Role of *Leishmania donovani* Exosomes from the Perspective of Host Macrophage Polari□ation by Metabolic Path□ay S□itching

A thesis submitted to the University of Hyderabad for the award of

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In the Department of Animal Biology

by

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DEPARTMENT OF ANIMAL BIOLOGY

DECLARATION

I, **Prince Sebastian**, hereby, declare that this thesis entitled **"Role of *Leishmania donovani* Exosomes from the Perspective of Host Macrophage Polarization by Metabolic Pathway Switching"** submitted by me under the guidance and supervision of **Dr. Radheshyam Maurya**, is an original and independent piece of research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that the thesis **"Role of *Leishmania donovani* Exosomes from the Perspective of Host Macrophage Polarization by Metabolic Pathway Switching"** submitted by **Prince Sebastian** bearing registration number **17LAPH23** in partial fulfilment of the requirements for the award of Doctor of Philosophy in the School of Life Sciences is a bonafide work carried out by him under my supervision and guidance.

The thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

The student has the following publications prior to submission:

- Radheshyam Maurya and **Sebastian P**. Extracellular vesicle-associated microRNA in human parasitic diseases. MicroRNA in Human Infectious Diseases. Academic Press, ELSVIER, London, United Kingdom. Editor-Vijay Kumar Parajapati. https://doi.org/10.1016/B978-0-323-99661-7.00013-8.
- Maurya R, **Sebastian P**, Namdeo M, Devender M, Gertler A. COVID-19 Severity in Obesity: Leptin and Inflammatory Cytokine Interplay in the Link Between High Morbidity and Mortality. Front. Immunol. 2021 18 June; 12:649359. PMID: 34220807. 10.3389/fimmu.2021.649359.
- Pandey RK, Ojha R, Devender M, **Sebastian P**, Namdeo M, Kumbhar BV, Sundar S, Maurya R, Prajapati VK. Febrifugine dihydrochloride as a new oral chemotherapeutic agent against visceral leishmaniasis infection. Exp Parasitol. 2022 May-Jun; 236237:108250. Epub 2022 04 April. PMID: 35390313. 10.1016/j.exppara.2022.108250.

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Further, the student has passed the following courses towards the fulfilment of the coursework requirement for a Ph.D.

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AS 803	Scientific Writing and Research Proposal	5	Pass

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List of Abbreviation	
□	Percentage
AFR	African Region
SEAR	South-East Asia Region
AAP3.2	Amino Acid Permease
ABC	ATP binding cassette
ADP	Adenosine Diphosphate
ADR	Arginine Deprivation Response
AFLP	Amplified fragment length polymorphism
AmB	Amphotericin B
AMR	American Region
AN□□A	Analysis of Variance
APC	Antigen-presenting cell
ARG	Arginase
ATCC	American Type Culture Collection
ATG	Autophagy-related Genes
BAFF	B-cell activating factor
BALB/C	Bagg Albino
BCA	Bicinchoninic Acid Assay
BCE	Before Common Era
B□D	Biochemical oxygen demand
C3b	Complement protein 3b
CD	Cluster of Differentiation
CDC	Centre for Disease Control
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxyfluorescein Diacetate Succinimidyl Ester
CL	Cutaneous Leishmaniasis
CL	Cutaneous Leishmaniasis
C□$_2$	Carbon Dioxide
C□□ 2	Cyclooxygenase 2
CR	Complement receptor

CTLA	Cytotoxic T lymphocyte antigen
C□L	Canine visceral Leishmaniasis
C□CL	*chemokine (C-X-C motif) ligand*
DAMP	Danger-Associated Molecular Patterns
DAPI	4', 6- diamidino-2-phenylindole
DAT	Direct Agglutination Test
DC	Dendritic cell
DD8	*Leishmania donovani* strain MHOM/IND/80/DD8
dH_2□	Distilled water
DLS	Dynamic Light Scattering
DMS□	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immuno Sorbent Assay
EMR	Eastern Mediterranean Region
EP2 receptor	Prostaglandin E_2 receptor 2
ER□1/2	Extracellular signal-regulated protein kinases
ETC	Electron Transport Chain
E□s	Extracellular vesicles
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
Foxp3	Forkhead box P3
FSC	Forward Scatter
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GP63	Glycoprotein63
H_2 DCFDA	2',7□dichlorodihydrofluorescein Diacetate
$H2S□4$	Sulphuric acid
$H_3P□_4$	Phosphoric acid
HBSS	*Hanks' Balanced Salt Solution*
HCL	Hydrochloric Acid

HGT	Horizontal Gene Transfer
HI□	Human Immune Deficiency Virus
HPLC	High-Performance Liquid Chromatography
HRP	Horseradish Peroxidase
HSP	Heat shock protein
H□L	Human visceral Leishmaniasis
iC3b	Inactive complement protein 3b
IC_{50}	Half inhibitory concentration
ICT	Immunochromatographic Test
IFAT	Indirect fluorescent antibody test
IFN-γ	Interferon-gamma
Ig	Immunoglobulins
ILs	Interleukins
iN□S	Inducible nitric oxide synthase
IP-10	Interferon-gamma inducible protein 10 kD
IRA□	Interleukin-1 receptor-associated kinase
IRE	Iron-Responsive Elements
IRP	Iron Regulatory Proteins
ISPF	α-isonitrosopropiophenone
□A□	Janus kinases
□A□	Janus kinase
□C1	5,5,6,6□tetrachloro-1,1□,3,3□tetraethylbenzimi dazoylcarbocyanine iodide
□TIM	Kinase tyrosyl–based Inhibitory Motif
L. donovani	*Leishmania donovani*
L. ethiopica	*Leishmania ethiopica*
L. major	*Leishmania major*
L. tropica	*Leishmania tropica*
L.infantum	*Leishmania infantum*
LAMP	Loop-mediated isothermal amplification
LAMP1	lysosomal associated membrane protein 1
LAT	Latex agglutination test

LC-MS	Liquid Chromatography-Mass Spectrometry
***Ld* MT**	*L. donovani* Miltefosine transporter
LIP	Labile Iron Pool
LPS	Lipopolysaccharide
LR□1	*Leishmania* RNA Virus 1
LR□1	*Leishmania* RNA Virus-1
LST	Lymphocyte Stimulation Test
m/□	Mass/charge ratio
M199	Medium 199
MAP□	Mitogen-activated protein kinase
MCL	Mucocutaneous Leishmaniasis
MCP	Membrane cofactor protein
MDR1	Multidrug resistance
MDSC	Myeloid-derived suppressor Cells
MFI	Mean Fluorescence Intensity
mg	Milli gram
MHC	Major histocompatibility
MIL	Miltefosine
ml	Milli litre
MLEE	*Multilocus Enzyme Electrophoresis*
mM	Millimolar
MMP	Mitochondria membrane potential
MnCl2	Manganese (II) chloride
M□	Milli Q water
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium bromide
M□B	Multivesicular bodies
MyD88	Myeloid differentiation primary response protein
NADH-□	Nicotinamide Adenine Dinucleotide dehydrogenase- Quionone
NaHC□3	Sodium bicarbonate
Na□H	Sodium Hydroxide
NASBA	Nucleic acid sequence-based amplification

ng	Nanogram
N□	Natural Killer Cells
N□T	Natural killer T cells
NLRP3	Nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3
nm	Nanometre
N□	Nitric Oxide
N□S2	Nitric Oxide Synthase
NTD	Neglected Tropical Disease
N□	New World
oC	Degree centigrade/Degree Celsius
□**s**□$_4$	Osmium tetroxide
□□	Old World
PAMP	Pathogen-associated molecular patterns
PAs	Polyamines
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD-1	Programmed Cell Death Protein -1
PDA	Photo Diode Array
PDI	Polydispersity Index
PE	Phycoerythrin
PFGE	Pulsed-Field Gel Electrophoresis
pg	Pico gram
PGE2	prostaglandins 2
pH	Potential of Hydrogen
PI	Propidium iodide
P□DL	Post Kala-azar Dermal Leishmaniasis
PMA	Phorbol 12-myristate 13-acetate
P□C	Protein quality control
PRR	Pattern Recognition Receptors
PTP1B	Protein tyrosine phosphatase 1B
RAPD	Random Amplified Polymorphic DNA

RBP	RNA Binding Protein
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
R□S	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RSD	Relative Standard Deviation
RSD	Relative Standard Deviation
Rt	Retention time
Rt	Retention time
RT	Room Temperature
RT-□PCR	Real time-quantitative polymerase chain reaction
SAG	Sodium antimony gluconate
SD	Standard Deviation
SDH	Succinate Dehydrogenase
SDS-PAGE	Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscope
SEM	Standard Error Mean
SGT	Small Glutamine-rich Tetratricopeptide Repeat-containing Protein Alpha
SHP-1	SH2 domain Containing protein tyrosine phosphatase
SLA	Soluble *Leishmania* Antigen
Slc3a2	Solute carrier 3a2
S□CS	Suppressor of Cytokine Signaling
SPD	Spermidine
SPDS	Spermidine Synthase
SSC	Side Scatter
ST11	Suppression Of Tumorigenicity 11
STAT	Signal Transducers and Activators of Transcription
Std	Standard deviation
TBS	Tris-buffered saline
TBST	Tris-Buffered Saline Tween

TEM	Transmission Electron Microscope
TfR1	Transferrin Receptor-1
TGF-β	Transforming growth factor-beta
THP-1	Human Leukaemia Monocytic Cell Line
TLR	Toll-Like Receptor
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
Tregs	T-regulatory cells
Tris-HCl	Tris-hydrochloric acid
US	United States
□L	Visceral Leishmaniasis
□ B	Western Blot
□ H□	World Health Organization
µg	Micro gram
µl	Micro litre
µm	Micrometre
µM	Micro molar
Ψm	Mitochondria

Chapter: 1
Introduction

The intracellular parasite *Leishmania* is a unicellular protozoan belonging to the trypanosomatidae family (WHO, 2010) and is transmitted through the female phlebotomine sand flies between vertebrate hosts. The dimorphic forms of parasites are motile flagellated promastigotes and non-motile non-flagellated amastigotes. The infective metacyclic promastigotes develop in the gut of sandfly vectors, inoculated into the mammalian skin during a blood meal. They gain access to the mononuclear phagocytes through phagocytosis and are transformed into round, non-motile forms called amastigotes (Bogdan et al., 2000). According to WHO, about 20 species of *Leishmania* infect more than 70 species of animals, including humans, and over 90 species of sandflies can transmit the parasites (WHO, retrieved on 12 January 2023). Globally, 70,000 to 1 million new cases are reported annually; 20,000 to 30,000 deaths occur yearly (WHO, 12 January 2023; retrieved on 01 October 2023). Primary clinical forms are: (i) Visceral Leishmaniasis (VL), and VL is invariably fatal if neglected medical care. Post Kala-azar Dermal Leishmaniasis (PKDL) is a complicated medical condition of the VL that occurs majorly after the treatment regime; (ii) chronic Cutaneous Leishmaniasis (CL) and is mostly self-healing; and (iii) Mucosal or Mucocutaneous Leishmaniasis (MCL), usually affect the mucosal membrane. Even though CL is endemic in the South Asian region, VL and PKDL are primary forms here. The causative species of Visceral Leishmaniasis (VL) by *Leishmania donovani (L. donovani)* and *Leishmania infantum; L. donovani* found in the Old World (OW), where it is associated with VL, mainly in the Northeastern region of the Indian subcontinent, and is a notifiable Neglected Tropical Disease (NTD) in India; Uttar Pradesh, Bihar, Jharkhand and West Bengal are four states in India endemic to Leishmaniasis (Alvar et al., 2012). *L. donovani* transmissions are mostly anthroponotic, and *L. infantum* transmission is mostly zoonotic, from canine reservoir hosts to humans (Lukeš et al., 2007). The VL affecting the visceral organs, particularly the spleen, liver, and bone marrow, can cause disease with different severity, including chronic, sub-acute, or acute forms. The symptoms include weight loss, irregular bouts of fever, anaemia, and hepatosplenomegaly. The parasite *L. donovani* is the causative species of VL, primarily in Northeastern India and the East African region. Besides, *L. infantum* is responsible for causing disease, mainly in Latin American and Mediterranean regions(Ready, 2014). Most VL cases are reported from India (90%), Ethiopia, South Sudan, Bangladesh, Sudan, and Brazil (Alvar et al., 2012). Asymptomatic patients and PKDL are the significant problems in the early diagnosis of the disease. In the absence of a promising human vaccine, the treatment option majorly depends on the chemotherapeutics, and it has limitations such as being time-consuming, expensive, and toxic to the drugs. PKDL is

the complication of the VL, a sequel of VL, and occurs like macular or nodular rashes. The majority are from East Africa and the Indian subcontinent, and the potential risk factor is people with PKDL, considered a potential source of *Leishmania* infection. Another potential threat of the disease is Leishmaniasis-HIV co-infection and sporadic reports of disease in non-endemic regions due to many factors (WHO retrieved on 01 October 2023).

The immune response is initiated from the moment of inoculation of the parasites into the host, and then opsonization and complement activation occur, leading to the lysis of the parasites. Parasite secretes various effector molecules to prevent this complement system-mediated lysis of the parasite (Filho et al., 2021). Moreover, sandflies also induce the immune response by various secretory molecules present in the saliva of the fly as well as secretory products of the parasite. Overall, this contributes to immune responses such as coagulation inhibition, vasodilation, and immunomodulatory effects, and the infection zone is invaded by immune cells (Andrade et al., 2007; Giraud et al., 2018). Then, the resident cells and various recruited immune cells initiate innate and acquired immune responses (Rossi Fasel, 2018). The PRRs (Pattern Recognition Receptors) are expressed by the various recruited immune cells, and they uphold the binding of the PAMPs (Pathogen-associated molecular patterns) and activate immune response cascades. These activated mononuclear phagocytic cells phagocytose the parasite and its secretory product, eliciting an oxidative stress response like reactive oxygen or nitrogen species formation (Filardy et al., 2014).The parasite and its effector molecule response induce various cytokines, especially IL-8, that mediate the recruitment of neutrophils and act as a trojan horse for the silent entry of the parasite to its primary host cells (Passelli, Billion, and Tacchini-Cottier, 2021).After the initial encounter with neutrophils, the mononuclear phagocytes (Goundry et al., 2018), including macrophages, are sentinel in the parasite clearance by inducing an immune defence against the parasites. It also plays an essential role as an innate resistant sensor. Furthermore, macrophages also participate in the adaptive immune response initiated by exposure of microbial peptides to the T cells by processing and antigen presentation (Kaushal et al., 2017).

Macrophages are crucial for the survival and proliferation of parasites. In contrast, it also involves the elimination of the parasites by their immune response, such as pro-inflammatory cytokines producing cell recruitment and production, and antimicrobial molecules such as ROS and NO production (Liew and Cox,1991; Horta et al., 2012). Parasite adopts diverse strategies such as poor antigenic presentation, large antigenic pools, and modulating immune responses

by secretory molecules for their survival inside the host macrophages. The parasites follow many evasion strategies to reach the macrophages and effectively inactivate the host immune defence by silencing macrophage activation. The few proposed strategies are the "trojan horse strategy" with neutrophils (Laskay, Van Zandbergen, and Solbach, 2003), the silent entry of parasites by "apoptotic death and mimicry" (Wanderley et al., 2020), proteophosphoglycan mediated arginase expression and survival (Rogers, 2012), another is immediately after the parasite infection, they intersect with the endocytic pathway but resist the fusion with early endosomes or lysosomes, thereby retarding endosomal maturation for better survival (Duclos Desjardins, 2000). In brief, *Leishmania* parasites gain entry into the host macrophages either by neutrophil-dependent or independent mechanisms (Ritter, Frischknecht, and van Zandbergen, 2009; Chouhan et al., 2014). Once inside the macrophages, promastigotes transform into amastigote form inside the phagolysosome vacuoles, a process for which hosts have levied a hefty fee as many of their microbicidal functions. The cell signalling pathways are down-modulated and impaired to favour *Leishmania* parasite survival inside the host cell. The Toll-like receptor (TLR) is a PRR that interacts with the PAMPs and damage-associated molecular patterns (DAMPs). TLR comes first as an innate immune response against pathogens by the host, so *Leishmania* manipulates the receptor cascade of TLR (Sauter et al., 2019). *Leishmania* exploits the negative regulator of TLR, that is, Interleukin -1 receptor-associated Kinase-1 (Hoogerwerf et al., 2012), and is inhibited by the direct participation of the SHP-1-containing SH2 domain through a Kinase Tyrosyl–based Inhibitory Motif (KTIM) for the better survival of parasite (Abu-Dayyeh et al., 2008). The NLRP3 protein inflammasome complex is another host innate immune response mechanism the *L. donovani* parasite exploits for safely residing inside the host cell (Saha, Basu, and Ukil, 2018). Innate immune responses are mediated by the inactivation of the gene family of Suppressor of Cytokine Signaling (SOCS) in human macrophages by *L. donovani* to curb cytokine production (Bertholet et al., 2003). The *Leishmania* parasite induces the host immunosuppressive molecules like prostaglandins 2 (PGE2) by Cyclooxygenase 2 (COX 2) dependant pathway (Rabhi et al., 2012). It creates a parasite-favouring environment through the Prostaglandin E_2 receptor 2 (EP2 receptor) of PGE2 (Saha et al., 2014). *Leishmania* preferably inhibits the host serine/threonine kinase, which is the AKT pathway for regulating apoptosis as well as immunosuppression (Yang et al., 2004). The inhibition of apoptosis and neutralizing antimicrobial molecules, especially ROS, is another parasitic survival strategy inside the harsh niche of the host cells (Paiva Bozza, 2014). Elevation in the free radical formation hampers

the major components of parasites, so maintaining redox homeostasis is the primary defence mechanism of parasites (Singh et al., 2016).

The humoral response is responsible for antigen-specific antibody production by B lymphocytes. B-1 lymphocytes are a sub-type of B lymphocytes taking part in the immune response of humoral immunity. It is characterized as regulatory B cells, presents antigens, and produces IL-10 (Firmino-Cruz et al., 2018). Previously, the scientific community believed that the antibody-mediated immune response has limitations in phagolysosomes residing as an obligatory intracellular parasite *Leishmania* (Vannier-Santos, Martiny, and Souza, 2005).However, recent studies show that the B lymphocyte-secreted antibody plays a major role in neutralizing, opsonizing, and activating the complement system as an immune response in *Leishmania* infection. Moreover, the cognate interaction of T and B cells in protective immunity is of utmost importance in *Leishmania* pathogenesis and immune response (Conde et al., 2022). The cell-mediated immune response is mediated primarily by T-cells and B-cells partly involved, and the characteristic feature of cell-mediated immunity is acquired immunity. Even though the B-cells participate in cell-mediated immunity, it is majorly contributed by the T cells of the CD4+ and CD8+ cell populations (Reiner Locksley, 1995). Cell-mediated immunity is crucial in *Leishmania* infection because it determines the cytokine production in the early innate immune response and its subsets, which link the innate and adaptive immunity of the host. Furthermore, it decides the disease's fate and duration and the infection's clinical manifestation (H. Rabb et al., 2002). The MHC-mediated antigen presentation of Antigen Presentation Cells (APCs) to the T cell, and the T cell recognizes the antigen and the naive T cells become activated. The T cells crosstalk with APCs, eliciting the secondary signal and introducing local cytokine secretion and CD4+ T lymphocyte memory sub-cells (Jawed, Dutta, and Majumdar, 2019). Moreover, the proliferating CD4+ T differentiated form of effector T cells are the Th1 and Th2 subtypes(Luckheeram et al., 2012). The CD4+ cell population takes part in a bidirectional function in Leishmaniasis, the disease persistence through IL-4 and other disease-progressing cytokines (Examples. TGF-β, IL-10) (Novais Scott, 2015), or the prevention of disease through IFN-γ mediated protective immunity (Kemp et al.,2000). In the case of VL, the predominant levels of IL-10 and TGF-β might significantly contribute more to the VL pathogenesis than other cytokines (Caldas et al., 2005; Kupani et al., 2021). A recent study demonstrated that the immunology of granuloma during VL. The sustained parasitic antigens or their effector molecule exposure enhances the inflammatory cytokines, stimulating the regulatory cytokine IL-10. Furthermore, it involves developing and proliferating cells that

produce IL-10 (Nylén Sacks, 2007). CD8+ T cells mediated degradation of infected macrophages and FasL-mediated apoptosis of macrophages are the other functions of the cellular immune response (Hill, Awwad, and North; St□ger and Rafati, 2012). The CD4+ and CD8+ cell-mediated immune response is highly debatable because of its controversial role in the pathology. In general, Th-17, IL-12+ CD4+ T cell, and CD8+ cell populations participated in a protective role during the *Leishmania* infection (Cardoso et al., 2015; Esch et al., 2013; Jawed et al., 2019), and few T cells especially IL-10+ $CD4^+$ T cells, and CD4+ TGFβ+ NKT and $FoxP3^+$ T reg cells progress the disease (Bunn et al., 2018; Hohman et al., 2021; Jawed et al., 2019). The T-cell immune response can resolve the infection and give protective, long-lasting immunity. On the other hand, it can exacerbate the diseases.

Macrophages are *Leishmania* parasite resident host cells, and their functional activation state decides the outcome of the infection. The functional activation of macrophages is distinct and is classical and alternative activation, and the microenvironmental stimuli decide their phenotypic fate. Macrophage polarization is a microbe-specific phenomenon, and the link between *Leishmania*-induced macrophage polarization and immune metabolic profile remains elusive. Macrophages are dynamic cells whose cellular metabolism decides their activation states (Verberk et al., 2022). The metabolic pathways are closely interconnected with the immune signalling pathways. The SAM (acetyl-CoA, *S*-adenosylmethionine), polyamines, and α-ketoglutarate are the major metabolites that are involved in the chromatin modification of many pathways, including major immune signalling pathways (Baardman et al., 2015). For example, polyamine governs the differentiation of CD4+ populations into distinct subsets, and the polyamine-hypusine axis maintains the fidelity of T_H cell differentiation through epigenetic regulation (Carriche et al., 2021; Puleston et al., 2021). The macrophage activation is strongly linked to the polyamine pathway; the inhibition of the polyamine-hypusine axis blunts the macrophage alternative activation and acts as a metabolic switch. Furthermore, the polyamine spermidine is involved in macrophage anti-inflammatory induction through AMPK pathway activation (Liu et al., 2020; Puleston et al., 2019). Metabolic modulations in immune cells are described in infectious diseases, and in particular, macrophage immunometabolism has a major impact on its phenotype and functional plasticity regulation (Van den Bossche, O'Neill, and Menon, 2017). Macrophages are the phagocytic cells that patrol most tissues, encounter pathogens, recognize the pathogen-associated molecular pattern, and activate the TLR pathways as the first innate immune defence. This molecular recognition and activation of downstream pathways of innate and cell-mediated immunity are the core immune response

against pathogens and their host metabolism regulations (Fitzgerald Kagan, 2020; Pollard, 2009). The metabolic rewiring of macrophages modulates cellular homeostasis in various diseases, including infections; the highly plastic macrophage phenotypes and their functional polarization from classically activated M1 to alternatively activated M2 is a major hallmark of pathogen elimination or sustainability (Sica et al., 2015).

Targeting immunometabolism pathways is a major emerging therapeutic approach such as editing the polarization status in various diseases like atherosclerosis, cancer, and obesity, including infectious diseases recently in COVID-19 infection (Geeraerts et al., 2017; O'Carroll & O'Neill, 2021). *Leishmania* is a pathogen that resides inside a hostile environment of macrophages that manipulates the host's metabolic pathways by metabolic reprogramming through immune response modulation. The parasite remodels the dynamic macrophage functionality by sensing the host nutrient or metabolite resources. The *Leishmania* metabolic dependency on the host and the molecular mechanism behind it remain elusive (Goldman-Pinkovich et al., 2020; Ferreira, Estaquier, and Silvestre, 2021). It is clear that the *Leishmania* parasite preferentially depends on oxidative phosphorylation rather than the glycolytic pathway, and it also prefers to live in an anti-inflammatory milieu. On the other hand, the parasite suppresses the inflammatory cytokines during infection by altering various immune metabolism pathways with effector molecules (Huang et al., 2016; Ty et al., 2019). The *Leishmania* granuloma immunometabolism perspective emphasizes the parasite dependency on host metabolism and the metabolic rewiring of major host metabolic pathways for the parasite survival by preventing the parasite-induced protective solid immunity of the host (Saunders McConville, 2020). Arginine metabolism is a major metabolic pathway that *Leishmania* manipulates the most, and it is a crossroad of parasite for life and death either by activation of the iNOS-mediated NO production and parasite elimination or the arginase-mediated polyamine synthesis for the parasite survival (Goldman-Pinkovich et al., 2020). Arginase I is a major enzyme that enforces the immune-metabolism interaction of the parasite and host for the disease progression. It is reported that the *Leishmania* infection induces the activity of Arginase I, and it positively correlates with the secretion of IL-10. The elevated level of IL-10 is a feedback loop that enhances the expression of arginase I (Mandal et al., 2017). The arginine supplement has improved the parasites' growth and fitness (Wanasen Soong, 2008).

Leishmania exosomes alter many signalling pathways of the host for its survival and multiplication (Tomiotto-Pellissier et al., 2018). The modulation of the host-pathogen interaction via exosome is another primary survival strategy of the parasite in the harsh niche of the host macrophage (Dong, Filho, and Olivier, 2019). Exosomes are Extracellular Vesicles (EVs) with a particle diameter size of ~30-150 nm that originate through an endosomal pathway and mimic the parental physiology (Coakley, Maizels, and Buck, 2015). The EVs involve various cellular processes such as chromosome silencing, splicing, and post-translational modifications. They are also involved in pathophysiological processes such as host-pathogen interaction, immune modulation by transcriptional and translational regulation of immune response genes, and drug resistance mechanisms. The small EVs produced by most prokaryotes and eukaryote cells, the *Leishmania* parasite also has exosomes through the endocytic exosome pathway retained in the evolution (Douanne et al., 2022). The *Leishmania* EVs associated molecular cargos, including proteins, lipids, nucleic acids, and metabolites, some of which have immunomodulatory properties (Douanne et al., 2022; Kusakisako et al., 2023; Marshall et al., 2018; Statello et al., 2018). These EVs associated with cargo play a major role in infection establishment and disease progress. A recent report shows that LRV1 (*Leishmania* RNA Virus 1) is a *Leishmania*-infecting non-enveloped double-stranded RNA virus that exploits *Leishmania* exosomes as a protective envelope. It enhances the disease sensitivity toward the parasitic diseases of the host by causing more severe and aggressive mucocutaneous Leishmaniasis (Atayde et al., 2015). Some studies report that the parasite-infected host EVs communicate with the neighbouring naive cell, making the cell more susceptible to diseases through intracellular communication (Dong et al., 2021). Furthermore, the EVs are efficient mediators of many virulence genes through the *Leishmania* parasite's Horizontal Gene Transfer (HGT)(Douanne et al., 2022).

The principal goal of the current investigation was to study the host macrophage polarization by *Leishmania* exosomes of both sensitive and resistant strains of the parasites from an immunometabolism perspective. Since *Leishmania* exosomes mimic the parasites, it could lead to polarization from the M1 to the M2 macrophage phenotype. The study also emphasizes the *Leishmania* exosome-induced immune-metabolic modulation as an immune inhibitory mechanism to dominate the host immune response.

Chapter: 2
Review of Literature

2.1 rigin and History of Leishmaniasis

Leishmaniasis is an age-old parasitic disease; the presence of *Leishmania*-like organisms was documented over the prehistoric era from the fossil ambers. Furthermore, fossil evidence is explored in the extinct sandflies, Cretaceous Burmese amber (100 million years old), and Dominican amber (20–30 million years old). Both preserved pieces of evidence are the proboscis and alimentary tract of the extinct sandfly *Palaeomyia burmitis,* with blood filled in the proboscis, called *Paleoleishmania neotropicum* (Poinar et al., 2004; Poinar., 2008). The fossil records prove that the neotropical sand fly was the vector for the *Leishmania*-like species. The speciation of different *Leishmania* species based on geography is an ongoing topic of debate. There are three main hypotheses: (i) The Palaearctic hypothesis of Lysenko in 1971 proposed the origin of *Leishmania* in the Palaeocene epoch of the Palaearctic region. The diversity of the NW (new world) *Leishmania* from the OW (old world) is explained by the adoption of vectors to the new host due to environmental factors and climate change (Kerr SF et al., 2000a; Kerr SF et al., 2000b; Lysenko, 1971). (ii) The Neotropical hypothesis was put forward in 1987 by Lainson Shaw (Noyes H, 1998)and further explained by Noyes in 1998 and its hypothesis that the Neotropical origin of *Leishmania*. The speciation and substantial diversity of NW *Leishmania* are because of climate change, wide and expanded host range, and isolated geographical areas in the Neotropical region. Further, in the Eocene period, the parasite entered through infected porcupines in the Nearctic region and later spread to the Palaearctic region. However, the hypothesis could not explain the two scientific facts: porcupines' presence in the Nearctic was reported in the late Pliocene period. Secondly, only the Neotropical sand fly vector, *Lutzomyia,* originated in the Oligocene in the Nearctic (Noyes et al., 1998; Lysenko, 1971). (iii) Supercontinent hypothesis by Momen Cupolilli in 2000, Africa is where the subgenera *Leishmania* and *Sauroleishmania* evolved, while the subgenus Viannia evolved in South America following the breakup of the supercontinent Gondwana during the Mesozoic era. Furthermore, this subspecies of *Leishmania* evolved into an American *Leishmania* species during the Eocene while travelling from Asia to the Neoarctic (Momen and Cupolillo, 2000). The ancient human history with leishmaniasis was with recorded evidence and descriptions of tablets in the 7th century Before the Common Era (BCE) library of Ashurbanipal of Assyrian King, for oriental sore (Manson-Bahr, 1996). The paleoparasitological study in West Thebes (2050–1650 BCE) of the Middle Kingdom tomb found *Leishmania donovani* mitochondrial DNA fragments in mummies of Egypt (Zink AR et

al.,2006). The Ebers Papyrus, an ancient Egyptian medical document of 1500 BCE, mentioned Leishmaniasis (Maspero G, 1910). There were also reports in 800 BCE Peruvian mummy specimens that *Leishmania* infected macrophages (Fr□as L et al., 2013)

In the 16th century, reports were there for Cutaneous and Mucocutaneous Leishmaniasis. A book published by Alexander Russell (1715–1768), a Scottish physician and naturalist, about different types of oriental sore, which is of zoonotic wet Cutaneous Leishmaniasis (CL) by *Leishmania major* (*L. major)* and anthroponotic dry lesion of CL by *Leishmania tropica* (*L. tropica*) (Schnur LF, 1987). The first Mucocutaneous Leishmaniasis (MCL) was reported in the Peruvian Andes and showed disfiguration of the lips and noses in571 (Lainson, 2010). In 1827, William Twining (1790–1835), the earliest reports of kala-azar in the 19th century mentioned in his article. Further, he noted the patients in Bengal, India, with symptoms of intermittent fever enlarged, acute anaemia, and spleen enlargement. Then, he published a book with more detailed signs of Kala-azar. Later, in 1824-25, the first outbreak of the disease was in the village of Mahomedpore in Bengal, India. In later years, the disease spread in Assam and West Bengal as an epidemic. Since the skin discolouration became grey, the disease was named Kala-azar in the 19th century. The word's literal meaning is 'Black disease' (Gibson, 1983). The search for a causative agent of the disease begins at the end of the 19th century. Scottish doctor David Douglas Cunningham (1843–1914) handled similar cases of the disease in Delhi Boil but could not explain the causative agent. Piotr Fokich Borovsky (1863–1932) was first recognized as the causative agent of the oriental sore lesion caused by a protozoan (Hoare CA, 1938). In the 20th century, William Boog Leishman (1865–1926), a Scottish Pathologist, was in the service as a British medical officer for an army wing and observed Leishmaniasis cases at Dum Dum town of Calcutta, India. The biopsy samples of the soldier spleen reveal the causative organism of the disease. The ovoid-shaped bodies he observed were trypanosomes, and the disease was termed Dum Dum fever. During the same period, Charles Donovan in the period of 1863–1951, an Irish doctor and professor at Madras Medical College, reported about the ovoid bodies that he found in splenic tissue samples of live and autopsy samples of Indian subjects with enlarged spleens and remittent fever. Donovan sent his tissue samples to a protozoan Biologist, Charles Louis Alphonse Laveran (1845–1922), to confirm the species. The observation was it's a new species of the genus Piroplasma. In 1898, by order of the Government of India, Ronald Ross (1857–1932), a British doctor, started an investigation on Kala-azar. His investigation reports through a paper commenting on Leishman and Donovan's observation that ovoid bodies are not degenerate trypanosomes but are also not the new species

of the Piroplasma genus. The novel protozoan causative agent resembles the clinical symptoms of Kalz-azar, and He named it *Leishmania donovani* (*L. donovani*) (Ross R,1903). Later, Charles Jules Henry Nicolle in the period 1866–1936, a bacteriologist of France, found *Leishmania infantum* (*L. infantum*), a similar causative agent of Visceral Leishmaniasis in dogs of Tunis and concluded that dog is a crucial reservoir host (Nicolle C, 1908). In later years, the discovery and classification of different species and subspecies were mentioned in the publication of Bray et al. in 1973, such as *L. tropica* and their subspecies called *L. tropica major* and *L. tropica minor* according to their epidemiology of the infection. The same report shows a new *Leishmania* species from Ethiopia named *Leishmania ethiopica* (*L. ethiopica*) (Bray RS et al.,1973). In the Brazilian state of Sao Paulo, Buru ulcers were dominant, and the examination of skin lesions of Buru ulcer patients by Antonio Carini (1872–1950 an Italian doctor also at the same period as Adolpho Carlos Lindenberg (1872–1944), the Brazilian doctor described NW parasites (Carini A, 1909). Alfonso Splendore (1871–1953), an Italian Bacteriologist, reported similar parasites in the mucocutaneous lesion of an Espundia patient (Splendore A,1911). It was found that the apparent morphological dissimilarities compared to the old-world *L. tropica* parasites by Brazilian clinical scientist Gaspar de Oliveira Vianna from 1885 to 1914. He named it *Lapsus calami Leishmania brazilienses,* now known as *Leishmania braziliensis,* and renamed it by Alfredo Augusto da Matta in 1916 (Vianna G, 1911).

2.2 Taxonomic Classification of Species *Leishmania* Parasites

The *Leishmania* species complex and the Vianna species complex are the two main taxonomic groups into which the parasite *Leishmania* genus is classified according to a hierarchy. The *Leishmania* species complex contains five species, while Viannia contains three (**Figure 2.1**

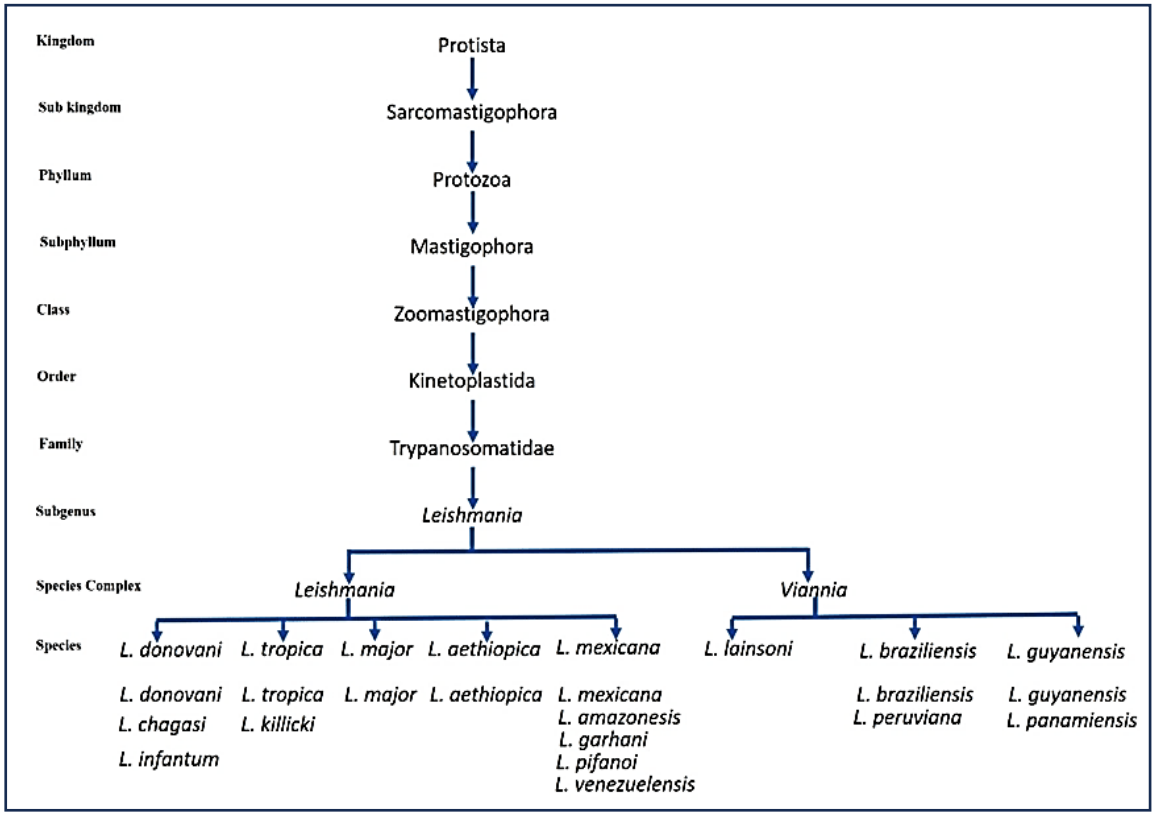


Figure 2.1: Represents the taxonomical classification of the *Leishmania* parasite (Raj et al., 2020)

2.3 Epidemiology and Geographical Distribution

Historically, the disease was limited to the tropical and subtropic regions; due to many factors such as climate change, deforestation, urbanization, tourism, migration, immigration, etc., it disseminates to various parts of the world. So, often, it is classified based on regions of occurrence; the Old World (OW) Leishmaniasis occurs in Africa, Asia, and Europe's southern part and exists in the Eastern Hemisphere as an endemic disease. The Western Hemisphere is home to the endemic NW leishmaniasis from Central Texas South to Central and South America (Kevric, Cappel, and Keeling, 2015).

The female sandflies belonging to the genera Phlebotomus (OW) and Lutzomyia (NW) are the carriers of *Leishmania* parasites. Until 1921, it was not clear, and French Biologists Etienne Sergent and Edmond Sergent proved this by applying the suspension of a sandfly to a volunteer and developing the oriental sore on his skin; the scientific communities did not accept it as proof that the sandfly was the mode of transmission of the parasites (Sergent, 1921). Then,

Saul Adler, in 1941, proved the mode of spread of *Leishmania* through the sandfly bite by experimentally infecting the sandfly with *Leishmania* parasites in the laboratory (Adler S and Ber M, 1941)

According to the Global Leishmaniasis Surveillance 2022, the Eastern Mediterranean Region (EMR) reported 76% of CL cases, and 18% are from the American Region (AMR). Eight countries, namely, the Syrian Arab Republic, Iraq, Brazil, Colombia, Iran (Islamic Republic), Afghanistan, Algeria, and Peru, have reported more than 5000 cases yearly and represent 85% worldwide. In 2022, 44% and 33% of new VL cases will be from EMR and the African Region (AFR), respectively. 14% is from AMR, and 8% is from South-East Asia Region (SEAR). The 11 countries, Eritrea, Ethiopia, India, Nepal, Somalia, Uganda, and Yemen, reported 95% of all VL cases globally. The demographic and geographical risk factors, as are the diversity and distribution of the host and pathogen biological concerns, are also notable. For example, high levels of leishmaniasis in Ethiopia are associated with people who are sleeping outdoors for their occupation. Similarly, Kenya and Argentina also reported increasing leishmaniasis cases due to vector exposure because of the living conditions and many other environmental factors (Gadisa et al., 2015; Ngere et al., 2020).

Table 2.1 Classification of Human Parasitic *Leishmania* Adopted and modified from the references *(Kevric, Cappel and Keeling, 2015; Steverding, 2017).*

Region	Subgenus	Species	Clinical Manifestation	Distribution
Old World	Leishmania	*L.aethiopica*	LCL, DCL	East Africa (Ethiopia Kenya)
		L. donovani	VL, PKDL	Central Africa, South Asia, the Middle East, India, China

		L. infantum (syn. L. chagasi)	VL, CL	Mediterranean countries (North Africa and Europe), South-east Europe, Middle East, Central Asia, North, Central and South America (Mexico, Venezuela, Brazil, Bolivia)
		L. major	CL	North and Central Africa, Middle East, Central Asia
		L. tropica	LCL, VL	North and Central Africa, Middle East, Central Asia, India
	Mundinia	*L. martiniquensis*	LCL, VL	Martinique, Thailand
New World	*Leishmania*	*L. amazonensis*	LCL, DCL, MCL	South America (Brazil, Venezuela, Bolivia
		L. infantum	VL	Mediterranean countries (North Africa and Europe), South-east Europe, Middle East, Central Asia, North, Central and South America

				(Mexico, Venezuela, Brazil, Bolivia)
		L. mexicana	LCL, DCL	USA, Ecuador, Venezuela, Peru
		L. venezuelensis	LCL	Northern South America, Venezuela
		L. waltoni	DCL	Dominican Republic
	Viannia	*L. braziliensis*	LCL, MCL	Western Amazon Basin, South America (Guatemala, Venezuela, Brazil, Bolivia, Peru)
		L. guyanensis	LCL, MCL	Northern South America (French Guinea, Suriname, Brazil, Bolivia)
		L. lainsoni	LCL	Brazil, Bolivia, Peru
		L. lindenbergi	LCL	Brazil
		L. naiffi	LCL	Brazil, French Guinea

		L. panamensis	LCL, MCL	Central and South America (Panama, Columbia, Venezuela, Brazil)
		L. peruviana	LCL, MCL	Peru, Bolivia
		L. shawi	LCL	Brazil
	Mundinia	*L. martiniquensis*	LCL, VL	Martinique, Thailand

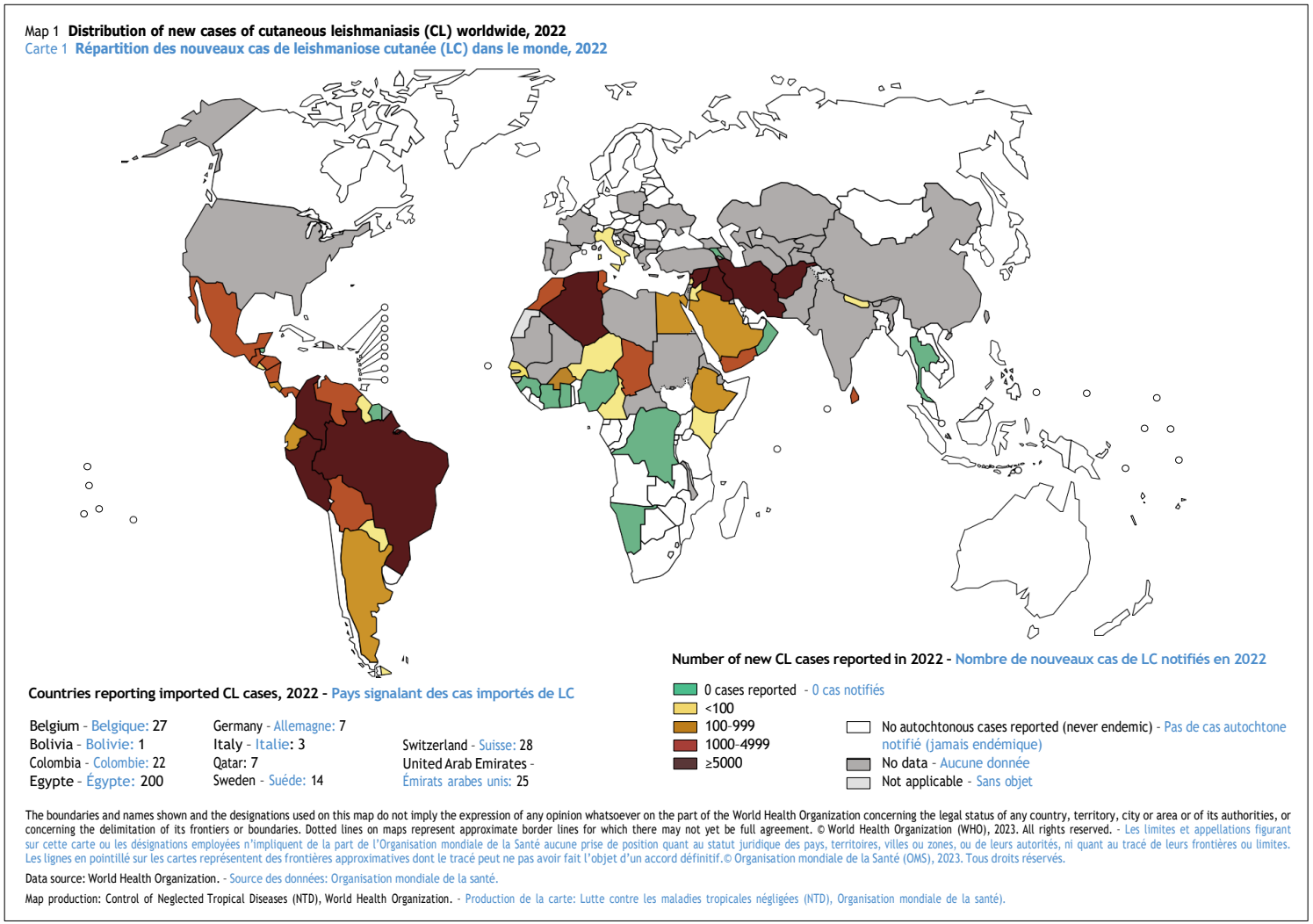


Figure 2.2: Schematic represents the Global Distribution of new cases of Cutaneous Leishmaniasis (CL),2022 (*Ruiz-Postigo et al., 2022*)

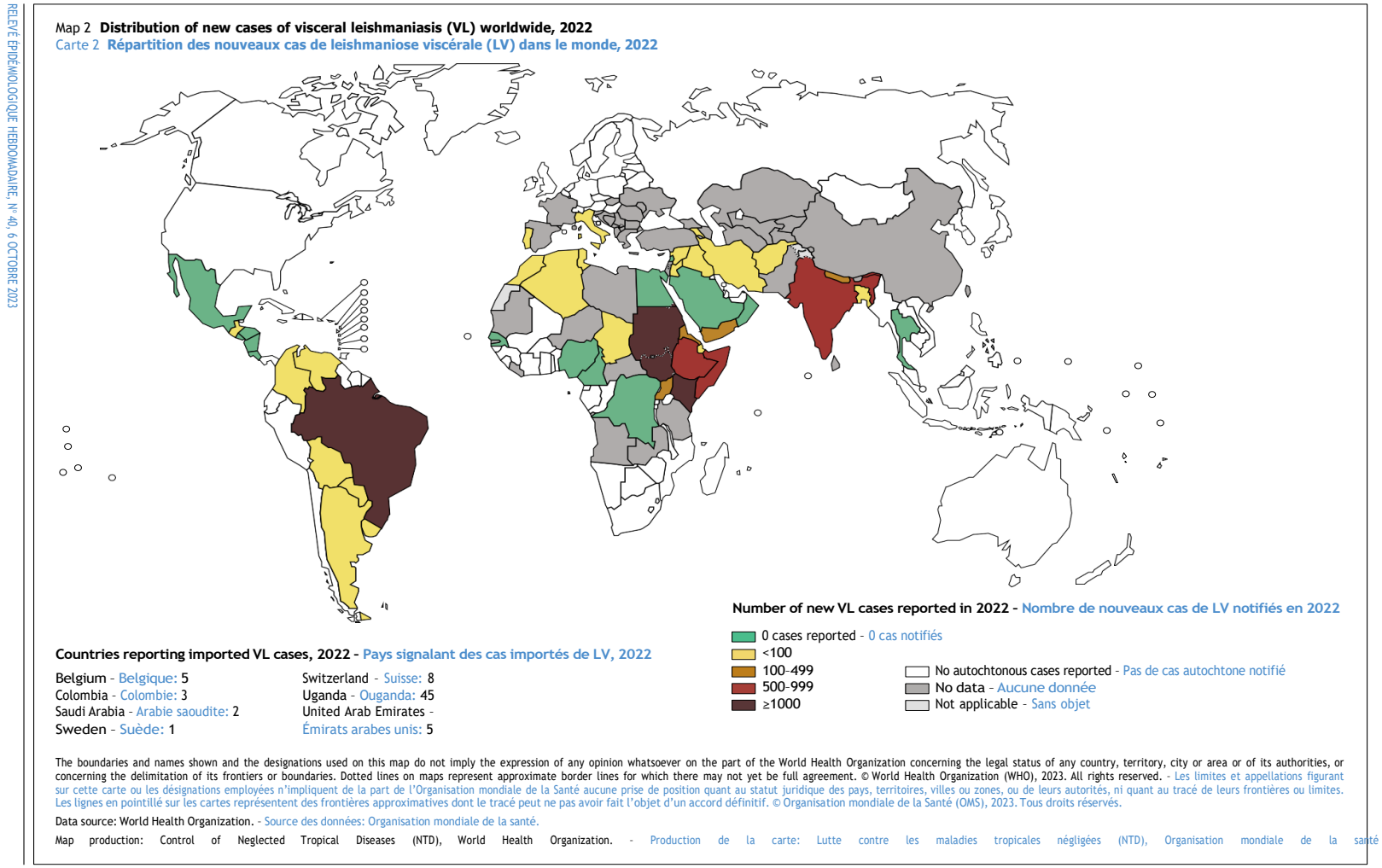


Figure 2.3: Schematic represents the Global Distribution of new cases of Visceral Leishmaniasis, 2022 (*Ruiz-Postigo et al., 2022)*

2.4 Global Burden of Leishmaniasis

Leishmaniasis is a public health threat among the top ten neglected tropical diseases. Americas, West and South-East Asia, East and North Africa are the world's four eco-epidemiological regions for leishmaniasis that continue to be a public threat. Worldwide, 99 (49%) countries are endemic to leishmaniasis. Among them, 90 (45%) were considered for CL and 80 (40%) for VL. The four countries, India, Sudan, Brazil, and Kenya, contribute more than 68% of cases of VL, and the nine countries report 85% of CL cases. The increment in cases of PKDL is another major issue in the clinical management of the disease. Globally, 771 PKDL cases were reported from 8 countries. Forty-two countries reported the Leishmaniasis-HIV co-infection that further intensified the disease burden (WHO, 12 January 2023; retrieved on 01 October 2023).

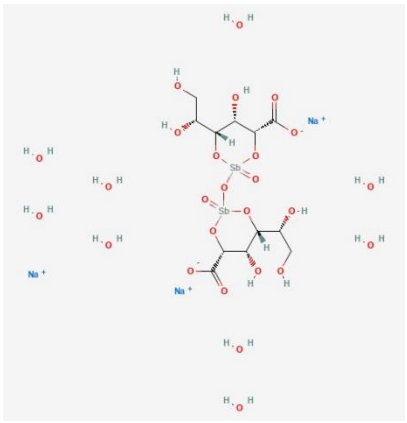
2.5 Diagnosis□prophylactic treatment□and vaccination of leishmaniasis

Leishmaniasis diagnosis is a major challenge for many reasons: the clinical resemblance of the disease's symptoms with other diseases, a broad spectrum of clinical manifestations, and co-infections (immunocompromised patients). The proper and scientific diagnosis of the disease is the primary step towards the sustainable preventive measure of disease management. Diagnosis of various forms of leishmaniasis is mainly based on clinical symptoms and parasitological or serological (rapid diagnostic tests) or its combination (WHO). Leishmaniasis diagnosis is majorly categorized into three categories: (i) Parasitological methods (Histopathology, microscopic examination, culturing techniques, isolation in experimental animals) are considered as a golden standard of diagnosis of leishmaniasis, but since it is an invasive procedure, risk is always associated and require technical experts to carry out. (ii) immunological methods (ELISA, DAT, ICT, immunoblotting, LST, FAT, and LAT), the diagnosis majorly depends on the specific humoral response of the diseases, and it is not always reliable in the case of CL and MCL since it shows a meager humoral response. Another major disadvantage is that the technique's sensitivity depends on the method; most serological assays do not distinguish the quiescent and active cases of infection. (iii) Molecular methods (PCR, LAMP, AFLP, NASBA, RAPD, MLEE) are more reliable than conventional methods because of their species-specific identification associated with disease. The information of DNA sequence-based PCRs are major techniques in molecular diagnosis, though others like gel electrophoresis for multi-locus enzymes pulse-field electrophoresis exist. The reliable molecular tools, accuracy, safety, and feasibility of the molecular method of diagnosis are used

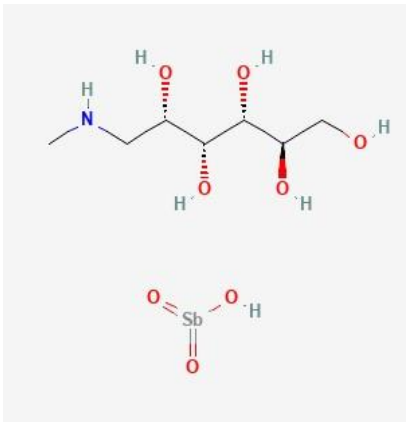
as supplementary or alternative methods of diagnosis in leishmaniasis, even though sophisticated equipment and skilled individuals are the prior requirements of this method (Thakur et al., 2020).

There are no promising vaccines for human leishmaniasis, so chemotherapy treats the disease in humans. Pentavalent antimonials of meglumine antimoniate and sodium stibogluconate are the first choices in the therapeutic approach. However, when first-line medications show poor efficacy in treating leishmaniasis, second-line medications should be considered (Croft, Seifert, and Yardley; Alviano et al., 2012; Sundar and Chakravarty, 2015). Other common medications that have been introduced for the treatment of leishmaniasis include amphotericin, miltefosine, and paromomycin. The first orally administered medication that was successful against VL was miltefosine. Even though the mechanism of the anti-leishmanial action of miltefosine is unknown, it has been reported that intracellular drug accumulation precedes apoptosis-like death by creating ROS. Additional potential pathways involve the inhibition of cytochrome c oxidase, resulting in immunomodulation and mitochondrial pathway regulations (Dorlo et al., 2012). The aminoglycoside antibiotic paromomycin has demonstrated encouraging outcomes when treating leishmaniasis, especially in its cutaneous form(Chouhan et al., 2014). Nevertheless, paromomycin cannot be used as a complete anti-leishmanial medication, and it might be a threat due to reports of the emergence of paromomycin-resistant parasites from *in vitro* research (Jhingran et al., 2009). In addition, the toxicity of paromomycin and miltefosine is a major concern (Sundar Chakravarty, 2015). Except for miltefosine, all other drugs such as antimonials (1st line of drugs), amphotericin B (AmB) (2nd line of drugs), and paromomycin require parenteral administration and continuous monitoring, which is a considerable disadvantage for leishmaniasis afflicted poverty-stricken population. The cure is often associated with awful side effects, and emerging drug resistance presents a grave issue, which has already led to the abolition of antimonials from Bihar, India (Morato et al., 2014). Anti-leishmanial chemotherapeutic treatments have transitioned from single-drug formulations to synergistic drug therapy approaches in the absence of a vaccine. These approaches were initially successful but were later shown sensitivity to the drugs. (Serezani et al., 2006). A major issue with the current systemic medications is the emergence of resistance against drugs and the severe side effects of the medications that are currently available. SAG alters the thiol profile by inhibiting the parasite's thiol metabolic enzyme trypanothione reductase. It also inhibits bioenergetics pathways such as glycolysis, fatty acid β-oxidation, and ADP phosphorylation to eliminate the parasite (Berman, Waddell, and Hanson, 1985; Berman,

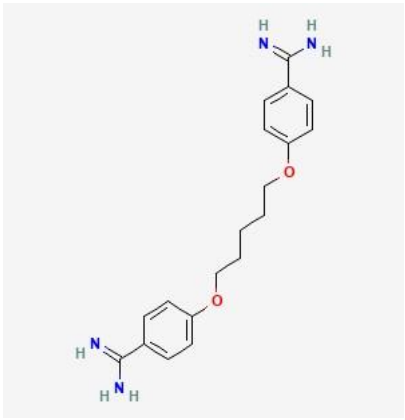
Gallalee, and Best, 1987; Haldar, Sen, and Roy, 2011; Singh et al., 2016). Adverse effects of SAG include vomiting, cardiac arrhythmias, hepatotoxicity, nephrotoxicity, nausea, cough, pain, and stiffness in the injection site muscles. Besides this, it shows unresponsiveness and relapse in previously treated patients (Sundar and Chakravarty, 2015; Singh et al., 2016). Amphotericin B is a primary second-line treatment drug, but the unresponsiveness of the SAG in endemic areas currently makes AmB a first-line drug. The possible mechanism of inhibition of AmB binds strongly with a high affinity to the parasitic membrane ergosterol and alters its permeability (Chattopadhyay & Jafurulla, 2011; Ramos et al., 1996; O. P. Singh et al., 2016). Nephrotoxicity, high fever, aches, vomiting, nausea, dysponea, hypokalemia, and thrombophilitis are the side effects of this second-generation drug. Amphotericin shows drug resistance by the up-regulation of thiol cascade protein and MDR1 (Chattopadhyay Jafurulla, 2011; O. P. Singh et al., 2016; Wasan et al., 2009). The aminoglycoside drug paromomycin is another drug for treating leishmaniasis, and it mainly acts against parasites by inhibiting ribosomes in protein biosynthesis (Chawla et al., 2011). Paramycin shows side effects such as nephrotoxicity, hepatotoxicity, and pains in the injection area muscles (Sinha et al., 2011). Overexpression of ABC transporter and protein phosphates 2A makes this drug resistant to the parasite (Bhandari et al., 2014). Miltefosine is the first oral drug effective against *Leishmania,* and its action mechanism might inhibit the biosynthesis of phosphatidylcholine; it also hampers the phospholipid and sterol composition (Dorlo et al., 2012). Parasites raise resistance against this systemic drug by lowering the expression of the LdMT efflux transporter and LdRos3 complex (Perez-Victoria et al., 2003 and 2006) and upregulation ABC transporter to pump out the drug (Castanys-Munoz et al., 2008). There are side effects of miltefosine and gastrointestinal problems such as vomiting and diarrhoea, nephrotoxicity, and hepatotoxicity, and it mainly acts as a teratogen (Dorlo et al., 2012).



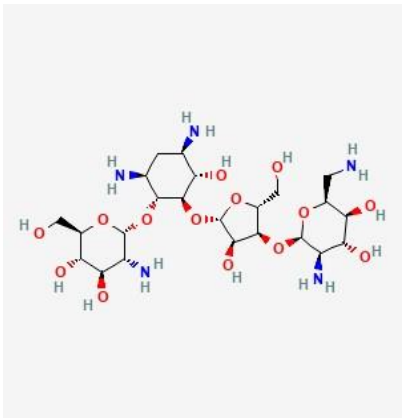
Sodium Stibogluconate



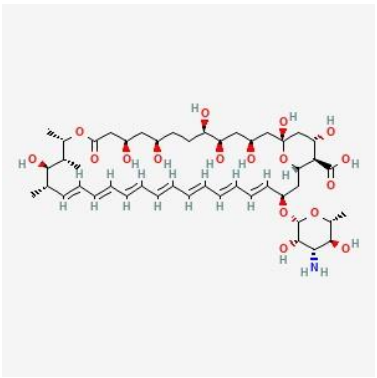
Meglumine antimoniate



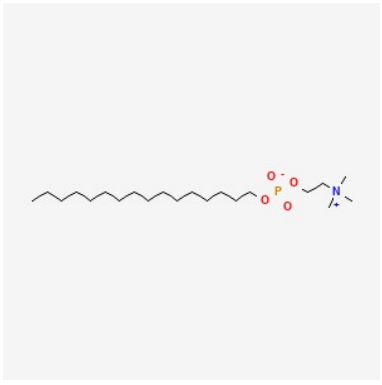
Pentamidine



Paromomycin



Amphotericin B



Miltefosine

Figure 2.4: The chemical structures of prophylactic chemotherapeutics of leishmaniasis

(*https://pubchem.ncbi.nlm.nih.gov*)

2.6 Prevention and control strategies

Preventing and controlling the disease leishmaniasis is a multifaceted process with a strategic approach. Preventive and control strategies such as (i) vector control are paramount in controlling the disease by reducing the transmission rate by reducing sandflies. Leishmaniasis typically affects the poorest and most marginalized populations with inadequate housing and domestic sanitation conditions. To attain sustainable vector control, integrated vector management and surveillance are necessary, including improving the housing and sanitation facilities with adaptation of screening for windows and doors, insecticide painting, and plastering. In many endemic regions of leishmaniasis, lack/unscientific waste management or open sewerage is a potential hindrance to vector control, which may increase the breeding of sandflies and provide resting sites for them. Deforestation and Climate change are implicated as primary risks associated with endemicity in a few regions. So, monitoring and preventing this may improve vector management. (ii) *Diagnosis and treatment:* leishmaniasis is a curable disease with early, precise diagnosis and proper treatment. In the absence of the promised vaccine, the field-friendly rapid and early diagnosis (rk39) and safe and effective anti-leishmanial medicines prevent disease transmission and burden. (iii) *Disease surveillance,* the treatment entirely relies on the effective surveillance of active and passive cases by early detection. (iv) *Prevention of disease by animal reservoir host control*. According to WHO, approximately 70 animal species, including humans, serve as the *Leishmania* parasite natural reservoir hosts. Preventing the zoonotic transmission of the disease is an integral part of the sustainable preventive strategy of the disease. (v) *Social mobilization and partnership*, as well as behavioural changes, have a significant impact on the prevention of diseases. Hence, implementing social awareness programs to educate the community about the disease is vital in eliminating and controlling the disease. Along with public mobilization, the various partnerships and collaborations in research and innovation and its implementation at the grassroots level, policy-making, and its practices, operational human resource networking for timely help and support for access and delivery of diagnostic aids, essential drugs, and other medical interventions (World Health Organization. Regional Office for South-East Asia, 2022). Vaccines are an imperative preventive strategy, although no promising vaccines have yet been approved for human use. Vaccines are available for canine leishmaniasis in many countries, such as Europe has CaniLeish□ and LetiFend□, and Leish-Tec□ in Brazil (Velez et al., 2020). There are many hindrances and difficulties in effectively controlling leishmaniasis, even when diagnosed in time, because of the limitations of the chemotherapeutics (Volpedo et

al., 2021). Research shows that those who are recovered from the infection are protected from subsequent infections. Interestingly, the protective immunity they achieve is lifelong and emphasizes the vaccine feasibility as an economically favourable preventive strategy. The recent mathematical model of VL transmission dynamics and effectiveness of the vaccination. The study was conducted in the anthroponotic transmission of the Indian subcontinent population, and it shows 60% vaccine efficiency, which means it is very close to the elimination target, which is less than one VL case per 10,000 people annually. Interestingly, the stimulated vaccine in this study shows the infected individual lower infectiousness to sandfly, reduces post-infection symptoms and is effective against PKDL cases. Here, there is a need for a prophylactic vector-based pan-*Leishmania* vaccine against the all-pathogenic *Leishmania* species (Cec□lio et al., 2020).

2.7 The *Leishmania* parasite

Leishmania, the unicellular protozoan trypanosomatid parasite with a dimorphic life cycle in a mammalian host as well as in sandfly vector host, motile flagellated promastigote, and immotile non-flagellated amastigote. Promastigote resides inside the sandfly midgut (*Phlebotominae* subfamily), serving as a vector and intermediate host. At the same time, amastigote resides in the mammalian host and replicates intracellularly. This stage is responsible for the symptoms in the infected host and is also involved in manipulating the host immune system and causing secondary infections (Kima, 2007). Leishmaniasis encompasses a spectrum of diseases in humans, and 20 species of *Leishmania* are pathogenic to humans. The various molecular and isoenzyme analysis distinguishes *Leishmania* at the species: *L. tropica, L. major, L. aethiopica, L. donovani, L. infantum,* and *L. chagasi*; three species make up the *L. donovani* complex; *L. amazonensis, L. mexicana,* and *L. venezuelensis*; and the Viannia subgenus, which contains species of *L. (V.) braziliensis, L. (V.) panamensis, L. (V.) guyanensis, and L. (V.) peruviana* (CDC retrieved on 01 October 2023).

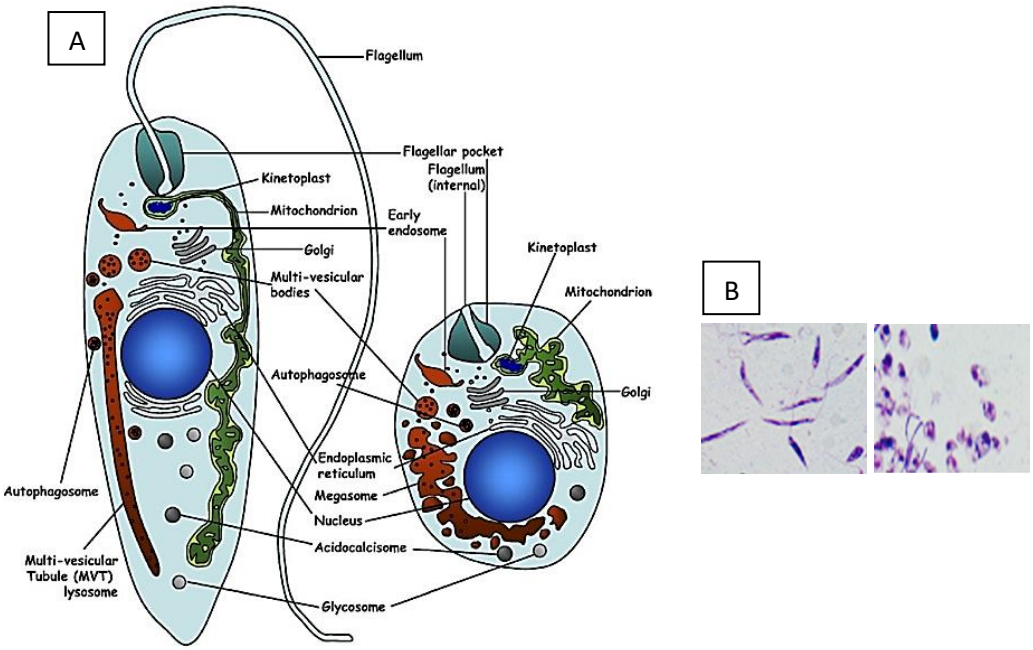


Figure 2.5: (**A**) Schematic description of promastigote and amastigote form of *Leishmania* parasite with intracellular organelles and flagellar pocket considers the anterior end of the cell (*adapted from* (Besteiro et al., 2007)). (**B**) Microscopic image of Giemsa-stained promastigote (left) and amastigote (right) images.

2.8 *Leishmania* Parasite Life Cycle

The female phlebotomine sandfly, a 2-3 mm-long vector insect of *Leishmania*, is how the disease is spread. During a blood meal, sandflies inject the infectious stage, or promastigote, into their mammalian hosts (WHO, retrieved on 01 October 2023). Host macrophages and mononuclear phagocytic cells phagocytized promastigotes on the skin wound. Then, amastigotes, or tissue stage, develop from promastigotes, and these parasites are divided by simple division and infect other phagocytic cells. The infection symptoms are based on the parasite, host, and other factors and develop as Cutaneous skin lesions or Visceral Leishmaniasis with parasitaemia. Sandfly takes blood from the host with active cutaneous lesions (in the case of CL) and parasitaemia (in the case of VL), and the sandfly becomes infected. The ingested amastigotes transform into infectious metacyclic promastigotes in the sandfly gut (the Viannia subgenus is in the hindgut, whereas the subgenus *Leishmania* is in the midgut) and migrate to the sandfly proboscis. Even though the dimorphic life stages and cycle

are similar in all forms of *Leishmania*, the disease pathology varies from species to species and with geographical location (Teixeira et al., 2013).

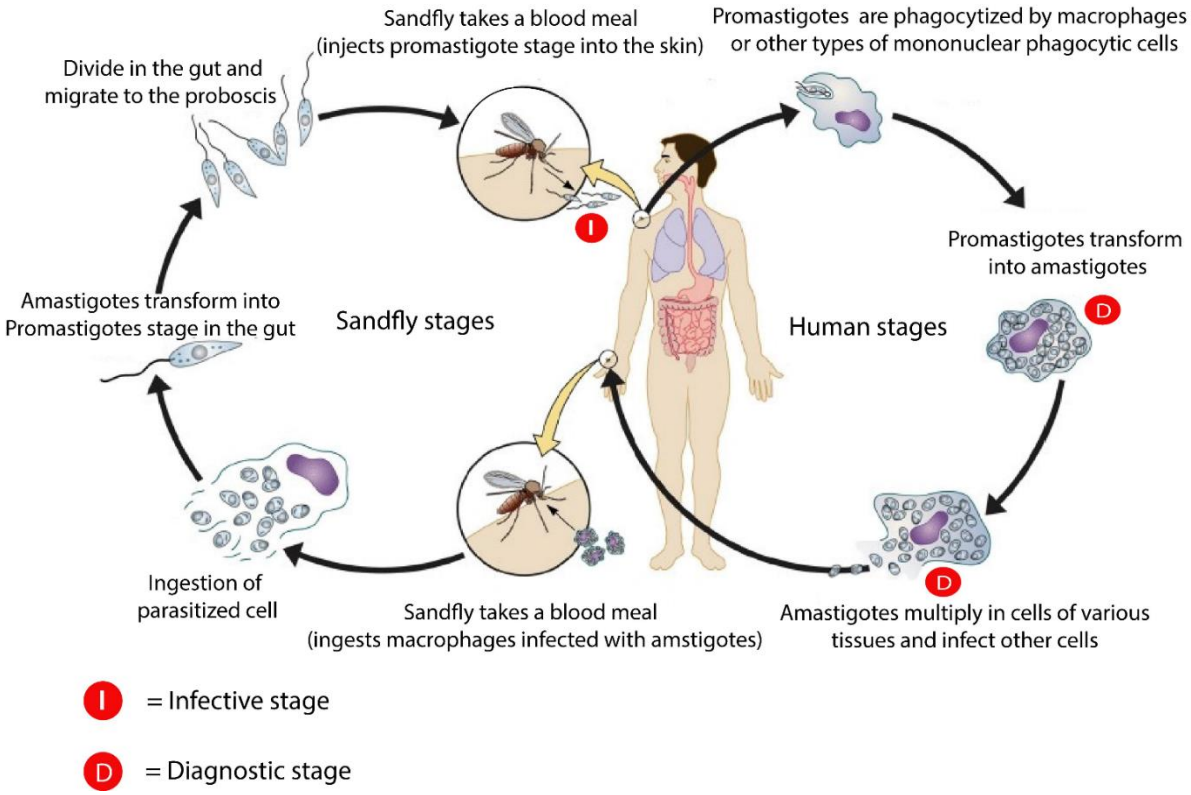


Figure 2.6: The life cycle of the *Leishmania* parasite. *(Adapted from Esch & Petersen, 2013).*

2.9 Clinical Forms of Leishmaniasis

Classified the different forms of disease based on the clinical symptoms, human leishmaniasis is clinically classified into three main types: (i) Cutaneous Leishmaniasis (CL), (ii) Mucocutaneous Leishmaniasis (MCL), and (iii) Visceral leishmaniasis (VL). Since the clinical manifestations differ with different types of diseases, identifying the specific causative species is important, and the host immunity and geographical location facilitate clinical management of the disease. CL is a form of the disease showing lesions or scars on the skin. It is primarily self-curable or chronic in □10% of patients (WHO). The symptom of CL starts with a papule on the skin, then grows as nodules with time and becomes an open ulcer. The symptoms are variable in patients since the misdiagnosis with other diseases that show skin malformation is a major problem of disease management. MCL is another type similar to CL; disfigurement of skin around the mucosal membrane, especially nose and mouth, is the major symptom. Even

though the CL and MCL are not lethal, the permanent scars and disability of the body create social stigma among the patients. VL is the most significant public health threat because more than 95% of cases are fatal if left untreated due to the systemic consequences. The clinical symptoms in the initial stages of VL are fever, weight loss, anaemia with reduction platelets, erythrocytes, and leukocytes, hepatosplenomegaly later it leads to death by sepsis from the secondary infection with a drastic reduction of platelets, severe anaemia, and rapid weight loss (CDC retrieved on 01 October 2023)

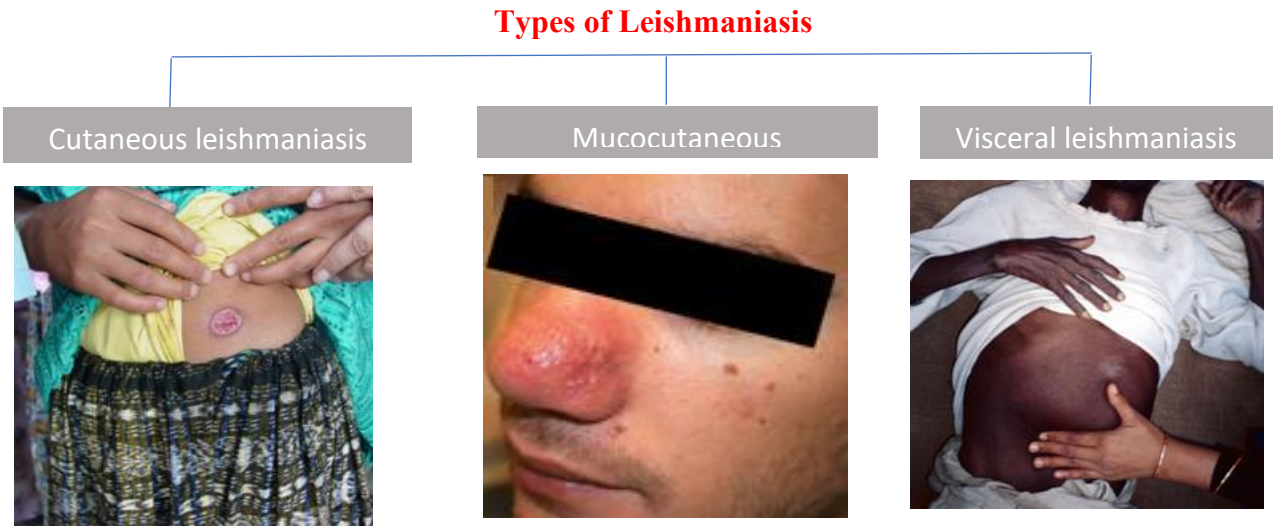


Figure 2.7: Clinical classification of ulcerative cutaneous leishmaniasis (left), mucocutaneous leishmaniasis with nasal skin erythematous patch (middle), and Visceral leishmaniasis with splenomegaly (Right).

(*adapted from* https://www.cdc.gov/parasites/leishmaniasis/disease.html).

2.10 Challenges of Leishmaniasis

Despite the milestone achievements in preventing and controlling leishmaniasis, the eco-epidemiological, socioeconomic, and biological challenges in eliminating the disease are considerable. According to a recent case study conducted in the Brazilian state of Sao Paulo, leishmaniasis and deforestation are related; in deforested areas, vector, canine, and human VL significantly increased by 2.63, 2.07, and 3.18 times, respectively. Additionally, the study discovered that the incidence of vector, CVL (Canine Visceral Leishmaniasis), and HVL (Human Visceral Leishmaniasis) decreased by 11%, 6.67%, and 29.87%, respectively, with a hypothetical 50% reduction in deforestation (Santos et al., 2021). Malnourishment (chronic

and acute), co-infections and co-morbidities, host genetics, parasitic adaptions, and virulence enhancement are the major biological challenges that accelerate the disease's progression (Volpedo et al., 2021). The currently available diagnostic tools are not adequate for the prevention of the disease; for example, the rk39 rapid diagnosis kit has a problem that cannot distinguish the current and past infection (false positivity rate is high), and it has limitations in detecting PKDL and the co-infection cases (WHO, regional report). Treatment relapse cases due to various reasons is a major challenge in disease control because of this sporadic occurrence in the non-endemic area (endemicity expansion), and drug-resistant, asymptomatic cases are continuously reported.

2.11 Immunology of Leishmaniasis

The immune biology of leishmaniasis, especially the immune defence against the parasite by the host, is complex. The coordinated contributions of several effector molecules and immune cells to manage the disease progress. The innate immunity is the front-line defence system of immunity against various pathogens. It is through the various mono and polymorphonuclear phagocytes, lymphocytes, and natural killer cells along with its effector functions such as TLRs and myeloid differentiation factor 88 (myd88) activation, complement system response, microbial effector molecules such as ROS, Chemokines and pro-inflammatory cytokines and nitric oxide (Carneiro et al., 2016; Tosi, 2005). The immune defence of the host starts at the bitten area of the sandfly; the promastigotes, along with their secretory products in the dermis of the mammalian host, elicit an immunological response by activating both the classical and alternative complement activation pathways by the interaction with serum and are very rapid and efficient (Mosser & Edelson, 1984).

The complement activation plays a crucial part in the opsonization of the parasite; C3b/opsonin binds to glycoprotein GP63 (Surface metallopeptidase of parasite) and turns C3b into iC3b, an inactive form (Hermoso et al., 1991). It helps invade the intracellular parasite *Leishmania* to the host through a broad range of phagocytic cells, including monocytes, neutrophils, and dendritic cells. However, the preferred primary host cells of *Leishmania* are macrophages. *Leishmania* invades the host cells and survives inside the macrophages. The recognition and invasion of parasites to the host by using the parasitic secretory product proteophoshoglycans and lipophosphoglycan is also a macrophage recruitment machinery stimulator (Giraud et al., 2018). *Leishmania* lipophosphoglycan signal to switch on the ERK 1/2 to enhance the production of IL-10 to counteract the secretion of IL-12, and this through MAPKs is a major

survival strategy of the parasite (G.-J. Feng et al., 1999; Mathur et al., 2004). After that, the transient infection occurs in neutrophils, which act as trojan horses to the parasite to escape from the host immunological defence and successfully interact with macrophages (Shapira & Zinoviev, 2011). The persistent initial interaction and infection in macrophages by intracellular survival factors released by parasitic flagellum and its zone is critical for the survival of the parasites (Halliday et al., 2020; Sunter et al., 2019). The choice of macrophage receptors such as CR1 and CR3 for the *Leishmania* uptake is another survival strategy of *Leishmania* to inhibit the inflammation as well as the oxidative damage of the parasite. The CR-mediated uptake accumulates the lysosome markers LAMP1 and Cathepsin D; it creates a favourable condition for the parasite inside the macrophage phagosome (Ueno Wilson, 2012). The phagocytized parasites were differentiated and replicated as amastigotes inside the host phagolysosome. The parasite prevents the cells of the host from apoptotic clearance. It spreads the infection to neighbouring uninfected macrophages through the membrane extrusions, which helps to attract and internalize uninfected macrophages (Real et al., 2014). The colonization of infected macrophages in CL is in the skin and mucous membrane in the case of MCL, whereas, in VL, it is majorly in the lymph node, spleen, bone marrow, and liver (V. Rodrigues et al., 2016).

The complex cross-talk of parasite distinct stages and immune cells, especially APCs of the host, is the deciding factor of the disease's after-effects and progression. TLR is a PRR expressed in phagocytes and APCs and recognizes the pathogen-associated molecular pattern. The macrophage TLR activation takes part in the *Leishmania* immunological response, and the *Leishmania* immunological response is categorized as a type 1 immune response, the T cell-mediated Th1 response by the APCs produced IL-12 via TLR signalling (Becker et al., 2003; Muller et al., 1989). These will initiate the macrophage-mediated ROS or NO production as an effector response (Carneiro et al., 2016). In addition, NK cell-activated chemokines mediated by IFN-γ are chemotactic protein (MCP)-1, lymphotactin, and protein-10 (IP) also produce the effector molecules against parasites (Nylén Sacks, 2007). The IFN-γ induced iNOS-dependent NO production and classically activated M1 phenotype, a major pathway involved in the parasite killing. In contrast, the arginase-mediated M2 polarization is involved in polyamine biosynthesis and parasite survival (Tomiotto-Pellissier et al., 2018). Numerous studies investigated the preventive role of TLRs in *Leishmania* infection. By way of illustration, in various species of *Leishmania,* TLR2 produced protective effector molecules of ROS and NO, TNF-α, IL-12, and NK cell activation via the NF-κB pathway (Becker et al.,

2003). Moreover, TLR2 induces Th1 and Th17 cells from CD4+ T cells and produces pro-inflammatory cytokines like CXCL1 and NO (Sacramento et al., 2017).

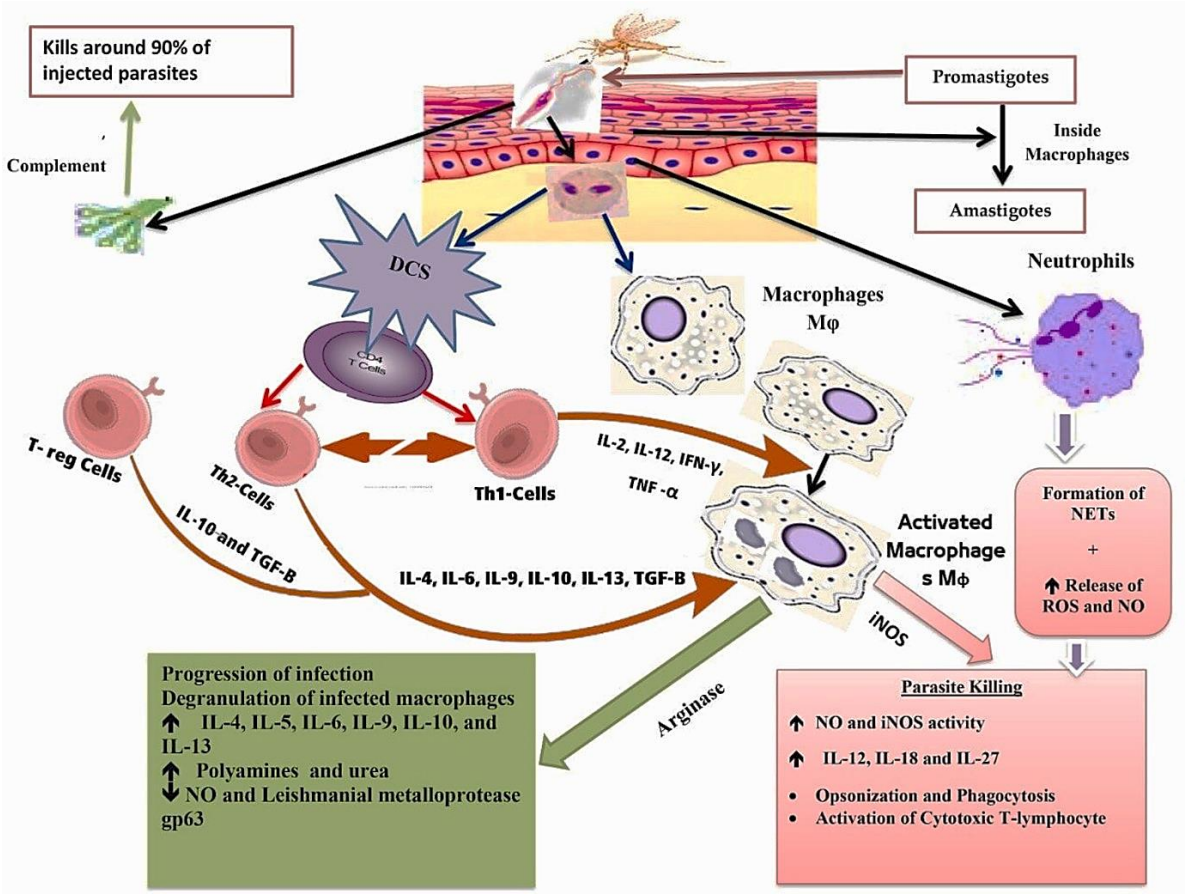


Figure 2.8: Immune biology of *Leishmania spp*. Schematic shows the various immune cells and their effector mechanism in *Leishmania* infection *(Elmahallawy et al., 2021).*

In leishmaniasis, innate and adaptive immunity plays a significant role, even though the humoral response has little effect on removing parasites, as the parasites reside inside phagolysosome as an obligatory intracellular pathogen. The divergent observations are that the humoral immune response, such as IgG2, is protective or exacerbation of pathogenesis. Indeed, some studies show the detection of *Leishmania* antigen-specific IgG, IgM, and IgE antibody titters during active leishmaniasis (Anam et al., 1999; Sacramento et al., 2017). Another study demonstrated that the B lymphocyte immune response favours some species of *Leishmania* infection (Firmino-Cruz et al., 2020). The B-cell deficient mice show a later appearance of symptoms and less disease severity (Smelt et al., 2000). Interestingly, animals deficient in B-cell activating factor (BAFF) efficiently control splenomegaly in the *L. donvani*-infected

experimental model of VL (Omachi et al., 2017). However, it takes part in a significant part in the serological diagnosis of VL by detecting antigen-specific antibody titters (Lévêque et al., 2020). Detailed and defined studies on humoral response might reveal its role in *Leishmania* pathogenesis.

The adaptive or cell-mediated immune response majorly decides the outcome of the Leishmania infection. The early innate immune response as cytokine secretions will shape the adaptive immune response either to progress the diseases or to eliminate the parasite. Removing the parasites and enduring memory to prevent re-infection are functional perspectives of the cell-mediated immunological response. The adaptive or cell-mediated immunity is indispensably by the T cells that are CD8+ memory and CD4+ effector cells, respectively (Reiner Locksley, 1995). Interestingly, the Treg (T regulatory) cells are a specialized subtype population of the CD4+ population of cells, and they suppress the immunological response for homeostasis and immune tolerance (O. R. Rodrigues et al., 2009). In the experiments in the murine model of Leishmaniasis, Treg cells suppress the *Leishmania*-specific T cell response of effector CD4+ cells and progression of diseases by persistence of parasites and loss of memory cells (Mendez et al., 2004; Sacks Noben-Trauth, 2002). In human leishmaniasis, it prevents cytokine production by hampering the proliferation of cytokine-producing effector cells Campanelli et al., 2006). The Foxp3-negative CD4+ effector cells are actively involved in the secretion of the regulatory IL-10 cytokine in VL (Nylén Sacks, 2007). The T cell-mediated Th1 response protects against parasites by majorly inducing the pro-inflammatory IFN-γ and TNF-α by host resistance. The Th2 response induces a susceptible phenotype with the persistence of the parasites (Kemp et al., 1994). The active VL cases predominantly induce the regulatory and ant-inflammatory TGF- β and IL-10 cytokines and sustained levels of cytokine IFN-γ (Kemp et al.,1994; Kupani et al., 2021). However, this polarization paradox is more complex in human leishmaniasis; for example, the Th1-mediated hyper-inflammatory state in diffuse cutaneous leishmaniasis leads to metastatic infection (Silveira et al., 2009). Furthermore, the IL-17-producing Th17 regulatory T cell population is also involved in leishmaniasis, and its response is mainly linked to the tissue or parasite species specificity. Recent reports reveal it plays a major role in CL disease progression and lesion formation(Gon alves-de-Albuquerque et al., 2017). The TGF- β and IL-10-producing Treg cells, such as $CD4^{+}CD25^{+}FoxP3^{+}$, are involved in the disease progression in VL patients (P. Kumar et al., 2017). The CD8+ T cell population in leishmaniasis depends on the disease's manifestations. It is mainly involved in a protective role by clearing parasites through IFN-γ

production (Nateghi Rostami et al., 2010). The CTLs of CD8+ T cells are majorly responsible for forming granulomas in *Leishmania* parasite infection (Rossi & Fasel, 2018). In contrast, it is observed that the IL-10-secreting CD8+ cells in disease development in MCL, DCL, and PKDL (Bourreau et al., 2007; Faria et al., 2009; Hernández-Ruiz et al., 2010; Mukherjee et al., 2019). The T cell response in leishmaniasis is highly divergent and complex and associated with the Th1/Th2 dogma of immune response. Overall, it elicits responses based on species and forms of the diseases as well as the host immune competence, and a large amount of data suggests the multifaceted role of T cell immunity is to balance the immunological response during the parasite infection.

2.12 Intracellular Survival Mechanisms of *Leishmania* Parasite by Modulation of immune-response of host

Survival of the parasites inside the hostile environment of the macrophages is through adopting various exploitive mechanisms. The dimorphic stage promastigote first comes in contact with host mononuclear phagocytic cells to escape the host's immunological response and propagate successfully by modulating the host's immune response (Matte Olivier,2002). Macrophages are the primary host cells for *Leishmania,* even though other mononuclear phagocytic cells take part in the initiation of the infection, so the modulation of host macrophages is predominant. The multifaceted modulation of immunological response includes regulation of the immune signalling, secretion of immune modulatory molecules or degradation of microbicidal molecules, regulation of transcription/translational factors, and poor antigen presentation (Liu Uzonna, 2012). The TLR-mediated early host innate immune defensive response against the parasite by the macrophage is initiated via the MyD88 independent or dependent pathway and leads to the activation of MAPK. The MAPK cascades produce pro-inflammatory cytokines (Soares-Silva et al., 2016). The pro-inflammatory cytokines eliminate the parasites, so the parasites exploit the TLR pathway for their better survival. The successful establishment of infection through the TLR pathway by using modulators or negative regulators that tightly regulate the TLR-mediated immune response of the host at the time of infection (Srivastava et al., 2012). The SHP-1 is a negative regulator protein encoded by the gene PTPN 6, and the phosphatase activity of the SHP-1 is to dephosphorylate the phosphate group from the tyrosine residue of IL1 Receptor-Associated Kinase 1 (IRAK1). SHP 1 and IRAK 1 interact through an evolutionarily conserved motif of IRAK 1, KTIM. This interaction will inactivate the IRAK 1 intrinsic kinase activity. Hence, the kinase cannot dissociate from the MyD88 complex, thereby

preventing the MyD88-TRAF6 association, leading to the inactivation of TLR signalling. The double knockdown of SHP-1 shows better IRAK 1 kinase activity (Abu-Dayyeh et al., 2008). The absence of SHP will induce the Th1 inflammatory molecules like IL-1β, IL-6, and TNF-α, eliminating parasites (Forget et al., 2005). The overexpression of TGF-□-1 down-regulates the expression of TLR4. The recombinant TGF-□-1 enhances the expression of the phosphatase SHP-1 and inactivates the IRAK1 and subsequent cascades (S. Das et al., 2012). The SOCS is a negative JAK-STAT pathway regulator, suppressing cytokine production. The SOCS family has eight members: CIS, SOCS 1 and SOCS 7 (Chandrakar et al., 2020). SOCS 1 has a well-known role in apoptosis, and it negatively regulates the apoptotic pathway by suppressing the apoptosis-inducing cytokines like TNF-α and IFN-γ, so it is majorly involved in infection (Delgado-Ortega et al., 2013). The virulence factor and various effector molecules, such as GP63 and EF-1α, to host cells through the exosomes activate phosphatase SHP-1 and PTP1B. These disrupt the signalling pathways of IFN-γ/Jak-STAT1; subsequently, they dampen the antigen presentation and create a presumptive environment for the parasite (J. M. Silverman et al., 2010). The efficient Protein Quality Control (PQC) machinery of intracellular protozoan parasites contributes to keeping the parasite's metastable aggregation-prone proteome functionally stable against hostile environmental challenges. Heat Shock Proteins (HSPs) are the major form of PQC, and in *Leishmania* parasites, many HSPs such as HSP90, HSP70, HSP60, HSP100, HSP40, HSP23, SGT, ST11, TCP20 have multifaceted roles of proteome protection by folding and degradation regulations. The autophagy machinery ATG 3 and ATG 4 are other components of the PQC network of the *Leishmania* parasite (Morales et al., 2010; Requena et al., 2015). The HSPs in the *Leishmania* exosome ensure the target-specific and protective export of its cargo proteins to the host (J. M. Silverman et al., 2010). Iron is paramount in the survival of intracellular pathogens and the host cells for their better survival, as it plays many roles in cellular mechanisms such as maintaining cellular homeostasis, erythropoiesis, and cofactors for many enzymes. *L. donovani* employs novel strategies to extract iron directly from the labile iron pool of macrophages. The parasite exploits the acquisition of sequestered iron of macrophage by altering the TfR1 (Transferrin Receptor 1 pathway) of the host cell. *Leishmania* infection induces the expression of TfR1 by sensing the iron depletion in the LIP of the host macrophage with iron sensory proteins that lead to the activation of IRP1 □ 2. After that, the IRP forms a complex with iron-responsive elements (IRE) that upregulate TfR1 by posttranscriptional regulation (Wilson et al., 2012). *L. donvani* directly scavenges the iron from LIP and enhances the production of intracellular iron through the TfR1 pathway mediated by the IRP-IRE interaction of host macrophages in both cell culture

and animal models (N. K. Das et al., 2009). The immune inhibitory/modulatory mechanisms are major survival mechanisms of parasites and are of two types: (i) extrinsic and (ii) intrinsic immune checkpoints. The extrinsic immune inhibitory agent, mainly by the T-regulatory cell's recruitment and production of anti-inflammatory and regulatory cytokines, counteracts the T-cell population-mediated host immunological response against the parasites (Kubo Motomura, 2012). MDSCs (Myeloid-derived suppressor Cells) are myeloid in origin and are natural suppressors that elicit a suppressive functional effect on the T cell population (Gabrilovich Nagaraj, 2009). The isolated MDSCs from BALB/c mice that are challenged with *L. donovani* parasites are highly immunosuppressive, and the repressive mechanism of MDSCs by the suppression of the T cell population function through depleting L-arginine and inactivation of protective cytokine production. MDSCs from *Leishmania*-infected mice show high-level expression of suppressive molecules such as cyclooxygenase-2 and Arginase I, iNOS, and PGE 2 (Bandyopadhyay et al., 2015). In the intrinsic inhibitory mechanism, the recruitment of inhibitory receptors such as CTLA-4, PD-1, CD47, CD300a, and CD200 and their subsequent binding sends an inhibitory signal through Tyrosine-based Inhibitory Motif (ITIM) (de Freitas e Silva von Stebut, 2021; R. K. Singh et al., 2018; Vaine Soberman, 2014).

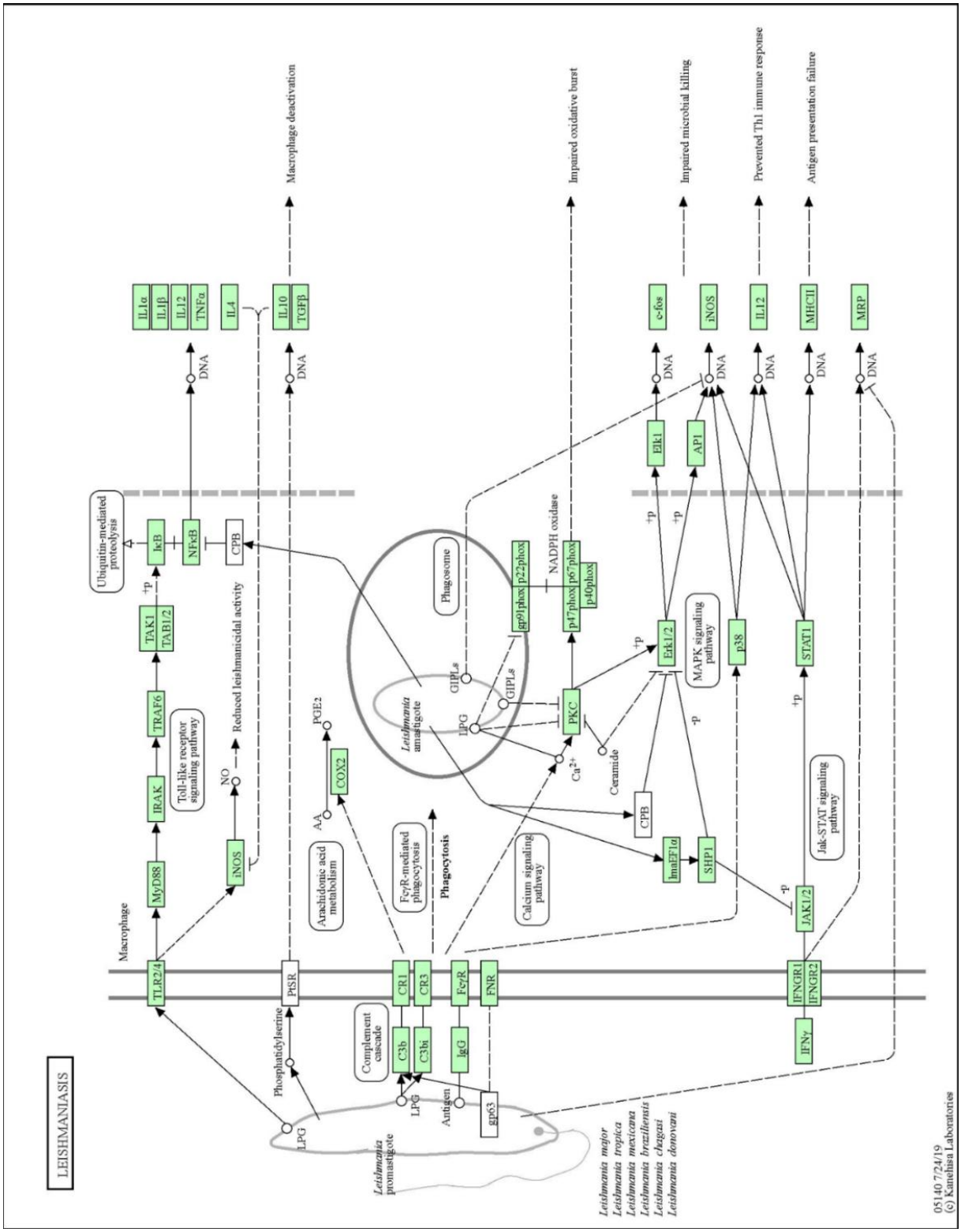


Figure 2.9: Schematic represents the *Leishmania* parasite intracellular evasion and molecular pathway alteration (https://www.genome.jp/pathway/hsa05140)

Over the decades, it has been well-defined that the immune cell and amino acid metabolism cross-talk play a significant role in immune cell functions, especially the arginine metabolism of macrophage activation to produce nitric oxide in inflammatory stimuli (Kung et al.,1977). However, there is no attention to the other side of the macrophage alternative activation through Arginase I. Later, it was clear that IL-4 and IL-13 inhibit NO production through the arginase (Doyle et al., 1994). Macrophages are represented as the primary cells for the innate immune response, and the *Leishmania* parasite uses these as the first choice of host cells. The primary effector cells phagocytose the parasite, activating the DC and complement system (Wozencraft et al.,1982). Macrophages are functionally plastic with distinct phenotypes: M1 is the pro-inflammatory subtype with microbicidal activity, whereas M2 is the anti-inflammatory subtype with tissue repair and resolution of inflammation and is proposed by Mills et al. (Mills CD et al. 2000; Tomiotto-Pellissier et al., 2018). The iNOS and ARG1 are two enzymes that use the same amino acid, which creates an 'arginine dichotomy' in the macrophages that classically activated M1 and alternatively activated M2. The relative expression of these genes is pivotal for their distinct functional phenotype (Kieler et al., 2021; Mori Gotoh, 2004). The classical activation of macrophages induces microbicidal molecules such as ROS, RNS, and NO and eliminates the parasite (Mosser Zhang, 2008).

On the other hand, the alternative activation of the macrophages drives the infection with parasite regulatory molecules such as TGF-β and IL-10, which dampens the effects of the microbicidal molecules (Rossi Fasel, 2018). The M1 phenotype upregulates the expression of iNOS, CD40, CD80, and CD86 and is involved in parasite killing (Takiguchi et al., 2021). M2 phenotype expresses CXCL14, CD163, CD206, and arginase I and promotes parasite survival (A. Kumar et al., 2018). Recently, it has been proven that many metabolic intermediates play a considerable role in cellular and molecular immunological events. Metabolic pathways are pivotal in the pathophysiology of many diseases, especially infectious diseases, and any dysregulations in the metabolic pathways might enhance disease susceptibility (Kaushal et al., 2017; H. Kumar, 2020). The highly dynamic interplay between metabolism and immunity is predominant in various infectious diseases, and it manipulates the immune cell population metabolism of the host for better survival. Immunometabolism of the macrophages is strongly linked to their state and fate, especially their functional phenotype (Chapman & Chi, 2022). The fate of the macrophage phenotype and functions mainly depends on the cues, such as obligatory metabolites or nutrients in the microenvironment, pathogens, and their effector molecules. Macrophages are highly versatile immune cells, and

immunometabolism reprogramming modulates their polarization. For example, the metabolic rewiring of L-arginine metabolism and functional plasticity of macrophages. The semi-essential L-arginine amino acid and its fate determine the state of the macrophages' classical activation or alternative activation (Thapa Lee, 2019). Interestingly, editing the macrophage polarization with immunometabolism modulators is an emerging therapeutic approach in many diseases (Geeraerts et al., 2017).

Immunometabolism in protozoan parasites is a budding area of research; the cross-talk between metabolism and immunity decides the disease resolution or progression. The harmonious symbiosis of intracellular pathogens residing inside the immune cells is largely due to the strategic immunometabolism exploitation of the host. The parasites regulate the immune-metabolic pathways of the host, or they rely on their metabolic enzymes to exploit the host metabolites (Moreira et al., 2018). *Leishmania spp.* manipulate the equilibrium of host immunometabolism and proliferate inside the host phagolysosomes. It demonstrated with an experiment that infection of human monocyte-derived macrophages with *L. amazonensis* or *L. donovani* shows an increased level of oxidative phosphorylation over glycolysis and diminished secretion of inflammatory cytokines, as the similar phenotypic pattern of M2. Interestingly, the pre-stimulation of LPS and IFN-γ significantly enhances the glycolysis and inflammatory cytokines. The results indicate that the M1 to M2 polarization of macrophages during the parasite infection might be due to the parasite and the effector molecules, which highly contribute to the strict M1 to M2 polarization and are also microbe-specific. *Leishmania*-mediated immunosuppression is due to the metabolic manipulation of the host macrophages (Huang et al., 2016; Ty et al., 2019). The metabolic reprogramming of the host macrophage decides the permissive or non-permissive host reservoir for the better infection of the parasite. The immunometabolism of *Leishmania* granuloma is composed of a heterogeneous population of infected and non-infected macrophages, and parasite survival depends on its functional fate, and it again depends on the nutrient/metabolite resources of the niche. The phagolysosome compartment is relatively nutrient or metabolite-limited and sometimes rich with host defence effector substances to overcome these hurdles through the metabolic adaptations of parasites (Saunders McConville, 2020). It is observed that in the phagolysosome compartment, there are various biomarkers and signal peptides for the vesicular fusion, suggesting that the highly dynamic compartment always fuses with endo-lysosome or various secretory vesicles to compensate for the energy requirement and parasites exploit it by rewiring of the various immunometabolism pathways (Young Kima, 2019). The

macrophage polarization is regulated by local effectors such as growth factors, chemotactic substances, cytokines, and nutrient or metabolite levels. It reprogrammed the metabolic and immune pathways and geared the cellular effector functions. The mTOR, AMPK and HIF1α are some major pathways that the *Leishmania* parasite alters for the polarization of the macrophages toward the parasite's favour (Batista-Gonzalez et al., 2020). The *Leishmania* granuloma model is excellent in that the immune cells meet the metabolic pathways for the defence and elimination of the parasite. However, the intimate interconnection of host and parasite alternatively activates many immunometabolism pathways, making naive cells more susceptible in the microenvironment. It impacts functionally highly dynamic macrophages to compromise, resulting in disease progression (Saunders McConville, 2020). Another study highlights that the early immune response is ineffective during splenic infection of *L. infantum* and is coupled with the down-regulation of metabolic markers of pathways such as AMPK, Adipocytokine, and icosanoid biosynthesis. It hampers the parasitic control (Palacios et al., 2023). The exposure of the parasite to the host makes immune cell recruitments, and its immune response is initiated to eliminate the parasites. The highly plastic macrophages exhibit various activation stages based on the signal from the local microenvironment that resides (Xue et al., 2014). *Leishmania* senses the activation state of the macrophage and its nutritional or metabolic resources and manipulates these local clues to regulate macrophage immune-metabolic pathways (Ty et al., 2019). The alteration in the metabolite availability and its concentration decides the outcome of infection by immune-metabolic modification of various signal transduction pathways. It initiates and gears the long-term epigenetic and transcriptional reprogramming of the M1 to M2 transition of the macrophages (Ivashkiv, 2013). The System biology approached mathematical models of macrophage polarization. It deals with the relevant immune-metabolic networks behind the Leishmania-mediated immunometabolism reprogramming. The transition state of the macrophage, i.e., M1 and M2 (**Fig**.**2.10**). The immunometabolism mathematical model of macrophage polarization shows that the arginase I mediated M2 polarization by conversion of arginine to polyamines, which are precursors of trypanothione for the parasite defence against ROS and NO. On the other hand, it inhibits the NOS2 activation by using various parasitic effector molecules (Bogdan, 2020).

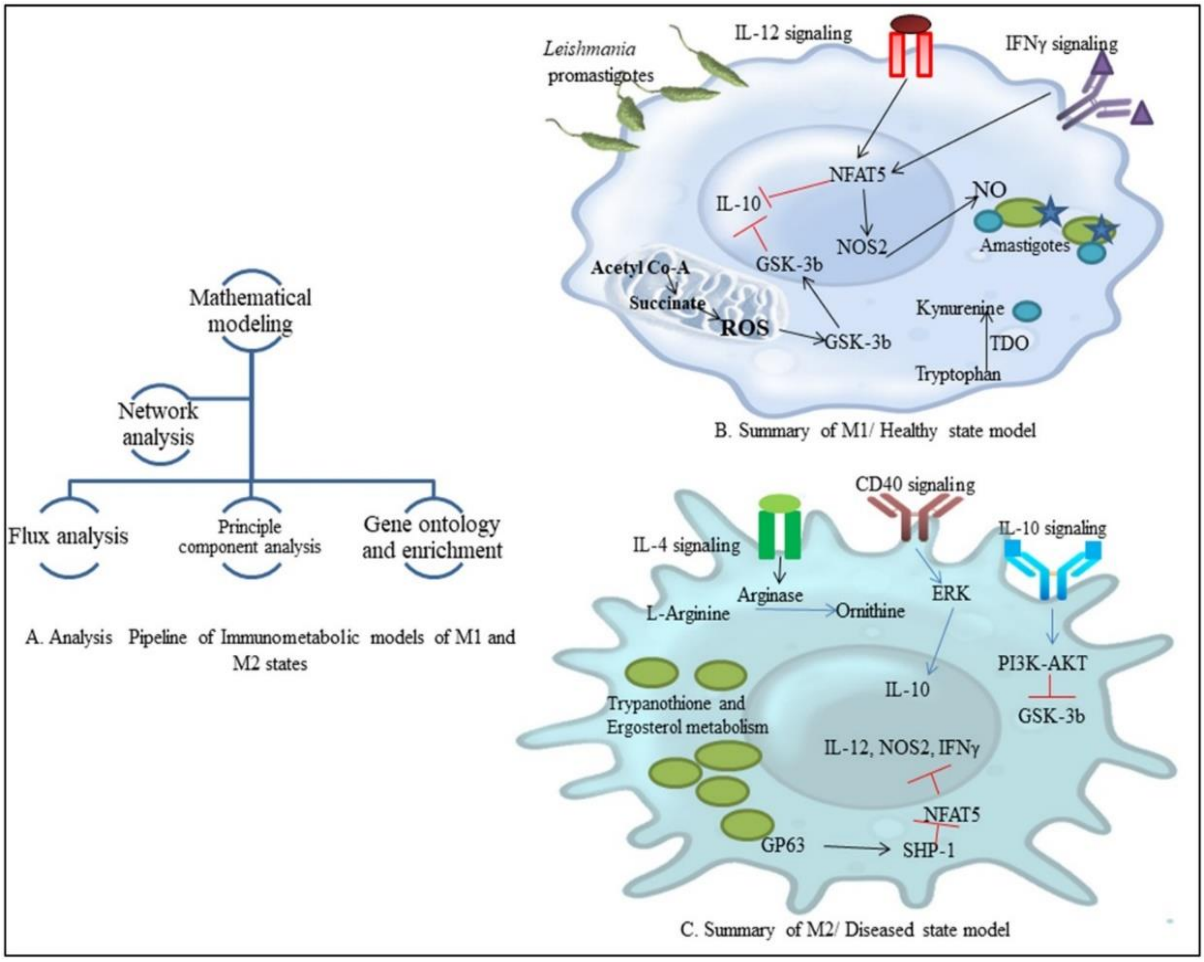


Figure 2.10: System biology approach of mathematical models of immunometabolism reprogramming of host pathways and macrophage polarization *(Khandibharad & Singh, 2023).*

Polyamines (PAs) are polycationic organic molecules with positively charged amine groups essential for all eukaryotes. Pathogenic protozoans use polyamines to survive and infect the host (Phillips, 2018). The metabolic state of immune cells influences immune modulation of the immune system. Spermidine is a crucial metabolite impacting the metabolic fitness of macrophages during protozoan parasitic infections (Mahalingam et al., 2023). The spermidine treatment induces the anti-inflammatory cytokines and their genes. Interestingly, the pretreatment of spermidine before the LPS treatment induces the iNOS gene and Nitric Oxide (NO) production (Choi Park, 2012). The reciprocal regulation of the iNOS and ARG I is crucial for the parasite's survival by driving the polyamine pathway (**Fig.2.11**).

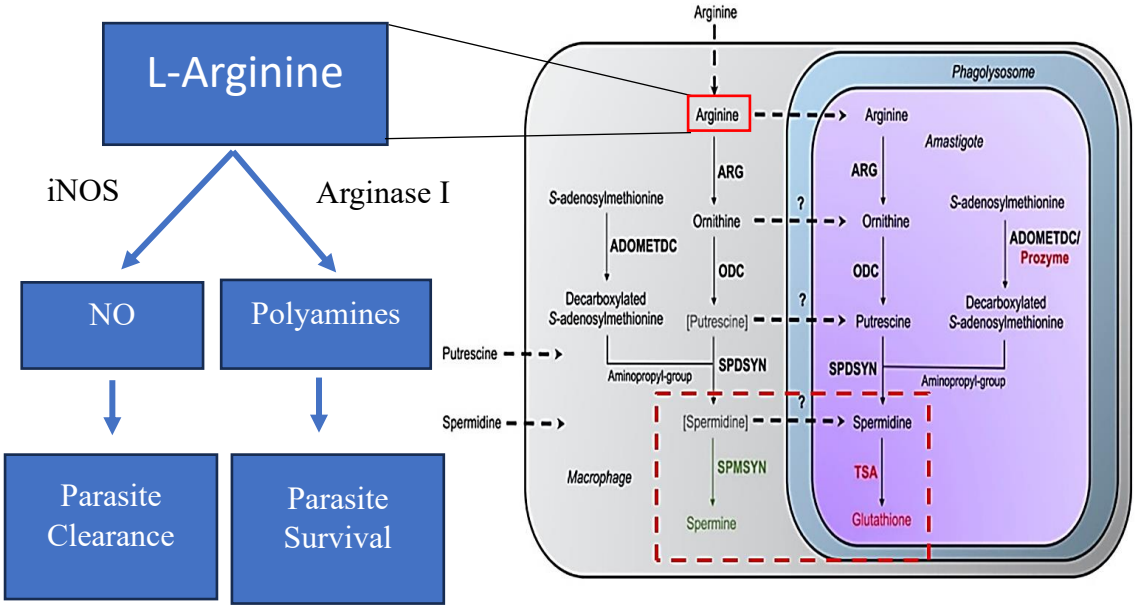


Figure 2.11: Schematic representation of "arginine dichotomy" of *Leishmania* parasite and immunometabolism cross-tal of host-pathogen interaction. *Leishmania* is auxotrophic for the semi-essential amino acid arginine and is very crucial in the intracellular survival of parasites by for the synthesis of polyamines. Moreover, the parasite lacks the *de novo* pathway for the polyamines and entirely depends on the host or the media. The phagolysosome compartment is a nutrient-deplete niche. Hence, inside the host macrophage, *Leishmania* is at a crossroads of win or lose the "hunger game". Arginine is a double-edged sword converted to highly microbicidal NO that eliminates the parasite or parasite-favouring polyamines that help parasite survival and disease progress. The polyamine biosynthetic pathway is distinct in *Leishmania* and host macrophages. In *Leishmania,* the pathway end product is spermidine (indicated in green), and they lack the back conversion enzymes for spermine to spermidine and spermidine to putrescine like other mammals. However, parasites have a unique conversion of spermidine into trypanothione (only in trypanosome parasites) as an oxidative stress-mediated free radical escape mechanism (indicated in red). (*adapted from Carter et al., 2022).*

In the host-pathogen interaction, the secretion of microbial effector components and its targeted delivery is theorized as a major part of the discussion on the pathophysiology of diseases (Gomez et al., 2009; Nandan et al., 2002; Nandan Reiner, 2005). EVs are one such kind that previously underappreciated organelle because they are considered a mechanical death of cells

or a result of apoptosis. In contrast, in the 20th century, it was firmly established that vesicles originated due to biological events and served as a communicator to enhance or dampen cellular responses (J. M. Silverman et al., 2010). EVs are nanometric membranes secreted by most prokaryotic and eukaryotic organisms (Gurung et al., 2021). The modulation of the host-pathogen interaction via EVs is a major survival strategy of the parasite inside the host macrophage's harsh environment. This might be due to the protective packaging and delivery of exosome contents to the host (Wei et al., 2020). The Scanning Electron Microscopy (SEM) based secretion of EVs by the *Leishmania* parasite was demonstrated (Hassani et al., 2011). EVs are formed by plasma membrane blebs and shedding to the extracellular space or secretion inside the Multi Vesicular Bodies (MVB) and secreted out to extracellular space by fusion of plasma membrane (Atayde et al., 2016; Tkach Théry, 2016). Different types of EVs are classified based on their origin, location, size, and morphology. Even though no sophisticated scientific technologies distinguish the exact classification of EVs (Raposo Stoorvogel, 2013; Tkach Théry, 2016). However, the provenance of EVs is from the cellular membrane and shed off to extracellular space, known as microvesicles or exosomes, with sizes 50 to 2000 nm. In contrast, exosomes are a small round or cup-shaped heterogenous population of EVs originating from an endocytic pathway with 30-150 nm in size (Tkach Théry, 2016). EVs can be fused to the plasma membrane of recipient cells or transfer the message by simple binding; for example, the exosomes expressing MHC II interact with the T cell populations without any internalization or fusion (Yang et al., 2011). Another important way of uptake was the receptor-mediated endocytosis or phagocytosis of the recipient cells (Bastos-Amador et al., 2012; D. Feng et al., 2010; J. M. Silverman Reiner, 2011). EVs play a major part in the intracellular communication of various infections, making the naive cells more susceptible to infection. However, extensive studies on EVs in infection are still progressing, and one such interesting role of exosomes in host-pathogen interaction is HIV infection. In the case of HIV infection, the infected cell producing the exosomes contains the HIV co-receptor that helps the virus enter into the neighbouring cells (M. Mack et al. 2000). It is also noted in many other intracellular pathogens such as *Mycobacterium* (Cheng Schorey, 2013), *Plasmodium yoelii* (Martin-Jaular et al., 2011), *Toxoplasma gondii* (Li et al., 2018). Protozoan pathogens, such as Trypanosoma (Li et al., 2018; Nogueira et al., 2015), *Leishmania spp.* (Atayde et al., 2015; Hassani et al., 2011; J. M. Silverman et al., 2010), can produce EVs with their cargos containing virulence components. *Leishmania* produces exosomes through the endocytic exosome pathway retained in the evolution time and is a small heterogenous population of EVs with sizes ~30-150 nm. The parasitic EVs mimic parental physiology and carry parasite-specific

cargo such as proteins, lipids, metabolites, and nucleic acids, some of which have immunomodulatory properties (Xu et al., 2019). The *Leishmania* RNA Virus 1 (LRV1) is a *Leishmania*-infecting double-stranded non-enveloped RNA virus that exploits the *Leishmania* exosome pathway. The LRV1 uses exosomes as a protective envelope to facilitate the effective transport and infectivity toward the host (Atayde et al., 2019). *Leishmania* alters many internal molecular mechanisms for its survival and multiplication. The *Leishmania* exosome-treated monocyte and dendritic cells show a high Th2 cytokine gene expression (J. M. Silverman et al., 2010). *The Leishmania exosome contains many RNA and RNA-binding proteins (RBP) that help the RNA*sack inside the exosome (Statello et al., 2018b). The highly enriched small fragments of rRNA and tRNAs inside the exosomes of many species of *Leishmania* suggest the specific and conserved mechanism of the RNA packaging exists and plays a significant role in the infection exacerbation (Statello et al., 2018). The virulence factors or drug-resistant proteins from the parental parasitic strain are specifically enriched in small EVs (Douanne et al., 2022). A recent report shows that the *Leishmania* drug-resistant genes are associated with EVs of the *Leishmania*, and it transfers drug-resistant genes through the EVs. Interestingly, the study demonstrated that the transfer assay of EVs from the resistant strain of parasites to naive parasites improved the growth and fitness of parasites and efficiently controlled oxidative stress. The study provides evidence that the *Leishmania* EVs are efficient mediators of genetic materials; they constitute the Horizontal Gene Transfer in parasites (**Fig. 2.12**) (Douanne et al., 2022).

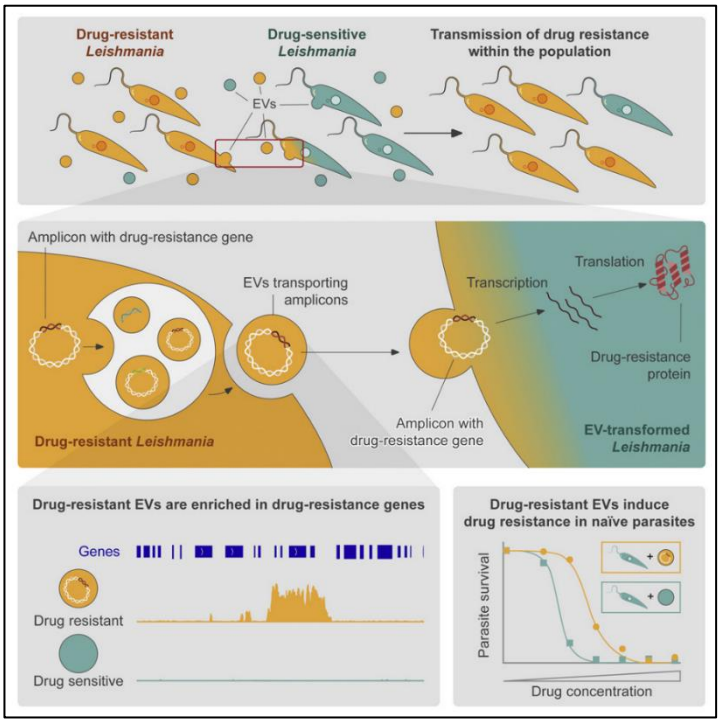


Figure 2.12: The *Leishmania* E s constitute the Hori ontal Gene Transfer HGT in *Leishmania* parasites. The transfer assay shows that the EVs associated with drug-resistant genes (amplicons) from the parental drug-resistant *Leishmania* parasite to the recipient drug-sensitive (naive) strain showed the exchanges of the amplicon. The drug-resistant genes enriched EVs induce drug resistance in the naive strain to encounter stressful environments *(Douanne et al., 2022).*

The *Leishmania* exosome membrane orientation is similar to the parasite's, following the same molecular interaction with the host cells. The *Leishmania* binds to CR3 and mannose receptors for phagocytosis, and *Leishmania* exosomes preferentially phagocytosed by the host cells as similar receptor-ligand interaction (D. Feng et al., 2010). An alternative hypothesis is that the *Leishmania* exosomes fuse to the host cell and release the cargo. The macrophages engulf the fluorescently labelled *Leishmania* exosomes mainly through an actin-based mechanism. *Leishmania* exosomes are delivery vehicles that deliver cargo to the host cells through receptor-mediated endocytosis (J. M. Axwell Silverman & Reiner, 2011). The studies demonstrated that *Leishmania* secretes exosomes as a mechanism of non-conventional secretion of protein and its delivery. The targeted delivery of the exosome cargos to long-distance delivery and immune modulation through intracellular communications. The studies prove, *in vitro* cell culture and *in vivo* models of animals, that the *Leishmania* exosomes mimicked the infection per se in the host (J. M. Axwell Silverman Reiner, 2011). The *L. donovani* exosomes stimulation of dendritic cells derived from monocytes failed to differentiate the naive CD4+ population of T cells to the Th1 subtype, dampening the inflammatory cytokines, especially IFN-γ. Furthermore, the exposure of parasite *L. donovani* exosomes to BALB/c mice and then parasite infection increases the parasite burden and exacerbation of the infection. The worsening of the diseases by producing high levels of IL-10 with Th2 polarization demonstrates that the *Leishmania* exosomes are predominantly immunosuppressive (J. M. Silverman et al., 2010). Macrophages are highly functionally plastic to the microenvironment stimuli and show phenotypic diversification. The distinct phenotypic diversification is known as polarization, as it is macrophages' response against the microbes or tissue repair. For example, *Leishmania*-infected cells derived EVs used to stimulate the peritoneal macrophages. Peritoneal macrophage exudate expresses transcripts of M2 polarization signature molecules (Emerson et al., 2022). In conclusion, *Leishmania* EVs play a major role in intracellular communication, non-conventional protein secretion, and its targeted delivery, packaging, and delivery of drug-

resistant genes and virulence factors (**Fig. 2.13**). Overall, it modulates the host's immune response for establishing successful survival in the host.

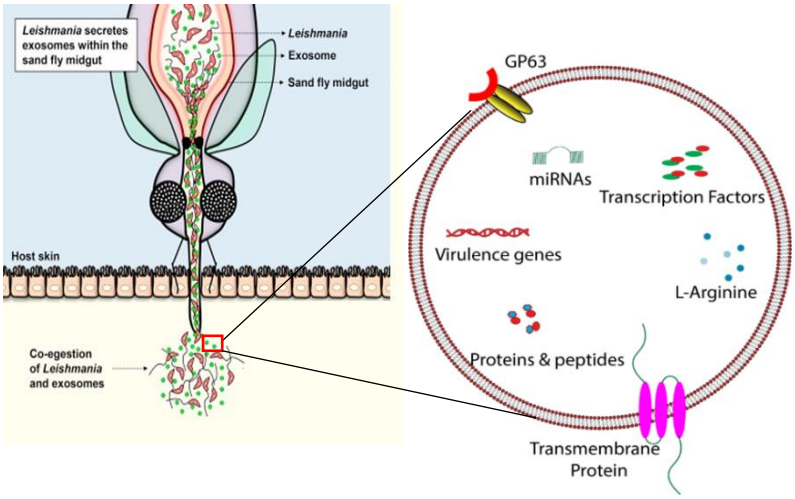


Figure 2.13: **Schematic representation of promastigotes derived exosomes and their cargos transfer to host during the blood meal of a sandfly**. *Leishmania* promastigote secrete exosomes inside the midgut of the sandfly and co-egestion to the host skin during the blood meal, and the magnified graphical representation shows the *Leishmania* exosomes with the lipid bilayer and its internal cargos (*Adapted from Atayde et al., 2015).*

Chapter: 3
Objectives

The secretory effector molecules of *Leishmania* parasites impact the disease fate and parasite survival. Exosomes are carriers of various effector molecules to the host and the parasite. According to WHO, asymptomatic cases of leishmaniasis are a noticeable threat. In addition, the PKDL and HIV-VL co-infection are important parasite reservoirs as per the WHO South-East Asia regional report 2022-2026 (World Health Organization. Regional Office for South-East Asia, 2022). The sporadic and rapid reports of chemotherapeutic resistance, comorbidities, and malnutrition-associated disease progress, relapse, and silent transmission through asymptomatic carriers are possibly linked with the immune-metabolic state of the host. The parasite invasion, disease establishment, and survival inside the host are strongly linked to the immune state of the host. Interestingly, recent studies emphasize the immune profile of the host is firmly connected to the metabolism status of the host. Since the *Leishmania* parasite survives in the macrophage phagolysosomes, and this microenvironment is harsh, the nutrient or metabolite is limited, and the parasite exploits various host immune-metabolic pathways to withstand and survive. In assumption, *Leishmania* exosomes are carriers of various effectors, including the essential metabolites that decide the intracellular parasite survival. Moreover, it also manipulates the host immunometabolism pathways for better survival and multiplication. The metabolic adaptation and the rewiring of host metabolism correlate to the *Leishmania* exosomes. We assumed that the polarization of macrophage towards the *Leishmania* favouring M2 state from the M1 state during the exosome stimulation is by the host immunometabolism reprogramming. We designed three primary objectives to investigate the *Leishmania*-derived exosomes and host immunometabolism status.

I. Characteri□ation of *L. donovani*- specific exosomes and metabolite enrichment in its cargo

a. Physio-chemical and molecular characterization of *L. donovani* exosomes

b. To identify the enriched metabolites in *L. donovani* exosomes

II. Studying the role of polyamines in *L. donovani* promastigote survival

a. Study of the pharmacological depletion of polyamine pathway in *L. donovani* promastigotes

III. Elucidation of the molecular mechanism of *L. donovani* exosome-mediated host macrophage polari□ation

a. Study of the uptake and infectivity of sensitive and resistant *L. donovani* exosomes by host macrophages

b. Study metabolic pathway switching during macrophage polarization from M1 to M2 by exosome stimulation

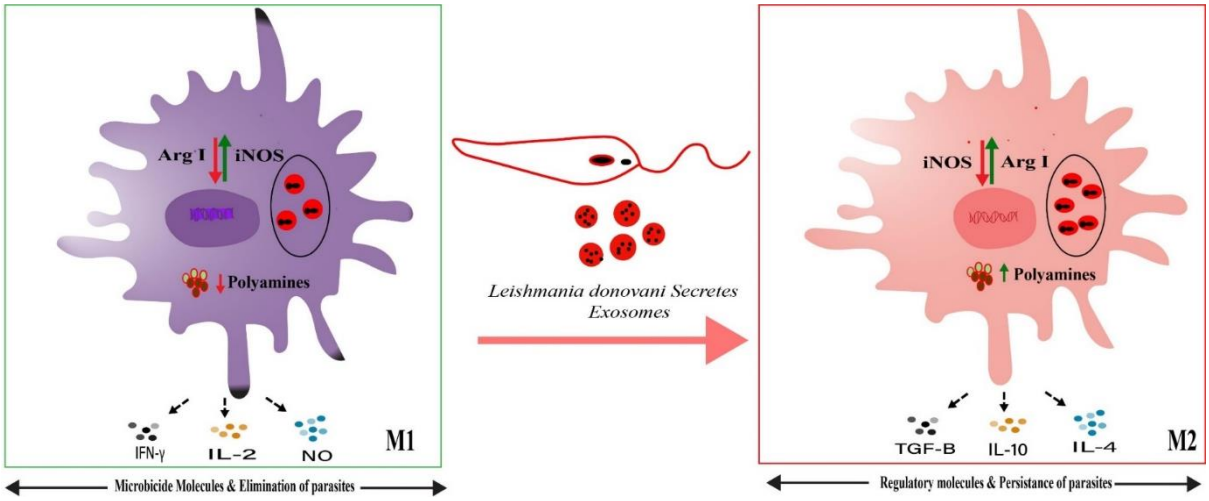


Figure 3.1: Schematic representation of the hypothesis. The scheme indicates that the *L. donovani* parasite produces exosomes and can polarize the macrophages from the M1 to M2 state. The microbicidal M1 to pro-parasitic M2 state is mainly through the polyamine biosynthetic pathway rewiring of the host immunometabolism.

Chapter: 4
Materials and Methods

4.1 Exosomes depletion of FBS to eliminate the cross-contamination of E□s

The functional EVs from the FBS might transfer various molecules while culturing the parasite for exosome isolation. So, it is important to deplete the exosomes of FBS before using it as a media supplement. The decomplemented FBS (Invitrogen) was briefly passed through a 0.2 □m membrane filter. After that, the FBS was ultracentrifuged at 120,000xg with an MLA 150 fixed angle ultracentrifuge rotor type (MAX-XP, Beckman Coulter Optima) for 18 hours (overnight). After that, the exosomes-free supernatant was carefully collected and used for the parasite culture (Shelke et al., 2014).

4.2 Media preparation for parasite and THP-1 cell culture

The RPMI and M199 media were used to culture macrophages and *Leishmania* parasites. For incomplete media preparation, 2.2 and 3.7g of $NaHCO_3$ were added in 1L Milli Q (MQ) water for RPMI and M199, respectively, and after that, autoclaved at 12^{o}C at 15lb for 20 minutes. After that, the bottle was kept for cooling at room temperature inside the laminar hood, and one pack (for one litter) of powdered RPMI/M199 medium was added. Then, the media was thoroughly mixed and stored at 4^{o}C until use. The incomplete media was used for complete media preparation by adding 10-20% fetal bovine serum (FBS), which was decomplemented in a water bath at 57^{o}C for 30 minutes. Antibiotics penicillin 100U/ml and streptomycin 100mg/ml (pen/strep) were added and filtered; the complete media was with a 0.22µM membrane filter by using a media filter assembly under a vacuum condition, the filter unit was autoclaved and dried in a hot air oven before use. Sterile 1N NaOH □ 1N HCl were used to maintain the complete media's pH (M199 pH-7.2 and RPMI pH-7.4) and stored at 4^{o}C for further use. The exosome-depleted FBS was used for the M199 media for parasite culture for the purification of exosomes.

4.3 Parasites culture

The *L. donovani* strain DD8 (MH0M/IN/80/DD8) was obtained from ATCC (USA), and Prof. Shyam Sundar of the Department of Medicine at the Institute of Medical Sciences at Banaras Hindu University in Varanasi, India, kindly gifted the Miltefosine drug-resistant *L. donovani* clinical isolate BHU875 parasite. The promastigote stage of *L. donovani* was cultured at 24^{o}C in the M199 medium. Parasite virulence was maintained in Hamster or BALB/c mice. Apart

from this, the parasite-free spent media for exosome purification from the stationary phase promastigote was cultured in exosome-depleted FBS-supplemented M199 media to avoid exosome cross-contamination from FBS.

4.4 Macrophage cell line culture

The THP-1 was cultured in the complete medium of RPMI-1640 (Sigma Aldrich, Cat. No. R4130-10X1L) containing 10% FBS at 37ºC in a humidified incubator with the supply of 5% CO_2.

4.5 Soluble *Leishmania*l Antigens SLA preparation

Soluble *Leishmania* Antigens (SLA) for the study were prepared from the stationary phase of promastigote parasites. Briefly, the promastigote parasites were washed 3-4 times with a sterile filtered 1X PBS by centrifuging at 2000-3000 rpm for 10 minutes at 4ºC to remove the spent medium. After that, 2 □ 10^8 promastigotes parasites per ml were resuspended into the lysis buffer, and lysis was carried out for 10 minutes at 4ºC, then freeze and thaw in liquid nitrogen for 3-4 times and sonicate for 2 minutes to extract the integral membrane protein out of the cell. Then, the lysed parasite suspension was centrifuged at 6000xg for 15 minutes at 4ºC. After that, the lysed supernatant was obtained, and the BCA method was used to estimate the protein concentration.

4.6 The *L. donovani* E s secretion by using Scanning Electron Microscopy SEM

The stationary stage parasites of *L. donovani* were processed as previously described culture conditions for SEM analysis, and we followed the method of Elizabeth R. Fischer et al. (Fischer et al., 2012) with minor modifications. Briefly, promastigotes were harvested at the stationary stage (2 □ 10^5 cells) and washed with gentle centrifugation of 1000xg for 10 minutes in a microfuge tube with filtered 1X PBS. Samples were then added to coverslips in a 12-well cell culture plate. Then, fixed in 2.5% glutaraldehyde solution in filtered 1X PBS of pH of 7.4 for 60 minutes at RT (Room Temperature) and washed with rinsing buffer for 3 □2 minutes. After that, post-fixation was done with a secondary fixative of 1% OsO_4 in sterile filtered dH_2O for 60 minutes at RT. Then, washed with dH_2O and dehydrated with a graded ethanol series by subsequent exchange of following dilutions in dH_2O, such as 25% for 1 □5 minutes, 50% for 1 □5 minutes, then 75% for 1□5 minutes, followed by 95% for 1 □5 minutes finally 100%

anhydrous ethanol 3 □ 10 minutes. Dehydrated samples were coated with gold metal and visualized using a scanning electron microscope (Philips).

4.7 Purification of *Leishmania*-derived exosomes by using ultracentrifugation

The cell-free supernatant of *L. donovani* of both sensitive and resistant parasites was collected at the stationary stage of the parasite culture and then purified the exosomes as per the method of Ricard J. Lobb et al. (Lobb et al., 2015)with minor modifications. Briefly, harvest the cell culture media and centrifuge at 2000xg for 30 minutes at 4^{o}C to remove the cells and debris. Filter the cell-free supernatant passed through a 0.2□m filter. The cell-free culture media was centrifuged at 150,000xg at 4^{o}C for 90 minutes with a rotor type MLA-150 fixed angle centrifuge rotor (MAX-XP, Beckman Coulter Optima instrument) to pellet exosomes. After that, the supernatant was discarded carefully without disturbing the pellet. The pellet was pooled from different tubes, resuspended in 1X filtered ice-cold PBS, and centrifuged at 150,000xg for 90 minutes at 4□C. Then, the resulting pellet was mixed in a filtered 1X PBS (ice cold) and stored in a deep freezer at -80^{o}C.

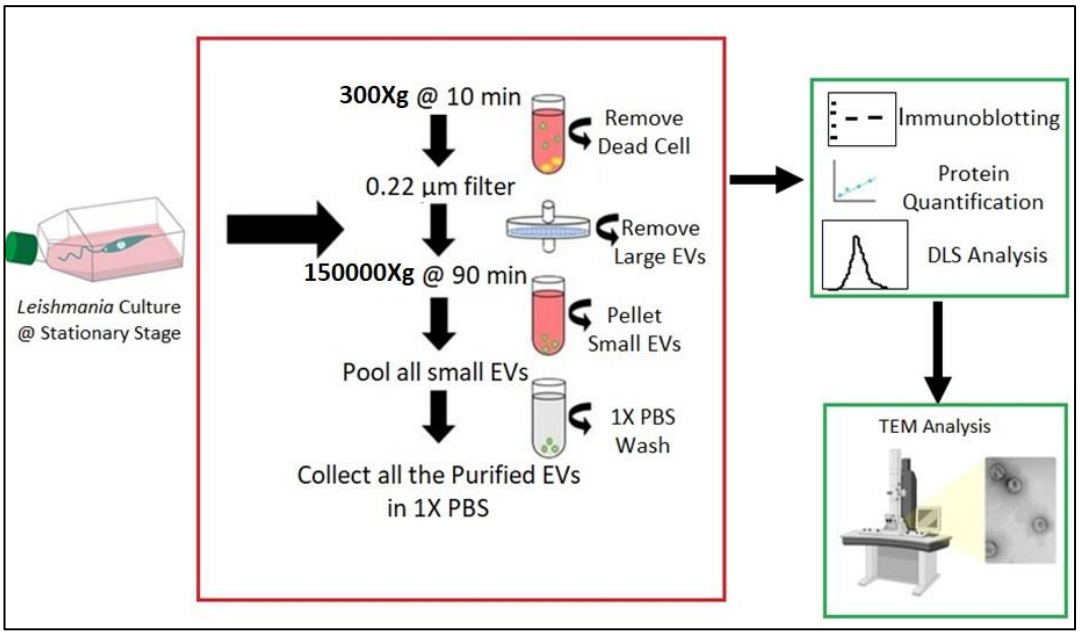


Figure: 4.1 Schematic representation of *L. donovani* exosomes purification and its characteri□ation. □*Adapted from* Gioseffi et al., 2020)

4.8 Physicochemical and Molecular Characterization of *L. donovani* Promastigote Derived Exosomes

4.8.1 Protein concentration estimation and Western Blot (WB)

The quantification of *Leishmania*-derived exosome concentration was obtained from the BCA assay using a reference protein concentration standard curve of the known quantity of BSA (Bovine Serum Albumin). The concentration obtained from the BCA was considered the concentration of the *Leishmania* exosomes. Western blot analysis was carried out to identify the exosome marker of the *Leishmania* parasite with anti-GP63 (Cat. No. MA1-81830, Invitrogen). The exosome sample was resolved in 12% SDS-PAGE, and a PVDF membrane was used to transfer the resolved protein bands by semidry blotting. The blot was blocked with 5% skimmed milk for one hour at RT in a TBS buffer (20 mM Tris and 150 mM NaCl with pH of 8.0) and then incubated with primary antibody anti-GP63 antibody (1:3000) overnight at 4^{o}C. After TBST buffer (20 mM Tris and 150 mM NaCl, 0.1% Tween 20), washes lasted for 10 minutes each twice, then incubated with HRP-conjugated secondary mice antibody in the dilution of 1: 5000 for one hour in RT. Then, wash the blot thrice, develop it using chemiluminescence reagents, and visualize it using the ChemiDoc instrument (Bio-Rad, California, USA).

4.8.2 Semi-quantitative flow cytometry characterization of *Leishmania* exosomes

The bead-based flow cytometry characterization of *Leishmania* exosomes was done by 4% w/v latex beads of Aldehyde/Sulfate (Cat. No. A37304, Thermo Fisher Scientific) with a slight modification of Suarez et al. (Suárez et al., 2017),and Biocompare protocols. Briefly, 0.5 µl of Aldehyde/Sulfate Latex Beads to 4-10 µl of exosomes in 1X PBS. Then, 1 ml of 1X PBS was added to the samples and was incubated on rotation overnight. Block the binding by adding glycine to the mixture and incubate for 30 minutes in RT. Bead-bounded exosomes were pelleted down at 2,000xg spin for 10 minutes of centrifugation, washed twice with 1X PBS supplemented with 0.5% exosome-depleted filtered FBS) and spun down again. The pellet was resuspended in 1X PBS containing 0.5% FBS. The samples were stained with anti-GP63 (Invitrogen, Cat. No. MA1-81830) as the primary antibody (1:1000). Then, it was washed once and incubated with a secondary antibody that conjugated with FITC (GeNei) for a time of 30 minutes at RT. After that, the samples were washed out twice to eliminate unbounded antibodies. Beads without antibody incubation served as a negative control, and the primary

antibody omitted bead-coated exosome suspension, which acted as a pre-absorbed antibody control (absence of primary monoclonal antibody). Data were acquired in a conventional flow cytometer (LSR Fortessa, BD Biosciences, USA) and analyzed with the analysis software FlowJo.

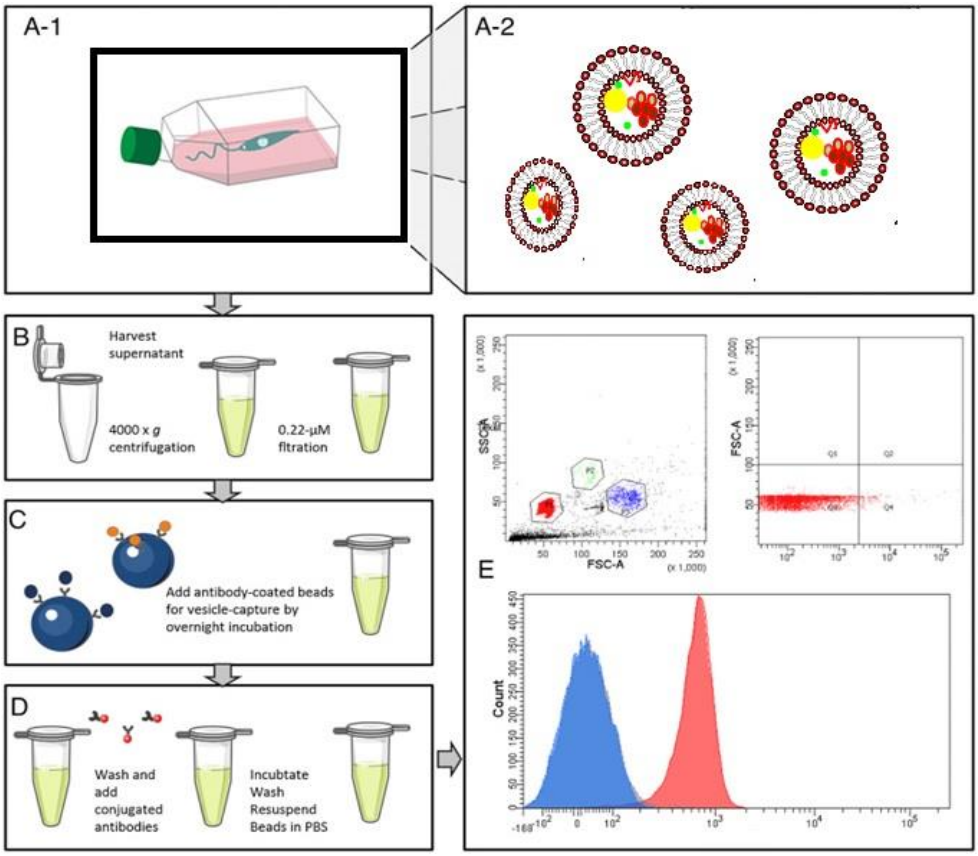


Figure: 4.2 Schematic representation of aldehyde-sulfate latex bead-based semi-□uantitative flo□ cytometry characteri□ation of *Leishmania* exosomes □*Scheme Adapted from* Suárez et al., 2017; Volgers et al., 2017).

4.8.3 Confocal microscopy of *L. donavani* exosomes

The Confocal Microscopy characterization of *Leishmania* exosomes was done by adding 1X PBS to the exosome suspension, and the sample was fixed with 4% paraformaldehyde. Then, it was incubated in 1X PBS for 20 minutes on rotation. After that, the samples were washed twice, adhered to the cleaned coverslips in a cell culture plate, and air dried. After drying, the slides were immersed in ice-cold methanol for 5 minutes and block samples by 1% BSA, made in 1X PBS for 20 minutes incubation. Then, the primary antibody of anti-GP63 in the dilution

ratio of 1:200 to the sample on a coverslip was dipped in a cell culture plate (6 wells). Then, it was washed for 1 hour and washed thrice with 1 ml of 1X PBS. The pre-absorbed antibody control sample was omitted, and primary antibody incubation was done. Then, a secondary antibody of anti-mouse IgG was conjugated with fluorochrome FITC in a dilution ratio 1:200 and incubated for one hour in the dark. Then, the unbound non-specific background antibodies were washed out with 1X PBS. Then, mounted on a slide with a mounting agent and analyzed in a confocal microscope (Leica, Wetzlar, Germany).

4.8.4 Dynamic Light Scattering (DLS) and Zeta potential measurements of *Leishmania* exosome

Physical characterization of *Leishmania*-derived exosomes was done with DLS to detect the approximate range of the size and zeta potential measurements. *Leishmania* exosomes (5µg/µl concentration) diluted in 1X PBS with a volume of 1 ml and determined the size distribution by using the DLS method in a particle analyzer (Zetasizer Nano ZS, Malvern, Herrenberg, Germany). Then, the Zeta Potential of *Leishmania* exosome by default settings of 25^{o}C is measured at a 632 nm laser with 120 seconds as an equilibration time.

4.8.5 Morphological characterization of *Leishmania donovani* exosomes by Transmission Electron Microscopy (TEM)

The detection of morphological characterization of the *Leishmania*-derived exosomes was done with TEM-negative staining, and we followed the method of Peter Cizmar and Yuana Yuana (Patrick et al.,2017) with minor changes. Briefly, the *Leishmania* exosomes (concentration of 1µg/µl) were fixed to a Formvar carbon-coated copper grid (Formvar/carbon Copper grids with mesh size 200, ~ 10 µl of the sample). The exosome-coated grids were dried in a culture dish at room temperature overnight. After that, the completely dried grids were applied to 2% uranyl acetate (2.5 µL) and the copper grids were washed thoroughly in ultra-pure water to remove uranyl acetate. Then, the grids were allowed to air dry and examined using the TEM facility (FEI Technai G2S-Twin).

4.9 Detection of PAs in *Leishmania* exosomes by chromatographic techni□ues □TLC and HPLC□

Leishmania-derived exosome polyamine was detected in TLC following the dansyl chloride conjugation method, and the samples for TLC were extracted with toluene and methanol. After that, the samples were quantified by HPLC with polyamines standards. We followed Dion and Herbst's protocol (Dion □ Herbst, 1997). Polyamines were extracted with toluene, and the organic solvent-mediated separated polyamine supernatant was collected and derivatized with dansyl chloride (3 mg ml $^{-1}$) using a flash rotatory evaporator. After that, proline (50 mM) was added to remove the unbounded dansyl chloride, and the evaporated and separated polyamine sample was dissolved in HPLC-grade methanol. Polyamines standards (Sigma Aldrich), such as spermine, spermidine, and putrescine, were also derivatized, as previously mentioned. The polyamine was detected in the Shimadzu HPLC instrument with a C-18 Luna (5□m, 250 □4.6 mm) column containing the PDA (Photo Diode Array) detector. A linear gradient technique separates the polyamines; the mobile phase was 1% (v/v) acetic acid in water (solvents A) and acetonitrile: methanol: 4:1 (solvent B) with a 0.8 ml/minutes flow rate. The polyamines are detected and confirmed with the alignment of standards, peak retention time and absorption.

4.10 □uantification of spermidine levels in *Leishmania* exosomes by Li□uid Chromatography □LC-MS□

To extract the metabolites for LC-MS analysis, we followed Palviainen et al. (Palviainen et al., 2019). Briefly, Metabolites were extracted from exosomes of DD8 sensitive (MH0M/IN/80/DD8) and drug-resistant (BHU875) parasites exosomes by adding 400µl acetonitrile to 100µl each sample, mixed thoroughly by vertexing. After that, the samples were centrifuged for 10 minutes at 16000xg, and the supernatant of the centrifuged sample was analyzed for metabolites in LC-MS (Agilent instrument for total metabolites and Shimadzu instrument for targeted quantification).

4.11 Semi-□uantitative flo□cytometry for spermidine of exosomes

The bead-based flow cytometry characterization of *Leishmania* exosomes was done by Aldehyde/Sulfate Latex Beads 4% w/v (Thermo Fisher Scientific□Cat**.** No. A37304) with a slight modification of Suárez et al. 2017 protocol. The analysis scheme is represented (**Fig. 4.2**). Briefly, 0.5□l of Aldehyde/Sulfate Latex Beads to 4-10□l of exosomes in 1X PBS. Then,

it was mixed with 1 ml of 1X PBS, and the sample was incubated on a rotor for rotation. Block the binding by adding glycine to the mixture and incubate for 30 minutes in RT. Centrifugation at 2,000xg for 10 minutes was used to pellet bead-coupled exosomes. After that, they were twice washed with 1 ml of 1X PBS supplemented with 0.5% exosome-depleted filtered FBS and centrifuged again. The pellet was resuspended in 1X PBS+0.5% FBS and stained with anti-spermidine antibody (Novus Biologicals, Cat. No. NB100-1847) as the primary antibody in the dilution ratio of 1:1000. Then, it was washed twice and incubated with FITC-conjugated secondary antibody in the dilution ratio of 1:500 (GeNei) for 30 minutes at RT. After that, the unbounded antibody was washed out twice. Negative control with incubating the beads and the exosome suspension with a pre-absorbed antibody control (absence of primary monoclonal antibody). Data were analyzed and acquired in conventional flow cytometers (LSR Fortessa, BD Biosciences, USA).

4.12 MTT: metabolic cell viability assay upon polyamine depletion

The metabolic viability of parasites upon hypericin treatment was done with the MTT index. The MTT was transformed to purple formazan crystals by mitochondrial reductase, and the OD was calculated at 570 nm to determine the concentration and extrapolate the metabolic activity. We followed the protocols (Kumar et al., 2018; Minor, 2004). Briefly, exponentially grown *L. donovani* miltefosine sensitive and resistant strains (1 □ 10^6/ml) were seeded in a 96-well microplate with 100µL M199 medium. Then parasites were treated with different concentrations of hypericin (diluted with M199: 0, 3.12, 6.25, 12.5, 25, 50µM), miltefosine (PBS pH-7.4, 0, 3.12, 6.25, 12.5, 25, 50, 100µM), polyamine media supplement (1, 0.5, 0.25,0.125, 0.062 and 0.031X concentrations) for 24 hours. Miltefosine was used as the standard reference drug, and 0.01% DMSO was kept as Vehicle Control of hypericin. Following the drug treatment, 20µL of MTT (5mg/ml) was added, and the suspension was incubated at 26□C in the dark for 4 hours. Then, the parasites were spun for 10 minutes at 3000 rpm, the supernatant was discarded, and the pellets were collected. The formazan crystals are water-insoluble, and they were dissolved in DMSO. A multimode plate reader (Molecular Devices, USA) was used to measure absorbance at wavelength 540nm. The following formulae were used to determine the viability percentage of the parasites:

$$\text{Parasite viability} = \frac{\text{Test sample absorbance} - \text{Control absorbance}}{100}$$

4.13 The growth curve of MIL-resistant *L. donovani* BHU875 during hypericin-based depletion of polyamines: a cell number-based viability assay

The effect of hypericin on the parasite growth pattern, the parasite growth curve was analyzed, and we followed the protocol of (Perdeh et al., 2020) with minor modifications. We performed a growth curve by exponentially grown parasites (MIL sensitive and MIL Resistant) 1×10^5 cells/ml and seeded in 12-well plates with MI99 media. Parasites were treated with IC_{50} concentration of drugs for five days. The Live parasites were counted in a hemocytometer with Trypan blue dye for 24, 48, 72, 96, and 120 hours. Then, a Giemsa staining and microscopy-based cell morphology study on drug treatment was done.

4.14 Giemsa staining of hypericin-treated *L. donovani* promastigote parasites

The stationary stage parasite was treated with IC_{50} concentration of hypericin (18 μM), Vehicle control (DMSO 0.01%), and media supplement of the polyamine (1X concentration) served as a control. After the treatment, the parasites were harvested and stained with Giemsa staining (Himedia, Cat. No. S011). We followed the staining protocol of the manufacturer. Briefly, the thin film of the parasite sample smear is made in a clean glass slide and allowed to dry; then, the slides are dipped in methanol for 3-5 minutes. We followed the staining of slides with Giemsa Stain stock solution (0.67 ml) to diluted stain (~ 30 ml volume distilled water). Then, the slides were dipped into a container with diluted stain for 30 minutes to stain the sample smear on the slide. After that, the slides were washed with distilled water to differentiate the stain of the prepared slides, and washing was done for ~ 1 to 3 minutes. The slides were air-dried and examined under the light microscope (Leica, Wetzlar, Germany) with a 100X magnification.

4.15 Intracellular ROS analysis with H_2DCFDA upon pharmacological inhibition with hypericin

The damage to DNA, RNA, proteins, and lipids by the oxidation of free radicals is detrimental. The ROS roles and consequences in several pathological conditions are essential in basic and clinical research. Detecting and measuring ROS levels is complicated because of its short half-

life. Flow cytometry is a simple, fast, and affordable technique that gives information on cell viability and the percentage of ROS-producing cells. The Carboxy-H_2DCFDA is the dye to detect intracellular ROS. The non-fluorescent reagent oxidized in the presence of ROS, emitting green fluorescence. Then, the green fluorescence's Mean Fluorescent Intensity (MFI) was measured. Here, we performed ROS analysis for hypericin-mediated polyamine-depleted parasites. The complete cell culture media was added by adding 10% decomplemented FBS and 1% penicillin-streptomycin antibiotic. Prepared the media fresh on the day and harvested the cells. After that, 5 □ 10^5 cells/well with different control groups and experiments were incubated at 26□C. The exponentially grown parasites were divided into different groups, and a fresh medium containing the drug of choice was used for 6 hours. The naive, polyamine supplemented, hypericin, vehicle control for hypericin (0.01% DMSO), and miltefosine (standard reference drug) are served as different sample groups. Then, the parasites were harvested by aspirating the supernatant and washing the parasites in the HBSS buffer. Then, the parasites were stained with the Carboxy-H_2DCFDA dye at a final concentration of 10µM in a regular culture medium (~500 □l in volume). After that, the parasites were incubated in a conventional BOD incubator for 30 minutes in the dark (26□C), and stained parasites were protected from light. After that, the carboxy-H_2DCFDA-containing medium was removed and washed twice with HBSS. Then, we detected and measured the ROS by flow cytometry using the channel with green fluorescence (Here, we used fluorochrome FITC with 495 nm / 519 nm excitation and emission, respectively, in the BD LSR Fortessa instrument.

4.16 Propidium Iodide □PI□upta□e study of parasite upon hypericin treatment

To study the membrane integrity and parasite death upon pharmacological inhibition for polyamine depletion mediated by the hypericin, follow the protocol of Lisa C. Crowley et al.(Crowley et al., 2016) with modification. Both strains of parasites (2 □ 10^6 parasite/well) were treated with hypericin (18□M) and naive, Vehicle Control (DMSO), miltefosine treated (positive control), and polyamine media supplement (1X) as different groups for 24 hours treatment. After the treatment, the parasite was harvested in a microcentrifuge tube and centrifugated for 5 minutes at 700xg. Then, parasites were incubated in HBSS buffer containing PI (20□l from the 0.5mg/ml stock) for 15 minutes in the culture condition of promastigote parasite (25^{o}C in a BOD incubator) to avoid stress-induced death. A flow cytometry instrument (BD FACS LSR Fortessa) determined the percentage of PI-positive parasites in different groups.

4.17 JC-1 mediated mitochondrial membrane potential ΔΨm or MMP upon polyamine depletion

To understand the Mitochondrial membrane potential status upon Hypericin treatment, we performed a Mitochondrial membrane potential assay using JC1 dye (Invitrogen, Cat. No. T3168). The mitochondrial potential-dependent accumulation of the aggregates is demonstrated by the specific dye called JC-1. This cationic carbocyanine dye indicates the accumulation of aggregates by changing the ratio of the fluorescence emission from green (~525 nm) to red (~590 nm). The changes in the ratio, such as a reduction in the red/green ratio of fluorescence intensity, indicate mitochondrial membrane depolarization; if it increases, it shows mitochondrial hyperpolarization. The concentration-dependent red fluorescent J-aggregates are responsible for the potential changes mediated by colour shift. So, JC-1 dye is an excellent indicator of the mitochondrial membrane potential. Exponentially grown promastigotes were treated for 48 hours with polyamine (1X), hypericin, and miltefosine with the IC_{50} concentrations determined earlier. Miltefosine is used for positive control as its treatment reduces mitochondrial potential. After treatment, parasites were washed in 1X HBSS buffer, and the mixture was resuspended thoroughly with pipettes. After that, it was stained with 10μM JC1 dye for 10 minutes at 26°C. Further, the wash was repeated, resuspended in 1ml HBSS buffer, and then analyzed in flow cytometry (BD FACS LSR Fortessa). The dye's MFI (fluorescence ratio of Red 560 nm and Green 530 nm fluorescence) is represented as a ΔΨm (MMP). The DMSO (0.01%) was kept as a vehicle control for the hypericin.

4.18 *L. donovani* exosomes uptake of host macrophage THP-1 and confocal microscopy

To differentiate into macrophages, the THP-1 cells (3×10^6 cells/ml) were seeded in 6-well plates, differentiated with PMA (10 ng/ml) for 24 hours, and rested for an additional 24 hours at 37°C and 5% CO_2. The differentiated macrophages were then stimulated with 10 μg/ml exosome of the resistant parasite strains (BHU875), sensitive (MH0M/IN/80/DD8), and unstimulated macrophages as a control. Following exosome stimulation, adhered cells were detached from the culture plate using a 0.25% trypsin EDTA solution, and excess exosome and trypsin were removed using 1X PBS wash before the detachment of the cells. After that, the cells were fixed for 15 minutes in a 2% paraformaldehyde solution. 0.1% Triton-X was added to PBS and allowed to permeabilize the cells for 15 minutes at RT. Then, the PBS containing Triton X (0.1%) and 3% BSA was used to wash the fixed, permeabilized cells by spinning the

cells for 5 minutes at 2000 rpm. Next, the cells were incubated with a 1:200 antibody dilution of the primary antibody, Anti-GP63, for 1 hour at RT. After that, washed three times, the cells were incubated in the dark for 30 minutes at RT with a secondary antibody of fluorochrome FITC-conjugated (1:200 dilution). The cells were rinsed three times with PBS following incubation. As previously stated, exosome-stimulated THP-1 macrophage samples (3 □ 10^6 cells/well) were used for the immunofluorescence assay. Thus, coverslips on glass slides were allowed to air dry after fixed cells were washed three times in PBS for 5 minutes, and ice-cold methanol (-20^{o}C) was poured over these coverslips. After three more washings, the coverslips were positioned in Vectashield antifade mounting medium onto the glass slides containing the nuclear stain DAPI. The fluorescence on the slides was inspected using a confocal microscope (Leica).

4.19 The □uantitative exosome inta□e of macrophages by flo□ cytometry analysis

The THP-1 cells (3 □ 10^6 cells/well) were seeded, and PMA (10 ng/ml) for 24 hours to differentiate the monocyte cell line. Then, it was washed and rested for the remaining 24 hours at 37^{o}C and 5% CO_2. After that, stimulated the differentiated macrophages with a concentration of 10□g/well exosomes of the resistant (BHU875) and DD8 sensitive (MH0M/IN/80/DD8) strains of parasites. Unstimulated macrophages as a control. After exosome stimulation, the adhered cells detached from the culture plate with trypsin EDTA solution (0.25%), washed out of excess exosomes and trypsin with 1X PBS. Then, macrophages were washed thrice with 1X PBS to remove the non-internalized exosomes. After that, the exosome internalized cells were incubated with anti-GP63 as the primary antibody for 1 hour at room temperature with an antibody dilution ratio of 1: 200. Following three 1X PBS washes, the cells were exposed to a 1:200 dilution of the FITC conjugated secondary antibody for 30 minutes at RT in the dark. After the incubation, cells were rinsed 1X PBS three times, resuspended in 500□l 1X PBS, and subjected to analysis with the BD FACS LS Fortessa flow cytometer.

4.20 Phagocytic activity in exosomes stimulated macrophages

To analyze phagocytic activity in exosomes-stimulated macrophages, we followed the protocol of (Dayakar et al., 2017) with minor modifications. The monocytes were differentiated into macrophages, 5 □ 10^5 THP-1 cells/well were seeded in 6-well cell culture plates and treated with PMA (10 ng/ml) for 24 hours at 37^{o}C and 5% CO_2 and additional resting for 24 hours. Then, 50□g/ml exosomes of the DD8-sensitive (MH0M/IN/80/DD8) and resistant (BHU875)

parasite strains were used for macrophage stimulation. After that, it was co-cultured for 6 hours with 5 □ 10^6 parasites/well or a 10:1 parasite to macrophage MOI (multiplicity of infection ratio). Subsequently, fresh, complete RPMI media were used to incubate for an additional 18 hours. After that, the non-internalized parasites and exosomes were thoroughly washed off with 1X PBS. Following incubation per the manufacturer's protocol, the cells adhered to the coverslips were stained with Giemsa (Himedia). Using a microscope (Leica), the infected macrophage count and phagocytosed parasite count per a total of 50 macrophages were determined. The following formulae are used to determine the phagocytic index of macrophage:

Phagocytic index □PI□□ □Total number of internali□ed parasites/number of macrophages containing internali□ed parasites□□ □number of macrophages containing internali□ed parasites /total number of counted macrophages□□ 100

4.21 The CFSE-based infectivity assay upon *Leishmania* exosome stimulation

The infectivity of *Leishmania*-derived exosomes stimulated human monocyte differentiated THP-1 was determined by CFSE-based flow cytometry analysis. We followed the method of Agostinho Goncalves Viana et al. (Viana et al., 2018) with minor modifications. Briefly, the stationary stage of promastigote forms from *L. donovani* was stained with the carboxyfluorescein diacetate succinimidyl ester dye (Invitrogen, Cat. No. C34554). Then, *Leishmania* parasites (2 □ 10^7 parasites/ml) were stained in a tube at a final 5□M CFSE dye concentration, and the mixture was well resuspended. The parasites were incubated for 20 minutes at 37oC in a CO2 incubator for staining. After that, the parasites were centrifuged three times at 200xg using ice-cold 1X PBS to wash parasites. After that, the CFSE staining was quenched by adding 10% decomplemented fetal bovine serum (Invitrogen) and resuspended in complete RPMI. Then, the PMA-stimulated THP-1 macrophage cell line (2 □ 10^6 cells/well) was infected with CFSE-labelled parasites (2 □ 10^7 parasites/well) in a complete RPMI medium in a MOI ratio of 1:10 (THP-1: parasites) for 24 hours. Un-infected, unstained exosome stimulation, then infection (Both sensitive and resistant parasite exosomes), and exosome stimulation without parasite infection served as controls. Samples were run on the flow cytometer (BD FACS LSR Fortessa) to determine the infectivity of the various experimental groups.

4.22 Detection of intracellular Reactive □xygen Species □R□S□of THP-1 macrophages by carboxy- H_2DCFDA

Detecting and measuring ROS levels is complicated because of its short half-life. Flow cytometry is a simple, fast, and affordable technique that gives information on cell viability and the percentage of ROS-producing cells. Carboxy-H_2DCFDA is the dye that detects intracellular ROS. The generation ROS, the non-fluorescent reagent, oxidizes and emits green fluorescence. Then, the green fluorescence, or Mean Fluorescent Intensity (MFI), was measured. The THP-1 was grown in the complete cell culture media with 10% decomplemented FBS and 1% Antibiotic (pen/strep). Prepared the media fresh on the day and harvested the cells. After that, seeded 5 □10^5 cells/ well with different groups of experimental variables such as unstained, naive stained, DD8 exosomes stimulated (50□g/ml), BHU875 exosomes stimulated (50□g/ml), polyamine supplemented and lipopolysaccharide (100ng/ml LPS), and incubated in 37ºC temperature with the supply of 5% CO_2 in an incubator, and harvested the macrophages by supernatant aspiration. Wash the cells once with incomplete media. Then, the cells were stained with the Carboxy-H_2DCFDA dye at a final concentration of 10µM in a regular culture medium (~500µl in volume). After that, the cultures were placed in a conventional incubator in the dark for 30 minutes (37ºC, 5% CO_2). Detected and measured the ROS by immediate acquisition of the samples by flow cytometry using the channel with green fluorescence (Here we used fluorochrome FITC with 495 nm / 519 nm excitation and emission, respectively, in LSR Fortessa instrument)

4.23 Estimation of Nitric □xide □N□□production upon *Leishmania* exosomes stimulation to macrophage cell line □THP-1□

NO was estimated using the Griess assay method (Invitrogen Griess Reagent Kit, Cat. No. G7921). We followed the manufacturer's protocol. Briefly, THP-1 macrophages (5 □10^5 cells /ml) were seeded and stimulated with PMA (10ng/ml) at 37ºC in a supply of 5% CO_2. It was stimulated with exosomes of both strains (50µg/ml), naive and polyamine supplemented, and LPS (100 ng/ml) kept as controls. Then, the samples were incubated at 37ºC temperature with a 5% CO_2 supply for 72 hours in an incubator. The cultured supernatant was used for the Griess assay-based NO estimation. In brief, the culture supernatant (100□l) was mixed with 100□l of the Griess reagent (Invitrogen Griess Reagent Kit, Cat. No. G7921) and was incubated for 10

minutes in RT. After that, the OD-based absorbance at 548 nm will be measured in a microplate reader. Nitric oxide (□M) was calculated from a sodium nitrate standard curve.

4.24 Macrophage polari□ation study of *Leishmania* exosomes □ith anti-arginase I by flo□ cytometry analysis

The seeding volume of 2 □ 10^6 cells/ml of THP-1 cell line in 6-well cell culture plates was differentiated using PMA (10ng/ml) for 24 hours at 37ºC and 5% CO_2 to convert into macrophages. After that, PMA differentiated macrophages with a concentration of 10□g/well exosomes of the BHU875 resistant and DD8 sensitive (MH0M/IN/80/DD8) strains of parasites and unstimulated macrophages as a control. After 24 hours of exosome stimulation, cells were detached mechanically/ chemically and washed with 1X PBS. The fixation and permeabilization were then done with 4% paraformaldehyde and 0.1% triton X for 20 minutes. After that, the primary antibody, i.e., anti-arginase antibody (Rabbit Monoclonal Antibody of CST, Cat. No. 93668), was added in a 1:200 dilution. Then, it was rinsed twice with 1X PBS, incubated with a secondary antibody in a 1:500 dilution of fluorochrome FITC, and incubated for 30 minutes at RT in rotation. Then, the cells were washed thrice, and 0.5 ml of staining buffer was added to each tube. The flow cytometry analysis proceeded in the flow cytometer (BD LSR Fortessa).

4.25 Macrophage polari□ation study of *Leishmania* exosomes □ith anti-arginase I by □ B Analysis

The THP-1 cells (2 □ 10^6 cells/ml) were in 6-well plates and differentiated with stimulation of PMA (10ng/ml) for 12 hours at 37ºC and 5% CO_2. After that, PMA differentiated macrophages with a concentration of 10□g/well exosomes of the BHU875 resistant and DD8 sensitive (MH0M/IN/80/DD8) strains of parasites and unstimulated macrophages as a control. After exosome stimulation, cells were chemically detached by trypsin EDTA (0.25%) treatment and rinsed using 1X PBS. The RIPA buffer was used to lyse the macrophages (containing 1% Proteinase Inhibitor Cocktail), 12% SDS-PAGE technique-based resolving of proteins and transferred the proteins that resolved in the gel onto the nitrocellulose membrane by voltage-mediated semidry-blotting. The membrane was blocked in 5% skimmed milk that lacked fat for 1 hour in RT in TBS buffer. Then, the incubation of anti-arginase I antibody (1:3000) was overnight at 4ºC. The blot was washed thrice with TBST, lasting 10 minutes each, and incubated with an anti-rabbit HRP-conjugated antibody as a secondary antibody (1:5000) for

1 hour at RT. After that, wash the blot thrice in TBST, develop it using chemiluminescence reagents, and visualize it using the ChemiDoc instrument (Bio-Rad, California, USA).

4.26 Arginase activity in exosomes stimulated THP-1 macrophage

The activity of the arginase I upon *Leishmania* exosome stimulation was done by following the method of (Dayakar et al., 2017) with minor modifications. Briefly, THP-1 macrophages (5 □ 10^5 cells/ml) were seeded in a cell culture plate by PMA (10ng/ml) at 37ºC in 5% CO_2 to convert monocytes to macrophages. Then, stimulated with *Leishmania* exosome of both strains (50µg/ml), naive and polyamine supplemented, and LPS (100ng/ml) kept as a control and incubated in a 5% CO_2 supplying CO_2 incubator at 37ºC for a time of 24 hours. Then, the treated macrophage was lysed with RIPA buffer (100µl) containing 1% PIC (proteinase inhibitor cocktail) and kept overnight at -80ºC. The 100µl of cell lysates (5×10^5 cells/ml) was added with the volume of 10µl $MnCl_2$ (10 mM) and incubated for a time last for 10 minutes at 56ºC in a dry bath to arginase enzyme activation. The 100µl L-arginine (0.5 M, pH- 9.7) was hydrolyzed by incubating the activated lysates at 37ºC for 20 minutes. The 900µl of H_2SO_4 (96%)/ H_3PO_4 (85%)/H2O (1v/3v/7v) ratio was used for terminating the reaction and followed by 9% α-iso nitroso propiophenone (40µl) incubation was done (Made in 100% ethanol) at 95ºC for 30 min. L-arginine hydrolysis produces urea as an end product by arginase enzymes. The intensity of the urea's colour was detected and measured at wavelength 540nm in a spectrophotometer. The urea concentration from arginase activity was calculated from a urea standard curve of known concentration.

4.27 Flo□cytometry-based intracellular spermidine levels upon *Leishmania* exosome stimulation

The PMA (10ng/ml) differentiated THP-1 macrophages of 5 □10^6 cells/ml were prepared in complete RPMI 1640 and incubated at 37ºC temperature condition with the supply of 5% CO_2 in an incubator. Then, it was washed and re-poured with fresh media and rested for 24 hours. After that, the cells were stimulated with 50□g of *Leishmania*-derived exosomes for 24 hours for the intracellular secretion of spermidine. Following the manufacturer's protocol, the fixation and permeabilization were done with cytofix/cytosperm (BD Biosciences). Briefly, the harvested cells were added with cytofix/cytosperm solution and incubated at 4ºC for 30 minutes. Subsequently, the macrophage was thoroughly washed with wash buffer (BD Biosciences) and spun at 200xg for a time lasting for 5 minutes. After that, the primary

antibody, i.e., anti-spermidine antibody, was added in 1:200 dilution. Then, it was rinsed twice with cytosperm wash buffer (BD Biosciences), incubated with Alex flour conjugated secondary antibody in 1:500 dilution, and incubated the antibody for 30 minutes at 4ºC. Then, the macrophages were washed thrice, and 0.5 ml of staining buffer was added to each tube. The flow cytometry analysis proceeded in the flow cytometer (BD LSR Fortessa).

4.28 Gene expression analysis upon exosome stimulation

The targeted gene expression of our study of *Leishmania*-derived exosomes-stimulated THP-1 macrophage by (RT-qPCR). Briefly, the THP-1 cell lines (1.2 □ 10^7 cells/ml) were stimulated to differentiate into macrophages by PMA (10ng/ml). The different experimental variables were naive, DD8 exosomes stimulated (50□g/ml), BHU875 exosomes stimulated (50□g/ml), polyamine supplemented, and lipopolysaccharide (100ng/ml LPS). After that, they harvested the cells, washed the pellets twice with sterile 1X PBS, and isolated the RNA using the RNA isolation kit protocol (Qiagen). Then, the RNA was quantified by nano spectrophotometer (Thermos Scientific), and complementary DNA (cDNA) was prepared from 2µg of the template by using the kit method (Takara). The appropriate primers for the study were used and listed in the table (**Table 2**). The cDNA of targeted genes with an SYBR green Premix Ex Taq (2X) (Takara) was amplified in the condition of 50ºC primary incubation for 2 minutes, then the denaturation at 95ºC for 10 minutes and kept for 40 cycles of 30 seconds at 95ºC, 60ºC for 1 min, and 72ºC for 45 seconds. The reaction was done in Sequence Detector (ABI Prism 7300). The CT values were obtained, relative fold expression (2-ΔΔct) was calculated by normalizing with GAPDH control, and un-modified RNA was retained as a negative control.

Table 4.1: Human gene-specific primers used in the present study for the expression analysis of various genes amplification of the target mRNA by the qRT-PCR

Sl.No.	Gene	Sequence (5'-3')
1	Human RT Slc3a2	**FP: GCATTGCGGCTTGGTTTTCT** **RP: CAGCTCAGAACAGGGTTAGA**
2	Human RT iNOS	**FP: TGCAGACACGTGCGTTACTCC** **RP: GGTAGCCAGCATAGCGGATG**
3	Human RT IL-10	**FP: GTGATGCCCCAAGCTGAGA** **RP: CACGGCCTTGCTCTTGTTTT**
4	Human RT TGF-β FP	**FP: TGCAGACACGTGCGTTACTCC** **RP: GGTAGCCAGCATAGCGGATG**
5	Human RT IFN-γ	**FP: TCAGCTCTGCATCGTTTTGG** **RP: GTTCCATTATCCGCTACATCTGAA**
6	Hu RT GAPDH	**FP: CCCATGTTCGTCATGGGTGT** **RP: TGGTCATGAGTCCTTCCACGA**

4.29 Data Interpretation and Statistical Analysis

The experiments of the current study were repeated twice, and technical duplicates/triplicates were kept. The statistics software GraphPad Prism 7.0 was used for the statistical analysis and data representation. The significance of a P value p□ 0.05 was considered as the significance levels of different experiment groups, and it was carried out using parametric tests (Unpaired students t-test or ANOVA). Data is presented in mean □standard deviation (SD).

Chapter: 5
Results

5.1 *L. donovani* parasites secrete Extracellular □esicles □E□s□ *Leishmania* exosomes are small vesicles of cellular communicator

Leishmania is a eukaryotic protozoan parasite that produces EVs through evolution (Douanne et al., 2022); the heterogenous population of vesicles is round or cup-shaped and contains a cytosol and lipid membrane layer similar to the parental cell that it originated (J. M. Axwell Silverman □ Reiner, 2011). *L. donovani* promastigotes released EVs, and the SEM images confirmed the bleb of vesicles throughout the membrane surface of the parasite (**Fig. 5.1**). The studies indicate that the vesicle secretion induced the mimic of the infection-like condition such as 37ºC and acidic pH ~5.5 (J. M. Axwell Silverman □ Reiner, 2011). However, our study used the stationary phase of the *L. donovani* parasite cultured under the axenic condition that mimics the sandfly midgut physiological conditions. Briefly, the temperature at 26–27ºC in M199 media containing EV-depleted decomplemented fetal bovine serum and the stationary stage of parasites were used for the experiment. It is proven that altering the culture condition is strongly related to the intracellular packaging of cargo. The biogenesis mechanism of plasma membrane blebs and exosomes results in cytosol within them, and their outer surface is coated with the extracellular plasma membrane leaflet, which is also the luminal leaflet of the origin of intracellular vesicles.

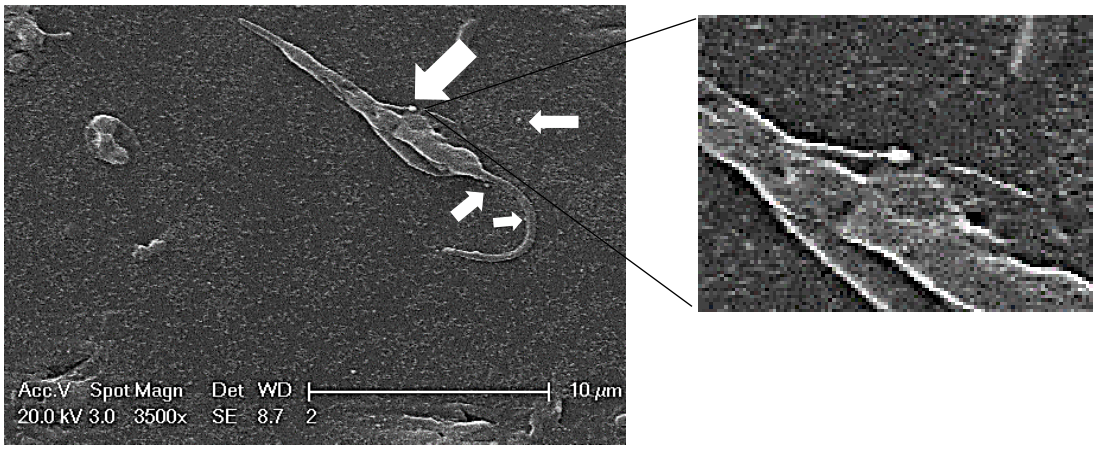


Figure 5.1: Scanning Electron Microscopic image of E□s release of *L. donovani* Promastigote. The arrows indicate EVs on the surface of the parasites, and the right panel is the zoomed image of the vesicle at the membrane surface of the parasite. The axenic stationary phase parasite was cultured at 26ºC, similar to the sandfly midgut condition.

5.2 The parasite metallopeptidase GP63: beyond the virulence factor□it confirms the parasitic specific exosomes

L. donovani parasites secrete exosomes inside the sandfly gut, axenic culture condition, and mammalian host. The secretion of exosomes is intended to function as intracellular communicators. So, it carries many parasitic virulence factors, including the metallopeptidase GP63 (Atayde et al., 2015). In our study, the scanning electron microscopic images presume that *L. donovani* secretes the extracellular vesicles, and it confirms that the axenic promastigote culture isolated exosomes were authentic *Leishmania* exosomes; we confirmed with a western blot of GP63, a parasite-specific marker. The *L. donovan*i parasite exosomes were obtained, SDS-PAGE was performed (**Fig. 5.2 A**), and then probing with *Leishmania* exosome markers such as GP63 by western blot assay. The detection of GP63 was seen in exosomes of the *L. donovani* parasite (**Fig. 5.2 B**). The total extract from *L. donovani* was used as a positive control. Interestingly, *L. donovani* exosomes carry many parasite proteins, as observed in the SDS-PAGE (**Fig. 5.2 A**). The enrichment of GP63 in the parasite's exosomes was especially marked. Studies show that enriching GP63 protein in *Leishmania* exosomes involves macrophage functional plasticity.

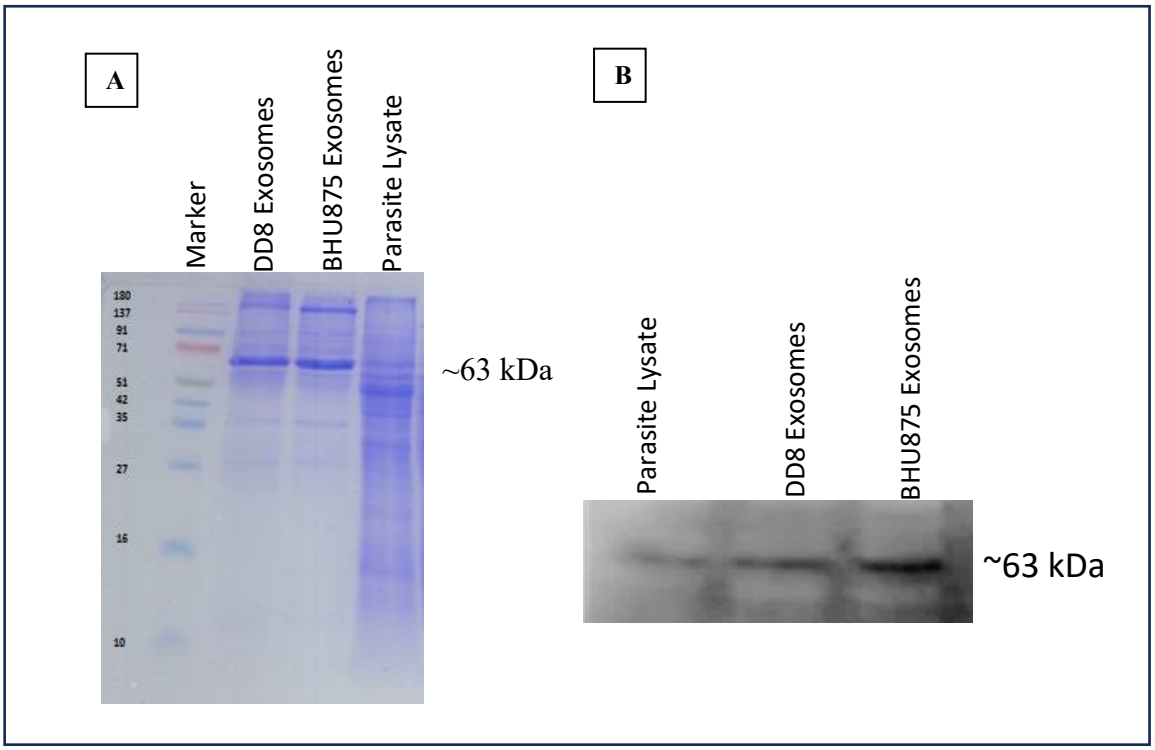
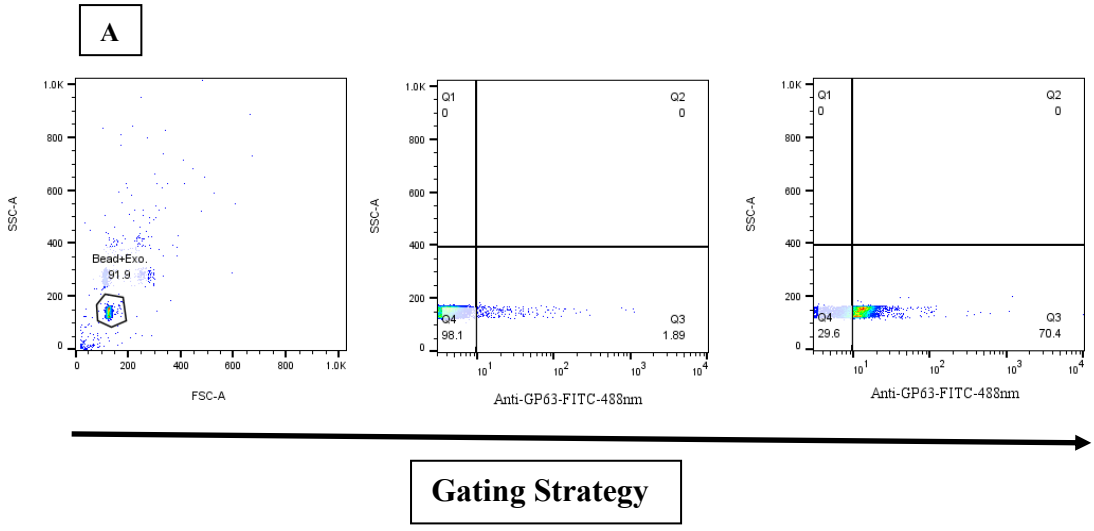


Figure: 5.2 *Leishmania*-derived exosomes SDS-PAGE and □ estern Blot. The 12.5 % SDS-PAGE of *L. donovani* DD8 (MH0M/IN/80/DD8) and resistant BHU875 strain promastigote

exosome lysate and a total extract of the parasite (**Figure: 5.2 A**□The image shows the western blot of *L. donovani* exosome and parasite lysate by the anti-GP63 antibody against *Leishmania* surface marker protein GP63 (*Leishmania* specific exosome marker) for the confirmation of *Leishmania*-derived exosomes □**Figure. 5.2 B**□

5.3 Bead-based semi-□uantitative flo□ cytometry: a □ay to □uantify the protein expression of nano molecules

The EVs originate from the parental cell's plasma membrane, so it is essential to show a specific transmembrane protein to characterize the EVs, and flow cytometry-based characterization is one of the suggestable methods as per the International Society for the Extra Cellular Vesicles guidelines (Tkach □ Théry, 2016). The conventional characterization techniques are restricted and have limitations in contaminating particles or protein aggregates because of their size and heterogenicity. The flow cytometry-based semi-quantification helps to overcome a few of these problems and is an additional characterization technique for homogenous and heterogenous populations of EVs. Bead-assisted flow cytometry was performed by incubating the samples with aldehyde/sulfate latex beads 4µm in diameter (Suárez et al., 2017). The isolated exosomes were characterized by bead-assisted flow cytometry using antibodies against *Leishmania* exosome-marker GP63 (**Fig. 5.3**). The background noise signal was resolved with pre-absorbed antibody and unstained bead-bound exosome control (**Fig. 5.3**). The 91.9 % of bead-bound *Leishmania* promastigote exosomes were gated for the quantification of GP63 positive exosomes (**Fig. 5.3 A**□among that 66.4 % population shows GP63 positive percentage as compared to the pre-absorbed antibody control that is 2.23% with p value□0.0038 (**Fig. 5.3 B** □ **C**□



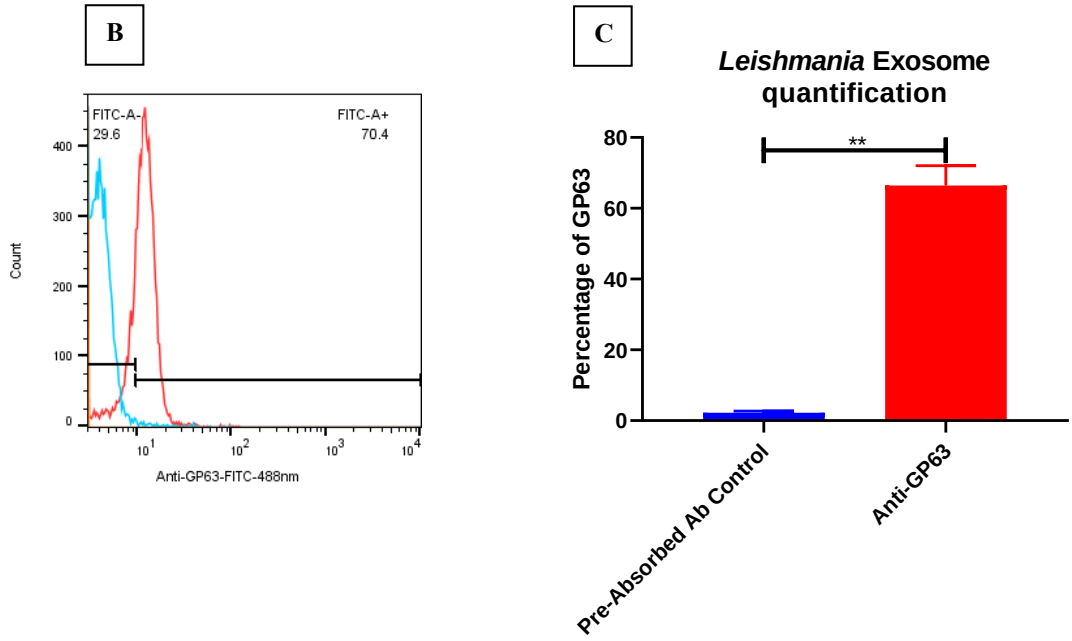


Figure. 5.3 Represent the bead-based semi-□uantitative flo□ cytometry analysis of *L. donovani* exosome. The dot plot shows the gating strategy of bead-bound exosomes and the antibody-based quantification; the first dot plot shows the total population gate; the other two show the pre-absorbed antibody control and antibody-labelled bead-bound exosomes. (**Fig. 5.3 A**). The bead-bound exosome population of promastigote was labelled with a *Leishmania* exosome-specific marker GP63, and the overlay histogram shows the shift of GP63-FITC.of pre-absorbed antibody control vs GP63-FITC stained *Leishmania* derived exosome (**Fig. 5.3 B**). The percentage of GP63 positive exosomes was quantified and shown in the bar graph with a p-value □0.0038 of □□P □0.05, a significant difference with pre-absorbed antibody control (**Fig. 5.3 C**) and the data represented in mean □SD.

5.4 Isolated *Leishmania* exosomes visuali□ation: single ob□ect Imaging in the heterogenic E□s population by fluorescence microscopy

The high-resolution fluorescence microscopy enables the single object visualization of the characterization of the highly heterogenic EVs with specific dye staining or antibody labelling, which would otherwise be a tedious task to visualize (Ter-Ovanesyan et al., 2017). Western blot and Flow cytometry confirmed *L. donovani* exosomes, and the visualization of exosomes through confocal microscopy was carried out. The primary anti-GP63 antibody was used to observe exosomes alongside the secondary anti-mouse IgG-FITC antibody. The absence of background noise of the FITC fluorescence was observed in the pre-absorbed antibody

(primary antibody omitted) control (**Fig. 5.4 A**). Exosomes are visualized as green fluorescence spots above the background (**Fig. 5.4 B**).

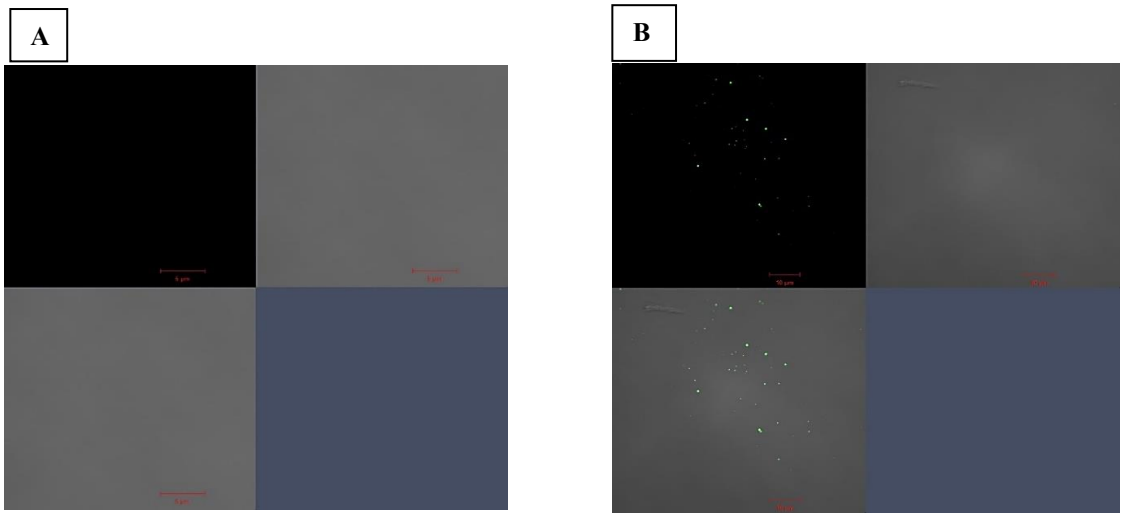
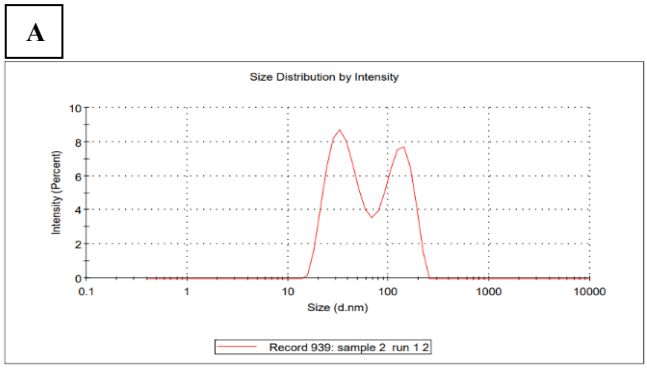


Figure: 5.4 Confocal microscopic images of *Leishmania* exosome (A) Pre-absorbed antibody control omitted with primary antibody to eliminate background noise of antibody **(B)** *Leishmania* promastigote exosome with anti-GP63 antibody shown as green fluorescent spots.

5.5 Physical characteri□ation of *L. donovani* exosomes by DLS and TEM analysis

The average size determination of isolated exosomes from the stationary phase parasite was done with DLS and TEM. The isolated exosomes were taken for physical characterization in DLS, and our result found that the particle size and the density were similar to exosomes. The average size of the isolated exosomes was 42. 75nm, with a moderate percentage intensity of 62.25% (**Fig. 5.5 A□B**). The zeta potential of the exosomes was 197.5 d. nm, and the Polydispersity index (PDI) was 0.299nm for the isolated exosomes (**Table. 5.1 B**). Although the TEM analysis shows that the global size distribution ranged from 10 to 200nm, the maximum number of exosomes was in the predicted size of 50-70 nm (**Fig. 5.5 C □ D**).

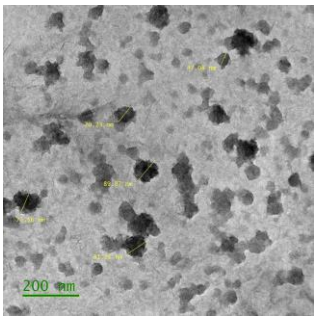


B

Table 5.1: The Dynamic Light Scattering Analysis

□-average □d. nm□	PDI	Intercept	Si□e □d. nm□	□ intensity	St.Dev.□d. nm□
146	0.299	0.98	42.75	62.25	15.99

C



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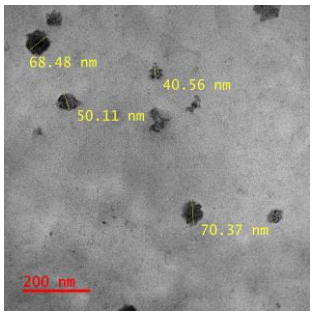


Figure: 5.5 Physical characteri□ation of *L. donovani* exosome □ith Dynamic Light Scattering and Transmission Electron Microscopy □TEM□□A□Size distribution plot with the intensity of the size of the exosomes in nanometre X-axis and percentage of intensity in the Y-axis. □**B**□The average of zeta potential, PDI, intercept, size (d. nm), percentage of intensity, and standard deviation of independent experiments were shown in the table (**Table. 5.1 B**). Representative images of TEM of parasite exosomes of sensitive DD8 and resistant BHU875 with average particle size dispersion in diameter (nm) (**Fig. 5.5 C □ D**).

5.6 *Leishmania* exosomes highly enriched in polyamines reflecting the exosomes mediated polyamine carrier in parasite

The *Leishmania* pathogen is signified by altering the host's metabolic pathways by manipulating host or parasite metabolomics. Recent reports emphasize the intra-exosome metabolome in the pathophysiology of *Leishmania*. The metabolic signatures of parasite exosomes have attracted far less attention. However, from the perspective of the immunometabolism approach in the *Leishmania* infection potentiates the dynamic changes induced by the small metabolites in the proteomic as well as genetic regulation of the pathogenesis of various diseases by intracellular communication (Dong et al., 2019). Interestingly, we spotted many metabolites in our LC-MS-based chromatographic study (**Fig. 5.7 A**); furthermore, metabolites are major contributors that decide the macrophage functional

fate and *Leishmania* disease progression, especially the arginine-mediated polyamine synthesis (Latour et al., 2020). Further, we focused on the polyamine biosynthesis pathway mediated by the *Leishmania*-derived exosomes. Our interest lies in the parasite and host polyamine pathway in the immunometabolism perspective of host-pathogen interaction. We checked the targeted metabolites of polyamine pathways to confirm that *Leishmania*-derived exosomes carry the polyamines. TLC has been used for the separation of *Leishmania* exosome polyamines. Dansyl chloride conjugated derivatization and extraction of *L. donovani* isolated exosome PAs (Madhubala, 1998). Interestingly, our result TLC supports the existence of polyamines in *L. donovani* exosomes. Also, the enrichment of polyamine is comparatively higher in exosomes than in the cell-free supernatant of the parasite (**Fig. 5.6 A**). Furthermore, we found that the polyamines are enriched in the *Leishmnia*-derived exosomes and need to be elucidated what the polyamines are in it. To address this, we did a High-Performance Liquid Chromatography (HPLC) of promastigote-derived exosomes of *L. donovani* parasites. The dansyl chloride conjugated derivative samples were injected, and two peaks were found in exosomes. The peaks were aligned with the standards of polyamines, and they adequately aligned with the standard peaks of spermidine and putrescine at an Rt value of 26.5 for spermidine and 28.0 for putrescine. *Leishmania* exosome shows peaks of spermidine and putrescine, the Y-axis represents the absorbance at 270 nm, and Rt (Minutes) is on the X-axis (**Fig. 5.6 B**). Our results found that the *Leishmania*-derived exosomes carry major and essential polyamine spermidine, the end product of the *Leishmania* polyamine biosynthesis pathway. Moreover, we also found its substrate putrescine. Surprisingly, as reflected in our result, the recent literature also shows that there is no report of the presence of spermine synthesis in the parasite. Also, the polyamine pathway of *Leishmania* lacks the back conversion enzymes of spermine to spermidine and putrescine like other mammalian cells (Carter et al., 2022).

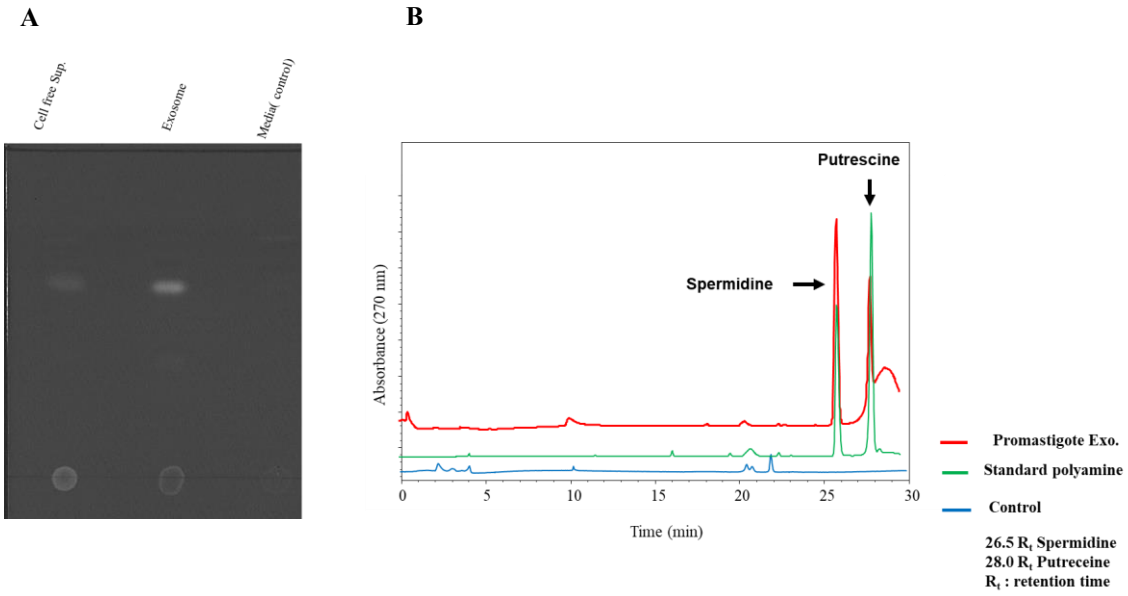
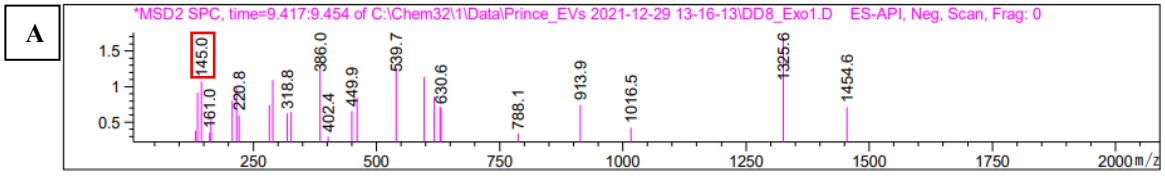


Figure 5.6 Detection of polyamine in *Leishmania*-derived exosome by Thin Layer chromatography and HPLC. Shows the dansyl chloride derivatized polyamine bands of cell-free supernatant, *Leishmania* exosome, and media as the negative control. PAs enrichment in *Leishmania* exosome compared to cell-free supernatant was in lane 2 of TLC (**Fig. 5.6 A**). The High-Performance Liquid Chromatography of the Promastigote exosome of the *L. donovani* parasite with control and polyamine standards (Spermidine and Putrescine). Peaks show the presence of polyamines spermidine and putrescine in *L. donovani* exosomes at a Retention Time (Rt) of 26.5 for spermidine and 28.0 for putrescine in the X-axis and the absorbance at 270 nm. The peak colour red indicates Promastigote exosomes, green is for polyamine standards of spermidine and putrescine, and blue shows the media control (**Fig. 5.6 B**).

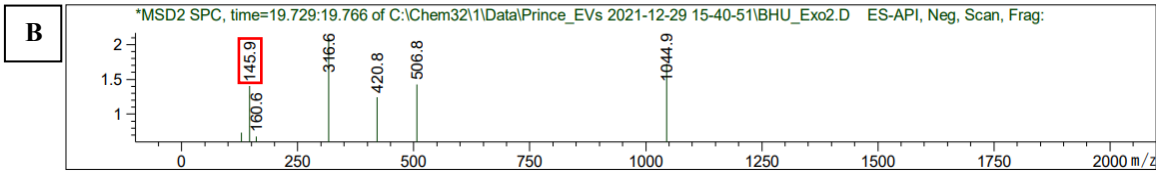
5.7 LC-MS-based qualitative and quantitative metabolic analysis revealed the presence and levels of spermidine in *L. donovani* exosomes

The chromatographic study-based exosome metabolic analysis is a sensitive technique that gives much input into the *Leishmania* parasite's immunobiology. The non-targeted LC-MS analysis was done for the metabolic profile analysis. Based on the mass charge ratio, we spotted the metabolite of our interest, especially our targeted metabolite spermidine, was there in our non-targeted metabolic chromatogram profile of both parasitic exosomes of sensitive DD8 (MH0M/IN/80/DD8) and resistant BHU875 strains spotted with many other metabolites. The chromatogram metabolic profiles A and B show the metabolites inside the exosomes of the sensitive and resistant strains, respectively, with an m/z value of 145 and the peaks marked as

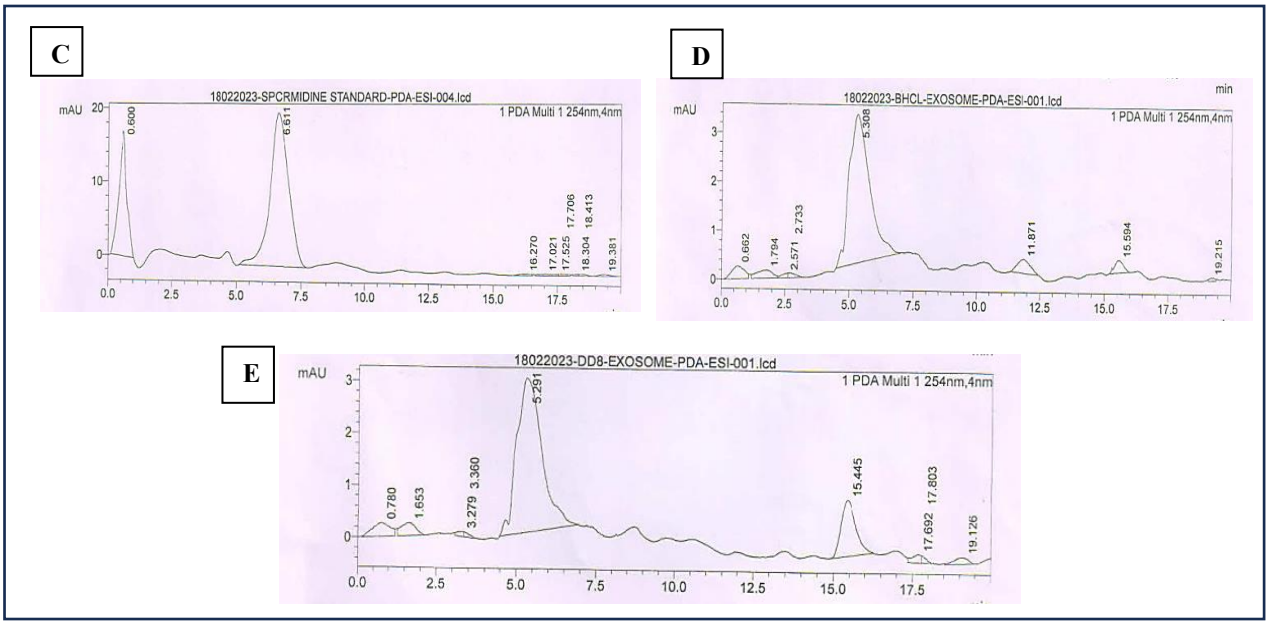
spermidine (**Fig. 5.7 A □ B**). The preliminary observation of the metabolic profile data reveals that many metabolites are carried over through the exosome, and their profile is varied in sensitive DD8 and resistant BHU875 strains. Based on the molecular weight and mass charge ratio, we spotted polyamine spermidine in both strains of parasitic exosomes. However, a detailed data analysis is needed to identify other specific metabolites carried in the exosomes of both strains and their quantification. Spermidine plays a significant role in *Leishmania* infection, and its exogenous supplement progresses the parasite's survival because spermidine is the end product of the *Leishmania* parasite (Zanatta et al., 2023). The targeted analysis of spermidine quantification in both parasite-derived exosome strains confirms the crucial polyamine spermidine in the targeted LC-MS with the known standard of spermidine. Biogenic spermidine of *Leishmania*-derived exosomes of both strains of parasites was determined and quantified by the Shimadzu LC-MS Solution instrument, using standard curves integrated with the particular curve obtained in the retention time and calculated the peak area and height and estimated the quantity of the spermidine. Biogenic polyamine spermidine in DD8 and BHU875 parasite exosomes are 15.458 and 16.073 mg. L^{-}1, respectively, with the retention time of the peaks 5.291 and 5.308. The percentage of RSD was considered a quality index of the analysis and represented the variations in the concentrations and Rt. The Guideline of the FDA (2001) recommends an RSD □15% for appropriate robustness of the analysis, and our results show a percentage of RSD value of ≤ 13.197 (**Table. 5.2 F**). Moreover, samples and standards show similar retention times in the chromatogram (**Fig. 5.7 C-E**). Overall, we confirmed the presence of spermidine in the *Leishmania*-derived exosomes and quantified it with LC-MS analysis. The result indicates that the *Leishmania* exosome carries the crucial polyamine spermidine, which might supplement the host during infection.



DD8 Chromatogram



BHU 875 chromatogram



F

Table 5.2 The LC-MS Analysis

Sample	Retention time (Rt)	Area	Height	Concentration (mg. L^{-1})
Spermidine Standard	6.611	1037267	20778	100
DD8 Exosomes	5.291	160336	2951	15.458
BHU875 Exosome	5.308	166717	3027	16.073
% RSD	13.197	110.927	115.158	110.927
Standard Deviation	0.757	504464	10270	48.634

Figure: 5.7 Sho□s the LC-MS analysis of parasite exosomes □MH0M/IN/80/DD8□sensitive vs. BHU 875 resistant□ The *L. donovani* LC-MS spectral images of (**Fig. 5.7 A □ B**) peaks, show polyamines spermidine with mass charge ratio (m/z) of 145 □1 in *L. donovani* exosomes of sensitive and resistant parasites, respectively. **Figure 5.7 C-E** shows the LC-MS

chromatograms of spermidine standard, sensitive and resistant parasite-derived exosomes peak of polyamine spermidine with retention time. The biogenic polyamine spermidine concentrations of DD8 and BHU875 parasite exosomes are 15.458 and 16.073 mg. L^{-1}, respectively, with the retention time of the peaks 5.291 and 5.308. The percentage of RSD value was ≤ 13.197 (**Table. 5.2 F**). The calculation was done with the area and height of a particular peak aligned with the standard peak of the spermidine Shimadzu LC-MS Solution software.

5.8 The presence and bead-based semi-□uantitative flo□ cytometry analysis of spermidine in the *L. donovani* exosomes □ith anti-spermidine antibody

The bead-based semi-quantitative flow cytometry analysis was performed, and positive signals from the anti-spermidine antibody-labelled exosomes were observed. The semi-quantitative flow cytometry analysis of the presence of polyamine with anti-spermidine antibody as primary and Alexa Flour 488 as secondary antibody. The total bead-bound promastigote parasite exosome was 98.4% in the dot plot gate (**Fig. 5.8 A□E**). The overlay histogram analysis shows an increment of spermidine labelled population with a percentage of 79.7 %. The Promastigote exosomes MFI (Mean Fluorescence Intensity) difference of spermidine with unstained bead and pre-absorbed antibody control was plotted. The MFI of anti-spermidine-FITC conjugated promastigotes exosomes shows a higher trend of MFI with 3456 compared to pre-absorbed antibody control of the MFI of 265 (**Fig. 5.8 C**). The background noise of the secondary antibody was eliminated by pre-absorbed antibody control. The auto-fluorescence of beads and exosomes was avoided with unstained bead control. It confirms the presence of spermidine in the exosomes by the specific binding of anti-spermidine antibodies.

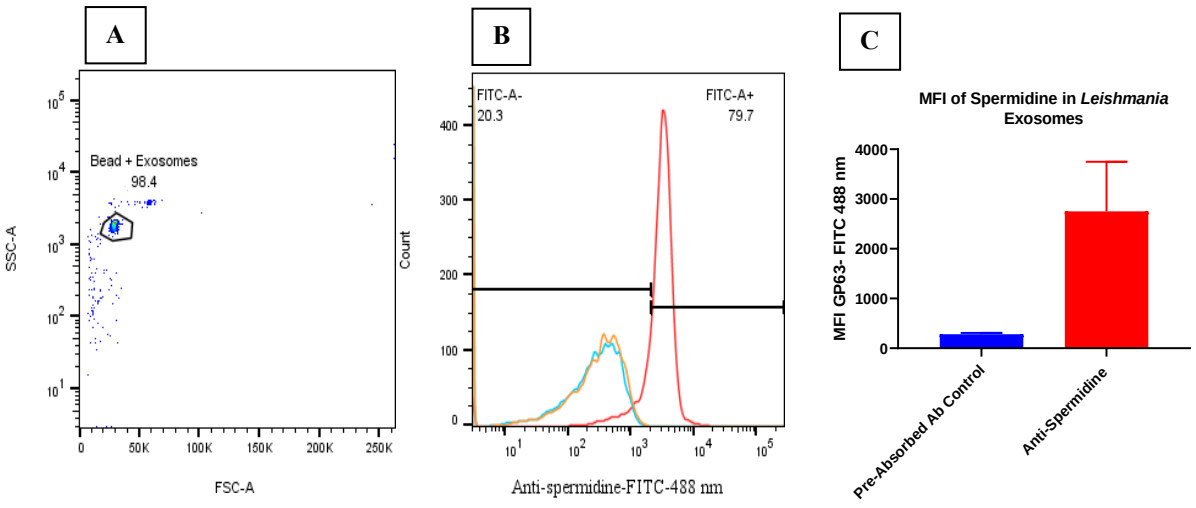


Figure 5.8 The bead-based semi-□uantitative flo□ cytometry analysis of *L. donovani* exosome. The dot plots (**Fig. 5.8 A**□show the bead-bound exosome population of promastigote was labelled with an anti-spermidine (SPD) antibody with a 98.4 % bead-bound population. The overlay histogram of the unstained bead control, pre-absorbed antibody control and stained exosomes of promastigote with primary anti-spermidine antibody and the secondary anti-FITC antibody shows a 79.7% positive population (**Fig. 5.8 B**□ The plot represents the MFI of pre-absorbed antibody control Vs anti-spermidine-FITC antibody labelled exosome bounded beads (**Fig. 5.8 C**).

5.9 Metabolic viability of the mitochondria during hypericin-mediated polyamine depletion

Hypericin is a plant-derived natural product and showed a competitive inhibition mode with its substrate putrescine. The *L. donovani* promastigote was inhibited by hypericin at an IC_{50} concentration of 18μM, and it was found to inhibit spermidine synthase activity (Singh & Dubey, 2016). In contrast, our result of metabolic cell viability assay of hypericin 18μM with the *L. donovani* promastigotes (2 x 10^5 cells/ml) of both sensitive and resistant parasites for a 72 hours treatment does not show any change in the MTT viability assay (**Fig. 5.9 B □ D**), it could be due to the higher activity of the mitochondria as a compensatory mechanism of spermidine depletion. In the treatment of the reference drug, the IC_{50} was 12.19□M for sensitive DD8 strain and 35.43□M for resistant BHU875 (**Fig. 5.9 A □ C**), whereas we are unable to calculate the IC_{50} for hypericin. Studies show that mitochondrial metabolic activity is high, and MTT won't be an excellent technique to check viability compared to cell number-based viability analysis (Murad et al., 2018). Even the higher concentration of the hypericin treatment does not show any changes in the metabolic viability index (**Fig. 5.9 B □ D**). So, we strongly presumed that hypericin reduces growth, but there is still no compromise in the metabolic activity in case of polyamine depletion due to the hyperpolarization of the mitochondria.

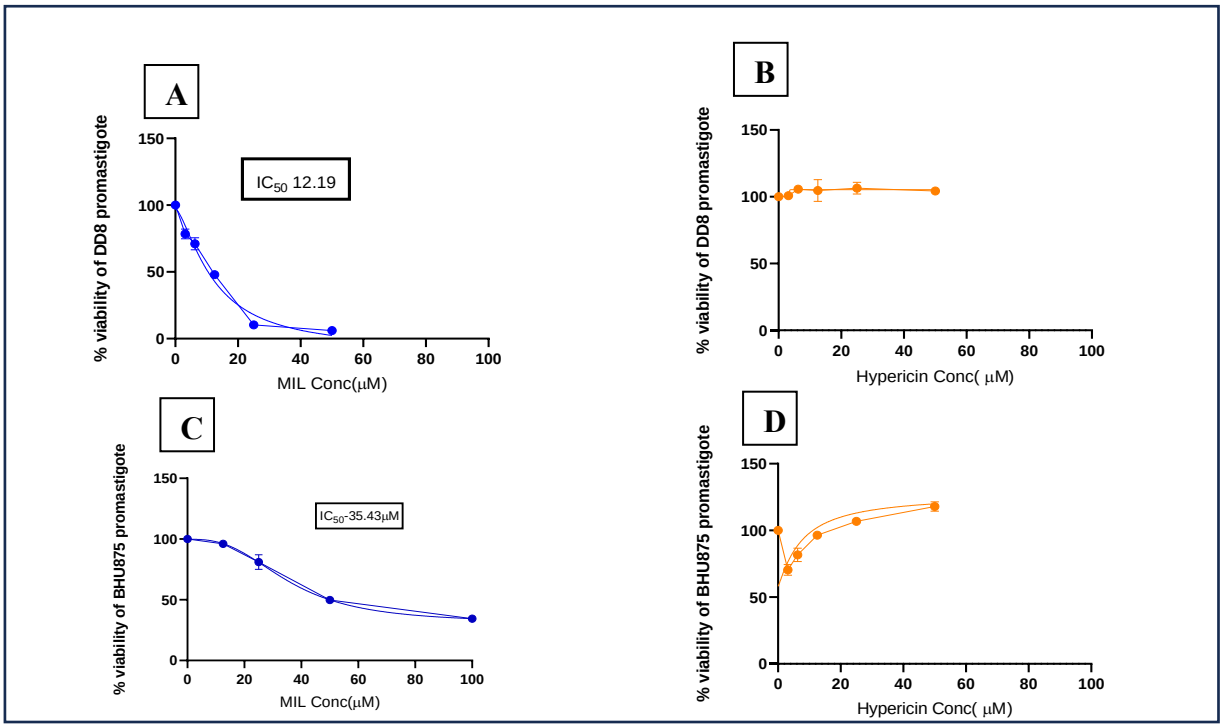


Figure: 5.9 Cell viability Assay by MTT Method on MIL sensitive and resistant *L. donovani* promastigotes upon hypericin 72 hours. The formazan crystals formed per parasite were quantified spectro-photometrically and depicted as a percentage of viability with different drug concentrations. The plot shows the percentage of viability and IC_{50} concentration of the reference drug miltefosine of sensitive and resistant BHU875, respectively (**Fig. 5.9 A C**) The plots show the parasite's metabolic viability by MTT while treating it with hypericin, and it did not show any significant differences compared to the control (**Fig. 5.9 B D**)

5.10 Cell number-based growth curve analysis shows the reduction of parasite growth upon hypericin-mediated polyamine starvation

As we noted, the mitochondrial metabolic activity by the MTT index does not show any changes in the viability of the *L. donovani* parasites (**Fig 5.9**). So, it is vital to cross-verify that the polyamine depletion of the parasites with hypericin reduces the parasite's growth by true numbers. The cell number-based viability assay was done for hypericin-mediated polyamine depletion and media supplementation of the polyamine over five days, with other experimental controls such as naive, vehicle control (0.01% DMSO), and miltefosine as an anti-leishmanial positive control. The proliferation of hypericin-treated *L. donovani* promastigote is reduced in

both parasite strains. The reduction was slightly higher in the case of BHU875 resistant strain of parasites (**Fig. 5.10 A □ B**). In contrast, the naive parasite, DMSO vehicle control (0.01%), and polyamine-supplemented (1X) parasites were grown optimally throughout the experiment for five days (**Fig. 5.10 A □ B**). Interestingly, hypericin (18□M) has shown an inhibitory effect till the 5th day constantly compared to control groups in both strains, it is important to note that the growth curve of MIL-resistant *L. donovani* BHU875 promastigotes has shown apparent sensitivity towards growth upon hypericin-mediated polyamine depletion as compared to DD8 sensitive strain (**Fig. 5.10 A □ B**). Our result of hypericin sensitivity towards the resistant strain interprets that the polyamines might have a solid link to the maintenance of virulence as well as the resistance of the parasites. Possibly, it is linked to trypanothione-mediated oxidative stress regulation. It is reported that the Sb (III) or arsenate resistance is linked to increased trypanothione levels in *Leishmania* (Author et al., 1996). The metabolic activity-based MTT index and true number-based growth curve results show contradictory results. So, further studies are suggested for the mitochondrial activity and the link of hypericin-mediated polyamine depletion.

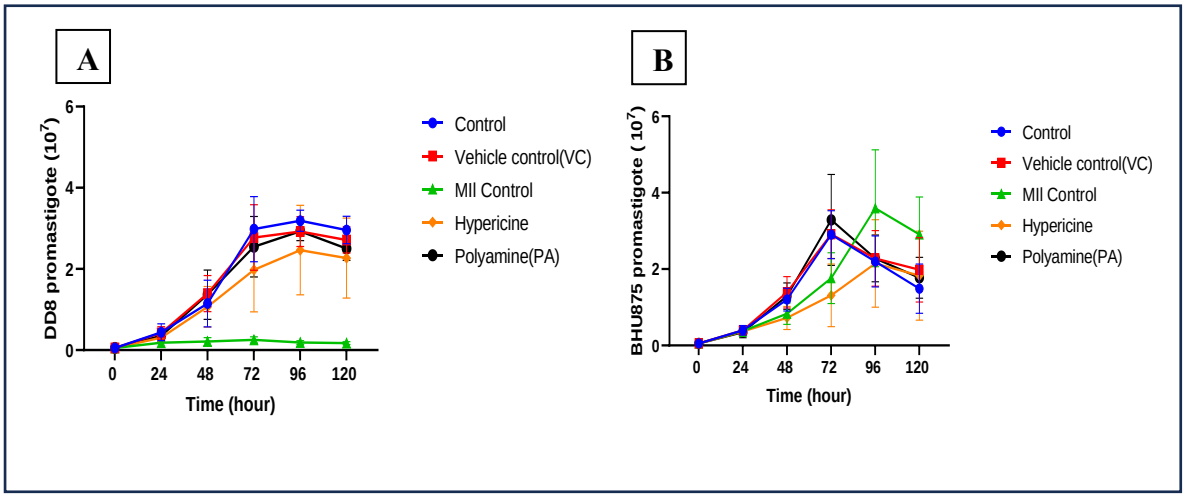


Figure: 5.10 The hypericin-mediated polyamine □Spermidine□starvation of *L. donovani* promastigote parasites. Promastigote parasites were seeded at 5 × 10^5 parasites/ml for 5 days, and the proliferation and number of parasites under a microscope were observed using a hemocytometer. The proliferation of different groups is plotted for both sensitive as well as resistant parasites (**Fig. 5.10 A □ B**). Hypericin (18□M) has shown an inhibitory effect till the 5th day constantly compared to control groups in both strains (**Fig. 5.10 A □ B**) line indicated with the colour yellow. The reference drug, shown with a green colour line, heavily affected the sensitive parasite compared to the resistant BHU875 (**Fig. 5.10 A**). The resistant strain

regained the growth fitness during the treatment period (**Fig. 5.10 B**). The polyamine supplementation shows a similar trend of both controls and the vehicle control (0.01% DMSO), indicated by the black line (**Fig. 5.10. A □ B**). Data from independent experiments with error bars representing standard error.

5.11 Hypericin-induced morphological changes in *L. donovani* promastigote parasite

Microscopy-based Giemsa stained hypericin treated parasites exhibited a round or spindle-shaped morphology with loss of flagellum (**Fig. 5.11 B □ E**). It is also observed that the treated parasite was less motile and formed the cluster. Meanwhile, the control and polyamine-supplemented parasites show normal morphology and motility (**Fig. 5.11 A□C□D□F**). Another important observation of our study is that the hypericin-treated parasites were more granular (**Fig. 5.11 B □ E**). Interestingly, as reflected in the growth curve, the phenotypical changes are more visible in the BHU875-resistant strain, showing that the resistant strain is more prone to hypericin-mediated polyamine depletion (**Fig. 5.11 E**).

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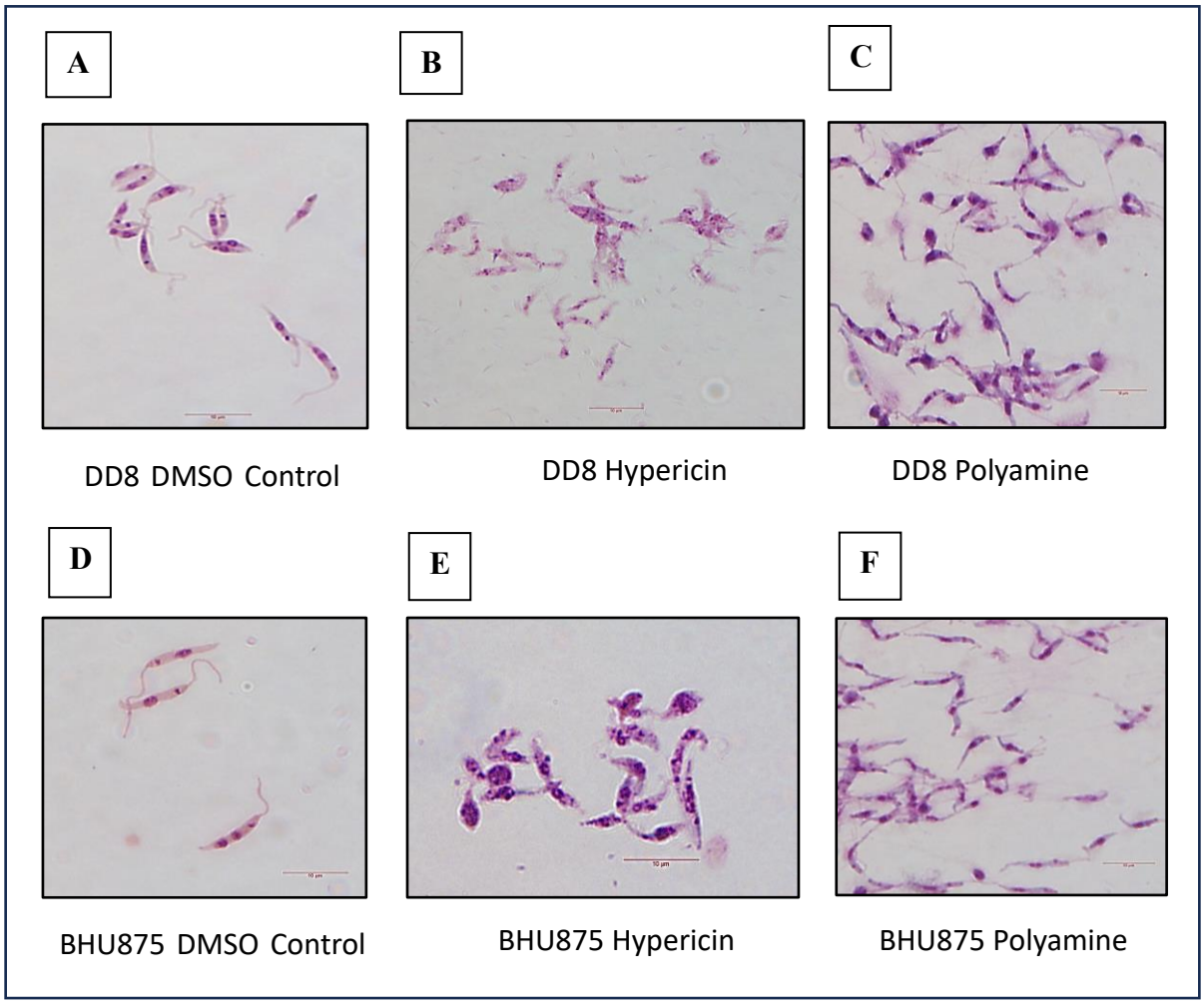
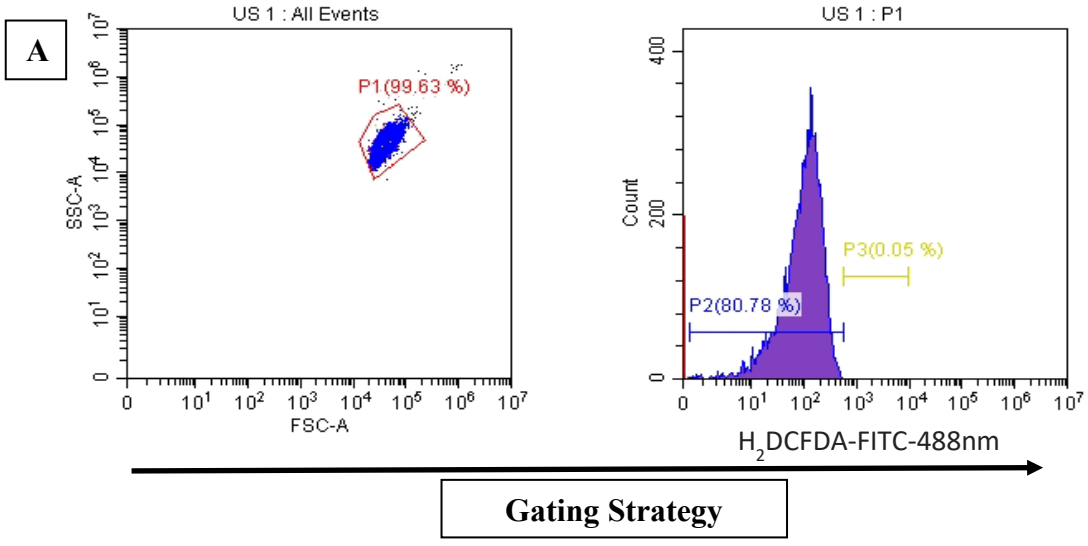


Figure: 5.11 Morphological changes of hypericin-mediated polyamine depletion. The representative bright-field images of Giemsa-stained parasites were taken on a Leica trinocular microscope after 72 hours of incubation of hypericin. DMSO vehicle control and polyamine-supplemented parasites were shown normal phenotypes (**Fig. 5.11 A□C□D□F**). In contrast, the hypericin-treated parasites were round or spindled in shape with loss of flagellum and were more granulated (**Fig. 5.11 B □ E**). The experiment was repeated more than three times, and visual observations were consistent.

5.12 Polyamine depletion mediated by hypericin in *L. donovani* triggered the generation of elevated levels of R□S

Promastigotes of *L. donovani* were treated with an IC_{50} dose of hypericin (18µM) for 6 hours, then analyzed with flow cytometry and dot plot and histogram shows the gating strategy of the experiment (**Fig. 5.12 A**). The bar graphs of both sensitive DD8 and resistant BHU875 strains show increased intracellular ROS levels compared to untreated *Leishmania* promastigotes with p-value □0.0014 for DD8 (sensitive strain) and p-value □0.0217 for BHU875 (resistant strain), respectively (**Fig. 5.12 B □ C**). It is associated with oxidative stress, stunted growth, and reduction of parasite numbers, as reflected in our previous results of the growth curve (**Fig. 5.10 A □ B**). The parasites under the starvation of polyamine were compared to those of parasites grown in the polyamine-supplemented condition. Polyamine supplementation did not induce reactive oxygen species similar to naive parasites (**Fig. 5.12 B □ C**). Indeed, it indicated that the hypericin induces elevated levels of ROS in the sensitive and resistant strains of the parasites. The reference drug control, the antileishmanial drug miltefosine, has been shown to exert its cytotoxic effect via the secretion of ROS in the *L. donovani* parasite.



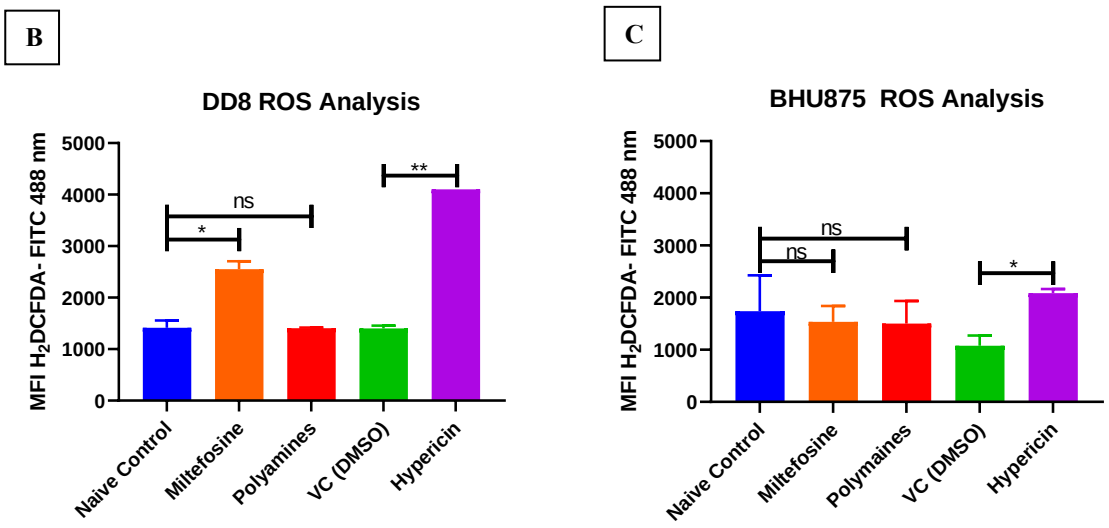


Figure: 5.12 The polyamine-depleted promastigote *Leishmania* parasite intracellular R□S estimation by H₂DCFDA mediated flo□ cytometry analysis. The exponentially grown parasites were treated with hypericin (18□M) for 6 hours and then stained with 10□M H₂DCFDA for analysis by flow cytometry. The plots represent the intracellular ROS upon polyamine depletion, especially spermidine, through hypericin treatment and naive control, vehicle control (0.01% DMSO), polyamine media supplement, and miltefosine (reference drug) serve as different experiment groups. The intracellular ROS in a fold change relative to the controls was measured. The dot plot shows the population gate of total macrophages (10,000 events), and the histogram represents the gating strategy for the MFI measurement (**Fig. 5.12 A**). The barograph shows the fold change of ROS in various experiment groups, and it shows a significant difference in the treatment hypericin of both parasite strains compared to the control groups (**Fig. 5.12 B**). The hypericin treatment leads to significance with p-value□0.0014 and indicated □□□p □0.0001; in the case of DD8, p-value □0.0217 and indicated with □p □0.05 in the case of BHU875 (**Fig. 5.19 C**). The standard reference drug miltefosine shows significance in the case of sensitive DD8 with p-value□0.0165 and indicated with □p □0.05. There is no significant difference in the case of BHU875 resistant strain and represented mean □ SD of independent experiments (n□2). H₂DCFDA□FITC excitation and emission range, i.e., 525/495nm.

5.13 Membrane permeability and viability assay of *L. donovani* parasites upon hypericin treatment

The promastigotes' viability of hypericin-treated parasites for 48 hours was analyzed with the dye propidium iodide (PI) based viability assay. Propidium Iodide (PI) is a stoichiometric membrane non-permeable dye, i.e., it binds in proportion to the amount of DNA present in the cell and is based on the membrane permeability of the cells. The parasite viability of promastigotes of both MIL-sensitive and MIL-resistant parasite strains was treated and stained with hypericin (18□M), naive, vehicle control (0.01% DMSO), polyamine supplement (1X), and miltefosine (reference drug) the different groups. The representative dot plots indicate the gating strategy of the experiment of sensitive and resistant strains, respectively (**Fig. 5.13 A □ B**). In the untreated naive promastigotes parasites, the binding percentage of PI was 1.30% and 1.34% in DD8 (sensitive) and BHU875 (resistant), respectively. In hypericin, treatment of sensitive DD8 promastigotes shows 11.7% PI-positive cells. The resistant BHU875 strain shows 31.40% as compared with the hypericin vehicle control, i.e., 1.08 % and 1.24 %, respectively, with p-value □0.0001 for DD8 and BHU875 (**Fig**. **5.13 C □ D**). Interestingly, upon hypericin treatment, the BHU875-resistant strain shows more sensitivity towards the drug than the sensitive strain. The polyamine supplement showed a similar percentage in naive parasites of both strains, 1.52% and 1.13%, respectively (**Fig. 5.13 C □ D**). Our results confirm that hypericin-mediated polyamine starvation compromises the membrane integrity and growth of the parasites and is more prominent in drug-resistant parasites than in sensitive parasites. It indicated that the polyamine is essential for the survival of drug-resistant parasites. The level of parasite growth is firmly connected to the polyamine metabolism of the parasites.

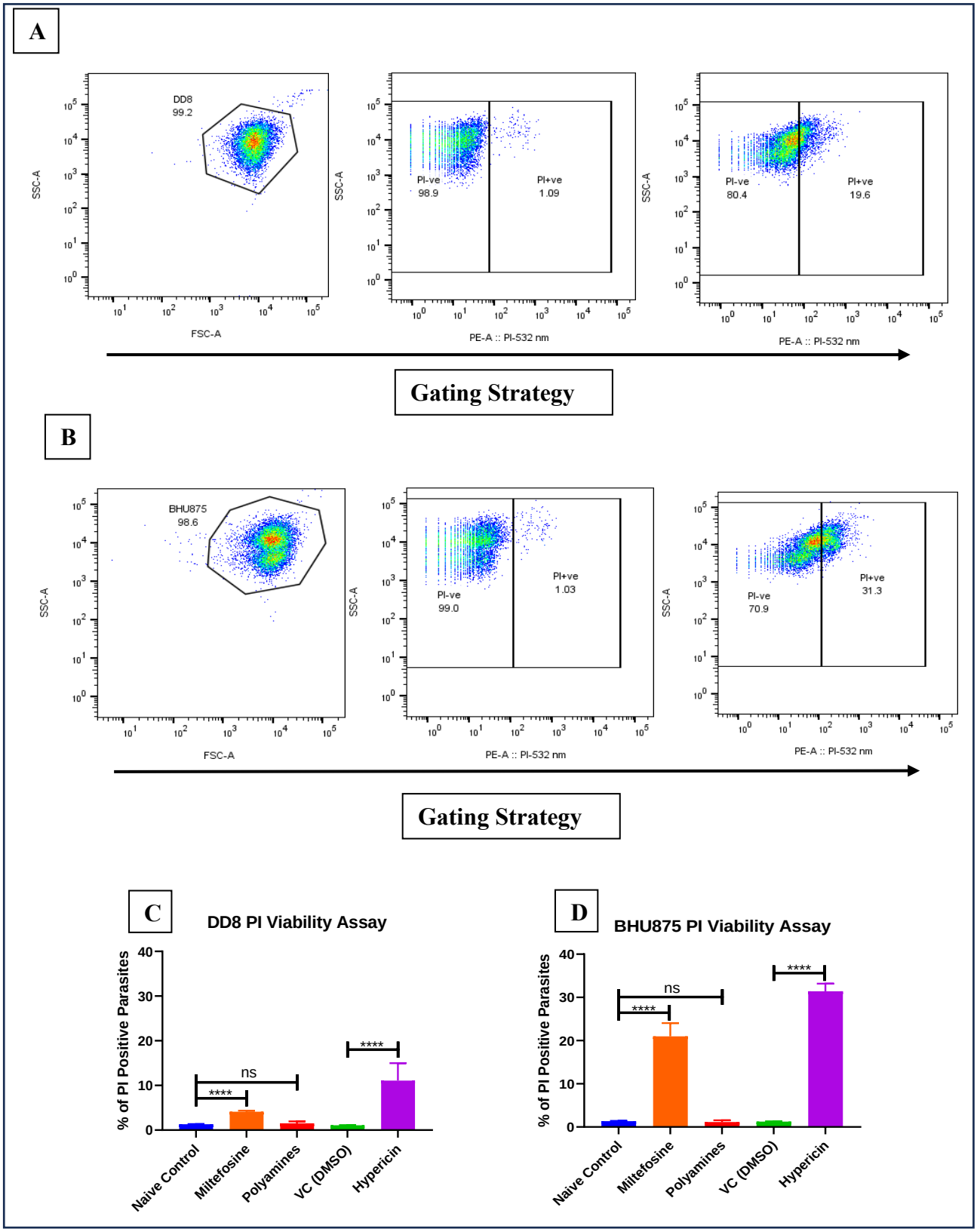


Figure: 5.13 PI-stained *Leishmania* promastigote parasites for viability assay. The exponentially grown parasites were treated with hypericin (18 M) for 48 hours and then

stained with PI for analysis by flow cytometry. The plots represent the viability index with the percentage of PI-positive parasites with permeable membranes of naive control, vehicle control (0.01% DMSO), polyamine supplemented, hypericin, and miltefosine as a reference drug. **Figure 5.13 A □ B** represents the gating strategy with a dot plot of total parasite gating and gating of PI negative and positive populations of unstained and naive control as a representative plot of groups. The bar diagram represents the percentage of PI-positive parasites of different groups of sensitive DD8 and resistant BHU875 parasite stains (**Fig. 5.13 C □ D**). The data depicts the percentage of PI-taken parasites of independent experiments (n□2) with mean □SD. The p-value □0.0001 indicated with □□□□ p□0.0001 compared to controls. PE in the FSC is excited at 565 nm, and its emission is 574 (PE laser channel used and PI excitation/emission maxima is ~532/674).

5.14 Hypericin modulates mitochondrial membrane potential (MMP): The link between polyamine depletion and mitochondrial energy metabolism

Since the MTT viability index doesn't show any effect upon treatment of hypericin, whereas an apparent growth compromise and membrane disruption were observed upon treatment, it was a hint to determine and link the mitochondrial action by the impact of hypericin on the mitochondrial membrane potential (ΔΨm). Moreover, mitochondria are a significant source of ROS, and *Leishmania* has a single mitochondrion. Therefore, mitochondrial energy metabolism is essential for parasite survival. So, the maintenance of MMP is crucial for the parasites. To evaluate the MMP, the exponentially grown promastigotes were exposed to IC_{50} hypericin concentration (18□M) to both strains of parasites for 48 hours. The experimental groups were naive parasite control, vehicle control (0.01 % DMSO), polyamine-supplemented control, hypericin, and miltefosine as reference drugs. The plots show the gating strategy of the experiment with total parasites and representative histograms of the JC-1 PI and FITC of the control (**Fig. 5.14 A □ B**). We observed that the miltefosine reference drug depolarizes the mitochondrial membrane potential as expected upon treatment with 1.249 □0.02 and 2.37 □0.23 compared to naive control, which is 9.89 □1.08 and 4.72 □1.48 for sensitive and resistant parasites, respectively (p-value □0.0001 for DD8 and p-value □0.0197 for BHU875). In contrast, the hypericin treatment increases the mitochondrial membrane potential with a red/green ratio of both sensitive and resistant strains of parasites were 4.728 □0.10 and 5.765 □0.74 in comparison to the vehicle control, i.e., 4.35 □0.14 and 4.38 □0.74, respectively with p-value □0.0079 for DD8 and p-value □0.0379 for BHU875 (**Fig. 5.14 C □ D**). However, no

significant changes were observed in the polyamine-supplemented parasites of both strains, and the ratio was 11.33 □1.432 and 4.10 □1.04, respectively, for both strains of parasites with naive control (**Fig. 5.14 C □ D**□ Our data red/green ratio for each strain shows a significant increment that indicates the parasite MMP led to the hyperpolarization of the mitochondria. It suggests that polyamine depletion demands a high energy requirement. In addition, oxidative stress-related changes are reflected in the mitochondrial energy metabolism upon hypericin treatment.

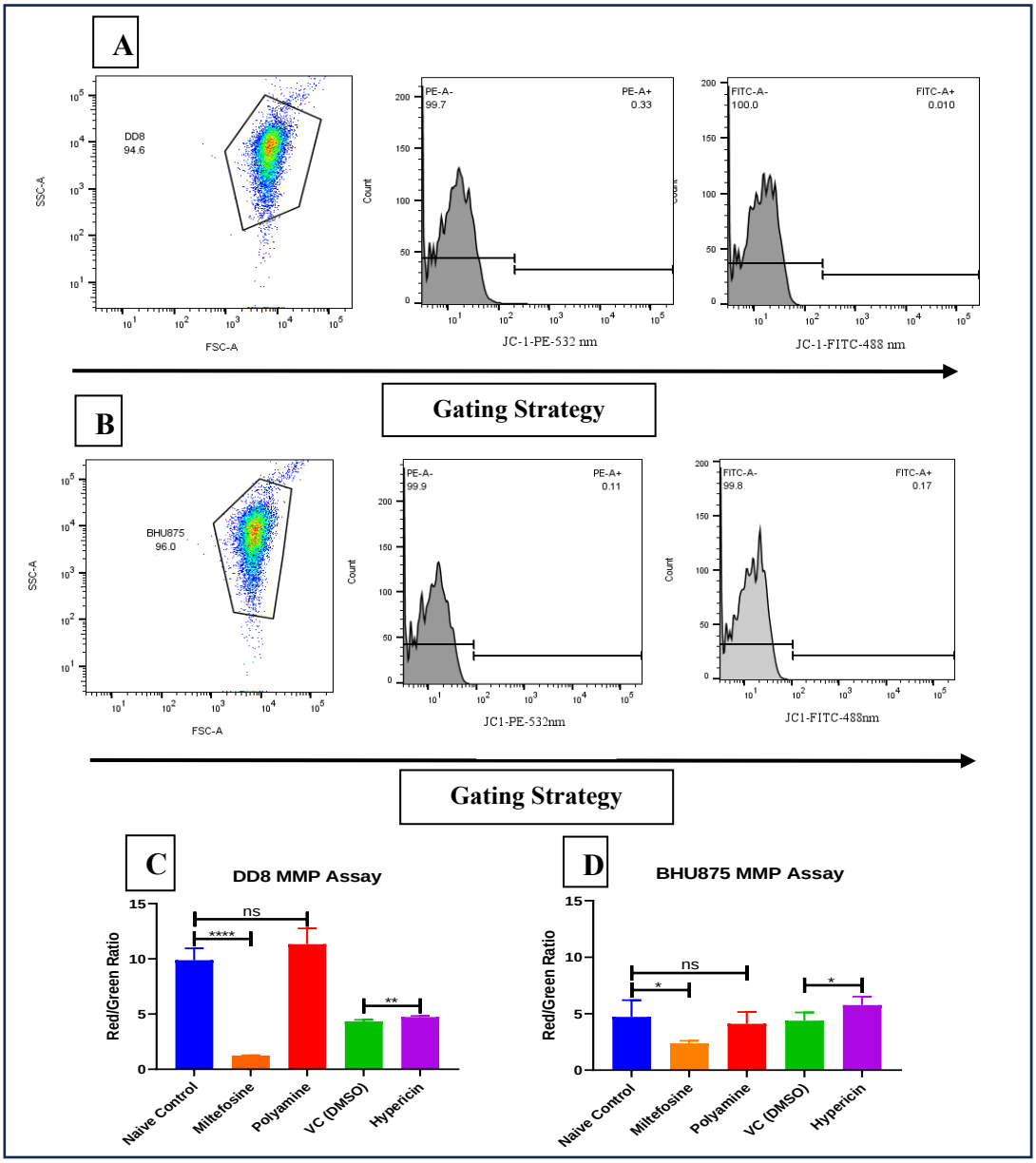


Figure: 5.14 The mitochondrial membrane potential (MMP) of *L. donovani* promastigotes upon hypericin treatment. The exponentially grown parasites were treated with IC_{50}

concentration of hypericin (18µM), naive parasites, polyamine media supplemented parasites (1X concentration), vehicle control (0.01% DMSO), and reference drug miltefosine as positive control serves as different groups. The parasites were treated for 48 hours, then the JC-1 dye (10µM) staining and observed the MFI of red/green ratio (590nm/530nm), the elevated red/green value is for hyperpolarization, and the lower levels of red/green ratio indicates the depolarization of the MMP (Ψm). The dot plots represent the gating strategy of the DD8 and BHU875 strains (**Fig. 5.14 A □ B**). The hypericin treatment shows an increment of red/green ratio in a sensitive and resistant strain of parasite upon hypericin treatment (**Fig. 5.14 C □ D**). The sensitive DD8 shows a significant increase in red/green ratio with p-value□0.0079 indicated with □□p□0.01(**Fig. 5.14 C**), and the resistant BHU875 show a p-value□0.0379 and indicated with □p□0.05 (**Fig. 5.14 D**) as compared with hypericin vehicle control (0.01% DMSO). The miltefosine reference drug shows depolarization with a reduction of red/ green ratio in sensitive DD8 p-value□0.0001 depicted with □□□□p□0.001(**Fig. 5.14 C**) and in resistant BHU875, it was p-value□0.0197 and defined with □p□0.05 (**Fig. 5.14 D**) as compared with naive parasite control. The data of independent experiments (n□2) are represented as mean □ SD.

5.15 *Leishmania* parasite-derived exosomes as a cargo carrier and communicator: an immunometabolism approach in the immunobiology of host-pathogen interaction

We investigated *L. donovani* exosomes from drug-sensitive (MH0M/IN/80/DD8) and resistant strain (BHU875) parasites and their role in the macrophage polarization from M1 to M2 state. Here, we approached the hypothesis using an immunometabolism of the host and the parasites. Since metabolites play a major role in the immune modulation by metabolic reprogramming of the host by the effector molecules of the parasites. So, here we explore the role of *Leishmania* exosomes in macrophage functional plasticity and phenotype changes during its stimulation as a survival strategy during the infection. Interestingly, the *Leishmania* exosomes act as a regulatory switch that rewires the host's immune-metabolism pathways. Arginine metabolism is the early investigated immune-metabolism interaction of host and parasite. However, the molecular insights of macrophage metabolic reprogramming and immune response regulation are poorly studied. This study focused on the polyamine biosynthesis pathway and its importance in the infection by an immunometabolism approach.

Leishmania secretes exosomes to extracellular space, and it is available to interact with host cells. The various studies of Hassani et al. and Silverman et al. prove that the vesicles of secretory supernatant carry the virulence factors of *Leishmania;* for example, GP63 is metallopeptidase, and it can be a biomarker for *Leishmania*-derived extracellular vesicle delivery to the host (Hassani et al., 2011; J. M. Silverman et al., 2010; J. M. Axwell Silverman Reiner, 2011). Another report by Gomez et al. explains the immunofluorescence of the punctate structure of GP63 inside the host cell, suggesting that GP63 was taken in vesicles (Gomez et al., 2009). There is a different way of vesicle-mediated cargo delivery proposed by various scientific studies, and the exosomes can bind to the recipient cell surface and deliver the cargo, plasma membrane fusion, or by receptor-mediated endocytosis (J. M. Axwell Silverman Reiner, 2011). Our results corroborate these observations and show that the *L. donovani* parasites secrete exosomes and deliver the exosomes cargos to the human monocyte differentiated macrophages (THP-1). We found the punctate structure of green fluorescence that the FITC labelled exosomes against the GP63 transmembrane protein inside the host macrophages. The images depict the confocal microscopy-based immunofluorescence analysis, and it was confirmed the uptake of *Leishmania* exosomes, and shows the naive macrophage unstained unstimulated control, pre-absorbed antibody control for DD8 (MH0M/IN/80/DD8) sensitive and BHU875 resistant strains, respectively (**Fig. 5.15 A B C**). The green fluorescence inside the macrophage indicates the uptake of the sensitive and resistant strain exosomes at 6 hours of stimulation **Fig. 5.15 D E** The fluorescence background noise was eliminated with pre-absorbed antibody controls (**Fig. 5.15 B C**

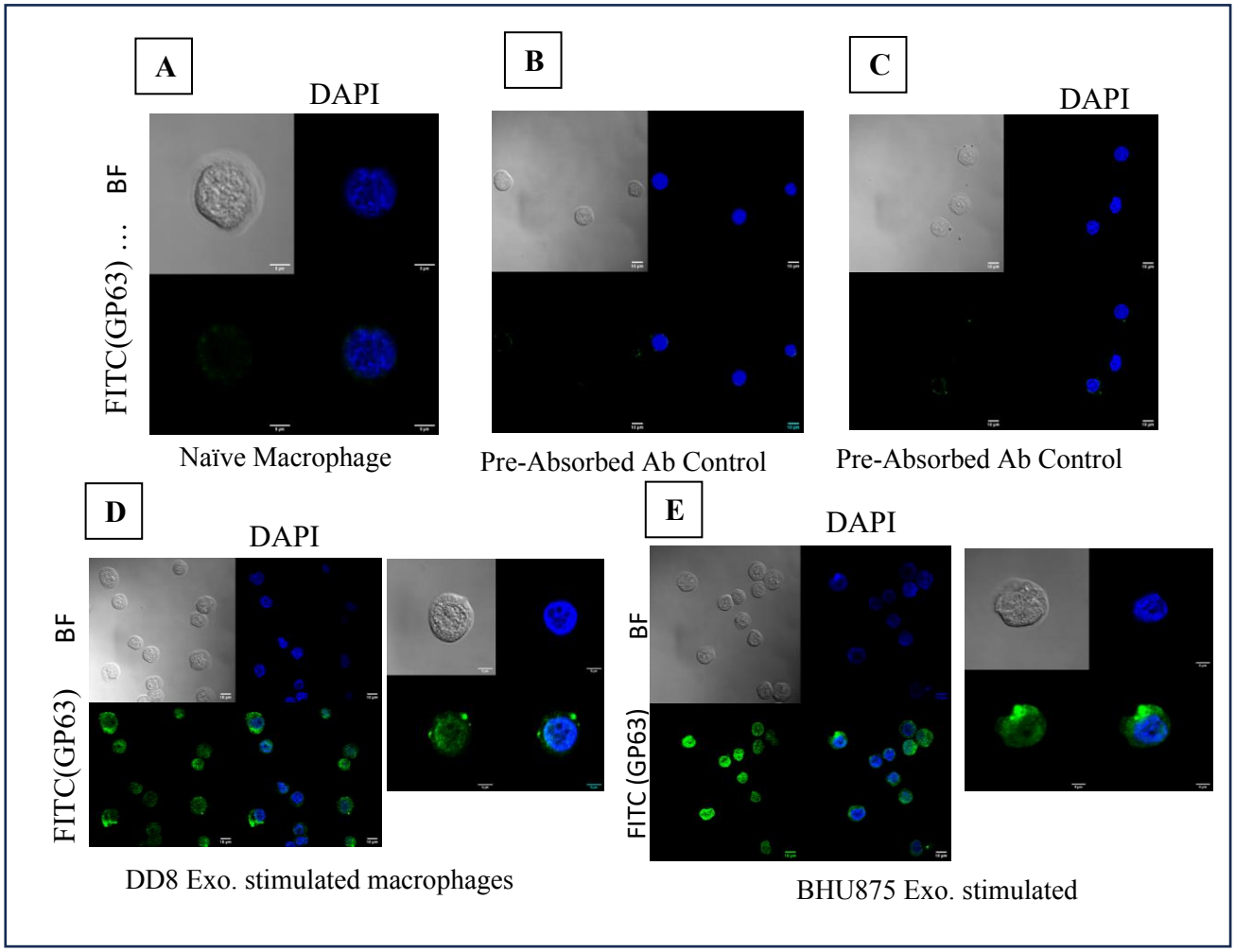


Figure: 5.15 Sho□s the macrophage □THP-1□ upta□e of *Leishmania* exosomes. The immunofluorescence image showing the naive macrophage control (**Fig. 5.15 A**) and images (**Fig. 5.15 B □ C**) show the pre-absorbed antibody control of sensitive Vs resistant parasites, respectively. The confocal images of (**Fig. 5.15 D □ E**) show the fluorescence of anti-GP63 labelled exosomes of *L. donovani* sensitive DD8 and resistant BHU875 strains inside the macrophage cells labelled with the nuclear stain DAPI.

5.16 *Leishmania* promastigote-derived exosomes a cargo carrier to the host macrophage

In corroboration with our confocal microscopy-based uptake study, we analyzed the flow cytometry data as supporting evidence, demonstrating that the exosomes were uptake by host macrophages and carried out with the *Leishmania* exosomes-specific marker anti-GP63 antibody. The FSC and SSC populations were gated on unstained control, doublets were eliminated, and singlets were chosen (**Fig. 5.16 A**□ The overlay histogram was shown with

different groups representing a shift of the peaks (**Fig. 5.16 B**). The background noise of the secondary antibody was eliminated by pre-absorbed antibody control. The uptake of exosomes by macrophages stimulated with DD8 (MH0M/IN/80/DD8) sensitive strain and BHU875 resistant strain (p-value 0.0018) (**Fig. 5.16 C**), and it was confirmed with FITC fluorescence of GP63 protein cargo inside the cells and exosomes are the cargo carrier. Our result suggests that the delivery is probably through receptor-mediated endocytosis because the macrophage expresses the ligand for the identification of the parasite-specific molecular patterns, especially GP63, which is a transmembrane protein that acts as a ligand for the endocytosis of the exosomes (J. M. Axwell Silverman Reiner, 2011).

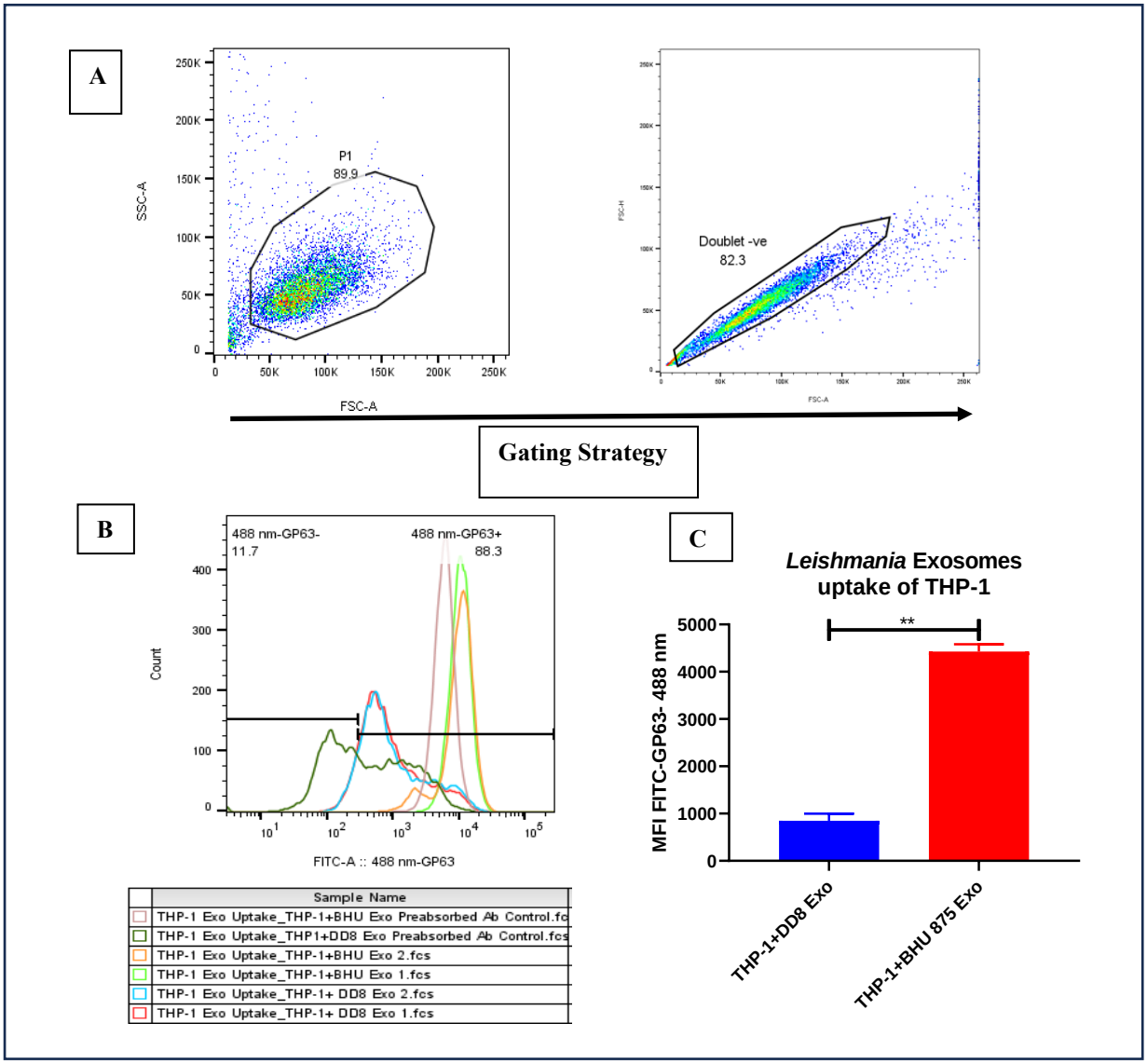


Figure: 5.16 Flow cytometry analysis of macrophage uptake of *Leishmania* Exosomes by human macrophage (THP-1) with specific *Leishmania* exosome marker anti-GP63. The

dot plots show the FSC and SSC population and doublet elimination, respectively (**Fig. 5.16 A**). The overlay histogram of different groups indicated the shift of GP63 positive THP-1 macrophages due to the uptake of sensitive DD8 and resistant BHU875 parasites exosomes (**Fig. 5.16 B**). The bar graph represents the MFI of GP63-FITC-488nm. It shows the pre-absorbed antibody control subtracted DD8 (MH0M/IN/80/DD8) sensitive and BHU875 resistant strain GP63 positive macrophage with green punctate structures of *Leishmania* exosomes (**Fig. 5.16 C**). The representative data of independent experiments (n□2) with mean □SD. The resistant BHU875 p-value □0.0018 and indicated with □□p □0.01 as compared with DD8 sensitive strain.

5.17 *Leishmania* promastigote-derived exosome stimulation induces the phagocytic activity of host macrophage

The *Leishmania*-derived exosome stimulation delivers the parasite effector molecules to create a presumptive environment for the parasites. It also might help the parasite intake to establish a successful infection. To analyze whether the promastigote-derived exosomes can enhance the *L. donovani* parasite phagocytosis by human monocyte differentiated THP-1 macrophages. The pre-stimulation of *Leishmania* exosomes on the macrophages was for 6 hours, and then the subsequent infection of *L. donovani* promastigotes parasites was for the remaining 18 hours. Then, the Giemsa staining was done, and the parasite-infected and uninfected macrophages were observed. After that, the intracellular parasites were counted through a trinocular light microscope (Leica). The phagocytic index was shown as 281.53 □48.75 and 161.88 □44.16 for sensitive DD8 and resistant BHU875 infection, respectively, without prior exosome stimulation in THP-1. Prior exosome (50□g) stimulation for 6 hours and subsequent parasite infection show the phagocytic index of 354.2 □0.69 and 535 □32.37 for the DD8 and BHU875 parasite, respectively. Interestingly, the BHU875-resistant exosome stimulation and parasite challenge increase the phagocytic index significantly with a p-value □0.0106 (**Fig. 5.17 E**). Overall, the *Leishmania*-derived exosome promotes macrophage phagocytic activity, and it helps the parasite evade the macrophages without any hindrance from the host immune defence.

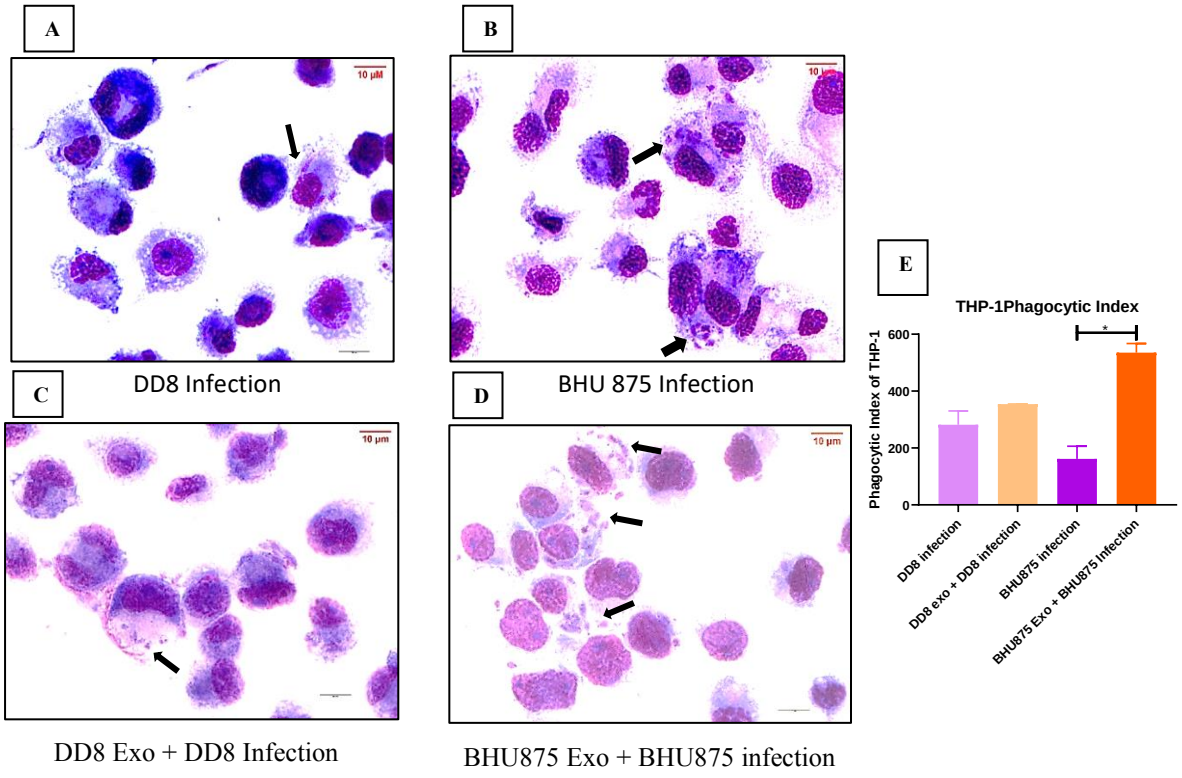


Figure: 5.17 Giemsa-Stained microscopic study of *Leishmania* derived exosomes stimulated and unstimulated human macrophage THP-1 ith parasite challenge. The human monocyte differentiated THP-1 cell line was stimulated with sensitive DD8 and resistant BHU875 *Leishmania* derived exosomes (50 g), then the respective parasite strain infection in the MOI ratio of 10:1 parasite to macrophage. Then, it was stained with Giemsa stain and counted in a trinocular light microscope with 100X magnification. The phagocytosed promastigotes were indicated with an arrow inside the macrophages. The representative images of post-infected macrophages of both stimulated and unstimulated exosomes of parasites from both strains (**Fig. 5.17 A B C D**). The representative microscopic image shows the post-infected macrophages with sensitive DD8 and resistant BHU875 without any prior exosome stimulation (**Fig. 5.17 A B**). microscopic image shows the post-infected macrophages with DD8 and BHU875 with prior stimulation of *Leishmania* derived exosomes (**Fig. 5.17 C D**). The bar diagram represents the calculated phagocytic index of macrophages with different groups, and a significant difference was observed in the BHU875 exosomes stimulation and parasite challenge with p-value 0.0106 and indicated with p 0.05 (**Fig. 5.17 E**) of independent experiments with mean SD.

5.18 *Leishmania*-derived exosome stimulation to the macrophages intensifies the infectivity of *L. donovani* parasites

Our results reveal that the phagocytic activity of the *Leishmania* parasites by macrophages increased upon exosome stimulation of the parasites. Next, we investigated the infectivity of the parasite upon exosome stimulation prior to the infection. The CFSE-based infection intensity (MFI) is a better method to measure the percentage of infection, and previous studies used this method to calculate the infectivity of *Leishmania*. Briefly, the *Leishmania* promastigotes were labelled with CSFE viable dye, then infected the macrophages, quantified the fluorescence intensity of the CFSE dye and correlated it with the infectivity (Viana et al., 2018). The infectivity was measured as a percentage of CFSE-positive cells within the total THP-1 macrophages, and the dot plot indicated the total population of macrophage gate and the representative gating strategy for CFSE-positive and negative population of both strains of parasite infection (**Fig. 5.18 A □ B**). The infection intensity was measured after 24 hours of infection with CFSE labelled *L. donovani* promastigotes. Our results show that both strains of *Leishmania*-derived exosome stimulation increase the infectivity. The CFSE+ THP-1 macrophage infection percentage with sensitive (DD8) parasites without exosome stimulation was 46.10%, and with exosome-stimulated infection for sensitive parasites was 54.80% with p-value □0.0019 (**Fig. 5.18 C**). The percentage of infectivity in the BHU875-resistant strain was 56.98% and 64.85 % unstimulated and stimulated, respectively, with p-value □0.0102 (**Fig. 5.18 D**). overall, our results found that the *Leishmania*-derived exosomes create a pro-parasitic environment that promotes the infectivity and survival of the parasites.

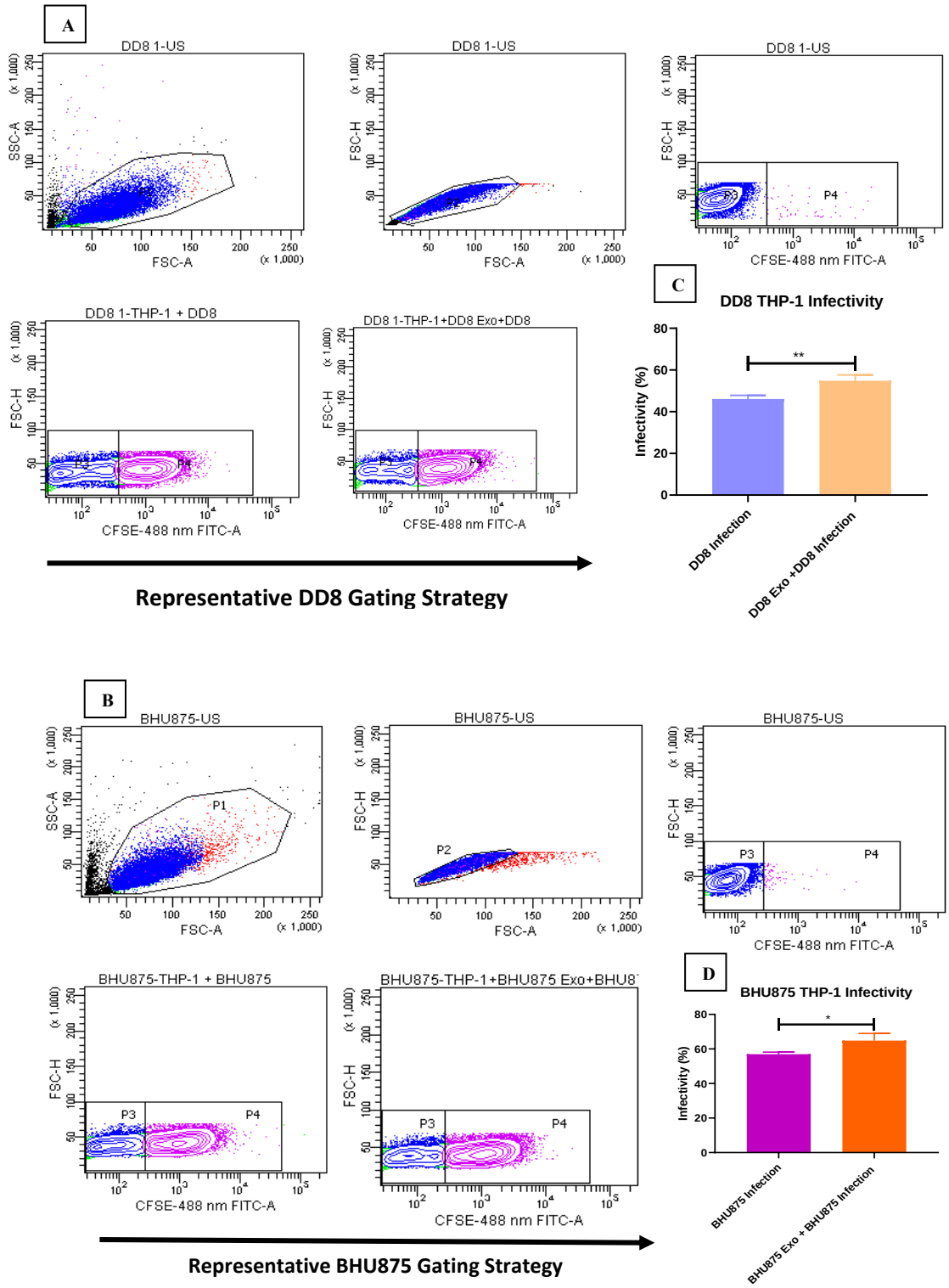


Figure: 5.18 CFSE dye-based *L. donovani* promastigote parasites THP-1 infectivity upon *Leishmania* exosomes stimulation. The macrophage cell line THP-1 was stimulated with *Leishmania* exosomes (50□g) for 6 hours and then challenged with CFSE-stained *L. donovani* promastigotes for 24 hours. Afterwards, the uninfected parasites were washed out and analyzed

in flow cytometry. The dot plots represent the gating strategy with the percentage of THP-1 infectivity with DD8 and BHU875 infection alone, and THP-1 stimulated with *Leishmania* exosomes prior and then infection with both strains of parasites. (**Figure 5.18 A □ B**□The bar graphs represent the percentage of THP-1 infection upon stimulation and without stimulation of exosomes of sensitive DD8 and resistant BHU875 strain of parasites. The results show that *Leishmania* exosome stimulation significantly increases the infectivity of both parasite strains (**Figure 5.18 C □ D**). The data depicts the percentage of THP-1 infectivity of independent (n□2) experiments with mean □ SD. The p-value □0.0019 for DD8 was indicated with □□p□0.01, and the p-value □0.0102 for BHU875 was indicated as □ p□0.05 compared to controls. CFSE dye is excited at 488nm, and we acquired 10000 events.

5.19 Reactive oxygen species □R□S□depletion by *Leishmania* exosome stimulation to avoid the detrimental side effects and create a presumptive environment for the infection

ROS and NO are the two primary microbial killing agents of host defence. In the case of *Leishmania* infection, ROS is negatively regulated by using parasite-specific secretory effector molecules, and one such molecule is *Leishmania* metallopeptidase GP63 (Isnard et al., 2012). The generation of ROS is strongly linked to the metabolic alterations of the host. For example, immediately after the phagocytosis of the pathogen, the respiratory burst and generation of ROS by NADPH oxidase, Electron Transport Chain (ETC). The metabolic effector response of glycolysis fuels ROS production and is a pattern of classically activated M1 polarized macrophages (Ganeshan □ Chawla, 2014). ROS production is induced earlier than NO, so ROS plays a significant role in the functional plasticity of the macrophages, and it is observed that the elevated level of ROS during M1 polarization substantially increases the pro-inflammatory cytokines (Kelly & O'Neill, 2015; Kieler et al., 2021; West et al., 2011). So, we investigated the *Leishmania*-derived exosomes and the role of ROS in the metabolic alterations of the host macrophage plasticity of function and phenotype. Our result suggests that the *Leishmania*-derived exosome-mediated metabolic alteration protects the parasite from the early innate response as a major prevention of ROS generation. We found no significant change in intracellular ROS during exosome stimulation of both parasite strains (**Fig. 5.19 B**). It indicates that the pattern of macrophage function and phenotype are more towards the alternatively activated M2 polarization. In contrast, the LPS-induced macrophages showed

significant levels of ROS production (p-value □0.0362), as the previously published reports (**Fig**. **Fig. 5.20 B**)

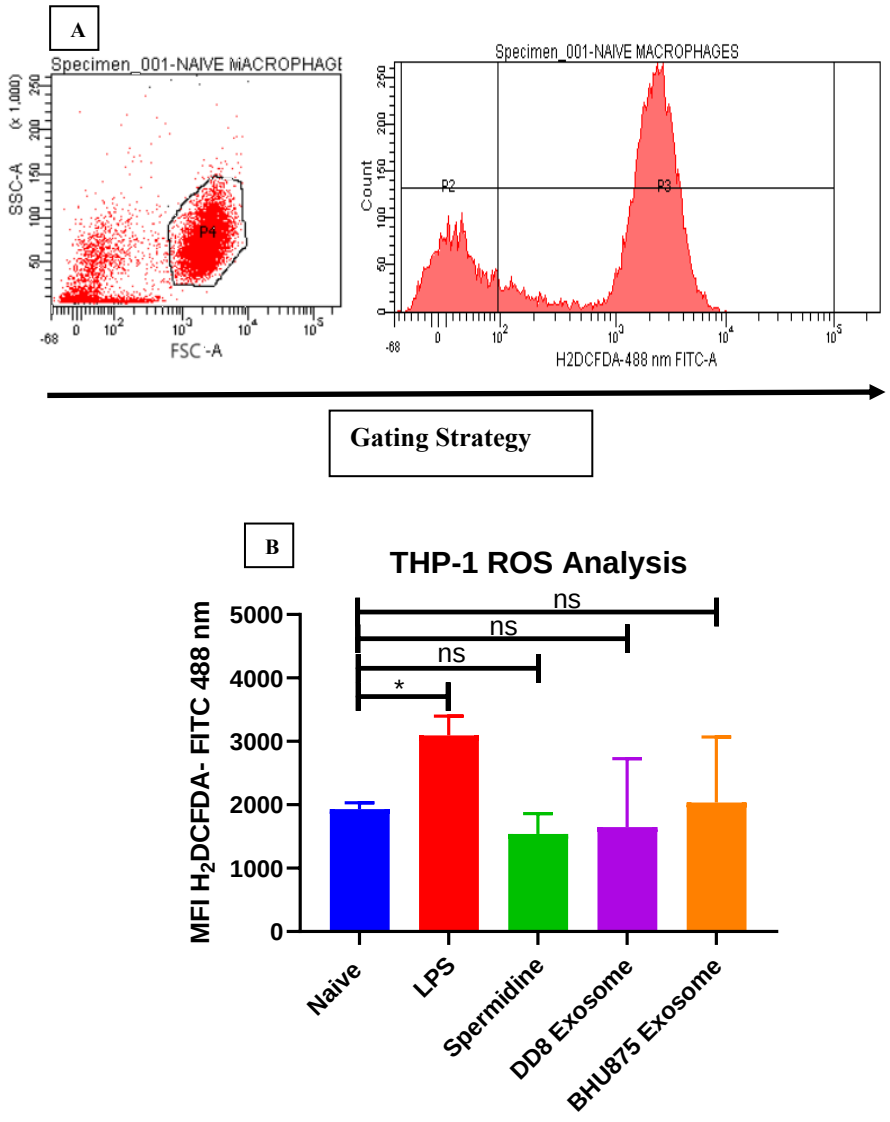


Figure: 5.19 Intracellular R□S analysis □ith H_2DCFDA upon *Leishmania* derived exosome stimulation by flo□ cytometry. The *Leishmania*-derived exosome of both strains, such as sensitive DD8 and resistant BHU875 (50□g) stimulated for 6 hours, was measured for generation of intracellular ROS in a fold change relative to the controls. The dot plot and histogram show the gating strategy of macrophages (10,000 events) (**Fig. 5.19 A**). The barograph shows the fold change of ROS in different experimental groups, and it did not show any significant difference in the stimulation of both strains of parasite exosomes (**Fig. 5.19 C**). The LPS stimulation leads to a significant difference (p-value □0.0362) and □p □0.05 (**Fig. 5.19**

C). The represented mean □ SD of independent experiments (n□2). H_2DCFDA□FITC excitation/emission range, i.e., 525/495nm, we used FITC 488nm laser.

5.20 Impaired nitric oxide □N□□production in the M2 polari□ation of the macrophage from the perspective *Leishmania* derived exosome stimulation

The innate immune effector molecule-mediated killing of the parasite is the major immune defence mechanism of the host in *Leishmania* infection; NO plays a major deciding molecule in the killing of the parasite (Carneiro et al., 2016). NO is the molecule that connects the immune system with metabolism, first presented by Drapier et al. (Drapier □ Hibbs, 1988). Then, the arginine-derived NO-mediated distinct metabolic phenotype macrophages were studied in detail. Another study shows that the lower level of NO in the VL patients' plasma and, in contrast, the elevated arginase activity strongly correlates with the metabolic rewiring of host macrophages during infection (Kupani et al., 2021). In this study, we checked the level of NO upon *Leishmania*-derived exosome stimulation of the human monocyte differentiated macrophage (THP-1). We discovered that the expression of the iNOS did not change significantly while stimulated with both strains of parasite-derived exosomes (**Fig. 5.20 A**). In continuous with this observation, we found that there are no significant shifts in the NO levels in the exosomes stimulated (50□g) macrophage supernatant (**Fig. 5.20 B**□, and a substantial change of NO level was observed in the LPS (100 ng/ml) stimulated control (p-value □0.0407) (**Fig. 5.20 B**). In general, our result indicates that the NO secretion is strongly impaired by the stimulation of *Leishmania*-derived exosomes of both strains of parasites, and it suggests that the metabolic alteration of arginine metabolism is the hallmark of *Leishmania* exosomes. This result supports the arginine dichotomy by a parasite and its effector molecules. Our result also suggests that this might lead to the activation of arginase and polyamine synthesis for the parasite survival by the alternative activation of the highly dynamic macrophage.

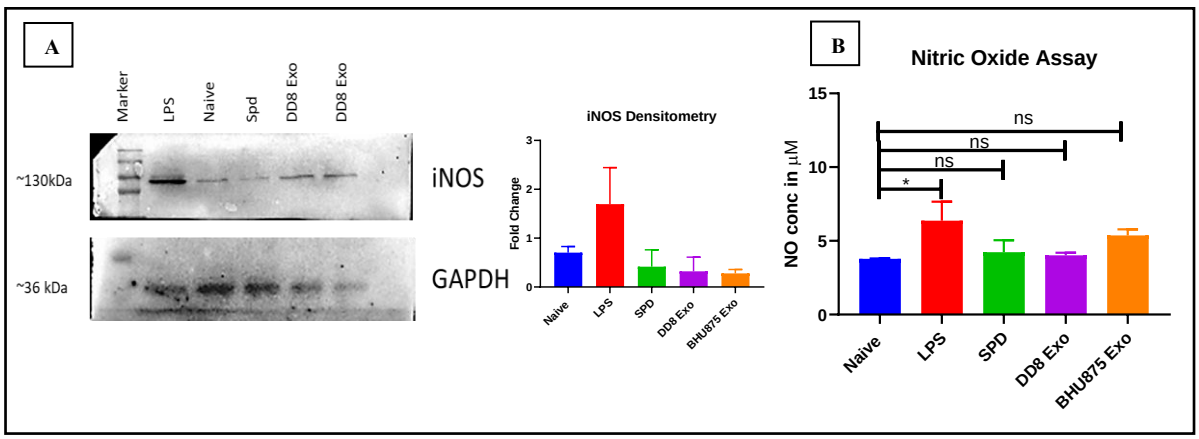
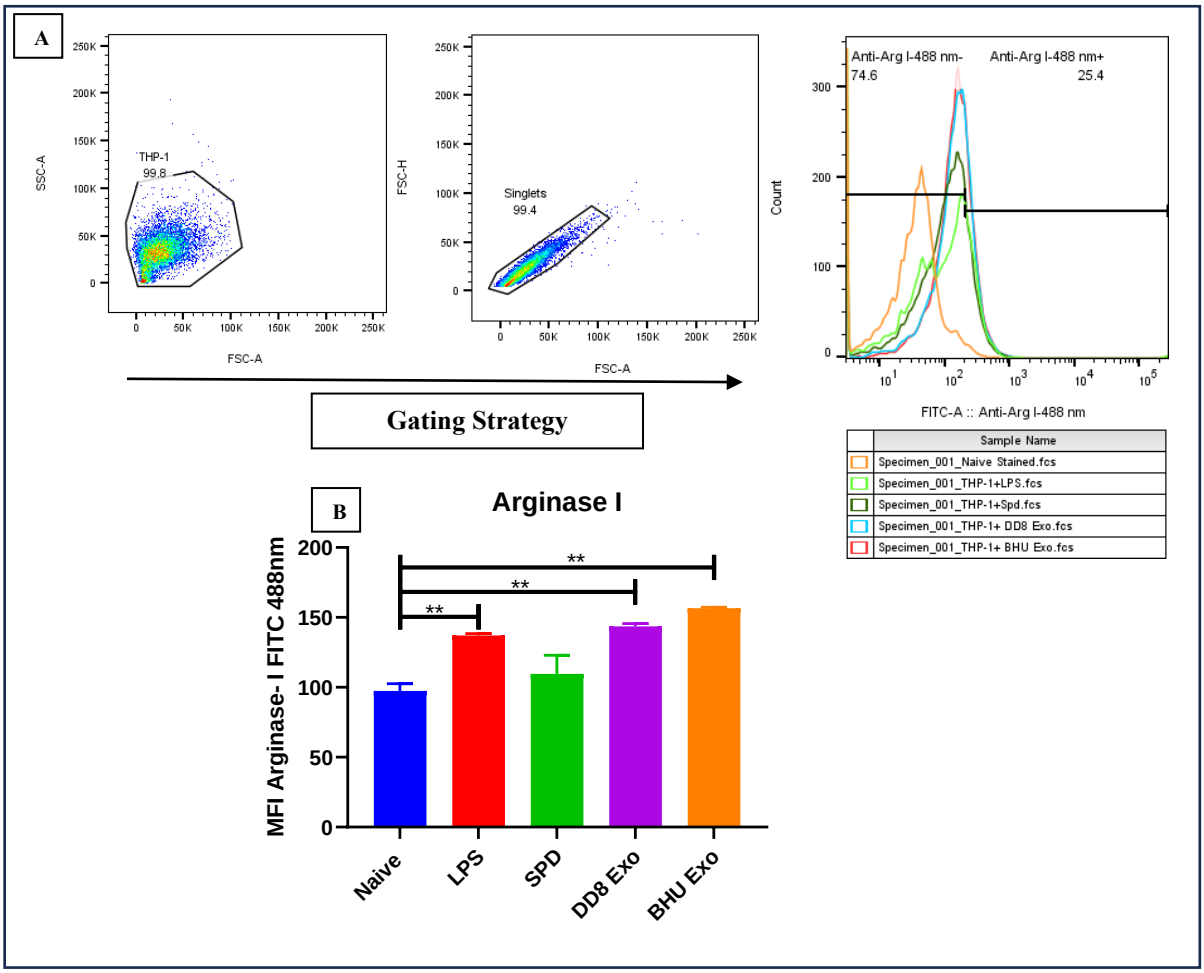


Figure: 5.20 The expression of iN□S and N□ production upon *Leishmania*-derived exosome stimulation. The western blot and its densitometric analysis show that upon stimulation of *L. donovani* exosomes sensitive DD8 and resistant BHU875 (50□g) for 12 hours did not show any significant change in the expression of iNOS as compared to the controls (**Fig. 5.20 A**). The same was reflected in the NO (□M) production in the human THP-1 cell line supernatant and showed no significant difference in the NO production during *Leishmania* exosome stimulation (**Fig. 5.20 B**). the LPS stimulation induces significant levels of NO production in the human THP-1 cell line. (**Fig. 5.20 B**). The data analysis with one-way analysis of variance of independent experiments (n□2) is represented as mean □ SD with p-value □0.0407 and indicated with □p □0.05.

5.21 *L. donovani* exosome-stimulated macrophages sho□ a higher level of Arginase I expression: an innate immune metabolic chec□point that drives polyamine biosynthesis

Arginine is a common substrate for the enzymes iNOS and ARG1; the reciprocal modulative activity of ARG 1 and iNOS decides the fate of macrophage activation (Modolell M and Munder M, Eichmann K,1950). The pattern of M2 polarization and its effector function is majorly dependent on the arginine to polyamine conversion by ARG1. Furthermore, an isotype tracing of arginine has proven that it acts as a major substrate for polyamines (spermidine and putrescine) (Miller-Fleming et al., 2015). Many pathogens exploit the arginase-mediated blunt of NO production to escape from the host's immune defence. It either induces the arginase expression in their genome or the pathogen co-opts macrophage arginase I expression and activity (El Kasmi et al., 2008; Gobert et al., 2001; Monin et al., 2015). The flow cytometry-based MFI, and western blot analysis of our study observed that the *L. donovani* exosome-stimulated macrophages show a higher level of Arginase I expression, showing the initiation of the polyamine pathway. Both strains (DD8 and BHU875) of parasite-derived exosomes show significant changes in the Mean Fluorescence Intensity (MFI) of arginase I in the macrophages (p-value □0.0072 for DD8 and p-value □0.0039 for BHU875) (**Fig. 21 B**). It also indicates a higher expression in the western blot and its densitometric analysis (**Fig. 21 C**). Surprisingly, we observed that the LPS (100ng/ml) shows a higher expression of arginase I (p-value □0.0090) (**Fig. 21 B □ C**). Reports indicate that stimulating LPS/IFN-γ or the bacteria-challenged macrophage induces the arginase I expression (Zhang et al., 2019). Another study demonstrated that the arginase I expression is dose- and time-dependent. While stimulated with LPS, the arginase I expression is significantly higher 24 hours post-stimulation of 20ng or

200ng/ml LPS. In contrast, the 2000ng/ml LPS stimulation does not show any significant changes in the expression of arginase I expression (Menzies et al., 2010).



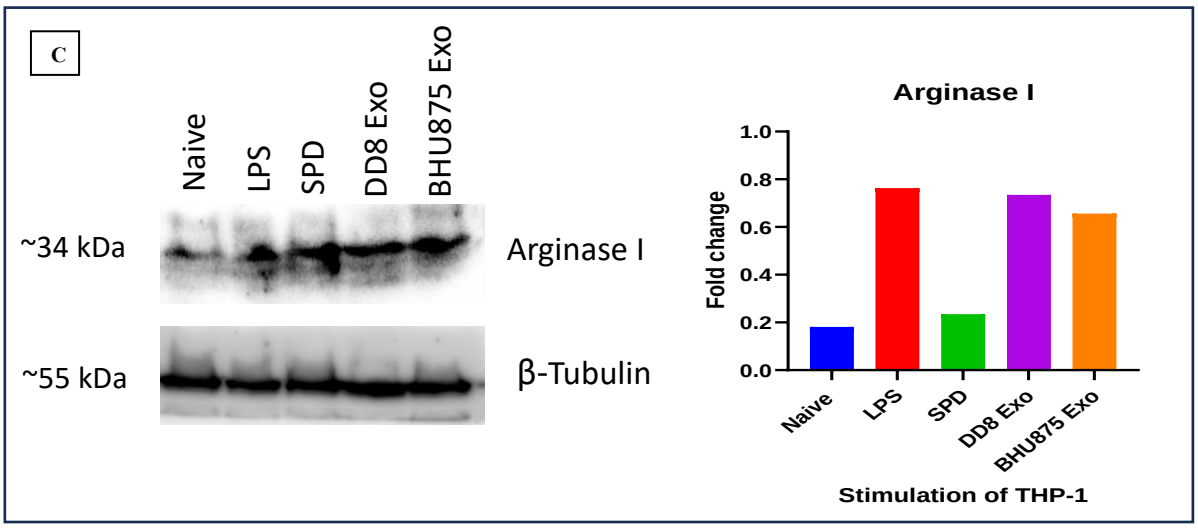


Figure: 5.21 *L. donovani* derived exosome stimulation to the host macrophage cell line THP-1 induces the arginase I expression. Shows the flow cytometry-based MFI of arginase I expression with anti-arginase I antibody (1:200 dilution). The first figure represents a dot plot and overlay histogram with different groups and their MFI (**Fig. 21 A** The stimulation of *L. donovani* exosomes from sensitive DD8 and resistant BHU875 were stimulated (50 g) with a human macrophage cell line (THP-1) and checked the expression of the arginase I. Both strains of parasite exosome along with LPS (100ng/ml) show a significant difference of expression with p-value 0.0090, p-value 0.0072 and p-value 0.0039 for LPS, DD8 and BHU875 exosomes stimulation depict with p 0.01 (**Fig. 21 B**) and the data of independent experiments (n 2) with representation of mean SD. The western blot and its densitometric analysis of *L. donovani* exosomes stimulated macrophage lysate by the anti-Arginase I (alternatively activated macrophage marker) antibody for confirming *Leishmania* exosomes mediated macrophage M2 polarization. The *Leishmania* exosome stimulation from both strains of parasite shows a trend of increment in the expression arginase I, β-tubulin act as a housekeeping control (**Fig. 21 C**)

5.22 *L. donovani*-derived exosomes stimulated macrophage metabolic adaptation and reciprocal regulation of iN S and ARG I

Arginase I is an alternative activated immune response marker gene, and more than that, its effector function plays a major role in the pathophysiology of various infections (Kieler et al., 2021). The arginase I activity drives polyamine biosynthesis and is involved in multiple downstream cell-intrinsic signalling pathways of the cell (Puleston et al., 2019). So, here we investigated the arginine dichotomy and its reciprocal regulation by iNOS-mediated NO production and ARG I activity. We found that naive macrophage's arginase I enzyme activity without exosome stimulation was 28.74 mU/mg protein. For sensitive parasite-derived exosomes stimulated macrophages, it was 33.39 mU/mg protein (p-value 0.0278) (**Fig. 22 A**). The case of resistant BHU875 parasite-derived exosome stimulation of macrophages shows a significant arginase I activity with 35.36 mU/mg protein compared to the naive macrophages without exosome stimulation (p-value 0.0130) (**Fig. 22 A**). The arginase activity increases during the *Leishmania* exosome stimulation condition, so there might be a hunger game to win for the host macrophage to produce NO. We have done a correlation analysis with the arginase I activity Vs NO production upon *Leishmania*-derived exosome stimulation, and we found a robust negative correlation with an r-value of -0.2893 (**Fig**. **22 B**). Our results from this study

confirmed a significant increment of arginase I activity during exosome stimulation, whereas the nitric oxide level showed a reduction. The result indicates that the exosome stimulation might drive the polyamine pathway instead of the iNOS pathway to create a pro-parasitic niche inside the host macrophage for better survival and proliferation of the parasite.

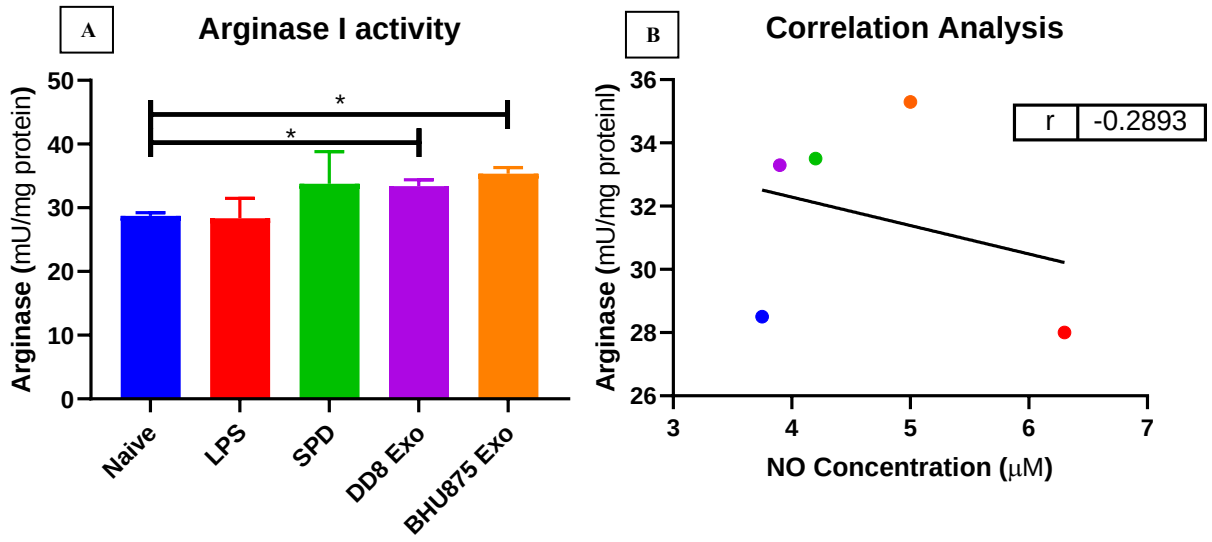


Figure: 5.22 The correlation analysis of arginase I en□yme activity □s N□ production upon *L. donovani* exosomes stimulation. The indirect arginase I enzyme activity (one unit of Arg I activity equal to the amount of enzyme-catalyzed to produce one □mol of urea) of the human macrophage cell line (THP-1) was measured with stimulation of parasite exosomes (50□g) from sensitive DD8 and resistant BHU875 parasites. Both strains of parasite exosome stimulation for 24 hours show a significant difference with p-value □0.278 and p-value □0.0130 for DD8 and BHU875, respectively. It represented as □p□0.05, and there are no changes in the naive macrophage and LPS (100ng/ml) control (**Fig. 5.22 A**). The increased level of arginase I activity is reflected in the production of NO, and it is observed that the arginase I activity Vs NO production correlation shows a robust negative correlation with r value -0.2893 (**Fig. 5.22 B**). The data of independent experiments (n□2) with mean □SD.

5.23 *L. donovani* derived exosome ensure the intracellular levels of polyamine in the host polyamine pool

The *Leishmania* parasite survival inside the host macrophage is strongly dependent on polyamine biosynthesis, particularly spermidine (SPD) because it is the end product of the

Leishmania polyamine biosynthesis pathway. Moreover, spermidine has a multifaceted role in the parasite survival inside the phagolysosome niche. For example, it serves as a precursor for trypanothione, a free radical scavenging molecule of the parasite during the macrophage infection. The spermidine uptake and *de novo* synthesis are needed for parasite survival and virulence maintenance (Mamani-Huanca et al., 2021). In the current study, we checked the intracellular spermidine level by spermidine antibody-based MFI calculation in the flow cytometry, and the dot plot represents the gating strategy (**Fig.5.23 A**). We found that the intracellular spermidine was significantly elevated in the *Leishmania*-derived exosomes from both strains of parasites while stimulating the macrophage as compared to the naive macrophage, which does not have any exosome stimulation with p-value □0.0473 for DD8 and p-value □0.0340 for BHU875 (**Fig. 5.23 B**). Interestingly, we observed that the stimulation of macrophages with recombinant IL-10 also significantly increases the intracellular spermidine levels in the host macrophages with p-value □0.0124 (**Fig. 5.23 B**). It is important to note that the polyamines reduce the NO production and shift the macrophage effector functions more towards an M2 polarized state, and our result of intracellular spermidine levels emphasizes the fact that macrophages are transporting the polyamine or it is actively inducing the intracellular polyamine pathway. However, the case of a higher level of polyamines enhances the macrophage to go in an alternative activation by subsides NO and favours the parasite's favourable condition. A recent study reported that at the time of *L. donovani* infection, the L-arginine transporter expression was upregulated, leading to a metabolic alteration of arginine metabolism and elevated polyamine levels, leading to an M2 polarization state (Mandal et al., 2017). So, we checked the polyamine transporter gene expression, that is, Slc3a2 of macrophage, and we observed that the expression is higher during the stimulation of *L. donovani* exosomes stimulation of DD8 with p-value □0.0115 and BHU875 shows a higher trend of increment of expression of the transporter (**Fig**. **5.23 C**) overall, the *L. donovani* derived exosome stimulation induced the intrinsic polyamine biosynthesis pathway. It also enhances the polyamine transporter expression to ensure enough polyamine supplementation for the parasite's survival inside the nutrient or metabolite-deplete environment of the phagolysosome compartment.

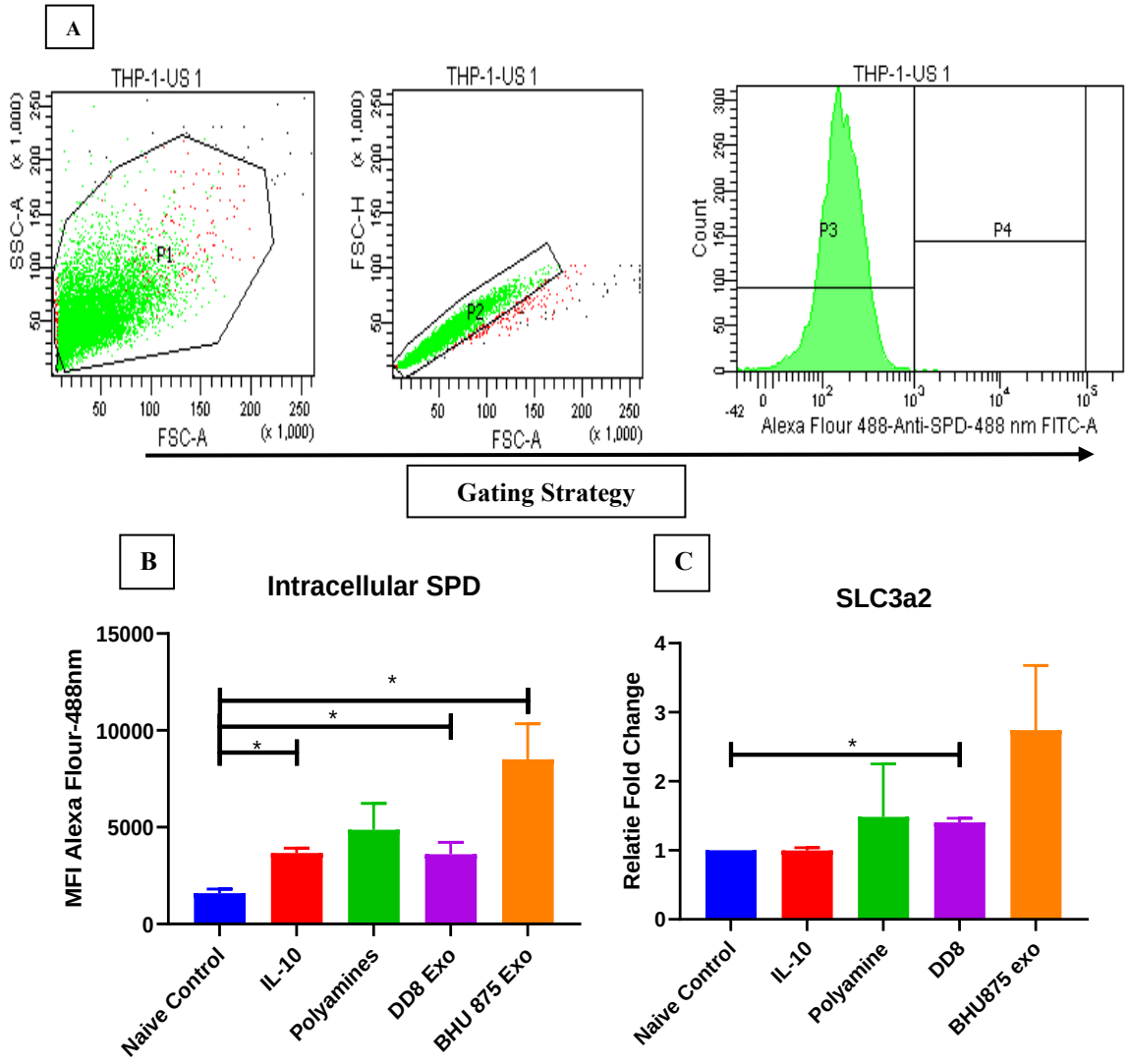


Figure: 23 The *L. donovani*-derived exosome contributes to the intracellular polyamine pool. Both strains of parasite that are sensitive as well as the resistant *Leishmania* exosome (50 □g) stimulation to host macrophage (THP-1) shows elevated levels of intracellular spermidine level and is measured by the detection of FITC fluorescence (MFI) of anti-spermidine antibody-based quantification. The MFI of spermidine was increased upon *Leishmania*-derived exosome treatment as well as recombinant IL-10 stimulation (20□M) with p-value □0.0124, p-value □0.0473, and p-value □0.0340 for IL-10, DD8 and BHU875 exosomes stimulation, respectively. The significance is represented with □p□0.05 (**Fig. 5.23 A**). The polyamine pool replenishment by the induction of the expression of polyamine transporter of the host by the *Leishmania* exosomes, the DD8 exosomes show significant difference of expression with p-value □0.0115 and represented as □p □0.05 and BHU875 show a higher trend of increment in

the expression of the transporter (Slc3a2) (**Fig. 5.23 B**). Data of independent experiment (n□2) and mean □SD.

5.24 Anti-Inflammatory Milieu by *L. donovani* derived Exosomes: Polari□ation of Macrophages from M1 to M2

The elevated levels of IL-10 and TGF- β in the clinical VL might play a major role in the pathogenesis than other cytokines, which regulate the arginase activity in various immune cells (Kupani M et al., 2021). The pathogen-induced autocrine cytokines of IL-10 and TGF- β mediate the induction of ARG 1 (Qualls et al.,2010). This study assumed that the *L. donovani*-derived exosomes might stimulate the autocrine production of cytokines TGF-β and IL-10. In turn, it induces the arginase expression as well as the activity. We found an increment of expression of these cytokines in the exosomes-stimulated macrophages (**Fig. 5.24**). Interestingly, it strongly corroborated with the results that we observed in the NO production upon exosome stimulation (**Fig. 5.20**). The *Leishmania* infection induces IL-10 and TGF- β cytokine that contributes to higher levels of arginase activity and elevated levels of NO production (Kupani et al., 2021; Mondanelli et al., 2017). Our correlation analysis also emphasizes that the higher activity of arginase I negatively correlates with NO production (**Fig. 5.22 B**). So, it is clear that the autocrine secretion of the IL-10 and TGF- β might enhance the arginase expression and activity, reducing or maintaining the basal levels of NO during *Leishmania*-derived exosome stimulation to the macrophage. On the other hand, our results interpret that the *Leishmania* exosomes of both strains significantly reduce the expression of iNOS and IFN-γ of macrophage with p-value □ 0.0001 and p-value □ 0.0006 for DD8 and BHU875 strains, respectively (**Fig. 5.24 A**). The clinical study on VL patients demonstrates that IL-10 and TGF- β are the major cytokines that blunt the activity of the IFN-γ (Caldas et al., 2005). This study correlated to our observation that the IFN-γ is not fully capable of exerting its action in the case of *Leishmania*-derived exosomes stimulation; further, it induces the counter-regulatory IL-10 that blunts the activity of the IFN-γ during persistent *Leishmania* exosomes stimulation (**Fig. 5.24 B**). Our results confirmed the crosstalk of IL-10 and TGF-β in arginase-mediated suppression of NO production. Interestingly, the reduction in the expression of iNOS and IFN-γ proved that the *Leishmania*-derived exosomes actively drive the polyamine pathway instead of iNOS pathway-mediated NO production. Furthermore, it induces the autocrine production of anti-inflammatory TGF-β and regulatory IL-10 cytokines upon exosome stimulation (**Fig. 5.24 C □ D**). These immunometabolism pathway switching

by *Leishmania*-derived exosomes create a pro-parasitic environment for the infection establishment as well as the sustained persistence of the parasite inside the nutrient or metabolite-depleted microenvironment of the host phagolysosomes.

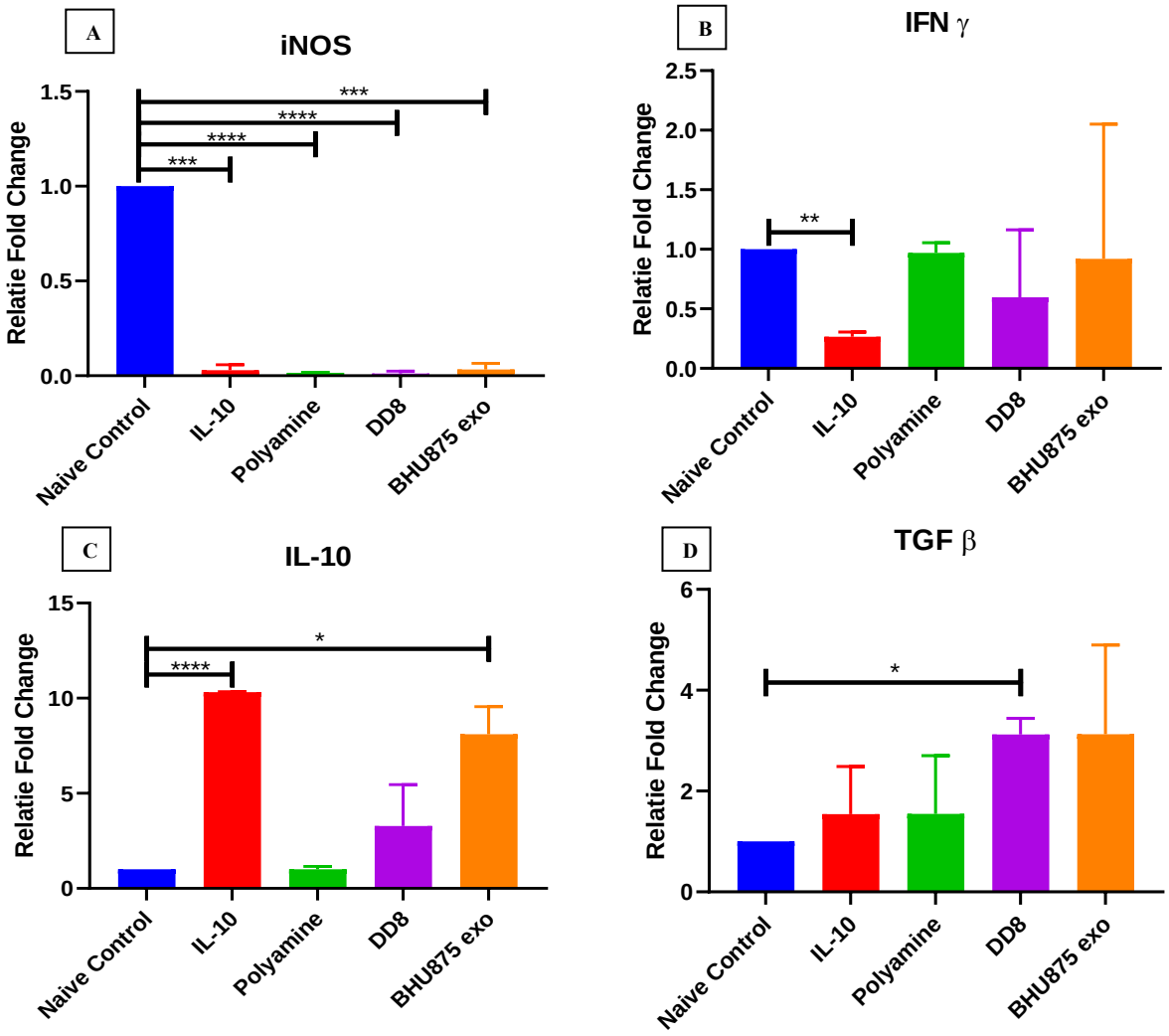


Figure: 5.24 M2 polari□ation state of macrophage cell line □THP-1□mediated by anti-inflammatory and regulatory cyto□ines and macrophage reduction of iN□S expression. The *L. donvani* exosomes stimulation (50□g) creates a pattern of alternatively activated M2 polarization state of macrophage by reducing the expression of iNOS (**Fig. 5.24 B**), and it did not change the expression of pro-inflammatory cytokine IFN-γ that initiates the M1 mediated immune response against the pathogen (**Fig. 5.24 A**). In contrast, the stimulation of parasite exosome creates a milieu of regulatory and anti-inflammatory cytokines such as IL-10 and TGF-β by elevating its expression levels (**Fig. 5.24 C □ D**). the data of independent expression (n□2) with p-values depicted in the plot indicates □p□0.05, □□p □0.01 and □□□p □0.001 and □□□□p □0.0001 respectively with mean □SD

Chapter: 6
Discussion

Extracellular vesicles (EVs) are nanosized membrane-bound vesicles produced by almost all the living kingdoms of organisms. EVs originated as endocytic or plasma membrane blebs and are involved in intercellular communication (Dong et al., 2021). EVs are categorized based on their origin or location of cellular provenance, morphology, and size; EVs shed from plasma membranes are exosomes or microvesicles, whereas endocytic origin is known as exosomes (Tkach Théry, 2016). Despite all the above features and information, scientific technologies still need to distinguish the types of EVs. In the literature, the exosomes are referred to as a small heterogenous population of EVs with sizes ranging from 30-150 nm (Dong et al., 2019). *Leishmania* produces exosomes through the exosome endocytic pathway retained in evolution, and it creates a suitable niche for infection by enrichment of parasites with virulence factors in the early infection and exacerbations of disease (Atayde et al., 2015; da Silva Lira Filho et al., 2022). Scanning Electron Microscope (SEM) of our study shows that the stationary phase of *L. donovani* promastigotes grows in the axenic conditions that mimic sandfly gut release extracellular vesicles to the extracellular space (**Fig. 5.1 5. 2**), and the physical characterization confirms that these vesicles are heterogenous population EVs with ~ 42.75 nm in size with average percentage intensity of 62.25% (**Fig. 5.5 A B**) same as reflected in the TEM analysis predicted size of 50-70 nm in diameter (**Fig. 5.5 C D**). studies show that the small EVs cargos of many parasites have the essential components of their life cycle that help in the survival. The transmission of virulence factors, adhesion to host cells and subsequent evasion. After that, immunological responses in host cells were initiated (Ofir-Birin Regev-Rudzki, 2019; Whitehead et al., 2020). The *Leishmania* metallopeptidase GP63 enrichment in the vesicles of the parasites, as well as the intra-macrophage vesicle clustering, confirms the secretion of the EVs or exosomes of the *Leishmania.* Then, it was demonstrated that the *Leishmania* exosome proteins were secreted non-conventionally, and they carry many virulence factors that help the establishment of infection and increase survivability inside the host (Atayde et al., 2019; Gomez et al., 2009; Hassani et al., 2011; Lambertz et al., 2015; J. M. Silverman et al., 2010; J. M. Axwell Silverman Reiner, 2011). Our SDS-PAGE also shows that the *Leishmania* exosomes carry many proteins, and western blot analysis displayed *Leishmania* surface protein GP63 enriched in the exosome of the parasite. It confirms the specificity of the origin of exosomes that are from *Leishmania* by serving as a biomarker (**Fig. 5.2 A B and Fig. 5.3 B C**). This result was significant. It is supportive of evidence for the third objective of the present study, as *L. donovani* exosomes polarize the macrophages from the classical M1 to alternative M2 activation owing to the surface protease GP63, which

is a major virulence factor of *Leishmania* (Chang et al., 1990). Furthermore, a study demonstrates that the GP63 deficient exosomes alter the packaging of its protein content and show a decreased immunosuppression in the host. It emphasizes that it is a crucial virulence factor of *Leishmania* (Hassani et al., 2014). The immunoblot of the *Leishmania* exosomes with GP63 is compelling evidence that the *in vitro* isolated exosome comes from the *Leishmania* parasites and is a biomarker for *Leishmania* EVs (Atayde et al., 2015)

Here, we assess the intracellular metabolites of *Leishmania* parasite exosomes indispensable for its viability, fitness, and growth. Interestingly, our preliminary study found that the polyamines (PAs) spermidine and putrescine were enriched in the *L. donovani* exosomes (**Fig. 5.6 A □ B□Fig. 5.7 A-F and Fig. 5.8 A-C**). These polyamines are critical for parasites that impact the metabolic fitness of the parasite. It is established that the null mutants of polyamine biosynthetic enzymes compromise the growth and virulence of the parasites (Gilroy et al., 2011; Perdeh et al., 2020). The polyamines bind to the RNAs and stabilize them, and also help polyamines to form the lipid curvature through the formation of vesicle aggregation (Acosta-Andrade et al., 2017; Trachman □ Draper, 2013). Another study demonstrated that in the 22v1 cells, the secretion of EVs is mediated by spermidine synthase as a key regulator (Urabe et al., 2020). Exosomes are one of the non-conventional ways of protein secretion in *Leishmania* parasites; their cargos and packaging can be discussed in the pathophysiology of the disease. Even though there is a lack of well-defined studies on the exact mechanism of delivery of vesicles to the recipient cells, the possibility of direct dumping of packaged cargo to the plasma or phagolysosomes membrane, the effector molecules or nutrients inside the vesicles might help the intracellular parasite (J. M. Axwell Silverman □ Reiner, 2011). In host-pathogen interaction, the requirement of parasite nutrients, especially in the host micronutrient environment, is a major concern. The phagolysosomes residing in *Leishmania* parasites are auxotrophic for many of these nutrients. Moreover, the limited nutrients and metabolites in the phagolysosomes raise an existential threat to the parasite, probably because the nutrients or metabolites are delivered to phagolysosome via the fusion of endocytic or phagocytic vesicles (Saunders □ McConville, 2020). Our result of enrichment of polyamines in the *Leishmania* exosomes, along with other effector molecules packaging in the exosomes (**Fig. 5.6 A □ B□Fig. 5.7 A-F□and Fig. 5.8 A-C**), might help in the better survival of the parasites inside the host by exosome-mediated polyamine supplementation and induction of polyamine production in the host. The Deprivation Response (ADR) pathway is activated during arginine deprivation in the host arginine pool and is sensed by the macrophage-residing

Leishmania amastigotes (Goldman-Pinkovich et al., 2020). The parasites would be exploiting the polyamine pathway for their protective and efficient survival inside the harsh environment in the host macrophage at the time of infection either by the parasite-derived specific effector molecules or by the parasite EV-induced effector molecules. The parasite-encoded arginase expression is increased in the alternatively activated macrophage, a major exploitive mechanism that the parasite adapted to enhance the polyamine biosynthesis for amastigote growth (Gaur et al., 2007; Naderer McConville, 2008). So, the crosstalk between *Leishmania* exosomes, polyamines, and macrophage polarization might be important in the *Leishmania* parasite's pathophysiology. The unique polyamine biosynthetic pathway of *Leishmania* from its primary host macrophages emphasizes its immune-metabolic crosstalk with the macrophages (Colotti Ilari, 2011). For example, the Δodc auxotrophic polyamine parasites are not overcome by supplementing spermine and other diamines (Jiang et al., 1999). Interestingly, *Leishmania* parasites lack spermine synthesis from spermidine and its back-conversion of spermine to spermidine and putrescine like other mammalian cells (Carter et al., 2022). These substantial differences, along with other structural and molecular differences, open a new avenue for polyamines biosynthetic pathway enzyme-based therapeutic targets, and it circumvents the drug distance issue in leishmaniasis treatment (Abirami et al., 2023; Carter et al., 2022). Even though various studies highlight the importance of polyamines for the *Leishmania* parasite survival, very few pharmacological approaches are there to elaborate the molecular mechanism behind the polyamine biosynthetic pathway inhibition. The integrated computational and biochemical drug study shows that hypericin, a plant-derived natural compound, has anti-leishmanial activity. It depletes spermidine production by specifically inhibiting the spermidine synthase of the *L. donovani* parasite and is rescued by spermidine supplementation (S. Singh et al., 2017). However, to our knowledge, there are no studies about the molecular events during the hypericin-mediated depletion of spermidine in the drug-resistant *L. donovani* parasites (clinically isolated BHU875). Our results show that hypericin-mediated polyamine depletion heavily affects parasite growth and fitness. interestingly, the BHU875-resistant strain shows more sensitivity toward hypericin treatment (**Fig. 5.10 A B and Fig. 5.11 B E**). It indicates that the synergistic anti-leishmanial drug approach might restore the sensitivity towards the available chemotherapeutic drugs that already have drug resistance. Polyamine biosynthetic pathway is linked to the drug resistance mechanism in *Leishmania,* and the alteration in the parasite anti-oxidative pathway is a major hallmark of drug resistance (Equbal et al., 2014; Kulshrestha et al., 2014; Ponte-Sucre et al., 2017). Miltefosine is an alternative or sometimes a combinational drug in treating antimony or

amphotericin-resistant leishmaniasis, and its resistance is linked to the polyamine pathway. The study elucidates the metabolome of the miltefosine-resistant and sensitive strains, and it shows the perturbation of polyamine metabolites such as arginine, ornithine, and *S*-adenosylmethionine in sensitive strains. In contrast, the increment of these metabolites, along with spermidine, was observed in the metabolic profile. The probable mechanism is to adapt to oxidative stress (Rojo et al., 2015). Spermidine is one of the major polyamines essential for the parasite and is the final product of the *Leishmania* polyamine biosynthetic pathway (Heby et al., 2007; Reguera et al., 2009). Spermidine synthase knockout (Δspdsyn) is auxotrophic for polyamines. It is required for the insect vector form growth, and the impaired spermidine production is negatively reflected in the mice infection (Gilroy et al., 2011). The trypanosomatid parasitic specific antioxidant molecule trypanothione is synthesized using spermidine as a substrate (Colotti Ilari, 2011). Additionally, inhibition of spermidine synthase shows parasitic death; spermidine supplementation rescued the parasite. Interestingly, the hypericin-mediated spermidine synthase inhibition does not rescue the parasite, indicating the multifaceted functions of spermidine in the parasite. Furthermore, the study found that the death is due to decreased trypanothione levels, leading to ROS generation and parasite killing (S. Singh et al., 2015). In correlation to this study, our result shows that the polyamine depletion induced intracellular ROS in both strains, possibly due to the impaired polyamine metabolic activity upon hypericin treatment (**Fig. 5.12 B C**). Unexpectedly, our result of the MTT viability assay does not show any significant changes. In contrast, clear morphological alterations such as granulation, loss of motility due to flagella loss, spindle-shaped morphology, and parasite growth and fitness are heavily hampered. Moreover, growth reduction was prominent in the resistant BHU875 strain of the parasite (**Fig. 11 B E**). A study demonstrated the relationship between cell number reduction (growth inhibition) And MTT assay (metabolic viability of mitochondria) in different tumorigenic and non-tumorogenic cell lines. Briefly, the cell lines were exposed to different ionizing radiation doses at different time points, and the cell numbers and MTT indexes were correlated. The correlation analysis shows that after the 48-hour irradiation, there was a 20-30% reduction compared to un-irradiated cell lines, whereas the cell number was reduced by 70-90% upon irradiation. Along with this study, many other studies indicate that the accurate indicator of growth inhibition is the number of cells rather than metabolic viability-based MTT assay (Rai et al., 2018; Stepanenko Dmitrenko, 2015). It indicates that the hypericin treatment might alter the mitochondrial activity. So, we further checked the MMP and found that the MMP is towards hyperpolarization (**Fig. 5.14 C D**). Subsequently, we checked the membrane integrity upon hypericin-mediated polyamine

depletion. It disrupted the membrane integrity (**Fig. 13 C □ D**), which might be due to the higher levels of ROS generation (**Fig. 5.12 B □ C**). Overall, our results emphasize that polyamines are essential metabolites for the survivability of the *Leishmania* parasites. Its starvation heavily affected both parasite strains and is more sensitive towards the resistant strain than the sensitive strain.

The growth and fitness of the parasite largely contribute to the establishment of successful infection, intracellular survival, and drug resistance. The molecular modification of parasites is a major survival clue for insect vectors and mammalian hosts. Besides, the secretory effector molecules have a tremendous role in parasite-to-parasite, vector-to-parasite, and parasite-to-host communication (Wu et al., 2019). The immunosuppressive role of *Leishmania* exosomes is well characterized; many effector cargos inside the vesicle contribute to the host-pathogen interaction. However, the exact molecular interaction of exosomes cargos initiated in the host cell remains elucidated. We elucidate the molecular mechanism of host macrophage polarization from M1 to M2 during the *Leishmania* promastigote-isolated exosome stimulation from both sensitive and resistant parasites. Our immunometabolism approach to the functionally plastic macrophages during exosome stimulation creates a presumptive microenvironment for the parasites. The macrophages and other mononuclear phagocytes efficiently take the parasite exosomes are well documented, and there are different ways of internalization occurs, such as phagocytosis, micropinocytosis, receptor-mediated endocytosis, and direct cargo release by simple fusion to the plasma membrane (Sabatke et al., 2023). Our fluorescent conjugated antibody-labelled exosomes confirmed the uptake of exosomes by the host macrophages. As shown in (**Fig. 5.15**), we found the presence of higher fluorescence in the flowcytometry-based Mean Fluorescent Intensity (MFI) calculation of exosomes-stimulated macrophages as compared with un-stimulated cells (**Fig. 5.16 B □ C**). The heterogenicity and small size made individual exosome visualization quite difficult in immunofluorescent imaging; nevertheless, the fluorescence of the punctuate structure was visible (J. M. Axwell Silverman □ Reiner, 2011). The same was reflected in the immunofluorescence imaging of our study; stimulated macrophages contained green fluorescence of labelled exosomes with punctate structures, and due to the multiple labelled exosomes accessing and appearing in the cytoplasm of the host cell, it confirmed the uptake of *Leishmania* exosomes by the human monocyte-derived macrophages (**Fig. 5.15 D □ E**). In the current study, our observation was that the *Leishmania*-derived exosomes are uptake by stimulated macrophages. Moreover, these results interpret that the exosomes deliver the

parasite cargo to the host to communicate with the broad and highly versatile host microenvironment. The stimulation of *Leishmania*-derived exosomes induces the production of IL-8 in the host (J. M. Silverman et al., 2010), and it recruits the neutrophils as an early response to infection. It supports the recently emerged "trojan horse model" of *Leishmania* infection (Peters et al., 2008). The *L. donovani* exosomes treatment before the infection in the C57BL/6 mice exacerbated the infection (J. M. Silverman et al., 2010). So, we checked the parasite phagocytic activity of macrophages stimulated with *Leishmania* exosomes before the infection. We observed the uptake of promastigotes to the macrophages in the early hours of infection. As expected, we found a higher trend of phagocytosis in the sensitive parasites and a significantly higher phagocytic index in resistant parasites (**Fig. 5.17 E**). It reassures that the parasite exosomes create a pro-parasitic environment, predominantly immunosuppressive, favourable to the successful establishment of infection and survival. It is important to note that only a few miltefosine clinical isolate was isolated and described in detail; two were from India, and another two were from an HIV infected patients from France (Cojean S et al., 2012; Mondelaers et al., 2016; S. Srivastava et al., 2017). According to WHO, as of 2021, 45 countries reported HIV-Leishmaniasis co-infection, and also there is a surge of asymptomatic cases. It indicates a warning sign that co-morbidities and immunosuppression are major risk factors for leishmaniasis infection. In connection with this, another study demonstrated that the infectivity and disease progression are higher in drug-resistant strains of parasites than in sensitive ones (Bulté et al., 2021). We found that the infectivity of human monocyte differentiated macrophages showed significantly higher infectivity upon *Leishmania*-derived exosome stimulation prior to the parasite challenge (**Fig. 5.18**). It might be due to the immunosuppressive effect of *Leishmania* exosome stimulation. Since ROS generation is the early innate response of the host and parasite, molecular adaptations against oxidative stress are a major survival strategy of the parasites (H. W. Murray, 2006). The initial burst of ROS during parasite exposure very much decides the successful establishment of infection and differentiation of promastigotes to amastigote conversion. The priming of an anti-oxidative defence system is correlated with virulence and the development of parasites for better tolerance (Alzate et al., 2007). Exosome exposure mimics the parasite infection condition and makes the silent phagocytosis of the parasite during infection by recruiting the neutrophils through IL-8 secretion (Peters et al., 2008).

The priming of exosomes also alters the signalling pathways, especially the JAK-STAT-mediated pathways. It subsidizes the microbicidal molecules such as TNF-α, ROS, and NO (J. M. Axwell Silverman Reiner, 2011). A recent report demonstrated that drug-resistant EVs exposed parasites are better tolerant to ROS-mediated stress than unexposed parasites (Douanne et al., 2022). So, we checked the macrophage intracellular ROS upon exosome stimulation, and there was no significant shift in the ROS levels. (**Fig. 19 B**). It is speculated that high ROS levels produce inflammatory cytokine response and M1 polarization (Kelly O'Neill, 2015; Kieler et al., 2021; Rendra et al., 2019; West et al., 2011). The Gch1 knockout of macrophage induces the production of cellular ROS (McNeill et al., 2015). So, ROS initiates the Electron Transport Chain (ETC) rewiring and M1 polarization of macrophages (Seim et al., 2019; Tan et al., 2016). At the same time, our result indicates no change in ROS levels, so the phenotype of macrophages is more towards the M2 than M1.

The present study explores the emerging area of immunometabolism in rewiring macrophage functional phenotype by the *Leishmania*-derived exosomes. This approach unveils the parasite and its secretory effector's crosstalk with the host macrophage immunometabolism and its modulation. For example, the *Leishmania* infection rewires the macrophage's energy metabolism, preferably the macrophage's increased oxidative phosphorylation over glycolysis. The same pattern of energy metabolism found in the M2 polarized macrophages and this strict phenotype change from M1 to M2 is majorly due to the metabolic rewiring of the parasitic effector molecules and the microenvironmental factors of the macrophage-deplete niche (Huang et al., 2016; P. J. Murray Wynn, 2011). The metabolic reprogramming of the polyamine biosynthetic pathway from the perspective of macrophage polarization is crucial to the parasite survival. Therefore, functional plasticity is strongly connected to the metabolic reprogramming of polyamine biosynthesis. The *Leishmania* parasite resides and proliferates inside the phagolysosome vacuole and is highly acidic (pH 5.5) and nutrient or metabolite-limited compartment. Hence, *Leishmania* auxotrophs for many nutrients or metabolites depend entirely on the host for their persistence and proliferation in the nutrient-depleted microenvironment. So, parasites must exploit the host metabolic pathways for their existence through the upregulation of parasitic or host metabolic pathways to wisely utilize the limited sources of nutrients or metabolites in the phagolysosome compartment. The semi-essential amino acid L-arginine stands at a crossroads of life and death of the intracellular parasite, and it is a double-edged sword that can resolve the infection by microbicidal action. On the other hand, it can synthesize the microbial regulatory molecules that enhance infection (Ilari et al.,

2015). It is also reported that the mutation in the promoter of arginine solute carrier (SLC7a2) from the macrophages of C57BL/6 mice reduced the *Leishmania* infection, mainly due to the unavailability of the arginine for the utilization macrophages for its classical activation (Sans-Fons et al., 2013). However, the reduction of the host arginine pool is a doubtful advantage for the parasite because the *de novo* pathway synthesis of arginine is absent in *Leishmania* and purely depends on the exogenous arginine (Shaked-Mishan et al., 2006). AAP3.2 is the *Leishmania* transporter for the exogenous arginine and the deletion of AAP3.2 mutant parasites unable to survive in both disease models *in vitro* and *in vivo.* Since *Leishmania* loses the "hunger game" of arginine for polyamine synthesis, intracellular survival is heavily hampered (Goldman-Pinkovich et al., 2020). The iNOS and arginase are the keystone enzymes in the arginine metabolism that decide the fate of the parasite because they are cross-inhibiting pathways (Mamani-Huanca et al., 2021). Our experiments demonstrated that the *Leishmania* promastigote-derived exosomes stimulation from both sensitive and resistant parasite strains to the human monocyte differentiated macrophages preferentially polarize towards the M2 phenotype, and it might be due to the driving of polyamine pathway instead of iNOS pathway by *Leishmania* exosomes. As shown (**Fig. 5.20 A □ B**), there are no significant changes in the expression of iNOS during the exosome stimulation, and it is reflected in the secretion of Nitric oxide. The arginine-derived NO is responsible for the reduction of mitochondrial complexes I (NADH-Q oxidoreductase) as well as the complex II (SDH) and concomitant increment of glycolytic activity (Drapier2 □ Hibbs, 1986). Later, the arginine dichotomy demonstrated that the polarization of the M1 phenotype is marked by the inhibition of mitochondrial complexes I and II and is mediated by NO (Kieler et al., 2021). Interestingly, LPS or IFN-γ mediated M1 polarized macrophage phenotype cannot be repolarized to M2 because of the NO-mediated rewiring of ETC complexes. In contrast, the knockout of the NOS2 gene or pharmacological inhibition of iNOS partially restored the macrophage plasticity of M1 to M2 (Van den Bossche et al., 2016). Here, the reciprocal regulation of the single substrate-dependent, independent pathway enzymes such as iNOS and arginase is important. Many intracellular pathogens strategically use this dichotomy. For example, the arginine depletion via arginase-mediated polyamine pathway in the *Mycobacterium* tuberculosis infection compromised the iNOS activity, which hampered the NO-mediated macrophage function against the *Mycobacterium* (Schreiber et al., 2009). In *Trypanosoma cruzi* infection promotes alternative activation of the macrophages by inducing the arginase I (Stempin et al., 2004). The alternatively activated macrophage-mediated intracellular polyamine by the arginase I provides a metabolic supplement to the intracellular *Brucella abortus* and is taken through the transporters that

express in the *B. abortus.* subsequently, it contributes to chronic infection in the host (Kerrinnes et al., 2018). Another study revealed that the specific inhibitors inhibiting NO production during *Trypanosoma cruzi* infection of macrophages show an M2 profile and enhance the parasite's survival rate (Sanmarco et al., 2017). Arginase I is a major enzyme in the polyamine biosynthesis pathway and is a marker for alternatively activated macrophages (Abdelaziz et al., 2020). Our result of flow cytometry-based and western blot-based arginase I expression analysis confirms that the expression of arginase I in *Leishmania* exosomes of resistant strain is high compared to the naive macrophage (**Fig. 5.21 B & C**). Furthermore, *L. donovani* exosome-stimulated macrophages show a significantly higher level of Arginase I activity showing that the initiation of the polyamine pathway (**Fig. 5.22 A**). It shows that *Leishmania* exosomes induce the co-opt mechanism of induction arginase I activity to macrophage polarize the macrophages from M1 (classically activated) to M2 (alternatively activated). It blunts the NO production and creates a pro-parasitic environment in the macrophages. Moreover, our result of correlation assay of arginase I activity with an intracellular level of NO for the cross verification of "the arginine paradox". Our data indicates that the correlation analysis showed a strong negative correlation with an r-value of -0.2893. The result suggested that the exosome stimulation drives the polyamine pathway instead of the iNOS pathway upon *Leishmania*-derived exosome stimulation (**Fig**. **5.22 B**). The study speculates that the LPS-mediated arginase I expression is time and species-dependent (Menzies et al., 2010). It is observed that bacteria-challenged and LPS/IFN-γ stimulated macrophages show arginase expression (Zhang et al., 2019). A study demonstrates that murine bone marrow-derived macrophages express arginase I in the case of both innate (LPS) and alternative (IL-4) stimulation. Still, it is in the later hours (≥24 hours) and indicates the arginase I expression is additive. It is suggested that this delay in the expression is the regulatory role of the arginase I, a highly endotoxic LPS-induced NO and its inflammatory spike (Nagasaki et al., 1999). However, ROS is the early innate response capable of showing the pattern of M1 polarization independent of NO production by an experiment; in the knockout that expresses iNOS but does not produce NO (McNeill et al., 2015). We observed in our study that there are no significant changes of ROS in the early hours of exosome stimulation (**Fig. 5.19**), and it shows that *Leishmania* exosomes initiated the polarization and shows preferably an M2 pattern of polarization, independent of iNOS-mediated NO. Similarly, in the later hours, the exosome stimulation reduces NO levels, which might be why the arginase I expression and activity enhance and polarize the macrophages into an M2 polarized state (**Fig. 5.20 B**). All these studies suggest that arginase I is more than an M2 marker; it significantly impacts the conversion of arginine to polyamines

and is involved in the effector function of the macrophage. Spermidine is a critical polyamine in macrophage polarization, and it is involved in the polarization of the macrophages (R. Liu et al., 2020; Puleston et al., 2021; Zanatta et al., 2023). Our results show that the *L. donovani*-derived exosomes stimulation of macrophages from both strains contributes to the host polyamine pool by elevated levels of intracellular spermidine (**Fig. 5.23 B**). The intracellular polyamine pool has a noteworthy effect on the intracellular survival of the *Leishmania* parasite in the metabolite as well as the oxidative stress-induced environment of the phagolysosome (Saunders McConville, 2020). A hunger game exists between the parasite and host to win the war for survival metabolites, especially in the arginine metabolism (Goldman-Pinkovich et al., 2020). There is the possibility that the parasite can supply its metabolites to phagolysosomes through the vesicles; it is reported that the various endo-lysosome and secretory vesicle markers and signal peptides for the fusion of phagolysosomes compartment (Young Kima, 2019). So, we strongly presumed that the metabolic cargos, especially the polyamine in the *Leishmania*-derived exosomes we found in our study (**Fig. 5.7 5.8**), possibly supplement to host polyamine pool. The other possibility is the induction of the polyamine pathway by parasite effector molecules, and we found that the *L. donovani* exosomes drive the polyamine pathway through arginase I and contribute to the polyamine pool of the host for the better survival of the parasite inside the polyamine-limited condition of the phagolysosome. Moreover, our results show that the host polyamine transporter (Slc3a2) expression elevated during the *Leishmania* exosome stimulation (**Fig. 5.23 B**The reports also suggest that during the time of infection of *Leishmania,* the polyamine transporter expression was higher, and the parasite could sense the deprivation of the polyamine resources. According to that, they metabolically adapt or regulate the various pathways to increase the polyamine pool or scavenge it from the host through transporters (Goldman-Pinkovich et al., 2020; Kerrinnes et al., 2018; Zanatta et al., 2023). Overall, polyamine is an essential metabolite needed for the *Leishmania* parasite, and it follows various immunometabolism mechanisms in the reprogramming of macrophages. Our results of this study also strongly corroborated that the parasitic effector molecules, especially metabolites inside the exosomes, and cargos from the parasite contribute to the polyamine biosynthesis of the host by supplementation, scavenging through elevated expression of transporters as well as drives the polyamine pathway through the induced activity of the arginase I. finally the elevated levels of polyamines shows a pattern of alternatively activated M2 phenotype and effector functions of highly dynamic macrophages by metabolic reprogramming and exploiting mechanism that parasite induces on the host immunometabolism pathways.

The pattern of alternatively activated M2 state of macrophage is hallmarked with elevated levels of various regulatory and anti-inflammatory cytokines. The classically activated macrophages generate a lot of ROS and NO as an effector response against parasites, and it geared the pro-inflammatory cytokine secretion (Martinez Gordon, 2014; Mosser Edwards, 2008). To prevent this, the parasite alters many signalling pathways to hamper the production of ROS and NO, and it also preferentially creates an anti-inflammatory milieu in the host. Interestingly, VL patient serum shows elevated cytokine IL-10 and TGF-β levels in various clinical samples of different studies (Caldas et al., 2005; Kupani et al., 2021). Our result also correlated with elevated TGF- β and IL-10 levels in the *Leishmania*-derived exosome-treated host macrophage cell line (THP-1) (**Fig. 5.24 C D**). On the other hand, we show that the reduction in the expression of iNOS (**Fig. 5.20 A and Fig. 5.24 A**) and there is not a noticeable difference in the levels of IFN-γ upon *Leishmania*-derived exosome stimulation to the macrophages (**Fig**. **5**.**24 B**) strengthened our hypothesis that the pattern of alternative activation of macrophage by *Leishmania* derived exosomes. It has already been reported that the IL-10 and TG-β in the VL induce the expression of arginase I during the infection, and in turn, the arginase I upregulation correlates with these cytokines production, acting as a positive feedback loop (Kupani et al., 2021; Mandal et al., 2017). Indeed, it is linked to the macrophage functional activation from M1 to M2 during exosome stimulation by driving the polyamine pathway.

Chapter: 7
Summary

In conclusion, *L. donovani* promastigotes produce exosomes and carry many virulence factors, especially the metabolites that are essential for the survival of the parasites. The immunometabolism perspective of our study focused on the polyamine biosynthesis pathway of a parasite and the host. Interestingly, we found that the essential metabolite polyamines (Spermidine and Putrescine) are enriched in the *L. donovani* exosomes, and it might be a carrier that transports and supplements the polyamine to the host to create a pro-parasitic environment in the metabolites and nutrient depleted niche of the phagolysosomes. Our pharmacological inhibition and polyamine depletion of drug-sensitive DD8 (MH0M/IN/80/DD8) and resistant BHU875 are heavily affected. Interestingly, the MIL-resistant *L. donovani* BHU875 promastigotes have shown more sensitivity towards growth upon hypericin treatment. Interestingly, the hypericin-mediated polyamine starvation hampered the mitochondrial metabolic fitness and observed a ROS-mediated necrotic death of the parasite. So, we conclude that the polyamine and its biosynthesis play a major role in parasite growth and fitness. Next, we investigated the metabolic reprogramming of the *L. donovani*-derived exosome from sensitive (MH0M/IN/80/DD8) and resistant (BHU875) from the perspective of host macrophage (THP-1) polyamine biosynthesis. Macrophages induce the innate immune response upon infection, and *Leishmania* preferentially resides inside the macrophages. So, studying the immune system crosstalk with metabolism is important to understand the metabolic adaptation and activation/deactivation of the immunometabolism pathways during the *Leishmania*-derived exosome stimulation. Since the *Leishmania* parasite survival is essentially linked to the polyamines, we primarily focused on the metabolic rewiring of the host polyamine biosynthesis pathway. We found that the *Leishmania*-derived exosome stimulation increases the phagocytic index and infectivity in the host macrophages upon infection with parasites. Then, we address the primary question of the present study: the metabolic reprogramming of the host polyamine biosynthesis. Our results found that the *L. donovani* exosomes induce the expression and activity of arginase I, which ensures that the polyamine biosynthesis pathway is active during infection and the exosome stimulation doesn't show significant changes in Nitric Oxide and ROS levels. It proves that the exosome stimulation drives the polyamine pathway instead of the iNOS pathway as reciprocal regulation of a single substrate for two enzymes. We also observed that *Leishmania* exosomes contribute to the intracellular spermidine pool, induce the expression of the polyamine transporter, and help in the hunger game of the parasite during metabolic adaptation. The *Leishmania* exosomes

stimulation creates a presumptive environment for the establishment of infection by driving the arginase I mediated polyamine synthesis, and it regulates the arginase I expression through the elevated levels of TGF-β and IL-10 and reduction of the iNOS and IFN-γ expression. In turn, it follows a pattern of alternatively activated M2 polarization of the highly metabolically dynamic macrophages.

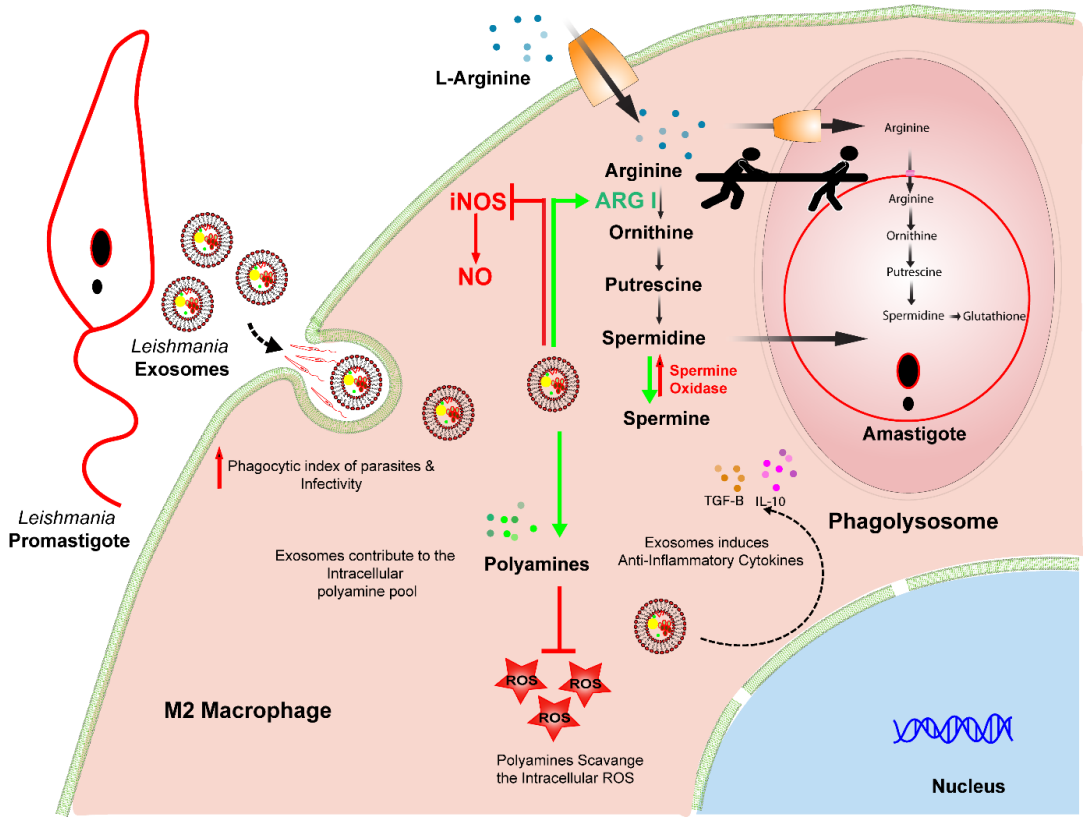


Figure 7.1: Schematic represents the summary of the study. The *L. donovani* parasite evolutionary produces an exosome and is uptaken by the host macrophage cell line. It rewires the immunometabolism of the macrophages by inducing the expression and activity of the arginase I and reducing the expression of iNOS. It hampers the production of NO. On the other hand, the arginase I-mediated polyamine biosynthesis pathway drives and contributes to the intracellular polyamine pool of the host. The *Leishmania*-derived exosomes also lower the early innate response by hindering the elevated production of intracellular ROS. The exosome from the parasite creates a pro-parasitic milieu by inducing various regulatory and anti-inflammatory cytokines (TGF-β and IL-10). In contrast, it lowers the expression of pro-inflammatory cytokine (IFN-γ). The *Leishmania* exosome contributes to the intracellular pool

of polyamine by supplementation, induction, and transport of polyamine by inducing the expression of the host polyamine transporter (Slc3a2). Overall, the *Leishmnia*-derived exosome manipulates the host immunometabolism by altering the polyamine biosynthesis, and it shows a pattern of alternatively activated macrophage effector function and phenotype. Moreover, it helps parasite evasion, infection establishment, and intracellular survival inside the nutrient or metabolite-deplete niche of the host macrophage.

Chapter: 8
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Publications

Chapter 12

Extracellular vesicle-associated microRNA in human parasitic diseases

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12.1 Introduction

Parasitic infections are a global problem in the tropics, subtropics, and temperate climates. There are three main types of parasites that infect humans and cause disease: helminths, parasitic worms (e.g., Schistosomes, *Wuchereria bancrofti*, etc.), protozoan unicellular eukaryotic parasites (e.g., *Plasmodium*, *Leishmania*, and *Trypanosoma*), and ectoparasites, an organism that lives on the skin of their hosts, such as *Sarcoptes scabiei*. Nearly 1 billion people worldwide suffer from various parasitic diseases. EVs are vesicles of endocytic origin secreted by all eukaryotic cells, which contain a variety of molecules, including lipids, proteins, and nucleic acids (RNAs), some of which have immunomodulatory properties. Extracellular vesicles (EVs) produced through the endocytic pathways are known as exosomes, whereas microvesicles are formed by the shedding of the plasma membrane. Exosomes usually range in size from 50 to 150 nm, have a distinctive cup-shaped morphology, and express markers related to their formation, which comprises the inward budding of multivesicular structures. However, extracellular fission and outward budding of the plasma membrane result in the formation of microvesicles, whereas apoptotic bodies occur when cells undergo apoptosis. These subtypes have a larger size range (50–2000 and 50–5000 nm) and lack the cup-shaped morphology unique to exosomes. Despite the above features, it is difficult to distinguish exosomes from other EVs, and there is no appropriate marker for their detection. Numerous studies have shown that the synthesis of EVs by parasites is an essential component of their life cycle and the progression of the infection; it follows that these are required for their survival. EVs offer a reliable delivery method to promote parasite development, the transmission of virulence factors, adhesion to host tissues, and the evasion of immune responses in host cells. They are successful in controlling the immune

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REVIEW
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COVID-19 Severity in Obesity: Leptin and Inflammatory Cytokine Interplay in the Link Between High Morbidity and Mortality

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Obesity is one of the foremost risk factors in coronavirus infection resulting in severe illness and mortality as the pandemic progresses. Obesity is a well-known predisposed chronic inflammatory condition. The dynamics of obesity and its impacts on immunity may change the disease severity of pneumonia, especially in acute respiratory distress syndrome, a primary cause of death from SARS-CoV-2 infection. The adipocytes of adipose tissue secret leptin in proportion to individuals' body fat mass. An increase in circulating plasma leptin is a typical characteristic of obesity and correlates with a leptin-resistant state. Leptin is considered a pleiotropic molecule regulating appetite and immunity. In immunity, leptin functions as a cytokine and coordinates the host's innate and adaptive responses by promoting the Th1 type of immune response. Leptin induced the proliferation and functions of antigen-presenting cells, monocytes, and T helper cells, subsequently influencing the pro-inflammatory cytokine secretion by these cells, such as TNF-α, IL-2, or IL-6. Leptin scarcity or resistance is linked with dysregulation of cytokine secretion leading to autoimmune disorders, inflammatory responses, and increased susceptibility towards infectious diseases. Therefore, leptin activity by leptin long-lasting super active antagonist's dysregulation in patients with obesity might contribute to high mortality rates in these patients during SARS-CoV-2 infection. This review systematically discusses the interplay mechanism between leptin and inflammatory cytokines and their contribution to the fatal outcomes in COVID-19 patients with obesity.

Keywords: COVID-19, leptin, obesity, inflammation, cytokine, mortality

INTRODUCTION

Obesity is marked as redundant fat accumulation in the body. Obesity is considered an increased circulating fatty acid that is causing low-grade chronic inflammation due to macrophages' chemoattraction and its expansion in the adipose tissue (1, 2). An individual with obesity presents with increased TNF-α cytokine, changed T-cell subset, and suppressed T-cell responses

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Febrifugine dihydrochloride as a new oral chemotherapeutic agent against visceral leishmaniasis infection

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ABSTRACT

Visceral leishmaniasis (VL) is the deadliest form of leishmaniasis without a safer treatment option. This study implies drug repurposing to find a novel antileishmanial compound, namely febrifugine dihydrochloride (FFG) targeting *Leishmania* antioxidant system. Starting with virtual screening revealed the high binding affinity and lead likeness of FFG against the trypanothione reductase (TR) enzyme of *Leishmania donovani,* followed by experimental validation. The promastigotes inhibition assay gave the IC50 concentration of FFG and Miltefosine (positive control) as 7.16 ± 1.39 nM and 11.41 ± 0.29 μM, respectively. Their CC_{50} was found as 451 ± 12.73 nM and 135.9 ± 5.94 μM, respectively. FFG has been shown to increase the reactive oxygen species (ROS), leading to apoptosis-like cell death among *L. donovani* promastigotes. Spleen touch biopsy resulted in 62% and 55% decreased parasite load with FFG and miltefosine treatment, respectively. Cytokine profiling has shown an increased proinflammatory cytokine response post-FFG treatment. Moreover, FFG is safe on the liver toxicity parameter in mice post-treatment.

1. Introduction

Visceral leishmaniasis (VL) or kala-azar is the fatal form of leishmaniasis caused by the obligate intracellular protozoan parasite of genus *Leishmania,* mainly *L. donovani* and *L. infantum* (Terefe et al., 2015). The former affects the population of the Indian subcontinent and Africa, while later affects the population of the Mediterranean basin, South and Central America (Torres-Guerrero et al., 2017). Sandflies, mainly *Phlebotomus argentipes* and *Lutzomyia longipalpis* are the vectors to spread this disease severity in the old world (Indian subcontinent and Africa) and the new world (Mediterranean basin, South and Central America), respectively (Maroli et al., 2013). VL targets the cells of the reticuloendothelial system, and increasing parasite load leads to the swelling of the liver and spleen (Alemayehu and Alemayehu, 2017). Its symptoms include fever, weight loss, anemia, hepatosplenomegaly, and thrombocytopenia (Lainson and Shaw, 1978). Even after such a severe clinical manifestation, none of the vaccine candidates have been registered to prevent this disease. The treatment of VL only relies upon the countable number of chemotherapeutic drugs. These drugs are pentavalent antimonials, amphotericin B, Miltefosine, and paromomycin.

Antimonial has been the first-line treatment for the VL since the 1970s; later on, a gradual decrease in its clinical efficacy and more than 70% resistance within only two decades restricted its use in the Bihar state of India. Later, amphotericin B (AmB) was introduced as a second-line treatment, yet soon it turned into a mainline treatment. Still, it has shown resistance and adverse events like myocarditis, infusion reaction, hypokalemia, and nephrotoxicity (Messori et al., 2013). Further, paromomycin was reported from Kenya in the 1990s, having good efficacy and showing side effects like pain at the injection site. At the same time, few patients experienced reversible ototoxicity (2%) and hepatotoxicity (6%) (Sundar et al., 2007). Later in 2002s, Miltefosine was approved as the first oral antileishmanial drug in India but was also associated with various adverse events like gastrointestinal toxicity, recurrent hepatotoxicity, nephrotoxicity, and teratogenicity (Sundar et al., 2012).

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