycosomal ATP/ADP and/or NAD^+/NADH balance is maintained by regulated enzyme distribution and cellular reservoir of metabolites[39]. It is widely agreed that hexoses such as glucose are preferred carbon sources during *Leishmania* life span[11]. Consequently, glycolysis, as a glucose utilization pathway, is an active metabolic process in *Leishmania*[40]. The expression pattern of *Leishmania* metabolic enzymes, however, reveals a relative downregulation of

glycolytic enzymes during differentiation. As such, the role of glycolysis in sustainig the energetic needs of *Leishmania* parasites seems to be more pronounced in promastigotes due to the constant exposure of this form to high sugar concentration in the sandfly host[34]. Glucose transporters in *Leishmania* robustly uptake the exogenous glucose and other hexoses to sustain parasite growth and functioning[41]. Feng *et al* reported that the proliferation of *L. mexicana* in axenic culture is severely abrogated in glucose transporter null mutants. Additionally, *L. mexicana* amastigotes with a defect in glucose transporters exhibit a significantly impaired survival and proliferation inside macrophages compared to wild type counterparts. These findings imply the significance of glycolysis in the generation of energy and essential metabolites vis glucose metabolism in both the life stages of *Leishmania* parasites[42].

1.5.3 Gluconeogenesis in *Leishmania*

Gluconeogenesis is a metabolic pathway by which glucose is synthesized from non-carbohydrate substrates such as lactate, amino acids, and glycerol[43]. *Leishmania* promastigotes are capable of importing exogenous glucose via transmembrane glucose transporters when available[42]. However, glucose depletion results in transient dependency to the gluconeogenesis pathway for *de novo* biosynthesis of sugar[44]. In *Leishmania* amastigotes, unlike mammalian cells, gluconeogenesis is constantly carried out even in the presence of an ample amount of glucose in environment[45 27]. Moreover, a significant increase in the protein level of key gluconeogenic enzymes such as fructose 1, 6 biphosphatase, PPDK, PEPCK, and gluconeogenic metabolites during differentiation suggests the significance of gluconeogenesis in the survival of amastigotes[34 39]. Despite the lack of detailed information about gluconeogenesis in Trypanosomatids, it is well known that this process is essential for the biosynthesis of glycoconjugates as well as DNA/RNA and oligosaccharides reserves in *Leishmania* [26]. Several studies have described PEPCK, GK, and PPDK as glycosome-localized involved in metabolism in *Leishmania*[46 27 47]. However, the contribution of these enzymes in gluconeogenesis has been recently shown via assessment of the cellular level of *Leishmania* reserve oligosaccharide 1, 2β mannan[28]. This carbohydrate is catabolized under glucose starvation and plays a role in the survival and virulence of *Leishmania* parasites[48]. Rodriguez *et al*. reported that deficiency of PEPCK, GK, and PPDK in *L. mexicana* results in a reduced level of mannan in promastigotes and amastigotes under glucose starvation[27]. The contribution of these enzymes in mannan

biosynthesis and by implication gluconeogenesis, supports the previous notion about the role of PEPCK, GK, and PPDK in entry and incorporation of aspartate, glycerol, and alanine, respectively, to gluconeogenesis pathway[45]. It is evident that the gluconeogenesis pathway maintains the redox balance ($NADPH/NADP^+$) via providing pentose phosphate shunt with hexose monophosphates metabolites[49]. Notably, the loss of redox flux due to the perpetuation of the gluconeogenesis pathway has been demonstrated to cause increased sensitivity of *Leishmania* parasites towards reactive oxygen species (ROS) in the activated macrophages[50 39]. Furthermore, gluconeogenesis seems to be an essential pathway in *Leishmania* amastigotes. In line with previous studies, it has been indicated that fructose 1, 6-biophosphatase (fbp) deficiency in *L. major* leads to an impaired proliferation inside macrophages. Moreover, fbp null mutants fail to develop progressive lesions in highly susceptible BALB/c mice. These findings imply that in a glucose-poor environment, an intact gluconeogenesis pathway provides the hexose requirements of *Leishmania* amastigotes for replication and virulence[28].

1.5.4 Phosphoenolpyruvate carboxykinase (PEPCK) in *Leishmania*

The enzyme PEPCK reversibly catalyses the conversion of oxaloacetate and ATP/GTP into phosphoenolpyruvate and CO_2. Although PEPCK contributes to carbon metabolism in various ways, it seems to be essential for the gluconeogenic flux of almost all organisms. In *Leishmania*, PEPCK is sequestered in the glycosome and excretes its function by the formation of PEP. It is believed that PEPCK supports the survival and proliferation of *Leishmania* parasites in limited glucose conditions via alternative utilization of amino acids for *de novo* synthesis of hexoses[27]. Moreover, PEPCK in combination with malic enzyme appears to maintain the glycosomal aspartate reservoir for gluconeogenesis. Among all gluconeogenic enzymes with an enhanced expression under glucose starvation, PEPCK exhibits the highest activity[39]. PEPCK as the first enzyme in glycosomal succinate fermentation plays a crucial role in maintaining the net charge of glycosomal ATP[51]. Likewise, PEPCK maintains the $NADPH/NADP^+$ hemostasis via activation of pentose phosphate shunt[49]. The loss of gluconeogenic flux and suppression of antioxidant machinery results in a high vulnerability to extracellular oxidative stress in PEPCK null mutants[39]. This supports the notion that PEPCK is essential for the survival and proliferation of *Leishmania* parasites inside macrophages. In this perspective, Sinai *et al* reported that PEPCK modulates the generation of intracellular ROS in limited oxygen conditions. Therefore, the high

accumulation of intracellular ROS, as present in glucose starved *L. donovani* PEPCK null mutants, positively correlates with increased sensitivity to oxidative stress in macrophages[39].

PEPCK has been recently identified as an immunodominant antigen that drives a robust and durable T cell-mediated immunity in both mice and human-infected *L. major* at the peak of infection. More than 80% identity in the PEPCK whole sequence in all pathogenic *Leishmania* as revealed *by* Several amino acid sequence alignments, shows that PEPCK is a highly conserved cross-species antigen[52]. Collectively, these findings suggest that PEPCK, as a multifunctional gluconeogenic enzyme and immunodominant antigen, might be in an ideal vaccine against leishmaniasis.

1.6 The disease

Leishmaniasis constitutes a spectrum of different and complex clinical presentations. The epidemiology and clinical manifestation of the disease vary with the parasite species, host factors, and environment. The disease is often categorized as cutaneous, mucosal, and visceral leishmaniasis[6].

1.6.1 Cutaneous leishmaniasis (CL)

Cutaneous leishmaniasis (CL) is the most prevalent form of this parasitic disease and is caused by several species of parasite[6]. The disease is most often associated with spontaneous healing that results in lifelong immunity against reinfection. Although non-life threatening, the disfiguring scars could be associated with social stigma if left untreated. A few numbers of nodular or papular lesions full of parasites appear on the site of parasite inoculation approximately 2-8 weeks after asymptotic period [5]. These nodules progressively ulcerate over the course of 2 to 24 weeks to become the ulcerative and excoriating lesions which are characteristic of local cutaneous leishmaniasis[5]. Depending on parasite species and type of host immune response to the parasite, the range of clinical presentations associated with the disease may vary[53]. The immunological spectrum in different individuals extends between a robust T cell response characterized by a delayed-type hypersensitivity (DTH) and high IFN-γ production to poor (or complete absence of) DTH response and production of high levels of antibodies[5]. Considering that eradication of intracellular *Leishmania* is largely mediated by the IFN-γ-activated macrophages not neutralizing antibodies, a strong T cell immune response is critical to control the parasite load. Therefore, chronic lesions are observed in patients with an intermediate

level of cellular and humoral immunity. On the other hand, diffused cutaneous leishmaniasis and mucosal leishmaniasis are severe forms of cutaneous diseases that develop in individuals with lack of T cell responses and excessive immune response, respectively[54]. Although it was previously believed that the outcome of infection is largely dictated by the differential development of Th1 and Th2-type response, new findings imply that a more sophisticated cellular response is perhaps involved in the outcome of infection[53].

1.6.2 Mucosal cutaneous leishmaniasis (MCL)

The cutaneous leishmaniasis caused by *Leishmania braziliensis* species, subgenus *Vienna* could turn into a tegumentary form of disease termed mucosal leishmaniasis (MCL) that is common in South America. This form of the disease, which is accompanied by massive metastatic lesions as well as tissue damage in the mucosal surface of upper respiratory and digestive tracts, often occurs due to infection of *Leishmania* belonging to subgenus Vienna. Sex (male>female), malnutrition, lesion size, and inefficient therapeutic measures are among the most significant factors involved in the emergence of mucosal leishmaniasis[5]. This form of the disease, is associated with specific T cells and inflammatory monocytes in the lesions despite few parasite burden[55]. A significantly high percentage of activated IFN-γ and IL-17-producing $CD4^+$ T cells in circulation and a reduced secretion of IL-10 are responsible for the progression of mucosal leishmaniasis. Therefore, the lack of balance between inflammatory and regulatory cytokines is considered the major factor that skews the immune response towards the hypersensitivity pole and tissue destruction during the course of mucosal leishmaniasis[56].

1.6.3 Disseminated cutaneous leishmaniasis (DCL)

Although mucosal leishmaniasis was previously thought to be the metastatic form of cutaneous leishmaniasis a significant increase in the cases of disseminated cutaneous leishmaniasis (DCL) caused by *Leishmania Vienna braziliensis* (*L.V. braziliensis*) has recently been observed[57]. Dozens to hundred pleomorphic lesions scattered in distinct regions of the body as well as the involvement of nasal mucosa are the hallmarks of the DCL. Metastasis of parasites from original lesions occurs due to a lack of lymph nodes enlargement. This could be in part attributed to the role of bloodstream in the rapid and wide dissemination of parasites[5]. Unlike cutaneous leishmaniasis, the level of Th1 cytokines is significantly low in DCL. This could be possibly due to the lack of massive migration of antigen-specific T cells to lesions, as present in cutaneous

leishmaniasis[5]. A marked difference has been reported between the level of pro-inflammatory cytokines in CL and DCL patients infected with different strains of *L.V. braziliensis*. Surprisingly, a slight difference between the genetic makeup of these strains seems to significantly alter the production level of pro-inflammatory cytokines and consequential manifestations of disease in CL and DCL patients[58].

1.6.4 Visceral leishmaniasis

Visceral leishmaniasis is mainly caused by *L. infantum* and *L. donovani*. These protozoan parasites are transmitted following the bite of the infected sandfly during a blood meal. Visceral leishmaniasis-associated mortality is estimated as 400,000 cases annually. *L. donovani*, as one of the causative agents of kala-azar or black death, is a substantial health problem in the Old World, particularly in the poor rural regions of Northeast India and East Africa. In these areas, humans are believed to be the major reservoir of the parasite. In contrast, *L. infantum* is prevalent in the Mediterranean and dried regions of Latin America where the parasite is transmitted from a canine reservoir to humans. The asymptotic incubation period often lasts between 2 weeks up to 18 months. Visceral inflammation, as well as skin lesions, develop within 2-8 months, whereas the clinical manifestations of visceral leishmaniasis might be latent for years. The persistent systematic infection including prolonged fever, hepatosplenomegaly, and loss of appetite and weight, if left untreated, is lethal in 75-95% of cases. As similar to other forms of leishmaniasis, parasites proliferate in mononuclear phagocytic cells, preferentially macrophages. Hence, the enlargement of spleen and liver, which is the most prevalent clinical feature of the disease, could be attributed to the high frequency of infected macrophages in these organs[59 60]. After recovery, some patients develop a chronic condition called post-kala-azar dermal leishmaniasis (PKDL) which is considered a complication of visceral leishmaniasis. PKDL is prevalent in the India subcontinent and Sudan where the causative agent of the visceral disease is *L. donovani*[60 7]. PKDL is characterized by hypo-pigmented erythematous rashes in face and trunk that can turn into non-tender plaques over time[61]. The rate of PKDL progression varies between different regions and is estimated to occur within 6 months in patients in Sudan and 2-3 years in patients in India. One of the key clinical features of PKDL is the presence of heavily parasitized lesions on the face which could serve as the putative reservoir of parasite for anthroponotic transmission[5 62].

1.7 Diagnosis of cutaneous leishmaniasis

The treatment of cutaneous leishmaniasis (CL) is prolonged and complicated and an early diagnosis is a critical measure for the control of the disease. However, the resemblance between clinical manifestations of CL and other infectious diseases is a major obstacle involved in the diagnosis of CL[63]. In non-endemic regions, travel-related leishmaniasis is the major source of diagnostic dilemma which could be explained by unfamiliarity with the particular symptoms of the disease. However, in endemic regions, the clinical manifestations of CL could provide adequate evidence to make the disease distinguishable from other clinically similar lesions[3]. Thus, multiple techniques ranging from microscopical examination of the parasite to molecular and serological tests have been established for the diagnosis of CL.

1.7.1 Microscopy

CL could be diagnosed via direct visualization of parasites in Giemsa-prepared smears of the lesion under microscope[3]. The needle-aspirated material from the margin of ulcers has the highest diagnostic value than commonly used lesion smears of scraping[69]. Microscopically, lesion-derived amastigotes are characterized as 2-4 μm round bodies with distinctive kinetoplasts and nuclei[3]. Notably, microscopic diagnosis of CL lacks a high level of specificity and sensitivity in comparison with other diagnostic methods[6].

1.7.2 Parasite culture

The cultivation of *Leishmania* parasite for diagnostic purposes is feasible via the addition of fluids or skin scrapings/biopsies of suspected lesions into the Novy-MacNeal-Nicolle medium. However, this diagnostic method is time-consuming and requires a high level of technical expertise due to the susceptibility of the medium to contamination[64]. Additionally, the culture seems to significantly lack sufficient sensitivity [65]. The newly developed mini and micro-culture technologies offer the benefits of being less expensive and easy-to-handle as it requires a considerably lower amount of medium. Moreover, the high sensitivity of this method facilitates the diagnosis of CL in the absence of a high number of parasites in lesions[64]. The diagnostic significance of parasite culture is to provide sufficient numbers of parasites required for sub-species identification tests such as polymerase chain reaction (PCR)[66].

1.7.3 Polymerase chain reaction (PCR)

Numerous molecular diagnostic methods have been developed over the last decades as these tests are thought to offer higher specificity and sensitivity compared to conventional diagnostic methods[67]. Kinetoplasts DNA sequences[73] or ribosomal DNA internal transcribed spacer I sequence of *Leishmania* are among the most popular genes targeted by different PCR assays[68]. Additionally, other PCR-like real-time assays that uses a high-tech fluorescence resonance energy transfer are under examination. PCR tests employed for CL diagnosis require the least invasive sampling such as aspirated fluids or skin biopsies[72]. The disadvantage of different PCR methods is the nonetheless lack of a defined protocol accepted by diagnostic laboratories which impede the possibility of inter-laboratory comparisons[69].

1.7.4 Serologic tests

Indirect fluorescent antibody, enzyme-linked immunosorbent assay (ELISA), western blot, and direct agglutination assays are among the diagnostic serologic tests used for CL. However, these tests are not extensively used for diagnostic of CL due to the low sensitivity. This could be attributed to the lack of a strong host humoral-mediated immune response after infection with *Leishmania* parasites[70]. Furthermore, in the aforementioned serologic tests, total parasite lysate or whole promastigotes are used as antigens. However, the incorporation of *Leishmania* specific antigens such as heat shock protein (HSP83) has been shown to increase the specificity and overall diagnostic performance of these serologic tests for CL[71]. De varies *et al* have recently developed a qualitative membrane-based immunoassay, termed CL Detect™ Rapid Test for reliable detection of CL-relevant *Leishmania* species[3].

1.8 Treatment

All efforts made to introduce a universal and effective anti-*Leishmania* remedy that possesses minor toxicity during multiple local and systemic treatments to the clinic have so far failed[72].. Systemic treatment is used to address complications including immunodeficiency, multiple lesions on hands and face, and joints involvement[73]. Approximately two-third of cutaneous leishmaniasis are self-healing if left untreated[6]. The parental administration of 20mg/kg pentavalent antimonial drugs for 28 days is considered the first choice of treatment for most forms of leishmaniasis. However, the emergence of resistant species has not only created some limitations in drug efficiency but also pushed treatment strategies towards species-specific

approaches[73]. The advent of novel diagnostic methods has facilitated rapid parasite identification. Nevertheless, the circulating species of the endemic area need to be used as a guide in the treatment regimen due to an increase in world travels. The high toxicity and side effects associated with systemic administration of the antimony drugs, financial burden, and the growing number of resistant species indicate the necessity of searching for novel therapeutic approaches[63 74]. Below is a brief introduction of various anti-leishmanial drugs based on the route of administration.

1.8.1 Physical Treatment

Cryotherapy, local heat, surgery, curettage and electrodesiccation, and CO_2 laser are among the widely used physical interventions to treat lesions caused by Old World *Leishmania* species. As reported by Bassiouny *et al*[82], the thermos-sensitive lesions up to 2mm margins could be frozen within 30-60 seconds resulting in a cure rate of %30-84. Another notable treatment in this category is radio-frequency induced heat therapy in which the skin temperature is raised to 55 °C for 5 min. This physical modality has proved efficacious as it induces an immune response which promotes the healing process of other lesions within 5-6 days[76]. However, the high cost of treatment and the lack of adequate information regarding the recurrence of lesions limits the utilization of this method in endemic areas[76].

1.8.2 Chemotherapy

1.8.2.1 Topical agents

A healing rate of 75% in New World cutaneous leishmaniasis has been reported following topical application of paromomycin and methylbenzethonium for 20 days[77]. The combination of this therapy with antimony drugs has shown to improve the efficacy of treatment up to 90%[77]. A topical administration of amphotericin B has provededeffective for treating *L. major* –infected individuals in Israel[78].

1.8.2.2 Intralesional antimonial

The intralesional administration of antimonial drugs seems to accelerate lesion healing. Moreover, this route of administration has several advantages such as reduced toxicity and low cost[79]. Solomon *et al* reported a 91% cure rate in adults infected with New World-related*L. major* speciesfollowing an average of 3 times intralesional administration of sodium

stibogluconate for a period of 3 months[80]. However, the benefit of this administration route for curing cutaneous leishmaniasis caused by Old World species is under-studied[63].

1.8.2.3 Subcutaneous and intramuscular drug delivery

Both subcutaneous and intramuscular drug injections have been employed in the treatment of leishmaniasis. The liposomal form of several anti-leishmanial drugs including sodium stibogluconate, meglumine antimoniate, miltefosine, and paromomycin has recently been developed. However, only the oral form of these drugs has demonstrated to be therapeutically effective. As such, the efficiency of subcutaneous use of liposomal anti-leishmanial drugs in reducing the lesion size and parasite load is under evaluation[81]. Although painful, the intramuscular administration of pentavalent antimonial drugs has been proved effective for both Old and New World leishmaniasis[73].

1.8.2.4 Intravenous drug delivery

Despite the advent of new anti-leishmanial medications such as amphotericin B, miltefosine, and pentamidine to , the intravenous administration of pentavalent antimony drugs is the treatment of choice for New World leishmaniasis[82]. However, in the case of broad resistance to antimony drugs, amphotericin B is the next anti-leishmanial drug. Amphotericin B mediated its therapeutic action through enhancing the permeability of parasite cell membranes which leads to an elevated ion flux and death in most *Leishmania* species. This drug is widely used in developed countries where its possible side effects including renal function disturbances, anemia, thrombocytopenia, and central nervous system toxicity could be simply monitored and addressed. Amphotericin B has also shown superior efficacy in reducing the *Leishmania*-related hospitalization cases compared to other therapeutic agents [63 83].

1.8.2.5 Oral drug delivery

The oral administration of miltefosine has reported effective against both Old and New World leishmaniasis[72]. Several studies have indicated the cure rate of 75-100% for cutaneous leishmaniasis caused by *L. major* and *L. braziliensis* following oral use of 133-150 mg/kg/ for 4 weeks as compared to placebo group[84]. The administration of ketoconazole has shown 80 and %89 efficacy in adults and children infected with Old World-related species in Iran and Kuwait[74]. Allopurinol is another oral agent that exerts its effect through inhibition of purine

synthesis in *Leishmania*. Allopurinol is known to be less toxic yet more effective against leishmaniasis than antimonial drugs[63].

1.9 Prevention

In the lack of effective prophylactic and therapeutic approaches against leishmaniasis, this neglected disease remains a global health issue. Although not a particularly efficient treatment and/or immunization has been so far developed, there are a few practical options to reduce the chance of infection[63]. Avoiding outdoor activities from dusk to dawn in endemic regions, use of insect repellents bed nets with sufficiently fine mesh are among the most effective preventive measures. The inoculation of parasite might occur unnoticed as the sandflies are noiseless when they fly and the bite is not mostly painful[85]. Notably, it has been reported that the colonization of parasites in the sandfly midgut does not occur following the intake of yeast or bacteria during a meal. This suggests that removing sandflies as the parasite vector, although seems impractical, might prevent parasite transmission into mammalian host[86].

1.10 Immunity in Cutaneous leishmaniasis

1.10.1 Murine models of cutaneous leishmaniasis

Murine models have been widely used to further study the immunology and disease pathogenesis of cutaneous leishmaniasis. Similar to humans, the interplay between the infecting parasite species and host immune response largely dictates the clinical disease pattern observed in murine cutaneous leishmaniasis[87]. The severity and outcome of *Leishmania* infection in mice is highly dependent on the quality of the innate and adaptive immune responses which is influenced by the genetic makeup of different mouse strains[54]. The th2-type immune response is responsible for the susceptibility of BALB/c mice to *Leishmania* infection. On the contrary, the resistant phenotype seen in C57LB6 mice is the result of the differentiation of naïve $CD4^+$ T cells into Th1-type cells[53]. The interaction between antigen-specific T cells and antigen-presenting cells leads to the production of inflammatory cytokine (IFN-γ and TNF-α) by Th1 cells which ultimately activate and induce a parasiticidal state in infected macrophages. The activated macrophages subsequently proceed to the intracellular killing of parasites through the production of nitric oxide[88]. The effectiveness of parasite elimination and the magnitude of the immune response in mice model of *Leishmania* infection is determined by the level of nitric oxide released and suppressive role of IL-10 expressed by T-regulatory cells, respectively[53]. In addition to the

resolution of cutaneous lesion, the immune response may play a detrimental role in mediating the pathology observed during the course of the disease. It has been shown that $CD4^+$ T cell-deficient CL57B/6 mice, despite the harboring high parasite burden, display very little tissue destruction and pathology[89].

Leishmania spp.	Human disease	Mouse disease			
		C57BL/6 mice		BALB/c mice	
		Type of disease	Dominant immune response	Type of disease	Dominant immune response
Leishmania major	Self-healing or chronic cutaneous leishmaniasis usually caused by a single skin lesion	Self-healing	T_H1	Chronic	T_H2
Leishmania major Seidman strain	Chronic cutaneous leishmaniasis	Chronic	T_H1	Chronic	T_H2
Leishmania amazonensis	Self-healing or chronic cutaneous leishmaniasis usually caused by a single skin lesion, and diffuse cutaneous leishmaniasis	Chronic	T_H1 and T_H2	Chronic	T_H2
Leishmania mexicana	Healing or chronic cutaneous leishmaniasis usually caused by a single skin lesion, and diffuse cutaneous leishmaniasis	Chronic	T_H1 and T_H2	Chronic	T_H2
Leishmania braziliensis	Healing or chronic cutaneous leishmaniasis usually caused by a single skin lesion, and mucosal leishmaniasis	Self-healing	T_H1	Self-healing	T_H1

The murine infection with *L. major* is considered a valuable dichotomy to delineate the mechanisms underlying the polarization of type 1 and type 2 $CD4^+$ T cells. However, the murine cutaneous disease caused by other species of *Leishmania* does not exactly resemble what is observed in *L. major*-caused CL. As an illustration, infection with *L. Mexicana* and Siedman strain of *L. major* promote a chronic infection in most mouse strains whereas the outcome of infection by *L. braziliensis* in wild type mice is small self-healing lesions[90] **(Table. I)**[53].

Table I. The frequently used *Leishmania* spp. in experimental studies of mouse and human leishmaniasis

1.10.2 Innate immune response to cutaneous leishmaniasis

1.10.2.1 The role of macrophages

Upon deposition of flagellated promastigotes into the skin via the bite of the infected sandfly, they are rapidly internalized by different mononuclear cells including macrophages, monocytes, and dendritic cells (DCs) at the site of inoculation. Promastigotes delivered to macrophages phagolysosome differentiate into the non-flagellated form of parasites also known as amastigote[91]. Several studies have reported that the unique biochemical structure of macrophages sustains the nutrient requirements of amastigotes. This explains why macrophages are the most permissive niche for the intracellular life stage of *Leishmania* among all phagocytic cells of innate immunity[92 29 26]. The reactive oxygen species, produced by respiratory burst during phagocytosis as well as nitric oxide (NO), produced by inducible NO synthase (iNOS) by IFN-γ activated macrophages are two main effector mechanisms that contribute to killing *Leishmania* parasites. Despite the sensitivity of parasite to ROS, non-activated macrophages are unable to kill the parasites due to the inadequacy of respiratory burst. Thus, IFN-γ seems to be an essential factor for better control of parasites as its activation leads to increased respiratory burst in macrophages resulting in the production of reactive oxygen and nitrogen species[53]. Additionally, TNF-α, as a pro-inflammatory cytokine, works in concert with IFN-γ to up-regulate the production of iNOS leading to optimal activation of macrophages and more effective parasite control[93]. Whereas the role of NO in parasite killing in human cutaneous leishmaniasis remains to be unequivocally demonstrated, NO appears to be the main mediator to control the parasite proliferation in *Leishmania*-infected mice. Gaur *et al*[94] demonstrated that NO-deficient mice, despite developing a Th1-type immune response, are significantly more susceptible to *Leishmania* infection as compared to wild type mice. Also, treatment of the highly resistant mice with iNOS inhibitor results in susceptibility to *L. major* infection and a concomitant loss of infection-induced immunity[95].

1.10.2.2 The role of neutrophils

Neutrophils are among the first innate immune cells recruited to the site of infection[30]. Neutrophils have been shown to play a controversial role during the course of infection. This role is largely determined by the parasite species and host genetic background Novais *et al* reported that neutrophils mediate the elimination of *Leishmania braziliensis* and *L. amazonensis* via

neutrophil extracellular trap (NET)[96]. However, apoptotic neutrophils engulfed by less activated macrophages and DCs at the onset of infection promotes parasite survival through suppressing Th1-type response and cross presentation-dependent activation of $CD8^+$ T cells[98]. Studies on neutrophil-depleted mice reveal that unlike neutrophils-deficient C57BL/6 mice, depletion of neutrophils in BALB/c mice significantly reduces the Th2-type response via suppression of IL-4 early burst after infection with *L. major*[99]. However, to further uncover the dual *in-vivo* role of neutrophils in *Leishmania* infection, development of monoclonal antibodies (mAb) which specifically recognize neutrophils, as well as use of neutropenic Genista mice, seem to be required[100].

1.10.2.3 The role of DCs and monocytes

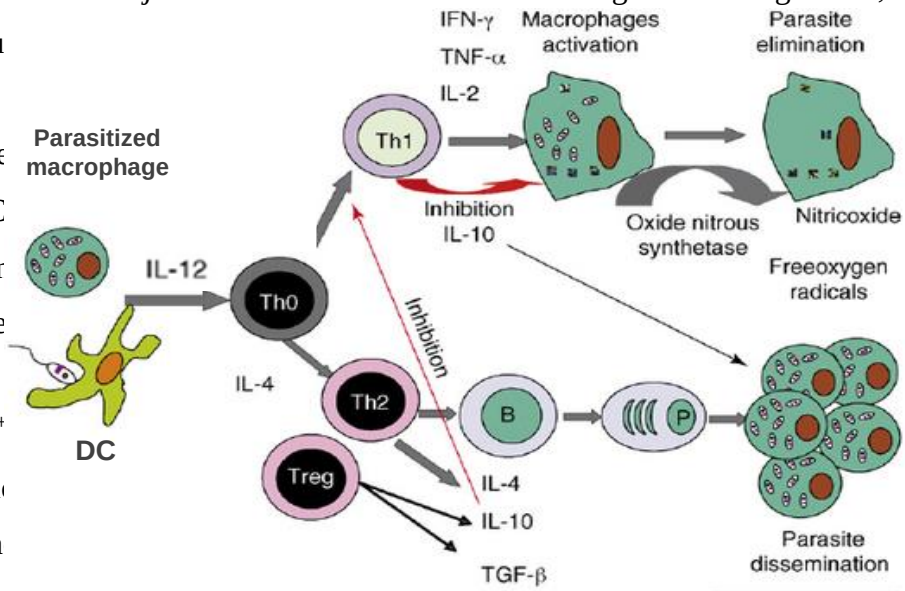
Inflammatory monocytes and DCs are considered the most abundant cells infected with *Leishmania* within a few days after the inoculation of the parasite into the host skin[53]. In addition to the synergic act of DCs with macrophages and monocytes in phagocytosis of parasites, DCs are mostly known for their substantial role as antigen-presenting cells (APCs)[101]. DCs, which are the main source of IL-12, contribute to the initiation of the antigen-specific protective immune response to *Leishmania*[102]. The differentiation of naïve T cell to Th1 cells is primarily mediated by IL-12 produced by lymph node resident DCs. However, the inflammatory monocyte-derived DCs found in lesions constitute the majority of lymph node resident DCs[103]. Before the differentiation of naïve T cells into protective Th1 cells, NK cells within the paracortex of the lymph node are the primary source of IFN-γ which increases the production of IL-12 by DCs[104]. Skin-resident murine DCs are classified into 5 various subsets (dermal-epidermal Langerhans cells, $CD11b^+$ DC, $Langerin^+CD11b^{neg}$, dermal DC $XCR1^+CD11b^{neg}$ DC and $Langerin^{neg}XCR1^{neg}$ double-negative DC). The various DC subsets elicit different T-helper responses that is believed to determine the outcome of *L. major* infection in C57BL/6 and BALB/c mice[105]. However, some studies revealed that DCs isolated from the skin of different mice strains have a similar ability in the induction of IL-12 production and expression of co-stimulatory molecules[101]. It has been shown that epidermal Langerhans cell internalize *L. major* and present the derived peptides to antigen-specific T cells following migration to the draining lymph nodes[106]. DCs express different co-stimulatory molecules on their surfaces such as CD40, CD80, CD86, and MHC II that function differently in T cell activation[105]. CD40 contributes to the optimal production of IL-

12 which mediates the differentiation of naïve $CD4^+$ T cells into Th1 cell[107]. CD80 (B7-1) and CD86 (B7-2) are known to participate in shaping the outcome of *Leishmania* infection by providing the second signal for T cell activation[108]. The Th2-type immune response in BALB/c mice and early protective Th1 response in C57BL/6 mice are both dependent on CD86. In contrast, the upregulation of CD80 following *Leishmania* infection induces the secretion of IFN-γ and IL-4 from Th1 and Th2 cells, respectively[108].

The production of CC chemokine ligand 2 (CCL2) by platelet-derived growth factor-activated cells is believed to mediate the early recruitment of monocytes to the site of infection[109]. The role of monocytes in *Leishmania* infection remarkably differs from that of macrophages. Notably, unlike macrophages which need to be activated by IFN-γ to kill the parasites, monocytes play a more efficient role in parasite control via promoting a robust respiratory burst following infection. Hence, in contrast to the dual role of neutrophils, inflammatory monocytes appear to have a central role in the elimination of parasites[53 109].

1.10.3 Adaptive immune response to cutaneous leishmaniasis

During experimental *L. major* infection in mice with different genetic backgrounds, developing resistance or su rential development of ved in promoting the de duced primarily by DC y Th1 cells. Several ar 12 for differential deve ads to parasite control f non-protective $CD4^+$ *mania* infection in mi ting a susceptible phen



1.10.3.1 T cells

T cells, also called T lymphocytes, are one of the vital components of the adaptive immune system. T cells originate in the bone marrow and develop in the thymus where they proliferate and differentiate into different T cell subsets such as regulatory, helper, cytotoxic, or memory T cells. $CD4^+$ and $CD8^+$ T cells are the most well-studied T cell subsets which exert their functions through activating macrophages leading to the recruitment of inflammatory cells to the site of infection. Moreover, T cells promote differentiation of B cells into antibody-producing plasma cells via secretion of effector cytokines. T cell receptors (TCRs) are only capable of recognizing short peptides presented in the context of MHC molecules. Therefore, once detected by antigen-presenting cells of the immune system, pathogens will be engulfed and digested by APCs to form short peptides that are recognizable by T cell receptors (TCRs). These peptides presented in the context of MHC molecules will be subsequently presented by cell surface molecules MHC I and MHC II which are critical for $CD8^+$ and $CD4^+$ T cell recognition and activation, respectively. MHC I molecules are ubiquitously expressed by nearly all somatic cells. However, MHC II molecules are expressed on the surface of professional antigen-presenting cells such as dendritic cells, mo ating immune itute the major components of cell-mediated immunity, T cells are indispensable for control of obligate intracellular pathogens such as *Leishmania*. The protective role of T cells in the

Figure III. The interplay between Th1 and Th2-type immunity in leishmaniasis

elimination of obligate intracellular pathogens has been corroborated by different studies. T cell-deficient mice on a resistant background are susceptible to *L. major* infection and the adoptive transfer of functional T cells from healed mice confers the lost protection to these mice[53].

1.10.3.1.1 T helper cells 1 and 2

Differentiation of naïve $CD4^+$ T cells to various T cell subsets is a multifactorial event that is modulated by the strength of TCR-antigen interactions as well as the cytokine milieu in the environment[115]. Naïve $CD4^+$ T cells differentiate into Th1 cells following recognition of antigens presented by APCs and/or the presence of a sufficient amount of IL-12 produced by these cells[116]. $CD4^+$ T cells subsequently contribute to the development of cell-mediated immunity against the intracellular pathogens by secreting pro-inflammatory cytokines such as IFN-γ and TNF-α. However, it is assumed that determinants other than cytokines produced by APCs, and the presentation of peptides by their MHC II molecules are involved in the differentiation of naïve $CD4^+$ T cells into Th1 cells immunity. In this respect, the interaction of T cell surface molecules such as CD28 and CD40L with APCs co-stimulatory molecules (CD40, CD80, CD86) is known to be required for efficient activation and consequent differentiation of naïve $CD4^+$ T cells into Th1 cells[117]. Various studies show the role of IL-4 and its downstream transcription factor signal transducer and activator of transcription (STAT) 6 in developmental differentiation of naïve $CD4^+$ T cells into Th2 cells[118]. Furthermore, mast cells, basophils, and eosinophils, as the major sources of the early IL-4, are believed to contribute to differential development of Th2[119 84]. Exposure of $CD4^+$ T cells to stimuli such as OX40 ligand during activation promotes their differentiation into IL-4 producing Th2 cells [112].

According to the Th1/Th2 dichotomy in leishmaniasis, a robust Th1 immune response mediated by IFN-γ production confers resistance to *Leishmania* infection in C57BL/6 mice. On the other hand, increased susceptibility to CL in BALB/c mice is mediated by IL-4-associated expansion of Th2 cells. This is exemplified in experimental cutaneous leishmaniasis where the outcome of the disease in mice is greatly determined by a balance between Th1 and Th2 cytokines. Resistance to CL is attributed to the development of IFN-γ-producing $CD4^+$ T cells whereas susceptibility is promoted by the early burst of IL-4 secreted by $CD4^+$ Th2 cells [53]. Moreover, it is widely believed that the preferential development of Th1 and Th2 cells in resistant and susceptible mice, respectively, is largely directed by the responsive of naïve $CD4^+$ T cells to IL-

12 produced by DCs during the early onset of infection. Biedermann *et al* further explained that as opposed to resistant C57BL/6 mice, DCs from susceptible BALB/c mice produce a low amount of IL-12 and their T cells exhibit a poor response to this cytokine as a result of low expression of IL-12Rβ1 chain[120]. In fact, the administration of IL-12 in BALB/c mice in the initial phase of infection confers them resistance, while treatment of C57BL/6 mice with anti-IL-12 antibodies renders them susceptibility to infection.

Although IL-12 is regarded as the single dominant factor that drives Th1 development and resistance, the factors that mediate Th2-type immunity and susceptibility in CL are not well defined[121]. One of the major arguments in the field of T helper cells is the effect of antigen dose on *in vitro* and *in vivo* development of Th2 cells[122]. Although *in vitro* studies have shown that a low dose of antigen promotes the development of Th2 cells, *in vivo* studies demonstrate the opposite. Uzonna *et al*[122] have addressed this discrepancy as they found that the low parasite dose leads to the induction of a transient Th2 response but umlitimately healing in C57BL/6 mice. The advent of protective Th1-cells in this model was partly attributed to the development of IFN-γ-producing $CD8^+$ T cells that mediate lesion healing in these mice.

The signature effector cytokine of Th1 immune response (IFN-γ) exerts its function by binding to its receptor on the surface of parasitized macrophages and activate them. The activation of macrophages is characterized by the upregulation of iNOS signaling leading to increased production of NO and effective elimination of Leishmania amastigotes in C57BL/6 mice. In contrast, in BALB/c mice, the expression of Th2 cells (and their production of IL-4 and IL-10) is known to repress the iNOS pathway resulting in deactivation of macrophages and parasite growth[123]. To further support the differential roles Th1 and Th2 cells in promoting resistance and susceptibility to *L. major* infection, SCID mice become resistance and susceptible and to leishmaniosis following adoptive transfer of Th1 and Th2 subsets, respectively[124]Unlike previous finding on the role of IL-4 in the induction of susceptibility in mice, emerging evidence has otherwise revealed the necessity of IL-4 to drive a solid Th1 immune response via an enhanced DC function[120]. However, further investigation is needed to illuminate the factors that influence the differentiation of naïve T cells into Th2 cells. Collectively, an increased understanding of the $CD4^+$ T cell differentiation pathway is needed to develop novel therapeutic/preventive approaches in leishmaniasis and other infection diseases driven by Th2 immune response.

1.10.3.1.2 T Helper 17

T helper 17 (Th17) cells are a unique subtype of $CD4^+$ T cells characterized by the production of IL-17. Although Th17 cells participate in host defense, they play a major role in promoting the pathogenesis of multiple autoimmune and inflammatory diseases. Th17 cells activity exerts a strong stimulatory effect on stromal cells leading to their production of inflammatory cytokines. Furthermore, Th17 cells recruit neutrophils and other inflammatory leukocytes following the production of IL-17[125]. Unlike the development of Th1 and Th2 cells, there are fundamental differences between mice and human Th17 differentiation. IL-1β plays a significant role in the differentiation of Th17 in humans, whereas IL-23 is the major inducer of Th17 effector functions in mice[126]. Moreover, TGF-β, which strongly supports IL-17 expression in mice, has been shown to have an inhibitory function in the development of Th17 cells in humans. TGF-β is also known to suppress the production of IL-4 and IFN-γ to reduce the dominant role of Th1 and Th2 cells in the downregulation of IL-17 production[127]. Two diverse effector phenotypes with a different cytokine profile and transcription factors have been identified in Th17 cells: ‘’alternative’’ Th17 cells, also referred as pathogenic Th17, are formed when IL-6, IL-23, and TGF-β are present and ‘’classical’’ Th17 cells which are induced by IL-6 and TGF-β in the absence of IL-23[128].

Lopez Kostka *et al* have shown that in comparison to susceptible BALB/c mice, Th17 cells from C57BL/6 mice secrete significantly lower levels of IL-17 following infection with *L. major*. Likewise, IL-17 deficient BALB/c mice infected with *L. major* develop less severe lesion with relatively lower parasite burden[129]. Interestingly, the contribution of Th17 cells in the pathogenesis of *Leishmania* is also determined by parasite species. It has been shown that Th17 cells promote susceptibility to leishmaniasis after infection with *L. major*[130], whereas they induce resistance in visceral leishmaniasis following infection with *L. donovani*[131]. Gonzales *et al* showed that increased induction of Th17 cells in IL-10 deficient ($IL\text{-}10^{-/-}$) BALB/c mice results in an extensive immunopathology due to neutrophil accumulation in lesions after infection with *L. major*. Given that IL-1β is often essential for IL-17 production, they also reported a reduced level of IL-17 and an abrogated pathology in $IL\text{-}10^{-/-}$ mice following blockade of IL-1β using neutralizing antibody. These results suggest a critical role for IL-10 in the modulation of IL-17-dependent neutrophil recruitment and enhanced immunopathology in *L. major*-infected mice[130]. Despite the unclear role and mechanism of IL-17 in controlling cutaneous disease caused by *L.*

braziliensis[132] [133], several mechanisms have been defined for the protective role of Th17 in visceral leishmaniasis[131]. IL-17 induces GM-CSF and G-CSF production leading to an elevated production of chemokines such as CXCL10 and CXCL8[134]. These chemokines are known to serve as potent chemoattractants for Th1 cells and neutrophils, respectively. Additionally, IL-17 stimulated IL-6 production which mediates pro-inflammatory and regulatory effects on immune response[135]. Finally, IL-17 and IL-22 co-expressed by Th17 cells, synergistically work to upregulate the production of β-defensins by epithelial cells[136]. As with the effector functions of Th1 cells, the effector activities of Th17 cells are impaired by anti-inflammatory cytokines expressed by Th2 cells. Castilho *et al* reported that administration of IL-4 and IL-13 neutralizing antibodies led to an increased IFN-γ and IL-17 and parasite elimination in *L. panamensis*-infected mice. Thus, it is conceivable that the deficiency of inhibitory cytokines promotes the concurrent expansion of Th1/Th17 cells and resistant to *Leishmania* infection[137].

1.10.3.1.3 Cytotoxic $CD8^+$ T cells

$CD8^+$ T cells are a distinct subset of T cells that, similar to $CD4^+$ T cells, originate from bone marrow and mature in the thymus. $CD8^+$ T cells contribute to mediating f adaptive immune response following their activation in an MHC I-restricted manner. Activated $CD8^+$ T cells, also termed cytotoxic lymphocytes, exert their functions via secreting cytokines such as IFN-γ as well as effector molecules like granzymes and perforins[122]. $CD8^+$ T cells are believed to actively participate in promoting cell-mediated immunity against intracellular microorganisms particularly viruses. The role of cytotoxic T lymphocytes in modulating host immune response against parasitic infections is controversial[138]. It is generally accepted that anti-leishmanial immunity is largely dependent on IFN-γ producing $CD4^+$ T cells. However, $CD8^+$ T cells are speculated to participate in shaping an optimal cell-mediated anti- *Leishmania* immunity through IFN-γ secretion [122] [139]. On the other hand, various studies have shown that deficiency of MHC I molecule in mice, which prevents $CD8^+$ T cell activation, has no impact on primary control of *L. major* infection[140]. The function of $CD8^+$ T cells in shaping a vaccine-induced immunity is well defined. In this respect, vaccination with LACK results in protective immunity in vaccinated animals which is mediated by LACK-specific $CD8^+$ T cells[141]. Recently, Uzonna *et al* reported that injection of low dose *Leishmania* antigens to C57BL/6 mice results in a sustained Th2 response in the absence of $CD8^+$ T cells characterized by an uncontrolled parasite proliferation[122]. The persistent Th2 response, however, could be skewed toward Th1 immune

response upon administration of rIL-12 or anti-IL-4 antibodies. These findings suggest that $CD8^+$ T cells suppress the early developmental differentiation of $CD4^+$ Th2 cells by secreting IFN-γ[122]. On the other hand, $CD8^+$ T cell effector functions seem to cause pathology in the host as MHC I deficient mice infected with *L. amazonesis* exhibit smaller lesions in the course of infection. Likewise, RAG1-deficient mice following infection with *L. major* develop smaller lesions which are reversed following adoptive transfer of $CD8^+$ T cells to these mice[142].

1.10.3.1.4 T Regulatory Cells

T regulatory cells (Tregs) are a subpopulation of T cells that are believed to play a crucial role in maintaining tolerance to self-antigens, regulating or suppressing the magnitude of host immune response, and preventing autoimmune disease[143]. There are two major subtypes of T regulatory cells: naturally occurring T cells and induced T regulatory cells. Tregs were previously characterized by CD25 as their classic surface marker. However, recent findings have demonstrated Foxp3 to be a major marker of naturally occurring Tregs[143]. During infection with *L. major*, Tregs accumulates at the dermal site of infection and plays a dual role by reducing immune-mediated pathology in on hand and promoting the survival of persistent parasites on the other hand[144]. Tregs are believed to regulate immune response through the production of TGF-β and IL-10. These immunoregulatory cytokines significantly suppress the effector functions of protective Th1 cells and are critical for maintaining the pool of antigen-specific T cells that are essential for durable immunity to subsequent exposure to parasites[148]. These findings imply that host and pathogen could both benefit from a state in which opposite forces have reached a balance [53]. Nevertheless, the high frequency of IL-10-secreting Tregs which support an uncontrolled proliferation of parasites has proved harmful to the host[146]. The expression of alpha and beta integrin, CD103, and chemokine receptor CCR5 seem to be essential for migration and homing of Tregs to the infection site[147]. Yurchenko *et al* reported that CCR5 deficient mice exhibit an increased resistance to *Leishmania* infection which is associated with a markedly high number of IFN-γ-producing $CD4^+$ T cells and consequent reduction of parasites at the site of infection[148].

1.10.3.2 B cells and antibodies

B cells are a distinct type of lymphocytes produced in the mammalian bone marrow or bird's bursa of fabricius from a common lymphoid progenitor shared with T cells, NK cells, and some

DC subsets[149]. After the early stage of maturation and gene re-arrangement in bone marrow, pro and pre-B cells express Ig surface receptors and migrate to the different peripheral niches where they continue their maturation[150]. B cells, as one of the major antigen-presenting cells, are capable of activating naïve $CD4^+$ T cells in an MHC II-restricted manner[151]. Upon activation and differentiation into plasma cells, B cells become effector cells that produce antibodies. Antibodies which constitutes the humoral arm of adaptive immunity can bind to specific antigens to activate other components of immune system[152]. Although the detection of *Leishmania*-specific antibodies is regarded as an important diagnostic tool, the protective role of B cells in immunity against *Leishmania* infection remains a matter of debate. *Leishmania*, as obligate intracellular parasites, resides in macrophages phagolysosome where they are not easily tagged by antibodies[153]. It has been shown that B cells play a role in the exacerbation of disease as BALB/c mice secrete high amounts of *Leishmania*-specific antibodies during the course of *Leishmania* infection[154]. Similarly, Miles *et al* found that anti-*Leishmania* IgG complexes enhance IL-10 production in macrophages and this was associated with reduced parasiticidal activity and enhanced parasite growth[155]. While some studies strongly support a pathogenic role for B cells and antibodies, other studies suggest a protective role for B cells during *Leishmania* infection. For instance, *Scott et al* in a contrary finding reported that selective deletion of B cells through either targeted gene deletion or administration of anti-IgM antibody leads to disease exacerbation in an otherwise resistant C57BL/6 mice[53]. Additionally, Woelbing *et al* showed that IgG produced by activated B cells in C57BL/6 mice facilitates Fc gamma receptor III-dependent phagosytosis of parasites by DCs during *Leishmania* infection culminating in an enhanced antigen presentation by DCs and a consequential robust Th1 immune response[156]. Furthermore, B cell deficiency in C57BL/6 mice has been shown to severely suppress the production level of IFN-γ which could be reverted following infection with IgG opsonized parasites[156].

1.10.3.3 Role of cytokines

The clinical outcome and immunopathogenesis of *Leishmania* infection are partly dictated by the equilibrium and dynamic changes in various cytokines produced in an infected host. Pro-inflammatory cytokines such as IFN-γ and IL-12 play a significant role in the establishment of protective Th1 immune response. However, IL-4, as an anti-inflammatory cytokine, mediates parasite growth and susceptibility to *Leishmania* infection. On the other hand, immunoregulatory

cytokines such as IL-10 limit the possible damages caused by excessive inflammatory responses[53 157].

1.10.3.3.1 Interferon gamma

Interferon gamma (IFN-γ) is a pro-inflammatory cytokine that plays a central role in both immunoprotection[2] and immunopathology[138] in cutaneous leishmaniasis. Th1 $CD4^+$ T cells[88], cytotoxic T cells[138], and natural killer cells[158] are the major secretory sources of this cytokine. IFN-γ is the key cytokine required for the upregulation of the iNOS pathway which results in activation of macrophages for a parasiticidal state and eventually elimination of parasite[53]. Moreover, IFN-γ stimulates differentiation of naïve $CD4^+$ T cells to Th1 cells which is essential for subsequent suppression of Th2 cytokines and stimulation of protective anti-*Leishmania* host immune response. There is ample evidence that selective impairment of IFN-γ in C57BL/6 mice results in a higher susceptibility to *Leishmania* infection which is characterized by larger lesions, enhanced parasite load, and establishment of Th2-type response which is associated to enhanced pathogenesis[121]. Similar observations were documented utilizing recombinant IFN-γ. Administration of recombinant IFN-γ after infection with *Leishmania* leads to a delayed onset of disease and formation of smaller lesions in an otherwise highly susceptible BABL/c mice[159]. Likewise, deficiency in either IFN-γ production or IFN-γ receptor expression results in loss of resistance to *Leishmania* infection in C57BL/6 mice. The mice with a defect in expression of IFN-γ receptor, the produced IFN-γ fail to activate macrophages and, as a consequent, iNOS-dependent parasites elimination leasing to susceptibility[160].

1.10.3.3.2 Interleukin 4

Interleukin 4 (IL-4) is primarily produced by Th2 $CD4^+$ T cells. Studies on the murine model of leishmaniasis have established that IL-4 plays a central role in the progression of infection[111]. IL-4 participates in B cell-mediated IgE class-switching leading to a humoral immune response in favor of disease. In susceptible BALB/c mice, elevated IL-4 level inhibits IFN-γ producing $CD4^+$ T cells leading to alternative activation of macrophages[161]. The alternative activation of macrophages is characterized by reduced NO and ROS production which results in parasite survival[112]. In addition, IL-4 exerts its pathogenic function through differentiation of naïve $CD4^+$ T cells into Th-2 type cells, as cutaneous lesions in BALB/c mice resolve following

administration of IL-4 neutralizing antibody [161]. Although IL-4 deficient BALB/c mice are susceptible to *Leishmania* infection[162], genetic ablation of IL-4 receptor renders resistance to these mice[163]. These findings suggest that IL-4 receptor is shared between IL-4 and another cytokine which also mediates susceptibility to *Leishmania* infection[163].

1.10.3.3.3 Interleukin 10

Interleukin 10 (IL-10), as a pleiotropic cytokine, is mainly produced by T regulatory cells, monocytes, macrophages, and DCs[161]. It was previously believed that IL-10 is a Th2-type cytokine that inhibits the development and expansion of Th1 cells. However, IL-10 has proved to have a potent inhibitory effect on both Th1 and Th2 $CD4^+$ T cells[108]. The spontaneous development of the autoimmune disease such as enteritis has been reported in mice with a defect in IL-10 production or IL10 receptor expression. This suggests that IL-10 cytokine plays a critical role in the suppression of excessive or inappropriate immune response in mice[164]. In combination with TGF-β, IL-10 is known to be a key factor in the progression of *Leishmania* infection in mice[53]. Th2 $CD4^+$ T cells and alternatively activated macrophages are major sources of IL-10 in mice infected with *L. major*[165]. IL-10 as an immunosuppressive cytokine inhibits IFN-γ production by $CD4^+$ T cells leading to insufficient macrophage activation and reduced oxidative burst. In addition, IL-10 suppresses T cell activation through the downregulation of both MHC II and co-stimulatory molecules on macrophages[166]. The susceptibility of BALB/c mice to *L. major* infection is thought to be highly dependent on IL-10 as susceptibility to *Leishmania* infection in IL-4Rα deficient mice could be circumvented following treatment with IL-10R neutralizing antibodies[167]. Although BALB/c mice with a defect in IL-10 secretion are resistant to infection with *L. major*[167], IL-10 deficiency in C57BL/6 mice does not render resistance to *Leishmania amazonensis* infection[168]. Additionally, IL-10 deficient BALB/c mice develop a progressive disease with slightly less severe lesions following infection with *L. mexicana*[169]. Therefore, the role of IL-10 in the pathogenesis of murine *Leishmania* infection seems to be dictated by mice's genetic background and parasite species[168]. The resolution of primary infection with *L. major* in C57BL/6 mice is followed by parasite persistence. However, the eradication of parasites in IL-10 deficient C57BL/6 mice supports this notion that protracted parasite control in *L. major* infection after lesion healing is attributed to the production of IL-10 by regulatory T cells[170]. Collectively, these finding strongly suggests that IL-10 plays a

significant role in pathogenesis, immunopathology, and parasite persistence of cutaneous leishmaniasis in mice.

1.10.3.3.4 Interleukin 12

Interleukin 12 (IL-12) is critically involved in the development of host-protective Th1 response during infection with *L. major*. IL-12 promotes the production of IFN-γ by $CD4^+$ T cells and reduce the expansion of IL-4-producing Th2 $CD4^+$ T cells[171 121]. The major sources of IL-12, macrophages, and DCs, are also antigen-presenting cells essential for activation of $CD4^+$ T cells[172]. It has been shown that macrophages exhibit a compromised IL-12 secretion throughout *Leishmania* infection/phagocytosis[173]. However, DCs seem to retain the capability of IL-12 production at all times following infection resulting in their ability to activate $CD4^+$ T cells to enhanced IFN-γ production and macrophage activation[174]. The significance of IL-12 in the development of resistance against *Leishmania* infection has been determined by antibody treatment and knockout mice. The blockade of IL-12 through monoclonal neutralizing antibody leads to an enhanced Th2 response and loss of resistance to *Leishmania* infection[175]. Conversely, treatment with recombinant IL-12 confers genetically susceptible BALB/c mice protection against *Leishmania* infection[176]. Bioactive IL-12 is composed of p35 and p40 subunits which equally contribute to resistance to *L. major* infection[177].

1.11 Gene editing tools

Gene editing refers to a group of methods that have been recently evolved to exploit DNA repair mechanisms to make precise alternations in the genome. An increase in the efficiency and accurateness of the editing process as well as the development of novel methods to deliver genetic components to target cells hold great promise in the treatment of various diseases at the DNA level [178]. Until quite recently, the process was mostly dependent on the breaks that randomly occur in the DNA. However, the new technological advancements in the field have revealed that the incorporation of a template sequence into the site of DNA damage could make the generation of genetic disease models feasible. Also, through particularly targeting changes in the genome, the opposite processes such as the generation of mutants and repairing DNA damages have become remarkably possible[179]. Despite the efforts being made to develop efficient gene-editing tools, there is some disadvantages associated with the current systems such

as lack of adequate precision and efficacy as well as being laborious and time consuming[180]. Below is a brief description of the most versatile gene-editing systems with targetable nucleases.

1.11.1 Zinc-Fingers Nucleases (ZFNs)

In 1996, the advent of synthetic peptides called zinc finger nucleases (ZFNs) with the ability to make DNA specific breaks was a breakthrough in genome editing. This system was founded on the principle that zinc fingers are DNA binding domains in many proteins that specifically interact with nucleotide triplets in DNA sequences. The combination of several zinc finger domains is often designed to engineer highly specific DNA binding proteins. These ZFNs when joined to an endonuclease domain are capable of inducing targeted double-strand cleavage in FokI, a DNA cleavage domain. Although highly specific, of the most notable disadvantages of this gene manipulation tool is the relative complexity involved in designing of domains[178].

1.11.2 Transcription Activator-Like Effectors Nucleases (TALENs)

Shortly after the discovery of ZFNs, a similar yet more efficient genome editing system, termed TALENs or transcription activator-like effector nucleases (TALENs), was developed. TALENs comprise DNA binding motifs called TAL for bacterial transcription activator-like proteins which target a single nucleotide and generate highly specific DNA breaks upon fusion to a FokI endonuclease. TALENSs are relatively easier to design than ZFNs, however, their large repetitive structure may cause cytotoxicity following incorporation into *in vivo* delivery systems[178 181].

1.11.3 Clustered Regularly Interspaced Short Palindromic Repeat associated 9 (CRISPR-Cas9) system

CRISPR-Cas9 system is one of the most eminent discoveries that has made an immense impact on targeted genome engineering[182]. The simplicity in design and capacity of multiplexed genome editing, have turned the CRISPR-Cas9 into the most cost-effective and easy-to-use gene-editing technology system[182]. CRISPR-Cas9 is an adaptive immune defense of bacteria in which short RNAs are employed to direct hybridization and degradation of foreign nucleic acids within invading viral or plasmid DNA[183]. Jinek *et al* suggested that Cas9–CRISPR (cr)RNA complexes of *Streptococcus pyogenes* and *Streptococcus thermophilus* could be exploited as *in vitro* RNA-guided endonucleases [184]. CRISPR-Cas9 system is classified into three different classes based on

the type of Cas9 protein involved in the process. The most widely used CRISPR-Cas9 system consists of trans-activating (tra)crRNA, pre-crRNA, and Cas9 proteins. An expression vector is the most common way of ɔ the cells as a short guide RNAs (gRNAs). Tl ɔ enzyme to identify the specific motifs (NGG M) which is located next to gRNAs compleme l interrogation of the Cas9 complex to the DN the target sequence through the nuclease acti ding on the cell life cycle and existence of a ed following Cas-9-mediated DNA double-st which occurs in the absence of a repair templ otides to the broken DNA. NHEJ pathway ma codons. In contrast, the homology-directed r ıutants via targeted insertions of a repair temp

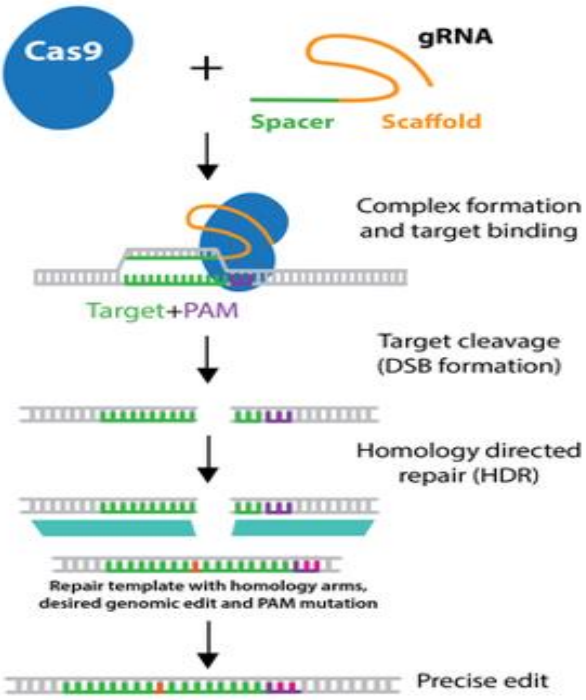


Figure IV. Gene editing using CRISPR-Cas9 gene-editing system and homology-directed repair (HDR)

1.11.3.1 CRISPR-Cas9 mediated genome editing system in *Leishmania*

Leishmania genome is comprised of about 8000 different genes, and little known about the functions of most them[187]. Conventional genome-editing tools such as homologous recombination have provided valuable insights into *Leishmania* biology and disease pathogenesis. Given that the RNA interference pathway is absent in *Leishmania*, homologous recombination-mediated genome editing requires the use of antibiotic selection markers which is nonetheless time-consuming, and in some cases, inefficient for introducing point mutations[188]. In recent years, the CRISPR-Cas9 system has emerged as a simple but yet powerful gene-editing tool, to define the function of *Leishmania* genes for novel drug and vaccine development[189 182]. Several stable and transient CRISPR systems have used in *Leishmania* to date[188 190]. The stable CRISPR-Cas9 system optimized by Zhang *et al* is well-suited for the deletion/disruption of single or multicopy *Leishmania* genes in various species including *L. major*, *L. donovani* and *L. mexicana*[191]. In this system, the *Leishmania* ribosome RNA promoter drives the constitutive co-expression of guide RNAs and Cas9 nuclease in procyclic promastigotes. Guide RNAs subsequently directs Cas9 nuclease to generate a double-stranded break at DNA target site[182]. Given that a functional non-homologous end-joining pathway is absent in trypanosomatids[192], the edited mutants are reliably isolated following the insertion of the repair template (antibiotic selection markers or fluorescent protein tags) in DNA break via microhomology-mediated end-joining pathway[188]. The transient expression system is nonetheless dependent on continuous expression of genome-integrated T7 RNA polymerase and Cas9 to by-pass labor-intensive DNA cloning procedures. Once a stable parasite line co-expressing T7 RNA polymerase and Cas9 is established, gene-editing could be attained via the introduction of guide RNAs and DNA repair templates into parasites[193 194]. Taken together, utilization of the CRISPR-Cas9 gene-editing system in *Leishmania* opens an avenue for the identification of virulence genes and improves our ability to further discover various aspects of *Leishmania* biology.

1.11.3.2 Off-target effects of the CRISPR/Cas9-system

CRISPR/Cas9 is An efficient genome editing system that is widely used to define the functions of different genes via a loss of function strategy. Nonetheless, the high frequency of unintended mutation, also known as off-target effects, is a major concern in particular for clinical and therapeutic studies[195]. PAM motif, delivery route of Cas9, double-strand repair pathway, etc., are

among the key factors associated with off-target effects in the CRISPR-Cas9 system. Different methods such as *in silico* prediction, T7E1 methods, and whole-genome sequencing have been developed to identify the off-target effects [196]. Despite the recent advances, the *in-vivo* detection of off-target effects by the above-mentioned approaches remains an ongoing challenge[195 201]. However, the following strategies can significantly reduce the frequency of off-target: i) the DNA-based elements of Cas9 complex such as gRNAs, enhancer and promoters should be chosen at a far distance from the target sequence ii) the wild type version of Cas9 should be replaced with D10 mutant version iii) the Cas9 enzyme should be fused to two gRNAs that each can cleave only one strand iv) the concentration of main components of CRISPR-Cas9 system, gRNAs and Cas9 nuclease should be assessed via titration. Moreover, the utilization of catalytically inactive Cas9 with a FokI nuclease domain has been shown to enhance the specificity of DNA breaks [198]. The frequency of off-target effects associated with CRISPR-Cas9 has been reported to be insignificant in *Leishmania* with correctly designed target-specific gRNAs[182]. However, it seems necessary to ensure that the observed phenotypic changes are solely attributed to the deletion of the targeted gene, not unintended mutations. Moreover, it is essential to examine whether the wild-type phenotype is reversible following the complementation of edited mutants with wild-type gene[199]. The generation of addback strain could be achieved via the introduction of an episomal plasmid encoding the wild-type gene into knockout mutants[200].

CHAPTER 2

2.0 Rationale, Hypothesis and Objectives

2.1 Rationale

Our lab recently identified phosphoenolpyruvate carboxykinase (PEPCK) is a highly immunogenic antigen that stimulates a strong cross-species T cell proliferation and IFN-γ production in both mice and human[52] PEPCK is broadly conserved among various pathogenic species of *Leishmania*[52] and constitutively expressed in both life stages of the parasite[201]. Additionally, PEPCK as a key gluconeogenic enzyme, which is involved in the conversion of oxaloacetate into phosphoenolpyruvate, has been shown to contribute to the virulence of *Leishmania*. In this respect, some studies have reported the loss of virulence in *Leishmania* parasites with a defect in gluconeogenic enzymes [27]. I, therefore, aimed to investigate the role of PEPCK in the immunopathogenesis of cutaneous leishmaniasis.

2.2 Hypothesis

Phosphoenolpyruvate carboxykinase (PEPCK) plays a critical role in the immunopathogenesis of cutaneous leishmaniasis

2.3 Overarching Aim

To assess the impact of PEPCK in virulence, disease pathogenesis and host immune response to *L. major*

2.4 Objectives

1. To generate PEPCK deficient *L. major* using CRISPR-Cas9 gene-editing technology
2. To determine whether the absence of PEPCK affects growth of *L. major* in axenic culture
3. To examine whether PEPCK deficiency affects the proliferation of *L. major* in macrophages
4. To investigate whether metacyclogenesis in *L. major* is influenced by the targeted loss of PEPCK
5. To examine whether PEPCK contributes in the pathogenesis of *L. major in vivo*
6. To assess the host immune response to *Leishmania* infection in the absence of PEPCK
7. To investigate whether infection with PEPCK$^{-/-}$ *L. major* will confer protection against secondary challenge with virulent *L. major* in highly susceptible BALB/c mice
8. To determine whether the glycolytic activity and oxidative phosphorylation are altered in PEPCK null mutants

CHAPTER 3

3.0 Material and Methods

3.1 Mice

Six to eight weeks female naïve C57BL/6 and BALB/c mice were acquired either from Manitoba Central Animal Care Services (CACS) breeding facility or purchased from the Jackson Laboratory, (Maine, USA). PEPCK TCR transgenic mice on the C57LB/6 background were also obtained from an in-house breeding colony from CACS. All mice were housed in a specific pathogen-free environment at the University of Manitoba (CACS) and were used according to the conditions stipulated by the Canadian Council for Animal Care.

3.2 Parasites

WT *Leishmania major* (*L. major*) parasites as well as PEPCK mutant ($PEPCK^{-/-)}$ and addback ($PEPCK^{-/+pepck}$) *L. major* parasites, all MHOM/80/Friedlin, were artificially grown in 25 cm culture flask containing 10 ml M199 complete medium (Hyclone, Logan, UT) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Cansera, Mississauga, ON, Canada), 100 UI/ml penicillin and 100mg/ml streptomycin in a 27 °C parasite incubator (Thermo Fisher Ottawa, ON). Throughout the study, the day-3 logarithmic phase parasites and day-7 stationary phase parasites were used for passage and infection, respectively. Briefly, the stationary phase parasites were transferred to a 50ml falcon tube and washed twice with sterile PBS at 3000 rpm

for 15 min. To count the parasite under an optical microscope (Fischer) at x100 magnification, 50 µl of parasite suspension was added to 450 µl of PBS in a 1.5 microtube (VWR) and vortexed for 5-10 seconds to produce a 1:10 dilution. Next, 10 µl of parasite suspension was transferred to a hemocytometer (Fisher Scientific, Whitby). Then parasites were resuspended in an appropriate volume of sterile PBS to make 1-5×10^6 parasite per 50 µl PBS depending on the route and dose of infection, as well as the objective of experiment and mouse strains.

3.3 Generation of PEPCK null mutant by the CRISPR-Cas9 system

PEPCK gene is available in two copies within a 7kb region on chromosome 27 of *L. major*. In order to delete all copies of this gene, two guide RNAs (gRNAa & gRNAb) targeting the upstream and downstream of the PEPCK genes were designed using Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool (EuPaGDT) as previously described[191 182]. The gRNA guide sequences were later cloned into the Bbs I sites of the *Leishmania* all in one CRISPR vector pLdCN (Addgene # 84290) to generate CRISPR plasmid pLdCN-LmPEPCKa&b. The resultant plasmid expressing CRISPR-Cas9 nuclease (responsible for DNA double-strand break) and gRNAs were introduced to WT *L. major* promastigotes via electroporation as described by Li *et al*[202]. Briefly, logarithmic-phase WT promastigotes were harvested at 3000 rpm for 15 min and counted using a hemocytometer. Promastigotes were washed twice and re-suspended inappropriate volume of ice-cold high-voltage Cytomix electroporation buffer (120 mM KCl, 0.15 mM $CaCl_2$, 9.2 mM K_2HPO_4, 25 mM HEPES, 2 mM EDTA, 4.75 mM $MgCl_2$, 69 mM sucrose, pH 7.6) to reach ~2×10^8 cells/ml. Thereafter, 400µl of suspensions were transferred into a 4-mm gap electroporation cuvette. Following incubation on ice for 10 min, 5–20µg chilled CRISPR-all-in-one plasmid DNA was added into a cuvette and mixed. Promastigotes were electroporated twice at 25 µF, 1500 V (3.75 kV/cm), pausing 10 s between pulses in a BTX ECM830 square wave electroporator. Following electroporation, promastigotes were allowed to sit on ice for 10 min to enhance the efficiency of transfection and then transferred to a flask containing 10 ml complete *Leishmania* culture media (M199) and placed in 27 °C in a $C0_2$-free incubator overnight. The next day, 10µl Hygromycin at a concentration of 50 mg/ml was added into each flask which resulted in a final concentration of 50 µg/ml. Promastigote culture was passaged twice and each time the same concentration of Hygromycin was added to the culture medium. In order to improve the episomal plasmid copy number and consequently expression of

gRNAs and Cas9 nuclease, the final concentration of Hygromycin was gradually increased to as high as 100 µg/ml in the next cultures. Upon establishment of a stable culture, the *L. major* promastigotes were provided with the Bleomycin resistant gene (584bp) as the donor template which was generated using a Bleomycin-containing plasmid as the template and Bleomycin specific primers: Lm271810BleF: 5' GCAACTCAGCGACCGTAAATCTAAAATCTTCATCGGATCGGGTAC and Lm271805BleR: 5' GACTTGTACCAACCTCGTACCGCTATCAGTCCTGCTCCTCGGCCA. The amplified fragment was later introduced to promastigotes via high-voltage electroporation as described above. The flanking homologues arms of Bleomycin resistance gene allowed for insertion of donor template in double-strand break created by Cas9 endonuclease using homology-directed repair (HDR) mechanism. In order to screen edited promastigotes in which PEPCK gene was replaced by Bleomycin resistant gene, culture media was supplemented with 50µg/ml Phleomycin (an antibiotic similar to Bleomycin). The concentration of Phleomycin was gradually increased to as high as 100µg/ml following three passages over the course of two weeks. Once transfection culture established, to further confirm the targeted deletion of PEPCK gene in Phleomycin resistant-promastigotes, PCR analysis was performed using post-electroporation primers (F2: 5' CGTGTCTTTCCTCCACGAAT and R2: 5'CAAACAAGCAGTGAGCCAAA). To generate *L. major* Addback strain, termed as PEPCK$^{-/+pepck}$, PEPCK-/- promastigotes were transfected with a PEPCK expression vector (pLPHygPEPCK)using high-voltage electroporation as described above.

3.4 Isolation of metacyclic *L. major* promastigotes

Metacyclic *L. major* promastigotes were isolated by peanut agglutinin (PNA) as Alcolea *et al* previously described[203]. Briefly, logarithmic-phase and stationary-phase promastigotes were centrifuged at 3000 rpm for 15 mins and parasite-containing sediment was re-suspended in 3ml complete DMEM growth culture at ~1×10^7/ml. Parasites were agglutinated with containing 50 µg/ml PNA for 30 minutes at room temperature. Fractions were further separated by centrifuging at 600 rpm for 10min and the sediment containing procyclic promastigotes (PNA^+ promastigotes) was discarded. The supernatant was centrifuged at 3000 rpm for 15 min to obtain metacyclic promastigotes (PNA^- promastigotes) in the sediment. To determine morphological differences,

the obtained metacyclic and procyclic promastigotes were subsequently stained with Giemsa stain and analyzed under optical microscopy.

3.5 Immunoblotting assay to assess PEPCK expression

L. major promastigotes at 1×10^6/ml were lysed in 2x Laemmli buffer containing 5% 2-mercaptoethanol (Sigma-Aldrich, St Louis MO) by heating at 95 °C for 5 min and vigorous vortexing. Cell debris was separated following centrifugation at 3000 rpm for 15 min. Lysates containing parasite proteins were loaded on a 12% SDS-PAGE gel and allowed to run for 1.5 hr at 100V. Proteins were transferred into an activated PVDF membrane using a semi-dry western transfer system (Bio-Rad, Mississauga, ON Canada) at 20V for 1h. The membrane was then blocked in tris-buffered saline (TBS) solution containing 0.5% tween and 5% non-fat milk (pH 7.4) for 1h at room temperature. Next, the membrane was incubated with a polyclonal rabbit anti-PEPCK antibody (1/1000 dilution) in the blocking solution (TBS supplemented with 3% BSA) overnight at 4 ^{o}C. Following 3 times with TBS solution supplemented by tween 20% (TBS-T) for 15 min in total, the membrane was incubated with mouse anti-rabbit horseradish peroxidase-conjugated secondary antibody (1/10000 dilution) in blocking solution (TBS supplemented with 3% BSA) for 1 hr at room temperature. Thereafter, the membrane was washed 3 times with TBS-T for 30 min in total and developed using ECL blocking agent (GE Healthcare, Mississauga, ON Canada) according to the manufacturer's protocol to visualize the protein bands. A Bio-Rad ChemiDoc™ Imaging System was utilized to capture luminescent images.

3.6 Infection

Naïve C57BL/6 and BALB/c mice were primarily infected in the left footpad via subcutaneous injection of 1×10^6 and 2×10^6 in 50 µl sterile PBS WT, PEPCK$^{-/-}$ or PEPCK$^{-/+pepck}$ strains, respectively. Some infected C57BL/6 mice were allowed to heal their primary lesion over the course of 8 to 12 weeks. BALB/c mice infected with WT and PEPCK$^{-/+pepck}$ strains were sacrificed at week 5 post-infection under the Ethics Protocol humane endpoint. BALB/c mice infected PEPCK$^{-/-}$ parasites that do not develop lesions were sacrificed at week 10 post-infection.

3.7 Lesion size measurement and determination of parasite burden

Lesion size was measured utilizing a Digital Vernier caliper weekly. The difference between the size of uninfected and infected footpads was used to calculate the lesion size. Parasite burden in infected footpads was quantified by performing limiting dilution assay as described by Titus *et al* [204]. Briefly, infected footpads of mice sacrificed by isoflurane and cervical dislocation were cut off by mouse surgical scissors and kept on ice in 2% pen/streptomycin containing PBS (PBS/PenStrep). To avoid bacterial infection, footpads were initially placed into 70% EtOH, chlorhexidine disinfectant, and 70% EtOH, each one for 5 min then rinsed in PBS/PenStrep. Afterward, toes were cut off and skin was peeled off through an incision made on footpads. The footpads were subsequently cut into smaller pieces and transferred to a grinder containing 2 ml PBS/PenStrep then ground until homogenized. The parasite suspension along with the homogenized tissues was subsequently transferred to a sterile 15ml conical tube and kept on the ice each time following the addition of 2ml PBS/PenStrep for three times. The grinding tool was washed out with PBS and the collected fluids were centrifuged at 600 rpm for 5 min to separate tissues from parasite suspension. The resultant supernatant was decanted and parasite suspension was transferred into a new conical tube and was immediately centrifuged at 3000 rpm for 15 min. Afterward, the pellet re-suspended in 2 ml complete Schneider media (Graces insect media) (Invitrogen, Life Technologies, Burlington, Ontario) complemented by 20% FBS, 2mM L-glutamine, 1% penicillin/streptomycin (1000X) and 25 mm HEPES. Twenty microliters of the resultant parasite suspension were added to the first row of a 96-well flat-bottom plate containing 180ul complete Schneider media and mixed by pipetting up and down for 10 times. In order to obtain a 10-fold serial dilution, 20 µl of the parasite suspension from the first row was added to the second row, mixed and serially diluted until the last row. Twenty microliters of parasite suspension were discarded at the last row. Plates were properly wrapped and incubated in 27 °C for 5 to 7 days. Thereafter, parasite growth was assessed under a light microscope at x40 magnification.

3.8 Assessment of parasite proliferation in axenic culture

One million WT, PEPCK$^{-/-}$ and PEPCK$^{-/+pepck}$ logarithmic-phase promastigotes were added to the 10 ml M199 media and incubated at 27 ^{o}C. The number of parasites was counted under an

optical microscope for 7 days consecutively. The culture media containing PEPCK$^{-/-}$ promastigotes, in some cases, was complemented with glucose (1 mg/ml).

3.9 Generation of macrophages from bone marrow stem cells

To generate macrophages, bone marrow stem cells were obtained as described above. The resultant cells were differentiated into macrophages by seeding 5×10^5/ml cells in 100 × 15 mM Petri dish containing 10ml complete RPMI 1640 media supplemented with 30% L929 cell culture supernatant at 37 °C. After 4 days, 10ml fresh L929 containing RPMI 1640 media was added to the Petri dish. Under these conditions, more than 70% of bone marrow macrophage progenitors were differentiated into a homogenous population of macrophages within 10 days. Since macrophages are adherent to the culture dish, the differentiated cells were detached from the surface using a cell scraper and gentle wash with 5-10ml PBS. The macrophages were then centrifuged at 1200 rpm for 5 min, counted, and kept on ice for subsequent use.

3.10 Infection of bone marrow-derived macrophages

Macrophages were generated from bone marrow progenitor cells of naïve BALB/c mice as described above. The bone marrow-derived macrophages (BMDMs) were reconstituted with complete RPMI 1640 media at 1×10^6/ml. To infect BMDMs with *L. major* promastigotes, WT, PEPCK$^{-/-}$, and PEPCK$^{-/+pepck}$ parasites were grown in M199 medium supplemented with 20% FBS, 100 µg/ml streptomycin and 100U/ml. Stationary-phase promastigotes (day 7) were centrifuged at 3000 rpm for 15 min. The supernatant was discarded and the parasite-containing sediment was later re-suspended and washed with PBS. The promastigotes centrifuged at 3000 rpm for 15 min were dissolved in an appropriate volume of PBS to make 1×10^6 promastigotes per 100 µl. For an *in vitro* infection of BMDMs, the *L. major* promastigotes were added to the cells (ratio of 1 cell:5 parasites). To remove the parasites which did not infect the cells (free parasites), the parasite/cell suspension was washed with PBS twice then span down at 600 rpm for 5 min, 6 h post-infection. During incubation of cell/parasite suspension at 37 °C, samples were taken 4 times at 6, 24, 48, and 72 hr following infection. The infected BMDMs have then spread on glass slides the following centrifugation at 1200 rpm for 5 min in Cytospin. Slides were subsequently stained with Giemsa stain and the number of parasites inside the macrophages was counted under 100x objective of optical microscopy. The percentage of infectivity was calculated as the number of intracellular parasites per 100 macrophages.

3.11 Carboxyfluorescein succinimidyl ester (CFSE) assay

Carboxyfluorescein diacetate succinimidyl ester (CFSE) was used to monitor T cell proliferation. Single cells isolated from spleen and draining lymph nodes (dLNs) were transferred to a 50ml conical tube and centrifuged for 5 min at 1200 rpm. The cells later re-suspended in 15ml warm PBS at 3×10^6/ml. To make a 1:2000 dilution, 2.5 µl CFSE from the stock solution (5mM in DMSO) was added to 15 ml warm PBS. The cells were labeled by pouring CFSE solution to the cell suspension followed by gentle inverting of the aluminum foil-wrapped 50 ml conical tube for 5 min. CFSE labeling reaction was then quenched by adding 15 ml warm FBS to the cell-CFSE solution. The labeled cells were immediately centrifuged for 5 min at 1200 rpm and resuspended in complete DMEM growth media counted, and kept on ice until used.

3.12 Isolation of cells from spleens and lymph nodes

Infected or disinfected mice were sacrificed at different time points following infection by isoflurane and cervical dislocation. Then spleens and draining lymph nodes (dLNs) were excised and collected in a 15 ml conical tube containing complete DMEM growth media. The organs were subsequently placed into a 70 µm cell strainer and ground using a 1ml syringe plunger. The homogenate was passed through the strainer and flushed out into a 50 ml conical tube containing 5 ml DMEM incomplete growth media. The cells were later centrifuged for 5 min at 1200 rpm, resultant supernatant was decanted and sediment was re-suspended in 3 ml (dLNs) and 10 ml (spleens) DMEM complete media. Then cells were diluted with PBS at 1:10 ratio and counted using a hemocytometer.

3.13 Cytokine enzyme-linked immunosorbent assay (ELISA)

For *in vitro* recall response, single cells obtained from the procedure described above, were seeded at 24-well plate (BD Falcon) at $2\text{-}4\times10^6$/ml and stimulated with or without 50 µg/ml soluble *Leishmania* antigen (SLA). The plate was later incubated at 37 °C with 5% CO2 for 3 days. Enzyme-linked immunosorbent assay (ELISA) was used to assess the production level of IL-10, IL-4, and IFN-γ in the cell culture supernatant. ELISA plates were coated with primary antibodies (100 µl/well) diluted in bicarbonate coating buffer (pH 9.6) and kept at 4 °C overnight. The following day, plates were washed with a washing solution (1X PBS, 0.05% tween 20, pH 7.4) 6 times. Upon addition of 200 µl/well ELISA blocking solution (5% heat-

inactivated FBS in 1X PBS), plates were incubated at 37 °C for 2 hours, then washed with washing solution 6 times. Afterward, the recombinant cytokines (all Peprotech) at different concentrations (see table 1) were added to the plates and serially diluted 2 folds in assay diluent (5% heat-inactivated FBS in 1X PBS) for 11 wells to provide the standard curve. Before adding the samples, a diluent assay was applied to the remaining wells (100 µl). Samples were later added to the wells containing diluent assay (100 µl/well) and tittered serially in 2 folds. Plates were later incubated at 4 °C overnight. Next day, after 8 times wash with washing solution, 100 µl/well biotin-conjugated secondary antibody was added to all wells and followed by a 2-hour incubation at 37 °C. Plates were washed with 10-12 times and the streptavidin-conjugated horseradish peroxidase at a concentration of 1:10000 was added to all wells. Plates were subsequently read at 405 nm (Spectra Max) after a 10-15 min incubation at 37 °C and appropriate color development.

Cytokine	Standard (pg/ml)	Sample dilution	Sensitivity
IFN-γ	2000	1:5	30.14
IL-4	2000	1:2	31.2
IL-10	4000	1:2	15.12

Table 1: List of cytokines, starting recombinant standard dilution, sample dilution and sensitivities of the sandwich ELISA

3.14 Intracellular cytokine detection by flow cytometry

Mice were sacrificed at indicated times after infection and single cells were obtained from spleens and lymph nodes as previously described. The expression of various markers including CD3, CD4, IFN-γ, IL-4, and IL-10 and was later examined by flow cytometry. Briefly, single cells counted and seeded in 24-well plates were stimulated with a stimulation cocktail comprised

of PMA (20 ng/ml), Ionomycin (1 µM) and Brefeldin A (10 µg/ml) (All from Sigma) for 5 hr at 37 °C. The stimulated cells were harvested and transferred into flow cytometry tubes and centrifuged for 5 min at 1200 rpm. The resultant supernatant was decanted and excess fluid was blotted out. Following a wash with FACS buffer (PBS containing 0.1% FBS and 0.1% sodium azide) 100ul TruStain FcX™ Antibody (BioLegend) was added to the cells for 5 minutes on ice, so as the name suggests, prevents non-specific binding of antibodies to Fc receptors of antibodies. The cells were thereafter stained using various fluorochrome-conjugated antibodies against the surface markers of interest and fixed with 2% paraformaldehyde. In order to permeabilize the cells, 1ml 0.1% saponin (Sigma) FACS buffer was added to the tubes, and cells were kept on ice for 15 minutes. After wash, cells were intracellularly stained with fluorescence conjugated antibodies against cytokines such as IFN-γ, IL-4, IL-10 at 20 µl/tube (all from eBioscience) for 30 minutes on ice. The stained cells were later rinsed with 1ml saponin containing FACS buffer then with FACS buffer alone to close the membrane pores. The cells were then re-suspended in 300 µl FACS buffer and acquired with FACS Canto II flow cytometer (BD Bioscience, Mississauga, ON, Canada). FlowJo software (TreeStar) was subsequently used for data analysis.

Antibody	Clone	Fluorescence
CD3	145-2C11	PE/cy7
CD4	GK 1.5	Pacific blue
IFN-γ	XMG1.2	PercCp/cy5.5, APC
IL-4	11B11	APC, PE
IL-10	1C10	APC, PE

Table 2: List of flourochrome conjugated antibodies used for flow cytometry

3.15 Measurement of mitochondrial respiration

Mitochondrial respiration in *L. major* promastigotes was measured as oxygen consumption rate (OCR) using a method previously described by Mejia *et al*[205]. Briefly, the Cell-Tak solution (Fisher Scientific, Mississauga, ON, Canada) was made by adding 23 µl Cell-Tak to 0.1M

$NaHCO_3$ buffer to reach the concentration of 20.26 µg/ml and added to XF 24 microplates for 20 min at room temperature. Thereafter, Cell-Tak was aspirated and the microplate was washed with 200 µl UltraPure DNase/RNase-free water to remove Cell-Tak residues followed by 10 min air drying. Logarithmic-phase promastigotes were harvested by centrifuging at 3000 rpm for 15 min and re-suspended at $5x10^7$/ml in an appropriate amount of at glucose-free XF assay media (pre-warmed in 37° C, pH 7.4 and filter-sterilized utilizing a 0.22 µM filter) supplemented by 1 mM sodium pyruvate and 11 mM D-glucose. One hundred microliters of XF assay medium suspension containing $5x10^6$ to was added to corresponding Cell-Tak coated microplate (6 replicates per parasite strain). Then, the microplate was centrifuged at 3000 rpm for 10 min and incubated at 37 °C for a maximum of 20 min to further settle the promastigotes at the bottom of the microplate. Meanwhile, ports A to C of an XF sensor cartridge were loaded with 75 µl oligomycin, 83 µl carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, and 93 µl rotenone/antimycin A (all from 10 µM solutions in XF assay medium), respectively to each a 1 µM final concentration of compounds in each well. The loaded sensor cartridge plate was put into the Seahorse XF24 analyzer (Agilent) and calibrated. Following 20 min incubation at 37 °C, wells were topped off with 575µl XF assay medium supplemented with sodium pyruvate and D-glucose and incubated at 37 °C until put into XF 24 analyzer.

3.16 Measurement of glycolysis

The rate of glycolysis in *L. major* promastigotes was measured as an extracellular acidification rate (ECAR) using XF 24 Seahorse analyzer. The same parasite number and method was used as described above with some minor modifications. In this assay, XF assay media (pre-warmed in 37° C, pH 7.4, and filter-sterilized utilizing a 0.22 µM filter) was complemented with 2 mM L-glutamine. The sensor cartridge was loaded in ports A to C by different components (all prepared in XF assay medium at 10 µM) including 80ul glucose (final concentration = 11 mM), 80 µl oligomycin (final concentration = 11 µM) and 80 µl 2-DG (final concentration = 22 mM). After calibration of the sensor cartridge, an additional volume of XF assay medium (460 µl) supplemented with 2 mM L-glutamine was added to each well following by placing it into the instrument.

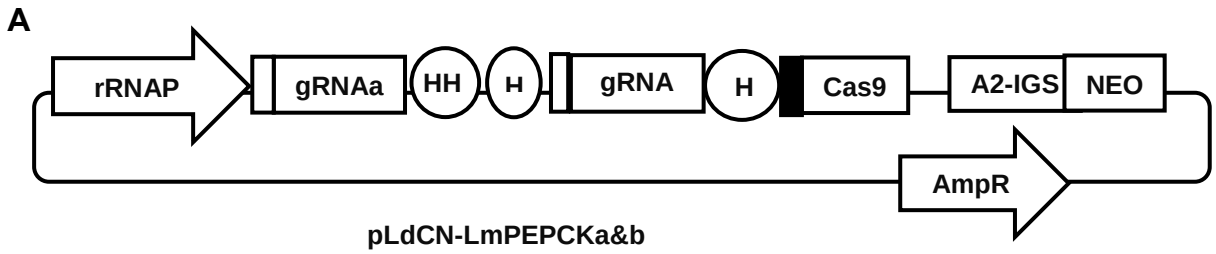
CHAPTER 4

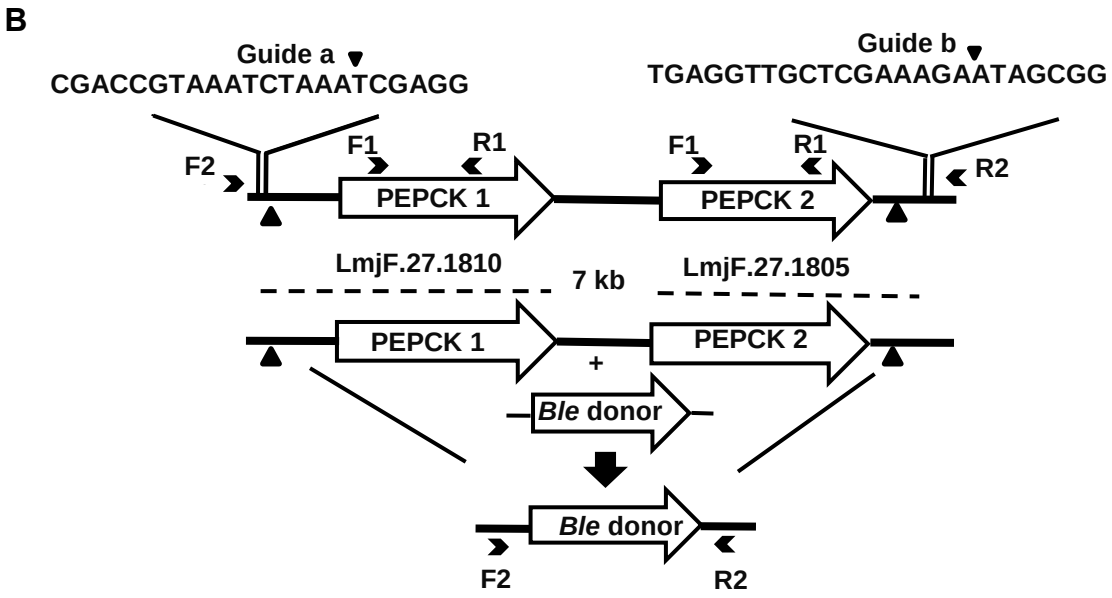
4.0 Results

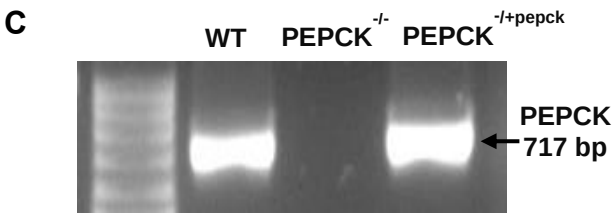
4.1 Generation and characterization of *L. major* null mutants

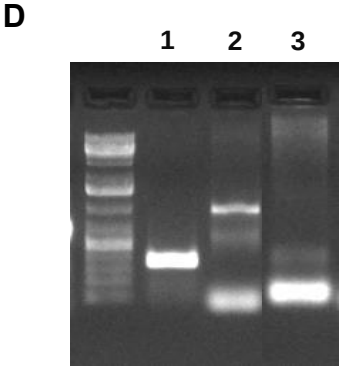
CRISPR-Cas9 gene-editing tool has been widely used for genetic manipulation of various *Leishmania* species[188] [182]. In this study, to define the role of PEPCK in the pathogenesis of *Leishmania major*, I generated specific PEPCK null mutants, referred to as PEPCK$^{-/-}$, using CRISPR-Cas9 gene manipulation system **(Figures 1A&1B)**. The deletion of PEPCK gene was confirmed by performing PCR analysis on genomic DNA isolated from PEPCK$^{-/-}$ promastigotes **(Figure 1C)**. Similarly, the inserion of Bleomycin gene in PEPCK$^{-/-}$ *L. major* at the given short guide RNAs target site was validated using Bleomycin specific primers (483bp amplicon) , as well as post-electroporation primers (925bp amplicon), repectively. **(Figure 1D, lines 1 & 2).** Additionally, Western blot analysis using the specific anti-PEPCK antibody against recombinant PEPCK confirmed undetectable expression of PEPCK protein **(Figure 1E)**. Our lab recently showed that at the peak of infection, PEPCK-specific CD4^{+} T cells from *Leishmania*-infected C57LB/6 mice comprises 20% of all *Leishmania*-reactive CD4^{+} T cells. In this respect, we also

generated a tetramer solution which facilitates detection of $CD4^+$ T cells specific to an immunodominant peptide derived from PEPCK[52]. I, therefore, used this reagent to further validate the deletion of PEPCK by infecting C57BL/6 mice with WT and $PEPCK^{-/-}$ *L. major* and quantifying the frequency of PEPCK specific-$CD4^+$ T cells 4-weeks post-infection. Flow cytometry analysis revealed that $CD4^+$ T cells undergo a massive expansion in spleen and dLNs of mice infected with WT promastigotes which express PEPCK. However, no detectable expansion was observed in $CD4^+$ T cells of tissues obtained from $PEPCK^{-/-}$-infected mice **(Figures 2A-2C)**. To further confirm PEPCK deletion, I adoptively transferred CFSE-labelled $CD4^+$ T cells from TCR transgenic mice into naïve mice and infected them with WT and $PEPCK^{-/-}$ promastigotes. Next, I assessed proliferation and IFN-γ production of $CD4^+$ T cells in recipient mice 5-days post-infection using flow cytometry. I found that $CD4^+$ T cells show a significant proliferation and IFN-γ production following infection with WT promastigotes and MHC II-restricted presentation of PEPCK. However, I did not detect a noticeable proliferation and production of IFN-γ in $CD4^+$ T cells of mice infected with $PEPCK^{-/-}$ *L. major* **(Figures 2D-2F)**. These results strongly show that all copies of *PEPCK* have been successfully removed in $PEPCK^{-/-}$ promastigotes. Off-target effects, as unintended mutations that may lead to genetic variations and alter the functionality of normal genes, is one major concern involved in gene manipulation using CRISPR-Cas9 system[195]. In this regard, the generation of addback strain is one of the approaches that could be employed to ensure that the integrity of *L. major* genome is not affected by the procedures culminated in the deletion of the PEPCK gene. Therefore, I generated PEPCK Addback strain, termed $PEPCK^{-/+pepck}$ through transfection of PEPCK null mutants with PEPCK expression vector. The presence of the PEPCK gene, as well as PEPCK protein expression in PEPCK-/+pepck strain, were confirmed by PCR and Western blot analysis **(Figures 1C&1D).**




B
Guide a
CGACCGTAAATCTAAATCGAGG
Guide b
TGAGGTTGCTCGAAAGAATAGCGG
F2
F1
R1
PEPCK 1
F1
R1
PEPCK 2
R2
LmjF.27.1810
7 kb
LmjF.27.1805
PEPCK 1
PEPCK 2
+
Ble donor
Ble donor
F2
R2


C
WT
PEPCK-/-
PEPCK-/+pepck
PEPCK
717 bp


D
1
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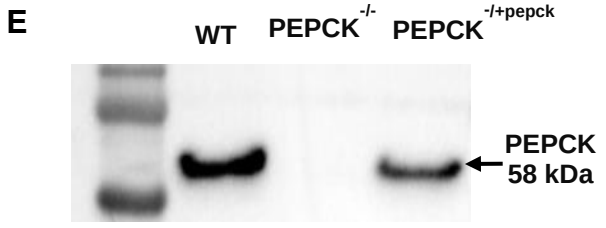
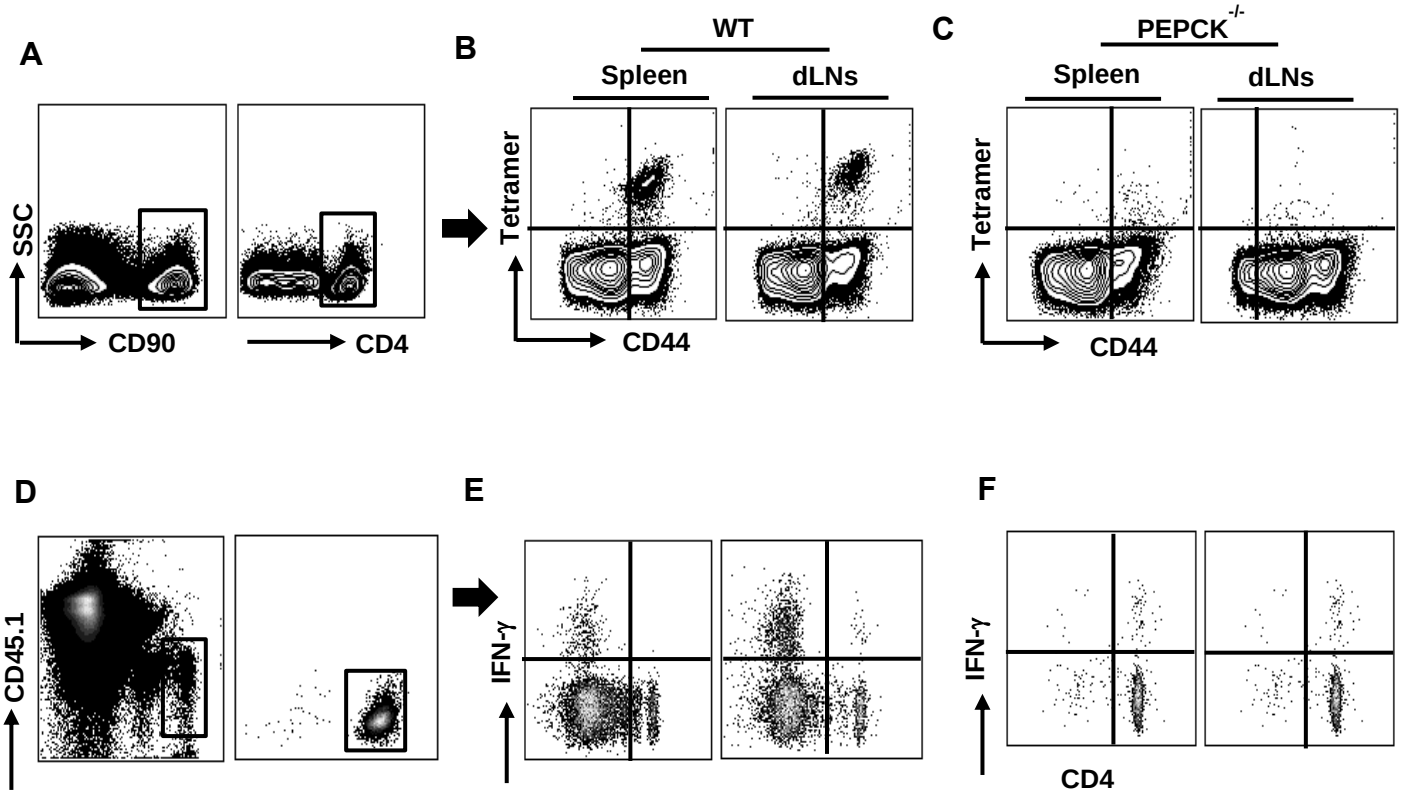

E
WT
PEPCK-/-
PEPCK-/+pepck
PEPCK
58 kDa

Figure 1. Generation of targeted PEPCK null mutants by CRISPR-Cas9 system. Map of *Leishmania* all-in-one CRISPR plasmid (pLdCN) expressing dual gRNAs targeting the *L. major* PEPCK gene flanking sequences (A). The *L. major* PEPCK gene locus on Chromosome 27 and the CRISPR strategy used to delete all four copies of PEPCK genes (B). PCR analysis showing that PEPCK gene sequence is absent in *L. major* PEPCK null mutant (PEPCK$^{-/-}$) but completely restored in the addback (PEPCK$^{-/+pepck}$) strain (C). PCR analysis showing that Bleomycin gene has been successfully inserted in *L. major* chromosome following the deleation of PEPCK in these mutants (line 2). Lines 1 and 3 are respresentative of the presence of *Bleomycin* and absence of *PEPCK* in PEPCK$^{-/-}$ *L. major,* respectively (D). Western blot analysis showing undetectable PEPCK protein expression in PEPCK null mutant (PEPCK$^{-/-}$) and complete restoration in the addback (PEPCK$^{-/+pepck}$) strain (E).



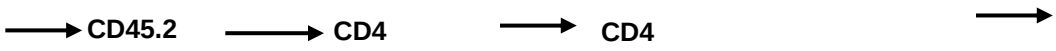


Figure 2. *In vivo* validation of PEPCK deletion in *L. major*. PEPCK$^{-/-}$ *L. major* does not activate PEPCK-specific CD4^{+} T cells *in vivo*. C57BL/6 mice were infected subcutaneously in the footpads with wild-type (WT) or PEPCK$^{-/-}$ parasites (A-C). After 4 weeks, the mice were sacrificed and cells from their spleens (B) and draining lymph node (C) cells were stained with IA^{b}-PEPCK$_{335-351}$ tetramer and analyzed by flow cytometry. PEPCK$^{-/-}$ *L. major* does not activate PEPCK-specific CD4^{+} T cell receptor transgenic cells *in vivo*. One million (1 x 10^{6}) CFSE-labeled CD4^{+} T cells from PEPCK TCR transgenic mice were adoptively transferred into naïve congenic (CD45.2) C57B/6 mice that were then infected with 2 x 10^{6} WT and PEPCK$^{-/-}$ promastigotes (D-F). After 4 days, mice were sacrificed and PEPCK-specific CD4^{+} (donor) T cells from spleens (E) and draining lymph nodes (F) were gated as shown (D) and assessed for proliferation and IFN-γ production by flow cytometry. Results presented are representative of 2 independent sets of experiment with similar results.

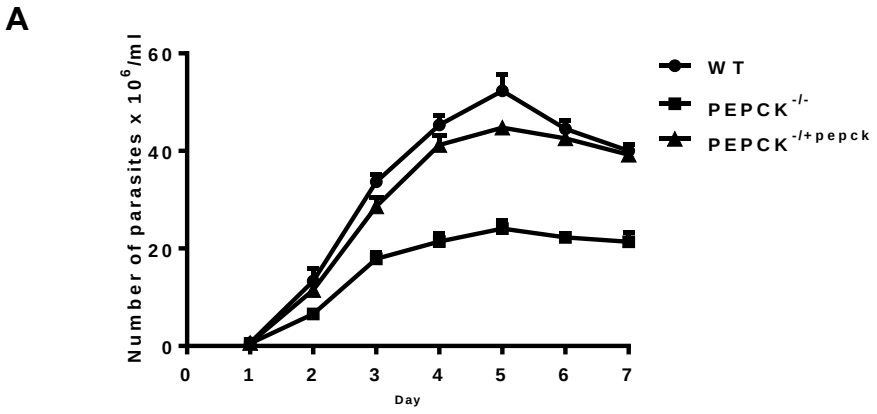
4.2 PEPCK deficient *L. major* exhibit a significantly impaired growth in axenic culture

Next, I aimed to determine whether PEPCK deficiency has an effect on the growth of *L. major* promastigotes. I, therefore, seeded an equal number of logarithmic-phase WT, PEPCK$^{-/-}$ and PEPCK$^{-/+pepck}$ promastigotes in fresh M199 media and counted the number of parasites for 7 days consecutively**.** Previous studies that have reported a similar growth for *Leishmania* promastigotes with a defect in gluconeogenic enzymes in synthetic media containing glucose as the carbon source[27]. However, I found that PEPCK$^{-/-}$ promastigotes exhibit an impaired growth in axenic culture following depletion of glucose compared to WT and PEPCK$^{-/+pepck}$ promastigotes. Moreover, the stationary-phase density of PEPCK null mutants is lower at 3 days post-passage. Interestingly, episomal complementation of PEPCK$^{-/-}$ promastigotes with a vector expressing PEPCK, fully restored the capability of these null mutants to grow in axenic culture and reach to a stationary-phase density similar to those of WT counterpart **(Figure 3A)**. Because it has been shown that glycolysis is an active pathway in promastigotes due to continuous exposure of

promastigotes to sugar in sandfly midgut[34], I supplemented the axenic culture containing PEPCK$^{-/-}$ promastigotes with glucose to determine whether their reduced growth could be restored. As shown in **(Figure 3B)** supplementation of axenic culture with glucose reversed the growth rate of PEPCK$^{-/-}$ promastigotes to a level similar to their WT and PEPCK$^{-/+pepck}$ counterparts.

4.3 Proliferation of PEPCK$^{-/-}$ *L. major* is highly compromised inside bone marrow-derived macrophages

Leishmania parasites are deposited into the skin of the host following the bite of an infected sandfly and phagocytosed by immune cells of innate immunity[6]. Among all immune cells, macrophages serve as the most permissive niche for the intracellular life stage of *Leishmania*. This is, in part, attributed to the biochemical composition of macrophages that could sustain the complex nutritional requirement of intracellular *Leishmania*[206 29]. To determine whether PEPCK participates in survival and proliferation of *L. major* parasites inside the macrophages, I differentiated bone marrow stem cells obtained from a naïve BALB/c mouse into macrophages and infected them with WT, PEPCK$^{-/-}$ and PEPCK$^{-/+pepck}$ promastigotes at a ratio of 1:5. Next, I counted the number of intracellular parasites in Giemsa-stained slides under optical microscopy at different time points (6h, 24h, 48h, and 72h) post-infection **(Figures4A&4B).** Interestingly, I found that at the early time point (6h post-infection), all parasite lines infect bone –marrow-derived macrophages to a similar level suggesting that *L. major* promastigotes retain their infectivity in the absence of PEPCK. However, as compared to of WT and PEPCK$^{-/+pepck}$, I detected a drastically reduced number of PEPCK$^{-/-}$ parasites inside macrophages at later time points following infection **(Figures 4A&4B).** These results clearly indicate that targeted loss of PEPCK leads to a severely compromised proliferation in these null mutants.



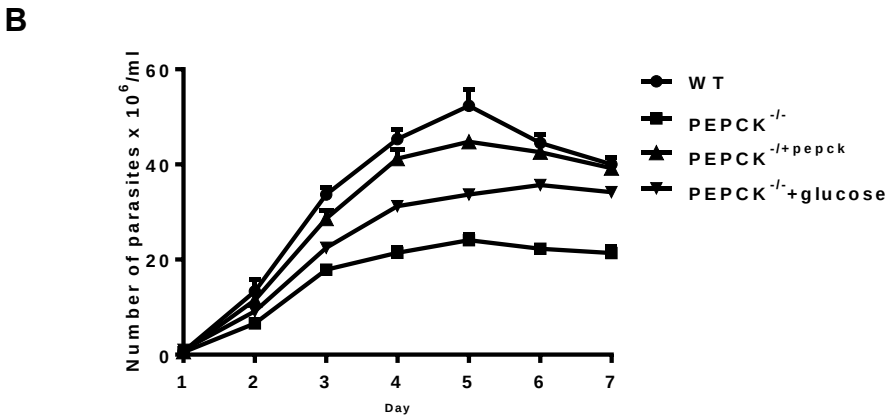
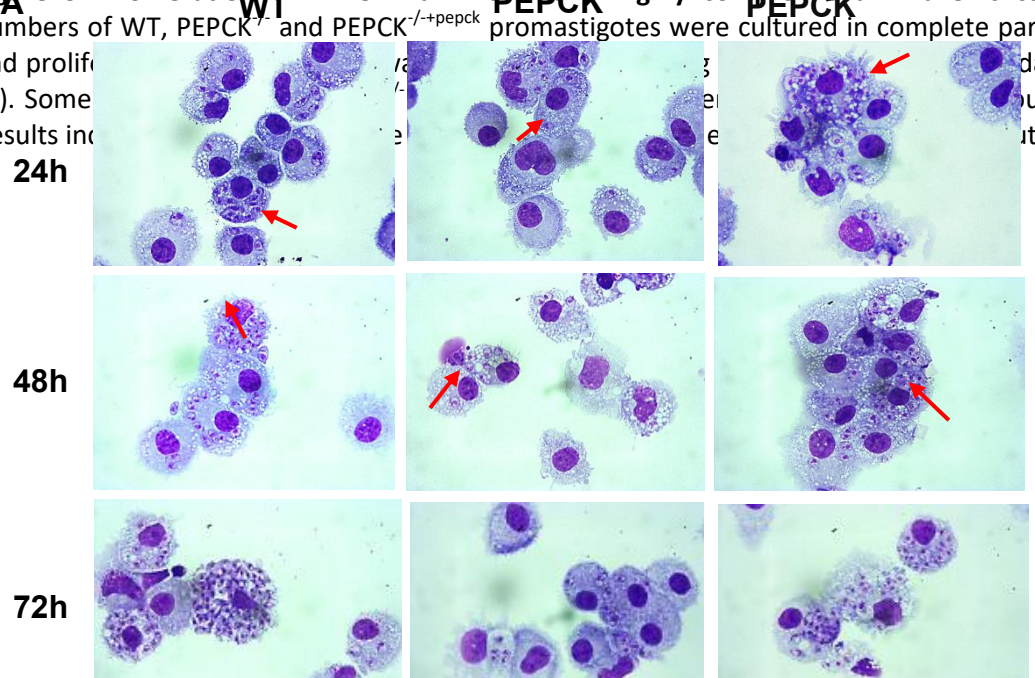
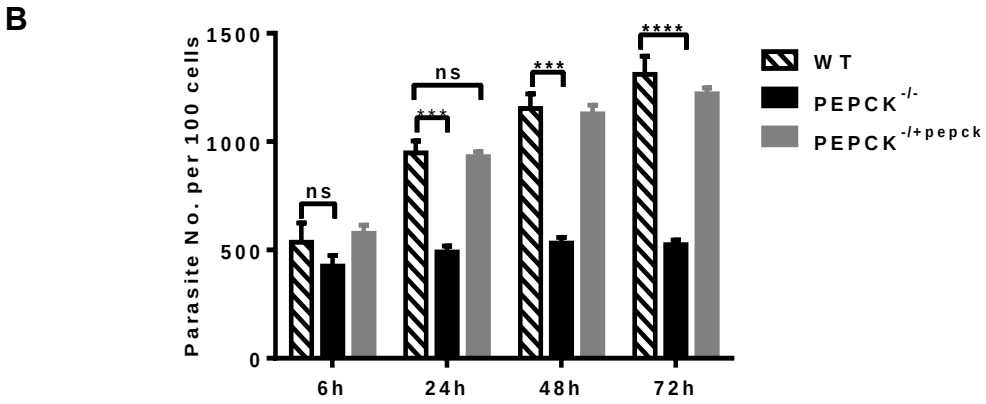


Figure 3. Proliferation of PEPCK null mutants is highly compromised in axenic cultures. Equal numbers of WT, PEPCK$^{-/-}$ and PEPCK$^{-/-+pepck}$ promastigotes were cultured in complete parasite medium and prolif daily for 7 days (A). Some us glucose (B). Results inc tcomes.



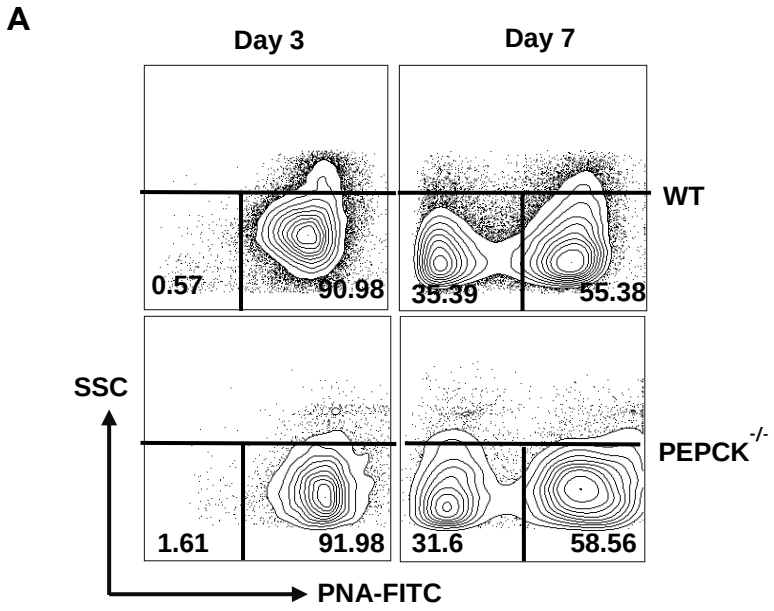


4.4 PEPCK deficiency does not impact metacyclogenesis in *L. major*

Metacyclogenesis, known as the conversion of metacyclic to procyclic promastigotes, is a transitional developmental stage that is characterized by modifications in morphology, infectivity, and structure of *Leishmania* parasites[203]. Among all structural changes, the addition of arabinose residues to galactose side chains of LPG (*Leishmania* major surface glycoconjugates) during metacyclogenesis. This modification, as the principle of negative purification methods used to isolate the infective form of *Leishmania*, prevents metacyclic promastigotes from being agglutinated by lectins such as PNA[15]. To understand the role of PEPCK in the metacyclogenesis of *L. major*, I used PNA-FITC to isolate procyclic and metacyclic forms of WT and PEPCK$^{-/-}$ promastigotes at 3 and 7 days following cultivation in axenic culture. FITC-conjugated PNA allowed for fluorometric assessment of procyclic and metacyclic forms in WT and PEPCK$^{-/-}$ promastigotes. Flow cytometry analysis revealed that

Figure 4. Proliferation of PEPCK null mutants is severely impaired in macrophages. Bone marrow-derived macrophages were infected with WT, PEPCK$^{-/-}$ and PEPCK$^{-/-pepck}$ promastigotes. At the indicated times, cytospin preparations were prepared, stained with Giemsa stain and infectivity was assessed by light microscopy (A). The number of parasites inside infected cells were counted and expressed as number of parasites per 100 macrophages (B). Results presented are representative of 3 independent sets of experiment with similar results. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

procyclic promastigotes constitute the majority of parasites (indicated as PNA-FITC^{+}) at logarithmic phase of axenic culture (day 3). However, during metacyclogenesis, a fraction of procyclic promastigotes convert to metacyclic from (indicated as PNA-FITC^{-}) in the course of 4 days. In addition, I found a comparable number of metacyclic promastigotes in both WT and PEPCK$^{-/-}$ experimental groups (Figure 5A). Notably, this finding is in line with *in vitro* data showing the similar level infectivity in all parasite lines. I further validated these finding through observation of isolated promastigotes using optical microscopy. As expected procyclic form of parasites display a small round-shape body with relatively shorter flagella, whereas metacyclic promastigotes exhibit a narrow body and clearly elongated flagella (Figure 5B). Collectively, these results suggest that metacyclogenesis and, as a result, infectivity of *Leishmania* parasites are not influenced by the targeted loss of PEPCK.



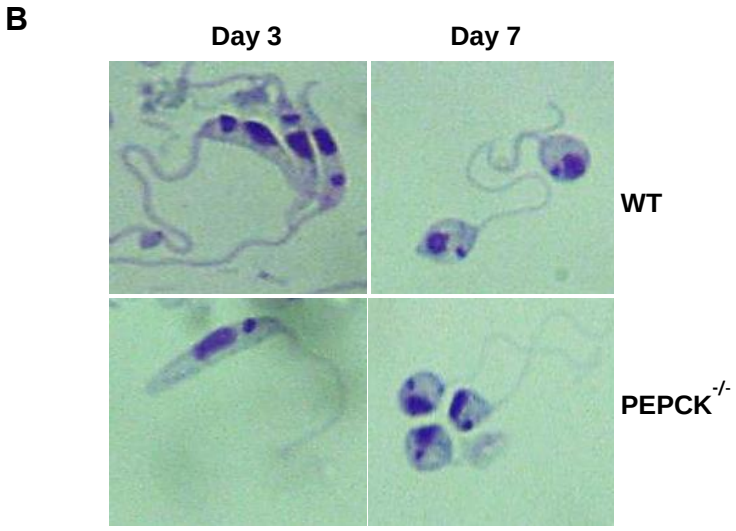


Figure 5. PEPCK deficiency does not affect metacyclogenesis in *L. major*. Equal numbers (2.5 x 10^5/ml) of WT and PEPCK$^{-/-}$ *L. major* promastigotes were cultured in axenic medium at 27 °C and at the indicated days assessed for the presence of metacyclics (PNA negative) by flow cytometry (A) or direct microscopy (B). Results presented are representative of 3 independent sets of experiment with similar outcomes.

4.!

The critical role of key gluconeogenic enzymes such as GK, PPDK, and FBP in virulence of *Leishmania* have been previously reported[27 28]. In line with this finding, in the current study, I found that PEPCK$^{-/-}$ parasites exhibit an attenuated phenotype manifested as an impaired proliferation in BMDMs. Therefore, I aimed to determine whether PEPCK also participates in *L. major* virulence *in vivo.* I infected BALB/c mice with 1×10^6 WT, PEPCK$^{-/-,}$ and PEPCK$^{-/+pepck}$ stationary-phase promastigotes and monitored the lesion size over time **(Figure 6A).** I also assessed parasite burden at the site of infection at indicated timepoints **(Figure 6B).** I found that mice infected with WT and PEPCK$^{-/+pepck}$ *L. major* develop uncontrollable cutaneous lesions as early as 3 weeks following infection, whereas PEPCK$^{-/-}$-infected mice remain without noticeable lesions at all-time tested. Expectedly, the progressive lesions in mice infected with WT and PEPCK$^{-/+pepck}$, harbor considerably higher number of parasites as compared to parasite burden in mice infected with PEPCK$^{-/-}$ parasites. Additionally, I infected C57BL/6 mice with 2×10^6 WT, PEPCK$^{-/-}$ and PEPCK$^{-/+pepck}$ promastigotes and measured lesion size weekly **(Figure 6C).** As in

BALB/c mice, I did not observe detectable lesion in C57BL/6 mice infected with PEPCK$^{-/-}$ parasites **(Figure 6C).** The lack of lesion formation in mice infected with PEPCK$^{-/-}$ parasites corresponded to significantly fewer number of parasites at the site of infection in these mice (**Figure 6D**). In contrast, mice infected with WT and PEPCK$^{-/+pepck}$ developed progressive lesions that peaked at 4 weeks post-infection. As expected, WT and PEPCK$^{-/+pepck}$ – infected mice achieved significant lesion resolution by 8 weeks following infection **(Figure 6D).** Altogether, these results suggest that genetic ablation of PEPCK leads to an *in vivo* attenuated phenotype in *L. major*.

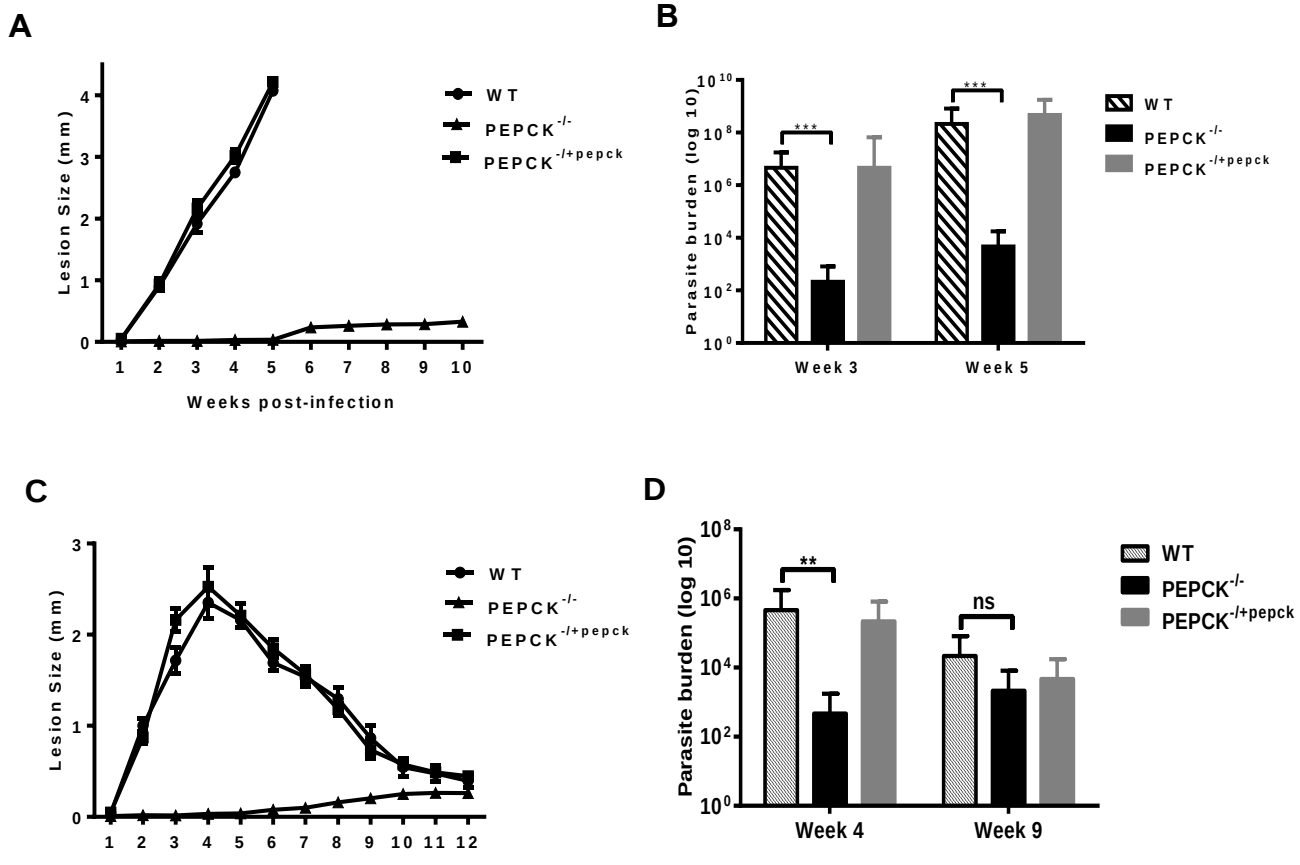
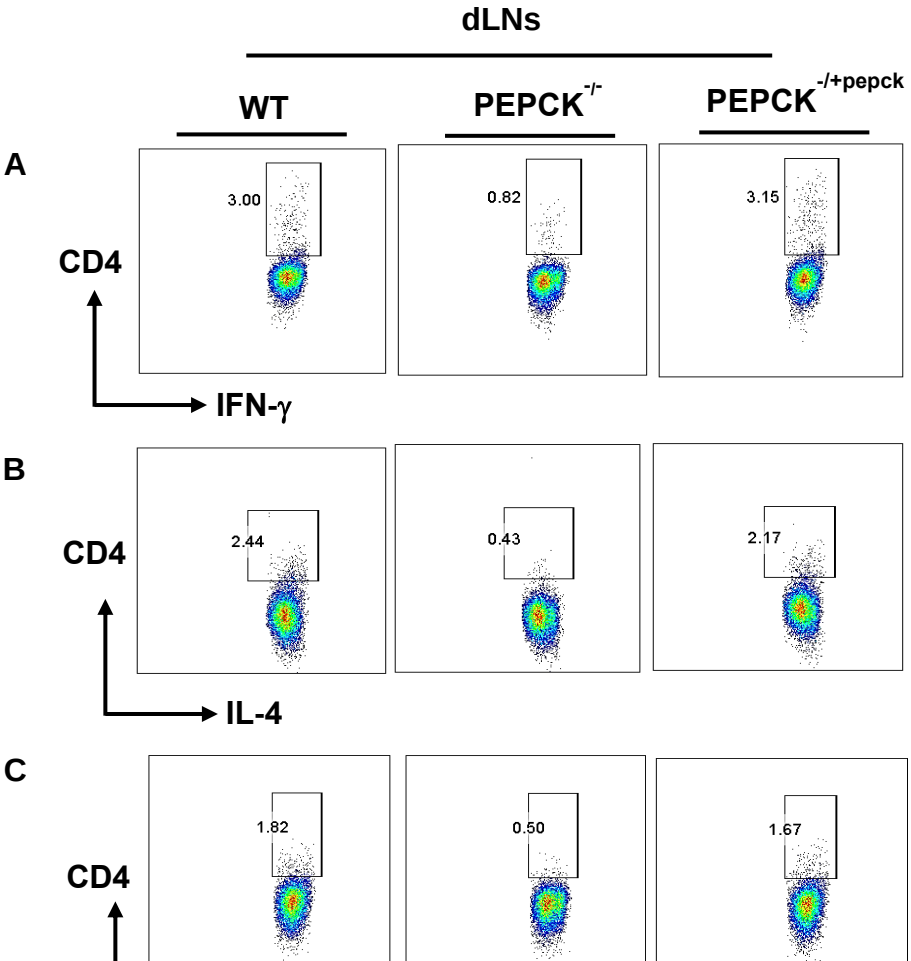


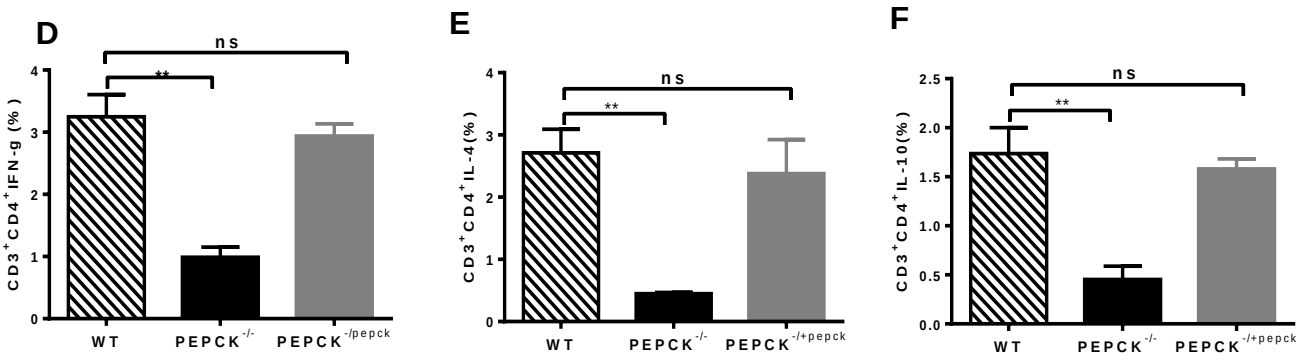
Figure 6. Deficiency of PEPCK results in attenuated pathology *in vivo.* BALB/c and C57BL/6 mice were infected with WT, PEPCK$^{-/-}$ and PEPCK$^{-/-+pepck}$ stationary phase promastigotes (8-10 mice per group) and the development of lesions in the infected footpads were monitored (A&C). At indicated times, mice were sacrificed and parasite burden in the infected footpads was determined by limiting dilution (B&D). Results presented are representative of 3 independent sets of experiment with similar results. **, $p < 0.01$; ***, $p < 0.001$.

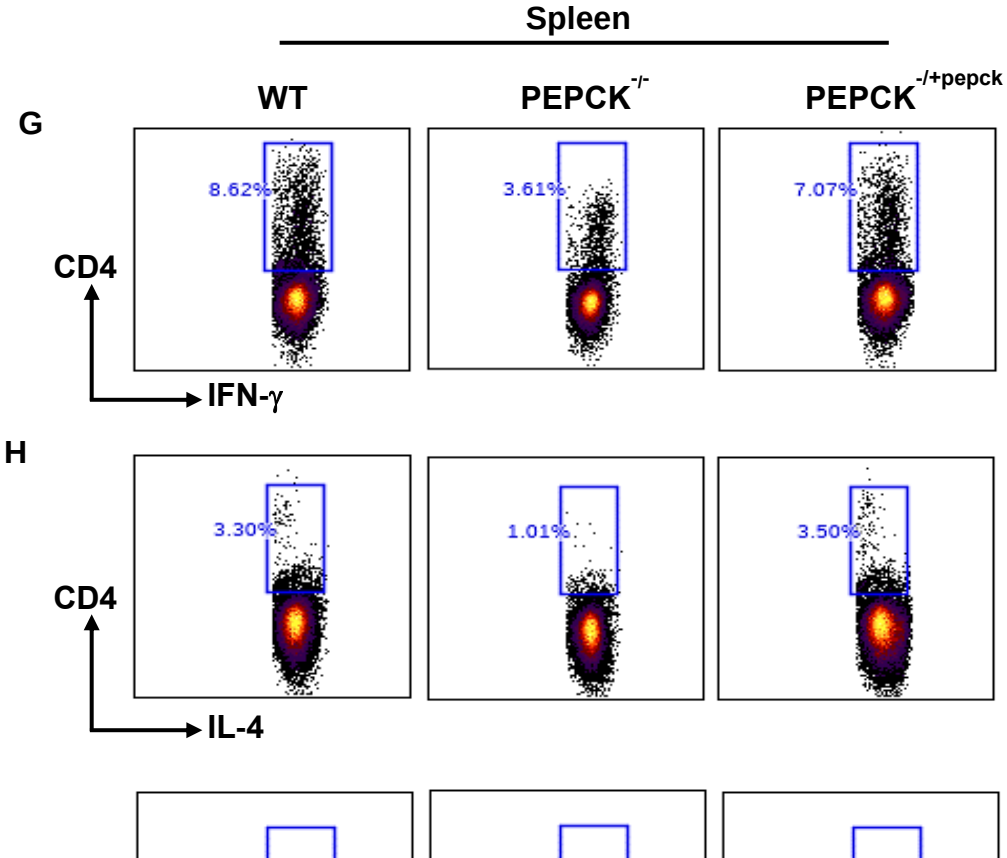
4.6 PEPCK deficiency results in a blunted host immune response

It is well established that immunity to *Leishmania major* infection, manifested as low parasite burden and self-healing of skin lesions is mediated by IFN-γ-dependent activation of macrophages. On the contrary, susceptibility in BALB/c mice is strongly associated with a Th2-type immune response as measured by the production of high levels of IL-4 and IL-10 by their CD4+ T cells[53]. Recently, our lab identified PEPCK as a highly immunodominant *Leishmania* antigen that induces a robust anti-*Leishmania* T cell-mediated response in both mice and humans [52]. Thus, to investigate the contribution of PEPCK in host immune response, I assessed the frequency of CD4^{+} T cells producing IFN-γ, IL-4, and IL-10 in spleens and dLNs of BALB/c mice infected with WT, PEPCK$^{-/-}$ and PEPCK$^{-/+pepck}$ promastigotes at weeks 3 and 5 post-infection. Direct *ex vivo* flow cytometric analysis shows that the frequency of cytokine (IFN-γ, IL-4 and IL-10)producing CD4^{+} T cells is drastically lower in both dLNs **(Figures 7A&7F)** and spleens **(Figure 7G-7O)** of mice infected with PEPCK$^{-/-}$ parasites than those infected with WT or PEPCK$^{-/+pepck}$ parasites The finding of significantly lower IFN-γ-producing cells were initially surprising because it is widely believed that resistant to *L. major* infection in mice(as observed in

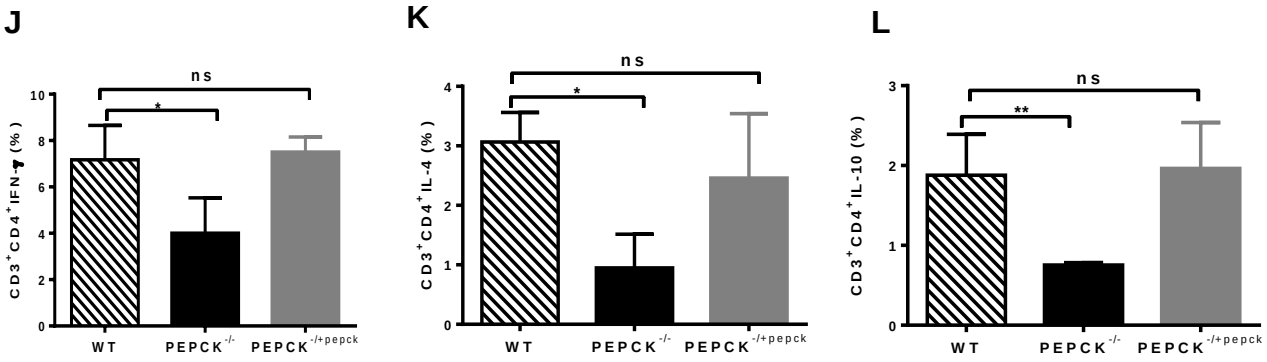
mice infected with PEPCK$^{-/-\ parasites}$) is associated with a high production level of IFN-γ leading to more effective macrophage activation. However, Uzonna *et al*[207]has previously shown that in the absence of progressive cutaneous lesions and a high amount of suppressive cytokines such as IL-4 and IL-10, the low levels of IFN-γ is sufficient for efficient activation of macrophages to a leishmanicidal state. I further confirmed the flow cytometry data by re-stimulating cells obtained from tissues of infected mice with SLA and assessing the production level of IFN-γ, IL-4, and IL-10 in culture supernatants by ELISA **(Figures 7P-7Y).** Similarly, I assessed the frequency of IFN-γ-producing CD4^{+} T cells in C57BL/6 mice infected with WT, PEPCK$^{-/-,}$ and PEPCK$^{-/+pepck}$ *L. major* at weeks 5 and 9 post-infection. Although I found an insignificant difference in the spleen production level of IFN-γ between mice infected with distinct parasite lines **(Figures 8A&8B)**, CD4^{+} T cells from the dLNs of PEPCK$^{-/-}$-infected mice produced a significantly lower amount of IFN-γ **(Figures 8C&8D)** compared to those infected with WT parasites. The flow cytometry findings were further validated by ELISA (**Figure 8E & 8F**). These results (blunted immune response in PEPCK$^{-/-}$-infected mice), confirms the previous findings by our group that PEPCK is an immunodominant antigen of *Leishmania major*[52].



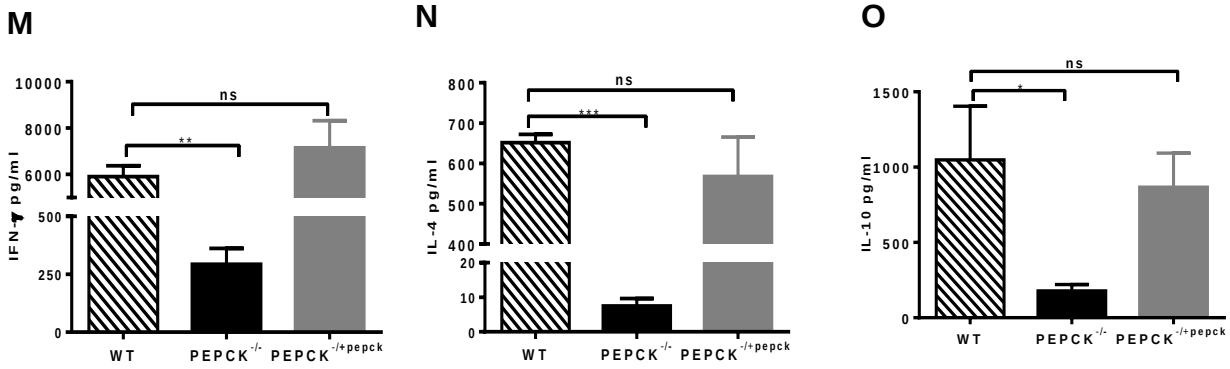

D
ns
**
CD3+CD4+IFN-g (%)
WT
PEPCK-/-
PEPCK-/pepck
E
ns
**
CD3+CD4+IL-4(%)
WT
PEPCK-/-
PEPCK-/+pepck
F
ns
**
CD3+CD4+IL-10(%)
WT
PEPCK-/-
PEPCK-/+pepck


Spleen
WT
PEPCK-/-
PEPCK-/+pepck
G
8.62%
3.61%
7.07%
CD4
IFN-γ
H
3.30%
1.01%
3.50%
CD4
IL-4

I



dLNs



Spleens

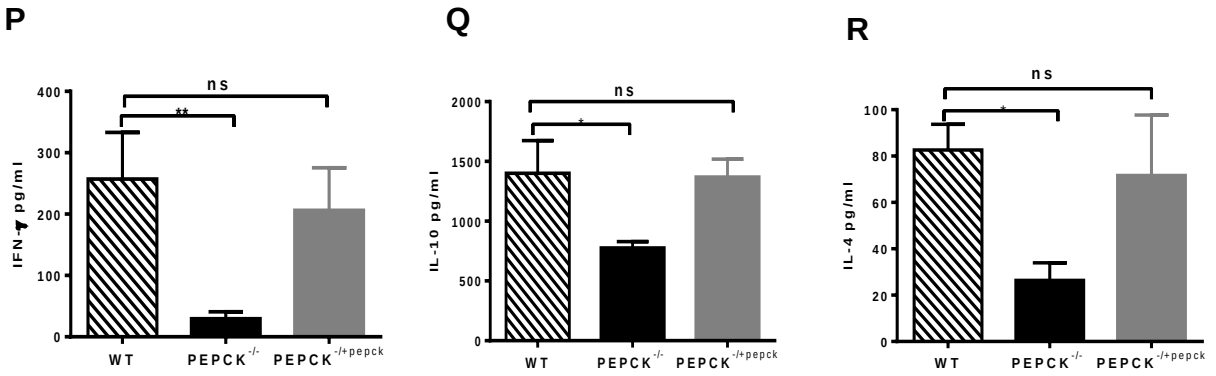


Figure 7. Deficiency of PEPCK results in blunted immune response. BALB/c mice (8-10 mice per group) were infected with WT, PEPCK$^{-/-}$ and PEPCK$^{-/+pepck}$ stationary phase promastigotes and at 5 weeks post-infection, mice were sacrificed and isolated from draining lymph nodes and spleen were stimulated for 5 hours with PMA, ionomycin and brefeldin A. Cells were then stained to assess the frequency of IFN-γ-, IL-4- and IL-10-producing CD4^{+} T cells in dLNs (A-F) and spleen (G-L) by flow cytometry. Some cells were re-stimulated *in vitro* with SLA (50 µg/ml) for 3 days and the production levels of IFN-γ, IL-4 and IL-10 in the culture supernatant of cells obtained from dLNs (M-O) and spleen (P-R) were determined by ELISA. Results pre[illegible]d are representative of 3 independent sets of [illegible] with similar results. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

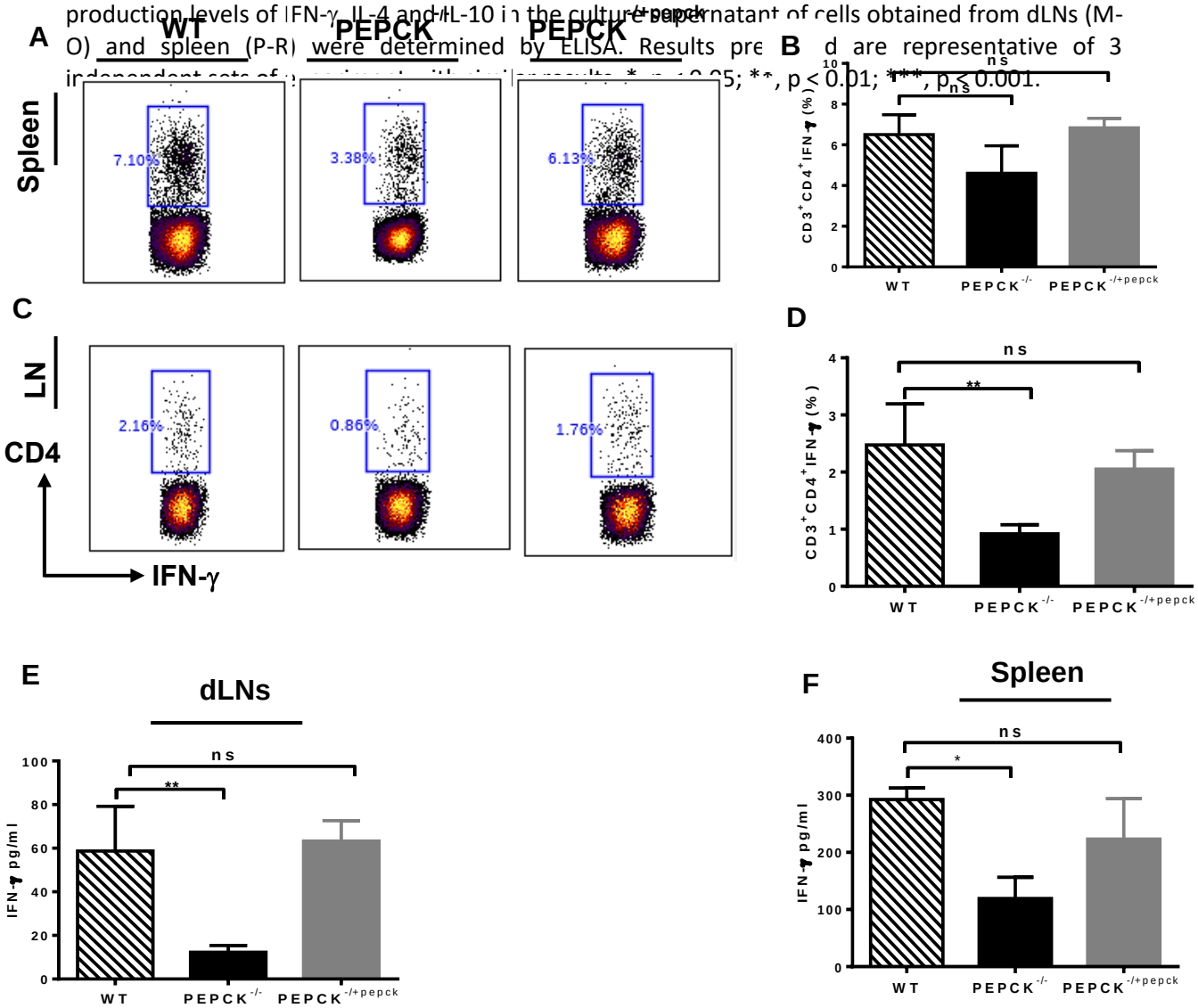


Figure 8. Deficiency of PEPCK results in blunted immune response. C57BL/6 mice (9 mice per group) were infected with WT, PEPCK$^{-/-}$ and PEPCK$^{-/-+pepck}$ stationary-phase promastigotes and at 5 weeks post-infection, mice were sacrificed and cells obtained from draining lymph nodes and spleen were stimulated for 5 hours with PMA, ionomycin and brefeldin A. Cells were then stained to assess the frequency of IFN-γ-CD4^{+} T cells in dLNs (A&B) and spleen (C&D) by flow cytometry. Some cells were re-stimulated *in vitro* with SLA (50 μg/ml) for 3 days and the production levels of IFN-γ, the culture supernatant of cells obtained from dLNs (E) and spleen (F) were determined by ELISA. Results presented are representative of 3 independent sets of experiment with similar results. *, $p < 0.05$; **, $p < 0.01$.

I a good live-attenuated vaccine candidate. To determine whether PEPCK$^{-/-}$ parasites would confer protection against virulent *L. major* challenge, I challenged BALB/c mice previously infected PEPCK$^{-/-}$ parasites as well as naïve age-matched mice on contralateral footpad with 5×10^5 virulent *L. major* at 10-week post-infection **(Figure 9A).** Delayed-type hypersensitivity was evaluated through measuring lesion size in the challenged footpad of both naïve and previously infected mice at 72 h after challenge **(Figure 9B)**. There was an insignificant DTH response at the site of virulent *L. major* challenge in both mice strains. The lack of DTH response in these mice is notable when compared with healed C57BL/6 mice t that exhibited strong DTH response (0.8-1.2mm) following secondary *L. major* infection[207]. Three weeks after challenge, naïve mice and previously PEPCK$^{-/-}$-infected mice developed detectable lesions at the site of the virulent challenge to a comparable level. Notably, parasite burden in the challenged footpads of PEPCK$^{-/-}$-infected mice was 2 folds lower than that of naïve control mice **(Figure 9C).** Interestingly, I found that virulent L. major challenge resulted in disease recrudescence in the primary infection sites of mice infected with PEPCK$^{-/-}$ parasites. To exclude the possibility that PEPCK gene has not been acquired back in PEPCK$^{-/-}$ *L. major* used in the primary infection, I performed PCR analysis on genomic DNA of promastigotes isolated from the site of primary infection. The result shown in **Figure 9D** confirms the absence of PEPCK in these null mutants. These results suggest that although infection with PEPCK$^{-/-}$ *L. major* induces an attenuated pathology in

BALB/c mice, vaccination with these null mutants provide moderate protection against *L. major* virulent challenge.

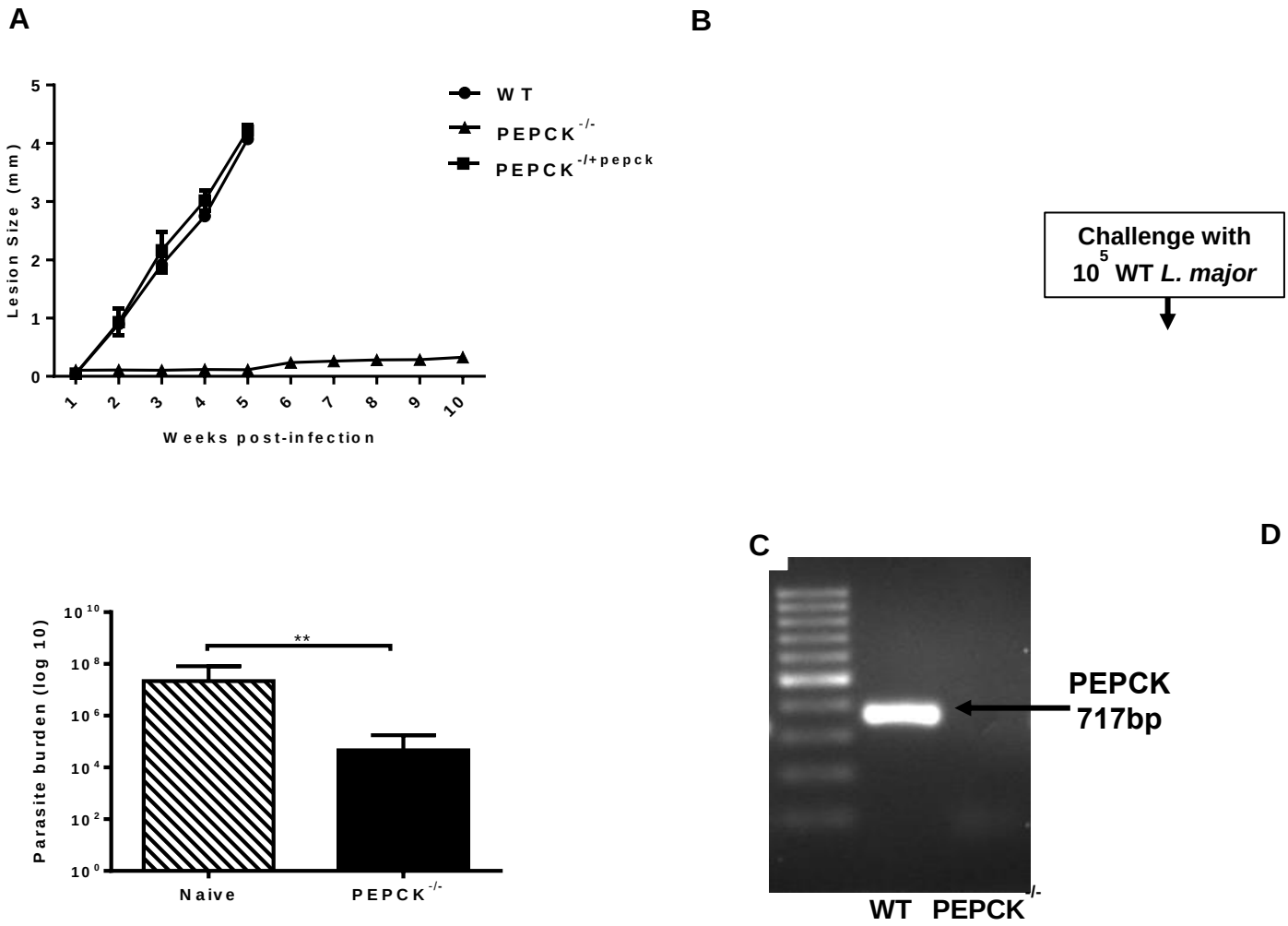


Figure 9. Vaccination with PEPCK deficient parasites induces modest protection against virulent challenge. BALB/c mice and naïve control mice (3 mice per group) were challenged with 5×10^5 virulent *L. major* on contralateral footpad at 10 weeks post-infection. Delayed-type hypersensitivity (DTH) response(A) and parasite burden(B) were assessed at 72 hr and 3 weeks after challenge, respectively. PCR analysis on genomic DNA of PEPCK$^{-/-}$ parasites isolated from primary site of infection confirming the absence of *PEPCK* in PEPCK$^{-/-}$ *L. major*(D). Results presented are representative of 3 independent sets of experiment with similar outcome. **, $p < 0.01$.

4.8 Modest protection in PEPCK null mutants is not associated with strong recall response

Next, I evaluated the antigen-specific recall response in PEPCK$^{-/-}$-infected mice to determine whether the protective immune response in these mice is driven by a strong Th1 response. Therefore, I stimulated dLN cells from primary or secondary (PEPCK$^{-/-}$-infected mice) that were challenged with virulent *L. major* with SLA and assessed the production level of IFN-γ, IL-4, and IL-10 by ELISA. I detected a lower production of IFN-γ as well as IL-4 and IL-10 in PEPCK$^{-/-}$-infected mice as compared with naïve mice **(Figure 10A-10C).** However, the IFN-γ/IL-4 ratio was significantly higher in the former group **(Figure 10D)**. This suggests that the modest protection to virulent challenge in mice infected with PEPCK null mutants is not associated with a strong Th1 recall response. However, the higher ratio of Th1 (IFN-γ) to Th2 (IL-4, IL-10), as observed in these mice, may be responsible for mediating the moderate protection to the secondary infection with *L. major* in PEPCK$^{-/-}$-infected mice.

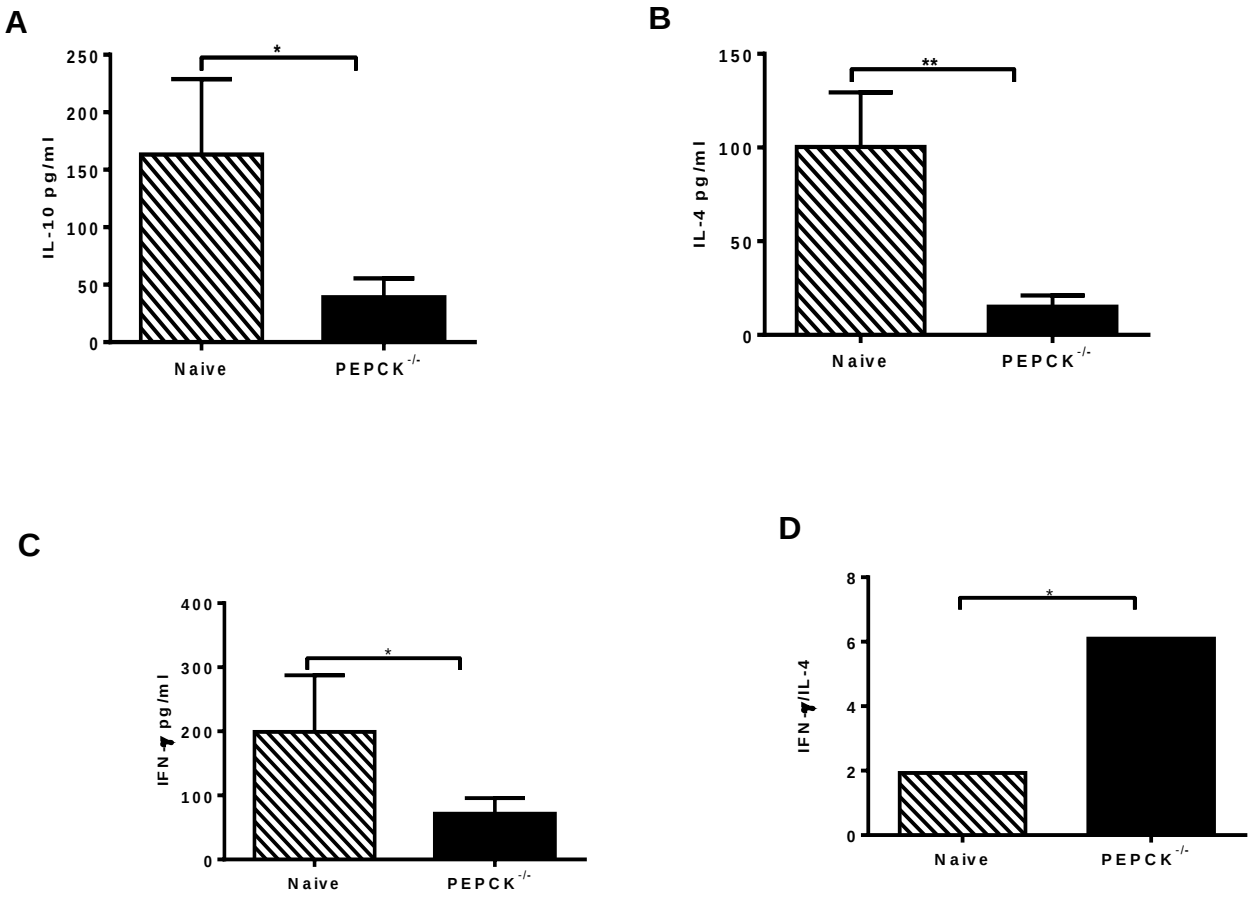

A
IL-10 pg/ml
250
200
150
100
50
0
*
Naive
PEPCK-/-
B
IL-4 pg/ml
150
100
50
0
**
Naive
PEPCK-/-
C
IFN-γ pg/ml
400
300
200
100
0
*
Naive
PEPCK-/-
D
IFN-γ/IL-4
8
6
4
2
0
*
Naive
PEPCK-/-

Figure 10. Modest protection in PEPCK null mutants is not associated with strong recall response. Cells obtained from dLNs were re-stimulated with SLA for 3 days and the level of IFN-γ, IL-4, and IL-10 was determined in resultant supernatant fluids by ELISA(A-C) and the IFN-γ/IL-4 ratio was accordingly determined (D). Results presented are representative of 3 independent sets of experiment with similar results. *, $p < 0.05$; **, $p < 0.01$.

4.9 PEPCK deficiency impacts glycolysis and mitochondrial respiration

Amastigotes and promastigotes are highly adapted to their respective environments. This suggests that the availability of different metabolites is the key factor to determine the metabolic pathways utilized by the different stages of the parasites[34]. Glycolysis is an active pathway in promastigotes as *Leishmania* is constantly exposed to an ample amount of glucose in sandfly midgut[25]. Therefore, to determine whether glycolysis is affected by the targeted loss of PEPCK, I assessed and compared glycolytic activity in different parasite lines by real-time measurement of extracellular acidification rate (ECAR) using Seahorse XF analyzer. I initially optimized the number of parasites in each well and I found that 5×10^6/ml promastigotes yielded a highly confluent monolayer in XF 24 microplates. I seeded WT, PEPCK$^{-/-}$ and PEPCK$^{-/+pepck}$ logarithmic-phase promastigotes in plates coated with Cell-Tak which minimizes the motility of promastigotes. Consequently, sequential injection of glucose, oligomycin and 2-DG allowed for assessment and concurrent measurement of ECAR in each group of parasites. I detected a significantly higher ECAR in PEPCK$^{-/-}$ promastigotes compared to WT and PEPCK$^{-/+pepck}$ parasites suggesting an elevated demand for ATP in promastigotes in the absence of PEPCK **(Figure 11A&11C).**

Next, I assessed the oxygen consumption rate (OCR) to investigate whether the absence of PEPCK alters mitochondrial respiration as the major source of cellular ATP. I observed a 5-fold higher basal OCR in PEPCK$^{-/-}$ promastigotes as compared to WT and PEPCK$^{-/+pepck}$ counterparts

(Figure 11B). Although this result primarily seems inconsistent with *in vitro* attenuated phenotype of PEPCK$^{-/-}$ promastigotes, it is well established that an elevated OCR is an indicator of ATP demand. In this perspective, I found a remarkable reduction in OCR only in PEPCK$^{-/-}$ promastigotes following the addition of oligomycin (complex V ATP synthase inhibitor) which is reflective of a low ATP coupling efficiency in parasites. This result suggests that as opposed to WT and PEPCK$^{-/+pepck}$ parasites, oxygen consumption is not firmly linked to ATP production in PEPCK null mutants **(Figure 11D).** The significantly lower ATP coupling efficiency, as present in PEPCK$^{-/-}$ promastigotes, is due to a severely high proton leak in these parasites **(Figure 11E).** Collectively, these results show glycolysis and oxidative phosphorylation, as the central metabolic pathways in *Leishmania,* are significantly altered due to ablation of PEPCK.

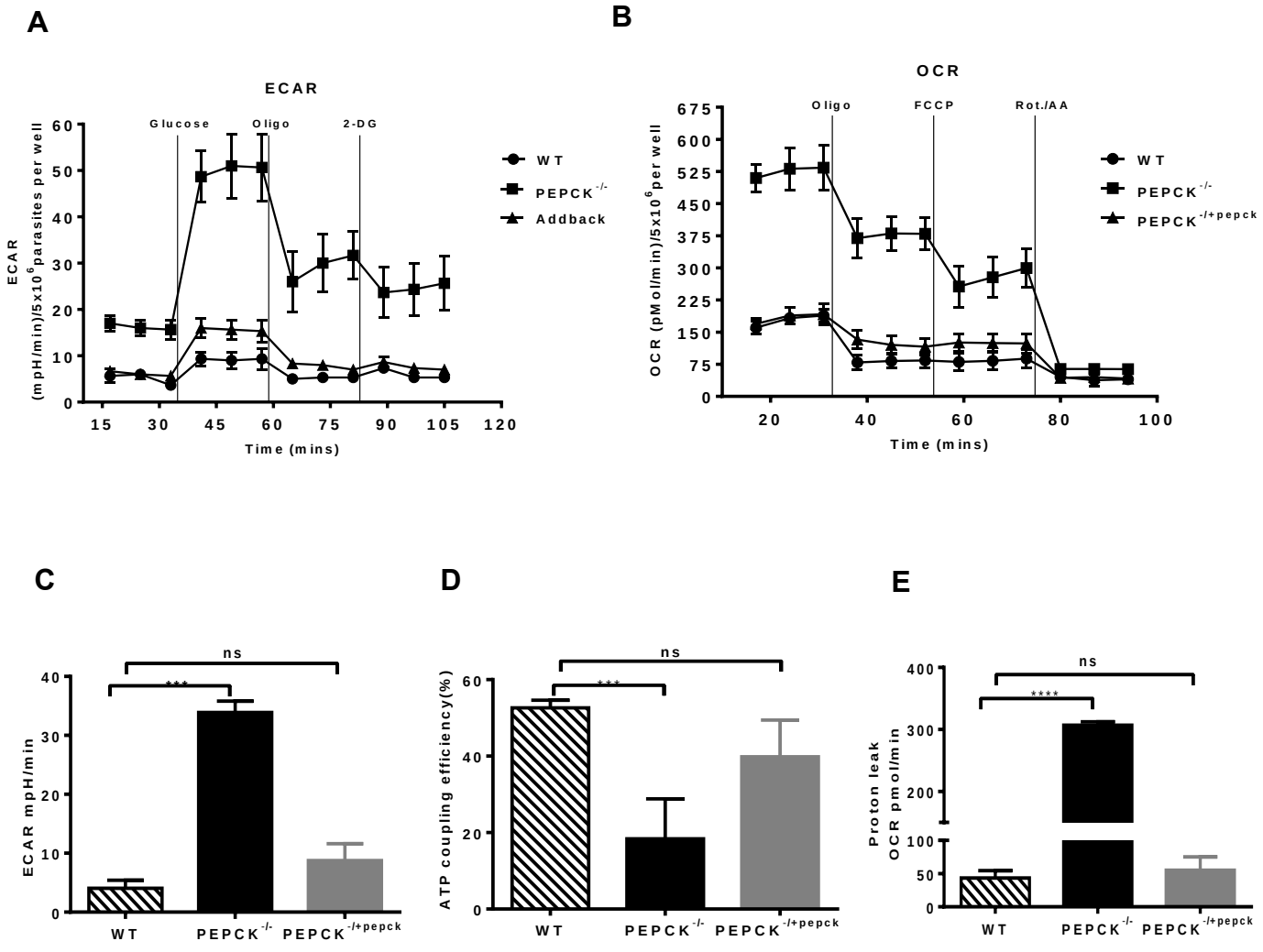


Figure 11. Deficiency of PEPCK affects glycolysis and mitochondrial respiration rates. Five million (5 x 10^6) WT, PEPCK$^{-/-}$ and PEPCK$^{-/-+pepck}$ promastigotes were immobilized unto XF24 microplates with Cell-Tak solution in the presence of glucose, oligomycin or 2-DG and the glycolytic activity was measured as extracellular acidification rate (ECAR) using XF24 analyzer (A) and calculated as the difference between ECAR at basal and ECAR following addition of glucose (C). In addition, immobilized parasites were treated with sequential injection of oligomycin, FCCP and rotenone/Antinomycin A (diluted in XF BASE medium) and mitochondrial function was determined as oxygen consumption rate (B). ATP coupling efficiency (D) and proton leak (E) of e different strains of the parasite were also measured following simultaneous injection of oligomycin into wells. Results presented are representative of 2 independent sets of experiment with similar results. **, $p < 0.01$; ***, $p < 0.001$.

5.1 Discussion

In the present study, I used the CRISPR-Cas9 gene-editing system to delete all copies of the PEPCK gene from *L. major* genome. Next, I compared *in vitro* proliferation, infectivity, and metacyclogenesis of WT, PEPCK$^{-/-,}$ and PEPCK$^{-/+pepck}$ *L. major*. Furthermore, I examined the effect of PEPCK deficiency on pathology and immune response in BALB/c and C57BL/6 mice. Finally, I evaluated the impact that deficiency of PEPCK has on glycolysis and oxidative phosphorylation of *L. major* promastigotes.

CRISPR-Cas9 has emerged as an efficient tool to facilitate sophisticated genome engineering in diverse microorganisms including the human protozoan parasite, *Leishmania major*[188]. The use of this approach to perform functional analysis of *Leishmania* virulent genes as well as identify potential drug targets to control the *Leishmania* infection has recently been prevalent[191]. In the current study, I employed a loss-of-function approach using the CRISPR-Cas9 gene-editing system to further understand the role of PEPCK in the pathogenesis of *Leishmania* infection. I used electroporation to introduce two key components of the CRISPR system, guide RNAs (gRNAs) and Cas9 endonuclease, which are essential to generate site-specific double-stranded DNA break in flanking sequences of PEPCK genes, in *L. major* promastigotes. Given that non-homologous end-joining (NHEJ), which commonly takes place in eukaryotic cells, often results

in undesired mutations[188], I concurrently transfected the promastigotes with a donor template (Bleomycin resistant gene) containing arms homologous to flanking sequences of Cas-9 target on *L. major* genome. Due to the lack of RNA interference pathway in *Leishmania*, specific gene manipulation in these microorganisms mostly depends on the use of antibiotic selection markers which is relatively labor-intensive and time-consuming[22]. Therefore, a more straightforward strategy seems to be necessary for high-throughput editing of the *Leishmania* genome. A stable Bleomycin resistant parasite line which constantly expresses Cas-9 nuclease was subsequently established following multiple rounds of selection on media containing the appropriate antibiotic. I primarily confirmed the deletion of PEPCK using PCR and Western blot analysis **(Figures 1C&1D).** A tetramer reagent, that efficiently identifies PEPCK-specific $CD4^+$ T cells in tissues of mice infected with *L. major*, was recently generated in our lab. Given that PEPCK-specific $CD4^+$ T cells constitute approximately 20% of *Leishmania*-reactive $CD4^+$ T cells at the peak of infection[52], I infected CL57B/6 mice with WT and $PEPCK^{-/-}$ *L. major* and assessed the frequency of PEPCK-specific $CD4^+$ T cells using flow cytometry. Despite the significant expansion of $CD4^+$ T cells specific to PEPCK in both spleen and dLNs of WT-infected mice, no detectable PEPCK-specific $CD4^+$ T cells were observed in tissues of mice infected with $PEPCK^{-/-}$ *L. major* **(Figures 2A-2C)**. $CD4^+$ T cells from TCR transgenic mice show a robust immune response upon exposure to their specific antigen[208]. Therefore, I also utilized the PEPCK TCR transgenic (PEG) mice that we generated to confirm the deletion of PEPCK in the parasites. All the $CD4^+$ T cells in PEG mice only recognize a peptide derived from PEPCK protein and upon such recognition proliferation and produce IFN-γ. I evaluated PEPCK specific immune response following the adoptive transfer of CFSE-labelled $CD4^+$ T cells from PEG mice (CD45.2) into naive C57BL/6 mice (CD45.1). Flow cytometry analysis revealed a massive proliferation and IFN-γ production in PEG $CD4^+$ T cells from WT*L. major infected*-mice. In contrast, PEG T cells in mice infected with $PEPCK^{-/-}$ parasites did not exhibit any detectable proliferation and IFN-γ production **(Figures 2D-2F).** Collectively, these observations strongly confirm the unequivocal targeted loss of the PEPCK gene in $PEPCK^{-/-}$ parasites.

It is well-established that *Leishmania* parasites are largely dependent on glucose and other hexoses as the essential source of carbon and energy to provide parasite redox balance and bioenergetics requirements[28 39]. Similar studies show that *Leishmania* promastigotes with a defect in metabolic enzymes rely on uptake of exogenous glucose to sustain their glycolytic

activities and biosynthesis of essential macromolecules (major surface glycoconjugates and β1, 2 mannan) at a comparable level with WT counterparts[27 28]. Some of these macromolecules such as LPG and mannan are critical for virulence and intracellular survival of both life stages of parasite[16 48]. However, upon glucose exhaustion in media which results in a reduction in glycolysis, WT promastigotes begin to rely on gluconeogenesis to address their cellular hexose requirement[25]. Gluconeogenesis pathway is known to scavenge non-glucose precursors for de-novo sugar biosynthesis through regulatory enzymes. PEPCK, PPDK and GK are among the most well-studied gluconeogenic enzymes that participate in entry and conversion of non-glucose precursors such as aspartate, alanine, and glycerol, respectively[27]. In addition to catalyzing the conversion of oxaloacetate to aspartate, PEPCK concomitantly plays a role in entering aspartate into the glycosomes by the combined action of malic enzyme. It is known that PEPCK is a key gluconeogenic enzyme which is constitutively expressed in all stages of the parasite, its genetic ablation leads to a significant perturbation of the central metabolic pathways in *Leishmania mexicana*[39 27]. However, no reports are showing the role of PEPCK in *in vitro* growth characteristics of *L. major*. therefore, I sought to compare the growth rate of WT, PEPCK$^{-/-,}$ and PEPCK$^{-/+pepck}$ *L. major* in axenic culture. After the inoculation of an equal number of WT, PEPCK$^{-/-}$,and PEPCK$^{-/+pepck}$ promastigotes to the fresh media, I counted the number of parasites for 7 consecutive days. I found that unlike WT and PEPCK$^{-/+pepck}$ promastigotes, PEPCK$^{-/-}$ *L. major* exhibited a reduced level of growth as early as day 3 which coincides with depletion of glucose from culture media. However, the addition of glucose to PEPCK$^{-/-}$ culture fail to rescue the promastigotes growth to a comparable level with WT and PEPCK$^{-/+pepck}$ counterparts. These results indicate that once PEPCK is disrupted, depletion of glucose during the late culture stage leads to compromised growth of promastigotes in axenic culture. Interestingly,the impaired gluconeogenesis in PEPCK$^{-/-}$ promastigotes can not be circumvented by the enhanced glucose-mediated glycolytic rate in these parasites. It shows that gluconeogenesis is a multifunctional pathway that its perpetuation is accompanied by detrimental sequences in *Leishmania* parasites. It further suggests an essential role for gluconeogenesis pathway in *L. major* promastigotes in the absence of sufficient amount of glucose in the environment.

Amastigotes, as the replicative form of parasites, inhabit within mature phagolysosome in the macrophages of their mammalian hosts where they multiply. Amastigotes cause a broad

spectrum of clinical manifestations in the host ranging from an acute infection (self-healing cutaneous lesions) to a chronic or latent disease[92]. Although this life stage of parasite plays a significant role in perpetuating the disease, (and hence serves as a potential target of antileishmanial drug), the metabolic properties of amastigotes, as well as the biochemical structure of phagolysosome, are comparatively less understood[29]. In phagolysosome where there is a rare amount of free sugars, it seems that other carbon sources rather than glucose will supply the energy requirement of amastigotes. Given that protein degradation actively takes place in phagolysosome, this compartment appears to be an accessible reservoir of amino acids as the carbon source for intracellular amastigotes[30]. Promastigotes are internalized by a variety of host immune cells including macrophages, neutrophils, dendritic cells, and fibroblasts. However, the unique biochemical composition of phagolysosome makes it the ideal niche which addresses the complex nutritional requirements of the intracellular life stage of parasite[209]. Several studies have reported that in contrast to the vast majority of intracellular pathogens, *L. major* can not scavenge acetyl Co-A (the major product of fatty acid oxidation)[210]. Consequently, in the absence of an ample amount of glucose, *L. major* mainly relies on a multitude of permeases and hydrolases to salvage the essential metabolites from the existing pool of phagolysosome exogenous and endogenous amino acids[25 211]. Feng *et al*, have shown that developmental differentiation of promastigotes into amastigotes stimulates a stringent metabolic response that leads to the marked downregulation of glucose transporter and consequent reduction in glycolysis[42]. Indeed, the metabolic alteration that occurs during the transformation of parasites would be a reasonable explanation for emerging gluconeogenesis as an essential pathway in amastigotes. This notion is supported by proteomic studies that report the substantial upregulation of gluconeogenic enzymes such as PPDK and PEPCK concurring with the marked downregulation of glycolytic activity during the transformation of promastigotes to amastigotes[20]. However, thus far, transcriptome analysis has failed to find a significant difference in mRNA abundance of aforementioned enzymes[212]. Previous reports suggesting a link between gluconeogenesis and virulence of different *Leishmania* species stimulated my interest to examine whether the PEPCK has an impact on virulence of *L. major* amastigotes. I, thus, infected bone marrow-derived macrophages with stationary phase WT, $PEPCK^{-/-}$ and $PEPCK^{-/+pepck}$ promastigotes, the ratio of 1:5. Next, I counted the number of phagocytosed parasites under optical microscopy at indicated timepoints. Despite the equal number of parasites

in infected bone marrow-derived macrophages at 6 h after infection, in comparison with WT and PEPCK$^{-/+pepck}$ counterparts, I observed a drastically lower number of PEPCK$^{-/-}$ parasites within macrophages at later time points (24, 48 and 72h post-infection). The former observation as well as isolation of a comparable number of PEPCK$^{-/-}$ parasites at the site of infection at all times after infection, suggests that *L. major* promastigotes retain the ability to infect macrophages and differentiating into amastigotes in the absence of PEPCK. However, within hexose-poor phagolysosome, gluconeogenesis turns into a critical pathway to sustain the propagation of parasites at a rate sufficient for inducing mature lesions in infected animals. Collectively, I found that proliferation of *L. major* amastigotes is severely impaired both *in vitro* (within bone-marrow-derived macrophages) and *in vivo* (infected animals) due to genetic ablation of PEPCK. However, in the absence of PEPCK, the viability and growth of *L. major* in a glucose-poor milieu are thought to be partially attributed to the redundant role of different gluconeogenic enzymes in preserving the ATP homeostasis of glycosomes. In this perspective, the contribution level of distinct gluconeogenic enzymes seems to be largely dictated by the life stage of the parasite and various metabolite trafficking between glycosomes and cytosol in promastigotes and amastigotes[27] [20]. PEPCK in combination with malic enzyme participates in sustaining glycosomal level of aspartate used for gluconeogenesis in promastigotes[213], whereas in amastigotes PPDK plays the major role in entry of lactate and alanine to fuel the pathway[26] [211]. Interestingly, amastigotes and promastigotes exhibit a different degree of pliability to ablation of central metabolic pathways. Despite a lack of knowledge on transport systems in glycosomes, it has been suggested that amastigotes have a higher rate of pyruvate trafficking between cytosol and glycosomes compared to promastigotes[27].

Leishmania species experience a wide spectrum of morphological, biochemical, and molecular sequential transitions during their life cycle[214]. Among these developmental changes, metacyclogenesis which is the conversion of poorly infective procyclic to highly infective metacyclic promastigotes occurs in sandfly midgut[215]. Although metacyclogenesis naturally occurs within the midgut of the sandfly, this process could be conveniently replicated in axenic culture. Logarithmic- phase promastigotes resemble procyclic form, whereas upon entry to stationary phase, a proportion of parasites differentiate into metacyclic form and authentically simulate the characteristics of sand fly-developed metacyclic promastigotes[216].

In addition, metacyclogenesis is known to associate with other changes in parasites including modifications in lipophosphoglycan (LPG) structure, morphology[217], and enhanced resistance to human serum[16 218]. Procyclic promastigotes express a longer LPG which facilitates midgut binding. However, the compositional modifications of LPG throughout metacyclogenesis lead to the expression of a smaller LPG in which galactose side chains are capped by arabinose residues[15]. This modification, which is the basis of current purification methods for characterization of metacyclic promastigotes, is a crucial step for many experimental procedures [219]. Considering that arabinose residues prevent lectins such as PNA from binding to LPG, a negative selection method can be utilized to isolate metacyclic promastigotes[15]. In the current study, I aimed to understand if metacyclogenesis and consequently infectivity of *L. major* is influenced by PEPCK deficiency. Therefore, I used FITC-conjugated PNA for purification of both metacyclic and procyclic forms of WT and PEPCK$^{-/-}$ parasites. I found (as expected) that PNA-FITC^{+} parasites that represent procyclic promastigotes comprise the majority of parasites at day 3 post-passage. However, within 4 days, a fraction of WT and PEPCK$^{-/-}$ procyclic promastigotes (~ 33%) differentiated into metacyclic forms which are indicated as PNA-FITC^{-}. To further validate this result, I prepared Giemsa-stained slides of isolated promastigotes and amastigotes to observe the stage-specific morphology of either form under optical microscopy. In this observation, metacyclic parasites with a narrow body and elongated flagella were expectedly distinguishable from procyclic promastigotes that mostly display a small round-shape body with relatively shorter flagella. Collectively, the insignificant difference in the number of WT and PEPCK$^{-/-}$ metacyclic promastigotes (PNA-FITC^{-}) suggests that the absence of PEPCK does not affect metacyclogenesis and infectivity of *L. major* promastigotes. Indeed, this data is consistent with one of the earlier findings of the current study that shows all parasite lines infect bone marrow-derived macrophages to the same degree **(Figures 4A&4B).**

LPG is thought to mediate a resistance against oxidants and serum complement[15 220]. Moreover, LPG in combination with β1,2 mannan oligosaccharide, has an implication in the survival of *Leishmania* within macrophages through interfering with host signaling pathways[48]. However, the conclusion that LPG itself is a virulent factor of *Leishmania* is compromised by the fact that all LPG structural domains could be found in other major surface and secreting molecules (biochemical redundancy)[221]. The expression level of β1,2 mannan gradually increases during differentiation of *Leishmania* species and comprises 90% of cellular carbohydrate in lesion-

derived amastigotes[222]. It has been reported that *Leishmania* parasites under glucose starvation break down mannan, suggesting a reserve role for this carbohydrate[48 211]. This finding has been supported by multiple reports that show the genetic ablation of enzymes involved in mannan biosynthesis leads to an attenuated phenotype in *Leishmania* parasites in glucose-poor milieus such as phagolysosome[48]. Interestingly, the gluconeogenic pathway also contributes to the biosynthesis of *Leishmania* surface macromolecules such as mannan and LPG which have been proved to play a role in parasite virulence[28]. Naderer *et al* have reported that WT *L. major* promastigotes cultivated in either glycerol or glucose-containing media accumulate a high level of mannan[28]. On the contrary, synthesis of these oligosaccharides in promastigotes in the absence of fructose-1, 6-biphosphatase (a key gluconeogenic enzyme) is largely reliant on the availability of glucose in synthetic media. This study also reported that WT and fructose-1, 6-biphosphatase (fbp) deficient promastigotes express a comparable level of mannan and infect macrophages to the same degree. Nevertheless, gluconeogenic enzyme null mutants exhibit reduced proliferation within macrophages[27 28 30]. Indeed, fbp mutants are viable but the intracellular reservoir of mannan is significantly low [92]. These results are consistent with the finding of the current report that showed intracellular replication of *L. major* amastigotes is severely compromised following targeted genetic ablation of PEPCK. In this respect, measurement of intracellular level of mannan in PEPCK$^{-/-}$ promastigotes different parasite lines could bring novel insight into the current study in the future.

The type of early adaptive immune response is the key to determine immunity (resolution of skin lesion) to cutaneous leishmaniasis[121]. It is well-established that following infection with *L. major*, developmental differentiation of IFN-γ producing CD4^{+} Th1 cells, which leads to activation of macrophages, mediates resistant to infection. In contrast, high production of Th2-type cytokines such as IL-4 and IL-10 is associated with susceptibility [53]. We previously showed that PEPCK is an immunodominant antigen that induces a robust Th1-type immune response in mice and humans[52]. Therefore, I investigated whether the ablation of PEPCK alters the immune response following *L. major* infection. I infected highly susceptible BALB/c mice with WT, PEPCK$^{-/-}$ and PEPCK$^{-/+pepck}$ promastigotes and quantified the number of CD4^{+} T cells producing IFN-γ, IL-4 and IL-10 at 3 and 5 weeks post-infection. Interestingly, the direct *ex-vivo* analysis revealed that the number of IFN-γ-producing CD4^{+} T cells in the dLNs and spleens of mice infected with PEPCK$^{-/-}$ *L. major* is significantly lower than those infected with WT and PEPCK^{-}

$^{/+pepck}$ promastigotes. A similar result was obtained in the numbers of IL-4- and IL-10-producing $CD4^+$ T cells in mice infected with PEPCK null mutants. To further validate the flow cytometry data, I assessed the production level of these cytokines by ELISA. Thus, I re-stimulated cells obtained from dLNs and spleens of mice infected with distinct parasite lines with SLA for 3 days and determined the production level of in culture supernatant fluids by ELISA. I detected that cells from $PEPCK^{-/-}$-infected mice produce the minimal level of IFN-γ, IL-4, and IL-10 in comparison to the mice infected with WT and $PEPCK^{-/+pepck}$ *L. major*. The blunted immune response against infection with $PEPCK^{-/-}$ *L. major* is expected as Mou *et al* have previously characterized PEPCK as a highly immunodominant antigen of *Leishmania*[52]. Consistent with similar study by Uzonna *et al*[122 207], in the current report, I found that despite the low level of IFN-γ in mice infected with $PEPCK^{-/-}$ *L. major*, the ratios of IFN-γ to inhibitory cytokines are higher in comparison with mice infected with WT and $PEPCK^{-/+pepck}$ counterparts[207]. IFN-γ-dependent activation of macrophages is an important mechanism to control the replication of parasites in *Leishmania* infection. However, the reduced production level of IFN-γ as a pro-inflammatory cytokine is possibly due to lack of progressive cutaneous lesions in $PEPCK^{-/-}$-mice. Altogether, these findings suggest that the observed *in vivo* attenuated phenotype of $PEPCK^{-/-}$ *L. major* is not entirely associated with a robust T cell-mediated immunity driven by a high level of IFN-γ and activated macrophages.

To date, no effective vaccine that stimulates a robust and durable immunity against infection with *L. major* exists[207 52]. This is mostly attributed to the lack of knowledge about the *Leishmania* immunogenic antigens as well as immunological correlates of protection[223]. However, live-attenuated vaccines have been proved to closely resemble the natural course of *Leishmania* infection[121]. Indeed, live-attenuated vaccines seem to elicit a similar host immune response as they deliver the maximum number of parasite antigens compared to a subunit or recombinant vaccines[224]. One of the key features of potential live-attenuated vaccines is to persist inside the mammalian host without causing actual infection[207], as found with $PEPCK^{-/-}$ *L. major*. In the current study, I aimed to determine whether vaccination with $PEPCK^{-/-}$ parasites would confer protection to secondary virulent challenge in BALB/c mice. Therefore, I challenged previously $PEPCK^{-/-}$-infected BALB/c mice and naïve age-matched controls with virulent *L. major* on the contralateral footpad. The minor DTH response on the challenged footpad as well as relatively low production level of IFN-γ in LN cells of the $PEPCK^{-/-}$-infected

mice are suggestive of moderate protection in these mice. However, the use of PEPCK$^{-/-}$ *L. major* as a live-attenuated vaccine is precluded due to the isolation of a reasonable number of parasites from challenged footpad of mice infected with these parasites. Several related studies have demonstrated that genetic ablation of gluconeogenic enzymes leading to perturbation of central metabolic pathways results in an *in vitro* and *in vivo* attenuated phenotype in these *Leishmania* null mutants[27]. Gene and protein expression and consequent metabolic profile of promastigotes and amastigotes are in part dictated by the carbon source in the environment that they reside. Glycolysis is an active pathway in promastigotes due to constant exposure of this form of the parasite to sugar in the sandfly midgut. However, gluconeogenesis is thought to be a more essential pathway for proliferation and virulence of amastigotes as they inhibit phagolysosome which is an amino acid-rich compartment[34]. Despite the wealth of knowledge on stage-specific gene expression in promastigotes and amastigotes[212], it is relatively less known about the metabolic pathways that underlie virulence in *Leishmania* parasites. Therefore, I evaluated the central metabolic pathways in WT, PEPCK$^{-/-,}$ and PEPCK$^{-/+pepck}$ promastigotes using Seahorse XF analyzer to assess extracellular acidification rate (ECAR) and oxygen consumption rate (OCR). Notably, the following results are the first report on the assessment of ECAR and OCR in *Leishmania* promastigotes as this method is widely used in cancer-related studies to determine the metabolic profile in mammalian cells. In this assay, following seeding of WT, PEPCK$^{-/-}$ and PEPCK$^{-/+pepck}$ promastigotes, XF 24 microplate and sensor cartridge were loaded into the analyzer. The serial compound injection allowed for assessment of glycolytic rate and mitochondrial respiration through ECAR and OCR under certain conditions, respectively. I found that PEPCK$^{-/-}$ promastigotes exhibit a significantly higher ECAR compared to WT and PEPCK$^{-/+pepck}$ following addition of glucose. Given that an intact gluconeogenesis pathway could address all hexose requirements of *L. major* promastigotes[39], the elevated ECAR as present in PEPCK mutants could be possibly explained by a higher demand to maintain ATP homeostasis in theses parasites. On the other hand, the high glycolytic activity reflected as an elevated ECAR might be a redundancy mechanism employed by PEPCK null mutants to compensate for the insufficient reservoir of hexose phosphates. These metabolites are known to be precursors for biosynthesis of surface glycoconjugates and reservoir carbohydrates essential for survival and infectivity of parasites[222 28]. It is notable that the source of ECAR before addition of glucose and in the absence of an active glycolysis pathway is CO_2 produced in TCA cycle. As a result, the

elevated ECAR in PEPCK$^{-/-}$ parasites before addition of glucose to the media is suggestive of higher TCA cycle activity in these parasites compared to WT and PEPCK$^{-/+pepck}$ counterparts. The enhanced TCA cycle activity appears to be a compensatory mechanism exploited by *L. major* promastigotes to compensate for the insufficient amount of phosphoenolpyruvate in the absence of an intact gluconeogenesis pathway caused by PEPCK deficiency. Remarkably, fructose-6-phosphate derived from the gluconeogenic pathway is a key substrate for the biosynthesis of mannan with an established role in survival and virulence of *Leishmania* parasites[222]. This suggests that the targeted loss of PEPCK leading to impaired gluconeogenesis might be partially circumvented by increased glycolytic activity in these parasites. It is anticipated that a high ECAR is accompanied by the accumulation of large amounts of acidic metabolites (pyruvate and lactate) as the final products of the glycolysis pathway. In the presence of glucose as the carbon source, in addition to glycolysis, mitochondrial respiration seems to be involved in energy generation in *Leishmania* promastigotes[25]. Thus, to determine whether mitochondrial respiration is influenced by the loss of PEPCK, I directly measured OCR. Unlike WT and PEPCK$^{-/+pepck}$ promastigotes, I detected a significantly higher basal OCR in PEPCK$^{-/-}$ promastigotes. Considering that OCR is indicative of mitochondria respiration and oxidative phosphorylation[225], this result initially seemed in contrast with this notion that *in vitro* attenuated PEPCK$^{-/-}$ promastigotes are metabolically quiescent. However, it should be noted that basal mitochondrial respiration is attributed to ATP demand. Further analysis revealed that the targeted loss of PEPCK results in an enhanced proton leak. This phenomenon, which refers to ATP synthase independent migration of proton to the matrix, is thought to be one the hallmarks of dysfunctional mitochondrial respiration[226]. Moreover, there is a well-established negative correlation between proton leak and ATP coupling efficiency. In the current assay, the coupling of ATP synthesis and substrate oxidation was measured following the injection of ATP synthase inhibitor oligomycin into media[226]. Collectively, this data suggests that a remarkably high proton leak in PEPCK deficient promastigotes leads to a drastic reduction in ATP coupling efficiency and the production of oxygen reactive species. Furthurmore, the impaired oxidative phosphoriliation that leads to insufficient amount of cellular ATP seems to be mainly responsible for the observed *in vito* attenuated phenotype of PEPCK$^{-/-}$. Remarkably, even the elevated glycolytic rate as demonstrated by the enhanced ECAR **(Figures 9A&9C)** in PEPCK null

mutants is unale to sustain the energytic requiremtns of these parasites to function and proliferate in the axenic culture **(Figures 3A&3B).**

5.2 Caveats and Limitations

The most notable caveat of this study and other studies using animal models is that, regardless of disease category, animal experimentation poorly mimics disease pathogenesis and outcome in humans. Animal models of leishmaniasis offer the benefit of having control over host genetic background, however, none of them perfectly mirrors the clinical and immunological features of human leishmaniasis[227]. Although I observed a remarkably less pathology (reduced parasite proliferation, lesion formation, and blunted immune response) is highly susceptible BALB/c mice infected with PEPCK$^{-/-}$ *L. major*, it remains to be determined whether the same result would be achievable following infection of humans with these mutants. Moreover, the size and nature of inoculum is another factor that greatly contributes to the disparity between experimental and natural *Leishmania* infection[228]. In this study, I always used a certain number of culture-derived metacyclic promastigotes for a single subcutaneous injection of mice in the footpad. Nevertheless, it is widely agreed that sandfly saliva plays a role in enhancing *Leishmania* infection[1]. In this regard, several studies have shown the capability of sandfly saliva in modifying the host immune system possibly through suppression of T cell activation and nitric oxide production[229 9]. Additionally, numerous exposures to bite of sandfly lead to the secretion of neutralizing antibodies against salivary proteins, and this is thought to adversely affect the evolution of *Leishmania* infection[230]. In this perspective, the development of novel approaches to improve experimental models of leishmaniasis seems essential. The amastigotes are the clinically relevant form of parasites which perpetuate *Leishmania* infection in permissive hosts[92]. However, the majority of studies on biochemistry and molecular biology of *L. major* as well as testing leishmanicidal compounds have been conducted on promastigotes. Unlike many other *Leishmania* species, the generation of *L. major* axenic amastigotes is not simply possible through adjustment of pH and temperature of the culture condition. Furthermore, axenic amastigotes do not closely resemble characteristics of lesion-derived amastigotes[38 36]. In this report, to assess glycolytic activity and mitochondrial phosphorylation of parasites in the absence of PEPCK, I seeded logarithmic-phase promastigotes in XF microplates. Despite using a significant amount of Cell-Tak, I could not entirely immobilize the highly motile promastigotes at the bottom of the

well. Given that measurement of ECAR and OCR using XF analyzer requires a confluent monolayer of cells[231], a high variability between replicates was observed following data analysis. To address this, it would be necessary to repeat these studies using lesion-derived or axenic amastigotes which are considerably less motile compared to promastigotes. However, despite many efforts, I failed to generate *L. major* axenic amastigotes. Additionally, due to the remarkably reduced replication of PEPCK$^{-/-}$ amastigotes in infected mice, isolation of sufficient number of lesion-derived amastigotes was not possible. Given that assessment of central metabolic pathways in amastigotes could provide the study with new insights, further effort to establish an effective *in vitro* system for isolation of *L. major* axenic amastigotes seems necessary.

5.3 Future directions

5.3.1 Determine the intracellular level of β1,2 mannan in PEPCK null mutants

We have shown that despite viability, PEPCK$^{-/-}$ amastigotes exhibit a remarkably reduced proliferation in the bone marrow-derived macrophages. It is well agreed that gluconeogenesis pathway participates in the production of hexose phosphates required to fuel other metabolic pathways. *Leishmania* depends on hexoses such as fructose-6-phosphate (a key metabolite of the gluconeogenesis pathway) to sustain biosynthesis of β1, 2 mannan oligosaccharides, and other key metabolites. However the contribution of PEPCK in biosynthesis of mannan (a dynamic reservoir carbohydrate essential for virulence and intracellular life of *Leishmania* parasites) is not clear[222]. It is conceivable that the the lack of enough mannan reservoir in PEPCK$^{-/-}$ parasites is responsible for the observed *in vitro* attenuated phenotype in these parasites. To address this assumption, WT, PEPCK$^{-/-}$ and PEPCK$^{-/+pepck}$ promastigotes will be cultivated in synthetic media containing either glucose or aspartate as the main carbon source. Next, mannan will be extracted from different parasite lines and analyzed by high-pH anion-exchange chromatography (HPAEC). It is expected that unlike WT and PEPCK$^{-/+pepck}$ counterparts, PEPCK$^{-/-}$ promastigotes growing in aspartate-containing media would accumulate a lower amount of mannan due to an impaired gluconeogenesis pathway caused by PEPCK deficiency.

5.3.2 Determine the role of IFN-γ in the attenuated phenotype of PEPCK null mutants *in vivo* through inhibition of IFN-γ signaling

The resolution of *Leishmania* infection has been proved to be mediated by the elaboration of IFN-γ leading to the activation of macrophages. IFN-γ exerts its function by binding to its receptors on the surface of macrophages and inducing an intracellular signaling cascade. However, in this study, we observed a reduced production of IFN-γ in mice infected with PEPCK$^{-/-}$ *L. major*. This observation could be possibly explained by the targeted loss of PEPCK (which is an immunodominant antigen), and the absence of progressive lesions and high level of Th2 cytokines in PEPCK$^{-/-}$-infected mice at all times following infection. However, the role of IFN-γ-associated activation of macrophages as the key mechanism of resistance to *Leishmania* infection could not be simply ruled out. Therefore, to further assess the role of IFN-γ in parasite killing, the IFN-γ will be depleted from mice following weekly injection of anti-IFN-γ monoclonal neutralizing antibody (as previously done)[121]. These mice will be subsequently infected with PEPCK$^{-/-}$ promastigotes and the lesion size and parasite burden will be monitored. It is expected that the attenuated pathology in PEPCK$^{-/-}$-infected BALB/c mice would be reproduced in IFN-γ-deficient mice following infection with PEPCK$^{-/-}$ parasites.

5.3.3 Measure ADP/ATP ratio, evaluate the integrity of mitochondria inner membrane and morphology of PEPCK null mutants

In this study, I observed an elevated ECAR in PEPCK$^{-/-}$ promastigotes as indicative of a high ATP demand in these null mutants. Therefore, the quantitative determination of ADP to ATP ratio in WT and PEPCK$^{-/-}$ promastigotes could provide further explanation on increased glycolytic rate and impared oxidative phosphorylation observed in these parasites. I also found a reduced ATP coupling efficiency and inhanced proton leak that may cause increased level of endogenouse ROS and loss of mitochondrial membrane potential in PEPCK$^{-/-}$ *L. major*, respectively. I will check WT and PEPCK$^{-/-}$ parasites mitochondrial membrane potential using MytoTracker dyes. The use of confocal microscopy will help to determine the loss of mitochondrial innermembrane potential in these parasites. Finally, real-time observation of different parasites with electron microscopy could provide valuable details on morphology and possible structural changes in different organelles of PEPCK$^{-/-}$ *L. major*.

5.4 Major scientific advances

Prior to this work, it was widely believed that resistance to *Leishmania* infection is associated with elevated production of IFN-γ which results in the activation of macrophages to a leishmanicidal state[232]. Results from this study strongly suggest that targeted loss of PEPCK can lead to attenuated pathology in the highly susceptible host, which is manifested as a lack of progressive cutaneous lesions and low parasite burden even in the absence of a strong T cell-mediated response. Although the observed *in vivo* attenuated phenotype may be attributed to an altered metabolic activity due to PEPCK deficiency, the role of IFN-γ in activation of macrophages and eradication of parasites could not be completely excluded.

5.5 Global summary and conclusion

In the current study, I successfully generated a PEPCK (a key gluconeogenic enzyme) deficient *L. major* and showed that genetic ablation of PEPCK results in a compromised growth in axenic cultures following depletion of glucose from culture media. Additionally, I demonstrated that although PEPCK deficiency does not have an impact on metacyclogenesis and infectivity of *L. major* promastigotes, it negatively regulates the proliferation of amastigotes inside bone marrow-derived macrophages. I also found a permanent inability to induce skin lesions in mice infected with PEPCK$^{-/-}$ promastigotes despite the persistence of low numbers of parasites. This finding suggests that *in vitro* attenuated phenotype observed in the PEPCK$^{-/-}$ *L. major* is also reproducible *in vivo.* In line with the previous finding of our lab, I showed that PEPCK is an immunodominant antigen of *L. major* and its targeted loss leads to a blunted immune response and consequently less pathology in the susceptible mice. Surprisingly, I detected a minor DTH response and low production level of IFN-γ in previously PEPCK$^{-/-}$-infected mice following challenge with virulent *L. major* which is indicative of moderate protection to secondary challenge in these mice. The lack of a robust host Th1-type immune response directed me to evaluate central metabolic pathways in these null mutants through assessment of glycolytic activity and mitochondrial respiration. I found a significantly high ECAR and OCR in PEPCK$^{-/-}$ promastigotes which are indicative of high ATP and hexoses demand. Similarly, these parasites exhibited a remarkably high OCR following oligomycin inhibition of ATP synthase, which is linked to a high proton leak leading to a low ATP coupling efficiency. Collectively, studies

reported in this thesis show that PEPCK plays an indispensable role in the pathogenesis of cutaneous leishmaniasis and could be a potential target for antileishmanial drugs.

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