THE ROLE OF ALKYLDIHYDROXYACETONE PHOSPHATE SYNTHASE (ADS) ON HOST IMMUNE RESPONSE AND VIRULENCE OF *LEISHMANIA MAJOR*

BY

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ABSTRACT

Introduction: Leishmaniasis is a spectral disease with clinical manifestations ranging from mild self-healing skin ulcers to chronic mucocutaneous lesions and severe and fatal systemic disease. While drugs are available for treating the disease, most are expensive or highly toxic, and there is emergence of drug-resistant strains. Interestingly, recovery from *Leishmania* infections leads to long-lasting protective immunity, suggesting that the disease can be prevented through vaccination. A key challenge is determining the antigens that could either be used as recombinant vaccine candidates or targeted for the generation of attenuated parasites to be used as a live-attenuated vaccine. Alkyl-dihydroxyacetone phosphate synthase (ADS) is the critical enzyme involved in the biosynthesis of glycerol-containing ether lipids, which is required for synthesizing glycosylphosphatidylinositol (GPI). GPI is important for anchoring lipophosphoglycan (LPG) and gp63, which are major virulence factors of the parasite, to the cell membrane. A deficiency of ADS synthesis leads to impaired synthesis of GPI-anchored molecules resulting in attenuated virulence. However, the impact of ADS deficiency on the immunopathogenesis of cutaneous leishmaniasis has not been studied.

Methods: The growth kinetics of ADS deficient (ADS KO) *L. major* parasites in axenic culture were compared to wild-type (WT) and Add-back (ADS-AB) parasites in axenic culture. Also, bone marrow-derived macrophages were infected with WT, ADS KO and ADS-AB parasites. The infectivity and parasite proliferation were measured and compared at different times using cytospin preparation and Giemsa staining. Balb/c mice were infected intradermally with the other parasites at different time points; parasite burden, lesion size, and cell response in the ear were measured. Balb/c mice were also vaccinated with ADS KO parasites and were also re-

challenged with WT parasite; their DTH response, lesion size, parasite burden, and cytokine response were measured.

Results: Our study shows that deficiency of *ADS* enzyme affects the growth kinetics of *L. major* in axenic culture. In addition, ADS KO parasites showed lower macrophage infectivity *in vitro* compared to their wild-type (WT) controls. Mice infected with ADS KO parasites had a significantly reduced lesion size and parasite burden compared to those infected with WT and ADS AB parasites. ADS KO-infected mice had reduced IFN-γ, IL-4 and IL-10. Mice vaccinated with the ADS KO parasite had a significant DTH response, Parasite burden and lesion size.

Conclusion: Deficiency of ADS in *Leishmania* major affects parasite growth rate in axenic culture, infection rate and proliferation in macrophages *in-vitro. ADS-deficient* parasites have impaired virulence in mice and induce protective immunity in re-challenged mice confirming the critical role of GPI-anchored molecules in parasite proliferation, infectivity and host immune response.

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DEDICATION

I dedicate this thesis to Almighty Allah, the beneficent and the most merciful, for making it possible for me to carry out this fantastic project. Alhamdulilah.

TABLE OF CONTENT

ABSTRACT .... **II**

ACKNOWLEDGEMENT .... **IV**

DEDICATION .... **VI**

CHAPTER 1 .... **1**

1.0 INTRODUCTION **1**

1.1 BACKGROUND 1

1.2 THE VECTOR 3

1.3 LEISHMANIA PARASITE LIFE CYCLE 3

1.4 MORPHOLOGY OF LEISHMANIA PARASITE 6

1.4.1. Leishmania surface coat (Glycocalyx) *6*

1.4.1.1 Glycoinositol phospholipids (GIPLs) / Glycosylphosphatidylinositol (GPI) 7

1.4.1.2 Lipophosphoglycan 7

1.4.1.3 Proteophosphoglycan 8

1.4.1.3 Leishmanolysin (gp63) 9

1.4.1.5 Synthesis of Leishmania surface coat 9

1.4.1.6 Akyldihydroxyacetonephosphate synthase 10

1.5 CLINICAL MANIFESTATION OF LEISHMANIASIS 12

1.5.1 Visceral leishmaniasis (VL) *12*

1.5.2 Mucocutaneous leishmaniasis *13*

1.5.3 Cutaneous leishmaniasis *13*

1.6.1 Microscopy *14*

1.6.1 Parasite culture *14*

1.6.2 Serologic tests *15*

1.6.3 Polymerase chain reaction (PCR) *15*

1.7 TREATMENT 15

1.7.1 Local Treatment *16*

1.7.2 Topical agents *16*

1.7.3 Intralesional drug *17*

1.7.4 Systemic Therapies *17*

1.7.4.1 Oral drugs 17

1.7.4.2 Subcutaneous and intramuscular drug delivery 18

1.7.4.3 Intravenous drug delivery 18

1.8 PREVENTION AND CONTROL OF LEISHMANIASIS 18

1.9 MURINE MODELS FOR CUTANEOUS LEISHMANIASIS 19

1.10 IMMUNE RESPONSE AGAINST CUTANEOUS LEISHMANIASIS IN MICE 20

1.10.1 Innate Immune response *20*

1.10.1.1 Neutrophils 20

1.10.1.2 Macrophages 21

1.10.1.3 Dendritic cells 23

1.10.2 Adaptive Immune response *24*

1.10.2.1 T cells 24

1.10.2.1.1 T helper 1 and 2 cells 25

1.10.2.1.2 T helper 17 cells 27

1.10.2.1.3 Cytotoxic CD8+ T cell 28

1.10.2.1.4 T regulatory cells 30

1.10.2.2 B cells and antibodies 31

1.10.2.3 Cytokines 32

1.10.2.3.1 Interferon- γ 32

1.10.2.3.2 Interleukin-12 33

1.10.2.3.3 Interleukin-4 33

1.10.2.3.4 Interleukin-10 34

1.11 GENE EDITING 35

1.11.1 Gene editing tools *35*

1.11.1.1 Zinc-Fingers Nucleases (ZFN) 36

1.11.1.2 Transcription Activator-Like Effectors Nucleases (TALENs) 36

1.11.1.3 Clustered Regularly Interspaced Short Palindromic Repeat associated protein 9 (CRISPR-Cas9) system 37

1.11.1.4 Leishmania gene editing using CRISPR/Cas9 39

1.12. THESIS OVERVIEW 41

1.12.1 Rationale *41*

1.12.2 Hypothesis *41*

1.12.3 Study aims *41*

1.12.4 Objectives *42*

CHAPTER 2 43

2.0 MATERIALS AND METHODS 43

2.1 MICE 43

2.2 PARASITES 43

2.3 MOUSE INFECTION 45

2.4 ASSESSMENT OF PARASITE PROLIFERATION IN AXENIC CULTURE 47

2.5 ISOLATION OF BONE MARROW CELLS 47

2.6 GENERATION OF MACROPHAGES FROM BONE MARROW CELLS 47

2.7. GENERATION OF DENDRITIC FROM BONE MARROW CELLS 48

2.8. *IN VITRO* INFECTION OF BONE MARROW-DERIVED MACROPHAGE AND DENDRITIC CELLS WITH *L. MAJOR* AND STIMULATIONS 48

2.9 CYTOSPIN 49

2.10 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) QUANTIFICATION OF CYTOKINES 49

2.11 RNA ISOLATION AND REAL-TIME PCR 50

2.12 FLOW CYTOMETRY 51

2.13. STATISTICAL ANALYSIS 52

CHAPTER 3 53

3.0: RESULTS 53

3.1 GENERATION AND CHARACTERIZATION OF NULL MUTANTS IN *L.MAJOR* 53

3.2 ADS DEFICIENT *L. MAJOR* PARASITE SHOWS IMPAIRED GROWTH IN AXENIC CULTURE 56

3.3 ADS GENE-DEFICIENT PARASITES HAVE IMPAIRED ABILITY TO INFECT AND PROLIFERATE IN MACROPHAGES.. 57

3.4 ADS-DEFICIENT PARASITE HAS IMPAIRED VIRULENCE *IN VIVO*... 60

3.5 ADS MAY BE CRITICAL FOR HOST IMMUNE RESPONSE *IN VIVO*.. 62

3.6 VACCINATION WITH ADS DEFICIENT PARASITE INDUCES PROTECTION AGAINST WT RE-CHALLENGE 67

CHAPTER 4 .... **71**

4.1 DISCUSSION 71

4.2 CONCLUSION.. 76

4.3 IMPACT OF STUDY 76

4.4 LIMITATIONS 77

4.5 FUTURE DIRECTIONS 78

REFERENCES **80**

APPENDIX **101**

LIST OF FIGURES

Figure 1: Geographical distribution of Cutaneous leishmaniasis. 2

Figure 2: The life cycle of Leishmania parasites... 5

Figure 3: Diagram of gRNA and Cas9 complex... 38

Figure 4: Diagram showing the CRISPR -Cas9 mediated double-stranded break and different repair mechanism... 39

Figure 5:Generation of ADS null mutants using CRISPR-Cas9 system. 55

Figure 6:The growth of ADS KO parasites is highly compromised in axenic cultures........ 56

Figure 7: ADS deficient parasites have impaired ability to infect and proliferate in macrophages... 59

Figure 8: ADS deficiency results in impaired virulence in vivo. 61

Figure 9: ADS gene product may be important for host immune response in vivo. 63

Figure 10: ADS gene product may be important for host immune response in vivo. 64

Figure 11: ADS gene product may be important for host immune response in vivo. 65

Figure 12: ADS gene product may be important for host immune response in vivo 66

Figure 13: Vaccination with ADS deficient parasite induces protection against WT re-challenge.... 68

Figure 14: Vaccination with ADS deficient parasite induces protection against WT re-challenge.... 69

Figure 15: Vaccination with ADS deficient parasite induces protection against WT re-challenge.. 70

LIST OF TABLES

Table 1: List of Primers used for PCR.. 45

Table 2: List of cytokines, starting recombinant standard dilution, sample dilution and sensitivities of the Sandwich ELISA 50

Table 3: List of Primers used for qPCR.. 51

ABBREVIATIONS

Acronym	**Definition**
AmphB	Amphotericin B
ANOVA	Analysis of Variance
ADP	Adenosine Diphosphate
APC	Antigen-presenting cell
ATP	Adenosine Triphosphate
ADS	Alkyl-dihydroxyacetone phosphate synthase
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
CL	Cutaneous Leishmaniasis
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
CTL	Cytotoxic T cell
DC	Dendritic cell
DCL	Diffuse Cutaneous Leishmaniasis
DHAPAT	Dihydroxyacetone phosphate acyltransferase
dLNs	Draining Lymph nodes
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
EP	Ethanolamine-phosphate
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FCγR III	Fc gamma 3 receptor
FITC	Fluorescein isothiocyanate

Foxp3	Forkhead box p3
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
GIPLs	Glycoinositol phospholipids
HDR	Homology-directed repair
HEPES	Hydroxyethyl-1-piperazineethanesulfonic acid
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
K2HPO4	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
MgCl2	Magnesium Chloride
MHC I	Major Histocompatibility Complex I
MHC II	Major Histocompatibility Complex II
ml	Millilitre
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
NETs	Neutrophil extracellular traps
NHEJ	Non-homologous end-joining
ng	Nanogram
NK	Natural Killer
NO	Nitric Oxide

PAM	Protospacer Adjacent Motif
PBMC	Peripheral Blood Mononuclear Cells
PI	Phosphatidylinositol
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pg	Picogram
PKDL	Post-kala-azar dermal *leishmaniasis*
PMA	Phorbol Myristate Acetate
PMNs	Polymorphonuclear neutrophil
PPG	Proteophosphoglycan
RCDP	Rhizomelic Chondrodysplasia Punctata
rIL-12	Recombinant Interleukin twelve
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute Medium
sAP	Acid phosphatase
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
TRM	issue-resident macrophages
VL	Visceral Leishmaniasis
WT	Wild Type
µg	Microgram
ZFN	Zinc-Fingers Nuclease

CHAPTER 1

1.0 INTRODUCTION

1.1 BACKGROUND

Leishmaniasis is a tropical disease caused by an obligate intracellular protozoan parasite belonging to the genus *Leishmania*. There are over 20 species of *Leishmania* that can infect humans and animals. *Leishmania* is transmitted through the bite of infected female phlebotomies in need of a blood meal to produce eggs[1]. Leishmaniasis is a complex disease that involves clinical manifestations ranging from a mild cutaneous/ skin lesion (cutaneous leishmaniasis (CL)) to a more deadly systemic infection (visceral leishmaniasis, VL, also known as kala-azar)) and mucocutaneous manifestation (mucocutaneous leishmaniasis (MCL))[2]. About 700,000- 1.6 million new leishmaniasis cases are reported annually from almost 100 endemic countries[2].

There are currently an estimated 12 million cases globally, with 350 million people at risk of infection[3]. Cutaneous leishmaniasis is the most common form of the disease, with 95% of the cases occurring in South America, the Mediterranean Basin, the Middle East, and Central Asia. Visceral leishmaniasis is mainly distributed in South Asia, Sub-Saharan Africa, and South and Central America[4]. Over 90% of new cases recorded in 2020 occurred in Brazil, East Africa, India and China[5]. There have been records of complications arising in patients with leishmaniasis; an example is post- kala-azar dermal leishmaniasis (PKDL), a skin condition that develops post-visceral leishmaniasis treatment. An estimated 5-10% of patients with visceral leishmaniasis develop PKDL in India[2]. There is also an increased incidence of *Leishmania*-HIV co-infection[6]. Patients with this condition are at a high risk of developing clinical symptoms, relapse, and death.

These cases have been reported in 45 countries, including India, Brazil, Ethiopia, etc., with India reporting the highest cases[2].

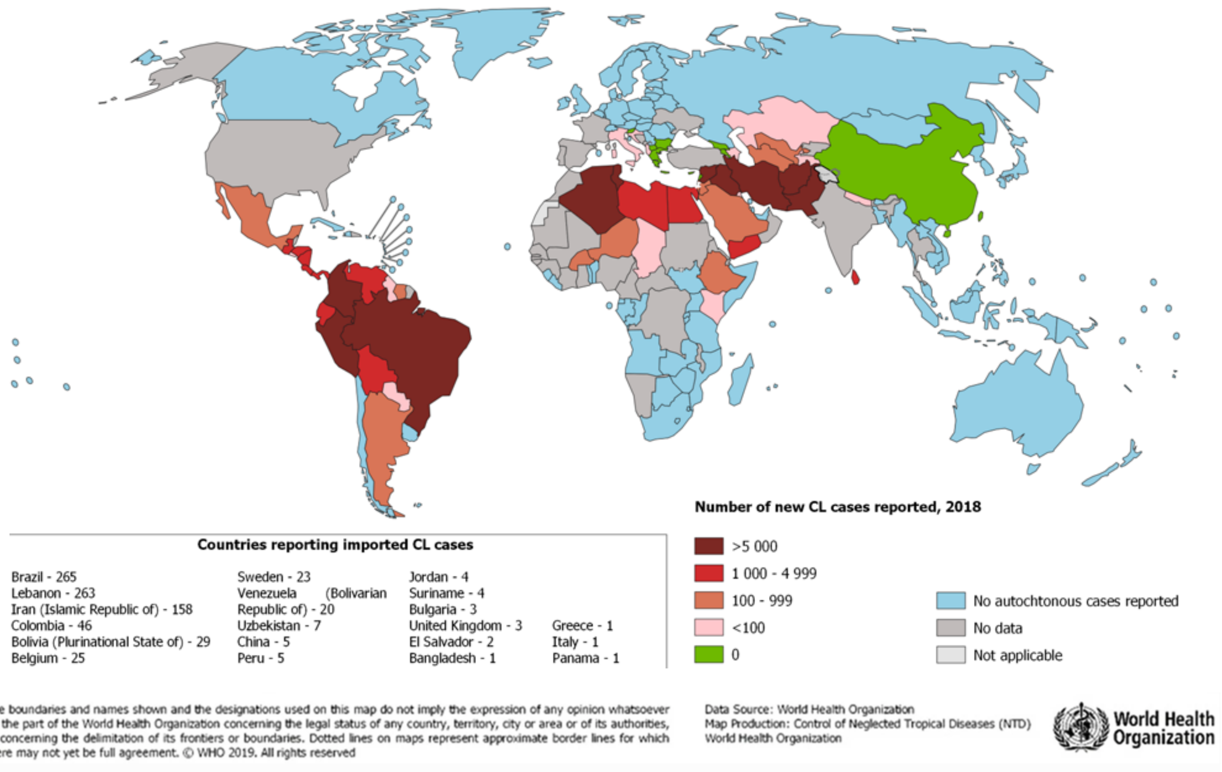


Figure 1: Geographical distribution of Cutaneous leishmaniasis.

The map shows the status and endemicity of cutaneous leishmaniasis worldwide in 2018

1.2 The Vector

Female sandflies transmit *Leishmania* parasites. They belong to the subfamily Phleobotominae. Out of about 800 known species of sandflies, 70 are proven vectors capable of transmitting *Leishmania,* and they belong to the genera Phlebotomus in the old world and Lutzomyia in the New World[7]. Sandflies breeds in habitats with high levels of humidity and organic matter. An adult sandfly is about 2-4mm long with a yellowish hairy body and is nocturnal. Both sexes of sandflies feed on plant juice, but females need blood to lay eggs[8]. Environmental and climate changes have influenced the distribution of sandflies, hence the spread and epidemiology of *Leishmania* parasites. Changes such as urbanization and deforestation can disrupt sandflies' habitat, causing their reach to new regions. Temperature changes can also allow increased transmission of leishmania parasites to new areas. Lack of knowledge on the precise localization of breeding sites of many sandfly species is also a significant constraint for controlling the spread of leishmaniasis[8].

1.3 Leishmania parasite life cycle

Leishmania species have a digenetic lifecycle that involves alternating between a mammalian host and a phlebotomine sandfly host[9]. The developmental stages that *Leishmania* parasites undergo are relatively consistent amongst their species, alternating between a flagellated promastigote to a non-flagellated amastigote. *Leishmania* parasites undergo tremendous biological changes in the mid-gut of sandflies to become flagellated promastigotes. These metacyclic promastigotes (infectious form) are deposited into the skin of the mammalian host through the bite of an infected sandfly during a blood meal. These promastigotes are rapidly picked up by antigen-presenting cells

such as macrophages and dendritic cells present at the site of the infection[10]. Once inside the phagocytic cells, the promastigote undergoes developmental stages inside the phagosome of the cell, where they become non-flagellated amastigotes. These amastigotes survive in macrophages by preventing phagolysosome fusion. Amastigotes rapidly proliferate in infected cells and eventually rupture the infected cell, allowing them to be picked up by another phagocytic cell. Inside the mammalian host, *Leishmania* remains in this form for the rest of its lifecycle. The parasite's lifecycle completes when the amastigotes are picked up during a blood meal on an infected mammalian host.

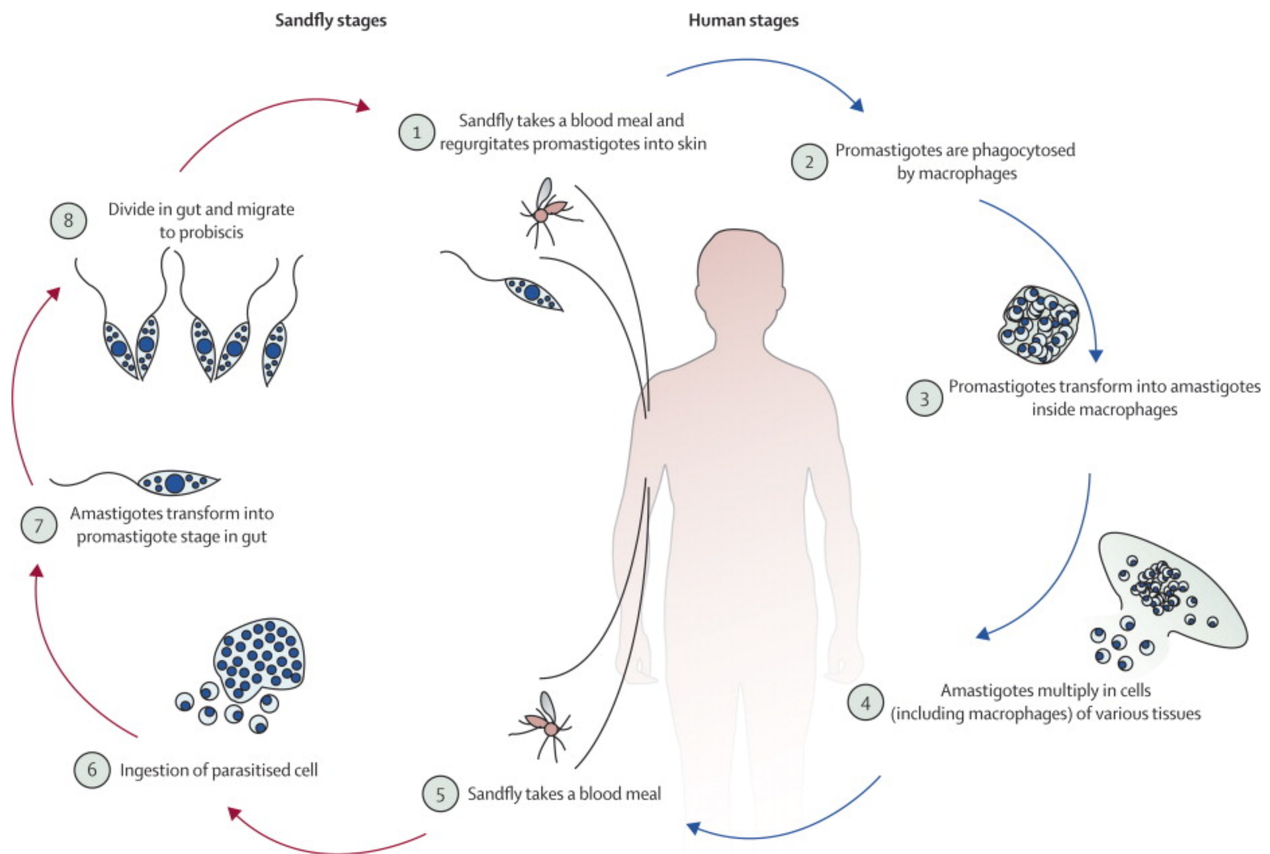


Figure 2: The life cycle of Leishmania parasites.

Leishmania parasites are transmitted by the bites of infected female sandflies, which inject a small number of infectious-stage, metacyclic promastigotes into the skin. These forms are opsonized and taken up by macrophages, where they reside in phagolysosomes and transform into replicating amastigotes. Sandflies take up infected macrophages during blood feeding; they are lysed in the fly midgut, releasing parasites that transform into rapidly dividing, non-infectious-stage promastigotes. These forms undergo a process of attachment to the midgut wall, release and anterior migration accompanied by their differentiation to non-dividing, metacyclic promastigotes that can be transmitted when the sandfly takes another blood meal.

1.3 Morphology of Leishmania parasite

Leishmania parasites have two major morphologies throughout their lifecycle, the promastigote form in sandflies and the amastigote form in mammals. The fundamental cellular architecture is conserved between the two parasite forms[8]. The amastigote form is a small spherical non-flagellated cell ranging from 2-4µm in diameter with a nucleus, and a small ring of vacuolated cytoplasm surrounds the kinetoplast. The promastigotes have slender, elongated cells about 5-14µm long and 1.5-3.5µm in width with a lance-like shape. The elongated cells have anterior kinetoplast and free flagellum. The morphology of the parasite is conserved amongst all *Leishmania* species.[11]

1.4.1. Leishmania surface coat (Glycocalyx)

Leishmania parasite is covered by a thick surface coat (glycocalyx) comprised of glycoconjugates that are important for the parasite's survival and pathogenesis in sandfly and mammalian hosts. The presence of the conspicuous fuzzy surface coat was first detected using ultrasound[12,13]. Biochemical studies of this coat have shown that it comprises Glycosylated proteophosphoglycan (PPG), gp 63 metalloproteinase, glycophosphatidylinositol lipids (GIPLs) and protein-free Lipophosphoglycan (LPG), which are anchored to the parasite surface through the glycosylphosphatidylinositol (GPI) anchors[14]. Under the GPI anchored compounds, a layer of densely packed free GPI glycolipids covers the parasite's cell surface. In addition, protein-linked Proteophosphoglycan (sPPG) and acid phosphatase (sAP) are also secreted by the parasite[15]. Collectively these molecules form a dense protective barrier that protects the parasite from host defence mechanisms such as complement-mediated lysis, oxygen radicals and hydrolases in the sandfly vector and mammalian host[16,17].

1.4.1.1 Glycoinositol phospholipids (GIPLs) / Glycosylphosphatidylinositol (GPI)

GPI is the most abundant glycoconjugate present on the surface coat of the *Leishmania* parasites.[18] GPIs are glycolipids and can serve as anchors for other macromolecular glycoconjugates found on the parasite surface coats. GPI that does not anchor glycoconjugates are called free-GPIs, and they can form densely packed glycocalyx above the plasma membrane. The glycan head groups found in the free-GPIs differ from GPI's, which act as anchors. However, free GPI can have glycan head groups like the GPI anchoring proteins or those anchoring LPG or have glycan headgroup identical to both. Free-GPIs also contain distinct lipid moieties and undergo unique glycan or ethanolamine-phosphate modifications[19]. Glycoconjugates on the surface coat are differentially expressed in the different developmental stages of the leishmania parasite.

GPI-anchored glycoconjugates are highly downregulated in intracellular amastigotes, while the expression of free-GPI remains highly expressed in both promastigote and amastigote forms[20]. The major glycolipid found on the amastigote's cell surface is free-GPIs. However, they can acquire glycosphingolipids from the host macrophage, incorporating them into the exoplasm leaflet above their plasma membrane[21]. The host glycosphingolipids may be incorporated in the same abundance as the free-GPIs[22]. This may represent a method employed by leishmania to evade the host immune response by mimicking the host cell surface membrane.

1.4.1.2 Lipophosphoglycan

Lipophosphoglycan (LPG), a heterogenous lipid-containing polysaccharide compounds[23], is one of the most abundant surfaces glycoconjugates of *Leishmania*, with each parasite containing about six million copies[23]. It has four main domains, 1-0-alkyl-2-lyso-phosphatidyl(myo)inositol, which acts as a lipid anchor for the polysaccharide portion of LPG. In addition, a phosphosaccharide is attached to the inositol anchor, which is the core of LPG[24]. This glycan core

consists of unacetylated glucosamine, mannose, galactose-6-phosphate, galactofuranose and galactopyranose[25].

The lipid anchor and glycan core structure are conserved in all *Leishmania* species. LPG also contains a repeating phosphorylated saccharide region. These repeating units have a PO4-6Gal(b1,4) Man (1) backbone, which is conserved, but some species of *Leishmania*, for example, *L. mexicana* and *L. major*, can have additional sugar molecules branching off the backbone structure[26]. Eighty-seven percent (87%) of galactose residue in *L. major* are substituted with smaller saccharides side chains of glucose or pentose arabinose[27]. The number of repeating units found in LPG directly depends on the growth stage of the promastigote[28].

The final domain of LPG is the capping oligosaccharides; these are small neutral oligosaccharides containing either galactose or mannose. These sugars terminate LPG molecules at the non-reducing ends[29]. Although *L. major* has the most complex repeating unit domain of LPG, they have the simplest capping structure exclusively made up of Man (1,2) Man (1)[30]. On the other hand, *L. donovani* and *L. mexicana* LPG are capped by a complex trisaccharide Gal(b1,4) [Man (1,2)] Man (1)[31] . This variation in cap structure could account for species-specific epitopes observed in serological studies. LPG undergoes much modification during the life cycle of Leishmania parasites, and a lot of these modifications are species-specific[32].

1.4.1.3 Proteophosphoglycan

Proteophosphoglycan(PPG) is a mucin-like glycoprotein that is majorly secreted and found on the surface of the *Leishmania* parasite[33]. PPG is the second major phosphoglycan produced in the *Leishmania* after LPG. PPG is a phosphoglycan like LPG but has a protein core (backbone)[25]. Promastigotes secrete PPG in the mid-gut of sand-fly vector and a medium containing aggregated parasites. PPG is a fibrous filament found at the center extending from the parasite's flagellar

pocket[34]. PPG found in species such as *L. major* have repeating units of amino acids such as serine[25]. Most of these serine amino acids are glycosylated through a phosphodiester bond and sugar molecules similar to LPG[35]. These amino acids play a vital role in the ability of the parasite to attach to host cells[33].

1.4.1.3 Leishmanolysin (gp63)

Leishmanolysin (gp 63), also known as the primary surface protease (MSP), is a zinc-dependent metalloprotease found abundantly on the surface of *Leishmania* parasites. gp63 molecules are attached to the parasite surface through the GPI-anchor, making up about 1% of the parasite cellular protein [36]. Although gp63 is primarily found in extracellular spaces, intracellular accumulation of the gp63 can occur due to extracellular triggers[37]. The cleavage of the gp63 anchor by phospholipase C results in the continuous shedding of the protease to the extracellular spaces. In addition, the protease can also be secreted and exported to extracellular space through the flagellar pocket. The study of the gp63 genomic sequence in various *Leishmania* species has shown a substantial sequence similarity and expression pattern among the species[38,39]. However, the slight differences in the genome sequence and pattern of expression of the gp63 gene across different species are yet to be fully characterized.

1.4.1.5 Synthesis of Leishmania surface coat

The *Leishmania* surface coats (LPG, GPI anchors and free GPI) biosynthesis pathways have yet to be well studied in *L. major*. The synthesis of the precursors for these molecules takes place in the glycosome[40]. Like in every other eukaryotic organism, synthesizing these precursors occurs through the sequential addition of monosaccharides and ethanolamine-phosphate (EP) to phosphatidylinositol (PI). This process of assembly occurs in the endoplasmic reticulum (ER). Phosphatidylinositol containing different lengths of alkyl chains are incorporated differently into

protein anchors, GPI anchor and free GPI. Three pathways are involved in the assembly of the surface coat; one of these pathways affects the addition of a minor pool of PI molecule species containing a very long alkyl chain (20-24 carbon atoms) into mature protein anchor precursors[41]. The second pathway involves incorporating a similar PI species into the LPG anchor precursor (GPI anchors)[40]. In contrast, the final pathway involves a larger pool of PI molecular species containing alkyl chains with 18 carbon atoms incorporated into the free GPI, which makes up 20% of the total PI in *Leishmania* parasites [40]. The different species of Akylacyl-PI lipids are selectively incorporated into GPI anchor, and free-GPI has been said to signify the specificity of enzymes in the biosynthesis pathway of some of these lipid's substrates or that the sequestering of these lipids to a specialized compartment in the endoplasmic reticulum[42]. These pathways have a similar intermediate mannose$_1$-glucosamine (Man$_1$-GlcN)-PI).

1.4.1.6 Akyldihydroxyacetonephosphate synthase

Ether phospholipids, also called ether glycerophospholipids are a subset of phospholipids that contains alkyl or an alkenyl bond at the sn-1 position of the glycerol backbone. The most abundant form of this group of phospholipids are plasmalogens synthesized in the peroxisomes[43]. Apart from their structural roles in cell membranes, ether lipids play essential roles in various cell functions such as signal transduction, neurotransmission, migration, adhesion, vesicular trafficking, and post-translational modifications [44]. Several studies have shown that disruption of lipid metabolism has been related to the onset and progression of some human cancers[45]. Reduced production of ether lipids has been associated with Alzheimer's disease[46].

Alkyl-dihydroxyacetone phosphate synthase (ADS) is one of the two key enzymes involved in ether phospholipid biosynthesis. Dihydroxyacetone phosphate acyltransferase (DHAPAT) and Alkyl-dihydroxyacetone phosphate synthase (ADS) are found in the peroxisome or glycosome.

DHAPA catalyzes the acylation of dihydroxyacetone phosphate (DHAP) to form acyl-DHAP. ADS enzyme is important for the formation of the ether bonds found in alkyl and alk-1 enyl glycerolipids[47]. In addition, ADS catalyzes the conversion of the cleavage and modification of the fatty acid ester of acyl-dihydroxyacetone phosphate (DHAP) to alcohol resulting in the formation of Alkyl-dihydroxyacetone phosphate (DHAP)[48]. ADS is an important enzyme as it catalyzes the formation of the substrate (1-alkyl-DHAP) needed for the synthesis of all ether glycerophospholipids. ADS functions by utilizing a FAD cofactor to exchange the acyl group with an alkyl group[49].

ADS enzyme has been found in all organisms that synthesize ether lipids using the DHAP pathway[50]. In higher eukaryotes, lack of ADS has been associated with impaired sperm biogenesis and eye defects. Mutations in the genes encoding these enzymes have been associated with a human disease called Rhizomelic Chondrodysplasia Punctata (RCDP), characterized by skeletal dysplasia and mental retardation[51, 52]. The depletion of ADS activity in cancer triggered by high levels of ether lipids resulted in the reduced survival and proliferation of aggressive cancer cells and tumor growth[53].

The role of the ADS enzyme and its gene has been characterized in lower organisms. For example in *Trypanosoma brucei*, the ADS enzyme is involved in the biosynthesis of ether lipids in all species and the deletion of the ADS gene did not affect the function and growth of the parasites[54]. In *Leishmania* parasite, ADS enzyme has been linked to the parasite's virulence as null mutants of ADS enzyme have impaired synthesis of their surface molecules such Lipophosphoglycan, which has been associated to virulence[30]. ADS deficiency has also been linked to impaired proliferation and growth of the *Leishmania* parasite as well as delayed onset

of lesions in mice[30]. However, the role of ADS on the host immune response and immunopathogenesis of *Leishmania major,* both *in vitro* and *in vivo* are yet to be studied.

1.5 Clinical manifestation of Leishmaniasis

The clinical manifestations of leishmaniasis are complex and vary depending on the parasite species, host immune system and the environment. These manifestations are further discussed below:

1.5.1 Visceral leishmaniasis (VL)

Visceral leishmaniasis is the deadliest form of Leishmaniasis when left untreated. It is mainly caused by *L. infantum* and *L. donovani*. The disease has varying incubation periods ranging from as low as 10 days to as long as 24 months, with an average of 2-6 months before the infected individual begins to show symptoms[55] This form of Leishmaniasis is also referred to as kala-azar. It is characterized by fever, leucopenia, weight loss, pallor, and hepatosplenomegaly. In severe cases, patients develop respiratory and gastrointestinal disturbances such as vomiting and diarrhea, which leads to malnutrition and lower limb edema. The classic symptoms and signs of VL or clinical presentation of VL are generally the same in endemic regions with slight differences in some specific areas; for example, enlarged lymph nodes are common in Sudanese VL patients, while hyper-pigmentation (kala-azar) is common in the Indian VL patients [56]. Post-kala-azar dermal leishmaniasis (PKDL) is a complication of VL that occurs mainly in patients in East Africa and the Indian subcontinent. It appears as a macular, papular, or nodular rash on the face, upper arm, trunk, and other parts of the body[8]. This group of patients is considered a potential source of new *Leishmania* infection[2].

1.5.2 Mucocutaneous leishmaniasis

Mucocutaneous Leishmaniasis or Mucosal leishmaniasis is a manifestation of leishmaniasis that affects the skin and mucosa around the nose, mouth and tracheal mucosa[44]. This form of leishmaniasis is caused by *L. panamensis*, *L. braziliensis*, and *L. guyanensis*, and it is characterized by ulcerated lesions on exposed parts of the mucosae[58]. This form of Leishmaniasis causes complete or partial damage to the upper respiratory and digestive tract mucosa resulting in lifelong scarring.

1.5.3 Cutaneous leishmaniasis

Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis[59]. This can occur due to infection with over 15 *Leishmania* species, but the most common ones are *L. major*, *L. tropica*, and *L. mexicana*[60]. CL causes clinical manifestations ranging from nodules to ulcerative skin lesions, which leads to lifelong scarring and is sometimes accompanied by local lymphadenopathy. The incubation period of this disease is about 2-8 weeks before the onset of clinical signs and symptoms[61]. The currently available treatments are not very effective, with many side effects and there are also reports of increasing drug resistance[62]. Not much progress has been made toward developing a vaccine for CL. This lack of progress may be due to the complexity of the disease[2]. The course of the CL infection is primarily determined by the specie of parasite and host immune response which is influenced mainly by the host genetics. These factors determine the clinical presentations of CL in different patients[63]. In most patients, the lesion induced after the first infection with CL spontaneously heals and gives rise to lifelong immunity against the disease. In some cases, this immunity might be limited to the specific *Leishmania* species that caused the initial infection or could give a broader coverage against other species of *Leishmania*[64].

1.6 Diagnosis of Cutaneous Leishmaniasis

The diagnosis of cutaneous leishmaniasis is based on clinical features and laboratory testing; however, the accuracy of the existing laboratory-based diagnostics varies significantly. The current methods that have been described include direct parasitological examination, which involves microscopy and parasite cultures. There is also an indirect method of testing that requires serology and molecular diagnostics[65]. The diagnostic test used for patients, especially in endemic regions, is influenced by the available resources, not accuracy.

1.6.1 Microscopy

The microscopic diagnosis of leishmaniasis is performed by identifying amastigotes in smears of biopsies, scraping, and impression smears from lesions and staining with Giemsa. Identified amastigotes are expected to be about 2-4um in diameter with a round or oval body with characteristic nuclei and kinetoplasts[66]. Direct parasitological diagnosis is the gold standard because of its high specificity. However, microscopy has low sensitivity compared to other diagnostic techniques[67].

1.6.1 Parasite culture

The parasite culture method uses a Novy-MacNeal-Nicolle medium to culture fluids or biopsies from suspected lesions in a tube. This process is arduous and requires a significant level of technical expertise[68]. In addition, the parasite culture method is prone to contamination, is not time-efficient, and is highly variable with low sensitivity[69]. However, the invention of mini and micro-culture equipment has allowed for more efficient use of this technique as they are cost-effective, rapid easier to use and more sensitive[68].

1.6.2 Serologic tests

The serological test used to diagnose cutaneous leishmaniasis includes enzyme-linked immunofluorescent assay (ELISA), western blot, lateral flow assay, direct agglutination assays and indirect fluorescence antibody. However, the poor humoral response induced by cutaneous leishmaniasis results in low sensitivity of these tests; hence, their limited use for diagnosis[70].

1.6.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is part of several molecular diagnostic tests that have been designed and developed to diagnose cutaneous leishmaniasis[71]. These molecular techniques are assumed to be more specific and sensitive and use a less invasive method for sample collection than the direct method of diagnosis[72]. PCR has been extensively explored as a single test, nested or qualitative assay. The most common target genes are kinetoplasts DNA sequence and ribosomal DNA internal transcribed spacer I sequence gene[73, 72]. Biopsy smear, tissue scrapping, and aspirated fluids from lesions are used for PCR diagnosis[74]. Other PCR-like methods, such as tryparedoxin peroxidase gene target and HSP70-based assays, are being evaluated to be used[75].

1.7 Treatment

Developing safe, efficacious, and affordable drugs to treat Cutaneous leishmaniasis is critical because only about 66% of CL self-heal without treatment or intervention[67]. In other cases where there are prolonged or non-healing lesions of more than 6 months, either due to immunodeficiency or the presence of multiple lesions, there is a need for treatment[76]. Although several methods have been developed to treat CL, such as local and systemic treatment, the most effective way is to use systemic therapies[77]. Pentavalent antimonial is currently the most effective systemic therapy available, but it gives only about 50% satisfactory clinical and

microbiological results with many side effects[78]. There is also increasing drug resistance amongst patients due to several factors such as HIV/AIDS co-infection[79]

1.7.1 Local Treatment

Several physical treatments such as thermotherapy, cryotherapy and the user of lasers (CO_2 lasers, Argon lasers etc.) have been recommended by the WHO as an alternative for systemic drugs for treating patients with multiple lesions with a minimum diameter of 4 cm that does not involve the face or joints[80]. Thermotherapy involves localized heat application by infrared light, laser, electrical stimulations, or radiofrequency. Radiofrequency is favoured over other local treatment methods because it can uniformly deliver heat waves that penetrate the lesions, allowing heat to kill the amastigote without damaging the surrounding skin. It has been reported that a single application at 50 ºC for 30 seconds is efficient and safe, with a cure rate of 87 to 98%.

1.7.2 Topical agents

Topical agents are very effective against cutaneous leishmaniasis[80]. These agents are usually a combination of Paromomycin, an antibacterial aminoglycoside, with another antibiotic[81]. Paromomycin combination with 12% methyl benzethonium chloride has shown 77% efficacy against Leishmania; a similar success rate was also observed when combined with gentamicin[82]. However, severe side effects such as irritability and intolerance are associated with methyl benzethonium, and mild localized side effects were observed in patients treated with the gentamicin combination[81,82].

1.7.3 Intralesional drug

Intralesional therapy was introduced to reduce the adverse effects of using antimonials systemically[83]. Pentavalent antimonials are effective when used to infiltrate cutaneous lesions locally. This delivery route is as effective as the systemic route in treating cutaneous leishmaniasis caused by both old and new world leishmaniasis[84]. A 75% and 77% cure rate was recorded when this delivery method was used for treating Old World leishmaniasis and New World leishmaniasis, respectively. In addition, an increased efficacy was documented when pentavalent antimonials were combined with other therapies such as sodium stibogluconate and meglumine antimonate[85, 86]. In addition to this method's high efficacy and reduced toxicity, another advantage is the low cost[87].

1.7.4 Systemic Therapies

Systemic therapies use antimonials, antifungals, antidepressants, amiodarone, and immunomodulators to treat cutaneous leishmaniasis. These drugs are administered orally, subcutaneously, intramuscularly, or intravenously[87].

1.7.4.1 Oral drugs

Oral administration of azoles such as ketoconazole, itraconazole and fluconazole has been shown to have variable cure rates in different studies[88]. Long-term treatment with Azole has been recorded to have high toxicity risks such as hepatoxicity[86]. Miltefosine has been reported to have 50% to 80% efficacy against leishmaniasis caused by *L. major* and *L. brazilienses* in different clinical trials[89]. Side effects of miltefosine include vomiting and diarrhea. Hepatic toxicity and nephrotoxicity have been recorded in about 13% of patients treated with this medication[90].

1.7.4.2 Subcutaneous and intramuscular drug delivery

Meglumine antimoniate (MA) is used as the first line of treatment against most leishmaniasis due to its high efficacy. It can be administered intravenously or intralesionally at a recommended dose of 20mg/kg /day for 20 days[91]. However, side effects of this drug include cardiotoxicity and an increase in liver function test markers such as creatinine, erythema, and anorexia[92,85]. The liposomal form of antileishmanial other medicines such as Miltefosine and paromomycin has also been developed, but the efficacy of these drugs is still under review[71].

1.7.4.3 Intravenous drug delivery

Amphotericin B is one of the primary drugs used for the systemic elimination of leishmaniasis[93]. This drug is a macrolide polyene antibiotic, a potent antifungal drug with a high anti-leishmanial activity that targets the principal membrane component of Leishmania spp[94]. Lipid formulations of Amphotericin B were developed to reduce the acute toxicity associated with it[85]. Liposomal Amphotericin B has an efficacy of 95-100% in visceral leishmaniasis and an 85% cure rate in immunocompromised patients treated for Old world cutaneous Leishmaniasis using varying dosages[95].

1.8 Prevention and Control of Leishmaniasis

The lack of an approved vaccine for human leishmaniasis and no effective therapy without severe adverse effects make controlling and preventing the spread of leishmaniasis reliant on combinations of strategies to either avoid exposure or limit the severity of the disease[96]. This strategy includes early diagnosis and prompt treatment, vaccination of animals to control reservoir hosts and strategies to control sandflies. In endemic regions, people are advised to wear clothes that cover as much skin as possible, use insect repellant on exposed skin, spray the indoor

sleeping area with insecticide, and avoid sleeping outdoors between dusk and dawn as sandflies are active during these times.

1.9 Murine models for cutaneous leishmaniasis

Over the last five decades, murine models have been used for the study of human diseases because of the existence of an extensive collection of inbred strain that have a diverse population with identical genetic clones allowing the collection and combination of results of several experiments overtime which enables phenotypic characterization of disease, which maybe difficult to achieve using other mammalian systems[98]. Cutaneous leishmaniasis has been extensively studied using these murine models, and it has been used to elucidate the immune response (cell type, signal transduction cascade and antileishmanial effector mechanisms) involved or necessary for the control of the infection, clinical resolution of the disease, vaccine development and requirements for resistance against secondary infections[99]. Experimental cutaneous leishmaniasis infection in mice presents signs like human cutaneous leishmaniasis. The study of *L. major* infection in mice has allowed for an understanding of the immunological mechanisms controlling resistance (C57BL/6 and C3H strains) and susceptibility (BALB/c strain) of different mice strains to infection[101]. The resistance (self-healing lesion) observed with C57BL/6 has been linked to their ability to develop a T helper type 1 (Th1) immune response which is marked by a strong production of IFN-γ needed for the induction of nitric oxide in macrophages, an important effector molecule for halting the proliferation as well as clearance of intracellular *Leishmania* parasite[102, 103]. In contrast, susceptible BALB/C mice develop severe and non-healing lesions, which have been associated with Th2 response characterized by the enhanced expression of IL-4, IL-10 and TGF-β[104, 105]. These cytokines deactivate macrophages and support the proliferation and survival of *Leishmania* parasites intracellularly.

Although the study of experimental murine model of leishmaniasis has provided great insight into the host response to infection, there are limitations in using this model. This is evident from the fact that disease outcome can be variable depending on the parasite species, tissue target (mice footpad, the base of tail or ear) and the dose of infection used in experiments. These changes generate a wide range of results that are not reproducible in natural human infection or cannot be extrapolated in human disease[106].

1.10 Immune response against cutaneous leishmaniasis in Mice

1.10.1 Innate Immune response

The innate immune response is the host's first defense against invading pathogens. The critical cells involved in the innate immune response against cutaneous leishmaniasis include macrophages, neutrophils, dendritic cells, and Natural killer cells. These cells play essential roles in determining the susceptibility or resistance of the infected mice to *L. major*[107].

1.10.1.1 Neutrophils

Neutrophils are the most abundant subset of leukocytes in circulation in the human blood[108]. These cells are among the first group recruited to the site of infection or injury, and they play significant roles in the innate immune response against pathogens. Once recruited to the area of infection, neutrophils phagocytose the invading organism and kill them using a variety of mechanisms such as the production of reactive oxygen species (ROS), which is toxic to microorganisms[109]. In addition, the formation of neutrophil extracellular traps (NETs), which are extracellular web-like structures that contain chromatin associated with antimicrobial granule protein, helps to limit the spread of pathogens[110]. These cells also have cytoplasm rich in

granules containing microbicidal proteins, which are rapidly released in the phagosome to kill phagocytosed pathogens.

In cutaneous leishmaniasis, neutrophils have been shown to play controversial roles depending on the host's species and genetic background. During the early onset of infection, the parasite uses neutrophils as Trojan horses to infect other phagocytic cells[111], suggesting that they play a negative role in disease pathogenesis. Neutrophils have also been shown to promote parasite killing by releasing NETs. They are recruited to the site of infection in different waves, and the maintenance of these cells at the site of infection varies depending on the parasite species[112]In contrast, the depletion of neutrophils in susceptible Balb/c mice leads to reduced Th2- type response and enhanced resistance[113].

1.10.1.2 Macrophages

Macrophages are a component of the innate immune system that specializes in detecting, phagocytosing, and destroying harmful pathogens in the body. Macrophages are widely distributed throughout the body in lymphoid and non-lymphoid tissues. These cells were initially thought to arise only from circulating blood monocytes which originate from the bone marrow and continuously traffic to different tissues to differentiate into macrophages[114]. However, it has been discovered that tissue-resident macrophages (TRM) are derived from the embryonic/ yolk sac and fetal monocytes progenitors[114]. These cells can maintain themselves independent of the circulating blood monocytes; when damaged, they can be replaced by the circulating monocytes[115]. In addition to phagocytosing foreign entities in the body, macrophages also function to maintain functional homeostatic balance in the body, which involves clearance of cellular debris, tissue repair, remodelling and serving as professional antigen-presenting cells to the adaptive immune cells[116].

Leishmania parasites infect and interact with various host cells, but macrophages and dendritic cells play essential roles in regulating the outcome of the infection. Macrophages are the primary resident cells for *Leishmania* parasites because they have unique biochemical structures that support the nutritional requirements of the amastigote form of the parasite. After the promastigotes are deposited into the host's skin, macrophages are recruited to the site of infection, and they pick up and internalize the parasites into the phagosome of the cell[117]. This phagosome fuses with lysosomes to form a phagolysosome, which contains antimicrobial enzymes creating a hostile environment that is expected to kill the parasites by design[110]. *Leishmania* is among the few microorganisms that have developed evasive mechanisms to thrive in this harsh environment.

Infected macrophages require adequate activation signaling to eradicate *Leishmania* parasites[100]. Depending on the type of activation signal that infected macrophages receive, they can either be classically activated (M1) or alternatively activated (M2). Classically activated macrophages receive a strong IFN-γ signal which is typically secreted by CD4+ Th1 cells, CD8+ T cells and NK cells. Upon IFN-γ stimulation, macrophages produce inducible nitric oxide synthase (iNOS), which breaks down L arginine to produce nitric oxide (NO)[118]. Nitric oxide is vital for killing intracellular pathogens, including *Leishmania*. In addition to IFN-γ, other cytokines such as TNF, IFN-α, IFN-β and IL-1 have been shown to classically activate macrophages and cause an upregulation of iNOS[119]. In contrast, alternatively activated macrophages receive Th2 cytokines such as IL-4 and IL-13, which have been shown to promote the production of polyamines through the upregulation of arginase that favors parasite survival within macrophages[120]. *Leishmania* parasite manipulates this pathway by preferentially expressing its endogenously

encoded arginase to enhance alternative activation of infected macrophages to avoid parasite killing and improve its survival and proliferation[121,122].

1.10.1.3 Dendritic cells

Dendritic cells (DC) are a heterogeneous family of professional antigen-presenting cells (APCs) that link the innate immune system to the adaptive immune system. Although these cells reside in all peripheral tissue in an immature state, they can rapidly undergo maturation and process antigen to be presented to naïve T cells in the T cell zone of the lymphoid organ, thereby modulating T cell response[123]. DCs, like macrophages, are infected by *L. major* and are regarded as the primary source of IL-12 cytokine during the early phases of infection. IL-12 is important for Th1 cell differentiation needed for protection during *Leishmania* infection[124].

Five different subsets of dendritic cells have been identified in the skin of mice: Langerin^{+}CD11b^{-}, dermal DC XCR1^{+}CD11b^{-} DC and Langerin^{-}XCR1^{-} DC, dermal-epidermal Langerhans cells[125]. These cells have been shown to function differently during T-cell mediated immunity and differences in the disease during *L. major infections* in BALB/c and C57BL/6 are believed to be a result of the differences in how the different DC sub-sets mediate T-cell response[126]. Epidermal Langerhans cells may play pathogenic roles during low-dose infection by inducing more regulatory T cells[127]. However, some studies have highlighted that DC isolated from the skin of the different mice showed a similar capability to produce IL-12 and express co-stimulatory molecules[128]. Dermal dendritic cells have also been shown to engulf parasites and traffic them into the lymph node draining the infection site to promote Th1 immunity field[129]. DC express co-stimulatory molecules such as CD40, C80, CD86 and MHC II on their surface, and these molecules have been shown to play essential roles in the outcome of infection[126].

The co-stimulatory molecules expressed on the DC are essential for T cell activation. CD40 expression is needed for optimal IL-12 secretion by DCs. Deficiency in CD40 expression has been demonstrated by Okwor et al. to lead to increased susceptibility to *L. major* infection due to reduced secretion of IL-12, which resulted in decreased IFN-γ production[130]. The roles of CD80 and CD86 in shaping the out come of *L. major* infection have also been reported in several studies[131]. Early Th1 response in C57BL/6 mice infected with *L. major* and Th2 response in BALB/c have been linked to CD86 expression, while CD80 expression has been shown to induce IL-4 and IFN-γ from Th2 and Th1 cells, respectively[132].

1.10.2 Adaptive Immune response

The adaptive immune response kicks in after the innate immune cells have been activated and APCs present *Leishmania* peptides to T cells or following recognition of *Leishmania* antigens to B cells. These events lead to activation of adaptive immune cells triggering *Leishmania*-specific immune response. The adaptive immune response involves a complex interplay between T-cells, B cells (antibody-mediated immune response) and the induction of immune memory[133]. These complex immune responses facilitate the resolution of cutaneous leishmaniasis and determine the phenotypes of the clinical presentations observed in patients. A good balance of adaptive immune response is vital for the resolution of cutaneous leishmaniasis[134].

1.10.2.1 T cells

T cells are essential to the adaptive immune system. They are produced in the bone marrow and undergo development in the thymus to uniquely rearrange their T cell receptors for antigen recognition. T cells are divided into subsets based on their helper, cytotoxicity and regulatory functions. CD4+ and CD8+ T cells are well-studied subsets of the T cells, which are associated

with adaptive immunity[135, 136]. They can proliferate and differentiate into effector cells and memory cells after activation. T cells coordinate complex immune responses, including activation of macrophages, which leads to an influx of inflammatory cells to the site of infection, enhancing B cell differentiation into antibody-producing plasma cells and production of effector cytokines.

Antigen recognition is essential for T cell activation and function. The T cell receptors can only recognize short peptides derived from an antigen, not the entire antigen. These short peptides are presented to T cells through a surface receptor called MHC I, or MHC II expressed on APCs[137]. Once APCs detect a pathogen, they phagocytose and break them down (process) into short peptides that the T cell receptor can recognize. Antigens presented on MHC class I or MHC class II molecules are essential for CD8+T cell and CD4+ T cell activation, respectively. MHC class I molecules are ubiquitously expressed on all cells in the host, while MHC class II is exclusively expressed on antigen-presenting cells[138].

T cells play a critical role in the resistance of the host against leishmaniasis because cell-mediated immunity is vital for controlling the proliferation and differentiation of intracellular pathogens like *Leishmania*. The role of the different subsets of T cells in the pathogenesis and control of leishmaniasis has been well documented in various studies[63].

1.10.2.1.1 T helper 1 and 2 cells

Upon activation, naïve $CD4^+$ T cells differentiate into different helper T cell subsets. This differentiation is dependent on factors such as the cytokines in the microenvironment and the strength of the signal from the TCR interaction with the antigen presented by the MHC-II molecule on APCs[139]. Naïve CD4+ T cell following exposure to antigen differentiates into Th1

cell when there is an optimal amount of IL-12 in the microenvironment[140]. Other factors apart from the antigen recognition and IL-12 have been shown to play essential roles in the activation and differentiation of naïve CD4+ T cell into a Th1 cell, including the interaction of co-stimulatory molecules CD40L and CD28 present on the T cell with CD40, CD80, and CD86 on APC[141].

Naïve CD4+ T cell has also been shown to differentiate into Th2 cells in the presence of IL-4. Mast cells, eosinophils and basophils are believed to be the initial source of this IL-4 needed for this differentiation[142,143,144]. In addition to IL-4, transcription factors STAT 6 and OX40 ligand have been shown to be essential to Th2 cell differentiation.[145,146]

During experimental cutaneous leishmaniasis in mice, a robust Th1 response marked by a high-level IFN-γ cytokine confers resistance; on the other hand, a high level of IL-4 produced by Th2 cells increases the susceptibility of the mice[63]. This Th1-Th2 interplay is seen in the outcome of the CL infection in C57BL/6 and BALB/C mice. The resistance of C57BLL/6 mice is associated with a strong Th1 response, while the susceptibility of BALB/C mice is promoted by early IL-4 secreted by CD4+ Th2 cells. IL-12 produced by DCs early during infection has been suggested to determine the preferential development of Th1 and Th2 cells in mice[147]. This was shown in a study where susceptible BALB/c mice became resistant to *Leishmania* infection after administering IL-12, while C57BL/6 mice became susceptible after anti-IL-12 antibodies administration. The dominant factor that mediates a Th2 immunity is not well understood. One of the factors that have been shown in studies to influence the development of a Th1 or Th2 type response is antigen dose.[148] The impact of antigen dose has been shown in studies done both *in vitro* and *in vivo*. In *in vitro* studies, a low antigen dose induced a Th2 response, while a high antigen dose enhanced a Th1 response. *In vivo* studies by Uzonna et al. showed that low-dose

parasite infection in C57BL/6 mice caused a transient Th2 response swiftly followed by a Th1 response, which was attributed to the IFN-γ produced by activated CD8 T cells; this eventually resulted in complete healing[149]. Additionally, a study done by Bretscher et al. showed that low-dose parasite infection in innately susceptible BALB/c mice resulted in resistance[150].

1.10.2.1.2 T helper 17 cells

T helper 17 (Th17) cells are a unique subset of CD4+ T cells that produce IL-17 cytokine. Naïve CD4+ T cell differentiates into Th17 cells in the presence of cytokines such as IL-6, IL-23, IL-1β and transforming growth factor-β (TGF-β) in the microenvironment[151]. Th17 was initially characterized as an effector CD4+T cell that promotes autoimmune and inflammatory disease because they stimulate stromal cells to produce inflammatory cytokines and recruit neutrophils and other inflammatory leukocytes[152]. The differentiation of naïve CD4+ T cells into Th17 is slightly more complex than Th1& Th2 differentiation in mice and humans. Th17 differentiation following exposure of naïve CD4+T cells to TGF-β and IL-6 is characterized by the expression transcription factor RORγt[153]. It has been reported that IL-4 and IFN-γ, the signature cytokines of Th1 and Th2, inhibit the development of Th17 cells, but TGF-β has also been shown to repress the expression of these cytokines. IL-23 was initially thought to be needed for Th17 development, but recent studies suggest it is required for the maintenance and expansion of Th17[154].

Th17 shares plasticity with T-regs, another subset of CD4+ T cells. The expression of RORγ-t suppresses the differentiation and proliferation of Foxp3+ T-reg and vice versa[155]. Essentially this suggests that depending on the cytokines present and the transcription factors expressed, a naïve CD4+ T cell can either become a Th17 or a T-reg. When TGF-β is abundant in the

environment, Foxp3 is expressed, resulting in T-reg differentiation. On the other hand, when IL-6 is abundant, RORγ-t is expressed, giving rise to Th17 cells[156].

Th17 has two distinct phenotypes; classical Th17 cells develop in the presence of abundant TGF-β but limited IL-23, while alternative Th17 cells develop when only IL-23 is abundant in the microenvironment[157]. These two subsets of Th17 cells have been shown in studies to function differently. Alternative Th17 was found to be more pathogenic than classical Th17 cells[158].

The role of Th17 cells and their effector cytokines in the immune response during *Leishmania* infection varies depending on the parasite species involved. IL17a has been associated with better clinical outcomes in patients with *L. braziliensis* infection because patients with high levels of IL-17A were almost or entirely asymptomatic[159]. This observation suggested that Th17 may play a protective role against cutaneous leishmaniasis. In line with this, the enhanced resistance to *L. major* infection in mice deficient in the expression of long pentraxin 3 (PTX3) was associated with increased frequency of Th17 cells leading to increased production of IL-17A (Gupta et al.). Treatment of pTX3 deficient mice with anti-IL-17 mAb abolished this enhanced resistance, confirming the role of IL-17 in this process (Gupta et al.). The protective role of Th17 is also seen during visceral leishmaniasis caused by *L. infantum* or *L. donovani* infection[160, 161]. In contrast, Th17 has been shown to mediate susceptibility to *Leishmania* infection caused by *L. major*[161,160].

1.10.2.1.3 Cytotoxic CD8+ T cell

CD8+ cells (also called cytotoxic cells) have CD8 receptors on their surface that help them to interact with antigenic peptides presented on the MHC I molecule of infected cells, leading to

their activation. CD8+ T cells carry out their effector functions through secretion of IFN-γ, granzymes and perforins [149].

The role of cytotoxic T cells role in modulating host immune response against parasitic infections, including *Leishmania* parasites, is controversial[162]. CD4 T cells are generally regarded as the champions of anti-*Leishmania* immunity because they produce IFN-γ. However, several studies have shown that CD8+T cells contribute to shaping this CD4+ cell-mediated immunity[163,149]. The role of CD8+ T cells in a vaccine-mediated immunity is well defined. Vaccination with leishmania homologue of activated C kinase (LACK) resulted in protective immunity in the vaccinated animals. This immunity was mediated by antigen-specific CD8+ T cells[68]. The recent study by Uzonna et al. showed that CD8+T cells mediated low-dose protection following *L. major* infection by suppressing a transient early Th2 response [149]. Low-dose parasite infection in CD8+ T cell-deficient C57BL/6 mice resulted in a persistent Th2 response and uncontrolled parasite proliferation, while the administration of recombinant IL-12 or anti-IL-4 resulted in a significant Th1 response[164]. Together these observations suggest that CD8+ T cells may modulate CD4+ T-cell's function through the secretion of IFN-γ[149]. CD8+T cells reconstituted RAG mice infected with *L. brazilliensis* developed lesions with uncontrolled parasites. These lesions had a high frequency of pro-IL1β positive cells, which led to increased secretion of IL-1β compared with RAG mice controls that did not develop any lesions. The activation of IL-1β was found to be dependent on the caspase -1 and NLRP3 -inflammasomes. The administration of treatment to inhibit the activation of NLRP3 inflammasome following leishmania infection in mice prevented the development of severe disease compared to untreated mice. Taken together, CD8+ T cells cause more pathology in cutaneous leishmaniasis, and this

cytotoxicity is mediated by inflammatory mediator (IL-1β) and activation of inflammasome complex (NLRP 3 inflammasome).[165]

1.10.2.1.4 T regulatory cells

T regulatory cells are a specialized subset of CD4+ T cells that play a role in regulating the immune response to maintain homeostasis and tolerance to self-antigens, which is important in preventing autoimmune diseases[166]. Regulatory T cells are classified into two groups, natural T-regs and induced T-regs. Foxp3 has been identified as the key identifier of regulatory T cells. CD25 was initially thought to be their classic marker[166].

Following infection, the host must modulate the magnitude of the immune response to avoid damage to host cells, and this process involves regulatory T cells. During *Leishmania* infection, Tregs at the site of infection plays a dual role in controlling immune response and promoting the survival of some parasites allowing them to persist long after the lesion has healed[167]. T-regs are known to carry out their effector functions through the secretion of TGF-β and IL-10[168]. These cytokines carry out the immunoregulatory function of suppressing the effector function of Th1 cells. The immunoregulatory function of T-regs is essential for maintaining the pool of antigen-specific T cells that would be needed to mount a robust immune response upon re-infection[169]. High-frequency of IL-10 producing T regs have been shown to promote an uncontrolled proliferation of parasites[170]. It has also been shown that p110d^{D910A} deficient mice developed hyper-resistance following cutaneous leishmania infection, these mice developed little to no lesions following infection and rapidly cleared their infection even with a suppressed Th1 and Th2 response. This enhanced resistance was associated with reduced function and frequency of Tregs and the resolution of inflammation regardless of the mouse background[171].

1.10.2.2 B cells and antibodies

B cells, also called B lymphocytes, develop in a complex microenvironment created by stromal cells in the bone marrow, where it receives stimuli and factors needed to initiate the series of cell signals required for its development[172]. An example of this is IL-17 augments the metabolism of fibroblastic reticulum cells in lymph nodes which enhances their proliferation and survival enabling them provide support for B cells during autoimmunity. B cells are at the centre of the humoral immune response of the adaptive immunity. They are responsible for mediating antigen-specific antibodies. After activation, B cells differentiate into antibody-producing plasma cells[173,174].

B cells are typically not considered to contribute to the protective immune response against cutaneous leishmaniasis because *Leishmania* are intracellular parasites that reside in vacuoles inside infected cells which may not be easily accessible to antibodies[175]. The non-protective role of B cell was demonstrated in a study where sub-lethally irradiated BALB/c mice infected with *L. major* were not protected when adoptively given B cell[176]or serum from healed mice. In contrast, these recipient mice showed resistance when they received CD4+T cells from healed animals[177]. In another study by Miles et al., the anti-*Leishmania* IgG antibody enhanced the production of IL-10 in macrophages, resulting in increased parasite survival and decreased resistance[178]. These observations suggest that B cells exacerbate the disease[178]. In contrast to this view, another study by Woelbing et al. showed that IgG antibodies produced by activated B cells enhance antigen uptake and presentation by DC through Fc gamma 3 receptor (FCγR III) during Leishmania infection resulting in resistance[128].

1.10.2.3 Cytokines

Cytokines are small proteins secreted by cells these proteins influence the interaction and communication of cells. Cytokine can act in an autocrine or paracrine manner. They are both pro-inflammatory and anti-inflammatory cytokines[179]. Different cytokines have been shown to play roles in the immune response to Leishmania infection.

1.10.2.3.1 Interferon- γ

Interferon- γ (IFN-γ) is a multifunctional molecule that has been associated with pro-apoptotic, antitumor and antiproliferative mechanisms. IFN-γ has a lot of immunomodulatory properties and is regarded as the central coordinator of the immune response[180].

IFN-γ is a crucial cytokine required for the optimal activation of infected macrophages to upregulate their nitric oxide (NO) production to effectively clear *Leishmania* parasites[181]. The protective role of IFN-γ was highlighted when anti- IFN-γ antibodies were used to treat C57BL/6 mice, resulting in the loss of their resistance[182]. C57BL/6 mice deficient in IFN- γ production or IFN- γ receptor expression lose their resistance to Leishmania infection. These mice are unable to activate macrophages, therefore, were unable to clear parasites using iNOS[183]. On the other hand, when *Leishmania* infected BALB/c mice were treated with recombinant IFN-γ, it resulted in delayed onset of disease[184]. This outcome is not surprising as IFN-γ is vital for stimulating the differentiation of naïve CD4+into Th1 cell, which is essential for control of infection and suppresses Th2 cytokine, which has been demonstrated in studies to favour parasite survival and enhanced pathogenesis. IFN-γ is produced by Th1 cells, NK cells and CD8+ T cells[185,186,149].

1.10.2.3.2 Interleukin-12

Interleukin-12 (IL-12) is a cytokine that helps the host's protective immunity against leishmaniasis by promoting the production of IFN-γ by CD4+ T cells. Macrophage and dendritic cells are the primary sources of IL-12, these cells are vital for CD4+ T cell activation[187,188]. However infected macrophages have been reported to have impaired IL-12 production, leaving DCs as the primary producers of IL-12 during *Leishmania* infection[189,190]. IL-12 has two functional subunits, p35 and p40 and both of these subunits are equally crucial for protection against *Leishmania* infection. When either of these subunits is altered, it increases the susceptibility to *L. major* infection[191]. The administration of the anti-IL-12 neutralizing antibody resulted in a Th1 deficient response in *L. major* infected mice, resulting in the loss of resistance in C57BL/6 mice[192]. In contrast, treatment of infected BALB/c mice with recombinant IL-12 enhanced Th1 response and resistance in an otherwise typically susceptible mouse strain[193].

1.10.2.3.3 Interleukin-4

Interleukin-4 (IL-4) is a cytokine that induces the differentiation of naïve CD4+ T cells into Th2 cells. IL-4 is produced by several cells, such as mast cells, basophils, and eosinophils, but the primary producer of IL-4 is Th2 CD4+T cells[194]. IL-4 is a potent immune regulator involved in several immune processes and plays a crucial role in mediating B cell IgE antibody class switching during immune response against helminths infection[195].

The role of IL-4 in the progression of murine *Leishmania* infection has been shown in several studies. High levels of IL-4 were recorded in BALB/c mice infected with *Leishmania* infection and was associated with their susceptibility[196]. This high level of IL-4 has been shown to inhibit IFN-γ-producing Th1 cells, leading to alternatively activated macrophages with reduced capacity to produce NO and ROS[197]. Additionally, alternatively activated macrophages enhance parasite

survival[195]. BALB/c mice treated with anti-IL-4 antibodies became resistant to *L. major* infection[196]. However, the complete deletion of the IL-4 gene in BALB/c mice did not result in resistance in these mice when infected with *L. major*[198], but IL-4 receptor α (IL-4R α) -deficient BALB/c mice are resistant to *Leishmania* infection[199], suggesting that other cytokines may also mediate susceptibility by signaling via IL-4Rα.

1.10.2.3.4 Interleukin-10

Interleukin 10 (IL-10) is a potent anti-inflammatory cytokine initially thought to be produced by Th2 cells to inhibit the development of Th1 cells[200]. Later, it was discovered that IL-10 could be produced by several cells such as macrophages, dendritic cells, mast cells and Tregs[201]. Mice with IL-10 deficiency were found to spontaneously develop enteritis, suggesting that IL-10 is critical for controlling excessive host immune response[202]. IL-10 deficient BALB/c mice were found to be resistant to *Leishmania* infection. Resistance to re-infection after the resolution of initial infection (known as infection-induced immunity) has been associated with the persistence of some parasites at the site of infection[203]. These parasites are thought to be essential for maintaining the pool of antigen-specific T cells[203]. Some studies have shown that *Leishmania* infection in IL-10 deficient C57BL/6 mice results in complete elimination of parasites, suggesting that IL-10-producing T cells may be responsible for the maintenance and persistence of the parasites[204] and indirectly to infection-induced immunity. However, the role played by IL-10 in determining the outcome of Leishmaniasis has been suggested to depend on the strain of the infecting parasite. In support of this view is a study that showed that IL-10 deficient C57BL/6 mice could not control infection with *L. amazonensis* even in the presence of a robust Th1 response compared to WT control[205]. Similarly, IL-10 deficient BALB/c mice infected with *L. amazonensis or L. mexicana* were also unable to control the progression of the disease[206],

these studies suggest that IL-10 plays a significant role in determining the outcome of *Leishmania* infection[207].

1.11 Gene editing

Gene editing, also known as genome editing, is a group of techniques and technology used to modify genes in living organisms. This area of science seeks to improve the knowledge and understanding of how specific genes function. Gene editing can delete, introduce, or correct almost any sequence in the DNA in different cell types and organisms[208]. Gene editing techniques have existed for decades, but several new methods are being developed to make gene editing much faster, cheaper, more precise, and more efficient. These improvements are essential for developing treatments for many genetic and acquired diseases[209]. The foundation of gene editing is based on the discovery of a natural cell-based repair mechanism that occurs when there is a break in the DNA. Gene editing allows researchers to mimic the repair process that occurs naturally in cells[210].

1.11.1 Gene editing tools

With the recent emergence of highly versatile gene-editing tools, investigators have introduced sequence-specific modification rapidly and economically into the genome of a wide variety of organisms and cell types[211]. The technologies or tools commonly used to facilitate gene editing include clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9), transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), and homing endonuclease.

1.11.1.1 Zinc-Fingers Nucleases (ZFN)

Zinc finger nucleases (ZFN) are site-specific endonucleases capable of binding and cleaving DNA at specific positions. These artificial restriction enzymes are generated by the fusion of a zinc finger DNA-binding domain to a DNA- cleavage domain. The DNA binding domain consists of eukaryotic transcription factors and the zinc finger[212]. In contrast, the DNA-cleavage domain consists of a FokI restriction enzyme that catalyzes DNA cleavage [213]. The combination of the binding domain and cleavage domain on a target DNA can introduce a double-stranded break at the FokI domain. The endogenous DNA repair mechanism in higher organisms allows these tools to be precisely used to alter the genome of higher organisms[212]. One of the concerns of using ZFNs for genome editing is off-target mutations, although several approaches have been developed to enhance their specificity. An example is the creation of obligate heterodimeric ZFN architectures that uses a charge-charge repulsion to prevent unwanted homodimerization of the FokI cleavage domain, thereby minimizing the potential of ZFNs dimerizing to an off-target site[214]. ZFN has not been widely adopted to be used in unspecialized laboratories because ZFNs lack target flexibility because they do not effectively recognize all the DNA triplet's variety, such as 5′-CNN-3′ and 5′-TNN-3′[215].

1.11.1.2 Transcription Activator-Like Effectors Nucleases (TALENs)

Transcription activator-like effectors nucleases (TALENs) were discovered in 2009 when the code used by the TALE protein was uncovered. TALEN is a fusion of transcription activator-like effector (TALE) and the catalytic domain of the restriction endonuclease FokI. Like Zinc Finger nucleases, TALENs can modify nearly any gene. Still, TALENs are easier to build and design to target any specific gene loci with high precision and efficiency. Unlike the Zinc fingers that recognize DNA triplets, TALE repeats recognize only a single base pair with little or no overlap.

One of the disadvantages of TALENs is their size; they are enormous with repetitive structure making their delivery into cells less efficient[216].

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

CRISPR- Cas is the most recent discovery in the field of gene editing. This system was discovered as part of the defence mechanism used by bacteria, where the system protects against invading viruses and plasmids by cleaving short segments of the foreign DNA, which are then integrated within the CRISPR locus of the infected bacteria[217,218]. Upon reinfection with the same virus, the fragment is transcribed into CRISPR (RNA) and then annealed into a trans-activating crRNA (tracr RNA) which is used for sequence-specific cleavage of the re-infecting virus DNA by the Cas protein. Doudna and her co-workers reported in 2012 that Cas9 protein only requires a seed sequence within the crRNA and a conserved protospacer-adjacent motif (PAM) 3 base pair upstream of the crRNA binding site for recognition of the target sequence[219]. CRISPR -Cas9 is currently the simplest, user-friendly, versatile, and precise gene-editing method. This tool has eliminated the need for engineering new proteins to recognize each section of the target site. Three CRISPR systems (type I-III) have been identified across a broad range of microbes, and these systems are differentiated based on the type of Cas protein[220]. The widely adopted CRISPR Cas9 system is a type II system, which has been simplified to consist of only Cas9 nuclease, a single gRNA that contains both the cr-RNA and tracrRNA elements which is essential for target recognition and a PAM motif which is recognized by the Cas nuclease. This PAM motif is located downstream of the gRNA target site and is critical for DNA cleavage[221]. The CRISPR Cas9 nuclease and its gRNA have been shown in several studies to be used *in vivo*[212]

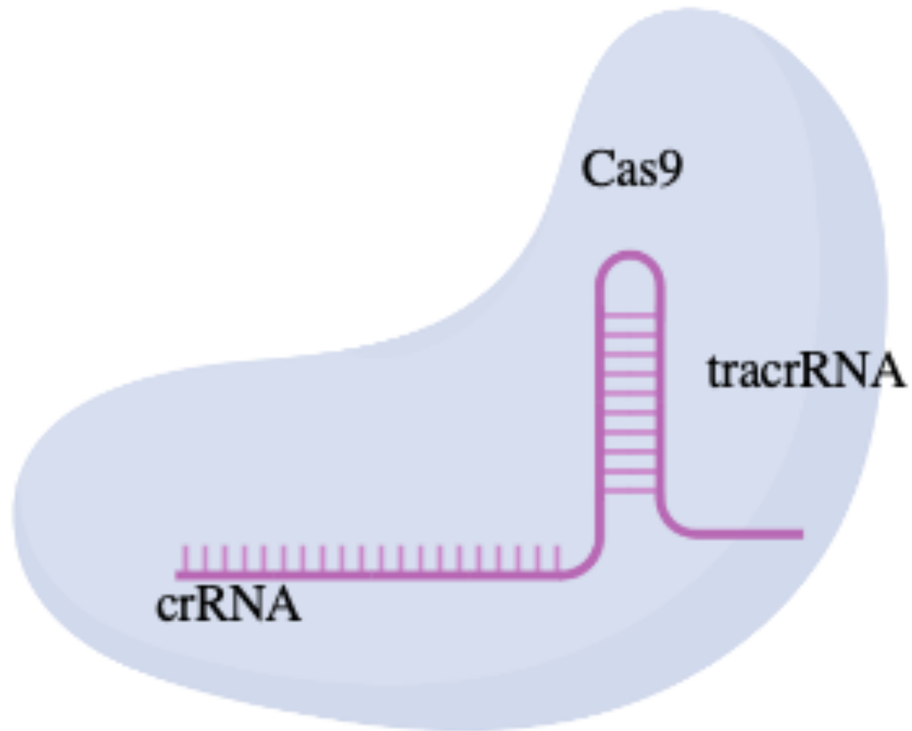


Figure 3: Diagram of gRNA and Cas9 complex.

The gRNA consists of crRNA and tracrRNA. While crRNA contains a 20-nt target-complementary sequence, the tracrRNA contains a scaffold sequence allowing the formation a complex with the Cas9 nuclease.

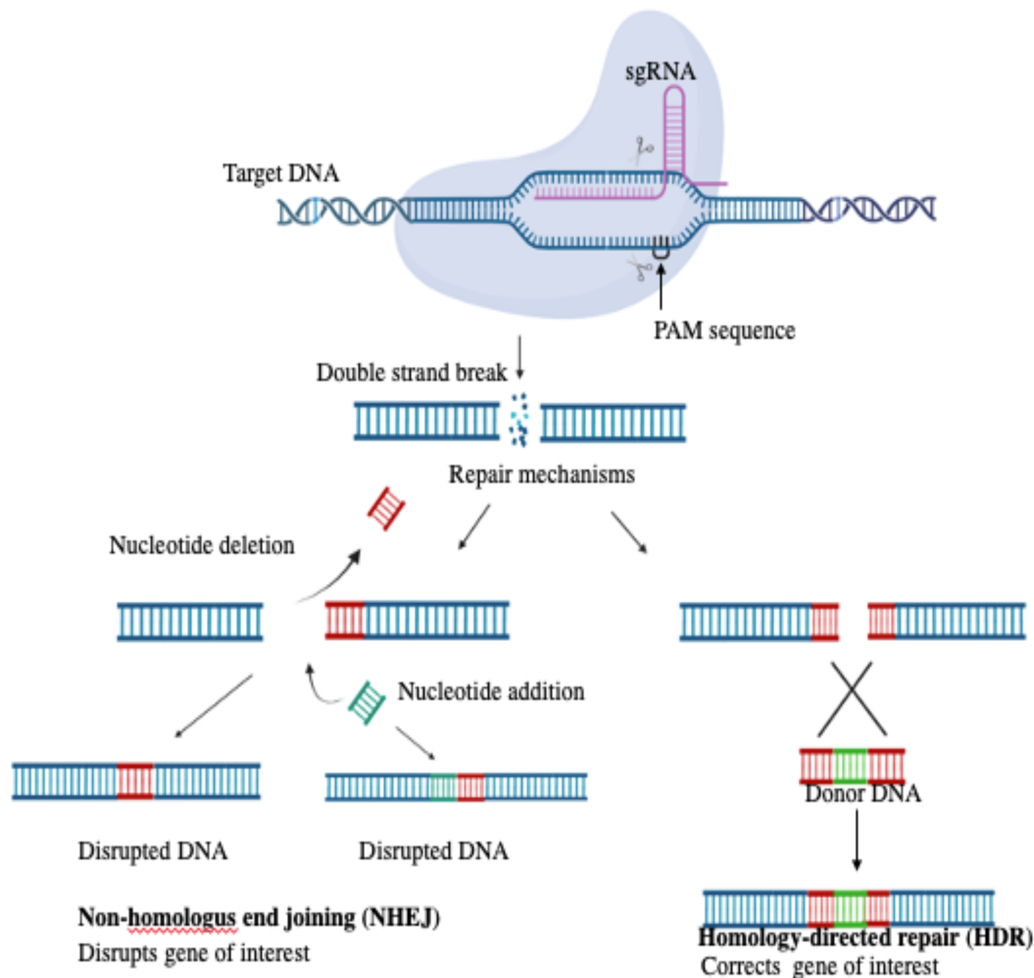


Figure 4: Diagram showing the CRISPR -Cas9 mediated double-stranded break and different repair mechanism.

There are two types of pathways for repairing Double-stranded breaks(DSBs): NHEJ and HDR. NHEJ pathway creates variations by inserting or deleting a few bases during the repair of the DSBs segment. On the other hand, the HDR pathway uses homologous sequences of donors, either in the form of dsDNA or ssDNA, which allows more accurate gene insertion than the other

1.11.1.4 Leishmania gene editing using CRISPR/Cas9

Leishmania has many genes with unknown functions, but gene-editing tools such as homologous recombination have allowed for the study of these genes to more correctly understand the biology of the parasite and the disease they cause[222]. However, homologous recombination is

time-consuming with a high chance of off-target effect. The recent introduction of the CRISPR-Cas9 system has made it easier and more efficient tool for studying the function of genes in *Leishmania*. Several stable CRISPR Cas9 system has been used for editing genes in the *Leishmania*. The CRISPR-Cas9 system was explicitly optimized to function in the Leishmania parasite by Zhang et al. this system has been used in gene deletion and disruption in different species of Leishmania parasite[223]. The expression of the gRNA and Cas9 nuclease in this system is driven by an RNA promoter; the gRNA directs the Cas9 nuclease on the site to induce the double-stranded break in the target DNA. This CRISPR Cas9 system contains an antibiotic resistance gene used for selecting mutants because *Leishmania*'s homologous directed repair mechanism lacks an RNA interference pathway[224, 225]. Mutant parasites are selected using antibiotic-resistant genes following the insertion of a repair template at the site of the DNA break via a microhomology-mediated end-joining pathway[223]. CRISPR Cas9 is an efficient tool that will allow for the identification and study of genes whose function in *Leishmania* biology was unknown[226]. Very little off-target effect has been recorded with the use of CRISPR Cas9 in the *Leishmania* parasite if the gRNAs are correctly designed[227].

This system is efficient enough to edit single or multicopy genes. Complementary add-back strains are generated on the mutant background by introducing an episomal plasmid containing the wild-type gene because it is essential to examine if the loss of wild-type phenotype is restored in the mutants[228,229].

1.12. Thesis Overview

1.12.1 Rationale

The components of *Leishmania* surface coat, such as LPG and gp63, have been associated with virulence[230]. Attempts to disrupt one or multiple members of this surface molecule have proved unsuccessful over the years as such attempts have either impaired the parasite's ability to infect mice or the parasite reverts to virulence without regaining the function of the disrupted gene[231,232]. ADS enzyme has been identified to be important for the synthesis of ether lipids needed for the assembly of the *Leishmania* surface coats[49] . In the previous work done by Zufferey et al. [30], ADS deficient parasites were confirmed to lack components of the surface coat that has ether lipid backbone, including LPG, GPI- anchored proteophosphoglycan and GPI. ADS deficient parasites were also reported to delay lesion appearance in mice, and the promastigotes could not induce infection in macrophages[30]. However, the impact of ADS deficiency on the host immune response and the potential to use the mutant parasite as a live attenuated vaccine were not investigated.

1.12.2 Hypothesis

ADS gene products regulate the host immune response to cutaneous leishmaniasis and is critical for virulence of *L. major.*

1.12.3 Study aims

To investigate the impact of ADS deficiency on host immune response and virulence of *L. major.*

1.12.4 Objectives

1. To generate ADS deficient *L. major* using CRISPR Cas9 gene-editing tool; developing ADS mutant parasites in a more virulent strain may allow the generation of a clearer phenotype compared to that observed in the LV39 parasite
2. To assess the impact of ADS deficiency on the virulence of *L. major;* this is important to determine if ADS deficiency could lead to impaired virulence in *L. major* similar to what was observed with LV39 parasites.
3. Determine the effect of ADS deficiency on the host immune response to the parasite; since the role and impact of ADS on the host immune is still unknown, it is unclear whether it will be critical for inducing a protective immune response or otherwise.
4. *Determining if vaccination with ADS deficient parasites will induce protective immunity against virulent L. is a major* challenge; if ADS deficiency is found to impair the virulence of the leishmania parasite and is critical for host immune response, it will be essential to test if the immune response generated can protect against WT rechallenge to examine the potential of these parasites to be used as an attenuated vaccine or leishmanization

CHAPTER 2

2.0 MATERIALS AND METHODS

2.1 MICE

Female BALB/C and C57BL/6 mice aged 6-8 weeks were purchased from the University of Manitoba Central Animal Care Services (CACS) breeding facility or Charles River Laboratories (Senneville, Quebec). All mice were used following the Canadian Council for Animal Care guidelines. The University of Manitoba Central Animal Services provided a pathogen-free environment, 12 hours of light-dark rhythm, water, and chow.

2.2 PARASITES

Wild-type (WT), ADS mutant ($ADS^{-/-}$) and complimentary addback ($ADS^{-/+ADS}$) *Leishmania major* parasites used in the study were either on the background of MHOM/80/Fredlin or LV39 strains. All parasites were grown in M199 medium (Hyclone, Logan UT) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Cansera, Mississauga, ON, Canada), 100Ul/ ml Penicillin- streptomycin antibiotics in a 27°C parasite incubator (Thermofisher Ottawa, ON).

2.3 GENERATION OF ADS NULL MUTANT BY CRISPR-CAS9 SYSTEM

The ADS gene is located on chromosome 23 in *L. major*. There is only one copy of the ADS gene in *L. major*, and it's made up of 1.8kb. To delete the ADS gene, two guide RNAs (gRNAa & gRNAb) were designed using the Eukaryotic Pathogen CRISPR guide RNA Design tool (EuPaGDT), targeting upstream and downstream of the ADS gene.

The gRNAs were then cloned into the Bbs I site of a *Leishmania* CRISPR expression vector pLdCN (Addgene #84290) containing a Neomycin resistance gene to generate an all-in-one

CRISPR plasmid called pLdCN-ADS. Double-stranded breaks were introduced in the DNA region coding for ADS by introducing the pLdCN-ADS plasmid into the logarithmic phase of WT *L. major* parasites using the electroporation technique as described by Li et al. [233]. The parasites were prepared by harvesting them at 3000 rpm for 15 mins and then washed twice with PBS. Following washing, the parasites were resuspended in high-voltage Cytomatrix 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) at 20×10^6 cell/ml and incubated on ice for 10 mins. Four hundred (400) µl of the parasite suspension was transferred into a 2-mm cuvette, and 5-20 µg of pLdCN- ADS was added and mixed. The parasites were then electroporated by six pulses at 900 V with 300 µs pulse length and 200-ms interval between pulses using a BTX ECM830 square wave electroporator. Following electroporation, the parasite was incubated on ice for 10 mins, then transferred into a flask containing 10 ml complete M199 media and allowed to recover in a 27 °C CO_2-free incubator overnight. Fifty µg/ml of Neomycin was added into the media of the recovered parasite on the following day. The electroporated parasites were passaged twice a week. The concentration of the Neomycin antibiotic added to the culture was gradually increased to 150 µg/ml over several passages to increase the copy number of plasmid and, in turn, improve the expression of the gRNA and Cas9 nucleases in the parasites. Once a stable culture was established, the parasites were re-electroporated as described above with the Bleomycin resistance gene (584bp) as a donor template generated using Bleomycin-specific primers to amplify Bleomycin from a plasmid. The Bleomycin-specific primers include nucleotides present upstream and downstream of the site cas9 endonuclease will cleave the gene of interest. The flanking of the bleomycin resistance gene will allow the insertion of the donor template at the site of the double-stranded break induced by the Cas9 endonuclease using a homology-

directed repair (HDR) mechanism. To screen mutant parasites to determine that the ADS gene has been deleted and replaced with a bleomycin resistance gene, the parasite culture medium was supplemented with 50 µg/ml of Phleomycin antibiotics (an analogue of Bleomycin). The concentration of Phleomycin antibiotics was gradually increased to 100 µg/ml over two weeks. Once a population of parasites resistant to hygromycin and phleomycin was established, the mutants were analyzed by PCR using primers specific to the ADS gene and the Bleomycin gene. Transfecting mutant parasites with a *Leishmania* expression vector containing the ADS gene (pLPHyg2ADS) using electroporation described above generated complementary Addback strains.

Table 1: List of Primers used for PCR

Target	Primer sequence
ADS (pLPHyg2ADS)	F- CTGCCGAATAGCCACAAGGA R- CTTTGGCGGTGCATGTGTAG
Bleomycin (LmADS Ble)	F- CGCCTCAAGTGGAATGGGTGGGGTGATCTTCATCGGATCGGGTAC R- TCAGCGCTGCGGCGCTCTCGTTCTCTCAGTCCTGCTCCTCGGCCA

2.3 MOUSE INFECTION

Six to eight weeks old female naive BALB/C mice were inoculated intradermally in the central ear pinna with WT, ADS KO or ADS AB (MHOM/80/Fredlin or LV39) promastigote parasites resuspended in 10 µl sterile PBS using a 30G needle. The parasites used for infection were prepared by isolating log phase parasites (day 7 culture) at 3000 rpm for 15 mins. The isolated parasites were washed twice with PBS and resuspended at 1×10^6 in 10 µl. Mice infected with MHOM/80/Fredlin were sacrificed 3- and 5-weeks post-infection.

2.5 LESION SIZE MEASUREMENT AND PARASITE BURDEN

Lesion size was assessed in the infected mice by measuring the diameter of the developing lesion weekly using a Digital Vernier calliper. The parasite burden in the ear was estimated by limiting dilution assay as previously described. Briefly, after sacrificing infected mice using isoflurane and cervical dislocation, the infected ear was neatly cut off at the base using surgical scissors and kept on ice in DMEM. For processing, the ears were placed for 5 mins each in 70% ethanol, chlorhexidine and 70% ethanol solution. After that, they were rinsed in sterile PBS containing 2% Pen/Strep solution. The dermal sheets of the ear were then carefully separated using forceps and incubated at 37 °C for 90 mins in PBS containing 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (PBS-Pen), all from Gibco, Grand Island, NY, USA) and 500 µg/ml Liberase CI (Roche, Basel, Switzerland). Following incubation, the ear tissue was ground in a tissue grinder using 2 ml of PBS-Pen, poured into a strainer and rinsed with 2X PBS-Pen. The recovered suspension was spun at 3000 rpm for 15 mins. The supernatant was discarded, and the recovered pellet was resuspended in 2 ml complete Schneider media (Schneider's medium supplemented with 25 mm HEPES, 20% FBS and L- glutamine, 1% penicillin/streptomycin (1000X). One hundred (100) µl of the resultant solution was added to the first roll of a 96-well flat-bottom plate containing 100 µl per well of complete Schneider media and the wells were mixed by pipetting up and down ten times. One hundred (100) µl from the first well was transferred to the second well and serially diluted until the last row. The plates were covered and wrapped in cling film and incubated at 27 ^{o}C for seven days, after which parasite numbers were quantified using a light microscope.

2.4 ASSESSMENT OF PARASITE PROLIFERATION IN AXENIC CULTURE

Five hundred (500) WT, ADS KO, and ADS AB log-phase parasites were cultured in 5 ml of complete M199 media and incubated for seven days at 27 ^{o}C. The parasites were counted under the light microscope every day for seven (7) days using the hemocytometer under the light microscope using x40 magnification.

2.5 ISOLATION OF BONE MARROW CELLS

Bone marrow cells were isolated from the hindlimb (tibia and femur) of mice sacrificed by isoflurane and cervical dislocation. The hindlimbs were collected by removing the fur to expose the limb and dislocating it from the hip bone. The muscle and tissue were cleaned off the tibia and femur, and the bones were separated. Each bone was cut open at one end, and bone marrow was gently flushed out using a 10 ml syringe with a 25G needle and RPMI medium. The bones were flushed until transparent. The cells were carefully transferred into a 15 ml conical flask and vortex to disperse the bone marrow into a single-cell suspension. The cells were centrifuged at 1200 rpm for 5 mins, and red blood cells were lysed using 1ml ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na2EDTA, pH 7.2-7.4) for 5mins. The cells were resuspended and washed in 10 ml RPMI; cells were counted using a hemocytometer and trypan blue dye.

2.6 GENERATION OF MACROPHAGES FROM BONE MARROW CELLS

The bone marrow cells prepared as described above were resuspended in complete RPMI (RPMI supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) at 4×10^6/ml. The cells were cultured in a conditional media (complete RPMI augmented with 30% L929 cell supernatant) in a 15×100 mm petri dish incubated at 37°C in a 5% CO_2 incubator. Each petri dish contained 9 ml of condition media and 1 ml of the cell

suspension,10 ml of fresh conditional media was added to the cell culture after 3 days of incubation. On day 7, the cells were fully differentiated and ready to use. The cells are collected gently using a cell scraper to detach the cells.

2.7. GENERATION OF DENDRITIC FROM BONE MARROW CELLS

In a 15×100 mm petri dish, bone marrow cells resuspended at 2×10^6/ml in complete RPMI were cultured with 10 ml complete RPMI containing 20 ng/ml of GM-CSF. The cells were incubated in a 5% CO_2 incubator at 37 °C. On day 3, after incubation, freshly prepared complete RMPI containing 20 ng/ml of GM-CSF was added to the cultured cells. On day 6, 10 ml of the culture media was carefully removed from the petri dish without dislodging the cells using a pipette. The media was spun down at 1000 rpm for 10 mins. The supernatant was discarded, and the recovered cells were resuspended in complete RPMI containing 20 ng/ml of GM-CSF. The resuspended cells were then added to the same petri dish and re-incubated for two days. On day 8, the differentiated dendritic cells are ready to be used. The percentage of purity of the cell was determined by flow cytometry using PE-conjugated anti-CD11c antibody, which showed 85-90% purity.

2.8. *IN VITRO* INFECTION OF BONE MARROW-DERIVED MACROPHAGE AND DENDRITIC CELLS WITH *L. MAJOR* AND STIMULATIONS

Bone marrow-derived macrophage and dendritic cells isolated from BALB/C or C57BL/6 mice were differentiated as described above. The cells were resuspended in complete RPMI (RPMI supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) at 2×10^6/ml. Stationary phase (day 7) WT, ADS KO and ADS AB parasites were grown in complete M199 and were harvested at 3000 rpm for 15 mins. The supernatant was removed, and the recovered parasites were washed with PBS twice at the same speed. The

parasites were then reconstituted in complete RPMI 1690 media at 10×10^6/ml. Bone marrow-derived macrophage and dendritic cells were infected with *L. major* at a five (5) parasite to one (1) cell ratio in a polypropylene tube. The parasites and cell culture were incubated for 6 hrs, and free parasite cells were removed by spinning down and washing the parasite and cell suspension with PBS twice at 600 rpm for 5 mins. The washed infected cells were then re-suspended in complete RMPI and further incubated for 72 hrs at 37 °C. The proliferation and Infection rate were assessed at 6, 24, 48 and 72 hrs after infection by microscopy and flow cytometry, respectively. In some experiments, the infected cells were stimulated with 100 μg/ml of LPS, 20 μg/ml of IL-4 and 20 μg/ml of IFN- γ.

2.9 CYTOSPIN

Infected cells were cytocentrifugated using labelled slides mounted with blotting paper and a cuvette coupled together with a metal holder. One hundred microliters of the infected cells suspension was loaded into the cuvette. The setup was spun at 1200rpm for 5mins. The slides were air-dried and stained with Giemsa stain (Hematoxylin and Eosin (H&E) solutions). The stained slides were examined under the microscope (Zeiss Primostar iLED)

2.10 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) QUANTIFICATION OF CYTOKINES

This study used Sandwich ELISA to quantify different cytokines produced by infected or stimulated cells. Ninety-eight (98)-well ELISA plates (Immulon VWR, Mississauga, ON) were coated using purified coating antibody diluted with ELISA coating buffer and incubated at 4°C overnight. The plates were washed 5-6 times using a wash buffer containing 1×PBS + 0.05% TWEEN-20 with a pH of 7.4. The wells of the plate were blocked using 100 μl of ELISA

blocking buffer (5% FBS + 1× PBS) and incubated at 37 °C for 2 hours. The incubated plates were washed, and 50 µl of cytokine standards/ samples were added to wells containing 50 µl ELISA blocking buffer. The standard and samples were serially diluted and incubated at 4°C overnight. The next day, the plate was washed, and 50 µl of detection antibody was added to each well and incubated for 2 hours at 37 °C. After incubation, the plate was washed, and 50 µl of Arvidin HRP was added to the plate and incubated at 37 °C for 45 mins. Fifty (50) µl of ABTS peroxidase substrate was added at room temperature and incubated for 15 mins away from light. The plates were read once the desired colour change was observed at 405nm (Spectra Max).

Table 2: List of cytokines, starting recombinant standard dilution, sample dilution and sensitivities of the Sandwich ELISA

Cytokine	Standard (pg/ml)	Sample dilution	Sensitivity pg/ml
IFN-γ	5000	1:5	30.14
IL-4	2000	1:5	30.14
IL-10	2000	1:5	15.12

2.11 RNA ISOLATION AND REAL-TIME PCR

RNA was isolated from the different strains of *L. major* parasites and stimulated infected cells using the method explained below. Two hundred (200) µl of trizol was added to 10^6-10^7 cells/parasites and the cells were pipetted up and down to avoid clumping in a 1.5 ml Eppendorf tube and allowed to lyse for 5 mins. Two hundred (200)µl of Chloroform per 1ml of Trizol used

for lysing the cells was added to the tubes, mixed and incubated for 3 mins and centrifuged at 12000× g at 4 °C for 15 minutes. The aqueous phase was transferred into a new Eppendorf tube, and 500µl of isopropanol/1ml trizol was added and incubated for 10 mins followed by 10 mins centrifugating at 12000× g at 4°C. One ml 75% alcohol was added to the RNA pellet after the supernatant was discarded, mixed and centrifuged for 7500×g for 5 mins at 4 °C. Thereafter, the supernatant was discarded, and the tubes were allowed to air dry. Extracted RNA was resuspended in 20 µl RNAase-free water and incubated in a water bath at 55 °C for 10 mins. The RNA was converted to cDNA using a cDNA synthesis kit (E6300L – New England Biolabs) according to the manufacturer's suggested protocols. PCR was performed using Luna universal qPCR master mix (M3003X – New England Biolabs) according to the manufacturer's suggested protocols.

Table 3: List of Primers used for qPCR

Target	Primer sequence
ADS	F- CTGCCGAATAGCCACAAGGA R- CTTTGGCGGTGCATGTGTAG
rRNA45	F- CCTACCATGCCGTGTCCTTCTA R- AACGACCCCTGCAGCAATAC

2.12 FLOW CYTOMETRY

Spleen and lymph nodes collected from infected mice were processed into single-cell suspension by crushing them in a cell strainer. The cells were spun down, and the red blood cells were lysed using ACK buffer, the reaction was stopped by adding incomplete DMEM. The cell is spun

down and resuspended to the desired concentration. The cells were stimulated with PMA (20 ng/ml), Ionomycin (1 μM) and Brefeldin A (10 μg/ml) (All from Sigma) for 4 hr at 37 °C. The cells were transferred into a flow cytometry tube (BD Falcon) and washed with FACS buffer (0.1% FBS, 0.1% sodium azide and PBS). No-specific uptake of antibodies via the FC receptor was prevented by adding 100 μl of 2.4G2 Hybridoma supernatant and incubated on ice for 10 mins. Thereafter, the cells were washed with FACS buffer, stained with Fluorochrome conjugated antibodies against surface markers such as CD90.2, CD4, CD11b, CD11c, MHCII, CD80, CD86, CD40 and F4/80 (eBiosciences) using 0.5μl antibody solution per tube. The cells were incubated on ice for 30 mins and washed afterwards using FACS buffer.

To assess the expression of intracellular cytokines, the cells were fixed using 2% paraformaldehyde (Sigma Aldrich), then permeabilized with 0.1% saponin (Sigma Aldrich) in FACS buffer and incubated on ice for 30 mins. The permeabilized cells were stained with Fluorochrome conjugated antibodies against the cytokines and/or molecules of interest including IL-4, IL-10, IFN- γ, iNOS, and Arginase dissolved in saponin buffer. Stained cells were washed using FACS buffer following incubation on ice for 30 mins and resuspended in 500 μl of FACS buffer. The cells were acquired using BD FACS Canto II (BD Bioscience, Mississauga, ON, Canada), followed by analysis using Flowjo software (Tree Star Inc, Ashland, OR).

2.13. STATISTICAL ANALYSIS

Results in this study were represented in bars and line graphs, and the statistical comparison was performed using T-test, One way or Two-way ANOVA using the Prism program (GraphPad Software Inc., CA, USA). If $p \leq 0.05$, the result is considered significant.

CHAPTER 3

3.0: RESULTS

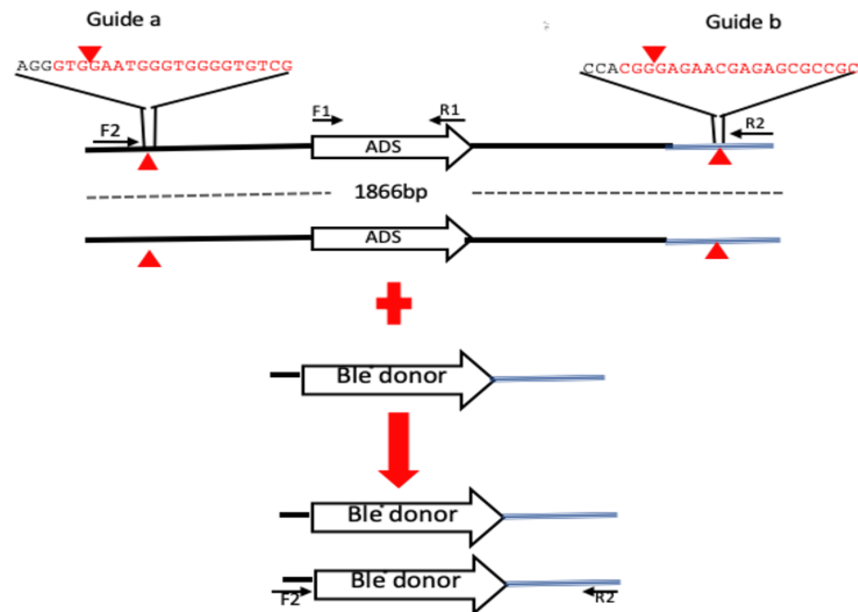
3.1 Generation and characterization of null mutants in *L.major*

The components of the surface coat or the glycocalyx of the *Leishmania* have been identified in different studies to play significant roles in protecting the parasite from microbicidal processes in the host. However, it has been shown that when these molecules were knocked out, or their expression is disrupted, as seen with lpg1 ko, the parasites were able to revert to virulence without regaining the expression of the deleted gene. In this study, we sought to explore the disruption of the entire surface coat by disrupting the synthesis of ether lipids needed to assemble the surface coat in a virulent *L. major* strain. This was done by deleting the gene that codes for the Alkyldihydroxyacetone phosphate synthase enzyme, the key enzyme required for the first steps of ether lipid synthesis. The CRISPR-Cas9 gene-editing tool was used to assess the role of Alkyldihydroxyacetone phosphate synthase (ADS) in the virulence of *Leishmania major* and host immune response. I generated ADS null mutants (ADS KO) using the CRISPR-Cas9 system (**Figures 1A and 1B**). ADS null mutants were confirmed by PCR analysis on the genomic DNA extracted from ADS KO parasites using the ADS-specific primers (601bp amplicon) (**Figure 1C**). Similarly, the Bleomycin gene was inserted into the ADS KO parasite at the site of the gRNA, which was confirmed by PCR using Bleomycin-specific primers (482bp amplicon), as shown in **Figure 1D**. The absence of the ADS gene band suggests that the ADS gene has been deleted in the electroporated parasites. The presence of the bleomycin gene indicates that the double-stranded break induced by the Cas9 enzyme has been successfully repaired.

Complementary add-back strains are an essential control to ensure that the integrity of the genome is not altered due to the deletion of the ADS gene, and hence any observed phenotype is not due to off-target effects. These complementary add-back parasites were called ADS AB, and they were generated by electroplating ADS KO parasites with a *Leishmania* expression vector containing the ADS gene. PCR confirmed the presence of the ADS gene. These were further confirmed by measuring the level of ADS mRNA expression in these parasites by RT-PCR **(Figure 1E).**

A

B



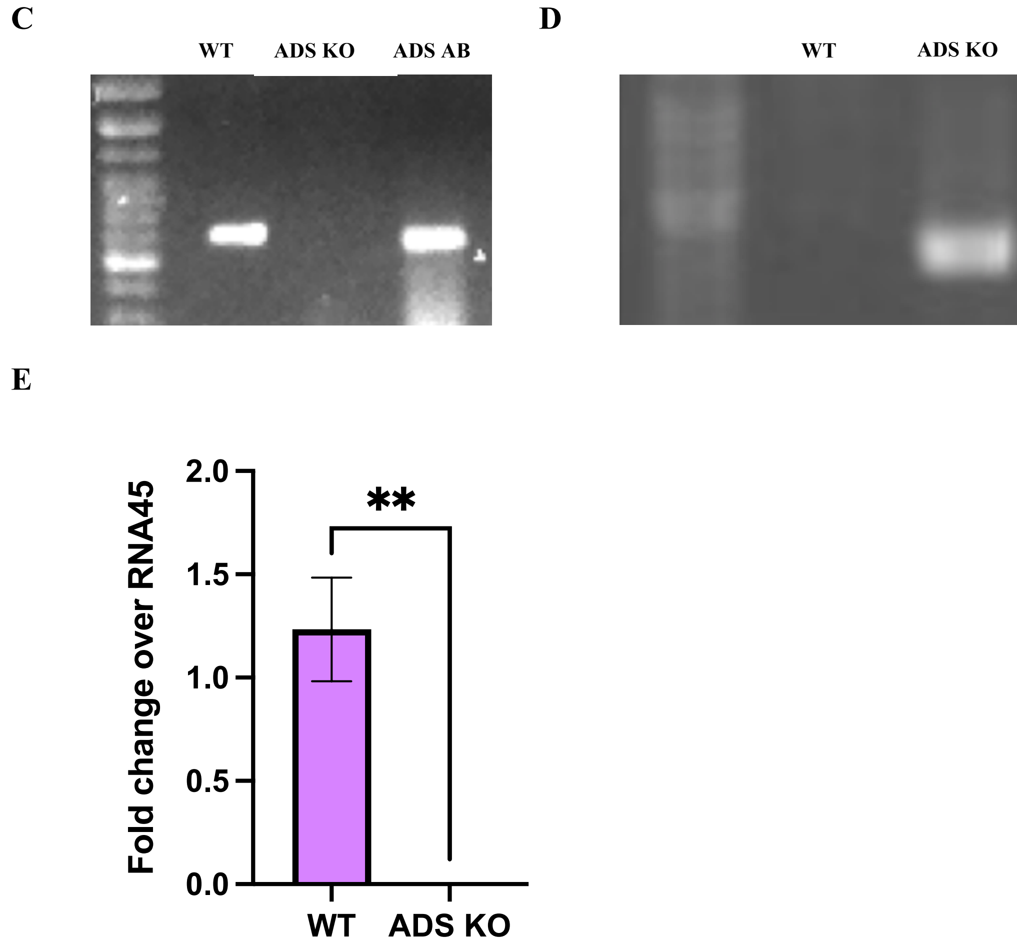


Figure 1: Generation of ADS null mutants using CRISPR-Cas9 system.

Map of *Leishmania* all-in-one CRISPR (pLdCN) expressing two gRNAs targeting the *L. major* ADS gene flanking sequence on both ends of the gene (A). The ADS gene loci on chromosome 30 of *L. major* and the CRISPR strategy used to delete the only copy of the ADS gene (B). PCR analysis showing that the ADS gene sequence is absent in *L. major* null mutant (ADS KO), which is completely restored in the complementary addback (ADS AB) strain (C). PCR analysis showing that the Bleomycin gene has been successfully inserted in *L. major* ADS null mutant (line 2) (D). qPCR quantification shows the ADS mRNA levels in the ADS KO parasite compared with WT (E). *ns = not significant;* ****, *$p < 0.0001$.*

3.2 ADS deficient *L. major* parasite shows impaired growth in axenic culture

This experiment aims to assess whether ADS deficiency will impact the growth of *L. major* in axenic culture. ADS KO parasites showed impaired growth in axenic culture compared to WT and ADS AB parasites **(Figure 2)**. Similar observations have been made in another study where ADS was deleted [30]. A slower growth rate is expected in gene-edited parasites and has been said to be one of the early indicators of successful gene deletion or editing[234].

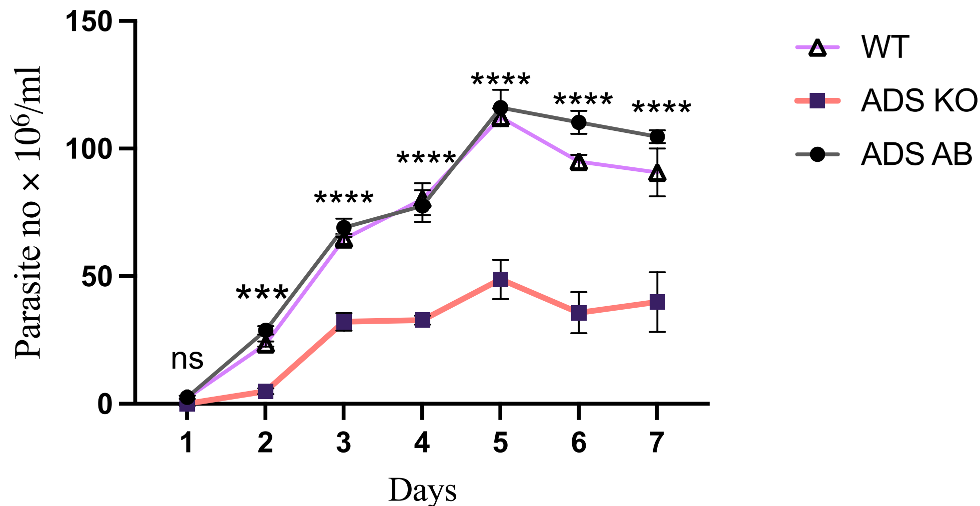


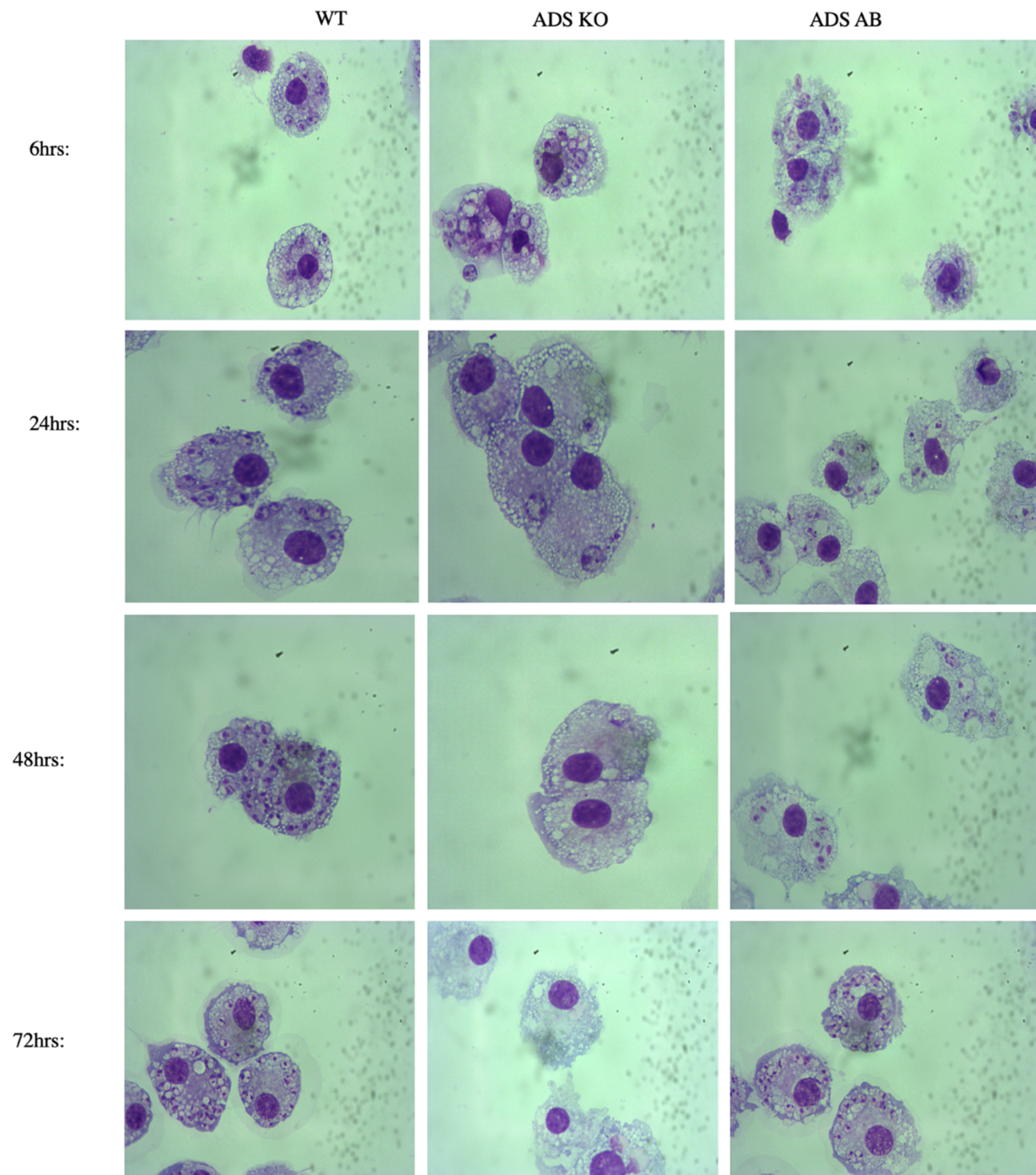
Figure 2: The growth of ADS KO parasites is highly compromised in axenic cultures.

Equal numbers of WT, ADS KO and ADS AB promastigotes were cultured in a complete M199 medium, and the proliferation of the parasites was measured daily for 7 days. *ns= not significant; ***, p<0.001; ****, p <0.0001.*

3.3 ADS gene deficient parasites have impaired ability to infect and proliferate in macrophages

This experiment was conducted to assess whether ADS deficient parasites would have an impaired ability to infect and proliferate in bone marrow-derived macrophages since they had impaired growth in axenic culture. I used bone marrow-derived macrophages in this experiment because *Leishmania* parasites preferentially infect macrophages in the mammalian host. These cells provide the most suitable niche for the parasites to thrive. Bone marrow-derived macrophages were then infected with WT, ADS KO and ADS AB promastigote parasites at a ratio of 1: 5. Cytospin preparations of infected cells were made on a slide at 6, 24,48 and 72 hrs after infection**,** stained using Giemsa stain and assessed under a light microscope **(Figures 3A-C)**. At 6 hrs, the infected cells had a similar number of parasites in them, suggesting that ADS deficiency does not affect the ability of the *L. major* to infect macrophages. However, the decline in the number of ADS deficient parasites found in infected cells later after infection indicates that ADS deficiency compromises the ability of these parasites to proliferate in macrophages (**Figure 3A-C**). Collectively these results suggest that ADS deficiency has impaired the proliferation of null mutants in macrophages.

A.



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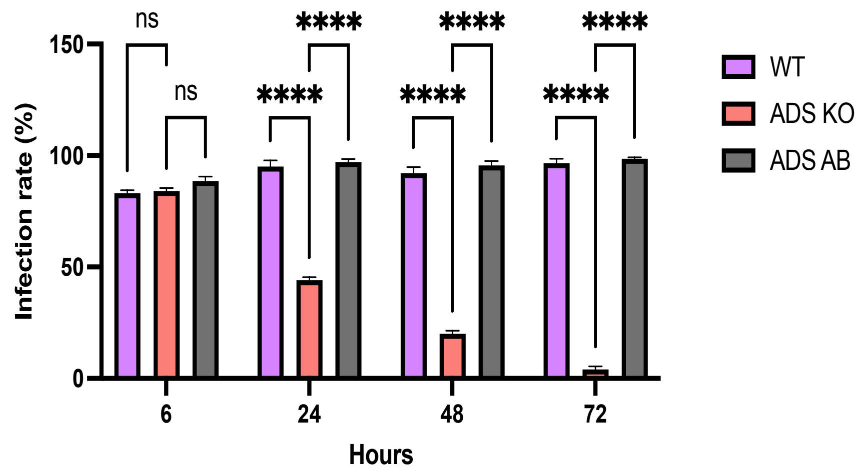


C

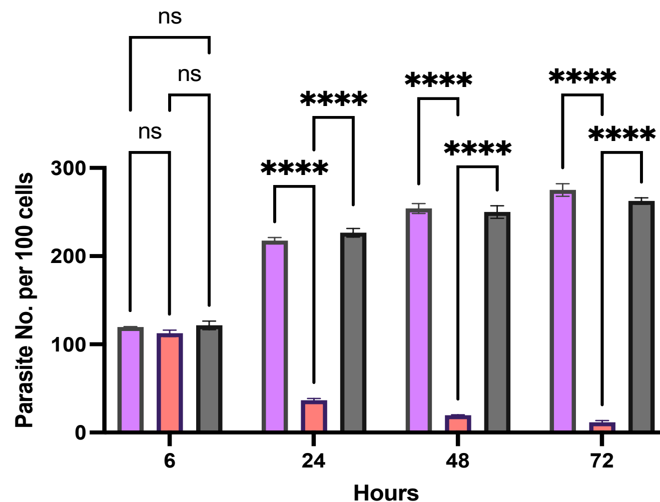


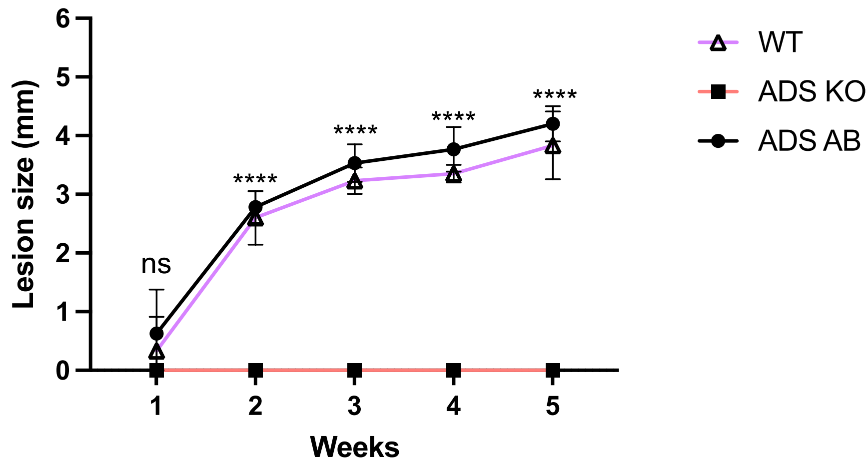
Figure 3: ADS deficient parasites have impaired ability to infect and proliferate in macrophages.

Bone marrow-derived macrophages were infected with WT, ADS KO and ADS AB promastigotes. Cytospin preparations of the infected cells were prepared at the indicated time (6, 24, 48 &72 hrs), stained with Giemsa stain, and the infected cells were assessed under a light microscope. The number of parasites inside the infected macrophages was also analyzed (A and B). The infection rate was also determined (C). These results represent three independent sets of experiments with similar results. *ns= not significant;****, p < 0.001.*

3.4 ADS deficient parasite has impaired virulence *in vivo*

Since ADS deficient parasites showed an attenuated phenotype in BMDMs, I wanted to examine if deficiency of ADS gene products affects the virulence of *L. major in vivo*. To assess this, BALB/c mouse were infected with 1×10^6 WT, ADS KO and ADS AB stationary-phrase promastigote and the lesion size were measured over time (**Figure 4A**). The parasite burden at the site was also assessed at specific time points (**Figure 4B**). Mice infected with WT and ADS AB parasites developed uncontrolled cutaneous lesions as early as 3 weeks after infection, while mice infected with ADS KO parasites did not develop any noticeable lesions throughout the experiments. As expected WT and ADS AB-infected mice had significantly higher parasite burden compared to mice infected with ADS KO parasites. Collectively these results suggest that ADS deficiency impairs the virulence of *L. major in vivo.*

A



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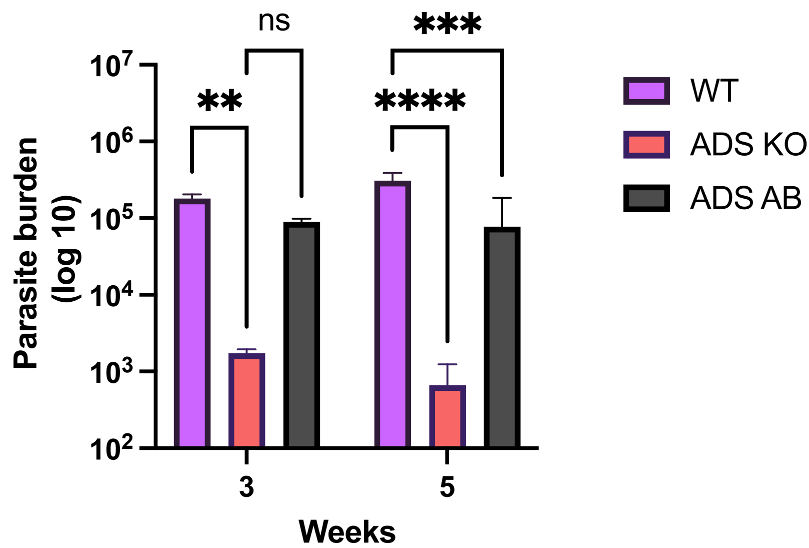

Figure 4: ADS deficiency results in impaired virulence in vivo.

BALB/c mice were infected with WT, ADS KO and ADS AB stationary phase parasites in the ear (9 mice per group). The lesion size of mice in each was measured weekly, and the parasite burden in the infected ears was assessed by limiting dilution (A & B). These results represent 3 independent sets of experiments with similar results. *ns= not significant*; **, *p<0.01; ****, p < 0.0001*

3.5 ADS may be critical for host immune response *in vivo*

Low parasite burden and self-healing skin lesion have been established as the hallmark of immunity to cutaneous leishmaniasis, typically mediated by IFN-γ -dependent activation of macrophages[235]. On the other hand, susceptibility has been marked by strong Th2 CD4+ T cells, which are associated with high levels of IL-4 and IL-10[236]. The impact of ADS deficiency on host immune response has not been investigated. To investigate the contribution of ADS to host immune response, I assessed the frequency of $CD4^+$ T cells producing IFN-γ, IL-4, and IL-10 in the spleens, draining lymph nodes and ears of BALB/c mice infected with stationary phase WT, ADS KO and ADS AB promastigotes at 3- and 5-weeks post-infection. Direct *ex-vivo* flow cytometry analysis showed that the frequency of IFN-γ, IL-4 and IL-10 producing $CD4^+$ T cells was slightly lower in the dLNs **(Figures 5A-5F)** and ears **(Figures 7A-7F)** of infected mice but not different in the spleens **(Figures 6A-6F)** of mice infected with ADS KO compared to those infected with WT or ADS AB parasites. Cells for the dLN **(Figures 8A- 8C)** and spleen **(Figures 8D-8F)** from infected mice were re-stimulated with SLA to quantify the production of IFN-γ, IL-4 and IL-10 in the culture supernatant by ELISA. Mice infected with ADS KO parasites had significantly low levels of cytokines (IFN-γ, IL-4, IL-10) compared with those infected with WT. Collectively these results suggest that ADS gene products may be important targets or stimulators of host immune response.

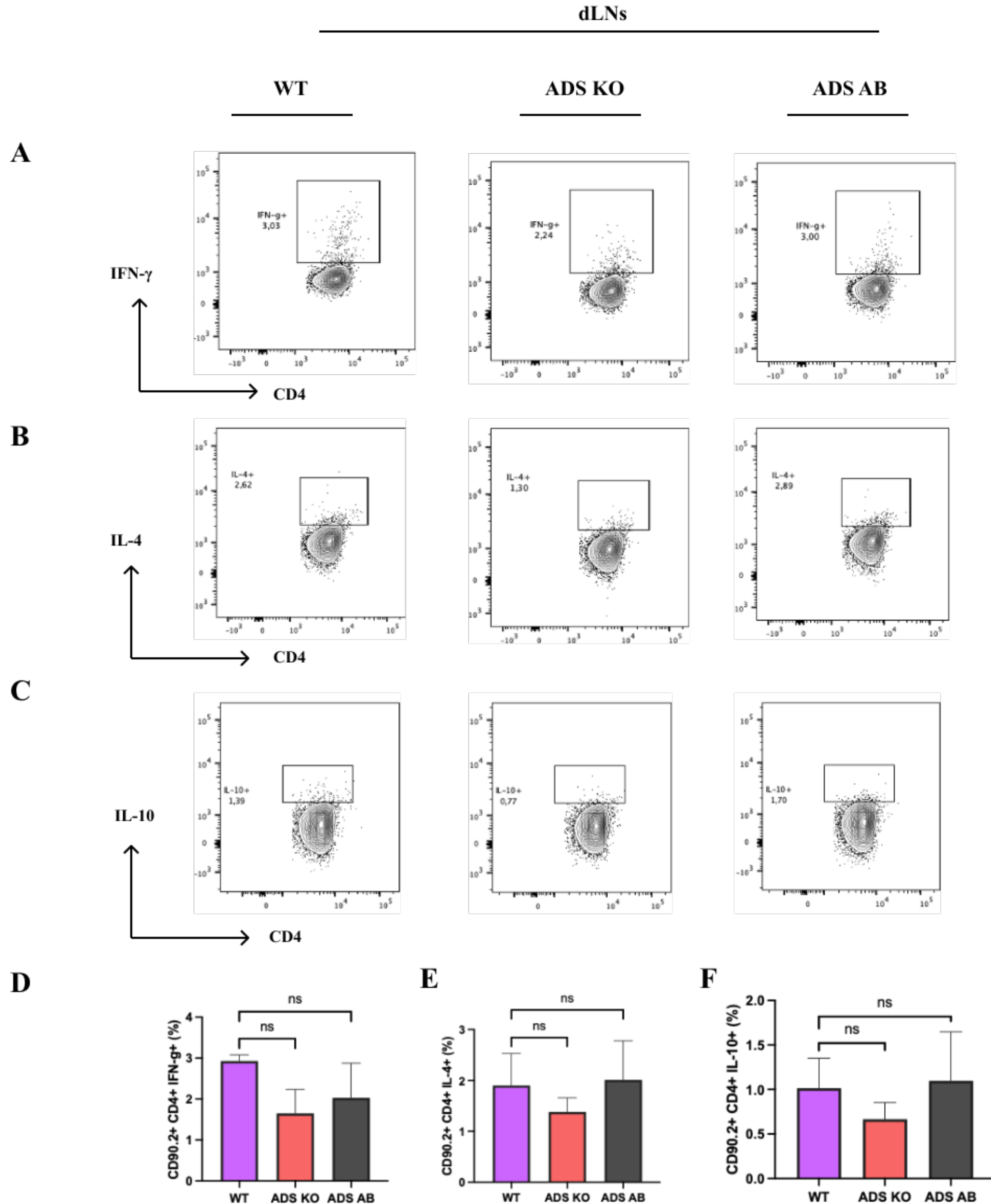

Figure 5: ADS gene product may be important for host immune response in vivo.

BALB/c mice were infected with WT, ADS KO and ADS AB with stationary phase promastigotes were sacrificed at 5 weeks post-infection. The frequency of IFN-γ, IL-4 and IL-10 producing CD4+ T cells in the dLNs (5A- 5F). These results represent 3 independent sets of experiments with similar results. *ns= not significant; *, $p<0.05$*

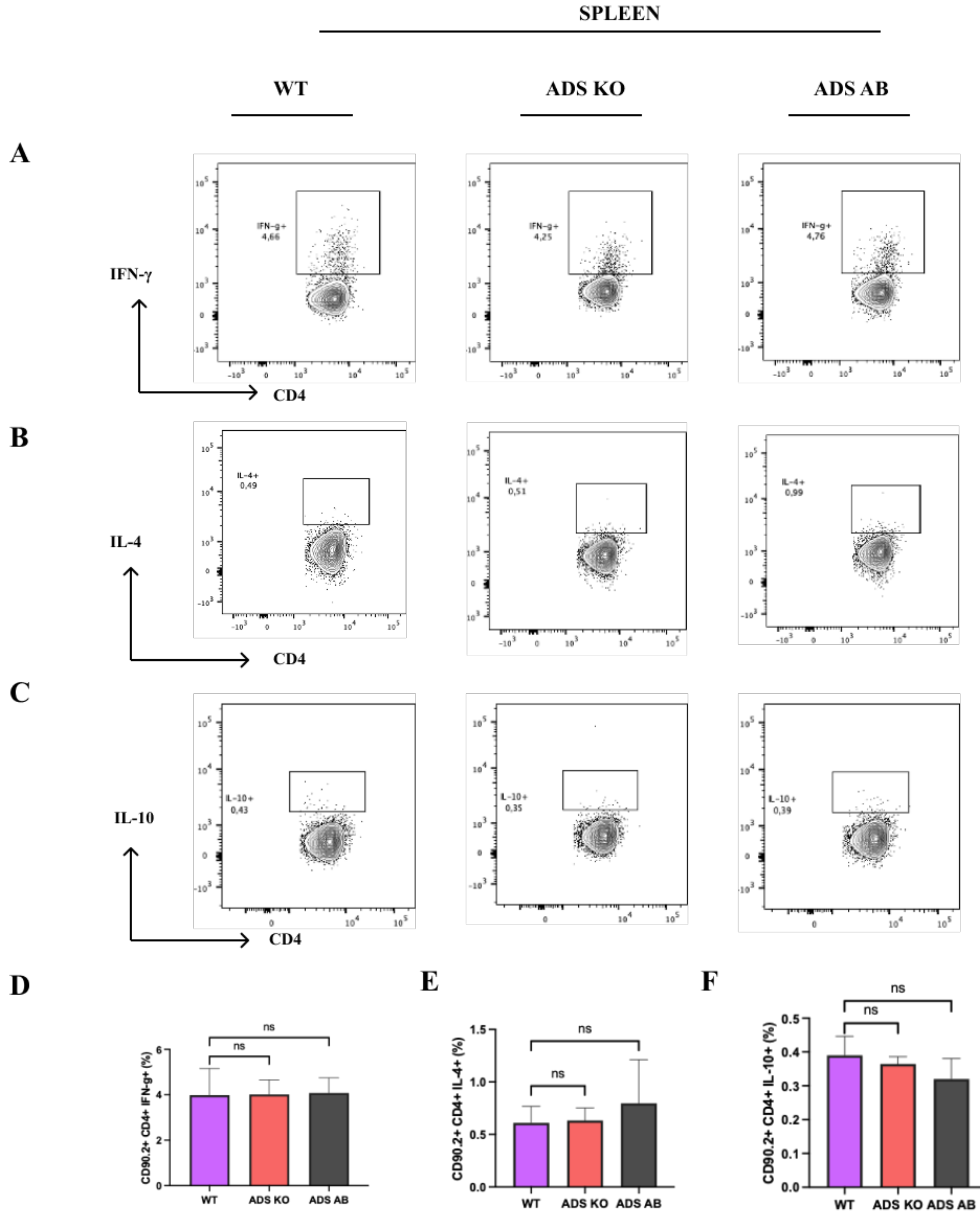

Figure 6: ADS gene product may be important for host immune response *in vivo.*

BALB/c mice were infected with WT, ADS KO and ADS AB with stationary phase promastigotes were sacrificed at 5 weeks post-infection. The frequency of IFN-γ, IL-4 and IL-10 producing CD4+ T cells in the spleen (6A-6 F). These results represent 3 independent sets of experiments with similar results. *ns= not significant; *, $p<0.05$*

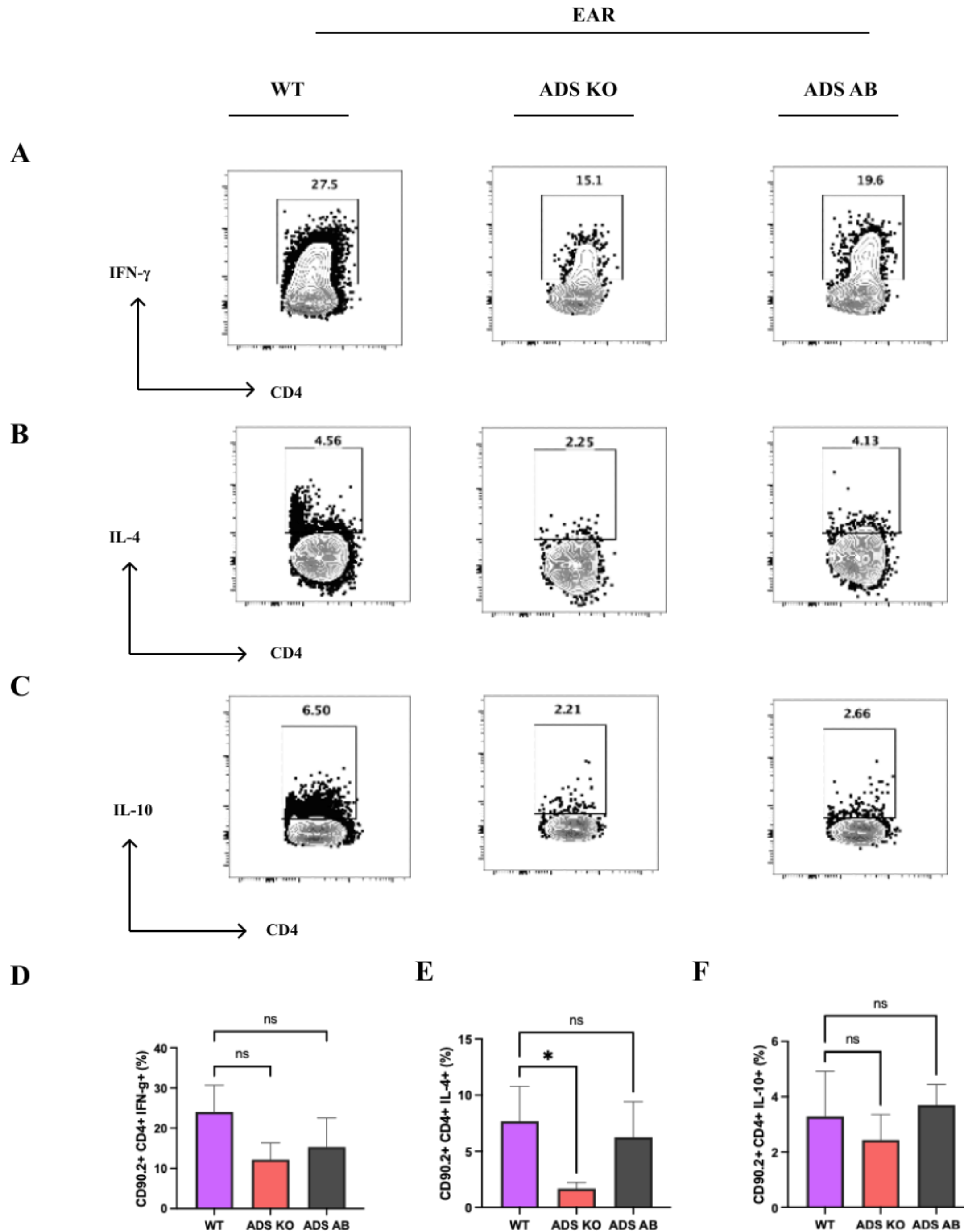

Figure 7: ADS gene product may be important for host immune response in vivo.

BALB/c mice were infected with WT, ADS KO and ADS AB with stationary phase promastigotes were sacrificed at 5 weeks post-infection. The frequency of IFN-γ, IL-4 and IL-10 producing CD4+ T cells in the ear (7A- 7F). These results represent 3 independent sets of experiments with similar results. *ns= not significant; *, p<0.05*

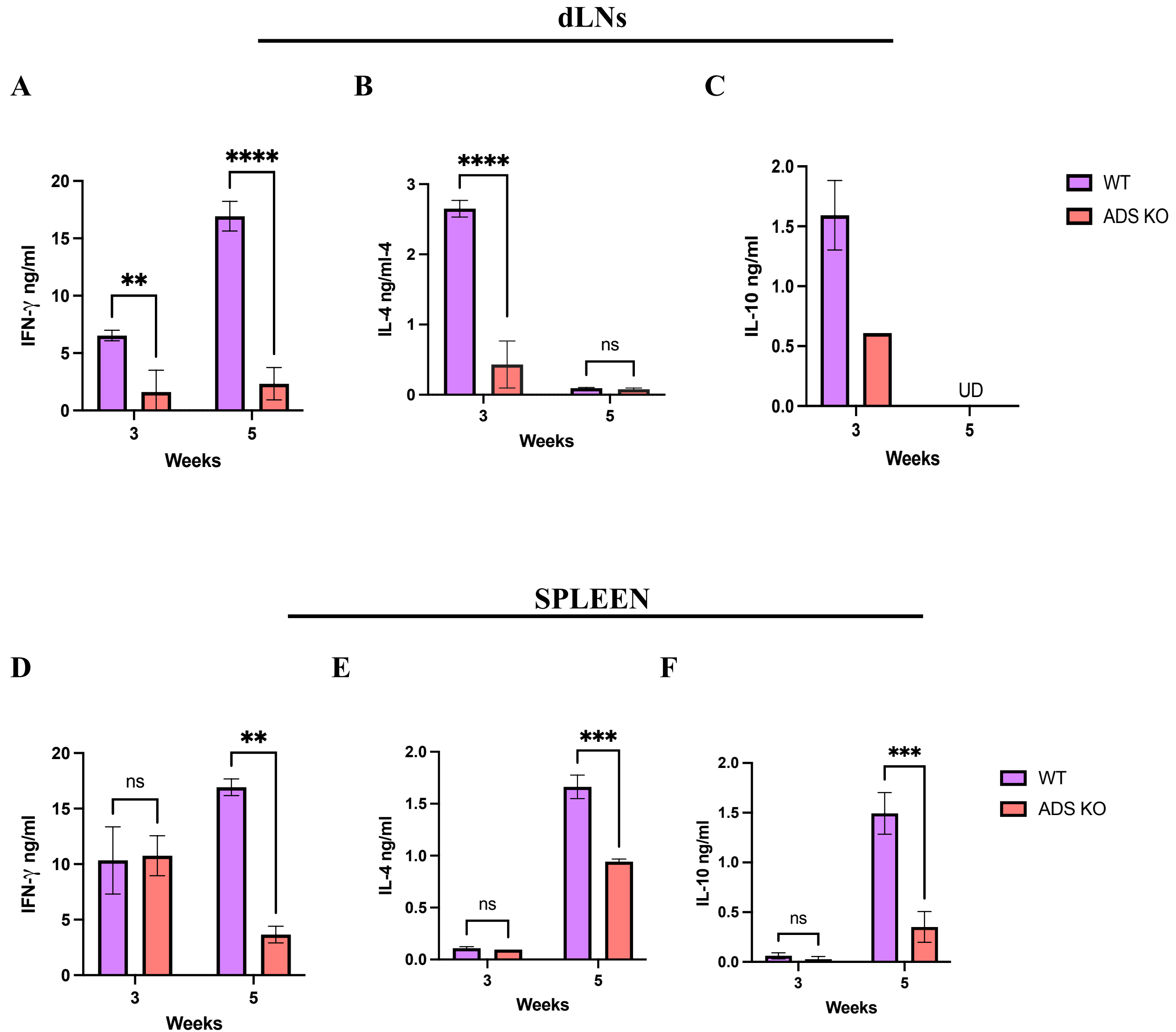

Figure 8: ADS gene product may be important for host immune response in vivo

Cells from the spleen and draining lymph nodes of the infected BALB/c mice from the experiments above were re-stimulated *in vitro* with SLA (50ug/ml) for 3 days. The supernatants from the cell cultures were to quantify the production of IFN-γ, IL-4 and IL-10 in the dLNs (6A-6C) and spleen (6D- 6F) using ELISA. These results represent 3 independent sets of experiments with similar results. *ns, not significant; **, p<0.01; ***, p<0.001; ****, p < 0.0001.*

3.6 Vaccination with ADS deficient parasite induces protection against WT re-challenge

Next, I wanted to determine whether ADS KO parasites would confer protection against WT *L. major* re-challenge. Mice previously infected in the ear with ADS KO parasites were re-challenged in the footpad with 2.5×10^5 WT *L. major* 5 weeks post-infection alongside age-matched naïve mice**.** Delayed-type hypersensitivity response was assessed by measuring the lesion size in the challenged footpad of both naïve mice and healed ADS KO (previously infected mice) 72h after the challenge. There was a significant DTH response at the site of rechallenge in healed mice **(Figure 9A)**. In addition, parasite burden in the challenged footpads of healed ADS KO mice was 100 folds more than that of naïve mice **(Figure 9B)** when analyzed 3weeks after the re-challenge. This experiment was repeated but this time the site of the re-challenge was in the ear (a distance far away from the primary infection site). As with challenge in the footpad, DTH response was noticed at early as 24 hours after the challenge at the site of the challenge over a period of 72 h **(Figure 9C).** Similarly, mice previously infected with ADS KO parasites had a lower parasite burden when compared with their naïve counterpart mice following challenge **(Figure 9D)**. The lesion size in the footpad 3 weeks post-infection in mice vaccinated with with ADS KO parasite was significantly lower compared to naïve mice (**Figure 9E**).

I was also interested in examining the immune response governing the protection in the healed mice by assessing the frequency of $CD4^+$ T cells producing IFN-γ, IL-4 and IL-10 in the cervical (**Figure 9E-9J**) and popliteal dLN (**Figure 9K-9P**) of mice from both groups. I found a similar frequency of cytokine-producing cells between the primary infection (naïve mice) and secondary infection (healed ADS KO). These results suggest that vaccination with ADS KO protects

against secondary infection with WT *L. major* in healed ADS KO. However, the mechanisms of this resistance remains unknown.

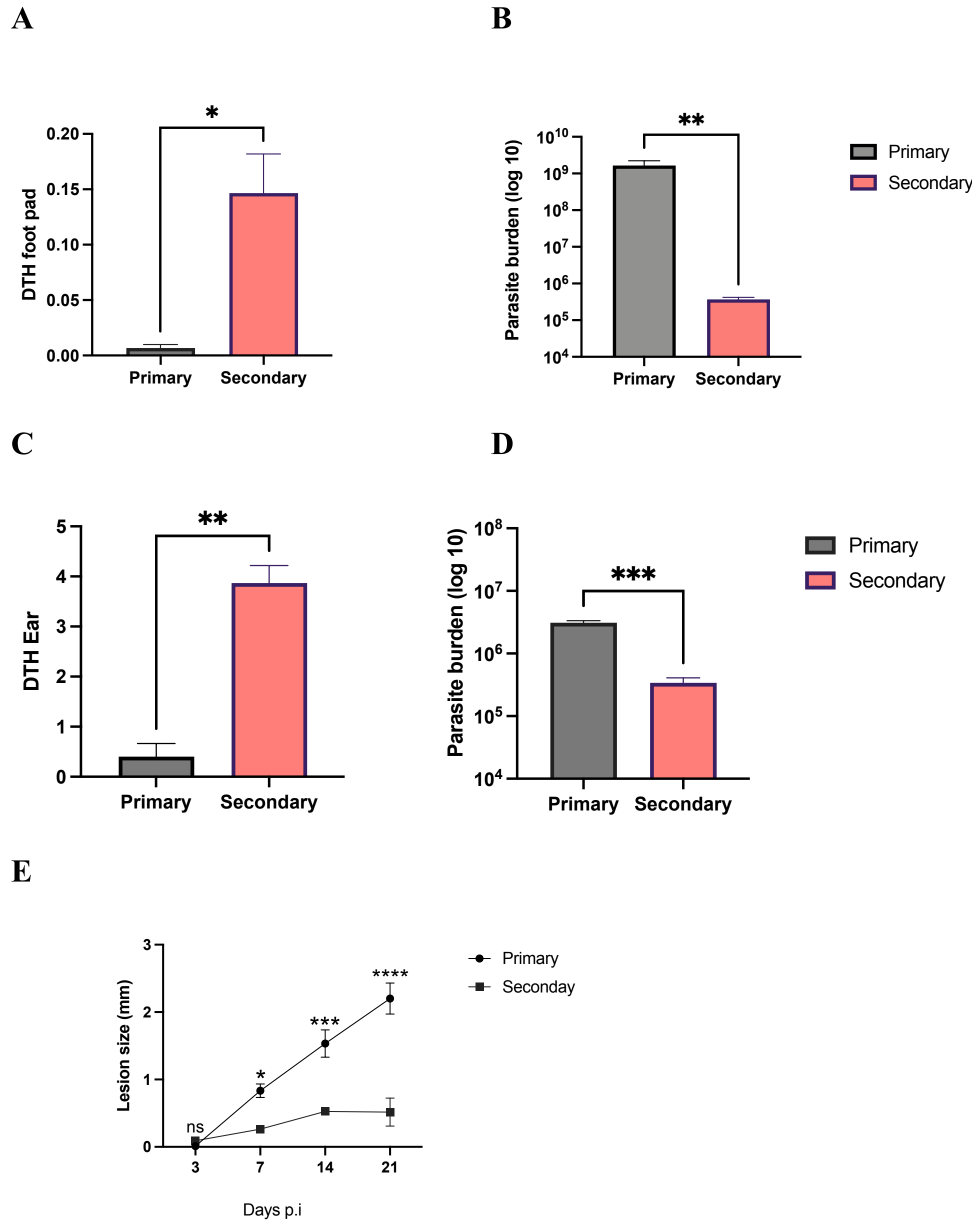

Figure 9: Vaccination with ADS deficient parasite induces protection against WT re-challenge.

ADS KO vaccinated BALB/c mice and naïve mice (control) were challenged with 2.5×10^5 WT L. major in the footpad and the ear. The DTH response and the parasite burden in the footpad (A & B) and ear (C &D) were assessed at 72 hrs and 3 weeks after the challenge. The lesion size in the footpad were measure weekly (E). *ns= not significant; *, p< 0.05, **, p< 0.01, ***,p<0.001; ****,p<0.0001*

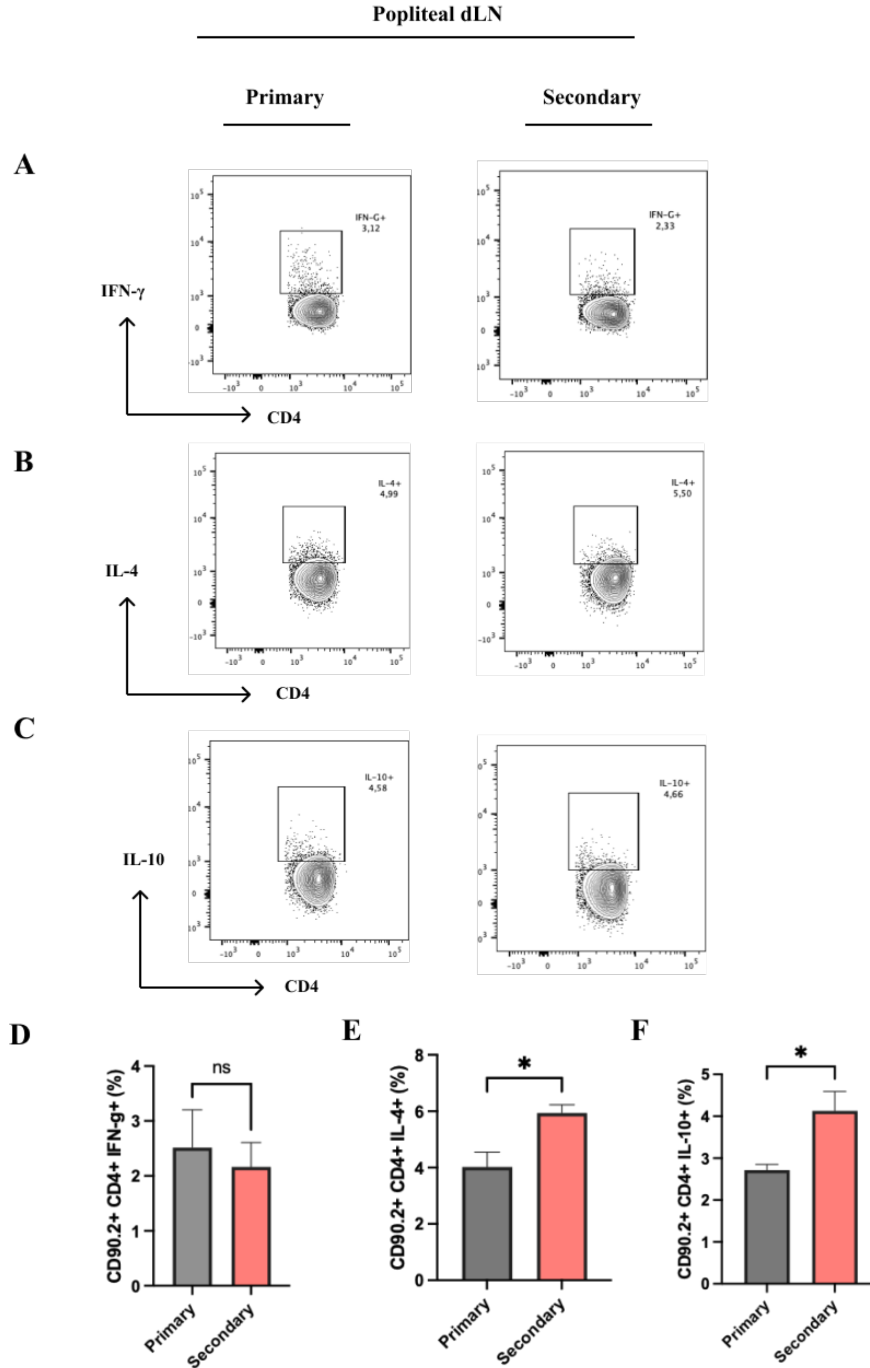

Figure 10: Vaccination with ADS deficient parasite induces protection against WT re-challenge.

ADS KO vaccinated BALB/c mice and naïve mice (control) were challenged with 2.5×10^5 WT L. major in the footpad and the ear. The frequency of IFN-γ, IL-4 and IL-10 producing $CD4^+$ T cells in the popliteal (A-F) dLNs from primary and secondary infection. *ns= not significant; *, p< 0.05.*

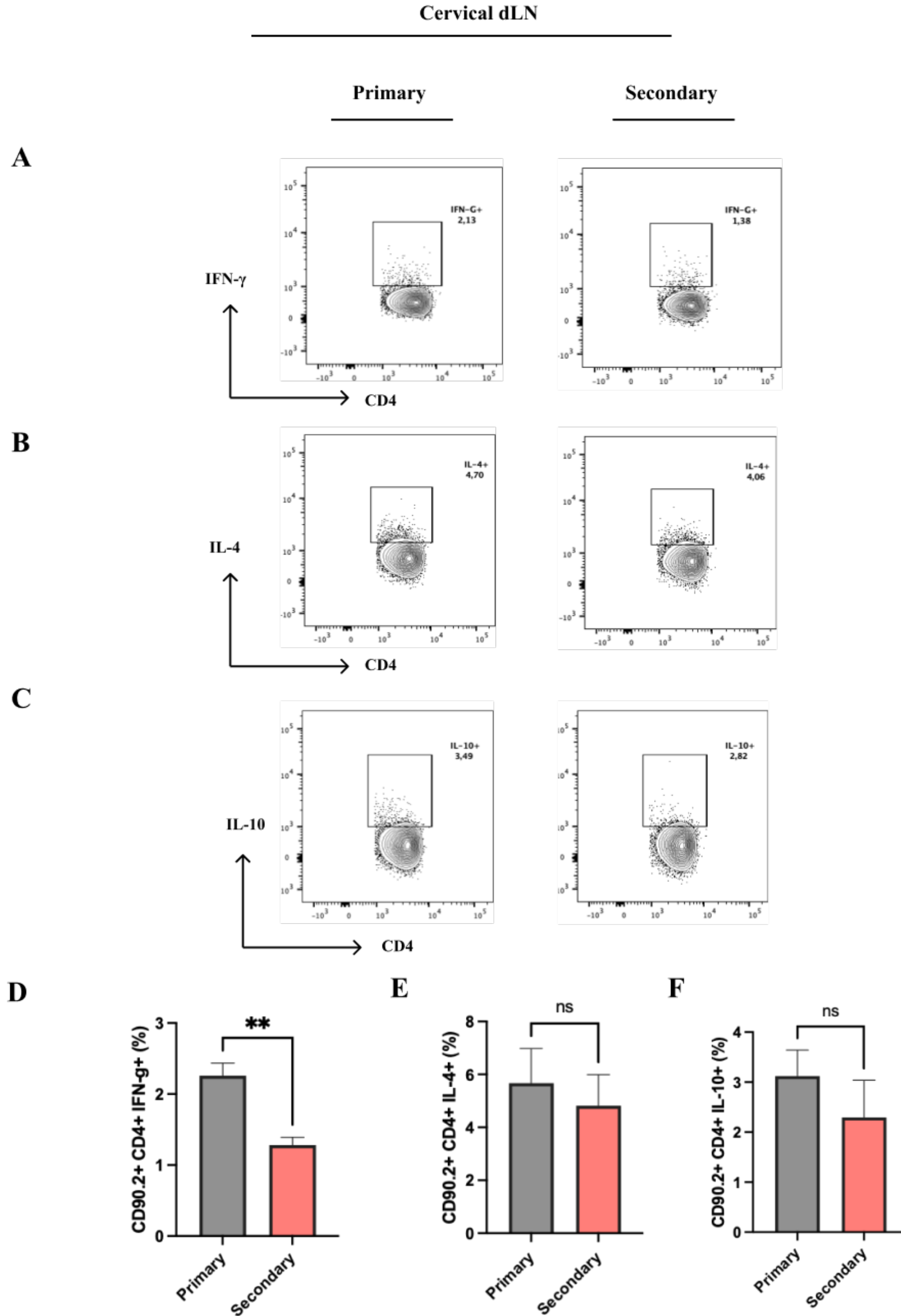

Figure 11: Vaccination with ADS deficient parasite induces protection against WT re-challenge.

ADS KO vaccinated BALB/c mice and naïve mice (control) were challenged with 2.5×10^5 WT L. major in the footpad and the ear. The frequency of IFN-γ, IL-4 and IL-10 producing $CD4^+$ T cells in the cervical dLNs (A-F) from primary and secondary infection. ns= not significant, **, p< 0.01

CHAPTER 4

4.1 DISCUSSION

Over the last few decades, attempts have been made toward developing an appropriate live-attenuated vaccine against leishmaniasis[237]. A challenge with this process has been identifying the correct antigen to target for vaccine development. Several attempts have been made to target the components of the glycocalyx, as these have been shown in several studies to contribute to the virulence of *Leishmania* parasite. The targeted deletion or disruption of genes to generate an attenuated phenotype that can be used as a live-attenuated vaccine has been unsuccessful over the years due to the generation of a completely impaired phenotype or the generation of parasites that are able to revert to virulence without regaining the function of the deleted gene suggesting the existence of compensatory mechanisms [14].

This study investigates the role of ether lipids and ether phospholipids of the surface glycocalyx in the virulence *L. major* and host immune response against the parasite. This involved the deletion of the only copy of alkyldihydroxyacetone phosphate synthase (ADS) gene present in *L. major,* using CRISPR-Cas9 gene-editing tool. CRISPR-Cas9 is an efficient gene editing tool that has been used in the genetic engineering of diverse organisms. This tool has enabled the functional analysis of virulent genes in *Leishmania* and the identification of possible drug targets to improve the treatment of *Leishmania* infections. With the help of the CRISPR Cas9 system, I introduced two components, the guide RNAs (gRNAs) and Cas9 endonuclease, which are essential to generate a double-stranded break at the site of the sequence flanking the ADS gene in the *L. major* genome, exactly three base pairs upstream of the place where the gRNAs binds. In eukaryotic organisms, double-stranded breaks in the DNA are repaired using the non-homologous end-joining pathway, but *Leishmania major* does not have the non-homologous end-joining pathway

because they lack an RNA interference pathway[238]. However, they use the microhomology-mediated end joining (MMEJ), single strand annealing (SSA) or homology-directed repair (HDR) pathway[239]. Repair through the MMEJ or SSA pathway in *Leishmania* often results in largely unpredictable deletions, which are hard to detect or verify[223, 239, 240]. Homology-directed repair allows mutation of the target gene and the introduction of a specific DNA sequence at the site of mutation[241, 223, 240].

To take advantage of the homology-directed repair mechanism, I concurrently transfected the promastigote parasites already expressing the CRISPR system with a donor template (Bleomycin resistance gene) flanked by the sequence of the site of the Cas9 target on the *L. major* genome. A stable population of parasites expressing the Bleomycin resistance gene and the Cas9 nuclease were established after several rounds of selection in media containing specific concentrations of antibiotics. The deletion of the target gene (ADS) and the repair with the bleomycin gene were first confirmed by PCR. For further confirmation, I quantified the level of ADS gene's messenger RNA at the genomic DNA level. ADS mutants had no expression of mRNA. These observations confirmed the ADS gene's targeted loss in the ADS KO parasite.

Alteration of gene sequence (deletion, disruption or point mutation) has resulted in phenotypic changes such as reduced growth rate, changes in size or motility[226]. The comparison of parasite growth between the three isolates in axenic cultures showed that the deletion of *ADS* impaired the capability of ADS KO parasites to efficiently replicate in contrast to the cultures of WT and ADS AB parasites (Figs. 2). This was evident as early as 24hrs after inoculating the parasites into the new media when compared with WT and ADS AB parasites, and a similar observation was made in work done by Zufferey et al, where the replication rate of ADS deficient parasites was found to be significantly slower compared with WT and ADS AB parasites[30]. The observation of a change in growth rate could indicate a change in phenotype since slower growth has been attributed to gene-edited parasites[242]. This could also suggest that the glycolytic pathway may be overactive, or there might be an

accumulation of by-products since the ADS enzyme acts on a substrate of the glycolytic pathway that is channelled into the lipid synthesis pathway[243].

To examine the potential ability of ADS mutants to survive and proliferate *in vivo*, I first compared the rate of infection and proliferation of the ADS KO parasites with WT and ADS AB in infected macrophages over a period of 72 hrs. I observed that ADS KO parasites had a significantly lower infection rate and compromised ability to proliferate in macrophages (**Figure 3 A-C**). The survival of the *Leishmania* parasite in macrophages rests on the parasite's ability to prevent a variety of intracellular mechanisms of parasite killing, one of which is dependent on ROS.[53] The parasite surface molecules, especially LPG, have long been known to play an important role in host-parasite interaction.[244,245, 15]

Biochemical studies of ADS mutant parasites compared with WT and ADS AB show that they lack surface molecules such as LPG, GPIL and other related molecules[30]. The absence of LPG may be responsible for the impaired ability of ADS mutants to survive since the lack of LPG in *L. major* and *L. donovani* through the targeted deletion of galactofuranosyl transferase gene (*LPG1*), which encodes LPG has been shown to strongly affect the survival of the parasite in sandfly host and macrophages[232]. LPG has been linked with impairing the microbicidal mechanisms associated with the phagolysosomes, such as NADPH Oxidase and the recruitment of the v-ATPase.[246,247] Another class of glycoconjugates, the GIPLs, have been shown to play an inhibitory role during *Leishmania* interaction with macrophages by suppressing their cytokine production. Not much is known about the roles of GIPLs during leishmaniasis infection across different species, especially in the New World Species[248]. The impaired ability of ADS KO parasites to survive and proliferate in macrophages may be due to the absence of glycoconjugates, making them susceptible to microbicidal processes in macrophages.

To understand the role of ADS in the virulence of *L. major*, I infected BALB/c mice with WT, ADS KO, and ADS AB promastigotes. I monitored the disease's progression by measuring the lesion size and quantifying parasite burden at different times post-infection. The results of the experiment showed that parasite burden in mice infected with ADS KO parasite was significantly reduced and these mice developed no visible lesion compared with those infected WT and ADS AB parasites (**Figures 4 A & B**).

Since the impact of ADS on host immune response is unknown, and surface glycoconjugates have been shown to have a strong inhibitory effect on cytokines and NO[249], I was interested in investigating whether the absence of ADS alters the host immune response following *L. major* infection. I quantified the frequency of CD4 T cells producing IFN-γ, IL-10, and IL-4 in the spleens and lymph nodes and ears of BALB/c mice infected with WT, ADS KO and ADS AB at 3 and 5 weeks post-infection. E*x-vivo* intracellular cytokine analysis revealed that the number of IFN-γ -producing CD4 T cells in ADS KO-infected mice was lower in the dLNs and ears but similar in the spleens compared to WT and ADS AB-infected mice (**Figures 5,6&7**). Similar patterns of results were observed when the frequencies of IL-4 and IL-10-producing CD4 T cells were assessed in ADS KO-infected mice. To further validate these flow cytometry observations, I quantified the total cytokine produced in the culture supernatant fluids of cells from dLNs and spleens restimulated with SLA for 3 days using ELISA. I observed that cells from ADS KO parasites infected mice have lower but significant levels of IFN-γ, IL-4 and IL-10 compared with those from WT infected mice (**Figure 8A-8F**). The lower parasite burden and cytokine level recorded in ADS KO-infected mice and the absence of lesions suggests that ADS is essential for virulence *L. major* and induction of host immune response in infected mice.

There is currently no approved vaccine against human cutaneous leishmaniasis due to the difficulty in identifying and selecting suitable immunogenic *Leishmania* antigen that will induce robust immunological protection without causing excessive pathology. Interestingly, recovery from natural or experimental infections leads to the development of long-lasting protective immunity,[250] suggesting that the disease can be prevented through vaccination if the antigens that drive this infection-induced immunity and the correlates of protection are known. It has been proposed that live-attenuated parasites are the ideal vaccine candidates against leishmaniasis because the maintenance of immunity following recovery from infection depends on parasites' persistence at the primary sites of infection.[148] The development of the suitable live-attenuated vaccine by several laboratories has been hampered by limited efficacy. In this study, I wanted to determine whether vaccination with ADS KO parasite would confer protection against secondary challenges with virulent WT parasites in BALB/c mice. Age-matched naïve mice were used as controls for this experiment. A significant DTH response was observed in the ears and footpad of mice previously infected with ADS KO parasites following rechallenge (**Figure 9A -9E**). The process of validating the protective capacity of vaccines is rigorous since there is an ambiguous immunological or other correlates of protection[251]. Some of the correlates or parameters used in previous studies include lower parasite burden, lesion size, strong DTH response, strong IFN-γ and reduced IL-4 and IL-10 cytokine response in the re-challenged mice.[252] Early studies have shown that a strong DTH response shortly after re-challenge indicates a significant TH1 response.[253] However, DTH response alone is insufficient to determine a vaccine's protective capacity.[251] The significantly reduced lesion size, reduced parasite burden, and DTH suggest that vaccination with ADS KO was protective.

To determine if the protection observed in mice vaccinated with ADS KO parasites was associated with enhanced immune response, I assessed the frequency of IFN-γ, IL-10 and IL-4 positive CD4 T cells in the lymph nodes (cervical and popliteal) draining the infection sites directly *ex-vivo* by flow cytometry. The frequency of IL-4+ $CD4^+$T cells in mice rechallenged in the footpad was more, but IFN-γ and IL-10 $CD4^+$ T cells were like naïve mice (**Figure 10A-10F**). For mice rechallenged in the contralateral ear, the frequency of IFN-γ+ $CD4^+$T cells in the cervical draining lymph node of the rechallenged mice was lower, but the frequency of IL-10 and IL-4 CD4 T cells was like the naïve mice (**Figure 11A-11F**). The increased frequency of IL-10 and lL-4+ $CD4^+$T cells with reduced IFN-γ + $CD4^+$T cells in the ADS-vaccinated mice may be a due resolution of the infection since the parasite burden in these mice had been significantly reduced.

4.2 CONCLUSION

In conclusion, my findings suggest that ADS deficiency negatively affects the virulence of *L. major* and ADS gene productions is essential for the host immune response. They also show that vaccination with ADS deficient parasites may induce protective immunity against virulent WT re-challenge, suggesting that these mutant parasites may be good candidate for life-attenuated vaccine.

4.3 IMPACT OF STUDY

This study highlights the effect of ADS deficiency on parasite virulence and host immune response in cutaneous leishmaniasis. The deficiency of ADS resulted in the generation of an attenuated phenotype of parasites that did not induce pathology but induced a sufficient immune response

that protected against virulent WT re-challenge/ re-exposure. Although more work must be done to examine the extent of these parasites' protective capacity, the initial results are promising.

4.4 LIMITATIONS

1. Mouse models do not mimic some aspects of human disease. This limits the translation of studies done using a mouse model to humans. An example of this is how the outcome of infection and the immune response is affected by the strain of the mouse used and the site of infection.
2. The expression of ADS in the mutant parasite generated was only done at the DNA and RNA level using PCR and RT-PCR; however, I could have also carried out a western blot to check for the expression of the gene at the protein level. This further validates the deletion of the ADS gene.
3. Sex and gender are biological variables that have been shown to affect the functions immune system[254]. These differences in immune response result in differential susceptibility to autoimmune disease, malignancies and infectious diseases, including leishmaniasis. I only used female mice in my experiments, I could have also used male mice because this would have allowed the assessment of gender and sex-based differences in the immune response and outcome of the disease.
4. Differences in strain have been identified to be a major determinant of the outcome of disease during Leishmania infection. Although, only one strain of *the Leishmania* parasite was used in this work, it would have been better if we could compare the impact of deleting the ADS gene on multiple strains since it would be more representative.

4.5 FUTURE DIRECTIONS

4.5.1 Assess the presence of the ether lipids in ADS KO parasites

The deletion of ADS in *L. major* is expected to impair the synthesis of ether lipids in the parasites. Biochemical analysis done ADS deficient parasites on the LV39 background confirmed that these parasites are deficient in ether lipids; it would be interesting to confirm the absence or presence of these ether lipids in the newly generated parasites; this could be done by staining the parasites using a monoclonal antibody to stain LPG and other PGs containing Gal(β1, 4) Man (α1-PO_4) repeating epitopes which can be imaged with the help of confocal immunofluorescence microscopy.

4.5.2 Check the impact of ADS deficiency on the metabolic pathways of L. major

ADS KO parasites are unable to persist in macrophages (**Figure 3**), suggesting that metabolism of the parasite may be impaired. It would be intriguing to determine the impact of deletion of the ADS gene on some key metabolic pathways, such as glycolysis since the ADS enzyme acts on a by-product in the glycolytic pathway. It will be interesting to understand how the deletion of the ADS enzyme may be affecting the glycolytic pathway and the parasite's mitochondrial integrity compared to the WT and ADS AB.

4.5.3 Delineate how ADS gene products regulate the host immune response

I had shown in my studies that ADS-deficient parasites are able to persist in mice without inducing any form of pathology (**Figure 4A&B**). This suggests that the absence of ADS gene products prevents excessive inflammation, which is the cause of pathology in cutaneous leishmaniasis. Understanding how ADS may control or influence the signals or pathways involved in host inflammatory responses would be exciting. The analysis of proinflammatory genes that are

differentially expressed in cells infected with ADS KO parasite compared with WT and ADS AB parasite may give great insight into how this process is being modulated.

4.5.4 Assess the early response to ADS KO in the infected ear

To further examine how ADS KO parasites can prevent pathology in infected mice, it would be interesting to assess if there is a population of cells that are responding differently to the ADS KO parasite compared with WT and ADS AB early on during the infection and how this differential interaction may be influencing the outcome of *Leishmania* infection.

4.5.5 Determine the role of other inflammatory cytokines in infected mice

Mice infected with ADS KO parasites could control the infection and did not show deleterious pathology, unlike those infected with WT parasites. In this study, the cytokine analysis was focused mainly on IFN-γ, IL-4 and IL-10. It will be fascinating to assess the levels of other pro and anti-inflammatory cytokines following infection with ADS KO parasites since other pro-inflammatory and anti-inflammatory cytokines have been shown to influence the outcome of infection with *L. major*.

4.5.6 Determine whether vaccination with ADS KO parasites can induce long-term protection

It is essential to carry out longitudinal studies on the ADS KO parasites since vaccines need to induce long-lasting immunity against WT re-challenge. The mice vaccinated in this study were only re-challenged after 6 weeks. It will be interesting to re-challenge these mice at different (long) time points to check if the protection persists. It is also important to repeat these experiments using C57BL/6 mice, the resistant strain to confirm that the protection observed in the preliminary experiments is due to protective immunity induced by the vaccination with ADS-deficient parasites. Additionally, it will be interesting to examine the presence and type of memory cells observed during the re-challenge.

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APPENDIX

LV39 parasites were also used in a similar experiment, as shown in chapter 3, and the results are compiled below.

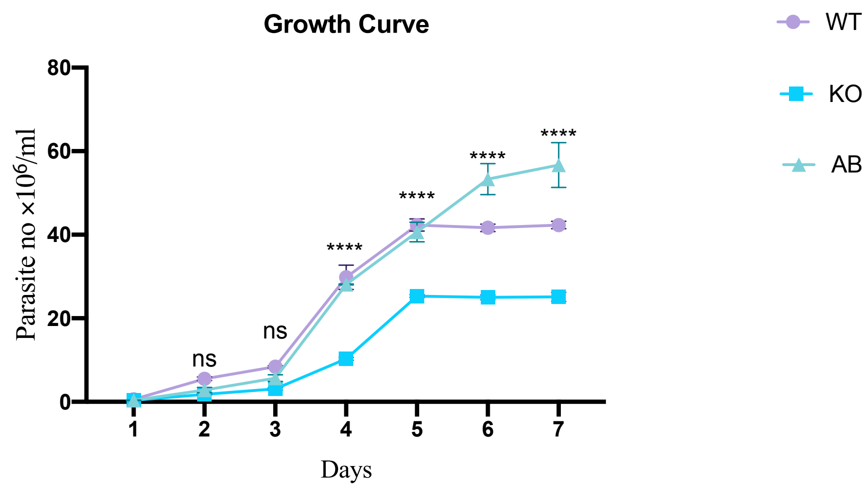
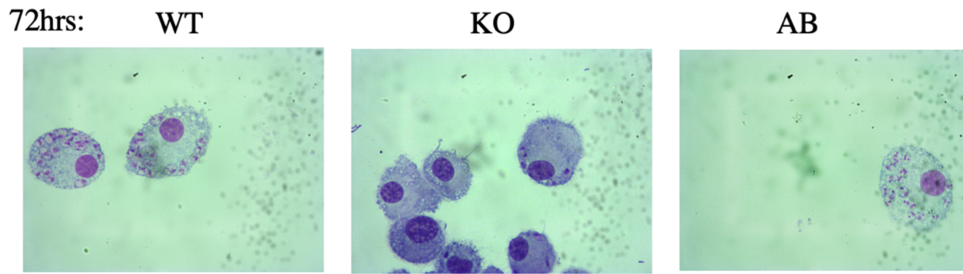


Figure 1: **The growth of ADS KO parasites is highly compromised in axenic cultures**. Equal numbers of LV39 WT, ADS KO and ADS AB promastigotes were cultured in a complete M199 medium, and the proliferation of the parasites was measured daily for 7 days. ns= not significant; ****, p <0.0001

A



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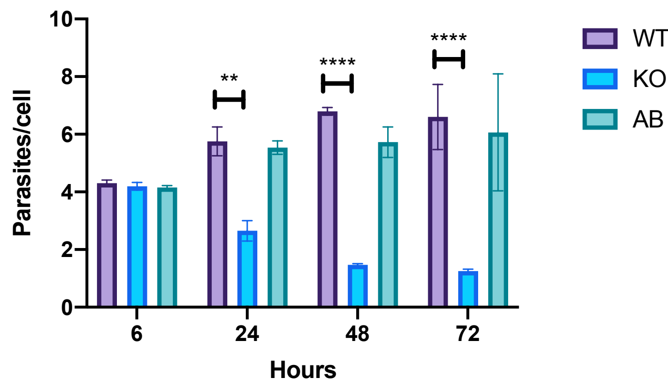


C

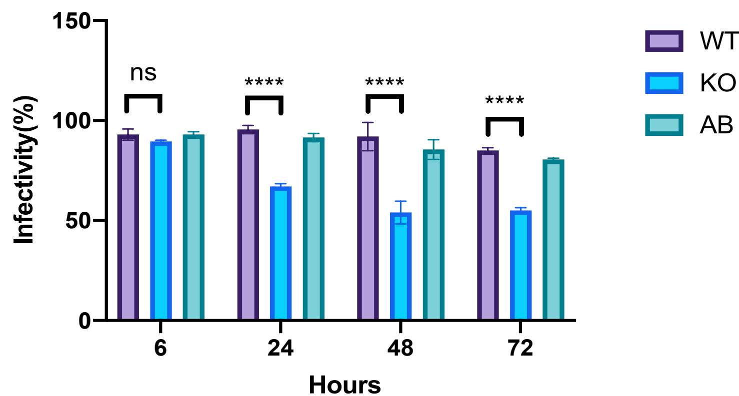
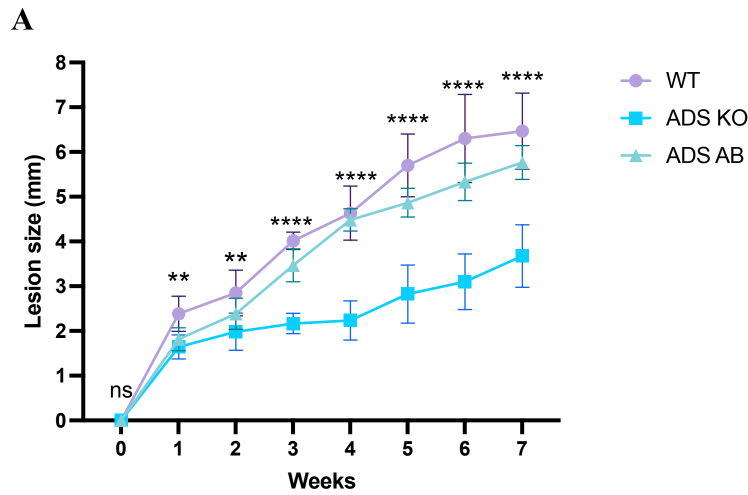


Figure 2: ADS-deficient **parasites have an impaired ability to infect and proliferate in macrophages**.

Bone marrow-derived macrophages were infected with LV39 WT, ADS KO and ADS AB promastigotes. Cytospin preparations of the infected cells were prepared at the indicated time (6, 24, 48 &72 hrs), stained with Giemsa stain, and the infected cells were assessed under a light microscope. The number of parasites inside the infected macrophages was also analyzed (A and B). The infection rate was also determined (C). These results represent three independent sets of experiments with similar results. *ns= not significant;* **, *p<0.01****, p < 0.001.*

A



B

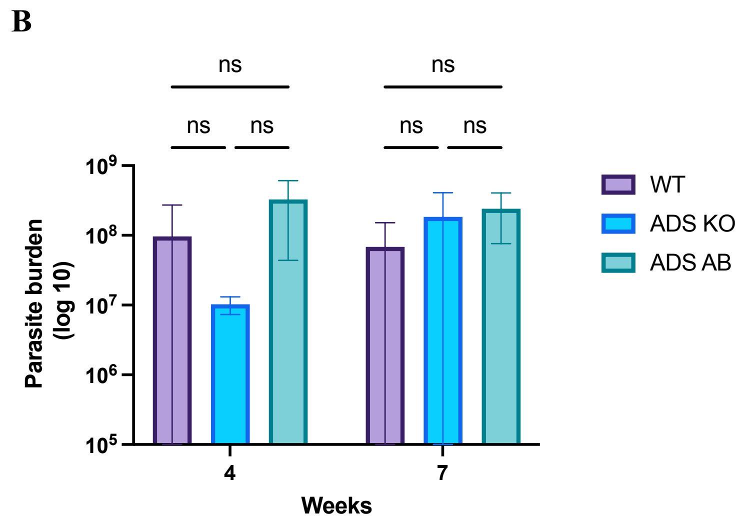


Figure 3: **ADS deficiency results in impaired virulence in vivo**.
BALB/c mice were infected with LV39 WT, ADS KO and ADS AB stationary phase parasites in the ear (9 mice per group). The lesion size of mice in each was measured weekly, and the parasite burden in the infected ears was assessed by limiting dilution (A & B). These results represent 3 independent sets of experiments with similar results. *ns= not significant*; **, *p<0.01; ****, p < 0.0001*

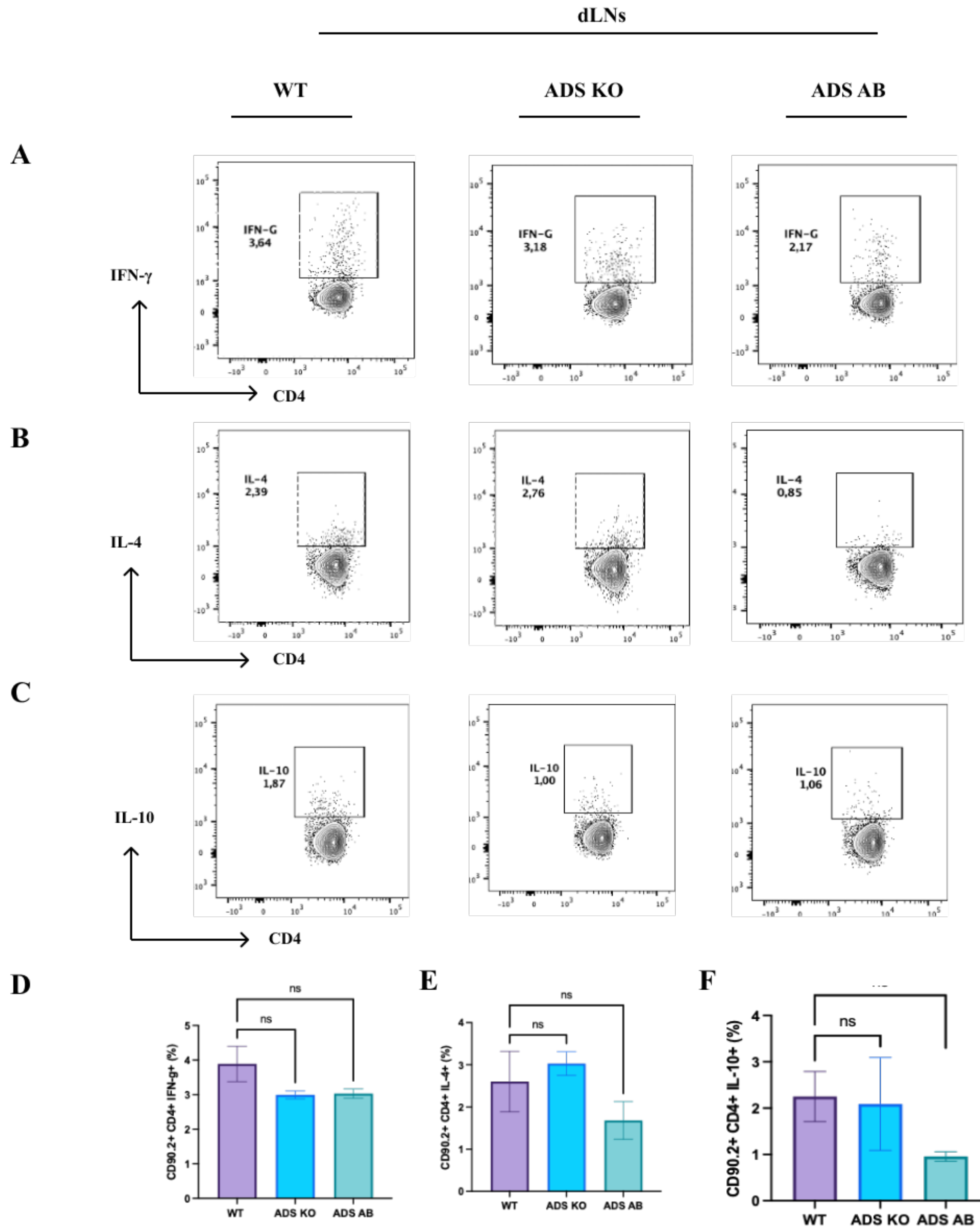


Figure 4: **ADS deficiency results in impaired virulence in vivo**.
BALB/c mice were infected with LV39 WT, ADS KO and ADS AB stationary phase parasites in the ear (9 mice per group). The lesion size of mice in each was measured weekly, and the parasite burden in the infected ears was assessed by limiting dilution (A & B). These results represent 3 independent sets of experiments with similar results. *ns= not significant*; **, *p<0.01; ****, p < 0.0001*

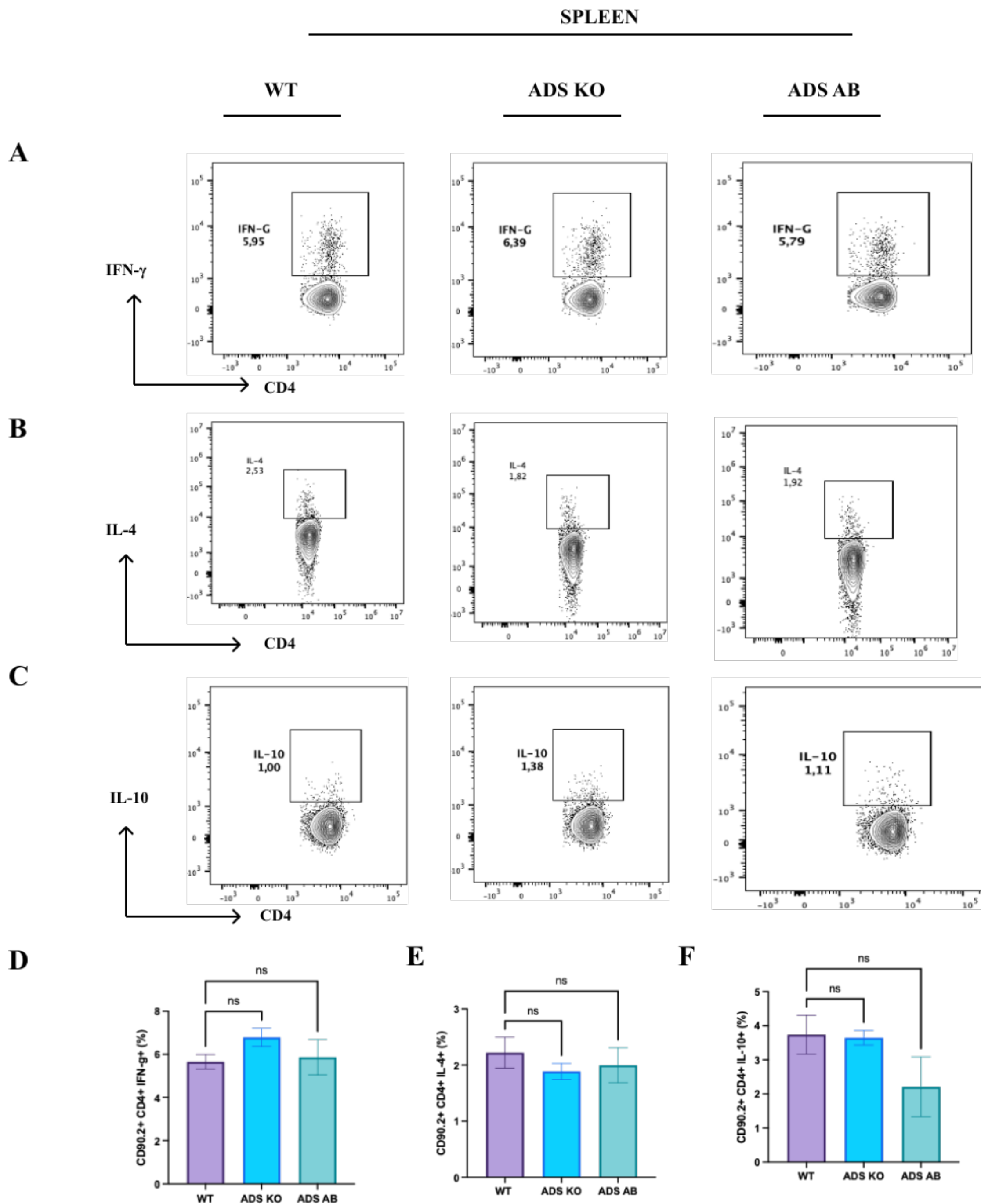


Figure 5: **ADS gene product may be necessary for host immune response *in vivo*.** BALB/c mice were infected with LV39 WT, ADS KO and ADS AB with stationary phase promastigotes were sacrificed at 4 weeks post-infection. The frequency of IFN-γ, IL-4 and IL-10 producing CD4+ T cells in the spleen (5A -5F) of infected mice. These results represent 3 independent sets of experiments with similar results. *ns= not significant.*

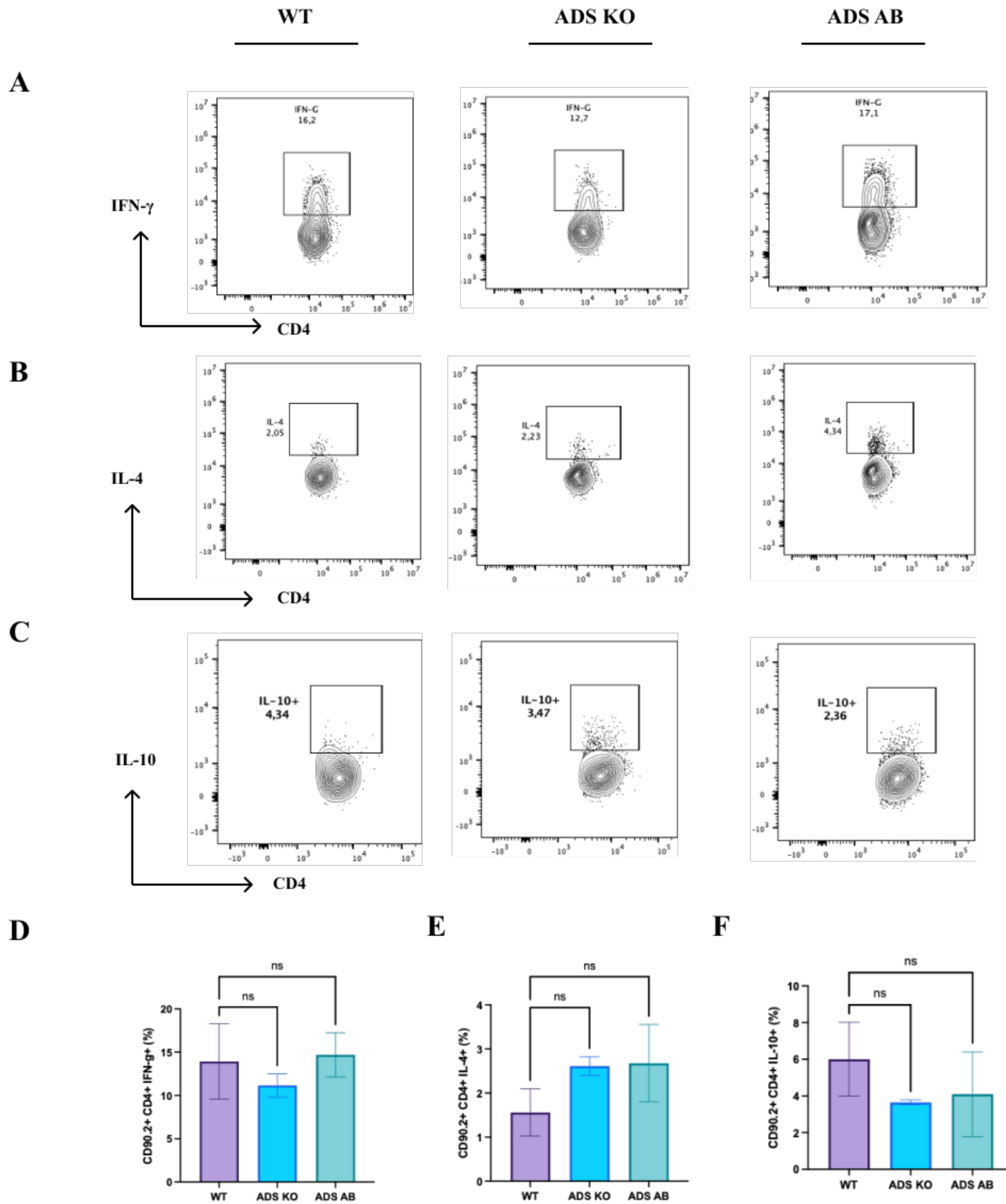


Figure 6: **ADS gene product may be necessary for host immune response *in vivo*.**
BALB/c mice were infected with LV39 WT, ADS KO and ADS AB with stationary phase promastigotes were sacrificed at 4 weeks post-infection. The frequency of IFN-γ, IL-4 and IL-10 producing CD4+ T cells in the ear (6A -6F) of infected mice. These results represent 3 independent sets of experiments with similar results. *ns= not significant.*

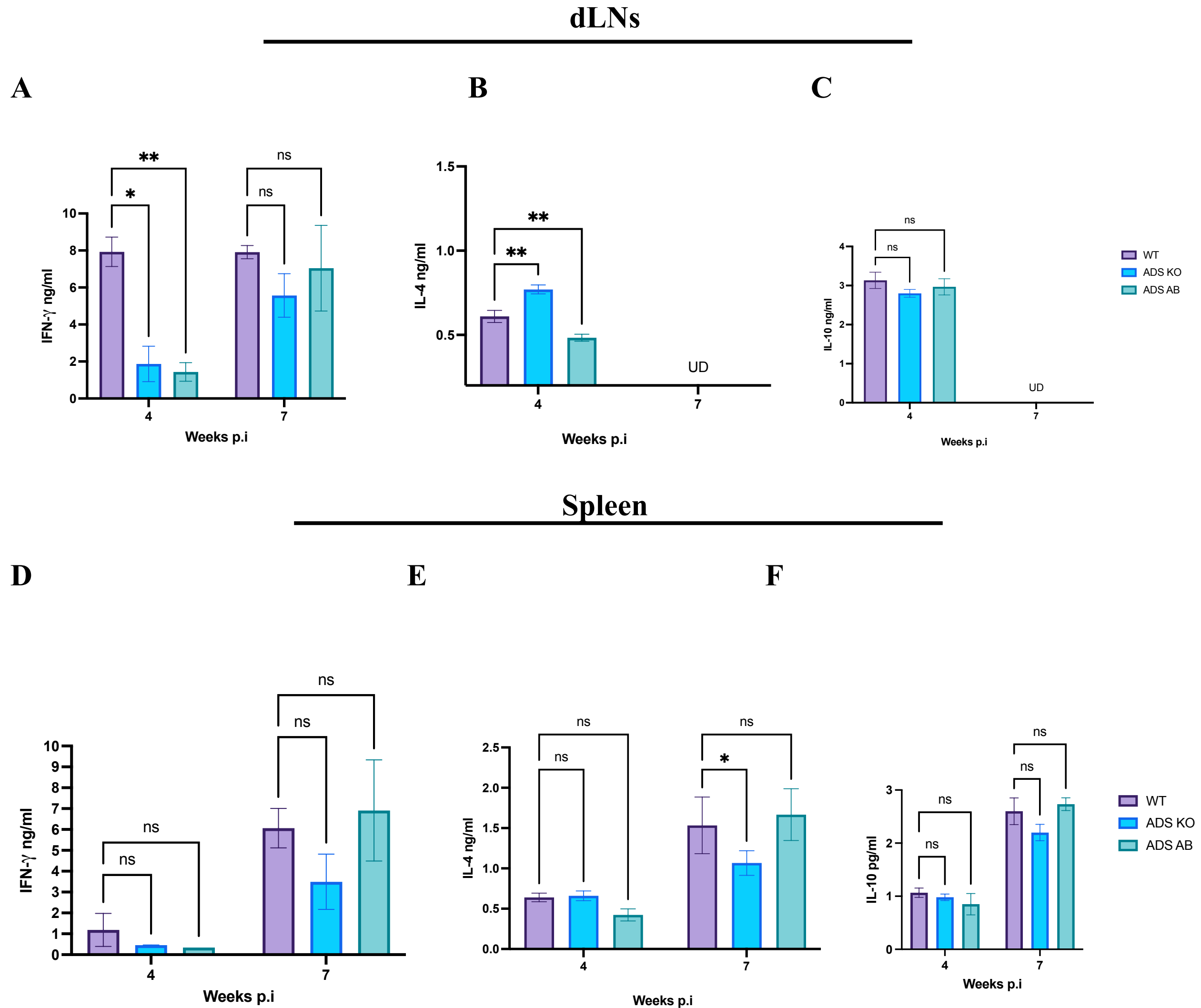


Figure 7: **ADS gene product may be important for host immune response *in vivo*.**
Cells from the spleen and draining lymph nodes of the infected BALB/c mice from the experiments above were re-stimulated *in vitro* with SLA (50ug/ml) for 3 days. The supernatants from the cell cultures were to quantify the production of IFN-γ, IL-4 and IL-10 in the dLNs (7A-7C) and spleen (7D- 7F) using ELISA. These results represent 3 independent sets of experiments with similar results. *ns, not significant; *, p<0.5 **, p<0.01.*