

Regulation of Immunity to Visceral Leishmaniasis by Regulatory T cells (Tregs) and Hepatic Stellate Cells (HSCs)

By

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ABSTRACT

Leishmaniasis is a vector borne disease that spreads through the bite of an infected sandfly and is caused by the intracellular parasite-*Leishmania*. An estimated 10-15 million cases of leishmaniasis occur worldwide, presenting as one of the three forms: cutaneous (CL), mucocutaneous (MCL) or visceral leishmaniasis (VL). The occurrence of leishmaniasis is increasing due to global traveling, emergence of drug resistant species and *Leishmania*-HIV coinfection. Therefore, there is an urgent need for the development of new therapies or vaccines against leishmaniasis.

Our laboratory previously showed that mice with an inactivating knock-in mutation in the p110 δ gene (known as p110 δ^{D910A}) are resistant to *L. major* (the causative agent of CL). Here, I demonstrate that signaling via the p110 δ also regulates immunity to *L. donovani* (the causative agent of VL) resulting in hyper-resistance to experimental VL. This outcome is dependent on the impact of p110 δ signaling on expansion and function of regulatory T cells (Tregs). I show for the first time that *L. donovani* can infect Hepatic Stellate Cells (HSCs) *in vivo* and *in vitro* and this infection leads to the production of cytokines that are known to induce Tregs. I also demonstrate that infection with *L. donovani* leads to dramatic expansion of HSCs in a PI3K-dependent manner, and this correlates with expansion of hepatic Tregs. I further show that *L. donovani*-infected HSCs can induce CD4⁺ T cells to become Tregs and this effect is dependent on p110 δ signalling. Targeted depletion of HSCs during infection caused a dramatic reduction in liver Treg numbers and proliferation, which was associated with a more efficient parasite control. I also demonstrate that prophylactic and therapeutic administration of CAL-101 (a pharmacological

inhibitor of p110 δ signalling) is associated with significant reduction in parasite burden, Treg numbers and cytokine production in both experimental models of VL and CL. More importantly, combination of CAL-101 with sub-therapeutic dose of Amphotericin-B leads to full cure from VL.

Collectively, these results provide novel understandings into the mechanisms involved in the development and regulation of protective immunity against VL, which could have direct implications for immunotherapy and drug/vaccine development against leishmaniasis.

DEDICATION

This thesis is dedicated to my

"Parents", "Mentors", "Sisters", "Friends" & "Partner in Life"

for constantly reminding me that:

"WHERE THERE IS A WILL, THERE IS A WAY"

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ABBREVIATIONS

<u>Acronym</u>	<u>Definition</u>
α -SMA	<i>Alpha</i> Smooth Muscle Actin (smooth muscle α -actin)
ACK	Ammonium-Chloride-Potassium
AIDS	Acquired Immune Deficiency Syndrome
Akt	Serine/Threonine-Specific Protein Kinase
Amph-B	Amphotericin-B
ANOVA	Analysis of Variance
AO	Aldehyde Oxidase
AP-2	Activator protein-2
APCs	Antigen Presenting Cells
B7-H1	Programmed Death Ligand-1 (PDL-1)
BCG	Bacillus Calmette–Guérin
BCR	B cell receptor
BMDC	Bone Marrow Derived Dendritic Cells
BMDM	Bone Marrow Derived Macrophages
BTLA	B and T Lymphocyte Attenuator
CCR7	Chemokine Receptor seven
CD103	Type I Transmembrane Glycoprotein known as α E Integrin
CD11b	Cluster of Differentiation 11b
CD11c	Cluster of Differentiation 11c
CD1d	Cluster of Differentiation 1d
CD25	IL-2 Receptor α -Chain
CD28	Cluster of Differentiation 28
CD3	Cluster of Differentiation 3
CD4	Cluster of Differentiation 4
CD40	Cluster of Differentiation 40
CD62L	Cluster of Differentiation 62 Ligand
CD8	Cluster of Differentiation 8
Cdc42	Cell Division Control Protein 42 Homolog
CFSE	5,6-Carboxyfluorescein Diacetate Succinimidyl Ester
CL	Cutaneous Leishmaniasis
CLL	Chronic Lymphocytic Leukemia
CNS	Central Nervous System
CP	Cysteine Proteinase
CpG	Cytidine-Phosphateguanosine
CRs	Complement Receptors
CTL	Cytotoxic T Cell
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
CXCR	Chemokine, CXC Motif, Receptor
CYP3A	Cytochrome P4503A
DC	Dendritic Cell

dLNs	Draining Lymph Nodes
DMEM	Dubelco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DTH	Delayed Type Hypersensitivity
<i>E. coli</i>	<i>Escherichia coli</i>
ECM	Extra Cellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EF-1 α	Elongation Factor 1 α
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ERK (1/2)	Extracellular Signal-Regulated Kinases (1/2)
FAK	Focal Adhesion Kinase
FasL	Fas Ligand
FBS	Fetal Bovine Serum
FFA	Free Fatty Acids
FGF	Fibroblast Growth Factor
FML	Fucose Mannose Ligand
Foxo	Forkhead Box Protein
Foxp3	Forkhead Box P3
FXR	Farnesoid X Receptor
GFAP	Glial Fibrillary Acidic Protein
GITR	Glucocorticoid-Inducible Tumor Necrosis Factor Receptor
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor
GP63	Glycoprotein 63
GPCR	G Protein-Coupled Receptor
H&E	Hematoxylin and Eosin
HGF	Hepatocyte Growth Factor
HIV	Human Immunodeficiency Syndrome
HSCs	Hepatic Stellate Cells
i.c.	Intracardial
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenously
IC ₅₀	Inhibitory Concentration
ICAM-1	Intercellular Adhesion Molecule-1;
IFN- γ	Interferon Gamma
IGF-I	Insulin-Like Growth Factor-I
IgG1 or G2	Immunoglobulin G1 or G2
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-4	Interleukin 4
iNOS	Inducible Nitric Oxide Synthase
IP-10	Inducible Protein 10

IRF-5	Interferon Regulatory Factor 5
ITAM	Immunoreceptor Tyrosine-Based Activation Motif
ITIM	Immunoreceptor Tyrosine-Based Inhibition Motif
iTregs	Inducible Regulatory T Cells
JAK1	Janus Kinase 1
JNK	Jun N-Terminal Kinase
KC/GRO (CXCL1)	IL-8 homologue in mice
kDNA	Kinetoplastic DNA
KI	Knock In mice
KMP11	Kinetoplastid Membrane Protein eleven
KO	Knock Out
LdCen ^{-/-}	Live-Attenuated <i>L. donovani</i> Lacking the Centrin Gene
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
LRAT	Lecithin Retinol Acyltransferase
LXR	Liver X Receptor
MA	Meglumine Antimoniate
MAPK	Mitogen-Activated Protein Kinase
MCL	Mucocutaneous Leishmaniasis
MCP-1 (CCL2)	Monocyte Chemotactic Peptide-1
M-CSF	Macrophage Colony Stimulating Factor
MFI	Mean Fluorescence Intensity
mg	Milligram
µg	Microgram
MHC I or II	Major Histocompatibility Complex I or II
MIP-1	Macrophage Inflammatory Protein-1
ml	Millilitre
mM	Millimolar
MMP(1/2)	Matrix Metalloproteinase (1/2)
mRNA	Messenger Ribonucleic Acid
MSCs	Mesenchymal Stem Cells
mTOR	Mammalian Target of Rapamycin
MyD88	Myeloid Differentiation Factor 88
MZ	Marginal Zone
NETs	Neutrophil Extracellular Traps
NF-κB	Nuclear Factor <i>Kappa</i> Light Chain Enhancer of Activated B Cells
ng	Nanogram
NGF	Nerve Growth Factor
NHP	Non-Human Primate
NK	Natural Killer Cell
NKT	Natural Killer T Cells
NO	Nitric Oxide
NTDs	Neglected Tropical Diseases
nTregs	Naturally Occurring Regulatory T Cells
P110δ ^{D910A} mice	Mice with an Inactivating Knock-In Mutation in the P110δ Gene

PAMPs	Pathogen-Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF-B	Platelet-Derived Growth Factor-B
PEPCK	Phosphoenolpyruvate Carboxykinase
pg	Picogram
PGs	Phosphoglycans
PH	Pleckstrin Homology Domain
PI3Ks	Phosphatidylinositol 3-Kinases (PI3Ks)
PIP	Phosphatidylinositol (3)-Monophosphate
PIP2	Phosphatidylinositol (3,4)-Biphosphate
PIP3	Phosphatidylinositol (3,4,5)-Triphosphate
PKC	Protein Kinase C
PKDL	Post Kalazar Dermal Leishmaniasis
PMNs	Polymorphonuclear Neutrophil
PPAR- γ	Peroxisome Proliferator Activated Receptor- γ
PRRs	Pattern Recognition Receptors
PTEN	Phosphatase and Tensin Homolog Deleted on Chromosome Ten
PTP	Protein Tyrosine Phosphatase
PXR	Pregnane X Receptor
Rac1	Ras-Related C3 Botulinum Toxin Substrate 1
RAE1	Retinoic Acid Early Inducible-1
RANTES (CCL5)	Regulated upon Activation, Normal T-cell Expressed, and Secreted (Chemokine (C-C motif) ligand 5)
RAR	Retinoid Acid Receptor
ROI	Reactive Oxygen Intermediates
RPMI-1640	Roswell Park Memorial Institute culture media
RTK	Receptor Tyrosine Kinase
RXR	Retinoid X Receptor
SAP	Secreted Acid Phosphatase
Sc111a1	Natural Resistance Associated Macrophage Protein-1 (NRAMP1)
scAb / C1-3	Human Recombinant Single-Chain Antibody (scAb termed C1-3)
SD	Standard Deviation
SHP-1	Src (Sarcoma) Homology 2 Domain Phosphatase-1 (tyrosine phosphatase)
SMAD	Similar to the Drosophila gene Mothers Against Decapentaplegic
SSG	Sodium Stibogluconate
STAT1	Signal Transducer and Activator of Transcription 1
Tcm	Central Memory T Cells
Tem	Effector Memory T Cells
TGF- β	Transforming Growth Factor Beta
Th1 or Th2	T Helper Cell Type1 or 2
TIMP1	Tissue Inhibitor of Metalloproteinase-1
TLRs	Toll-Like Receptors

TNF- α	Tumor Necrosis Factor- α
Tr1	Regulatory T cell type 1
Tregs	Regulatory T Cells
UGT1A4	Uridine 50- Diphospho-Glucuronosyltransferase 1A4
VEGF	Vascular Endothelial Cell Growth Factor
VL	Visceral Leishmaniasis
WHO	World Health Organization
WT	Wild Type

1 CHAPTER 1: Introduction

1.1 Leishmaniasis and worldwide distribution/ epidemiology

Leishmaniasis is a vector-borne neglected tropical disease (NTD) caused by an intracellular protozoan of *Leishmania* genus, belonging to the *Trypanosomatidae* family. The disease is one of the six major parasitic diseases recognized by the World Health Organization (WHO) as major causes of morbidity and mortality in affected people [1]. Leishmaniasis is endemic in 98 countries and three territories [2], and is mostly associated with social imbalance related to poverty including malnutrition, displacement, poor housing and lack of social resources [3]. It is mainly manifested as one of three forms: Cutaneous Leishmaniasis (CL), which is the most common form; Visceral Leishmaniasis (VL), which is the most serious form; or Mucocutaneous Leishmaniasis (MCL) [4]. The symptoms range from self-healing cutaneous ulcers in CL to a deadly severe systemic multi-organ disease characterized by anemia, fever, fatigue, weight loss, hepatomegaly and splenomegaly in VL [5].

The prevalence of leishmaniasis is dependent on several risk factors including man-made factors (such as migration, deforestation, urbanization), changes in the human host's susceptibility to infection (immunosuppression and malnutrition), and natural environmental changes [6]. According to WHO, it is estimated that 0.7–1.3 million new cases of CL and 200,000–400,000 cases of VL occur annually [3] leading to 30000-50000 deaths a year [7, 8]. Due to relatively incomplete epidemiological data, official figures are likely to underestimate the real prevalence of the disease.

Leishmaniasis is spreading throughout Southern Europe, North Africa, the Middle East, Central and South America, the Indian subcontinent and Australia [2, 9]. Zoonotic and anthroponotic forms of CL and VL occur in Afghanistan, Algeria, Bangladesh, Brazil, Egypt, Greece, India, Iran, Iraq, Jordan, Libya, Morocco, Nepal, Pakistan, Palestine, Peru, Saudi Arabia, Spain, Somalia, South Sudan, Sudan, Syria, Tunisia, Yemen [10-12]. The increase in prevalence and spread of leishmaniasis to many geographical areas is due to multiple factors including increased international travel, globalization, military conflicts (soldiers returning from duties in the endemic areas) [13], lack of effective vaccines [14] and difficulties in controlling the vectors [15]. In addition, *Leishmania*-HIV co-infection [16] and the development of resistance to chemotherapy [17] are increasingly emerging and complicate an already difficult problem. Although most studies on leishmaniasis have focused on the cutaneous disease, visceral leishmaniasis remains the most important disease in humans in terms of mortality and morbidity [18].

1.2 Leishmaniasis classification, vectors and reservoirs

Leishmania species are classified on the bases of eco-biological criteria, clinical manifestation and the patterns of polymorphism exhibited by kinetoplastic DNA (kDNA) markers, proteins or antigens [19, 20]. CL is mainly caused by *Leishmania major*, *L. tropica* and *L. aethiopica* in the Old World (southern Europe, Middle East, southwest and central Asia and Africa) and by *L. mexicana*, *L. venezuelensis* and *L. braziliensis* in the New World (Mexico and Latin America). *L. amazonensis* and *L. braziliensis* are the primary causative agents of MCL in the New World (South American continent). VL is caused by *L. donovani* and *L. infantum* (syn *L. chagasi*) in the Old World (Indian subcontinent, Asia, Mediterranean region, Africa, South

America) and by *L. chagasi* (syn *L. infantum*) in the New World (South American continent) [21, 22]. In rare instances, *L. infantum* and *L. donovani* have cause CL and *L. tropica* and *L. amazonensis* have caused VL in the Mediterranean and Middle East region [23]. A full list of *Leishmania* species causing different forms of leishmaniasis and their distribution is presented in Table 1-1 (CL) and Table 1-2 (VL and MCL)

As mentioned, Leishmaniasis is a vector-born disease, which spreads through the bite of infected female sand fly belonging to the genus *Phlebotomus* (divided into 12 subgenera) in the Old World and *Lutzomyia* (divided into 25 subgenera) in the New World [24]. Among the 500 known *phlebotomine* species, only 31 have been positively identified as vectors of pathogenic species of *Leishmania* and 43 as probable vectors. The sand fly species involved in the transmission of *Leishmania* vary from one geographical region to another but also depend on the species of *Leishmania* [25]. Different vectors spreading the various forms of CL, VL and MCL are listed in detail in Table 1-1 and Table 1-2.

Different orders of mammals (rodents, canids, edentates, marsupials, procyonids, primitive ungulates and primates) are considered as potential reservoirs of the disease. Humans are also possible hosts of these parasites [26].

Table 1-1: Cutaneous Leishmaniasis pathology, distribution and vectors.

Pathology	Leishmania spp.		Distribution	Vector
Cutaneous Leishmaniasis	L. tropica complex	Leishmania major	Old World (Southern Europe, Middle East, Southwest and Central Asia, Africa)	Phlebotomus papatasi Phlebotomus duboscqi Phlebotomus salehi
		Leishmania tropica		Phlebotomus sergenti
		Leishmania aethiopica		Phlebotomus longipes Phlebotomus pedifer
	L. mexicana complex	Leishmania venezuelensis	New World (Mexico and Latin America)	Lutzomyia spinicrassa
		Leishmania mexicana		Lutzomyia olmeca olmeca Lutzomyia columbiana Lutzomyia ayacuchensis Lutzomyia longipalpis Lutzomyia ylephiletor Lutzomyia olmeca Lutzomyia cruciate
				Leishmania amazonensis
	L. braziliensis complex	Leishmania braziliensis		Lutzomyia intermedia Lutzomyia ovallesi Lutzomyia spinicrassa Lutzomyia trapidoi Lutzomyia gomezi Lutzomyia whitmani Lutzomyia umbralitis
		Leishmania panamensis		Lutzomyia trapidoi Lutzomyia hartmanni Lutzomyia panamensis Lutzomyia gomezi
		Leishmania peruviana		Lutzomyia peruensis Lutzomyia verrucarum
		Leishmania guyanensis		Lutzomyia umbralitis Lutzomyia anduzei Lutzomyia ovallesi Lutzomyia whitmani
				L. shawi
		L. lainsoni		New World (South America)
	L. naiffi	Lutzomyia ayrozai Lutzomyia squamiventris		
	Leishmania donovani	Mediterranean and Caspian sea region	Phlebotomus argentipes Phlebotomus orientalis Phlebotomus martini	
	Leishmania infantum Syn (L. chagasi)		Lutzomyia longipalpis Lutzomyia evansi Lutzomyia olmeca olmeca	

(Summarized from [4, 5, 21-23, 27-29])

Table 1-2: Visceral and mucocutaneous Leishmaniasis pathology, distribution and vectors.

Pathology	Leishmania spp.	Distribution	Vector
Visceral Leishmaniasis	<i>Leishmania donovani</i>	Old World (Indian subcontinent, Asia, Africa)	<i>Phlebotomus argentipes</i> <i>Phlebotomus orientalis</i> <i>Phlebotomus martini</i>
	<i>Leishmania infantum</i> (Syn <i>L. chagasi</i>)	Old World (Mediterranean region, southwest and central Asia, South America)	<i>Phlebotomus ariasi</i> <i>Phlebotomus perniciosus</i>
	<i>Leishmania archibaldi</i> (Syn <i>L. donovani</i>)	Old World (African continent, Middle East, Mediterranean basin)	<i>Phlebotomus orientalis</i>
	<i>Leishmania tropica</i>	Old World (Middle East)	<i>Phlebotomus sergenti</i>
	<i>Leishmania amazonensis</i>	New World (South American continent)	<i>Lutzomyia flaviscutellata</i> <i>Lutzomyia carrerai carrerai</i>
	<i>Leishmania donovani</i>	Northwestern China (Xinjiang Region: Kashgar alluvial plain and Aksu oasis) and South American continent	<i>Phlebotomus longiductus</i>
	<i>Leishmania chagasi</i> (Syn <i>L. infantum</i>)		<i>Phlebotomus longiductus</i>
Mucocutaneous Leishmaniasis	<i>Leishmania braziliensis</i>	New World (South American continent)	<i>Lutzomyia intermedia</i> <i>Lutzomyia ovallesi</i> <i>Lutzomyia spinicrassa</i> <i>Lutzomyia trapidoi</i> <i>Lutzomyia gomezi</i> <i>Lutzomyia whitmani</i> <i>Lutzomyia umbralitis</i>
	<i>Leishmania panamensis</i>		<i>Lutzomyia trapidoi</i> <i>Lutzomyia hartmanni</i> <i>Lutzomyia panamensis</i> <i>Lutzomyia gomezi</i>
	<i>Leishmania guyanensis</i>		<i>Lutzomyia umbralitis</i> <i>Lutzomyia anduzei</i> <i>Lutzomyia ovallesi</i> <i>Lutzomyia whitmani</i>
	<i>Leishmania amazonensis</i>		<i>Lutzomyia flaviscutellata</i> <i>Lutzomyia carrerai carrerai</i>
	<i>Leishmania mexicana</i>		<i>Lutzomyia olmeca olmeca</i> <i>Lutzomyia columbiana</i> <i>Lutzomyia ayacuchenisis</i> <i>Lutzomyia longipalpis</i> <i>Lutzomyia ylephiletor</i> <i>Lutzomyia olmeca</i> <i>Lutzomyia cruciate</i>

(Summarized from [4, 5, 21-23, 27-30])

1.3 *Leishmania* Life Cycle and evasion mechanism:

When the sandfly bites an animal (reservoir) infected with *Leishmania*, the mammalian stage of the parasite (called an amastigote) is ingested along with the host's blood. In the sand fly's abdominal midgut, the amastigotes transform to procyclic amastigotes and replicate to nectomonad promastigotes. The nectomonad promastigotes then migrate to thoracic midgut and foregut of the sand fly, change to leptomonad promastigotes and replicate by binary fission and transform to metacyclic promastigotes. The metacyclic promastigotes live extracellularly in the sand fly's alimentary canal, reproducing asexually, and then migrate to the proximal end of the gut where they become poised for a transmission. After an infected sand fly bites an individual, the promastigotes are then introduced locally to the skin and invade macrophages and transform back into amastigotes that replicate inside the phagolysosome [31]. Infected host cells rupture to release the more amastigotes that are spread via the bloodstream to infect new macrophage, dendritic cells and fibroblasts [32].

Leishmania species undergo a variety of metabolic changes in the first 24 hr. of stage differentiation, which include the differential expression of proteins involved in stress response, amino acid metabolism, proteolysis, energy metabolism, phosphorylation processes, cell cycle control and proliferation [33]. Intracellular proteases have been implicated to actively participate in parasite survival and pathogenicity. Serine protease and metalloprotease, respectively, have been shown to down regulate the phagocytic activity of macrophages and play important roles in parasitic development [34].

1.4 Visceral leishmaniasis

Visceral leishmaniasis (VL), also known as kala-azar, black fever, and Dumdum fever, is the most serious form of leishmaniasis and if not treated will almost always result in death. Over 90% of cases of VL occur in six countries: Bangladesh, Brazil, Ethiopia, India, South Sudan, and Sudan. VL is highly endemic in the Indian subcontinent and in East Africa but is spreading to non-endemic areas mainly due to immigration and international travels [2]. About 200,000-400,000 new cases of VL occur worldwide annually and about 50,000 lead to death [3]. *L. donovani* is the primary cause of VL in India, Pakistan, China and Africa, while *L. infantum* is the causative agent in the Mediterranean region and *L. chagasi* in the New World [4]. A more detailed description of VL causes and vectors is presented in Table 1-2. There is however a controversy as to whether *L. infantum* (syn *L. chagasi*) and *L. chagasi* (syn *L. infantum*) are the same specie of *Leishmania* or different species [35].

VL has a very variable incubation period that ranges from 6 weeks to 9 years [36]. The symptoms consist of headache, fever, chills, sweating, cough, diarrhea, dizziness, vomiting, bleeding of gums, pains in the limbs, weight loss, enlargement of spleen (splenomegaly) and liver (hepatomegaly), anemia with leukopenia and lymphadenopathy [37]. The skin may become discolored, particularly dark gray, hence the name Kala-Azar or black sickness in India [38]. In some cases, VL mimicking or exacerbating various autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, autoimmune hepatitis and antimitochondrial antibody positive primary biliary cirrhosis have been reported [39]. If not treated, death may occur within few weeks to 2-3 years depending on whether the disease is acute (few weeks), sub-acute (within a year) or chronic (within 2 to 3 years) [36].

Because VL is a systemic infection and affects most of the host's internal organs particularly the spleen, liver and bone marrow [40], latent cases may remain undiagnosed for years to decades until the individual becomes immune-compromised. Since the parasites reside in macrophages [41], which constitute a major part of the host immune defense mechanisms, VL patients are invariably immunosuppressed and thus easily fall prey to secondary opportunistic infections [42]. Heterogeneous complications associated with immunosuppression including transplantation, rheumatologic, hematologic and oncologic disorders have been reported and these contribute to VL immunopathology [43]. In addition, VL-HIV co-infections are a major health issue where VL and HIV are both endemic [16]. Furthermore, both HIV and non-HIV cases of immunosuppression pose significant diagnostic and therapeutic challenges for VL.

1.5 Experimental models

The methods that are used for *Leishmania* drug screening and vaccine production both use several *in vitro* and *in vivo* systems that each has specific characteristics.

1.5.1 *In vitro* models

Available *in vitro* methodologies for anti-VL drug screening and their respective merits and demerits have been reviewed by Gupta [32]. In this review *in vitro* models and methods indicated are promastigotes, axenic amastigotes, intracellular amastigotes and reporter gene assays (Green fluorescent protein (GFP), β -galactosidase, β -lactamase and Luciferase) [32].

1.5.2 *In vivo* models

Many *in vivo* experimental models have been developed for anti-leishmanial drug and vaccine production screening and their respective advantages and disadvantages have also been reviewed [32, 36, 44, 45]. Most of these models, however, do not accurately reproduce what happens in humans. For *in vivo* testing several animal species have served as experimental model for VL.

1.5.2.1 Rodent models

Murine models (BALB/c, NMRI, DBA/1, C57BL/6) [36, 46] of VL have been used to study the pathogenesis of the disease and to test novel therapeutic agents. In mice, the outcome of visceral disease depends on the strain, the genetic background, route of infection and the dose of parasites administered [47]. Inbred strains of mice display susceptible, resistant and intermediate phenotypes that share some similarities with human disease. Outbred mice are generally resistant to infection with *L. donovani* [32]. It has been reported that the Sc111a1 (Natural Resistance Associated Macrophage Protein-1 (NRAMP1)) locus, determines the degree of early expansion of the parasites in the liver and spleen of mice. Mice with a wild type (WT) locus of Sc111a1 (formally called NRAMP1) (CBA mice) have an earlier parasite growth than those with the mutated Sc111a1 locus (BALB/c and C57BL/6 mice) [48, 49]. BALB/c, C57BL/10 mice are susceptible and C3H.HeJ, CBA, DBA/2 mice are resistant to *L. donovani* or *L. infantum* (syn *L. chagasi*) infections [50]. BALB/c mice infected with *L. donovani* or *L. infantum*, do not exhibit a high susceptibility resulting in a self-healing chronic infection [51].

Some rat models that have been used in the past include the cotton rat (*Sigmodon hispidus*) [52], the African white tailed rat (*Mastomys albicandatus*) [53] and the multi-mammate rat *M. natalensis* [54].

A rodent model for VL that provides a more synchronic infection in the liver and spleen that can develop into a chronic infection, leads to death and is more similar to human VL is the Syrian golden hamster (*Mesocricetus auratus*) [46, 55]. European hamster and Chinese hamster have also been used as *in vivo* experimental models [46]. *L. donovani* and *L. chagasi* infection of hamsters by using intraperitoneal (i.p.) or intracardial (i.c.) injection of amastigotes isolated from the spleen of a previously infected animals is very successful with symptoms close to human disease [56-59]. The hamster model could be a useful tool for the characterization of molecules and mechanisms involved in pathogenesis of VL, but immunological studies are limited because of the lack of available reagents and also defect in NO production, so these limitations make using hamster model quite challenging [47].

Passaging *Leishmania* species that cause VL through hamsters is an important method to maintain their virulence. These newly isolated parasites from an infected hamster can then be grown in culture for only several weeks before loss of function or phenotype. Lei et. al. have reported that saphenous vein inoculation and the use of cryopreserved parasite cells can decrease animal usage and stress [58].

1.5.2.2 Canine models of visceral leishmaniasis

Some dog populations (mongrel [60], boxer [61], doberman [62]) are the major reservoir of *L. infantum* in the Middle East and the Mediterranean region and *L. chagasi* in South America

[63] and China [64]. The infection of dogs with *Leishmania* reproduces the disease pathology similar to human infections, such as long period of asymptomatic infection, anaemia, enlarged lymph nodes, hepatosplenomegaly, strong tremors, sweating, leukopenia, lymphadenopathy, fever and weight loss. In some cases, skin lesions due to the proliferation of amastigotes in the skin are also present in infected dogs [65]. For experimental purposes German shepherd dogs give better results than beagles [66], some of the mixed breeds also give highly successful infection rates [63, 64, 67] .

1.5.2.3 Non-human primate model of visceral leishmaniasis

Non-human primate models of visceral leishmaniasis largely mimic the human disease. However, financial and ethical reasons limit their use in biomedical research. The owl monkey (*Aotus trivirgatus*), geoffroy's tamarin (*Saguinus geoffroyi*), tufted capuchin monkey (*Cebus paella*) and bearded saki (*Chiropotes satanus*) are susceptible to natural infection with pathogenic *Leishmania* species [68]. *Aotus trivirgatus* (owl monkeys) [69] and *Saimiri sciureus* (squirrel monkey) [70, 71] both develop an acute and fulminating, but short-lived, infection. The common marmoset (*Callithrix jacchus jacchus*) is susceptible to *L. donovani* [68]. *Macaca fascicularis* and *M. nemestrina*, [46] and African vervet (green) monkeys (*Cercopithecus aethiops*) [72] developed low and/or inconsistent infections. *Rhesus macaques* (*M. mulatta*) is a moderately susceptible host for *L. infantum* and *L. donovani* complex parasites [73]. The Indian Hanuman langur leaf monkey (*Presbytis entellus*) is a highly susceptible non-human primate model that has also been used for evaluation of potential anti-leishmanial drugs and vaccines but complicated to use due to cost and difficult handling procedures [74, 75]. Melta *et. al.* have

reported an incident of naturally acquired VL with *L. donovani* complex parasites in black-fronted titi (*Callicebus nigrifrons*) in a zoo in Brazil [68].

1.6 Leishmaniasis control and treatment

Current leishmaniasis control measures rely on transmission reduction (by vector and animal reservoir control in selected areas), which is accomplished at the cost of substantial environmental damage [76]. Since patients who recover from leishmaniasis (natural or drug induced) gain immunity against re-infection, it is believed that the development of a safe, effective and affordable vaccine would offer the best and most cost effective solution to control leishmaniasis [76].

In order to obtain effective and suitable protective responses, it is essential to understand the complete immunogenetics of leishmaniasis, how the parasite survives in the host macrophage phagolysosome and which parasite and host factors determine the type of T helper cell response [76]. These studies could reveal targets for new treatments and increase the choice of successful vaccine designs. The completion of the genetic sequences of both human and *Leishmania* spp. has accelerated the identification of the genes that regulate host susceptibility and resistance and helped identify genes that determine or enhance virulence [77].

Current drugs available for leishmaniasis treatment are: Pentavalent antimonials (available as sodium stibogluconate (SSG) and meglumine antimoniate (MA)), Amphotericin B (Amph-B, Fungizone), different liposomal formulations of Amph-B (Fangisome or AmBisome), Miltefosine, Pentamidine, Paromomycin (aminosidine), Sitamaquine, Azoles and ketoconazoles [22, 78]. Some of the first-line employed treatments reported for VL are organic Pentavalent

antimonials (Pentostam, Glucantime, Rhone Poulenc), Amphotericin B (Fungizone), Liposomal amphotericin B ((LAmph-B) Fangisome or AmBisome), Miltefosine and Paromomycin sulfate [4, 29]. These chemotherapies are still currently in use in various parts of the world including South Sudan and Europe [79], East Africa [80], United States [81], Brazil [82] and India [83].

SSG [81] and MA (which are pentavalent antimonials) administered as 20 mg/kg daily for 28-30 days has been shown to be effective in treating VL in most parts of the world except for India and Nepal [22, 79]. Anti-leishmanial activity of pentavalent antimonials might be due to its indirection effects on host macrophages [84, 85]. Miltefosine is an alkyl phospholipid and the first oral anti-leishmanial agent administered as 150 mg/day for 28 days [83], in India, Nepal and Bangladesh. Due to relatively high cost, need for monitoring of gastrointestinal side effects and occasional hepatotoxicity and nephrotoxicity, its use has been limited [22]. The possible mechanisms of action of Miltefosine include inhibition of ether remodeling, phosphatidylcholine biosynthesis, signal transduction and calcium homeostasis [85]. Paromomycin (aminosidine) is an aminoglycoside-aminocyclitol antibiotic, which has been used for the treatment of VL as 11-20 mg/kg/day for 28 days (administered intramuscular (i.m.)) [86]. Anti-leishmanial effect of paromomycin is thought to be through premature termination of mRNA translation in the mitochondria [85]. Pentamidine was used in early 1980s for the treatment of refractory VL in India. However, its use has been abandoned for VL due to its adverse side effects such as insulin-dependent diabetes mellitus, pain, sterile abscess at the injection site, nausea, vomiting, dizziness, myalgia, headache, hypotension, syncope, transient hyperglycemia and hypoglycaemia [22]. Pentamidine accumulates in the parasite and has inhibitory effect on kDNA binding [85]. Amph-B is an antibiotic of the macrolide class, derived from a strain of *Streptomyces nodosus* which is considered as the second choice of treatment for VL. Its mechanism of action is through

binding on ergosterol of fungi and protozoa cell membrane, leading to structural disorganization, creating pores which affect the intracellular potassium permeability, thus triggering the death of parasite by osmotic cell lysis [87]. Amph-B is very effective in disease treatment when administered intravenously (i.v.) at 1mg/kg alternate days for 30 days [83]. On the other hand, Amph-B is very toxic and is associated with diverse side effects, such as nausea, chills, phlebitis, anemia, thrombocytopenia, anorexia, hypocalcemia, renal impairment, nephrotoxicity, cardiac alterations, hemolysis and liver damage [22, 88]. To minimize the adverse events of Amph-B, various lipid formulations (Fangisome or AmBisome) have been introduced which are rapidly concentrated into organs, such as liver and spleen, and remain there for long period of time. Additionally, liposomes are phagocytosed by macrophages, the main infected cells, leading to a direct reaction between the drug and the parasite's ergosterol, thereby increasing therapy success and presenting a lower reaction to host cell cholesterol [89, 90]. In this way, tolerance is greatly improved and various adverse effects including nephrotoxicity are minimized, which allows for delivery of large doses of the drug over short periods of time (2 mg/kg for 5 days) [91].

Most anti-leishmanial drug regimens currently in use are not always successful and there are abundant drawbacks to each of the treatments, such as difficulty to administer, length of treatment, toxicity, cost and increasing drug resistance. In order to increase site-specific drug delivery and reduce side effects, several new delivery systems including liposomes, emulsomes, niosomes, polymeric particles, conjugates and plant products such as alkaloids, terpenes, phenolics and chalcones are being developed and their potential applications in VL treatment have been reviewed previously [92]. Combination therapies such as the administration of fluconazole, miltefosine and Picroliv [93] have been examined and proven to be useful in order to reduce treatment costs, increase treatment efficacy and tolerance, reduce treatment duration,

reduce burden on the health system and limit the emergence of drug resistance [94]. Some of the more recently developed compounds are alkylphosphocholines (phospholipid derivatives), amiodarone, Amph-B oral formulations, 4-arylcoumarins, arylimidamides, bisphosphonates, chalcones, dihydropyridine antihypertensives, hydroxybibenzyl compounds, hydroxyurea, ivermectin derivatives, nitroaromatic compounds, paromomycin topical formulations, peganine hydrochloride, pyrazinamide, pyrimidines and triazines, quinazolines, quinolines, rhodacyanine dyes, sterol metabolism inhibitors [95]. Arylimidamides, are oral treatments for *L. donovani* axenic amastigotes, which produced dose-dependent inhibition of liver, spleen and bone marrow parasite burden in mice and hamster models [96]. Systemic administration of cholesterol liposomes cures the *L. donovani* infection in hamster model. The cholesterol liposomes correct the decreased membrane cholesterol of the antigen presenting cells (APCs) which allow the APCs to properly stimulate T cells and clear the infection [97]. Combining amiodarone and miltefosine, affected the proliferation of intracellular amastigotes inside macrophages and led to a 90% cure in a *L. mexicana* infected mouse model [98].

Most drugs used for treatment of VL are associated with resistance of the parasite to therapy, undesirable side effects such as gastrointestinal symptoms, ototoxicity and hepatotoxicity [79] as well as variable cure rates. Therefore, currently used monotherapy needs to be reviewed and possibly replaced with multidrug/combination therapy to increase effectiveness through use of compounds with synergistic or additive activity acting at different sites. Additionally it could lead to shorter duration of therapy and lower dose requirement, thereby reducing chances of toxic side effects and cost, and preventing the emergence of drug resistance [22]. A summary of Monotherapy and available Multidrug/Combination therapy regimens in different parts of the world is listed in Table 1-3.

Table 1-3: Monotherapy and Multidrug/Combination therapy regimens for visceral Leishmaniasis in different parts of the world

Drug/Chemotherapy	Dose	Rout	Leishmania Spp./ Disease	Country/Region	Patients used for
Monotherapy					
Amph-B	15 mg/kg over 30 days	i.v.	VL	Indian subcontinent, East Africa and Yemen	immunocompetant patients, HIV-coinfected patients
Amph-B (Fungizone)	0.75–1 mg/kg for 15–20 doses (daily or alternate days) Or 7–20 mg/kg total dose for up to 20 days	i.v.	VL	Indian subcontinent, USA, and East Africa	immunocompetant and HIV-coinfected patients
LAmph-B (Ambisome)	7.5 mg/kg over 5 days or once	i.v.	VL	Indian subcontinent, East Africa	immunocompetant patients
LAmph-B (Ambisome)	21 mg/kg over 21 days	i.v.	VL	East Africa	immunocompetant patients
LAmph-B (Ambisome)	18 mg/kg over 10 days	i.v.	VL	Mediterranean	immunocompetant patients
LAmph-B (Ambisome)	3–5 mg/kg daily for 3–5 days	i.v.	<i>L. donovani</i> <i>L. infantum</i>	Worldwide	immunocompetant and HIV-coinfected patients
LAmph-B (Ambisome)	30 mg/kg total dose	i.v.	<i>L. donovani</i>	East Africa and Yemen	immunocompetant and HIV-coinfected patients
LAmph-B (Ambisome)	4 mg/kg/day on days 1–5, 10, 17, 24, 31, 38 up to 40 mg/kg	i.v.	VL	USA	HIV-coinfected patients
LAmph-B (Fungisome)	10 mg/kg or 15 mg/kg once or for 2 days	i.v.	VL	Indian subcontinent	immunocompetant patients
LAmph-B(Ambisome)	10 mg/kg over 5 days or once	i.v.	VL	Indian subcontinent	immunocompetant patients
Miltefosine	2.5 mg/kg/day for 28 days	oral	VL	Indian subcontinent	immunocompetant patients
Paromomycin	11 mg/kg/day for 21 days or 14 days	i.m.	VL	Indian subcontinent, East Africa	immunocompetant patients
Paromomycin	15 mg/kg/day for 17 days	i.m.	<i>L. donovani</i>	East Africa and Yemen	immunocompetant and HIV-coinfected patients
Pentavalent Antimony	20 mg/kg/day for 30 days	i.v.	VL	East Africa, Mediterranean	immunocompetant patients
Pentavalent Antimony	20mg/kg daily	i.m. or i.v.	<i>L. donovani</i>	East Africa and Yemen	immunocompetant and HIV-coinfected patients
Multidrug/Combination therapy					
LAmph-B (Ambisome) + Miltefosine	5mg/kg once + 2.5 mg/kg/day for 7 days	i.v.+ Oral	VL	Indian subcontinent	immunocompetant patients
LAmph-B (Ambisome) + Paromomycin	5mg/kg once + 11 mg/kg/day for 10 days	i.v. + i.m.	VL	Indian subcontinent	immunocompetant patients
Miltefosine + Paromomycin	2.5 mg/kg/day for 10 days + 11 mg/kg/day for 10 days	oral + i.m.	VL	Indian subcontinent	immunocompetant patients

(Summarized from [8, 22, 78, 99-102])

1.7 Leishmaniasis immunity and pathogenesis

The protective immune response to *Leishmania* infection is cell-mediated immunity. Th1 (T helper cell type 1) response correlates with resistance whereas Th2 response is associated with susceptibility to infection [103]. Several factors influence resistance or susceptibility to leishmaniasis including parasite strains, host genetics and environmental factors [40]. In general, the host determined antigen-specific T cell reactivity and cytokine secretion, the species and virulence of the infecting *Leishmania* species determine the ultimate clinical presentation of the disease. An overview of the host immune response to *Leishmania* is presented in Figure 1-1.

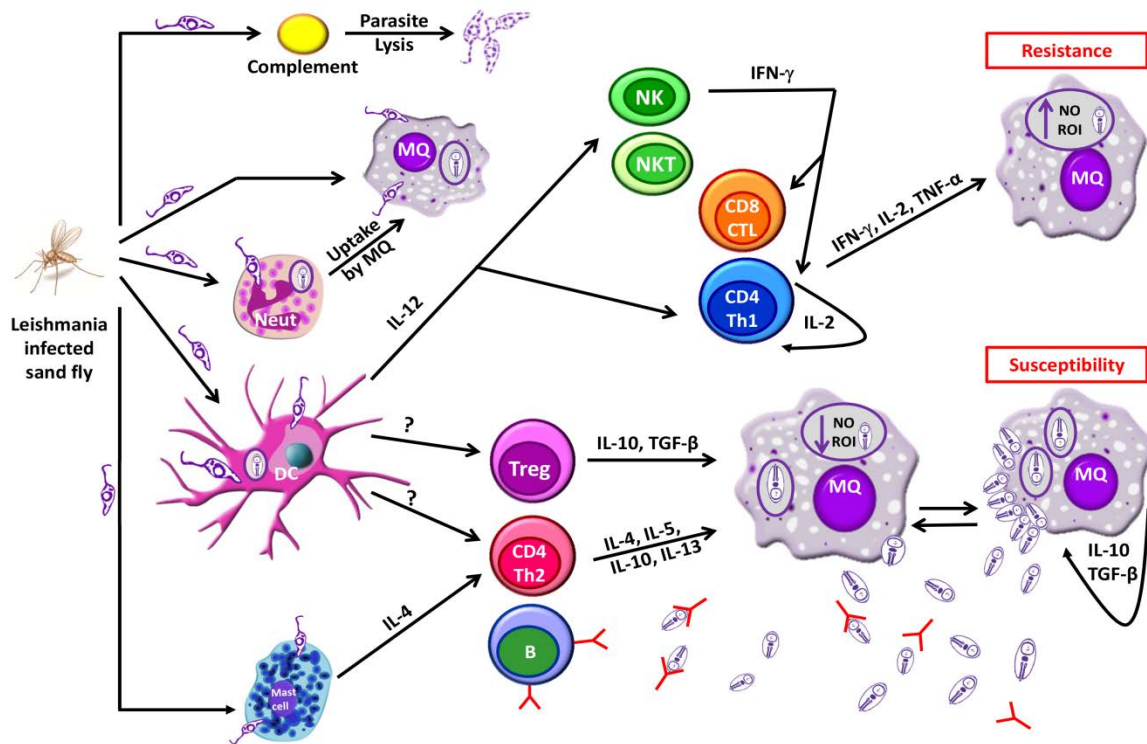


Figure 1-1: Innate and adaptive immune mechanisms underlying resistance/susceptibility to leishmaniasis.

During a blood meal, an infected sandfly deposits saliva containing metacyclic promastigotes into the dermis of a mammalian host. In addition to containing anti-coagulant properties, some salivary proteins may initiate local inflammatory responses that could either enhance parasite survival or enhance host immune response. The majority of parasites are lysed by the membrane attack complex of the complement system. The remaining parasites are phagocytosed by macrophages (MQ) where they transform into amastigotes. In addition to macrophages, some parasites may be taken up by neutrophils (Neut), dendritic cells (DC) and mast cells. Infection of neutrophils may facilitate escape from complement-mediated lysis and serve as a means (Trojan horses) for delivering parasites to macrophages. In resistant animals, infected DCs produce IL-12 that primes CD4⁺ T cells towards Th1 response and IFN- γ production leading to activation of infected MQs, the production of nitric oxide (NO) and reactive oxygen intermediates (ROI) and ultimately parasite destruction and resistance. IL-12 may also prime natural killer (NK) and (natural killer T) NKT cells to produce IFN- γ that further promote CD4⁺ Th1 and cytotoxic CD8⁺ T cell responses. The pathways leading to induction of CD4⁺ Th2 cells that results in susceptibility are not very well defined although IL-4 produced by non T cells (including mast cells) are thought to contribute to this process. Once CD4⁺ Th2 cells are induced, their cytokines (including IL-4, IL-5, IL-10 and IL-13) deactivate infected macrophages and downregulate NO and ROI production leading to unregulated parasite replication and persistence. In addition, IL-10 and TGF- β produced by CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) contribute to the downregulation of parasiticidal activities of infected macrophages. Furthermore, during infection, *Leishmania*-specific B cells are also activated to produce anti-*Leishmania* antibodies. The uptake of antibody-coated parasites by macrophages enhances their IL-10 and TGF- β production, which act in an autocrine manner to further downregulate parasite killing (adapted/modified from [40, 102, 104-111]).

1.7.1 Host immune response to visceral leishmaniasis

1.7.1.1 Innate immune response

Following the deposition of infective metacyclic promastigotes into the dermis, the skin innate immune system detects invading promastigotes, recruits inflammatory cells to sites of invasion within minutes and promotes the induction of adaptive immunity [112]. Initial sensing of the parasite involves pattern recognition receptors (PRRs) and complement receptors (CRs) present on different cell types including neutrophils, macrophages, dendritic cells (DCs) and natural killer (NK) cells. Several toll-like receptors (TLR) such as TLR2, TLR3 [113], TLR4 [114], TLR7 [115] and TLR9 [114] have been shown to contribute to innate sensing and recognition of *Leishmania* by various innate immune cells. This recognition leads to activation of intracellular signaling pathways that are necessary for the initiation of inflammatory responses and control of parasite proliferation by the innate immune response [116].

Neutrophils are essential cells involved in inflammatory response and contribute to phagocytosis and killing of microbial pathogens. Neutrophils are recruited to the site of infection shortly after parasites are introduced into the skin and have dual protective and permissive role during the establishment of infection and in chronic phase of disease [117]. It has been previously demonstrated that *Leishmania* parasites are able to delay the process of neutrophil apoptosis [118] and the infected neutrophils signal macrophages, through appearance of phosphatidyl serine [119] or via release of MIP-1 α and MIP-1 β [120], to engulf them. In this regard, neutrophils have been suggested to function as “Trojan horses” ensuring the entry of *Leishmania* into macrophages as their safe havens [121, 122]. Trojan horse notion has both been supported [123] and denied [124] in *L. major* infection models. However, reports indicate that

the Trojan horse theory might not be correct in case of *L. donovani* infection; as despite uptake of *L. donovani* promastigotes by human neutrophils, majority of the cells surprisingly die 3 hours after *in vitro* stimulation probably due to the release of neutrophil extracellular traps (NETs) [125]. Therefore, the precise role of neutrophils in VL remains to be addressed.

It has been previously shown that the turnover and activity of granulocytes including neutrophil and eosinophil are increased in human cases of VL [126]. Despite their efficient leishmanicidal machinery, *L. donovani* has developed some strategies to transiently survive inside neutrophils. For instance, *L. donovani* promastigotes inhibit activation of oxidative burst, avoid being targeted to lytic compartments such as lysosome via their surface lipophosphoglycans (LPG) and induce rapid release of neutrophil extracellular traps (NETs), which leads to containment of parasite at the site of inoculation thereby facilitating their uptake by mononuclear phagocytes [127]. Neutrophils also play an important role in early control of parasite growth in the spleen but not in the liver. Neutrophil depletion at the beginning of *L. donovani* infection leads to increase in parasite burden in the spleen and bone marrow but not in the liver, enhanced splenomegaly, a delay in the maturation of hepatic granulomas and a decrease in inducible nitric oxide synthase (iNOS) expression within granulomas [128]. It appears that the absence of neutrophils provided a perfect milieu for the development of non-protective Th2 responses as evident by a dramatic increase in serum levels of IL-4 and IL-10 and a significant increase in the ratio of *L. donovani*-specific serum Immunoglobulin G1 (IgG1)/IgG2a levels [128]. The importance of neutrophils in resistance to VL is also supported by observations in dogs, which suggest that there might be neutrophil dysfunction depending on the different disease stage. In moderate stage of canine VL, superoxide production is decreased without any alteration in neutrophil viability. In contrast, dogs in very severe stage of the disease

show increased neutrophil apoptosis and decreased superoxide production, which was associated with uremia [129].

The early interaction of *Leishmania* with macrophages and DCs and its influence on the host immune response have been reviewed previously [112]. Both macrophages and DCs phagocytose *Leishmania* and contribute to the initial decision processes that regulate resistance and/or susceptibility. For example, infected DCs produce interleukin-12 (IL-12), which is critical for the development of Interferon gamma (IFN- γ) producing CD4⁺ Th1 cells. Failure to produce functional IL-12 by DCs leads to progressive expansion of Th2 cells, upregulation of arginase activity in macrophages and parasite proliferation. In contrast to DCs, *Leishmania*-infected macrophages are unable to produce IL-12 and are therefore believed to play no significant role in the initiation of protective adaptive immunity [130].

Although promastigotes are capable of directly invading DCs and macrophages following their deposition by infected sandflies, several TLRs have been shown to contribute to this process and play vital role in the production of proinflammatory cytokines that are critical for immunity [103]. In particular, TLR2 and TLR3 participate in the phagocytosis of *L. donovani* promastigotes by macrophages [113]. Interestingly, during *L. donovani* infection, the parasite modulates TLR2 expression and signaling to suppress IL-12 production [131]. TLR3 is involved in the leishmaniacidal activity of IFN- γ primed macrophages [113]. A recent report showed that TLR7-mediated activation of interferon regulatory factor 5 (IRF-5) is essential for the development of Th1 responses to *L. donovani* in the spleen during chronic infection [115]. IRF-5 also indirectly regulates iNOS expression in infected cells [115].

In addition to the TLRs, CRs also contribute to detection of *Leishmania* infection and the initiation of inflammatory responses. Following injection of promastigotes into the skin, both the classical and alternative pathways of the complement system are activated [132] and within a few minutes after serum contact, more than 90% of all inoculated parasites are lysed via the membrane attack complex [107]. Interestingly, certain species of the parasite have evolved to exploit this pathway to enhance their infectivity and survival in the host. For example, metacyclic promastigotes of *L. infantum* (syn *L. chagasi*) enter macrophages via the CR3 and this transiently subverts macrophage activities [112].

During the initial uptake and phagocytosis of *Leishmania* promastigotes, macrophage produces superoxide anions. Following macrophage activation by IFN- γ , the second and most important anti-*leishmania* event occurs via nitric oxide (NO) generation. Failure to activate macrophages, resulting from the absence of a proinflammatory response and NO production, leads to invasion of *L. donovani* promastigotes within the mammalian host and their successful establishment [133]. Infection with *Leishmania* also stimulates the production of IFN- γ from NK cells via IL-2-dependent and independent pathways. Since DCs (and not macrophages) are the critical source of early IL-12 production following *Leishmania* infection, DC-T cell interaction are thought to provide the microenvironment for initial NK cell activation [130].

1.7.1.2 Adaptive immune response

The activation of the adaptive immune system during active VL in humans leads to the production of both macrophage-activating (IFN- γ and tumor necrosis factor- α (TNF- α)) and deactivating (IL-10 and transforming growth factor- β (TGF- β)) cytokines mainly in the spleen and liver [134]. The balance between these activating and deactivating factors ultimately dictates

the outcome of infection, pathology and the ensuing disease. Unlike murine models of most *Leishmania* infections, there is no clear dichotomy in Th1 and Th2 cytokines in human VL and their impacts on resistance and susceptibility is not clearly defined. However, patients with active VL have higher plasma levels of IL-10, IL-4, IFN- γ , TNF- α and IL-12 relative to asymptomatic and cured subjects [135]. *L. donovani* derived-exosomes have been recently implicated in this immunomodulatory process by promoting IL-10 production and inhibiting TNF- α production by human monocytes [136].

Experimental studies in mice suggest that the control of VL may be associated with the development of parasite-specific cell-mediated immune responses involving both CD4⁺ and CD8⁺ T cells [134]. These cells produce IFN- γ , which activates infected macrophages leading to the production of NO and other free radicals that kill the parasites. DCs activate CD8⁺ T cells through mechanisms that involve antigen cross presentation [137]. In contrast, susceptibility to *L. infantum* or *L. donovani* in infected mice is associated with the production of high amounts of IL-10, which is a potent immunosuppressive and anti-inflammatory cytokine [138]. Both conventional CD4⁺ T cells and regulatory T cells (Tregs) have been shown to contribute to IL-10 secretion and immunosuppression in VL [139]. In addition to IL-10, the production of TGF- β 1 by Tregs also contributes to disease pathogenesis and susceptibility [140]. In VL, there is a fine line between immune responses that effectively control parasite growth and induce long-term immunity and those that allow parasite persistence and associated disease. Thus, differences in splenic and hepatic tissue microenvironments dictate differences in the ability to generate effective immune responses and parasite control in these organs.

As in other forms of leishmaniasis, studies have shown that sandfly salivary proteins play a crucial role in shaping the quality of adaptive immunity in VL. For instance, vaccination with a DNA encoding an 11-kDa salivary protein from *Lutzomyia longipalpis* called LJM19, protected hamsters against an otherwise fatal challenge with virulent *L. chagasi* mixed with *L. longipalpis* saliva [141]. This protection was associated with high IFN- γ /TGF- β ratio, increased iNOS expression in the spleens and livers and a strong DTH response [141]. Interestingly, vaccination with LJM19 induced a rather weak response in the dogs, which is main reservoir of VL [142]. Reverse antigen screening studies have identified other salivary proteins (LJL143 and LJM17) of sandfly capable of inducing protection against experimental VL [142]. Vaccination with these proteins induces strong systemic and local Th1 cell-mediated immune response in dogs characterized by remarkable IFN- γ and IL-12 expression [142]. Notably, this protective response was recalled by sandfly bites and increased leishmaniacidal activity of macrophages *in vitro* [142]. It has been demonstrated that a proteophosphoglycan-rich mucin-like gel is secreted by *L. infantum* inside the midgut of *L. longipalpis* and this is regurgitated along with parasites during a blood meal [143]. Interestingly, Rogers *et al.* have demonstrated that *L. infantum*-derived proteophosphoglycans enhances parasite establishment at the site of inoculation suggesting that *L. longipalpis* saliva could synergistically enhance establishment of infection in the the skin [144].

1.7.1.2.1 Regulatory T cells (Tregs) and Leishmaniasis

Tregs are mainly viewed as critical mediators for immunosuppression and therefore are important for maintaining immune cell homeostasis and self-tolerance. However, recent studies have identified Tregs as cells that possess many diverse functions in immune regulation. Tregs

can be generally grouped into two categories based on cell surface markers or cytokine secretion profiles: naturally occurring Tregs (nTregs) and those induced in response to infectious challenge Tregs (inducible, iTregs) [145]. Conventional CD4⁺T cells can develop into T_R1 or T helper type 3 (T_H3) cells (examples of iTregs) by getting exposed to specific stimulatory conditions such as the blockade of costimulatory signals, deactivating cytokines or drugs. However, nTregs are developed during the normal process of maturation in the thymus and survive in the periphery as nTregs which express IL-2 receptor α -chain (CD25), type I transmembrane glycoprotein known as α E integrin (CD103), T cell inhibitory receptor cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), glucocorticoid-inducible tumor necrosis factor receptor (GITR) and unique transcription factor forkhead box P3 (Foxp3) [146, 147]. nTregs have the ability to respond to both self-antigens and antigens expressed by microbes [146]. Tregs not only have distinct immunosuppressive properties but have been shown to be able to become other types of effector Th cells (such as Th17 [148] or Th1 [149]) to promote and regulate immune responses. CD4⁺CD25⁺Foxp3⁺ nTregs (usually termed as Tregs) that express IL-10 and TGF- β consist of 5% – 10% of peripheral CD4⁺T cells in normal rodents and humans at steady-state conditions, have potent effects on the activity of both CD4⁺ and CD8⁺ T cells and play important roles in resistance to many pathogens [146, 150-156].

Published data from different investigators have shown that Tregs are involved in the direct induction of immunosuppression of effector immune response during chronic *Leishmania* infections [154], accumulate at the primary site of *L. major* infection in humans and mice [150, 157, 158] and mediate disease chronicity and their depletion leads to parasite clearance [146, 150, 158, 159]. Our laboratory has previously reported that lower number of CD4⁺CD25⁺Foxp3⁺ and antigen-specific IL-10⁺CD4⁺CD25⁺ T cells and defects in homing, expansion and/or

function of Tregs contribute to the resistance to *L. major* [160]. *L. infantum* infection has been shown to be associated with elevated levels of CD4⁺Foxp3⁺CD103⁺ Tregs producing TGF- β in the spleen and draining lymph nodes leading to inadequate Th1 and Th2 effector immune responses [139]. It has been demonstrated that CD40 can play differential roles in Treg differentiation and determine the course of *L. donovani* infection in BALB/c mice. DCs expressing low levels of CD40 were required for efficient Treg generation, whereas DCs expressing high levels of CD40 induced CD8⁺CD40⁺ effector T cells [161]. CD4⁺Foxp3⁺ Treg expansion has been demonstrated to be involved in hepatic *L. donovani* persistence in alymphoplastic *aly/aly* immunodeficient mice that lack lymph nodes and have disturbed spleen architecture [110]. Although both CD4⁺CD25⁺FoxP3⁺ Tregs and CD4⁺CD25⁺FoxP3⁻ effector T have been shown to produce IL-10 in VL patients; increased CD4⁺CD25⁺FoxP3⁺ antigen driven Treg expansion was observed in VL patients in the bone marrow during disease course and these Tregs persisted after successful chemotherapy [162].

Collectively, it can be concluded that Tregs play important roles in parasite persistence and disease reactivation/recurrence in both mice and human CL and VL due to changes in the host immune system (for example through HIV/AIDS), immunosuppressive treatment or ageing. We previously demonstrated that despite having an impaired Th1 and Th2 response, mice that lacks a functional PI3K (delta isoform) pathway were strongly resistant to CL due in part to having fewer numbers of CD4⁺CD25⁺Foxp3⁺ Tregs [160]. Whether this pathway also regulates Treg expansion and resistance to VL is unknown.

1.7.1.3 Liver vs. spleen immunity in visceral leishmaniasis

The development of new therapies or vaccines against VL is greatly hindered by the limited understanding of the specific immune mechanisms required for controlling parasite growth without inducing pathology in the liver and spleen. In most experimental models of VL, infection in the spleen remains chronic over a prolonged period of time whereas liver infections are self-resolving [115]. Resolution in the liver is associated with the development of controlled but effective granulomas that promote parasite clearance. In contrast, pathology is mediated by changes to the local tissue microenvironments that contribute to an inability to generate effective immune responses. Therefore, understanding the development of immunity in the liver may lead to potential means to improve parasite clearance in the spleen.

The development of inflammatory granulomas around infected liver macrophages leading to immunity is a T cell-driven event. This Th1-dominated response is mediated by TLR7, TLR8, TLR9, IL-1 and IL-18 via the Myeloid differentiation factor 88 (MyD88) signaling pathway [115]. An efficient granuloma formation involves the expression of iNOS by macrophages [163], which is regulated by several proinflammatory (Th1) cytokines such as IL-12, IFN- γ , TNF- α , lymphotoxin, and granulocyte/macrophage colony-stimulating factor (GM-CSF) [164] as well as intact and functional NK and NKT cells [105]. Interestingly, a report suggests that IL-4 (a prototypic Th2 cytokine known to inhibit granuloma formation) is also required for the resolution of hepatic infection and for priming of CD8⁺ T cells that are critical for long-term protection [137]. Although IL-10, TGF- β and IL-27 can suppress the control of *L. donovani* growth, they appear to have little impact on granuloma formation in the liver [165].

In VL, the spleen and bone marrow also become chronically infected by mechanisms that are less well understood. In experimental murine VL, the spleen becomes enlarged (akin to the condition in humans) and splenomegaly can account for up to 15% of the body weight of infected mice in as little as 6–8 weeks post-infection [106]. The persistence of parasites in the spleen is associated with changes in the splenic lymphoid microenvironment, concomitant increases in the rate of T cell apoptosis and decreased responsiveness to leishmanial antigens [106]. In addition, elevated levels of TNF- α in the spleen may contribute to this process since members of the TNF superfamily of cytokines are known to promote apoptotic cell death [166]. The production of IL-10 in the spleen contributes significantly to the establishment of infection and immunological dysfunction associated with the disease [106].

1.7.1.4 Subversion of host immune response in visceral leishmaniasis

Several pathogens including *Leishmania* have evolved mechanisms to subvert the host immune system in order to establish production infection and enhance their survival [167]. In the case of *Leishmania* that are obligate intracellular organisms, subversive events that enhance intracellular survival inside the mammalian host cells are important for their survival and disease pathogenesis. This is important given that *Leishmania* resides and replicates inside macrophages which are equipped with lytic enzymes capable of destroying infecting pathogens once the cell is appropriately activated. Hence, *Leishmania* parasites have evolved to actively subvert several aspects of the host cell signalling events, ranging from preventing the production of microbicidal molecules and protective cytokines to interfering with effective antigen presentation.

A number of virulence factors including surface and secreted molecules have been shown to actively interfere with macrophage activities *in vivo* and *in vitro*. These include fructose 1,6

bisphosphate aldolase, secreted acid phosphatase (SAP), elongation factor 1 α (EF-1 α), glycoprotein 63 (GP63), lipophosphoglycan (LPG) and peroxidoxins. For example, the interaction of *L. donovani* with EF-1 α secreted within exosomes and fructose 1,6 bisphosphate aldolase with host Src (Sarcoma) homology 2 domain phosphatase-1 (SHP-1) (tyrosine phosphatase) has been shown to inactivate macrophage [168]. Similarly, it has been proposed that lipid raft-dependent release of GP63 (a protease) by *L. donovani* promastigotes and its subsequent translocation into the macrophage cytosol leads to cleavage of several macrophage proteins including protein tyrosine phosphatase (PTP) thereby promoting macrophage deactivation [169]. In addition, *L. donovani* promastigotes and amastigotes secrete SAP, which have been shown to dephosphorylate host proteins [108].

To prevent activation of an effective immune response, *Leishmania* directly or indirectly suppress a number of cytokines involved in host immune response. For example, *L. donovani* promastigotes have the ability to inhibit IL-12 production in macrophages thereby impairing cell-mediated immune response and IFN- γ production [164]. Similarly, *L. chagasi* inhibits host immune responses by inducing TGF- β production in macrophages [170]. *Leishmania* also induces the production of IL-10 in macrophages, which causes suppression of IL-1, IL-12, TNF α and NO production and inhibits optimal expression of costimulatory molecules including B7-1/2 [171]. *L. donovani* inhibits antigen presentation by repressing the major histocompatibility complex (MHC) class I and II expression on macrophages [172]. Recently, our laboratory reported that *Leishmania* glycoprophosphate conjugates including phosphoglycans (PGs) and LPGs influence the host early immune response by inhibiting antigen presentation in DCs [173].

Another mechanism by which *L. donovani* limits leishmaniacidal activity of the host is by inhibiting the activities of some important kinases in macrophages. LPG from *L. donovani* strongly inhibits protein kinase C (PKC)-mediated intracellular signaling and its downstream functions [174, 175]. Similarly, *L. donovani*-infected macrophages are defective in their ability to phosphorylate Janus kinase 1 (JAK1), JAK2, and signal transducer and activator of transcription 1 (STAT1) proteins [108]. *L. donovani* also inhibits nuclear translocation of STAT1 α , activator protein 1 (AP-1) and nuclear factor *Kappa* light chain enhancer of activated B cells (NF- κ B) in macrophages [176]. *Leishmania* infection also leads to activation of the PI3K pathway in macrophages resulting in inhibition of macrophage apoptosis [177]. The enhancement of host cell survival consequently leads to pathogen replication, persistence and spread [177]. Furthermore, *L. donovani* infection of macrophage leads to alteration of mitogen-activated protein kinases (MAPK) pathway and promotes parasite survival and propagation within infected cell [177].

Manipulation of host PTP pathway is another mechanism by which *Leishmania* subverts the host immunity [108]. In *L. donovani*-infected macrophages, the activation of SHP-1 prevents IFN- γ dependent NO production via inactivation of JAK2 and extracellular signal regulated kinase 1/2 (ERK1/2) [178]. This results in failure of NF- κ B and AP-1 to translocate to the nucleus thereby inhibiting transcription of key genes including those involved in macrophage activation and production of proinflammatory cytokines and NO [178].

1.8 Phosphoinositide 3-kinases and leishmaniasis immunity

PI3Ks are a family of enzymes that phosphorylate phosphoinositides at the 3' position of the inositol ring, producing phosphatidylinositol (3)-monophosphate (PIP), phosphatidylinositol (3,4)-biphosphate (PIP₂), and phosphatidylinositol (3,4,5)-triphosphate (PIP₃). These generated phospholipid second messengers recruit and activate various intracellular enzymes involved in cellular functions such as regulating cell growth, differentiation, intracellular trafficking, motility, survival and apoptosis [179].

Based on structural similarities, substrate preference and coding genes, the PI3K family is divided into three classes (I, II, and III). The class I PI3Ks are further sub-divided into Class IA (PI3K α , PI3K β and PI3K δ) and Class IB (PI3K γ). The Class IA consists of heterodimers of one p110 catalytic subunit encoded by *PIK3CA* (p110 α), *PIK3CB* (p110 β) or *PIK3CD* (p110 δ) and one p85 regulatory subunit encoded by *PIK3R1* (p85 α), *PIK3R2* (p85 β) or *PIK3R3* (p85 γ). In contrast, Class IB contains a p110 γ catalytic subunit (encoded by *PIK3CG*) and a p101 or p84 regulatory subunit (encoded by *PIK3R5* or *PIK3R6*, respectively). PI3K α and PI3K β are ubiquitously expressed in all cells and tissues; however, PI3K γ and PI3K δ are mainly enriched in leukocytes [180].

Ligands acting through B and T cell receptors, cytokine receptors (e.g., IL-2), insulin receptor, insulin like GFR1 and TLRs activate the PI3K pathway an [181]. When activated, Class I PI3Ks are recruited to the cell membrane and bind to receptor tyrosine kinase (RTK), G protein-coupled receptor (GPCR), Ras or other adaptor proteins leading a subsequent conformational change. They switch to an active conformation and utilize PIP₂ to generate PIP₃. Consequently PIP₃ can bind to Pleckstrin homology (PH) domain containing molecules, such as

the protein kinase AKT and mediate membrane recruitment [182] and a mammalian target of rapamycin (mTOR) pathway [183]. Two major antagonists of PI3K are lipid phosphatases such as phosphatase and tensin homolog deleted on chromosome ten (PTEN) and the SH2 domain-containing inositol polyphosphatase (SHIP), which can remove the 3-phosphate and 5-phosphate from PIP3, respectively [184].

PI3Ks play an essential role in antigen receptor signaling in B and T lymphocytes [179]. The p110 δ and p110 γ isoforms play essential roles in immunity, and are important new therapeutic targets in inflammation and autoimmunity [185]. The PI3K pathway has been shown to either promote [186] or impede [187, 188] *Leishmania* parasite growth in macrophages in different experimental model systems. For example, *L. major* promastigotes can activate PI3K/Akt signaling in infected host macrophages and delay apoptosis, thereby giving ample time for parasites to complete their replication cycle [186] and therefore promote parasite growth. In contrast, macrophage-specific inhibition of PTEN [187, 188], abrogates efficient killing of parasites by infected macrophages [189] and inhibit parasite growth. Also, it has been shown that *L. donovani* parasites engage TLR2 receptor on macrophages and induce mTOR signaling in PI3K dependent and independent mechanisms thereby modulating TLR-induced IL-12 and IL-10 production [181].

Various genetic approaches have been used to investigate the functional importance of different PI3K isoforms. In this regard, several knockout (KO) or knock-in (KI) mice have been developed by using genetic approaches to target the catalytic or regulatory subunits of PI3K. Mice with an inactivating knock-in mutation in the p110 δ gene (termed as p110 δ^{D910A}) which have a catalytically inactive p110 δ due to change of Aspartic acid to Alanine at position 910, are

the focus of this thesis [190]. These mice have the following characteristics: (1) Have reduced phosphorylation of serine/threonine-specific protein kinase (Akt) and Forkhead box protein (Foxo) (a transcription factor that plays important role in regulation of gluconeogenesis and glycogenolysis by insulin signaling) [191]; (2) Are impaired in clonal expansion and helper T cell lineage differentiation into Th1 and Th2 subsets [191]; (3) Have impaired B and T cell antigen receptor signaling which leads to prominently reduced total number of peripheral mature T cells and decreased T cell proliferation [192-194]; (4) Produce reduced levels of IL-2, IL-4, and IFN- γ in response to stimulation with Ag [191]; (5) Exhibit altered Treg development, differentiation and function in the periphery. As such, although higher numbers of their thymocytes develop into Tregs in the thymus, the lymph nodes and spleens contain significantly reduced number of Foxp3⁺ Tregs [195]; (6) Do not have the ability to develop into CD38^{high} population (a transmembrane glycoprotein which has both enzymatic activity and which acts as a receptor) greatly suppressive Tregs [196]. Although all these findings are reported in regard to p110 δ ^{D910A} mice characteristics, but the role of PI3K pathway and its absence in immunity to parasitic infections is poorly understood.

Previous work from our laboratory show that p110 δ ^{D910A} mice are hyper-resistant to *L. major* (causative agent of CL), develop minimal or no cutaneous lesion, rapidly control parasite proliferation and mount suppressed Th1 and Th2 responses. The enhanced resistance of p110 δ ^{D910A} mice to *L. major* was independent of mouse genetic background and is associated with decreased numbers and function of Tregs at both the peripheral lymph nodes and cutaneous site of infection [160]. P110 δ ^{D910A} mice that healed their *L. major* infection display impaired secondary anti-*Leishmania* immune responses, manifested as poor delayed-type hypersensitivity (DTH) response, impaired IFN- γ recall response and the absence of faster and efficient parasite

control at the secondary challenge site. *Leishmania* reactive memory T cells from p110 δ ^{D910A} mice are unable to down-regulate cluster of differentiation 62 ligand (CD62L) expression upon secondary *L. major* challenge and failed to home to the site of infection due to their inability to convert central memory T cells (Tcms) into effector memory T cells (Tems) [197].

1.8.1 Phosphoinositide 3-kinase pharmacological inhibitors

Advances in PI3K pharmacological inhibitor development have been a major step forward in recent years and have become another powerful tool for functional studies in regard to PI3K activity. These inhibitors have permitted selective inhibition of individual PI3K family members (regulatory or catalytic subunits) *in vivo* and *in vitro* [198-200]. Different categories of PI3K inhibitors including (1) pan-PI3K inhibitors, (2) dual-PI3K/mTOR inhibitors and (3) isoform-specific PI3K inhibitors have been developed to date. Some of these inhibitors include: (1) Pan-PI3K inhibitors: BKM120 (Buparlisib), XL147, GDC-0941 (Pictilisib), BAY80-6946 (Copanlisib), PX-86644, CH5132799, Wortmannin and LY294002; (2) Dual-PI3K/mTOR inhibitors: XL765, BEZ235, GDC-0980, PF-04691502 and GSK2126458 and (3) Class I PI3K isoform-specific inhibitors: p110 α inhibitors: INK1117, BYL719 and GDC-0032, p110 β inhibitors: GSK-2636771, AZD8186 and TGX-221 and p110 δ inhibitors: CAL-101 (Idelalisib / GS-1101 / Zydelig), IPI-145, GSK2269557 and IC87114 [200-202].

Although some of these PI3K inhibitors have encountered problems in clinical trials due to limited efficacies as a monotherapeutic agent and relatively high rate of adverse side effects [202], but Pre-clinical or clinical trials are being conducted using them as potential targets for anti-inflammatory treatments or autoimmunity and cancer therapies. For example, the following inhibitors are in use in different stages of clinical trials: (1) BKM120 (phase 3) for metastatic

breast cancer (NCT01633060); (2) XL147 (phase 1) for malignant neoplasm (NCT01587040); (3) BYL-719 (phase 1/2) for recurrent or metastatic squamous cell carcinoma (NCT02145312); (4) GSK2269557 (phase 2) for chronic obstructive pulmonary disease (COPD) (NCT02294734); (5) GSK2126458 (phase 1) for pulmonary fibrosis (NCT01725139) and (6) BAY80-6946 (phase 2) for non-Hodgkin's lymphoma (NCT01660451) [203, 204].

In this study I focus on p110 δ specific inhibitors, IC87114 and the FDA approved CAL-10. Although IC87114 was the first p110 δ specific that was developed [205, 206] and still is being used in research [207, 208], CAL-101 has taken the lead in clinical and research applications because its inhibitory concentration (IC₅₀) is 2.5 nM, which is several hundred folds better than IC87114 (IC₅₀, 0.5 μ M). CAL-101 is also known as 5-fluoro-3-phenyl-2-[(1S)-1-(9H purin-6-ylamino)propyl] quinazolin-4(3H)-one) or C₂₂H₁₈FN₇O and has a low molecular weight of 415.4 g/mol [209]. There are two pathways involved in primary CAL-101 metabolism; one is via oxidation by aldehyde oxidase (AO) to its major circulating plasma metabolite, GS-563117; and the other is oxidation by cytochrome P450 3A (CYP3A) and glucuronidation by uridine 50-diphospho-glucuronosyltransferase 1A4 (UGT1A4) [209]. In addition to being a substrate for CYP3A, CAL-101 can also inhibit p-glycoprotein, organic anion transport proteins and CYP3A [210]. After a very successful phases I, II and III clinical trials, a major step forward has been the approval of CAL-101(also called idelalisib) for different B-cell malignancies [198-200]. Monotherapy for patients with relapsed follicular B-cell non-Hodgkin lymphoma [211] and small lymphocytic lymphoma, and CAL-101 and rituximab combination therapy for those with relapsed chronic lymphocytic leukemia (CLL) have been approved by US Food and Drug Administration (FDA) in 2014 [198]. The adverse side effects associate with CAL-101 are fatal and/or serious hepatotoxicity, diarrhea, colitis, and fatal and serious pneumonia or intestinal

perforation as indicate in the fact sheet provided by FDA [212, 213]. At the same time, very low IC_{50} of 2.5 nM has been associated with the consumption of CAL-101, which in turn allows for the administration of relatively higher doses leading to enhanced target and pathway suppression and efficacy [211, 214].

1.9 Hepatic stellate cells, fibrosis and leishmaniasis immunity

Hepatic stellate cells (HSCs) are liver stromal cells located in the sinusoidal space. They regulate sinusoidal blood flow, are involved in maintenance of hepatic architecture and production of various growth factors and cytokines [215]. HSCs also play critical role in deposition of extra cellular matrix (ECM) in chronic liver injury [216], liver fibrogenesis [217], excess collagen deposition during fibrosis [218], and liver repair [219]. Their contribution in liver repair stems from their exhibition of progenitor cell properties [220] and support of liver hematopoiesis after injury due to their mesenchymal stem cells (MSC) properties [221].

Several important parasitic infections are associated with fibrosis which is a pathological process arising from abnormal and continuous wound repair processes [222, 223]. Fibrosis occurs when the normal wound healing response, which after injury is initiated to synthesise new connective tissue, fails to terminate [224]. Following chronic liver injury, inflammatory lymphocytes infiltrate the hepatic parenchyma. Some hepatocytes undergo apoptosis which leads to the release of inflammatory cytokines and soluble factors and thus activation of Kupffer cells (KCs) and release of fibrogenic mediators which contribute to collagen synthesis. This inflammatory milieu stimulates the activation of resident quiescent vitamin A storing HSCs into fibrogenic myofibroblasts. HSCs proliferate and undergo a dramatic phenotypical activation, express smooth muscle α -actin (α SMA) and secrete large amounts of ECM proteins [223]

including type I collagen [218]. If the liver injury persists, it leads to tissue fibrosis, whereas if the cause of the liver injury is removed, fibrosis is resolved through apoptosis of activated HSCs, regeneration of hepatocytes [225] and increased activity of matrix metalloproteinases (MMPs) [223].

Although the roles of HSCs have been extensively studied in liver fibrosis, cirrhosis, hepatitis C and B infections and liver transplantation, there are not many studies performed on identifying the role of HSCs in parasitic diseases. Limited research on *Schistosoma japonicum* have revealed significant role of these cells in liver pathology associated with schistosomiasis. HSCs have been demonstrated to be present in the periphery of *S. japonicum* egg granulomas in murine and human infections and are likely involved in granuloma associated fibrosis [226] through expression of chemokines and recruitment of eosinophils, neutrophils, macrophages [227], increased proliferation, transdifferentiation into myofibroblasts, secretion of ECM molecules and ECM remodeling [228].

1.9.1 HSC localization, nomenclature and origin

HSCs are resident nonparenchymal cells located in the subendothelial space of Disse, between the basolateral surface of hepatocytes and the antiluminal side of sinusoidal endothelial cells. They comprise approximately one-third of the non-parenchymal cell population and ~10-15% of the total number of liver resident cells and ~1.5% of total liver volume in normal human liver [229-231].

HSCs have been identified and named by many different researchers since 1876. For example, they have been termed as sternzellen, perisinusoidal cells and vitamin A storing cells

by Kupffer [232] and Wake [233], hepatic collagen-producing lipocytes by Friedman [234], hepatic pericytes by Zimmerman, fat-storing cells and Ito cells by Ito [235, 236], and precursors to the fibroblasts by Kent [237]. Finally in 1996, investigators agreed to the current globally used nomenclature for these cells as hepatic stellate cells [230, 238, 239].

HSCs have been thought to be of embryologic or neural crest origin. Embryologic origin is supported by localization of these cells in the septum transversum mesenchyme during liver development [240] or by having a common endoderm origin with hepatoblasts [241]. Neural crest origin is supported by their expression of neural markers such as, glial fibrillary acidic protein (GFAP), nestin, neurotrophins and synaptophysin [242].

1.9.2 Quiescent HSCs vs. activated HSCs

The number, phenotype and cytokine profile of quiescent and activated HSCs in the liver is altered in different phases and conditions. There are fundamental differences between quiescent HSCs / HSCs in normal liver (Figure 1-2) and activated HSCs / HSCs in injured liver (Figure 1-3) which have been listed in Table 1-4.

Transdifferentiation of quiescent HSC to myofibroblasts is facilitated through autocrine and paracrine stimulation of different mediators produced by several liver resident cell types and migratory inflammatory cells with HSCs. Of these cells and the produced mediators, hepatocytes (producing IGF, ROS, TGF- β), KCs (producing TNF- α , ROS, TGF- β), existing myofibroblasts (producing TGF- β , PDGF), sinusoidal endothelial cells (producing VEGF, ICAM-1, TGF- β), CD8 cells, neutrophils (producing: ROS) and platelets (producing PDGF, EGF, TGF- β) can be named [243, 244].

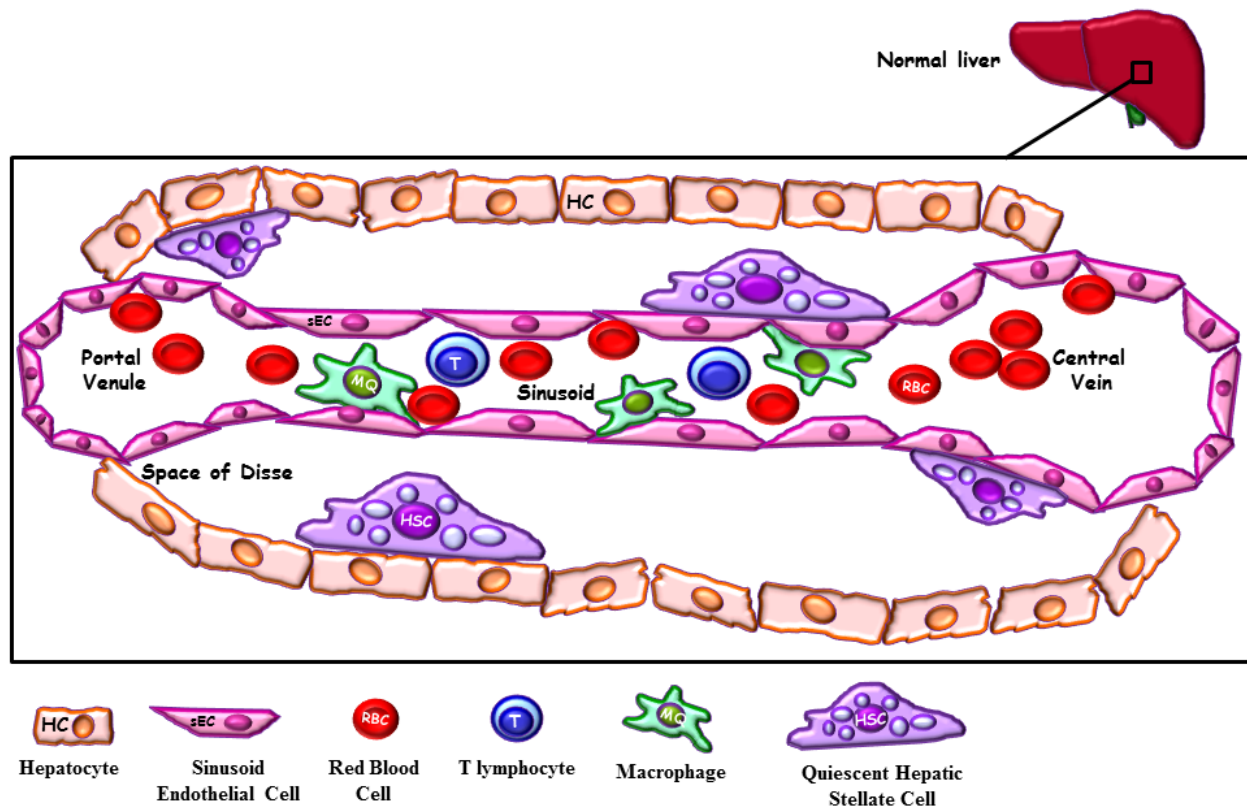


Figure 1-2: Quiescent hepatic stellate cells in normal liver

Quiescent HSCs are located in the subendothelial space of Disse, between the basolateral surface of hepatocytes and the antiluminal side of sinusoidal endothelial cells. They store 80–90% of liver vitamin A and have round appearance with autofluorescence due to the lipid droplets which are retinoid storing vacuoles. Since the different compartments of the liver are intact in a normal state, HSCs do not come in contact with red blood cells (RBCs) or leucocytes (for example: T lymphocytes and Macrophages) and therefore remain in their quiescent form.

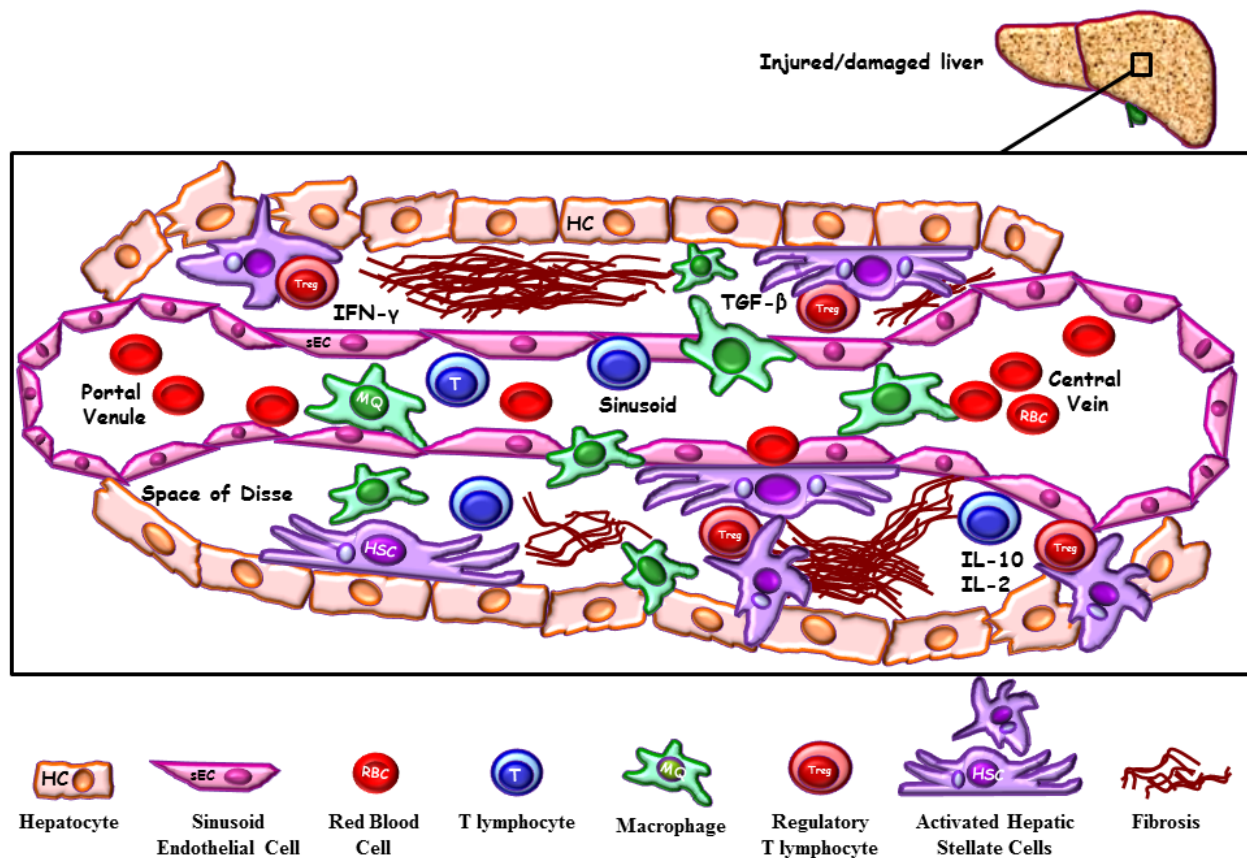


Figure 1-3: Activated hepatic stellate cells in injured/damaged liver

Activated HSCs are located in the space of Disse, between the hepatocytes and the sinusoidal endothelial cells. During liver injury, the hepatocyte and sinusoidal endothelial cell layers that surround HSCs become damaged and consequently the resident HSCs come in contact with leucocytes (for example: T lymphocytes and Macrophages) that enter the space of Disse. This cell-cell contact (i.e HSC-macrophage (KC) or HSC-Treg) and the cytokine milieu (i.e IFN- γ , IL-10, TGF- β and IL-2) that is produced by different cell types in the space of Disse allows for activation of HSCs. HSCs obtain a large flat appearance and this process leads to loss of lipid droplets and increased proliferation and ECM production by HSCs which ultimately results in fibrosis.

Table 1-4: Quiescent hepatic stellate cells (HSCs) in normal liver vs. activated HSCs in injured/damaged liver

Quiescent HSCs / HSCs in Normal liver	Activated HSCs / HSCs in injured liver	Ref
Store 80–90% of liver vitamin A in the form of retinyl esters in cytoplasmic lipid droplets which consist of ~50–80% of total retinoid of the body	Lose lipid droplets	[222, 242, 245]
Decrease fibrogenesis	Increase fibrogenesis	[222]
Have round appearance with autofluorescence	Have large flat appearance	[222, 239]
Proliferate less	Proliferate more via inducing PDGF- β , VEGF and FGF	[230]
Reduced ECM production	Increased ECM production	[230, 246]
Express transcription factors that maintain HSC quiescence (PPAR- γ , FXR, PXR, LXR, RXR)	Express transcription factors that regulate inflammation and apoptosis (NF- κ B), ECM deposition, collagen expression and fibrogenesis (SMAD2, SMAD3, SMAD4, NF-1, AP-2), proliferation and α -SMA expression (AP-1, PPAR β)	[222, 230, 231, 247]
Are involved in vasoregulation through endothelial cell interactions	Transdifferentiate into α -SMA expressing contractile myofibroblasts, which contribute to vascular alterations and increased vascular resistance	[231]
Maintain immune tolerance via storing vitamin A and producing TGF- β	produce intensified inflammation and immune regulation responses	[243, 248, 249]
Express high levels of surface class I MHC and low levels of T cell costimulatory molecules (CD40, CD80, and CD86)	Express high levels of class II MHC, adherent markers (ICAM-1 and VCAM) and programmed cell death markers (PD-L1 and Fas-L)	[250]
Are vital in development of intrahepatic bile ducts during development	Increased autophagy to induce fibrogenesis via lipid droplet mobilization, liberation of FFAs, and mitochondrial β -oxidation	[251, 252]
Promote maturation of hepatic progenitors through cell-cell contact in culture		[220] [253]

1.9.3 Primary HSC isolation techniques and HSC cell lines

Different techniques have been utilized to isolate rat, mouse or human HSCs, including: (1) *In situ* digestion using enzyme perfusion followed by density gradient centrifugation [254, 255] (2), Cell sorting, based on endogenous vitamin A fluorescence [256, 257] (the technology is costly and yields are lower), (3) Explant culture of activated human HSCs obtained from liver biopsy material (early events in cellular activation are not tracked) [258], and (4) Administration of CL2MDP [259] or GdCl₃ [250] to selectively eliminate or block activity of KCs from mouse livers (yields highly purified cells and is inexpensive). A major issue with all these HSC isolation techniques is that under normal cell culture conditions, quiescent HSC spontaneously activate into the myofibroblast phenotype [222] and this limits our understanding of the characteristics and function of these cells under physiologic conditions *in vivo*. There are two ways to possibly resolve this issue: one is to culture HSCs on plates coated with the basement membrane-like matrix (Englebreth Holm Sarcoma (EHS) matrix) which renders these cells to non-proliferative and non-fibrogenic cells [260]; the other solution is to study these cells direct *ex vivo*, which is the approach I have developed and used in this thesis.

Immortal HSC cell lines that have the *in vivo* activated phenotype features and overcome the need for primary cell isolation have been developed and offer a ready to use supply of cells in studies related to liver fibrosis. To name a few, HSC-T6, PAV-1 and PQ are rat HSC cell lines and L190, GRX, LX-1 and LX-2 are human HCS cell lines used to date [230, 261].

There are some advantages and disadvantages to using HSC cell lines. The advantages are that: (1) They grow continuously; (2) Have almost an unlimited lifespan which allow for longterm experiments; (3) Have a homogenous and specific phenotype; (4) They are easily

available; (5) Have simple culture conditions which are easily standardized among different laboratories. On the other hand, the disadvantages are that: (1) The cell lines are prone to genotypic, karyotypic and phenotypic drift during prolonged culture time; (2) Sub-populations may ascend by the selection of specific, more rapidly growing sub-clones that may cause cell line heterogeneity; (3) Differences in morphology, growth characteristics and irregularities of chromosome number and structure are also very problematic [262].

1.9.4 Markers used to identify HSCs

HSCs can be identified via a variety of markers such as (1) ectoderm origin markers (glial fibrillary acidic protein (GFAP), nestin, neurotrophins and their receptors, nerve growth factor (NGF), brain-derived neurotrophic factor, synaptophysin and N-CAM), (2) mesoderm origin markers (vimentin, desmin, α -SMA and hematopoietic markers) [231] and (3) Lecithin retinol acyltransferase (LRAT).

In this thesis, I have identified HSCs by GFAP, Desmin, α -SMA and LRAT markers; and synaptophysin was targeted in HSC depletion experiments. While GFAP is originally expressed by astrocytes in the central nervous system (CNS) and is required for blood-brain barrier repair following brain injury [263]; it is also expressed by HSCs and is involved in vascular remodeling in damaged hepatic tissues during the development of liver fibrosis [264] and can be used both as a marker for quiescent HSCs [265] or as an early marker of HSC activation [266]. Desmin, originally known as a muscle-specific type III intermediate filament, is expressed at low levels during early development of muscle cells and in higher levels as the cells near terminal differentiation [267]. Although reports indicate the presence of desmin positive HSCs around blood vessels in fetal liver [268], trans-differentiation of HSCs into myofibroblast has also been

shown to be associated with increased desmin synthesis and formation of desmin-containing intermediate filaments which is Vimentin dependent [269]. α -SMA, which is an actin isoform, was originally known to be a specific marker for smooth muscle cell differentiation [270], but due to the myofibroblastic phenotype of HSCs after activation and transdifferentiation, α -SMA has also been used as a marker for activated HSCs [271, 272]. Although recent reports argue their specificity in activated HSCs and injured liver and demonstrate that α -SMA can also be a marker of quiescent HSCs in normal liver depending on the actual definition of normal liver and the sensitivity of the detection techniques [273]. LRAT is a physiological retinol esterification enzyme that incorporates retinol into the retina and adjusts its concentration to maintain visual function [274]. It also plays a role in storing systemic retinoid in rodent and human liver [275]. Recently, this marker has successfully been used to specifically identify liver HSCs by different authors [275, 276]. Synaptophysin is a plasma membrane protein primarily associated with neural tissues and is responsible for release and/or uptake of neurotransmitters in the synapse [277].

In both human and rodent liver, synaptophysin is expressed on the surface of activated HSC-derived myofibroblasts [278]. Its extracellular domain can be internalized via endocytic vesicles. In the HSC depletion experiments performed, this is the mechanism by which myofibroblasts take up the human recombinant single-chain antibody (scAb termed C1-3 [279]) that is targeting the extracellular region of synaptophysin [280]. On the other hand, free gliotoxin has been shown to stimulate the apoptosis of liver myofibroblasts [281] and to a lesser degree the apoptosis of hepatocytes and KCs [282, 283]. Hence, conjugating the scAb to gliotoxin allows for specific depletion/apoptosis of synaptophysin positive activated HSCs/myofibroblasts. This is the method by which liver HSCs are depleted in this thesis

1.9.5 HSCs and the PI3K pathway

The PI3K pathway has been demonstrated to be very important in different aspects of HSC function and activation. For example: (1) PI3K is involved in increased expression of PDGF- β receptors that are markers of HSC activation and transdifferentiation into myofibroblasts [284]; (2) Fibrogenic response triggered by HSCs is stimulated by activation of PI3K/Akt pathway through inducing cell proliferation (transduced through focal adhesion kinase (FAK), PI3K, and Akt) and type I collagen expression [285]; (3) PI3K is required for HSC antioxidant response and myofibroblastic transdifferentiation in liver fibrosis [286, 287]; (4) Procollagen $\alpha 1(I)$ translation is induced in HSCs during fibrosis by leucine (a profibrogenic branched-chain amino acid) via ERK and PI3K/Akt/mTOR activation and ROS stimulation [288, 289] and also by leptin (profibrogenic hormone) mediated by the PI3K/Akt pathway through activated JAK1 [290]; (5) HSC survival during liver fibrosis is mediated by engulfment of apoptotic bodies in a JAK/STAT and PI3K/Akt/NF- κ B dependent manner [291]; (6) TGF- $\beta 1$ plays a crucial role in the development of hepatic fibrosis through activating HSCs via PI3K pathway [292]; (7) PI3K/Akt pathway activation plays a critical role in the early regenerative response of the liver after resection [293]; (8) Inhibition of PI3K signaling in HSCs during active fibrogenesis blocks the progression of hepatic fibrosis by inhibiting ECM deposition and proliferation through reducing expression of profibrogenic factors and type I collagen synthesis [218] and also by inducing apoptosis in HSCs [225]. Collectively, these observations indicate that PI3K signaling is critical for HSC activation and function in both normal and injured liver. However, the isoform of PI3K responsible for the effects observed in HSCs is not known and whether PI3K signaling influences HSC response during *Leishmania* infections has not been determined. This gap in knowledge is addressed in this thesis.

1.9.6 HSCs and hepatic immune regulation

Liver inflammatory response to viral diseases, alcohol consumption and autoimmunity is mediated by recruitment of different immune cells (such as macrophages and neutrophils) and production of cytokines and chemokines that lead to tissue regeneration and deposition of ECM by activated HSCs. Both adaptive and innate immune responses contribute to chronic inflammation and fibrosis and determine the outcome of liver injury [294]. Mild inflammatory responses are beneficial to the liver as they favor the reestablishment of tissue homeostasis, whereas, excessive or chronic inflammation aggravate liver injury by promoting fibrosis through HSC activation [295]. HSCs mediate immune regulation by acting as antigen presenting cells and producing proinflammatory and anti-inflammatory cytokines, chemokines and other factors involved in proliferation, fibrogenesis, chemoattraction, inflammatory responses, liver regeneration and apoptosis (listed in Table 1-5). HSCs have been shown to influence or affect liver immunity by different mechanisms that include: (1) Attracting IFN- γ secreting leukocytes to liver tissues by secretion of chemokines and proinflammatory as well as anti-inflammatory cytokines [296]; (2) Inducing NKT proliferation [297]; (3) Functioning as APCs via expressing retinoic acid early inducible-1 (RAE1), CD1d and MHC I and II and ultimately presenting peptides to CD4⁺ and CD8⁺ T cells and NKT cells [298, 299]; (4) Acting as regulatory bystanders by enhancing differentiation and accumulation of Tregs [300, 301]; (5) Expressing PRRs such as TLR-2 and TLR4 [302]; (6) Influencing CD4⁺ T cell differentiation by expanding pre-existing allogeneic CD4⁺CD25FoxP3⁺ Tregs in an IL-2-dependent manner (83) and generating *de novo* Foxp3⁺ Tregs from naive CD4⁺ T cells in (84); (7) Elimination of activated CD8⁺ T cells and expansion of Tregs in an IFN- γ dependent manner [217]; (8) Engulfing disease-associated CD4⁺, CD8⁺ and NK cells through activation of Ras-related C3 botulinum

toxin substrate 1 (Rac1) and cell division control protein 42 homolog (Cdc42) pathways, which leads to reduced CD4/CD8 ratio and NK cells in livers with advanced human fibrosis [303]; (9) Acting as liver profibrotic stimuli in the presence of IL-13 and IL-4 producing NKT and CD8 cells, IL-17 producing Th17 cells, IL-17 and ROS producing neutrophils, and TNF- α producing DCs and M1 macrophages [253]; (10) Functioning as antifibrotic agent activated by IFN- γ and TNF related apoptosis inducing Ligand (TRAIL) produced by NK and NKT cells, IL-22 produced by TH-17, IL-10 produced by M2 macrophages, Tregs, and bone marrow derived CD11b⁺Gr1⁺ immature cells, and MMP-9 produced by DCs [253]; (11) Mediating Fas-L induced death of allogeneic conventional CD4⁺ cells and MHC II dependent expansion of Tregs [250]. Despite all these findings in regard to the role of HSCs in liver immunity and the mechanism of action in different conditions, the impact of HSCs on liver immunity/pathogenesis in VL remains to be studied and identified. In this thesis, I have focused on the role of HSCs in liver immunity / pathogenesis following VL induced by *L. donovani* and its related mechanism of action.

Table 1-5: HSCs and hepatic immune regulation.

Function / Action	Example
Chemokine and chemokine receptor production [229-231, 252, 296, 304]	Secrete: MCP-1 (CCL2), MIP-1, RANTES (CCL5) Express: B and T lymphocytes and DC chemoattractant (CCR7, CCL21), neutrophil chemoattractant (CXCL1), Eosinophil chemoattractant (Eotaxin, CCL5), peripheral blood-derived DCs, CD34 ⁺ hematopoietic progenitor cell and activated/memory Th1 lymphocyte chemoattractant (CCR5)
Toll-like receptors (TLRs) expression [302, 305]	Express TLR4 and TLR2 in response to a range of PAMPs including LPS
Complement protein production [306]	Express: C4
Cytokine production [217, 249, 292, 300, 301, 307-309]	Secrete: IL-6, IL-5, IL-2, IFN- γ , TGF- β , TGF- α , M-CSF, TNF- α , IL-10, IL-1 β , IL-1- α , IL-17
Function as antigen presenting cells [250, 310]	Express: CD40, CD80, CD86, PDL-1, MHC-I, MHC-II
Growth factor production [311-313]	VEGF, IGF-I, PDGF-B, HGF, aFGF, bFGF
Transcription factor expression [247, 314, 315]	<u>Expressed highly by quiescent HSCs:</u> Nuclear hormone receptors (PPAR- γ , FXR, PXR, LXR, RXR, Vitamin D Receptor, Estrogen Receptor, Glucocorticoid Receptor) (maintain HSC quiescence) <u>Expressed highly by activated HSCs:</u> NF- κ B (regulate inflammation and apoptosis), SMAD2, SMAD3, SMAD4, NF-1, AP-2 (regulate ECM deposition, collagen expression and fibrogenesis), AP-1, PPAR β (regulate proliferation and α -SMA expression)
Antifibrotic activity [244, 316-318]	Downregulate: MMP2, TIMP1 Express: Adiponectin
Fibrosis induction [224, 234, 235, 243, 272, 289, 290, 294, 319-323]	Upregulate: TIMP1, MMP2, TGF- β 1, α -SMA, MCP-1 (by IL-8 producing Foxp3 ⁺ CD4 ⁺ Tregs) Express: Leptin, leucine, Type I and II Collagens
Exhibit properties of stem/progenitor cells and are liver resident mesenchymal stem cells (MSC) [220, 221]	Express: CD133 (stem cell marker)
Display Phagocytic properties [291, 303, 324, 325]	Extracellular galectin-3 and lymphocyte engulfment induce HSC phagocytosis which lead to fibrosis

1.10 Vaccines and vaccination strategies against visceral leishmaniasis

Despite the global public health importance of leishmaniasis, progress in developing vaccines against the disease has lagged because of some key technical hurdles and the fact that the disease occurs mostly in the world's poorest countries. The current status of scientific and technical progress in the development of novel NTD vaccines (such as leishmaniasis) and many *in vivo* experimental models for vaccine screening and their respective advantages and disadvantages have been thoroughly reviewed [32, 36, 326, 327]. Currently, there is no animal model that accurately reproduces all the unique features of human VL. Although the Syrian golden hamster (*Mesocricetus auratus*) provides a more synchronic infection in the liver and spleen resulting in chronic infection and death if untreated (akin to human VL) [58], the limited availability of reagents severely hampers its use for immunologic studies. In contrast, although reagents are commonly available for murine studies, inbred strains of mice show varying degrees of susceptibility to VL and variable clinical and pathological features with human disease. In general, several vaccination and/or control strategies have been used and/or proposed including leishmanization, vaccination with virulent or genetically modified live-attenuated parasites, killed parasites, whole-cell lysates/purified fractions, parasite protein components or subunits, whole native/recombinant parasite proteins, plasmid DNA and viral vector-based vaccine candidates encoding parasite virulence factors [328].

There are currently only four vaccines against leishmaniasis: a killed vaccine for immunotherapy in Brazil, a live vaccine in Uzbekistan, a recombinant vaccine for prophylaxis in dogs in Brazil [329] and an European canine vaccine (CaniLeish) prepared from purified excreted-secreted proteins of *L. infantum* [327, 330]. CaniLeish has been shown to induce a

strong and effective Th1-dominated anti-*L. infantum* response in vaccinated dogs, which was associated with significant reduction in parasite burden and disease intensity in vaccinated animals [330]. However, the impact of this vaccine on the overall immune system of the vaccinated dogs is currently unknown [331]. In addition, the efficacy of these vaccines remains controversial [329]. In order to develop a safe, effective and practical vaccine against VL, the identification of protective antigens and improvement of antigen delivery platforms and vaccine formulations that could induce effective T-cell responses are critical and challenging hurdles that must be overcome. In addition, a comprehensive proteomics approach is needed in order to identify cross protective *Leishmania* antigens that could protect against both cutaneous and visceral forms of the disease.

Several vaccination studies (at least in experimental models of the disease) point to the feasibility of developing an effective *Leishmania* vaccine. Vaccination with the *Leishmania* histone H1 protein has been reported to induce strong protection against VL caused by *L. infantum* in mice. The protection was associated with enhancement of IFN- γ production by T cells and a reduction in IL-10 production by DCs from vaccinated mice [332]. Recently, the safety and efficacy of a recombinant *Leishmania* polyprotein vaccine, Leish-F1 in MPL-SE adjuvant against VL was tested in India. The vaccine was shown to be safe and well-tolerated in subjects with and without history of previous *Leishmania* infection [333]. In addition, the vaccine was also strongly immunogenic and induced IFN- γ production by T-cells [333], suggesting that it may offer some level of protection against the disease. The efficacy of Leish-F1 vaccine has also been tested in CL in Brazil. As in VL, the vaccine was found to be safe and immunogenic in CL patients. Interestingly it shortened the time to cure in patients when combined with MA which is a pentavalent antimonial drug used for VL therapy [334]. Perhaps,

the most encouraging vaccine that has raised high hopes for potential development of a vaccine against human VL is Leishmune, a purified glycoprotein derived from fucose-mannose ligand of *L. donovani* promastigotes and formulated in a saponin adjuvant [335]. Leishmune is a transmission blocking vaccine that has been demonstrated to be effective against canine VL in both prophylactic and immunotherapeutic manners [336]. One of the major groups of virulence factors in *Leishmania* belongs to cysteine proteinase (CP) family. Considering the essential role of dogs in the pathogenesis of the VL, Rafati S. *et al.* have proposed that the combination of DNA and recombinant protein vaccination using CP type I and II of *L. infantum* can interrupt the domestic cycle of the disease [337]. In addition, they have reported that VL prime-boost vaccination based on CP type III induces appropriate humoral and cellular immune responses *in vivo* that leads to lower parasite load [338].

Because of the importance of live parasites in the maintenance of anti-*Leishmania* immunity [339] and the proven efficacy of leishmanization against CL [340] and VL [341], several studies have focused on live-attenuated organisms as viable vaccine candidates against leishmaniasis. Attenuated parasites have the advantage of being infectious but not pathogenic, and as such are able to be taken up by the host APCs in the same compartment as virulent organisms. In addition, they may persist long enough to induce appropriate immune response without causing disease [342]. In line with this, a genetically modified live-attenuated *L. donovani* lacking the centrin gene (termed LdCen^{-/-}) has been shown to induce protection in BALB/c mice and Syrian hamsters against homologous and heterologous *Leishmania* challenges [343]. A recent study also showed that LdCen^{-/-} parasites induce strong humoral and cell-mediated immune responses in vaccinated dogs [344]. Although protection against virulent

challenge was not assessed in this study, the induction of strong CD4⁺ and CD8⁺ responses suggested that this may be a viable vaccine candidate against canine VL.

One of the concerns of live-attenuated vaccine is the potential of the parasites to revert to virulence, which could lead to the development of active disease, particularly in immune compromised individuals. As a result, efforts are being made to utilize non-pathogenic parasites as potential vaccine candidates. The lizard *Leishmania* specie, *L. tarentolae* has been shown to activate DC maturation processes and induce strong protective CD4⁺ Th1 cell-mediated immunity against *L. donovani* challenge in mice [345]. In addition, recombinant expression of amastigote-specific *L. donovani* A2 antigen in *L. tarentolae* elicits protection against *L. infantum* challenge in mice [345]. Recently, a nanotechnological approach consisting of DNA/live and live/live prime-boost vaccination strategies against *L. infantum* infection in BALB/c mice was carried out using a recombinant *L. tarentolae* expressing the *L. donovani* A2 antigen along with CP A and B [346]. The results show that recombinant *L. tarentolae*-expressing tri-fusion proteins elicited a strong protective response and protected vaccinated mice against VL caused by *L. infantum* [346].

1.11 Immunotherapy of visceral leishmaniasis

Despite significant advances in the treatment of VL, there are still concerted efforts towards developing new therapies and treatment regimens. This is because most conventional anti-leishmanial therapeutic strategies have several drawbacks such as difficulty in administration, prolonged duration of treatment, toxicity, high cost of treatment, emergence of drug resistant strains and disease relapse [4, 40]. Therefore, immunotherapy, which is the use of biological substances to modulate or modify immune responses in order to achieve a

prophylactic and/or therapeutic goal, has been proposed as a rational treatment option for leishmaniasis. The rationale behind immunotherapy against VL stems from the belief that following infection, progressive disease occurs when there is either a failure, suboptimal or excessive host immune response against the parasite. Hence, it is reasoned that the induction of appropriate immune modulation or interventions using immunomodulatory agents or biological response modifiers could reverse or ameliorate disease progression [347]. In addition, immunotherapy prior to or in synergy with conventional therapy, may lower the required drug dose and treatment regimen, reduce drug toxicity, improve drug efficacy, reduce emergence of drug resistant strains and consequently reduce the chances of disease relapse [40].

Efforts to use immunotherapy as an alternative treatment for leishmaniasis have been mostly focused on human CL in South America. This involves the administration of killed parasites either alone or in combination with adjuvants (such as *Bacillus Calmette–Guérin* (BCG)) or chemotherapeutic agents to patients with chronic cutaneous disease. Such combination treatments were shown to be effective in curing American CL [348]. Due to of the similarities in certain aspects of the immunopathogenesis of CL and VL, it is plausible that similar immunotherapeutic endeavors utilizing killed parasites, adjuvants and anti-*Leishmania* compounds could also be beneficial in the treatment of VL. Indeed, it has been shown that a combination of killed parasites, BCG and SSG (which is a pentavalent antimonial VL therapy) was very effective in curing persistent post-kala-azar dermal leishmaniasis (PKDL) in East Africa [349]. The combined therapy successfully cured the disease in 87% of patients, which was significantly different from those that received the drug alone [349].

Studies in experimental CL have shown that vaccinating *Leishmania*-infected mice with DNA expressing immunogenic proteins could ameliorate the disease and in some cases lead to cure [350]. This cure was attributed to the vaccine's ability to shift existing disease-promoting Th2 cytokine towards a protective Th1 cytokine response [350]. Similarly, vaccination with a recombinant DNA expressing the *L. chagasi* nucleoside hydrolase protein (NH36-DNA) modulates the outcome of VL in BALB/c mice previously infected with *L. chagasi*, leading to survival from an otherwise lethal disease [351]. A single dose of a recombinant adenoviral vector containing *L. donovani* antigens, HASPB and KMP11, significantly reduced liver parasite burden in mice previously infected with *L. donovani*. This immunotherapeutic effect was associated with increased antigen-specific CD4⁺ and CD8⁺ T cell responses resulting in enhanced DTH [352]. Thus, the inherent adjuvant activity of the adenoviral vector provides a novel vaccination strategy for administering immunotherapeutic candidate genes to hosts with existing VL infection [352]. Collectively, these experimental murine studies suggest that DNA vaccines may induce immunomodulatory activities in human VL patients that could result in disease cure.

Experimental studies in mice suggest that the outcome of VL may also be altered by the administration of subunit vaccines. A vaccine containing the fucose mannose ligand (FML) protein induced a significant reduction in liver parasite load and decrease in IL-10 level in mice experimentally infected with *L. donovani* [353]. Similarly, Leishmune®, the first commercial licensed vaccine against canine leishmaniasis that consist of FML fractions in saponin adjuvant has been shown to lower clinical disease and parasite burden when administered several months after *L. donovani* infection [354]. This protection was associated with recovery of CD4⁺ T cell response and decreased serum antibody levels [354]. These few encouraging reports highlight the potential of DNA or subunit vaccines as immunotherapeutic strategy for the treatment of VL.

Another strategy with potential immunotherapeutic implication is targeting of cytokines. As with CL, protective immunity against VL is dependent on an IL-12-driven IFN- γ production by T cells. In contrast, susceptibility is usually associated with the preferential expansion of T cells that produce immunoregulatory cytokines [355]. Thus, targeting immune imbalances to preferentially expand protective T cell responses provides a rational strategy for treatment of VL. For example, high levels of TGF- β , a cytokine that suppresses lymphocyte activation, has been shown to be partly responsible for the immunosuppression observed in experimental leishmaniasis by inducing T cell apoptosis [356]. Therefore, targeting TGF- β may potentially minimize lymphocyte apoptosis and restore the suppressed cell-mediated immunity in the host, which could result in cure. Similarly, VL is associated with high levels of serum IL-10 as well as production of high levels of IL-10 by splenic cells [357]. Treatment of mice infected with *L. donovani* with anti-IL-10R mAb has been shown to result in significant amelioration of the disease [358]. Thus, targeting this cytokine could be potentially effective in treating human VL.

Because progression of VL is usually associated with increased Th2 and a concomitant decreased Th1 cytokine response, it is conceivable that a combination of Th1-inducing cytokines or immunostimulants with chemotherapy could enhance Th1 response and possibly lead to cure of clinical disease. For example, while treatment with IFN- γ alone was not beneficial in VL treatment in India [359], a combination of IFN- γ and MA in Guatemala was more effective than MA alone in treating patients infected with *L. braziliensis* [360].

Another mediator that could hold potential immunotherapeutic promise in treatment of leishmaniasis is interferon inducible protein 10 (IP-10), a CXC chemokine. Treatment of infected BALB/c mice with recombinant IP-10 induced a strong protective Th1 immune response that

was associated with marked decrease in TFG- β and IL-10-secreting CD4⁺ T cells. In addition, IP-10-mediated decrease in production of immunosuppressive cytokines was correlated with a marked reduction in the frequency of CD4⁺CD25⁺ Tregs [361]. Thus, a detailed mechanistic insight into IP-10-mediated regulation of Tregs and enhanced immunity during VL might be helpful in designing immunotherapeutic intervention strategies against the disease.

1.12 General project rationale, hypothesis and specific aims

1.12.1 Rationale

Despite numerous publications on host-pathogen interactions in visceral leishmaniasis, full understanding of the nature and mechanisms that regulate these interactions still remain unclear. Among the host factors essential in regulating disease pathogenesis in leishmaniasis, the phosphoinositide 3 kinase (PI3K) pathway, which is crucial for cell differentiation, proliferation, migration and survival, plays fundamental role in immune response. Our laboratory recently investigated the outcome of infection of mice with inactivating knock-in mutation in the p110 δ gene (p110 δ^{D910A} mice) with *L. major* - the causative agent of CL. The results revealed that p110 δ^{D910A} mice develop minimal or no cutaneous lesion and rapidly clear their parasite despite mounting suppressed T helper cell responses. The enhanced resistance in p110 δ^{D910A} mice to *L. major* was independent of mouse genetic background and was associated with decreased numbers and function of regulatory T cells (Tregs) at both the peripheral lymph nodes and cutaneous site of infection and dramatic amelioration of inflammatory response [160, 197]. These studies reveal that the inhibition of a kinase offers protection against Leishmaniasis. Other than going against the dogma that the quantity and quality of Th1/Th2 cell responses regulate the outcome of infection with *L. major*, our studies also highlight the importance of p110 δ isoform of PI3K signaling in the regulation of T cell-mediated immunity and suggest that targeting this pathway could be a viable alternative for treatment of leishmaniasis. Whether the PI3K pathway also controls resistance to *L. donovani*, the causative agent of VL, is not known. Furthermore, whether treatment of *Leishmania*-infected mice with a pharmacologic inhibitor of this enzyme would result in cure of leishmaniasis is unknown. As the resistance observed in p110 δ^{D910A} mice to *L. major* was in part due to impaired expansion and function of Tregs [160] and liver resident hepatic stellate cells (HSCs) have been reported to selectively expand or induce Tregs [219, 250, 319, 362] and the PI3K signaling pathway is important in HSC activation and function [218, 285], therefore, I also sought to investigate the role of HSCs in immunity to VL in this thesis.

1.12.2 Hypothesis

I hypothesize that PI3K regulates immunity to Visceral Leishmaniasis.

1.12.3 Specific aims

1. Investigate the role of PI3K in immunity to Visceral Leishmaniasis.
 - 1.1. Are p110 δ^{D910A} mice resistant or susceptible to *L. donovani* infection?
 - 1.2. What is the nature of immune response in *L. donovani*-infected p110 δ^{D910A} mice compared to wild type (WT) mice?
 - 1.3. What is the mechanism through which signaling via the p110 δ isoform of PI3K regulates resistance/susceptibility to *L. donovani*
2. Determine the contribution of hepatic stellate cells (HSCs) to pathogenesis of *L. donovani*-infection in WT and p110 δ^{D910A} mice?
 - 2.1. What is the number, activation and cytokine status of HSCs before and after *L. donovani* infection in WT and p110 δ^{D910A} mice?
 - 2.2. What is the effect of *in vivo* depletion of HSC on outcome of *L. donovani* infection in WT and p110 δ^{D910A} mice?
 - 2.3 Does *L. donovani* infect and proliferate in HSCs?
 - 2.4 What is the mechanism through which HSCs influence outcome of *L. donovani* infection by assessing its effects on Treg expansion/induction and function in *L. donovani* infected and uninfected WT and p110 δ^{D910A} mice?
3. Determine whether treatment of *Leishmania* infected mice with a pharmacological inhibitor of p110 δ will cure Leishmaniasis.
 - 3.1. Does treatment of mice with IC87114 or CAL-101 prevent/cure leishmaniasis?
 - 3.2. How does IC87114 or CAL-101 treatment with or without Amphotericin-B alter the host immune response in *Leishmania*-infected mice?
 - 3.3. Is combination treatment with CAL-101 and Amphotericin-B more effective in curing VL than single treatment with either agent alone?

2 CHAPTER 2: Materials and Methods

2.1 Mice

Female BALB/c mice were purchased from GMC, University of Manitoba. C57BL/6 (B6) mice that express an inactive form of p110 δ isoform of PI3K (termed p110 δ^{D910A}) were generated by introducing a germline point mutation into the p110 δ gene as previously described [190]. BALB/c p110 δ^{D910A} mice were bred at the GMC facility of the University of Manitoba and were originally generated by backcrossing B6 p110 δ^{D910A} mice onto the BALB/c background for more than 12 generations. All mice were maintained at the University of Manitoba Animal Care facility under specific pathogen-free conditions and used according to guidelines stipulated by the Canadian Council for Animal Care. The studies were approved by the University of Manitoba Animal Care and Use Committee (Protocol Approval number 12-072).

2.2 Infection and parasite quantification

Leishmania donovani (strain LV9) or *L. major* (MHOM/IL/80/Friedlin) were grown in 25 cm tissue culture flask (Corning, VWR, Mississauga, ON) containing 10 or 20 ml complete M199 insect culture medium (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT), 2 mM glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin for 7 days in a 27°C parasite incubator (Thermo Fisher, Ottawa, ON). On day 7 post culture, parasites were washed and centrifuged (Eppendorf 5810R, Mississauga, ON) at 3000 rpm for 15 minutes. The pellet was re-suspended in phosphate buffered saline (PBS) (Invitrogen). A 1: 100 dilution was made and parasites were counted with a haemocytometer (Fisher Scientific, Whitby,

ON), under a light microscope at x40 magnification. In most experiments, mice were injected i.v. with 5×10^7 stationary phase promastigotes or 1×10^7 amastigotes (isolated from spleen of 8-10 wk. infected hamsters) in 100 μ l PBS suspension. Parasite burden in the spleen and liver was determined by limiting dilution assay [363].

To quantify liver parasite burden, *L. donovani*-infected liver were collected, homogenized in a tissue grinder (Fisher) with DMEM medium, washed and centrifuged at 600 rpm for 5 minutes. The supernatant was collected and centrifuged at 3000 rpm for 15 minutes. The pellet was resuspended in 2 ml Schneider medium (Invitrogen) supplemented with 20% heat inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 25 mM HEPES (Invitrogen) (complete Schneider medium). Parasites (20 μ l) were seeded in the first row of a flat bottom 96 well plate, containing 180 μ l/well complete Schneider medium. The rest of the wells were filled with 100 μ l/well complete Schneider medium. Two-fold serial dilutions were made (in two plates) and the last 100 μ l was discarded from the second plate. Plates were wrapped in plastic wrap, incubated at 27°C and parasite growth was assessed by light microscopy on day seven post *in vitro* culture. To quantify spleen parasite burden, *L. donovani* infected spleen were collected, grinded and homogenized through a cell strainer with DMEM medium, washed and centrifuged at 3000 rpm for 15 minutes. The pellet was resuspended in 2 ml complete Schneider medium and parasite burden was determined via twofold serial dilutions as described above.

In some experiments, mice were infected with 10^4 7-days stationary phase *L. major* (MHOM/IL/80/Friedlin) promastigotes resuspended in 100 μ l PBS, into the right hind footpad. Lesion sizes were monitored weekly by measuring footpad swellings with calipers.

To determine footpad parasite burden, *L. major* infected feet were cut off above the ankle and transferred sequentially every five minutes into 70% ethanol, chlorhexiderm, (DVM Pharmaceuticals, Maimi, FL), 70% ethanol and finally into 100 µg/ml streptomycin in PBS (2x P/S) (Invitrogen). Toes and skin were removed from feet using stainless steel blade, homogenized in a tissue grinder (Fisher) with 2x P/S, washed and centrifuged at 500 rpm for 5 minutes. The supernatant was collected and centrifuged at 3000 rpm for 15 minutes. The pellet was re-suspended in 2 ml complete Schneider medium (Invitrogen) supplemented with 20% heat inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100µg/ml streptomycin, 25 mM HEPES (Invitrogen). Parasites (20 µl) were seeded in flat bottom 96 well plates, containing 180 µl/well complete Schneider medium. Ten-fold serial dilutions were made and the last 20 µl was discarded. Plates were wrapped in plastic wrap, incubated at 27°C and parasite growth was assessed by light microscopy on day seven post *in vitro* culture. To measure lymph node parasite burden, *L. donovani* infected lymph node were collected, ground and homogenized through a cell strainer with DMEM medium, washed and centrifuged at 3000 rpm for 15 minutes. The pellet was resuspended in 2 ml complete Schneider medium and parasite burden was determined via ten-fold serial dilutions as described above.

2.3 *In vitro* infection of bone marrow-derived macrophages (BMDMs)

Bone marrow cells were isolated from the femur and tibia of WT and p110δ^{D910A} mice. The cells were differentiated into macrophages (BMDMs) using RPMI complete medium (RPMI supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin) supplemented with 30% L929 cell culture supernatant. BMDMs were harvested on day 7 and infected at a cell-to-parasite ratio of 1:5. After 5 hr. of infection, free

parasites were washed away and infected cells were further cultured for 24-72 hr. and the level of infection was determined by counting Giemsa-stained cytospin preparations under light microscope at x100 (oil) objective.

2.4 Isolation of spleen, liver, lymph node and blood lymphocytes and flow cytometry

At different days post infection, mice were sacrificed and the spleens, livers, lymph nodes (popliteal, inguinal and mediastinal) and blood were collected according to experimental design. The spleens and lymph nodes were homogenized in 10 ml DMEM media using tissue grinders and centrifuged at 1000 rpm for 5 min and made into single cell suspensions. The livers were digested with collagenase D (125 µg/ml) for 30 min. in 37°C, homogenized in complete DMEM (DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin) and liver lymphocytes were separated using percoll gradient centrifugation. Liver cells were resuspended in 40% percoll, layered on top of 70% percoll and centrifuged at 750 g for 20 min at 22°C. After centrifugation, the interface layer containing lymphocytes was harvested and washed twice in complete DMEM medium [364]. Blood cells were depleted of red blood cells with Ammonium-Chloride-Potassium (ACK) lysis buffer and washed with complete DMEM medium.

All spleen, liver, lymph node and blood cells were counted and according to each experimental design, were directly stained *ex vivo* for CD3, CD4, CD8, CD25, CD127, GITR (extracellular staining) and Foxp3 (intracellular staining using BD Biosciences Foxp3 Staining Kit) expression for phenotypic flow cytometry analyses. In some experiments, cells were also

directly stained *ex vivo* for intracellular cytokine analysis according to each experimental design [160]. Briefly, cells were stimulated with 50 ng/ml PMA, 500 ng/ml ionomycin, and 10 µg/ml Brefeldin A for 4 hr, surface-stained with specific fluorochrome-conjugated mAbs against CD3, CD4 and CD8, fixed with 2% paraformaldehyde (Sigma-Aldrich), permeabilized with 0.1% saponin buffer (Sigma-Aldrich) and stained intracellularly for IFN- γ , IL-4, IL-17 and IL-10. For Foxp3 intracellular staining, the fixation and permeabilization buffers (available as a kit) and fluorochrome-conjugated antibody was obtained from eBioscience. Samples were acquired on a FACSCanto II cytometer (BD Bioscience, San Diego, CA) and analyzed using Flowjo software (Tree Star, Ashland, OR). A list of all the antibodies used for flowcytometry in this thesis is present in table.

Table 2-1: Antibodies used for flowcytometry

Antibody	Clone	Fluorochrome	Company
CD4	RM4-5 and GK1.5	FITC, PE, APC, APC-Cy7, perCP-Cy5.5, Pacific Blue, APC-Alexa Flour750, eFluor450, PE-Cy7, PE/Cy5	eBioscience
CD3	17A2 and 145-2C11	FITC, PE, APC, eFluor450, APC-Cy7, Pacific Blue	eBioscience
CD8	53-6.7	FITC, PE, APC, PE, eFluor450, PE/Cy5	eBioscience
CD49b	DX5	FITC	eBioscience
F4/80	CI:A3-1 and BM8	FITC, PE/Cy5	eBioscience
CD11c	N418	PE, APC, Pacific Blue, FITC	eBioscience
CD11b	M1/70	PE, APC, FITC, Pacific Blue, PE/Cy5	eBioscience
MHC II	M5/114.15.2	PE, APC, FITC, eFluor450	eBioscience
MHC I	SF1-1.1.1	eFluor450	eBioscience
CD80	16-10A1	APC, PE, FITC	eBioscience
CD 86	GL1	APC, PE	eBioscience
CD40	HM40-3 and 3/23	PE, APC, FITC, Pacific Blue	eBioscience, Biolegend
IL-4	11B11	PE, APC	eBioscience
IL-17	PAJ-17R	PE	eBioscience
IL-10	JESS-16E3	PE, APC, FITC	eBioscience
IFN- γ	XMG1.2	Pacific Blue, eFluor450, PE, APC, perCP-Cy5.5, Alexa Flour 700, FITC	eBioscience
CD25	PC61	FITC, PE, APC	eBioscience
GITR	YGITR 765	PE	eBioscience
FoxP3	FJK-16s	FITC, PE, APC	eBioscience
CD127	A7R34	PE, FITC	eBioscience
Desmin	Y66	Alexa Fluor488	Abcam
GFAP	1B4	PE, Alexa Flour488	BD biosciences
α -SMA	1A4	PE, APC	R&D Systems
LRAT	M34-P1F10		EMD Millipore Corporation
F(ab') ₂ Anti-Mouse IgG	polyclonal	PE	eBioscience
IgG2b K Isotype Control	eBMG2b and RTK4530	Alexa Flour488, APC, PE	eBioscience
IgG2a K Isotype Control	eBM2a and RTK2758	APC, FITC, PE	eBioscience
IgG1 K Isotype Control	P3.6.2.8.1, RTK4530 and RTK2071	eFluor450, APC, FITC, PE	eBioscience
IgG Isotype Control	HTK888	APC, PE	eBioscience

2.5 *In vivo* expansion of Tregs

Tregs were selectively expanded *in vivo* by injecting mice with IL-2-anti-IL-2 mAb immune complexes according to recently published reports [365, 366]. Briefly, rIL-2 (1 µg, PeproTech, Rocky Hill, NJ) was mixed with anti-IL-2 mAb (5 µg, clone JES6-1, BD Bioscience) and incubated at 37°C for 30 min. WT and p110δ^{D910A} mice were injected i.p. with the immune complex once a day for 3 days. Three days after the last injection, mice were infected with 5 x 10⁷ stationary phase *L. donovani* promastigotes. Thereafter, the immune complex was administered once weekly until mice were sacrificed.

2.6 *In vivo* Treg depletion

Mice were injected i.p. with anti-CD25 mAb (clone PC61) or isotype-matched control rat IgG1 antibody (100 µg/mouse) to deplete Tregs [367]. Three days after antibody administration, mice were infected with *L. donovani* and the antibody was administered once weekly for additional 2 or 4 wk.

2.7 *In vitro* recall responses and cytokine ELISA

Single cell suspensions of cells from the liver and spleen of infected mice were resuspended at 4 × 10⁶/ml in complete DMEM medium, plated at 1 ml/well in 24-well tissue culture plates and stimulated with freeze thawed *L. donovani* (10 µg/ml). After 72 hr., the supernatant fluids were collected and assayed for cytokines (IL-4, IL-12, IL-10, IL-17 and IFN-γ) by ELISA using paired antibodies (Biolegend, San Diego, CA) according to manufacturer's suggested protocols. Binding ELISA plates (Immulon® VWR, Mississauga, ON) were coated

with 50 µl/well primary antibodies (Biolegend, San Diego, CA) at a concentration of 1 µg/ml and incubated overnight at 4°C. Plates were washed 5 times with wash buffer (1x PBS, 0.05 % Tween 20 (Sigma) pH 7.4) using the automated ELISA washing machine BIOTEK ELX405 plate washer (Winooski, VT). Blocking buffer solution (5% new calf serum in PBS pH 7.4) was added to all wells (200µl) and incubated for 2 hours at 37°C, to block nonspecific bindings. Plates were then washed again with wash buffer using the automated washer. Recombinant cytokine (2 ng/ml, Preprotech) was applied to the plate and titrated 2 fold in dilution buffer. Samples were appropriately diluted in dilution buffer, titrated 2 folds and incubated overnight at 4°C. After plates were washed 50 µl of biotinylated detection antibody (2 µg/ml, Biolegend) in dilution buffer was added to all wells. Plates were incubated for 1-2 hours at 37°C, washed, and streptavidin horseradishperoxidase (1:3000 dilution, BD Pharmagen, San Jose, CA) in dilution buffer was added to all wells and incubated for 30 min. at 37°C. Plates were washed 10 times and two component ABTS substrate (Mandel Scientific, Guelph, ON) was added to plates. Plates were read at 405 nm (Spectra Max) after the appropriate color development. In some cases, the cytokine levels were determined by 13plex FlowCytomix Multiplex kit from BD Biosciences and were acquired on the FACSCanto II cytometer (BD Bioscience, San Diego, CA) according to BD Biosciences protocols and analyzed using the free BD Biosciences software.

2.8 Measurement of serum antibody levels and NO assay

At sacrifice, serum was obtained from infected mice and used to determine the levels of anti-*Leishmania*-specific antibody titers (IgG, IgM, IgG1 and IgG2a) by ELISA [368]. ELISA plates were coated with 10 µg/ml freeze thawed *L. donovani* overnight at 4°C. Coated plates

were then incubated with blocking buffer (2% BSA in washing buffer) for 2 hours at 37°C and then washed (PBS, 0.05% Tween 20, 0.02% NaN₃, pH 7.4). Serum samples and standards were serially diluted using ELISA dilution buffer (1:10 dilution of blocking buffer with wash buffer) and incubated at 37°C for 2 hours. Plates were washed and bound antibody levels were detected using biotinylated anti-mouse IgG, IgM, IgG1 or IgG2a (Southern Biotech). After incubating overnight at 4°C, plates were washed and incubated with streptavidin alkaline phosphatase for 1 hr. at room temperature. Following washing, p-nitrophenyl phosphate tablets (Sigma Aldrich) were dissolved in ELISA substrate solution and then added to each well. Absorbance was read at 405nm and 690nm using a Molecular Devices plate reader.

NO levels were measured in 72 hr. culture supernatant fluids of spleen and liver lymphocytes of *L. donovani*-infected mice that were stimulated with freeze-thawed *L. donovani* by measuring nitrite concentration using the Griess assay [369]. A solution of one part sulfanilamide (1%) in and one part Naphthylethylenediamine dihydrochloride (0.1%) in 2.5% H₃PO₄ was mixed with the sample in a 1:1 ratio. The intensity of the fuchsia colour developed in the samples due to the presence of nitrite and its reaction with the solutions, was determined spectrophotometrically at a wavelength of 600 nm.

2.9 Assessment of hepatic granuloma

The granulomatous response to infection in the liver was assessed in histologic sections stained with hematoxylin and eosin at 2, 4 and 8 weeks post infection [370, 371]. At each time point, sections from at least 6 individual mice were analyzed in each group. Granuloma formation was scored as follows: (1) Ineffective granulomas: large quantities of mononuclear cells forming adjacent to sinusoids with no mononuclear cell infiltration to the tissue; (2)

Developing granulomas: some functional mononuclear cellular infiltration at the parasitized focus; (3) Mature granulomas: a core of functional fused infected KCs surrounded by a well-developed epithelioid-type mononuclear cell mantle.

2.10 Hepatic stellate cell isolation and direct *ex vivo* characterisation by flow cytometry and confocal microscopy

HSCs were isolated from *L. donovani*-infected and naive WT and p110δ^{D910A} mice [372, 373]. Livers of sacrificed mice were perfused *in situ* through the inferior vena cava with solution I (10x HBSS, 100x HEPES, 100x NaHCO₃, 100x Glucose, 50x EGTA and double distilled water) followed by solution II (solution I supplemented with 100x CaCl₂ and 150 µg/ml of Collagenase D). Thereafter, the livers were excised, mashed and digested at 37°C with 10 ml solution III (Solution II supplemented with 0.4 mg/ml Pronase and 20 µg/ml DNAase). After 20 min., the tissue slurry was filtered, washed in RPMI and HSCs were isolated via an Optiprep (Nycondenz) gradient column, washed and their viability was assessed by trypan blue exclusion. Isolated HSCs were stained and characterised by flow cytometry directly *ex vivo*, or cultured at 37°C in RPMI supplemented with 20% fetal calf serum (FCS), penicillin, streptomycin, L-glutamine-200, 100x sodium pyruvate and 100x MEM NEAA (complete HSC medium) for different days at 37°C for use in different experiments. In most experiments, the purity of HSCs was also determined by typical light microscopic appearance of the lipid droplets [219]. For direct *ex vivo* characterization, HSCs were stained and assessed by flow cytometry for expression of various surface markers associated with HSC including GFAP [372], α-SMA [372], LRAT [276] and Desmin [250]. F4/80 and CD11b (macrophage and monocyte markers) and CD11c (DC marker) positive cells were excluded from the system and surface and costimulatory marker

(MHC class II and CD86) expression was assessed on HSCs. The following antibodies were used for HSC staining: APC-conjugated anti- α -SMA (clone #1A4, R&D systems) and PE-conjugated anti-GFAP (clone #1B4, BD Biosciences). In addition, LRAT was stained using mouse anti-LRAT antibody (clone M34-P1F10, EMD Millipore) and detected with PE conjugated goat anti-mouse IgG (polyclonal, eBioscience). Cytospin preparations of the stained HSCs were counter stained with DAPI (nuclei marker) and visualised by confocal microscopy on a Zeiss observer Z.1 confocal laser microscope (Zeiss, Melville, NY, USA) 63 (oil-immersion) lens.

2.11 Hepatic stellate cell detection in liver tissue by immunofluorescence

Formalin-fixed liver tissues from naïve or *L. donovani*-infected mice were immuno-stained [276]. Paraffin-embedded blocks were prepared in 5- μ m-thick sections, deparaffinized and rehydrated. Then antigen retrieval was performed in boiling sodium citrate buffer (pH 6.0) for 10 min. Sections were washed and incubated with blocking buffer (10% normal mouse serum in TBS) followed by overnight incubation with the following antibodies at 4 °C: PE-conjugated human α -SMA mAb (clone #1A4, R&D systems), mouse GFAP mAb (clone #1B4, BD Biosciences) or isotype controls: PE-IgG2A, (clone 20102, R&D systems) and PE-IgG2b (clone 27-35, BD Pharmingen). Slides were washed twice with TBS and incubated for 1 hr. at room temperature with biotin-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, Inc). Thereafter, the slides were extensively washed with TBS and incubated with streptavidin alkaline phosphatase for 30 min. at room temperature, developed with Fast Red (Sigma-Aldrich, Ontario, Canada) and counterstained with modified Mayer hematoxylin (Fisher Scientific, Fair

Lawn, NJ). Immunofluorescence microscopy was performed on a Zeiss axioscope mot plus laser microscope (Zeiss, Melville, NY, USA) using 20 or 63 (oil-immersion) lenses.

2.12 *In vitro* infection of HSCs

Primary HSCs were isolated according to section 2.10 and infected with 7-day stationary phase *L. donovani* promastigotes or lesion-derived amastigotes (isolated from spleens of 8-10 wk. infected hamsters) at a cell-to-parasite ratio of 1:10 for 10 hr. Free parasites were washed away and the infected cells were further cultured for 24 and 72 hr. at 37°C. At the end of the cultures, the level of infection was determined by counting H&E-stained cytospin preparations under Zeiss Primo Star (Zeiss, Melville, NY, USA) light microscope at 100x (oil) objective.

2.13 Direct *ex vivo* demonstration of *L. donovani* infection in HSCs

Freshly isolated HSCs from 1 or 2 wk. *L. donovani*-infected mice were stained directly *ex vivo* with APC-conjugated anti- α -SMA (clone #1A4, R&D systems) and Alexa 546-conjugated anti-Phosphoenolpyruvate carboxykinase (PEPCK) [374] according to routine laboratory flow cytometry intracellular staining protocols. Thereafter, cytospin preparations of the stained HSCs were counter stained with DAPI (nuclei marker) and infectivity was determined by confocal microscopy on a Zeiss observer Z.1 confocal laser microscope (Zeiss, Melville, NY, USA) using 20 or 63 (oil-immersion) lenses. *L. donovani*-infected HSCs were also visualized by H&E-stained cytospin preparations under Zeiss Primo Star (Zeiss, Melville, NY, USA) light microscope at 100x (oil-immersion) objective magnification.

2.14 Coculture of HSCs with CD4⁺ T cells

HSCs were isolated from livers of naïve or 2 wk. *L. donovani*-infected WT and p110δ^{D910A} mice and cultured overnight in the presence of 270 µM GdCl₃ (Sigma-Aldrich) to block the activity of any contaminating KCs [250]. CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells were purified from spleen cells of naïve WT mice by negative and positive selection using CD4⁺CD25⁺ selection kit (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's suggested protocols. Enriched CD4⁺ T cells (98% purity) were labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) dye. HSCs were co-cultured with CFSE-labeled CD4⁺CD25⁻ or CD4⁺CD25⁺ T cells (at 1:10 ratio) in 96-well flat-bottom plates at 37°C in the presence of soluble anti-CD3 and anti-CD28 mAbs (1 µg/mg). After 5 days, the percentage and proliferation of Tregs (CD4⁺CD25⁺Foxp3⁺ cells) were assessed by flow cytometry.

2.15 Quantification of cytokines produced by HSCs

HSCs from *L. donovani*-infected or naïve mice were resuspended at 2.5×10^5 /ml in complete HSC medium, plated at 1 ml/well in 24-well tissue culture plates and cultured at 37°C. After 3 or 5 days, the supernatant fluids were collected and assayed for cytokines (IL-1β, IL-10, IL-2, IL-4, IL-6 and TNF-α) by V-PLEX Plus Proinflammatory Panel1 (mouse) Kit (Meso Scale Discovery Rockville, MD) according to manufacturer's suggested protocols. IL-17 levels were assessed via normal ELISA protocols as explained in section 2.7. To measure TGF-β in samples, the Human/Mouse TGF-β 1 Ready-SET-Go! ELISA Set (Affymetrix, eBioscience, Inc., San Diego, CA, USA) was used according to the manufacturer's suggested protocols. This kit

contains plates and all the necessary reagents, standards, buffers and diluents. Plates were read at 405 nm (Spectra Max) after the appropriate color development.

2.16 *In vivo* depletion of HSCs in infected mice

HSCs were depleted *in vivo* by using the C1-3 scAbs fragment specific for an extracellular domain of synaptophysin, a protein uniquely expressed by activated HSCs as previously described [278, 280]. Polyhistidine tagged recombinant scAb–C1-3 was expressed in *E. coli* and purified by nickel chromatography and gel filtration. Following rigorous endotoxin removal, gliotoxin, a compound shown to stimulate apoptotic-resistant fibrogenic HSC, was conjugated to scAbs [375, 376]. Groups of WT or p110 δ ^{D910A} mice (n = 4-6) were injected i.p. with C1-3 scAbs conjugated to gliotoxin (C1-3-gliotoxin, 20 mg/kg), unconjugated C1-3 scAbs (C1-3, 20 mg/kg), gliotoxin (600 μ l/kg) or dimethyl sulfoxide (DMSO, 1.2 ml/kg) 24 hrs prior to *L. donovani* infection and on days 1, 3, 5, 7, 9, 11 and 13 post-infection. Mice were sacrificed at 2 wk. post-infection and the absolute numbers, percentage and phenotype of HSCs were assessed by flow cytometry. In addition, the percentage of CD11b⁺, CD11c⁺, F4/80⁺ and CD49b⁺ (NK marker) cells in the livers of C1-3-gliotoxin treated and untreated mice were also determined by flow cytometry after routine digestion with Collagenase D [377]. Parasite burden in the spleens and livers were determined by limiting dilution assay (according to section 2.2) and proliferation (as assessed by Ki67 expression) [312] and percentage of CD4⁺Foxp3⁺ Tregs were determined directly *ex vivo* by flow cytometry (according to section 2.4). In addition, the percentages of cytokine (IL-10 and IFN- γ)-producing cells were assessed directly *ex vivo* by flow cytometry (according to section 2.4).

2.17 RNA extraction and RT-PCR

Total mRNA was extracted from naïve and 2 wk. *L. donovani*-infected mice HSCs using TRIzol (Life Technologies) according to the manufacturer's instructions. cDNA was synthesized from total RNA by MultiScribe Reverse Transcriptase kit (Applied Biosystems) according to the manufactures' suggested protocol. Expression of the *Pik3ca*, *Pik3cd* and 18s was analyzed by RT-PCR by running in a thermocycler for 30 cycles at 60°C annealing temperature. PCR products were run on 2% wt/vol agarose gel electrophoresis and visualized by ethidium bromide staining. Specific primers are as follows: *Pik3ca*, forward 5'-TTCTCTGGAACTGCAGACC-3' and reverse 5'-GTGGACAGCATCCCTGTAAC-3'; *Pik3cd*, forward 5'-CTCTCATTTGCGGGTAAGAA-3' and reverse 5'-ATCAGTCCTGTCCGTCCATA-3'; 18s forward 5'-TGA CTC AAC ACG GGA AAC CTC A-3' and reverse 5'-ACC AGA CAA ATC GCT CCA CCA A-3'.

2.18 Collagen staining

Liver sections from *L. donovani*-infected & uninfected WT and p110δ^{D910A} mice were deparaffinized and hydrated by a descending ethanol series and washed in running water. The samples were stained with hematoxyl for 8 min. and then immersed in sirius red solution (0.1% in saturated picric acid, Sigma Aldrich) for 1 hr. The sections were then washed in acidified water, dehydrated and mounted [378]. The stained sections were visualized by AxioVision software (Carl Zeiss, Inc).

2.19 Assessment of *L. donovani* growth in the presence of p110δ

pharmacological inhibitors

L. major or *L. donovani* promastigotes (2.5×10^5) were cultured in 96 well flat bottom plates at 37°C in the presence of different concentrations (0, 100 nM, 1 µM and 20 µM) of either IC87114 or CAL-101. Parasite growth was monitored every day by counting parasites under light microscopy for seven days and parasite count plots were graphed and compared.

2.20 *In vitro* infection of macrophages in the presence of P110δ

pharmacological inhibitors

Retrovirus-immortalized bone marrow-derived macrophage (BMDM) cells (ANA-1) were grown in complete RPMI medium for 3 days at 37°C [379]. ANA-1 macrophages were then infected with 7-day stationary phase *L. donovani* or *L. major* promastigotes at a cell-to-parasite ratio of 1:5 for 5 hr. Free parasites were washed away and infected macrophages were further cultured for 24 and 72 hr. at 37°C in the presence of 0, 1 and 10 µM of IC87114 or 0, 0.1 and 1µM of CAL-101.[380-382] At the end of the cultures, the level of infection was determined by counting H&E-stained cytopsin preparations under Zeiss Primo Star (Zeiss, Melville, NY, USA) light microscope at 100x (oil) objective.

2.21 Prophylactic, therapeutic and combination treatment regimens in experimental models with P110 δ pharmacological inhibitors

For prophylactic treatment, mice were administered i.p. CAL-101 (Idelalisib / GS-1101 / Zydelig) [209], (0.05 mg/mouse) or IC87114 (0.5 mg/mouse), both from Selleck Chemicals LLC, TX, USA [383] twice a day, 24 hr. prior to getting infected i.v. with 5×10^7 7-day stationary phase *L. donovani* or subcutaneously with 10^4 7-day stationary phase *L. major*. Intraperitoneal injection of CAL-101 and IC87114 was continued every 12 hr. for two wk. (Figure 2-1 A). For therapeutic treatment, mice were infected i.v. with 5×10^7 7-day stationary phase *L. donovani* promastigotes. At one or two wk. post-infection, mice were administered i.p. with 0.05 mg/mouse CAL-101 every 12 hr. for a period of two wk. (Figure 2-1 B). For CAL-101 and low dose Amph-B combination therapy, mice were infected i.v. with 5×10^7 *L. donovani* promastigotes. Two wk. post *L. donovani* infection, mice were divided to four groups as follows and administered i.p. with the different treatments for 5 consecutive days and sacrificed one week after last treatment. Group 1: control (PBS: 100 μ l/mouse), group 2: CAL-101 (0.05mg/mouse), group 3: Amph-B (0.1 mg/kg) and group 4: CAL-101 (0.05mg/mouse) and Amph-B (0.1 mg/kg) in combination (Figure 2-2).

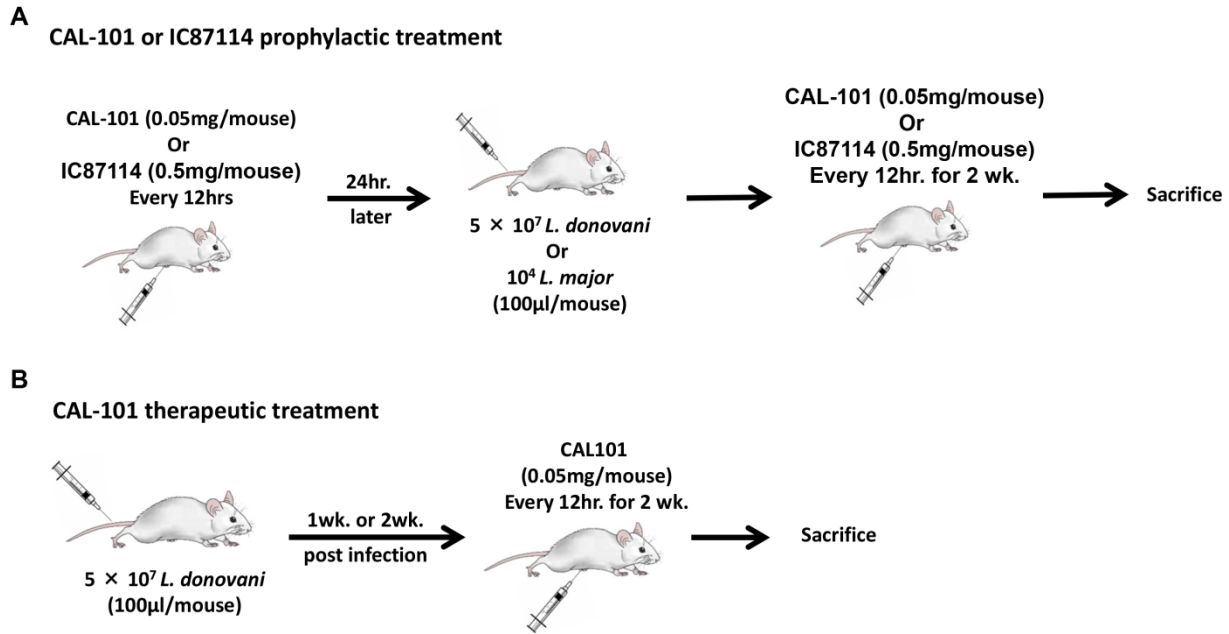


Figure 2-1: CAL-101 or IC87114 prophylactic treatment and CAL-101 therapeutic treatment regimens

For prophylactic treatment: mice were administered i.p. CAL-101 (0.05mg/mouse) or IC87114 (0.5mg/mouse), twice a day, 24 hr. prior to getting infected i.v. with 5×10^7 7-day stationary phase *L. donovani* or 10^4 7-day stationary phase *L. major*. Intraperitoneal injection of CAL-101 and IC87114 continued every 12 hr. for two wk. (A). For therapeutic treatment, mice were infected i.v. with 5×10^7 7-day stationary phase *L. donovani* promastigotes. One or two wk. post *L. donovani* infection, mice were administered i.p. with 0.05mg/mouse CAL-101 every 12 hr. for a period of two wk. (B)

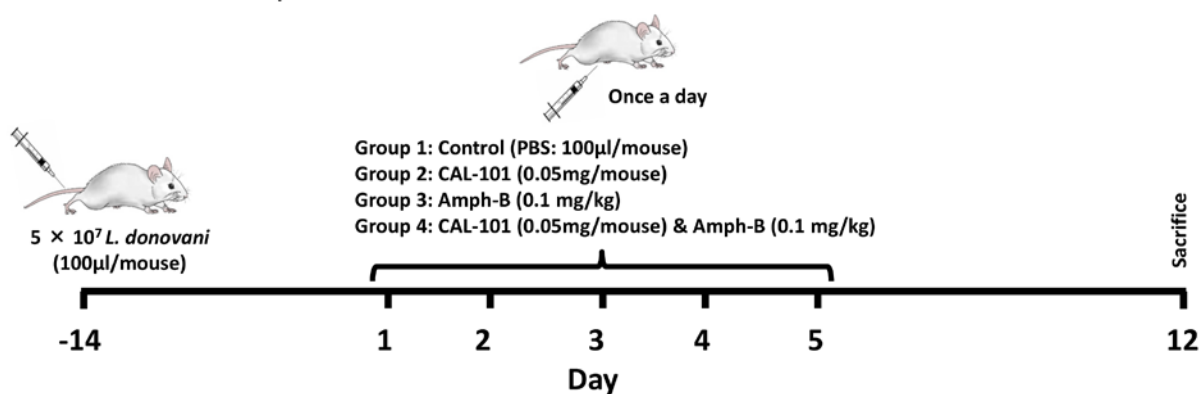


Figure 2-2: CAL-101 and low dose Amphotericin-B combination treatment regimens

Mice were infected i.v. with 5×10^7 *L. donovani* promastigotes. Two wk. post *L. donovani* infection, mice were divided to four groups as presented above and administered i.p. with the different treatments for 5 consecutive days and sacrificed one week after last treatment.

2.22 Assessment of hepatic and splenic Tregs and cytokine production after CAL-101 and Amph-B individual or combination therapy

At different times, mice were sacrificed and the spleens, livers and lymph nodes were collected. The spleens and lymph nodes were made into single cell suspension in complete DMEM medium. The livers were digested with collagenase D (125 µg/ml) for 30 min. in 37°C, homogenized in complete DMEM and liver lymphocytes were separated using percoll gradient centrifugation as previously described in section 2.4 [364, 384]. Cells were counted and directly stained *ex vivo* for CD3, CD4, CD25, Foxp3 and IFN-γ as described in section 2.4 [160, 384]. Samples were acquired on a FACSCanto II cytometer (BD Bioscience, San Diego, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR). Spleen, liver and lymph node cells were also resuspended at 4×10^6 /ml in complete DMEM medium, plated at 1 ml/well in 24-well tissue culture plates and cultured at 37°C. After 3 days, the supernatant fluids were collected and assayed for cytokines (IFN-γ, IL-10, IL-6, IL-4 and KC/GRO) by V-PLEX Plus Proinflammatory Panel1 (mouse) Kit (Meso Scale Discovery, MD) or ELISA according to manufacturer's suggested protocols.

2.23 Statistical analysis

Student T test was used to compare means and standard error of means (SEM) between two groups and nonparametric one-way or two-way analysis of variance (ANOVA) were used to compare means and standard deviation (SD) of more than two groups using Prism program (GraphPad Software Inc., CA, USA). Tukeys or Bonferroni post tests were used where there

were significant differences in ANOVA. Error bars indicate $\pm$ SEM and differences were considered significant when $p < 0.05$.

3 CHAPTER 3: Results

3.1 Deficiency of p110 δ Isoform of the Phosphoinositide 3 Kinase Leads to Enhanced Resistance to *Leishmania donovani*

3.1.1 Rationale

The overall clinical symptoms, resistance and susceptibility to VL depend on several factors including the strain and species of *Leishmania* and the nature of the host immune response [102], e.g. whether it is associated with the production of macrophage-activating cytokines such as IFN- γ and TNF- α or macrophage-deactivating cytokines such as IL-10 and TGF- β [134]. In general, susceptibility to *L. donovani* infection is mainly correlated with increased IL-10 production in humans [138] as well as in mice [385]. Both CD4⁺ and CD8⁺ T cells contribute to optimal protection against experimental *L. donovani* infection [386] by either regulating tissue damage or promoting parasite replication [40]. Tregs, which are CD4⁺ T cells expressing CD25 and Foxp3, have also been shown to play a critical role in determining the outcome of *Leishmania* infection in mice [110] and humans [162]. For example, Foxp3⁺ cells accumulate at the pathologic sites of infection and play a role in both murine [110] and human VL [162].

The class IA PI3Ks are a family of lipid kinases that control multiple cellular processes including cell differentiation, growth, proliferation, migration, metabolism, survival [179] and immune response [193, 195]. Mammals have 3 catalytic subunits of class IA PI3Ks [179, 387] with the p110 δ isoform being highly enriched in leukocytes [388]. The p110 δ isoform seems to be involved in T cell signaling and immune activation [179]. Indeed, naive CD4⁺ T cells from

mice with an inactivating knock-in mutation in the p110 δ gene (known as p110 δ^{D910A}), proliferated poorly and produce significantly less cytokines than cells from WT mice [389]. Interestingly, previous work from our laboratory, demonstrated that p110 δ^{D910A} mice were hyper-resistance to *L. major* (the causative agent of CL), develop minimal or no cutaneous lesion and rapidly clear their parasite despite mounting suppressed Th1 and Th2 responses [160]. This enhanced resistance was independent of mouse genetic background and was associated with dramatic amelioration of inflammatory response and decreased numbers and function of Tregs. Whether this pathway also controls resistance to *L. donovani*, the causative agent of VL is not known. Since regulation of host immunity to different *Leishmania* spp. may be highly variable, here, I investigated the outcome of infection of p110 δ^{D910A} mice with *L. donovani* and the underlying mechanism(s) that regulate such disease outcome.

3.1.2 Hypothesis

*I hypothesize that the p110 δ isoform of PI3K pathway also controls disease outcome in mice infected with *L. donovani* as it was shown to be responsible for resistance to *L. major* infection (causative agent of CL).*

3.1.3 Objectives

- a) Determine whether p110 δ^{D910A} mice are resistant or susceptible to *L. donovani* infection
- b) Define the nature of immune response in *L. donovani*-infected p110 δ^{D910A} mice in comparison to WT mice
- c) Investigate the mechanism through which signaling via the p110 δ isoform of PI3K regulates resistance/susceptibility to *L. donovani*

3.1.4 Results

3.1.4.1 Mice with inactive p110 δ PI3K are highly resistant to *L. donovani* infection

Our laboratory previously showed that despite significantly impaired T cell responses, p110 δ^{D910A} mice are highly resistant to *L. major* (causative agent of CL) [160]. To determine whether signaling via the p110 δ isoform of PI3K also regulates resistance to VL, I infected WT and p110 δ^{D910A} mice i.v. with *L. donovani* promastigotes or amastigotes at different times after infection, assessed parasite burden in the spleens and liver by limiting dilution assay. In agreement with our previous observation with *L. major* [160], *L. donovani*-infected p110 δ^{D910A} mice were more resistant than their WT counterparts. By two wk. post-infection, p110 δ^{D910A} mice harbored significantly fewer parasites than infected WT mice both in their spleens (Figure 3-1A and E, $p < 0.01$) and livers (Figure 3-1B and F, $p < 0.001$) and this trend was maintained for several wk. (up to 8 wk. post-infection). Consistent with this reduced parasite burden, the spleens (Figure 3-2A) and livers (Figure 3-2B) of infected p110 δ^{D910A} mice were significantly smaller than WT mice, indicating that hepatomegaly and splenomegaly, which are marked features of VL, were significantly controlled in *L. donovani* infected p110 δ^{D910A} mice. The reduction in splenic and hepatic sizes in infected p110 δ^{D910A} mice was correlated with significantly reduced numbers of cells in these organs (Figure 3-1C and D and Figure 3-1G and H), suggesting that deficiency of p110 δ might affect cellularity and/or increased cell proliferation or recruitment into these organs.

Because *L. donovani* is known to activate PI3K/AKT in macrophages [390], which might influence parasite replication, I determined whether the enhanced resistance of p110 δ^{D910A} mice was related to hyperactivity of their macrophages in restricting parasite growth. Both WT and

p110 δ ^{D910A} BMDMs were equally permissive to *L. donovani* following *in vitro* infection (Figure 3-3A to D), suggesting that as reported previously for *L. major* [160], the enhanced resistance of p110 δ ^{D910A} mice to *L. donovani* is not due to enhanced responsiveness or leishmaniacidal activities of their macrophages.

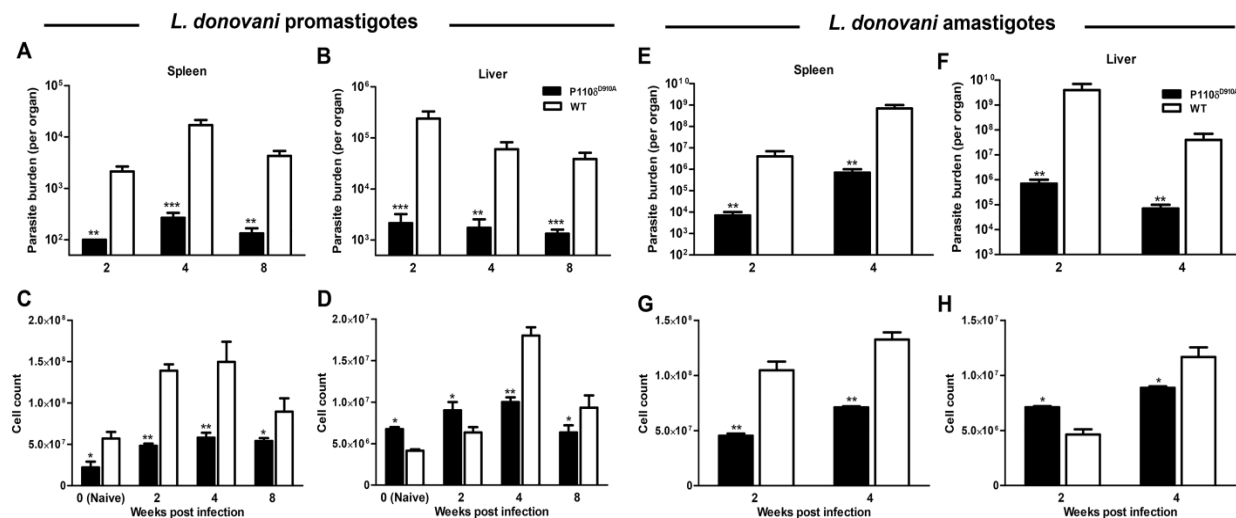


Figure 3-1: P110 δ^{D910A} mice are hyper-resistant to *L. donovani*.

(A and B) Kinetics of parasite burden in the spleens and liver of WT and p110 δ^{D910A} BALB/c mice. Mice were infected with 5×10^7 stationary phase promastigotes (A and B) or 1×10^7 hamster spleen-derived amastigotes (E and F) and sacrificed at different times (as indicated) to assess parasite burden in the spleens (A and E) and liver (B and F). Total number of cells in the spleens (C and G) and liver (D and H) of WT and p110 δ^{D910A} mice at different times post-infection with promastigotes (C and D) or amastigotes (G and H). Results are representative of 6 (A-D) and 2 (E-H) independent experiments (n = 4 mice per group) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

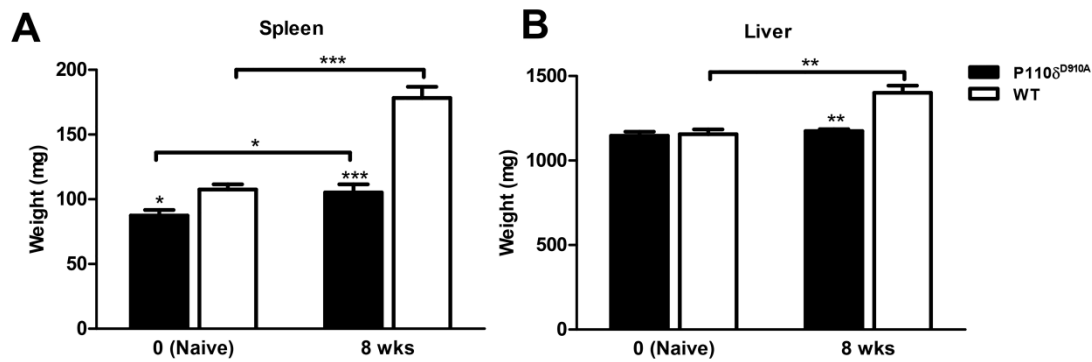


Figure 3-2: Reduced splenomegaly and hepatomegaly in infected p110 δ^{D910A} mice.

WT and p110 δ^{D910A} mice were infected with 5×10^7 stationary phase promastigotes of *L. donovani*, sacrificed at 8 wk. post infection and the spleens (A) and livers (B) of infected mice were weighed. Results are representative of 3 independent experiments (n = 4 mice per group) with similar results. Error bars, $\pm$ SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

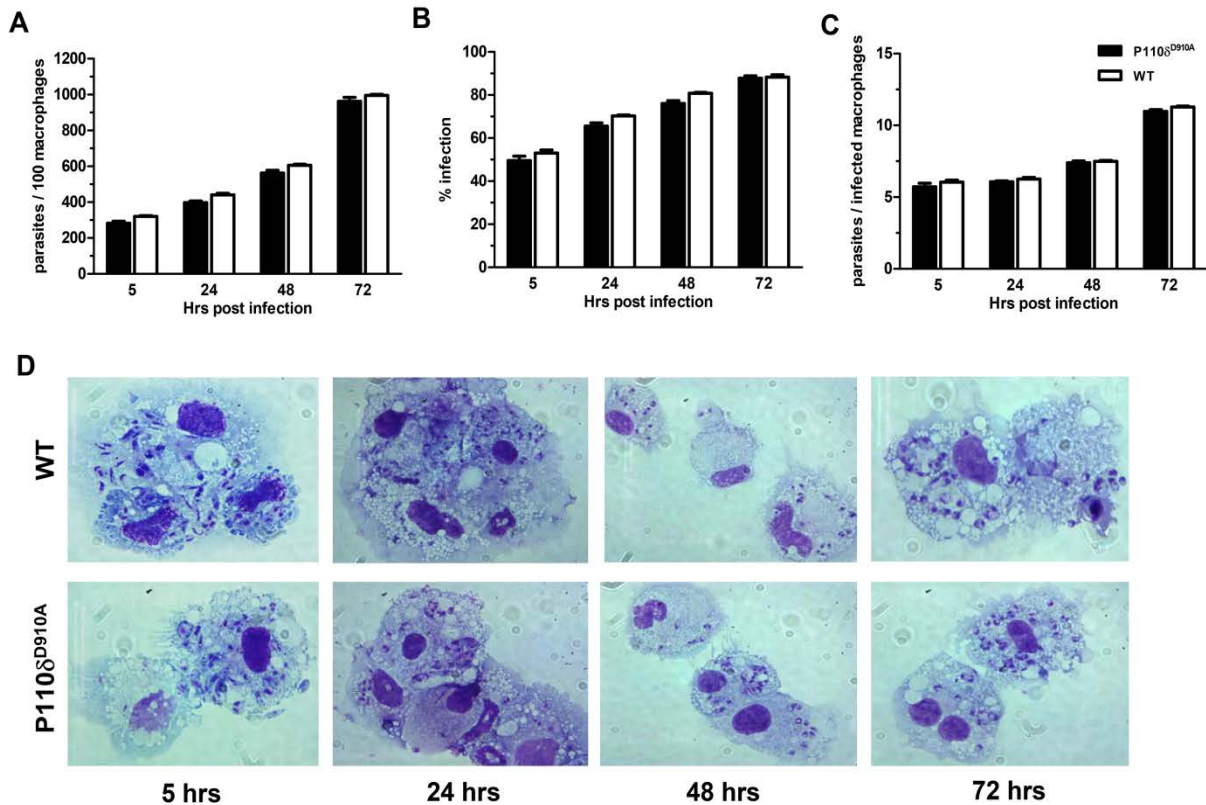


Figure 3-3: Enhanced resistance of p110 δ^{D910A} mice to *L. donovani* is not due to superior macrophage responsiveness.

Bone marrow-derived macrophages from WT and p110 δ^{D910A} mice were infected with *L. donovani* promastigotes at a cell-to-parasite ratio of 1:5. After 24, 48 and 72 hr., cytopsin preparations were made, stained with Wright-Giemsa stain and the number of parasites per 100 macrophages (A), percent infectivity (B) and number of parasites per infected macrophages (C) were determined. (D) Light microscopy images (at x100 (oil) objective) of infected macrophages in different time points. Results are representative of 2 independent experiments (n = 3 mice per group) with similar results.

3.1.4.2 Splenic and hepatic immune (cytokine) responses in *L. donovani*-infected

p110 δ ^{D910A} mice

The observation of enhanced resistance (lower parasite burden) in p110 δ ^{D910A} mice following *Leishmania* infection, prompted me to assess their T cell responses. Infected p110 δ ^{D910A} mice had fewer leukocytes than WT mice in the spleens during the course of infection (Figure 3-1C and G). Surprisingly, in the liver, the leukocyte count was slightly higher in the p110 δ ^{D910A} mice at 2 wk. post-infection and significantly lower at 4 and 8 wk. post infection compared to WT infected mice (Figure 3-1D and H).

To determine whether the enhanced resistance of p110 δ ^{D910A} mice was associated with superior effector cellular cytokine response, I assessed splenic and hepatic cells from infected mice for their cytokine response directly *ex vivo* (by flow cytometry) or after 3 days restimulation *in vitro* with *L. donovani* antigen by ELISA. At all-time points after infection, the percentages and absolute numbers of IFN- γ -producing (Figure 3-4A to D) and IL-4-producing (Figure 3-5A to D) cells in the spleens and livers of infected highly resistant p110 δ ^{D910A} mice were significantly lower than those from their infected WT counterpart mice. Interestingly, while CD4⁺ cells were the major producers of IFN- γ in both organs, IL-4 producing cells were mostly from CD3⁺ lymphocyte population (Figure 3-5A to D). Consistent with the flow data, splenic and hepatic lymphocytes from infected p110 δ ^{D910A} mice also produced significantly less IFN- γ , IL-4 and IL-10 in culture supernatant fluids compared to those from WT mice (Figure 3-6A to C and Figure 3-6E to G). Interestingly, while spleen cells from p110 δ ^{D910A} mice produced significantly less IL-12 in cultures compared to WT mice, their hepatic cells produced more of this cytokine than those from WT mice (Figure 3-6D and H). Similarly, while the levels of NO, key effector

molecule for killing *Leishmania* inside infected cells, were significantly lower in the spleen cell cultures from infected p110 δ ^{D910A} mice in the later time points , they were comparable in cultures from liver cells from infected p110 δ ^{D910A} and WT mice (Figure 3-7A and B). The slight increase observed in regard to the NO levels in the *L. donovani* infected p110 δ ^{D910A} in comparison to their WT counterparts in the early time points, could be due to the production of NO by DCs in the spleen and could be addressed further in the future. Collectively, these findings show that the loss of p110 δ activity is sufficient to reverse the susceptibility of infected BALB/c mice to *L. donovani* infection despite having impaired cytokine responses.

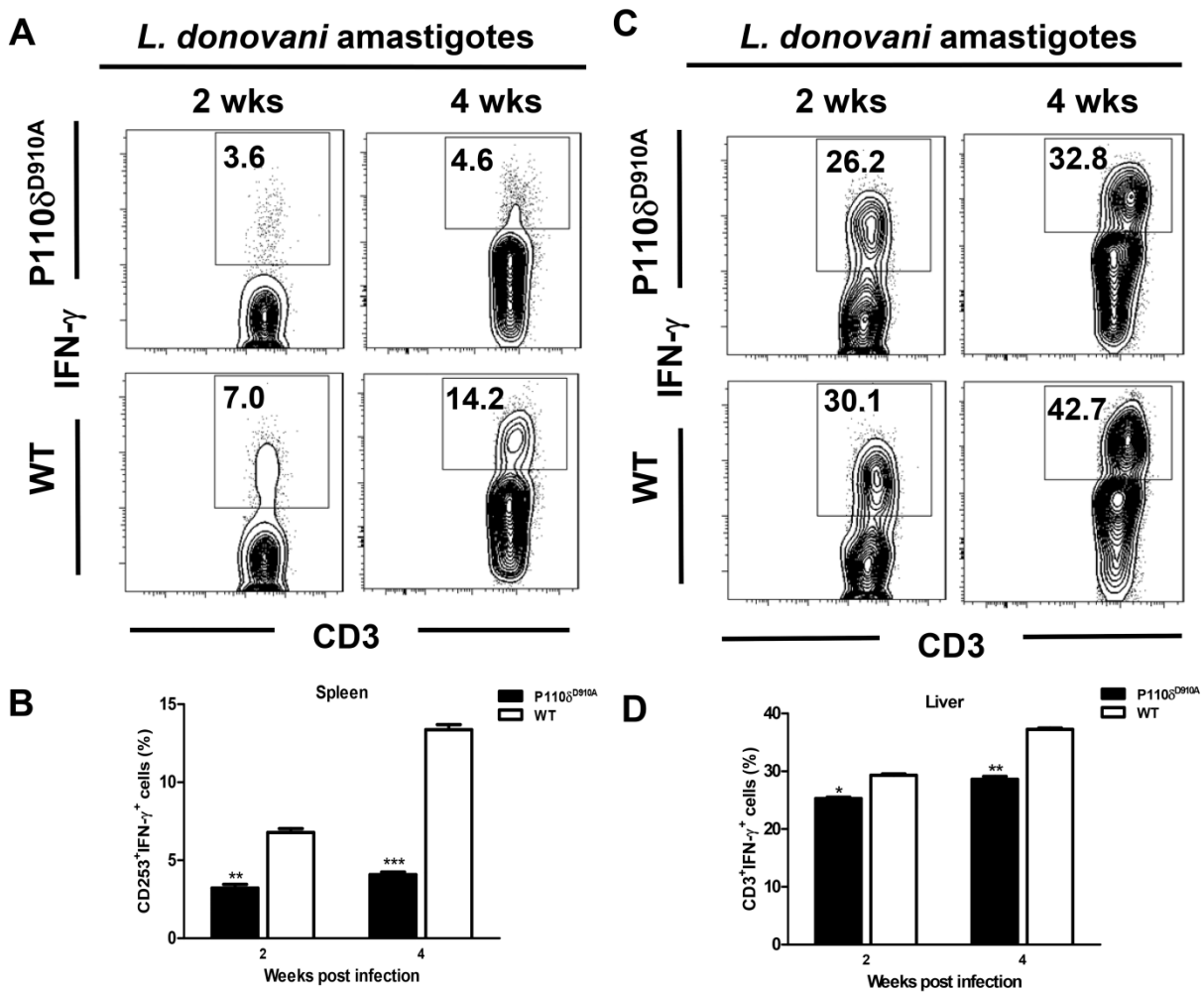


Figure 3-4: Spleen and liver lymphocytes from infected resistant p110 δ ^{D910A} mice produce less IFN- γ than those from WT mice.

Spleen (A and B) and liver (C and D) lymphocytes from WT and p110 δ ^{D910A} mice infected with *L. donovani* amastigotes were assessed directly *ex vivo* at 2 and 4 wk. post infection for IFN- γ production by flow cytometry. Results are representative of 2 independent experiments (n = 3 mice per group) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

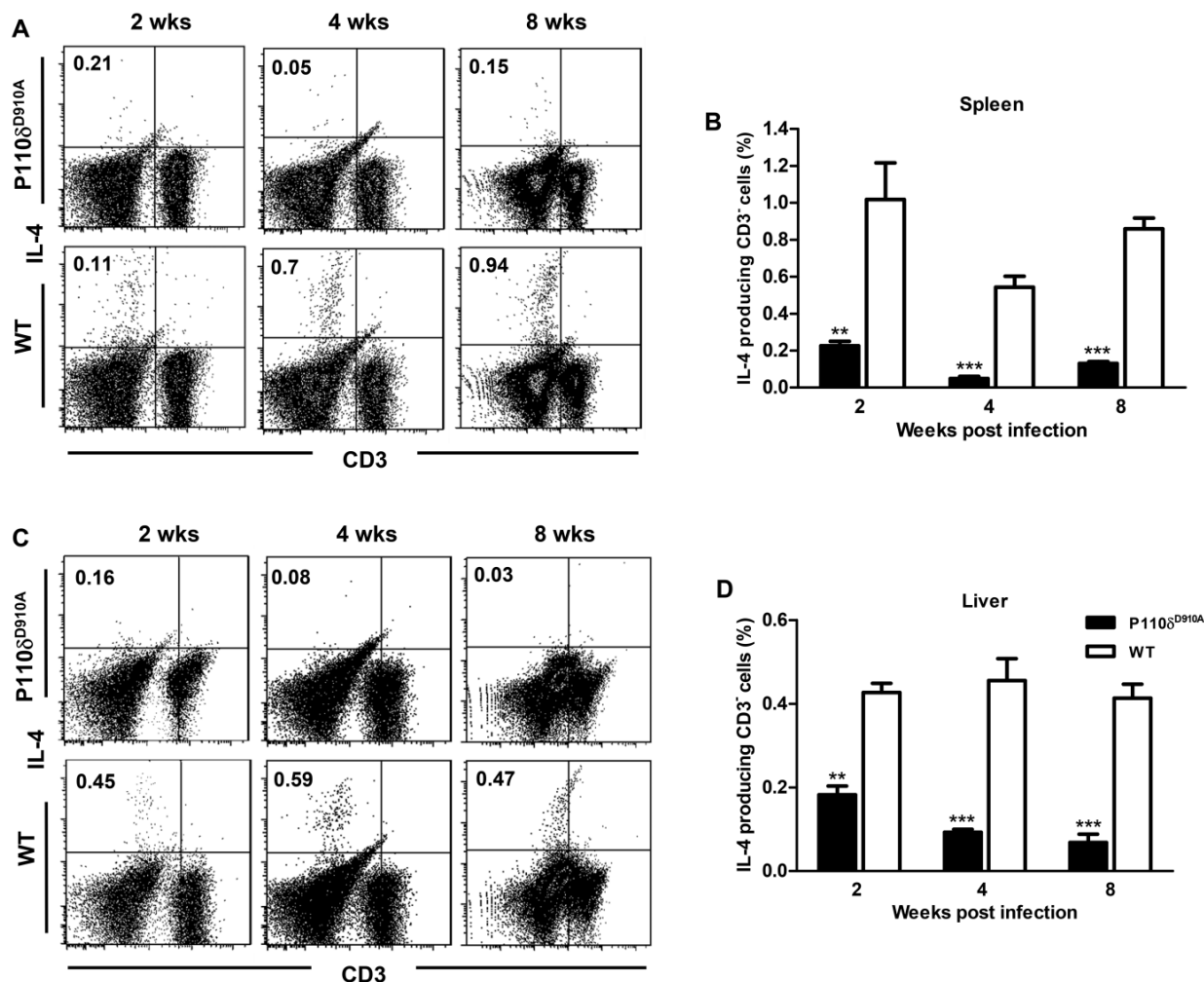


Figure 3-5: Non T cells (CD3⁺) are the major IL-4-producing cells in the spleens and liver of *L. donovani* infected WT and resistant p110 δ^{D910A} mice.

L. donovani promastigote infected p110 δ^{D910A} and WT mice were sacrificed at the indicated times and their spleen (A and B) and liver (C and D) lymphocytes were pulsed with PMA, ionomycin and brefeldin A (BFA) for 4 hr. and directly stained *ex vivo* for CD3, CD4 and IL-4. Results are representative of 3 independent experiments (n = 3 mice per group) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

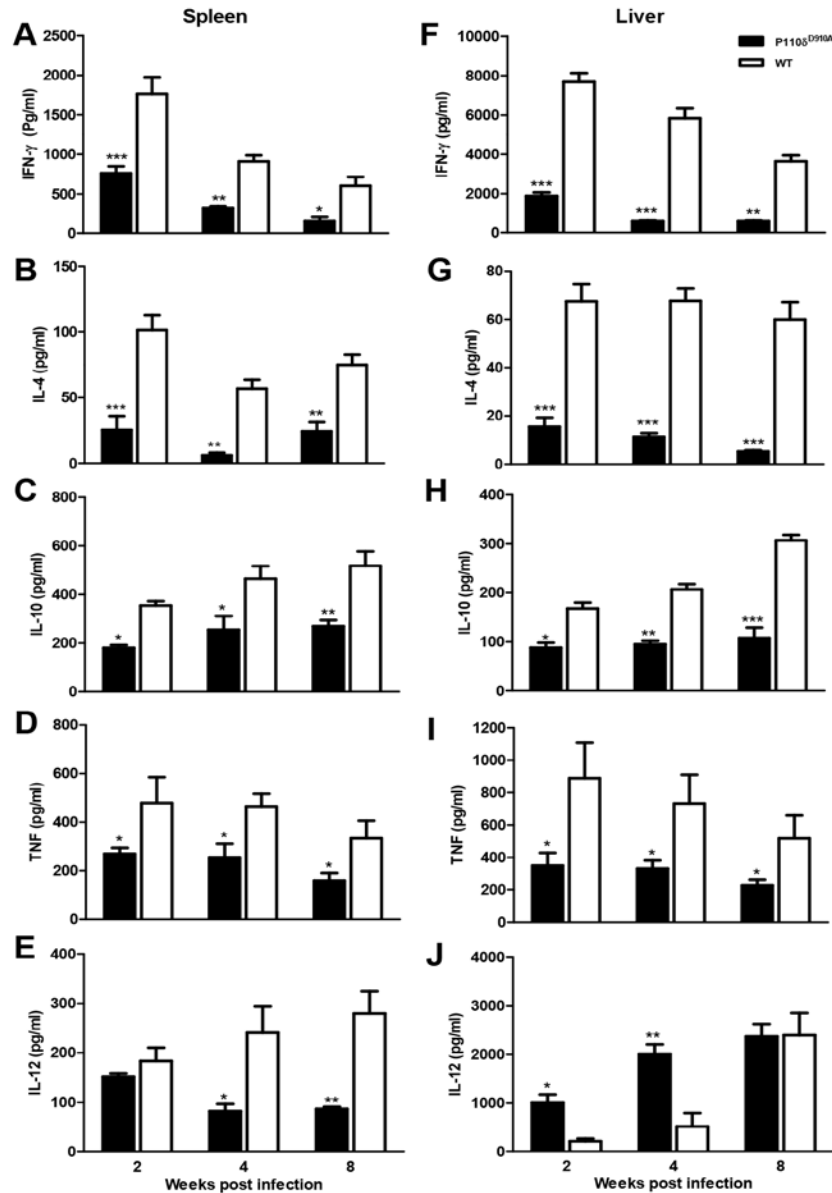


Figure 3-6: Impaired cytokine production by spleen and liver lymphocytes from *L. donovani*-infected highly resistant p110δ^{D910A} mice.

At the indicated times after infection, spleen and liver lymphocytes from WT and p110δ^{D910A} mice were cultured *in vitro* in the presence of *L. donovani* antigen for 72 hr. and the culture supernatant fluids were assayed for cytokines by Flowcytomix array. Shown are the splenic values for IFN-γ (A), IL-4 (B), IL-10 (C), TNF (D) and IL-12 (E) and liver values for IFN-γ (F), IL-4 (G), IL-10 (H), TNF (I) and IL-12 (J) at different times post-infection. Results are representative of 3 independent experiments (n = 4 mice per group) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

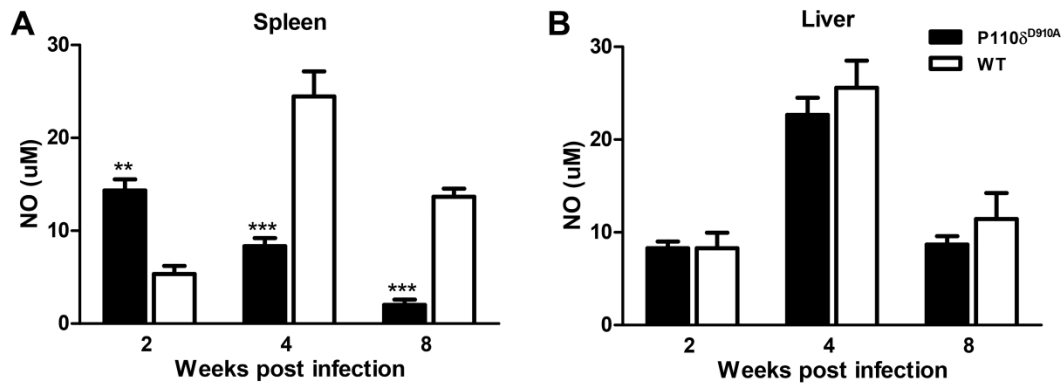


Figure 3-7: Enhanced resistance of p110 δ^{D910A} mice to *L. donovani* is not associated with high nitric oxide (NO) production.

NO levels were measured in 72 hr. culture supernatant fluids of spleen (A) and liver (B) lymphocytes of *L. donovani*-infected WT and p110 δ^{D910A} mice that were stimulated with freeze-thawed *L. donovani*. Results are representative of 3 independent experiments (n = 3 mice per group) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

3.1.4.3 Impaired antibody response in *L. donovani* infected p110 δ ^{D910A} mice

Previous reports show that p110 δ ^{D910A} mice have reduced numbers of peripheral B cells as well as impaired B cell signaling and a concomitant reduction in circulating plasma cells and serum antibody levels [190, 391, 392]. In addition, our laboratory previously found that the total IgG as well as parasite-specific IgG1 and IgG2a levels in the sera of *L. major*-infected p110 δ ^{D910A} mice were significantly lower than in WT controls [160]. Therefore I assessed whether infection with *L. donovani* was also associated with impaired B cell responses. As shown in Figure 3-8A-D, the parasite-specific IgG and IgM as well as IgG1 and IgG2a levels in the sera of *L. donovani*-infected p110 δ ^{D910A} mice were significantly lower than in WT controls during the course of infection.

Although impaired B cell response and/or antibody production is not responsible for the enhanced resistance of p110 δ ^{D910A} mice to *L. major* infection [160], in light of the current knowledge on the importance of B cells in the pathogenesis of visceral leishmaniasis, the same conclusion might not be correct in the case of *L. donovani* infection. It has been recently reviewed that in p110 δ null or catalytically inactive transgenic mice, there are defects in B cell receptor (BCR) signal transduction, basal immunoglobulin production and the loss of marginal zone (MZ) and B1 B cells [393]. In *Leishmania* infections, it has been shown that that depletion of MZB enhanced T cell responses and led to a decrease in the parasite burden but did not alter the generation of effector memory T cells [394]. Additionally, activation of MZB which are responsible for initiating protective T cell responses during the early stages of *L. donovani* infection by *L. donovani* result in disease exacerbation and mediate hypergammaglobulinemia, a feature of visceral leishmaniasis that contributes to immunopathology [395]. Therefore it could

be concluded that resistance to *L. donovani* infection in p110 δ ^{D910A} mice might in part be due to lower or impaired B cell responses and/or reduced antibody responses which needs further investigation.

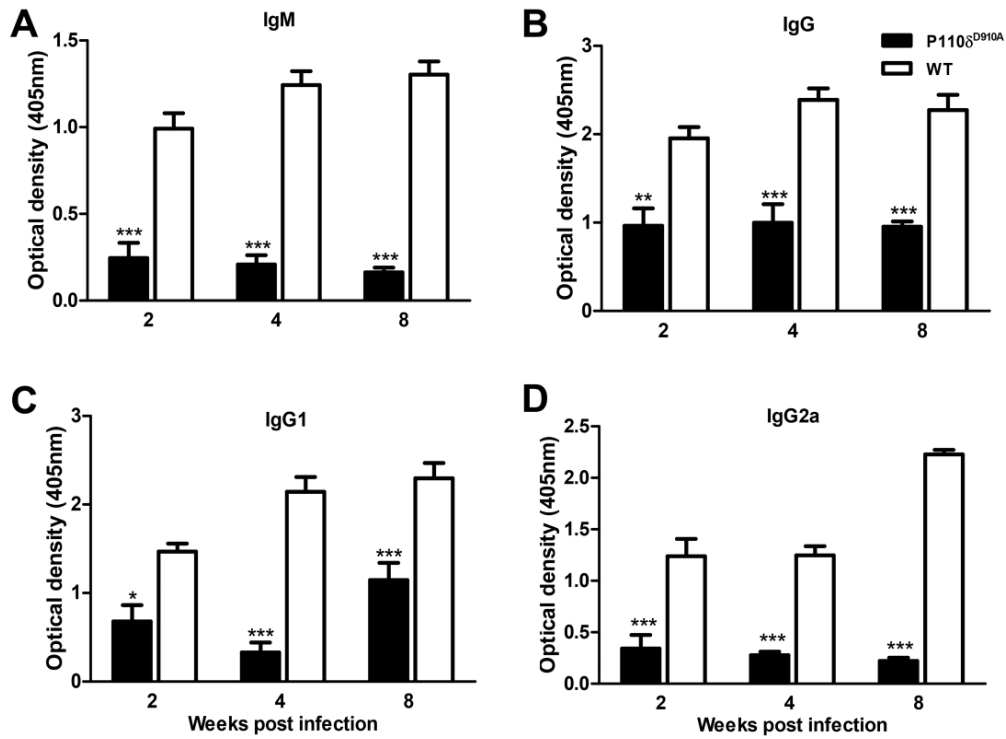


Figure 3-8: Impaired antibody response in resistant p110 δ^{D910A} mice.

Total antigen-specific IgM (A), IgG (B), IgG1 (C) and IgG2a (D) levels in the sera of infected p110 δ^{D910A} and WT mice. At different times after infection, mice were sacrificed and sera were analyzed for different isotypes of *Leishmania*-specific antibodies by ELISA. Results are representative of 3 independent experiments (n = 4 mice per group) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

3.1.4.4 Impaired granuloma formation in *L. donovani*-infected p110 δ ^{D910A} mice

Leishmania-specific immune response in the liver leads to the formation of granulomas that limit infection, kill and remove the microbial target and repair any accompanying tissue injury [371]. Enhanced resistance to *L. donovani* infection in mice has been linked to formation of effective granuloma [163, 396, 397]. As p110 δ ^{D910A} mice are strongly resistant to *L. donovani*, I hypothesized that this would be linked to more efficient and effective granuloma formation in their livers. Therefore, we assessed granuloma formation in H&E sections in these organs at different times after infection. By wk. 2 post-infection in WT mice, mononuclear cells were recruited to adjacent sinusoids and ineffective granulomas with no mononuclear cell infiltration were already formed. In addition, developing functional granulomas were starting to generate by parasitized Kupffer cells fusing together and this was surrounded by foci of infiltrating lymphocytes and monocytes. By wk. 4 post-infection, developing and/or mature granulomas were visible and involuting large epithelioid granuloma devoid of amastigotes were clearly present by wk. 8 post-infection (Figure 3-9A and B). In contrast, mostly ineffective granulomas and only very few developing functional granulomas were visible in tissues from infected p110 δ ^{D910A} mice by 4 wk. post-infection such that by 8 wk. post-infection, mononuclear cells were still remaining largely within adjacent sinusoids and significantly fewer numbers of developing or smaller mature granulomas were present (Figure 3-9A and B). Thus, contrary to the established dogma, enhanced resistance to *L. donovani* infection in p110 δ ^{D910A} mice was not associated with more effective granuloma formation in the liver.

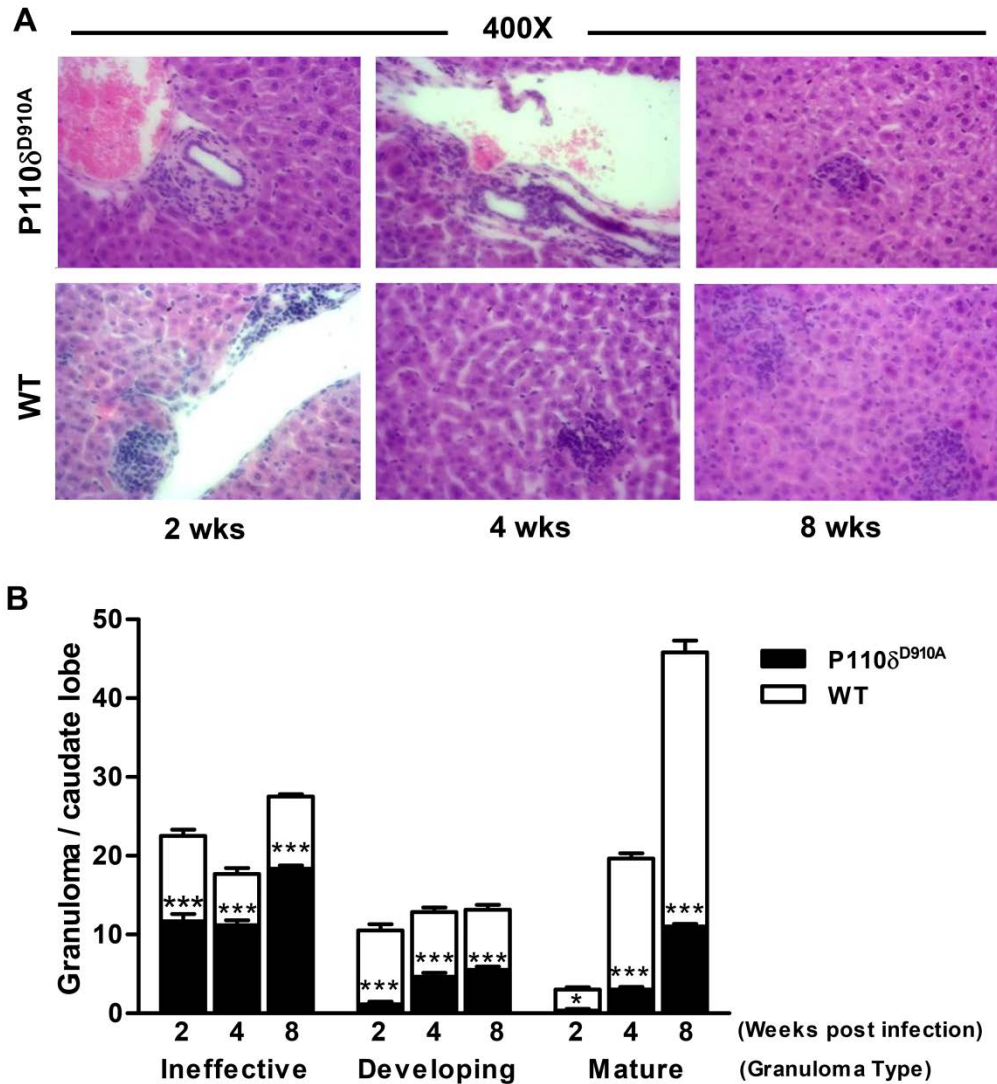


Figure 3-9: Enhanced resistance of p110 δ ^{D910A} mice is not associated with more robust granuloma formation.

Infected p110 δ ^{D910A} and WT mice were sacrificed at the indicated times and their liver were processed and stained routinely to assess granuloma formation (size, cellularity and maturation) as described in the materials and methods section. The H&E stained sections (A) were assessed and scored blindly by a pathologist for the presence/number of ineffective, developing and mature granulomas and represented as a bar graph (B). Results are representative of 2 independent experiments (n = 3 mice per group) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

3.1.4.5 Regulatory T cells in *L. donovani*-infected p110 δ^{D910A} mice

Tregs contribute to susceptibility to *L. donovani* infection [361, 398], in part by enhancing parasite persistence in infected organs [110]. In addition, previous reports show that p110 δ^{D910A} mice have impaired expansion of Tregs [190, 399] and this was in part responsible for their enhanced resistance to *L. major* [160]. To determine whether the enhanced resistance of p110 δ^{D910A} mice to *L. donovani* is related to impaired induction and/or expansion of Tregs, I compared the percentage (Figure 3-10A, B, D and E) and absolute numbers (Figure 3-10C and F) of CD4⁺CD25⁺Foxp3⁺ cells (Tregs) in the spleens of *L. donovani*-infected p110 δ^{D910A} and WT mice. At all times tested, the percentages and absolute numbers of Tregs in the spleens of infected p110 δ^{D910A} mice were significantly lower than in their WT counterpart mice. The data also show that in both WT and p110 δ^{D910A} mice, infection with *L. donovani* leads to increase in the number of Tregs, peaking around wk. 4 and returning to baseline by wk. 8 post-infection. However, this increase was significantly higher in WT than in p110 δ^{D910A} mice. Interestingly, most of the CD25⁺ T cells in infected mice also co-expressed Foxp3, suggesting that during *L. donovani* infection, most of activated CD25⁺ T cells are skewed towards a Treg phenotype. Taking together, these results suggest that impaired expansion and/or function of Tregs may be responsible for the enhanced resistance of p110 δ^{D910A} mice to *L. donovani* infection.

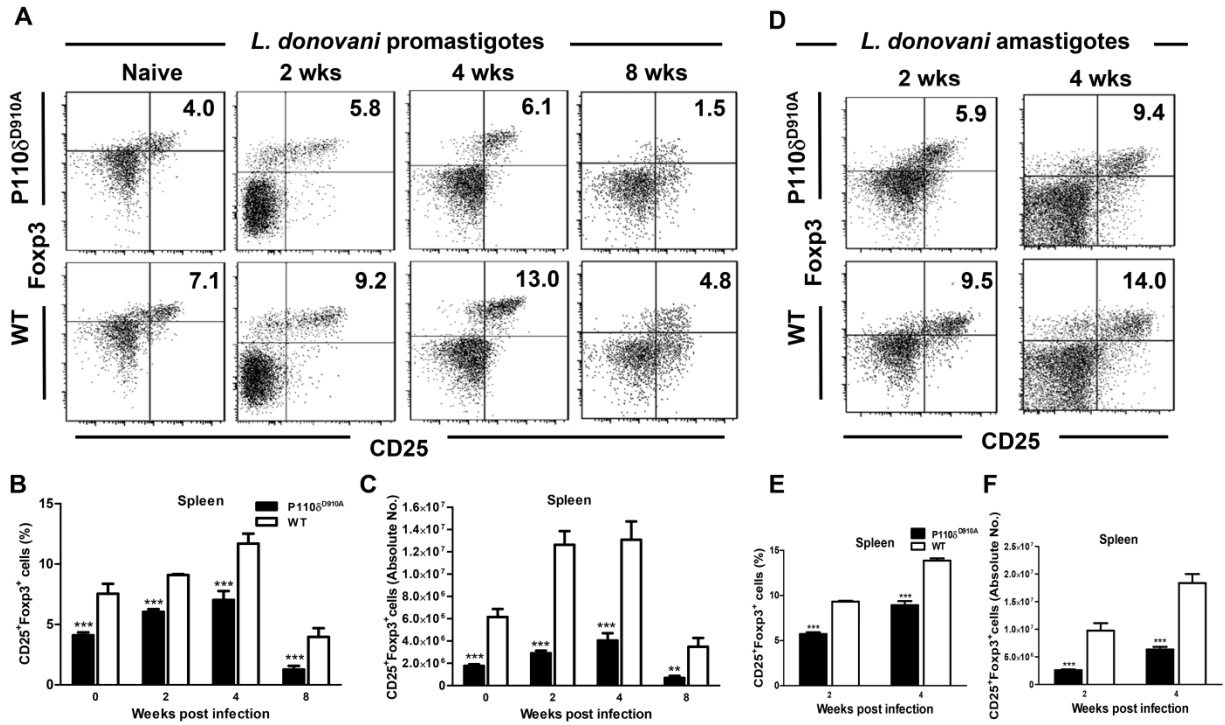


Figure 3-10: Reduced number of CD4⁺CD25⁺Foxp3⁺ T cells (Tregs) in p110^{ΔD910A} mice.

Flow cytometry showing the percentages (A and B) and absolute numbers (C) of CD4⁺CD25⁺Foxp3⁺ (Tregs) in the spleens of WT and p110^{ΔD910A} mice infected with *L. donovani* promastigotes at different times post-infection. The percentages (D and E) and absolute numbers (F) of Tregs in the spleens of WT and p110^{ΔD910A} mice infected with *L. donovani* amastigotes were also assessed. Splenocytes of uninfected (naïve) and infected mice were directly stained *ex vivo* for CD3, CD4, CD25 and Foxp3 at 2, 4 and 8 weeks post-infection. Representative dot plots (A and D) and bar graphs showing the mean $\pm$ SEM of the percentages (B and E) and absolute numbers (C and F) of CD25⁺Foxp3⁺ cells are shown after gating on CD3⁺CD4⁺ population. Results are representative of 3 independent experiments (n = 4 mice per group) with similar results. Error bars, $\pm$ SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

3.1.4.6 Systemic *in vivo* expansion of Tregs renders p110 δ^{D910A} mice susceptible to *L. donovani* infection

I speculated that the significantly lower numbers of Tregs after infection dampen Treg-mediated suppression of parasite killing leading to rapid clearance of parasites in infected p110 δ^{D910A} mice despite lower T cell response. Therefore, I hypothesized that increasing Treg numbers in infected p110 δ^{D910A} mice would abolish their enhanced resistance to *L. donovani*. To test this hypothesis, I utilized a novel *in vivo* approach for inducing rapid expansion of Tregs by injecting rIL-2/anti-IL-2 immune complex into naïve and infected mice. Consistent with previous reports [365, 366], this protocol led to rapid and comparable increase in the percentage and absolute numbers of Tregs in the spleen, liver, lymph node and blood of both uninfected (Figure 3-11A and B) and infected (Figure 3-11C) WT and p110 δ^{D910A} mice, suggesting that Tregs have the ability to expand in p110 δ^{D910A} mice. I found that the immune complex only expanded Tregs and had very little/minimal effect on other non-Treg cells such as CD8⁺ (Figure 3-12A) and CD4⁺CD25⁻ (Figure 3-12B) T cells in spleens of WT and p110 δ^{D910A} mice. This is consistent with the observations of Webster *et al.* who first reported this phenomenon [366].

Next, I infected WT and p110 δ^{D910A} mice injected with rIL-2/anti-IL-2 immune complex with *L. donovani* and followed up with weekly injection of immune complex to maintain high levels of Tregs. Strikingly, expansion of Tregs results in dramatic abrogation of enhanced resistance of p110 δ^{D910A} mice to *L. donovani* such that parasite burdens in the spleens and liver were significantly increased and indistinguishable from those of WT mice at 2 weeks (Figure 3-11D) post-infection. Although Treg expansion in WT also increases their susceptibility

to *L. donovani* infection, this effect is more pronounced in p110 δ ^{D910A} mice. One possible explanation could be that since p110 δ ^{D910A} mice have fewer Tregs to start with, therefore once they encounter higher levels of Tregs (due to induced Treg expansion) the impact is much more significant. Collectively, these results show that the enhanced resistance to *L. donovani* is related to the significantly reduced numbers of Tregs in absence of p110 δ signaling.

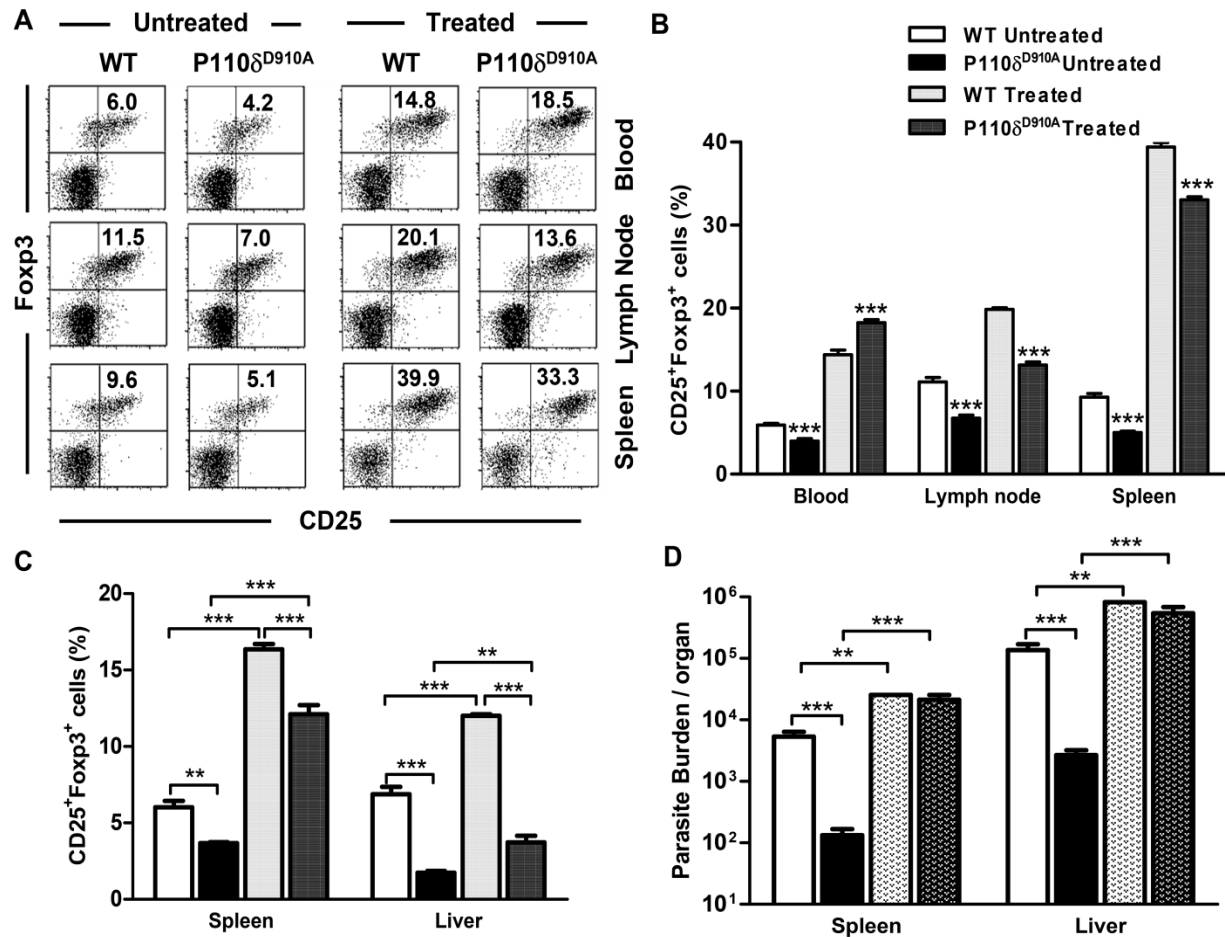


Figure 3-11: Systemic Treg expansion by administration of IL-2/anti-IL-2 immune complex leads to abrogation of enhanced resistance to *L. donovani* in p110 δ^{D910A} mice.

WT and p110 δ^{D910A} mice were injected i.p. with rIL-2/anti-IL-2 mAb immune complex (treated) once a day for three consecutive days. Control mice were injected with isotype-matched control antibody mixed with rIL-2 (untreated). Two days after the last immune complex injection, mice were sacrificed and the percentage of CD4⁺CD25⁺Foxp3⁺ cells (Tregs) in the blood, lymph nodes and spleens was determined directly *ex vivo*. Representative dot plots (A) and bar graphs showing the mean $\pm$ SEM of the percentages (B) of CD4⁺CD25⁺Foxp3⁺ cells in the blood, lymph nodes and spleens. In a different experiment, immune complex-treated (or untreated) mice were infected with 5×10^7 *L. donovani* and immune complex treatment was continued once a week for 2 additional weeks. Infected mice were then sacrificed and the percentages of CD4⁺CD25⁺Foxp3⁺ cells (Tregs) in spleens and liver tissues were assessed directly *ex vivo* by flow cytometry (C). At sacrifice, parasite burden in the spleens and livers was assessed by limiting dilution assay (D). Results are representative of 2 independent experiments (n = 4 mice per group) with similar results. Error bars, $\pm$ SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

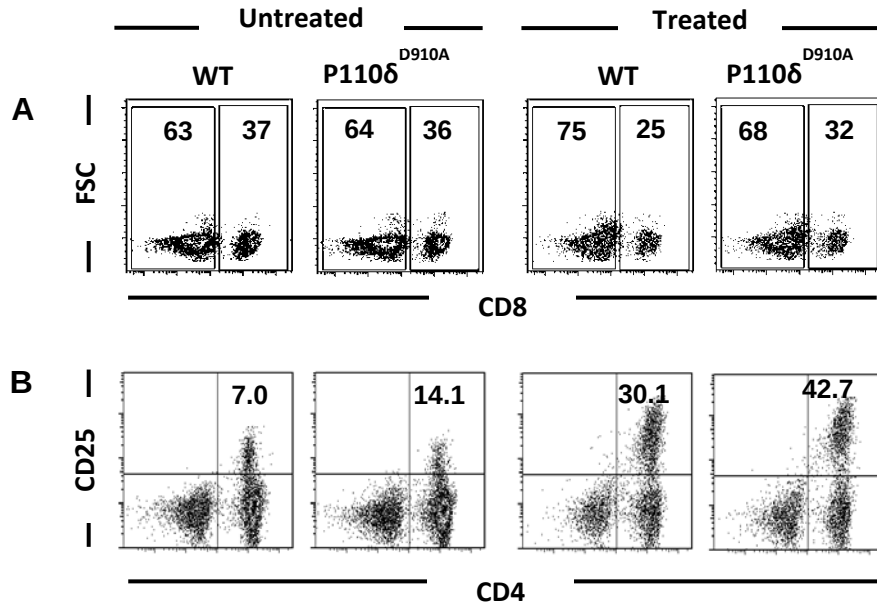


Figure 3-12: Immune complex injection did not increase the percentages of CD8⁺ and CD4⁺CD25⁺ T cells in spleens of WT and p110δ^{D910A} mice.

WT and p110δ^{D910A} mice were injected intraperitoneally with rIL-2/anti-IL-2 mAb (immune complex, treated) once a day for three consecutive days. Control mice were injected with isotype-matched control antibody mixed with rIL-2 (untreated). Two days after the last injection, mice were sacrificed and the percentages of CD4⁺ and CD8⁺ cells in the spleens were determined directly *ex vivo*. Representative dot plots showing the percentages of CD8⁺ cells (upper panel) and CD4⁺CD25⁺ cells (lower panel) are shown after gating on CD3⁺ population. Results are representative of 2 independent experiments (n = 4 mice per group) with similar results.

3.2 Hepatic stellate cells regulate liver immunity to visceral leishmaniasis through p110 δ -dependent induction and expansion of Tregs in mice Introduction

3.2.1 Rationale

VL is a systemic infection affecting the reticuloendothelial organs including spleen, liver and bone marrow resulting in severe splenomegaly and hepatomegaly [40]. The disease causes pathological and functional changes in the liver resulting in chronic mononuclear cell infiltrations, degeneration of hepatocytes and focal fibrosis [400]. Although the exact causes of these changes are unknown, they are speculated to be immunologically driven. The liver has important immunologic functions and contains immunologically active cells, including T and B lymphocytes, KCs, NK cells, NKT cells, DCs and hepatic stellate cells (HSCs) [401]. The role played by these cells (especially HSCs) in regulating immunity to VL is poorly understood.

HSCs are resident non-parenchymal cells located in the sub-endothelial space of Disse [231]. Although HSCs only represent ~10% of the total number of resident cells in normal liver and ~1.5% of total liver volume [230], they are very versatile and have immunomodulatory and regulatory [300] functions. Under normal physiological conditions, HSCs are quiescent and stores most (~80%) of the body's retinoic acid as retinyl esters [250]. In addition, they are also involved in vasoregulation of endothelial cell interactions, contribute to ECM homeostasis and are mediators of immune tolerance [231]. However, during inflammation and liver injury, HSCs become activated, which is characterized by the loss of retinoids, transdifferentiation into myofibroblasts and expression of α -SMA. Activated HSCs also contribute to vascular distortion,

fibrogenesis, matrix degradation and amplification of inflammation [231]. HSCs have also been shown to display antigen-presenting [304] and phagocytic properties [324], produce numerous cytokines with proinflammatory and anti-inflammatory activities [307] and play important role in the immunopathogenesis of schistosomiasis [222]. However, whether HSCs are infected by *L. donovani* in vivo and whether or how they contribute to the pathogenesis of VL is unknown.

The class IA PI3Ks are heterodimeric enzymes composed of 85-kDa regulatory and a 110-kDa catalytic subunit that play important physiologic roles including cell differentiation, growth, proliferation, migration, metabolism and host defense [179]. The p110 δ isoform is uniquely expressed in leukocytes [388], suggesting they may play important role in regulating host immunity. Our laboratory was the first to report that mice with inactivating knock-in mutation in the p110 δ gene (p110 δ^{D910A} mice) display enhanced resistance to cutaneous leishmaniasis [160]. I also found that p110 δ^{D910A} mice are highly resistant to visceral leishmaniasis (see section 3.1.4.1 above). This enhanced resistance was related in part to impaired expansion and function of Tregs in the infected mice [160, 384]. Several reports show that PI3K signaling pathway regulates HSC activation and function, including proliferation, migration and survival [218, 285]. As HSCs have been reported to selectively expand or induce Tregs [219, 250, 319, 362], I investigated the role of HSCs in Treg expansion/induction following *L. donovani* infection and thus the pathogenesis of experimental VL.

3.2.2 Hypothesis

I hypothesize that the differential resistance of WT and p110 δ^{D910A} mice to L. donovani is related to differences in expansion and function of their HSCs.

3.2.3 Objectives

- a) Determine the number, activation and cytokine status of HSCs before and after *L. donovani* infection in WT and p110 δ^{D910A} mice
- b) Identify the effect of *in vivo* depletion of HSC on outcome of *L. donovani* infection in WT and p110 δ^{D910A} mice
- c) Determine whether *L. donovani* infects and proliferates in HSCs
- d) Investigate the mechanism through which HSCs influence outcome of *L. donovani* infection by assessing its effects on Treg expansion/induction and function in *L. donovani* infected and uninfected WT and p110 δ^{D910A} mice

3.2.4 Results

3.2.4.1 Infection with *L. donovani* is associated with increased number of Tregs and HSCs in the liver

Tregs accumulate at the pathologic sites of infection in both murine [110] and human VL [162] and contribute to susceptibility to experimental *L. donovani* infection [384]. Consistent with our previous observations [160, 384], infection with *L. donovani* was associated with increased percentage (Figure 3-13A and B) and absolute numbers (Figure 3-13C) numbers of Tregs in the livers at 2 and 4 wk. post-infection. Majority of the Tregs were uniformly CD127^{lo} and expressed high levels of GITR, which is consistent with these cells being *bona fide* Tregs (Figure 3-14A to D). The increase in Treg numbers was directly correlated with increased parasite burden (Figure 3-13D). Treatment of infected mice with anti-CD25 mAb led to dramatic reduction in Treg numbers and parasite burden in the livers of infected mice (Figure 3-13E and F).

Although HSCs are important in the progression of parasite-induced liver fibrosis and disease [222] and have been shown to regulate Treg induction [362] and expansion [250], no study has investigated the role of these cells in the immunopathogenesis of VL in the liver. Using immunofluorescence, I found that HSC numbers and their activation increase during *L. donovani* infection (Figure 3-15A). To further confirm these observations, I utilized a novel flow cytometry approach to assess HSCs using GFAP, α SMA, Desmin and LRAT expression directly *ex vivo*. The purity of HSCs (as assessed by GFAP, α SMA, Desmin and LRAT expression) was > 97% and the purified HSCs did not significantly express CD11b, F4/80 or CD11c molecules (Figure 3-16A and B). I further confirmed the authenticity of the HSCs by showing co-expression of α SMA and GFAP, and α SMA and LRAT by flow cytometry and confocal microscopy (Figure 3-16C and D). Consistent with the immunofluorescence results, the absolute numbers and mean fluorescence intensities (MFI) of GFAP⁺ (Figure 3-15B), α SMA⁺ (Figure 3-15C), Desmin⁺ (Figure 3-15D) and LRAT⁺ (Figure 3-15E) HSCs increased significantly ($p < 0.05$ - 0.01) in *L. donovani*-infected mice. Interestingly, the increase in HSC numbers correlated directly with increase in Treg numbers ($r = 0.897$, $p < 0.0001$; Figure 3-17).

Collectively, these findings indicate that *L. donovani* infection is associated with expansion and activation of HSCs in the liver.

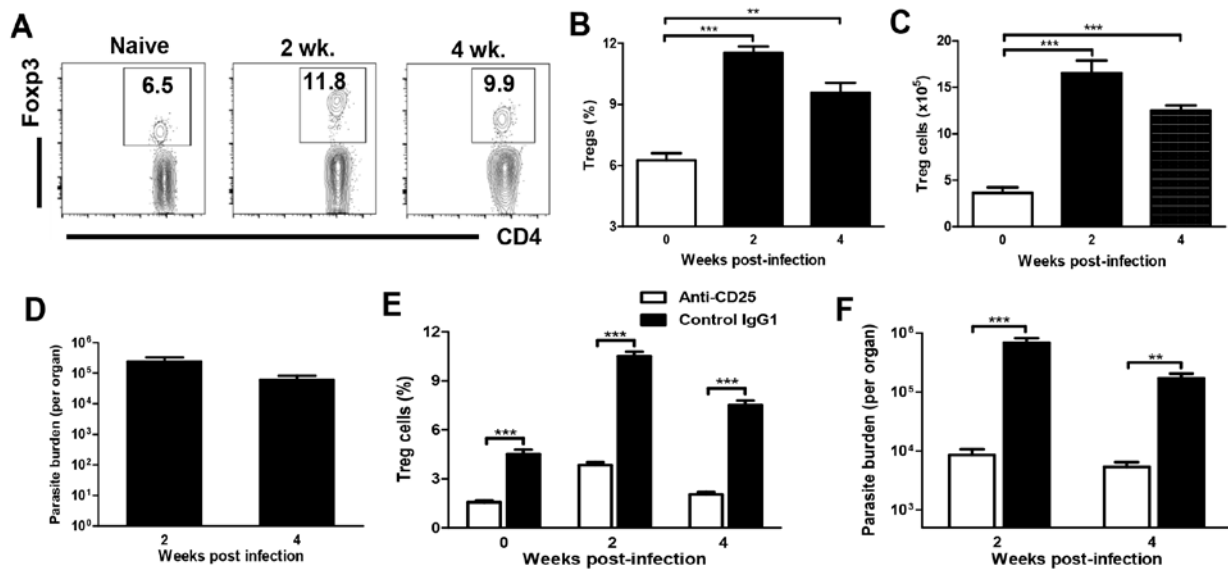


Figure 3-13: Tregs mediate impaired parasite control in the liver of *L. donovani*-infected mice

BALB/c mice were infected with 5×10^7 stationary phase *L. donovani* promastigotes. At 2 and 4 wk. post-infection, mice were sacrificed and the percentages (A, B) and absolute numbers (C) of Tregs (CD4⁺Foxp3⁺ cells) in the livers of infected mice were determined directly *ex vivo* by flow cytometry and compared to those of uninfected mice. Parasite burden in the liver was determined by limiting dilution (D). Naïve mice were injected i.p. with anti-CD25 mAb (clone PC61, 100 µg/mouse) or isotype-matched control rat IgG1 and infected with *L. donovani* 3 days later. Antibody treatment was administered once weekly, and at indicated times, mice were sacrificed and the percentage of Tregs (E) and parasite burden (F) were determined. Results are representative of 3 independent experiments (n = 4 mice per group per experiment) with similar results. Error bars, +/- SEM; **, p < 0.01; ***, p < 0.001.

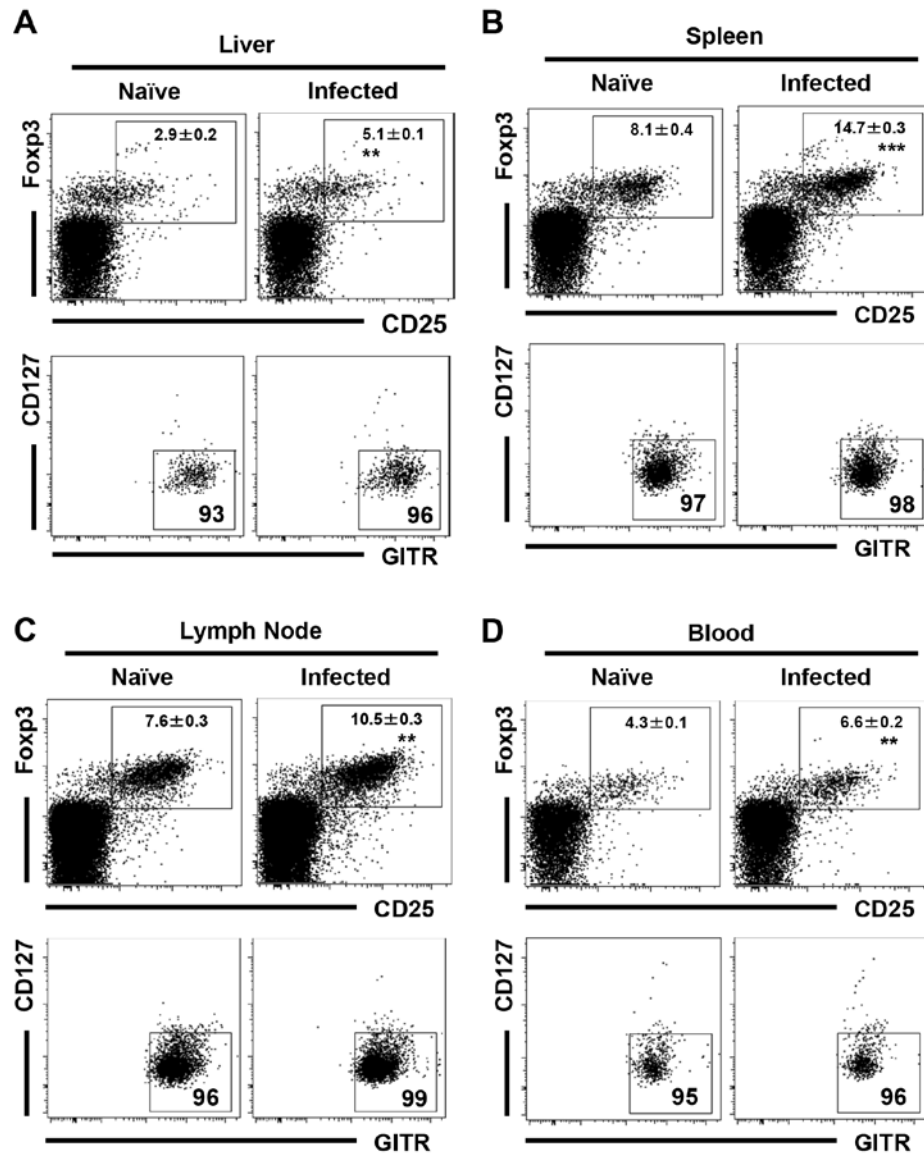


Figure 3-14: Increase in hepatic and periphery Tregs following *L. donovani* infection

Naïve and 2wk. *L. donovani*-infected WT mice were sacrificed and the percentages of Treg (CD25⁺Foxp3⁺) cells in the liver, spleen, pooled peripheral lymph nodes, (popliteal, inguinal and mediastinal) and blood were determined directly *ex vivo* by flow cytometry after gating on CD3⁺CD4⁺ cells. The Tregs (Foxp3⁺ population) were further analyzed for the expression of CD127 and GITR. Shown are representative dot plots displaying the mean $\pm$ SEM of the percentages of CD25⁺Foxp3⁺ (upper panels) and mean percentages of CD127^{low}GITR⁺ (lower panels) cells in the liver (A), spleen (B), lymph nodes (C) and blood (D). Results are representative of 2 independent experiments (n = 4 mice per group) with similar results. **, p < 0.01; ***, p < 0.001.

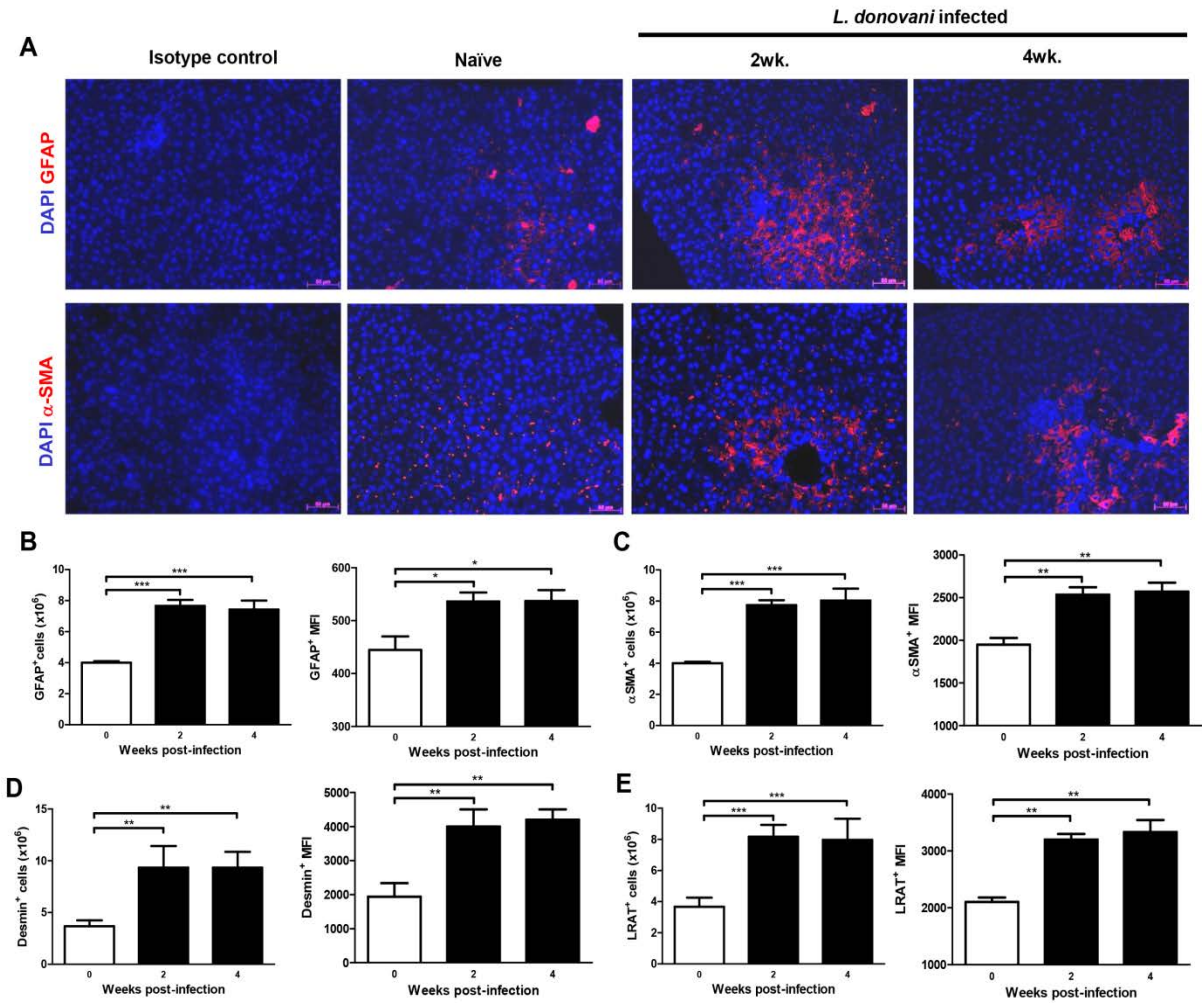


Figure 3-15: *L. donovani* infection leads to expansion of HSCs in the liver

Immunofluorescence staining of formalin-fixed liver tissues from naïve or 2 and 4 wk. *L. donovani*-infected mice (Red = GFAP and αSMA; Blue = DAPI) (A). Slides were visualized at 20x magnification. Bar graphs of freshly isolated HSCs from naïve or 2 and 4 wk. infected mice showing the absolute numbers (left panels) and mean fluorescence intensities (MFIs, right panels) of GFAP⁺ (B), αSMA⁺ (C), Desmin⁺ (D) or LRAT⁺ (E) cells as assessed directly *ex vivo* by flow cytometry. Results are representative of 4 (A-C) and 2 (D) independent experiments (n = 3 mice per group per experiment) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

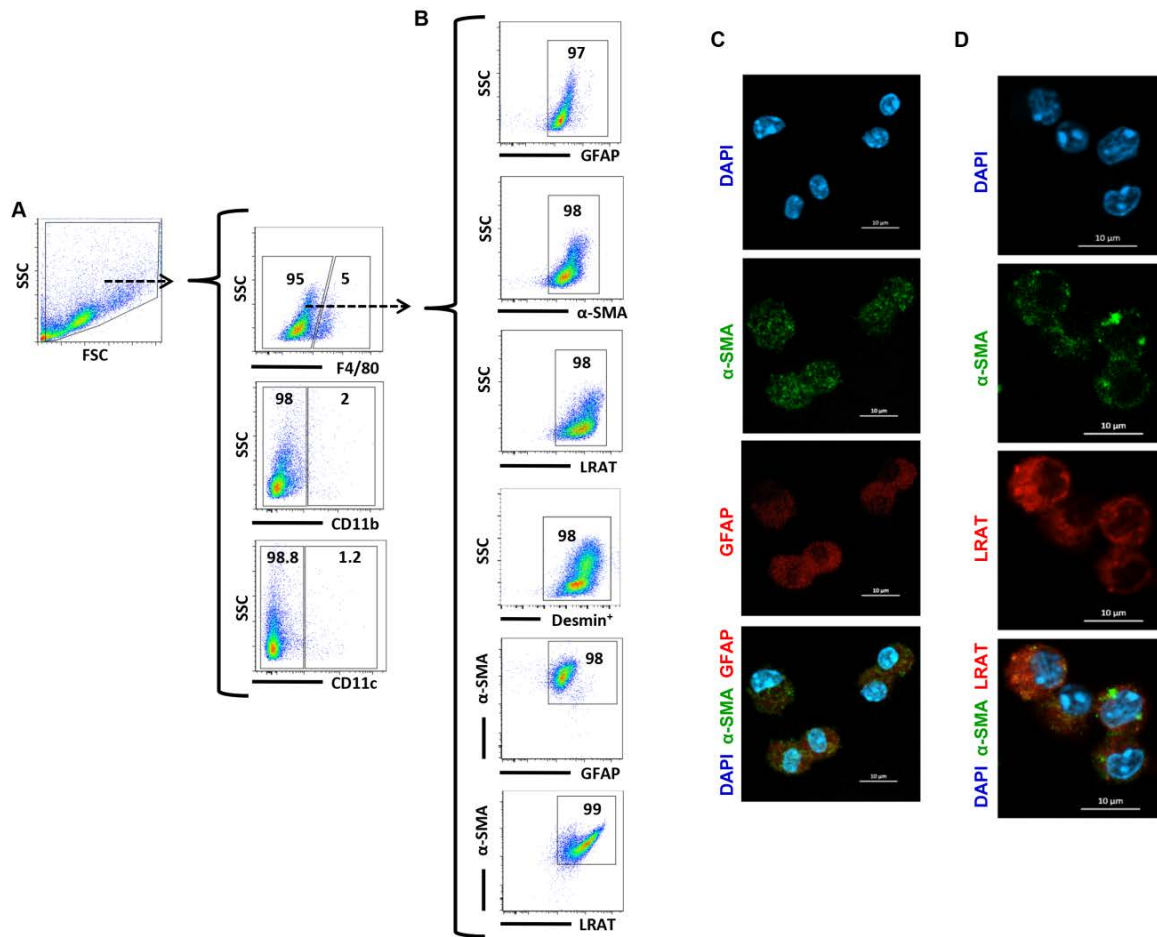


Figure 3-16: Flow cytometry analyses and confocal microscopy showing the gating strategy and purity of HSCs

HSCs isolated from naïve or *L. donovani*-infected WT mice were directly characterised *ex vivo* by flow cytometry and assessed for expression of F4/80 (macrophages/monocytes), CD11c (DCs) or CD11b (monocytes) molecules (A). Following exclusion of cells expressing these molecules, the purity of HSCs was further assessed based on expression of GFAP, α-SMA, Desmin and LRAT (B). Cytospins were prepared from freshly isolated HSCs stained directly *ex vivo* according to flow cytometry protocols for DAPI, GFAP and α-SMA (C) or DAPI, α-SMA and LRAT (D) and visualized under confocal microscopy with 63 oil-immersion lenses. Results are representative of 4 (C) and 2 (D) independent experiments (n = 3 mice per group per experiment) with similar results.

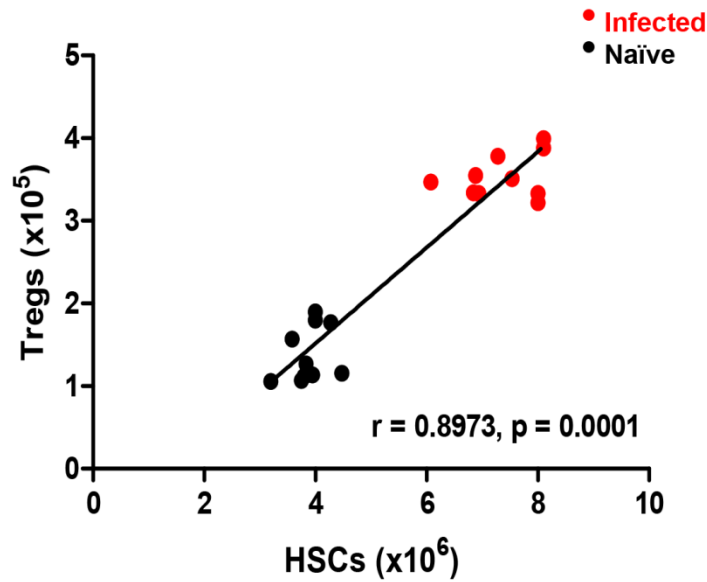


Figure 3-17: Direct correlation between increase in Treg and HSC numbers following *L. donovani* infection

Naïve or 4 wk. *L. donovani*-infected mice were sacrificed and the numbers of Tregs and HSCs in the livers were determined by flow cytometry. The correlation between HSC and Treg numbers in naïve and 4 wk. *L. donovani*-infected mice was determined by linear regression. Results are representative of 3 independent experiments (n = 3 mice per group per experiment) with similar results ($r = 0.8973$; $p = 0.0001$).

3.2.4.2 HSCs are infected by *L. donovani* *in vitro* and *in vivo*

Leishmania parasites have developed various strategies to evade the host immune defenses. One of these strategies includes invasion of cell types lacking leishmanicidal effector mechanisms and using them as safe havens to replicate [402]. Although a recent report suggested that quiescent and activated human HSCs may be permissive to *L. donovani* with no apparent impact on HSC function [403], there is no report regarding whether *L. donovani* parasites can actually infect and replicate in mouse HSCs *in vivo* or *in vitro*. Therefore, I isolated HSCs from naive mice, infected them with *L. donovani* promastigotes or amastigotes and determined the level of infection at various times after infection under light microscopy. For the first time, I unequivocally show that *L. donovani* amastigotes (Figure 3-18A and B) and promastigotes (Figure 3-18C and D) can infect and replicate in HSCs *in vitro*. As a control, infection of BMDMs with *L. donovani* promastigotes *in vitro* resulted in high infectivity (Figure 3-19) consistent with our previous report [384].

Next, I investigated whether HSCs are also infected with *L. donovani* *in vivo*. I prepared cytopsin preparations of HSCs isolated directly from *L. donovani*-infected mice (without *in vitro* culture), stained them with Hematoxylin and Eosin (H&E) and assessed for infection by microscopy. The data presented as Figure 3-18E show that HSCs are infected with *L. donovani* *in vivo*. I further confirmed *in vivo* infection of HSCs by immunofluorescence, which shows co-localization of *L. donovani* (PEPCK⁺ and DAPI⁺) in α -SMA⁺ HSC cells (Figure 3-18F).

Collectively, these results show that *L. donovani* infects and replicates in HSCs *in vitro* and *in vivo*, suggesting that these cells could influence liver immunity following infection.

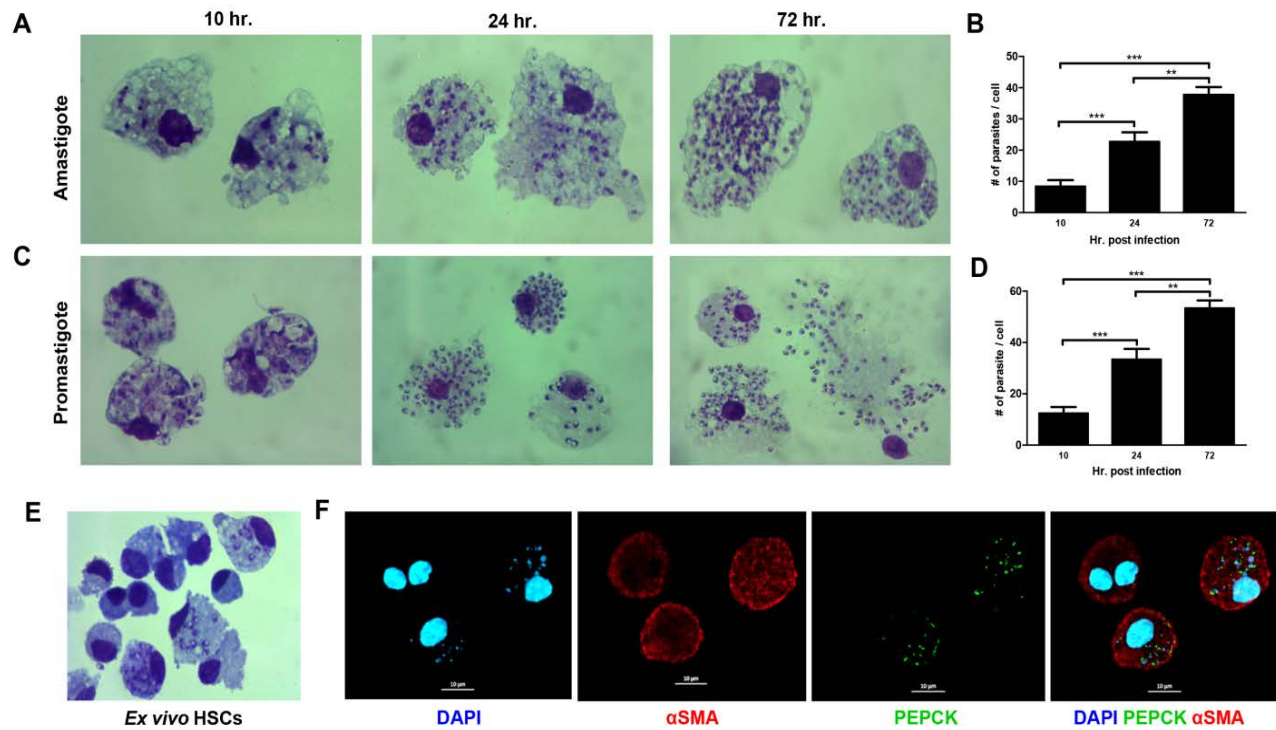


Figure 3-18: *L. donovani* can infect and replicate in HSCs *in vitro* and *in vivo*

HSCs were isolated from naive BALB/c mice and infected with *L. donovani* amastigotes (A and B) or promastigotes (C and D) in polypropylene tubes at a ratio of 1:10. After 10 hr., free parasites were washed off and at the indicated times, infectivity was assessed by counting H&E-stained cytopsin preparations under the light microscope at x100 (oil) objective. Cytopsin preparations of freshly isolated HSCs from 2 wk. *L. donovani*-infected mice were stained with H&E and visualized under light microscope (E). Some cells also were stained for DAPI (nucleus marker), α-SMA (HSC marker) and PEPCK (*L. donovani* marker) and pictured under confocal microscopy with 63 oil-immersion lens (F). Results are representative of 2 (A-D) and 3 (E and F) independent experiments (n = 3 mice per group per experiment) with similar results. Error bars, +/- SEM; **, p < 0.01; ***, p < 0.001.

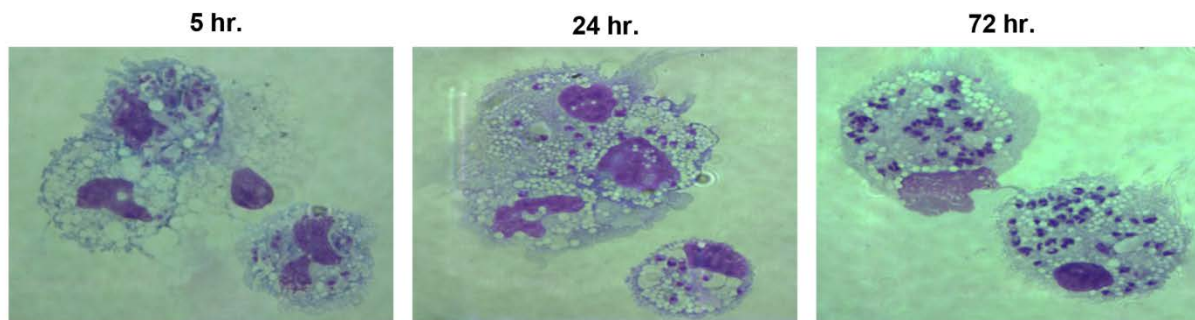


Figure 3-19: Infectivity of *L. donovani* promastigotes in bone marrow-derived macrophages *in vitro*

Bone marrow-derived macrophages (BMDMs) were differentiated *in vitro* from marrow cells and infected with 7-day stationary phase *L. donovani* promastigotes at a cell-to-parasite ratio of 1:5. After 5 hr., free parasites were washed off and the infected cells were cultured at 37°C. At indicated times, the level of infection was determined by assessing H&E-stained cytospin preparations under the light microscope at 100x (oil) objective.

3.2.4.3 HSCs from *L. donovani*-infected mice produce immunoregulatory and proinflammatory cytokines and induce CD4⁺ T cells to become Tregs

Previous studies have shown that activated HSCs produce cytokines with proinflammatory/anti-inflammatory activities [307]. Therefore, I assessed and compared cytokine production by HSCs from naïve or *L. donovani*-infected mice *in vitro*. As shown in Figure 3-20A, HSCs from *L. donovani*-infected mice produce significantly higher levels of TNF- α , IL-1 β , IL-6, IL-4, IL-2, IL-10, and TGF- β than those from naïve (uninfected) mice *in vitro* as detected by ELISA. IL-17 levels were also detected by ELISA and there were no significant difference between the naïve or *L. donovani*-infected mice Figure 3-20A. I further confirmed the ability of HSCs from infected mice to produce IL-2 (Figure 3-20B and C) and IL-4 (Figure 3-20D and E) directly *ex vivo* by flow cytometry.

IL-2 and TGF- β have been associated with the conversion of CD4⁺ T cells into inducible Tregs in an MHC class II dependent manner [250]. Indeed, I found that freshly isolated HSCs from *L. donovani*-infected mice, unlike those from naïve mice, express high levels of MHC class II (Figure 3-21A) and CD86 (Figure 3-21B) molecules. Given that HSCs have been implicated in antigen presentation to CD4⁺ T cells [304], I determined whether their expression of costimulatory molecules and production of IL-2 and TGF- β could be associated with induction or expansion of Tregs. Therefore, I co-cultured HSCs from naïve or *L. donovani*-infected mice with CFSE labeled CD4⁺CD25⁻ or CD4⁺CD25⁺ (>89% Foxp3⁺) T cells from naïve mice, stimulated them with soluble anti-CD3/anti-CD28 mAb and assessed Treg induction/expansion after 5 days. I found that although HSCs from both naïve and infected mice were able to induce CD4⁺CD25⁻ T cells to become Tregs, HSCs from infected mice had significantly ($p < 0.01$)

greater ability to induce Tregs compared to those from naïve mice (Figure 3-21C). Furthermore, *L. donovani*-infected HSCs induced significantly more proliferation of Tregs than those from naïve mice (Figure 3-21D). I also observed HSC-dependent proliferation of CD4⁺CD25⁺ T cells although HSCs from both naïve and infected mice had comparable effects (Figure 3-21E).

In conclusion, these results show that the immunoregulatory and proinflammatory cytokine milieu produced by *L. donovani*-infected HSCs in the liver, is associated with conversion of CD4⁺ T cells into inducible Tregs.

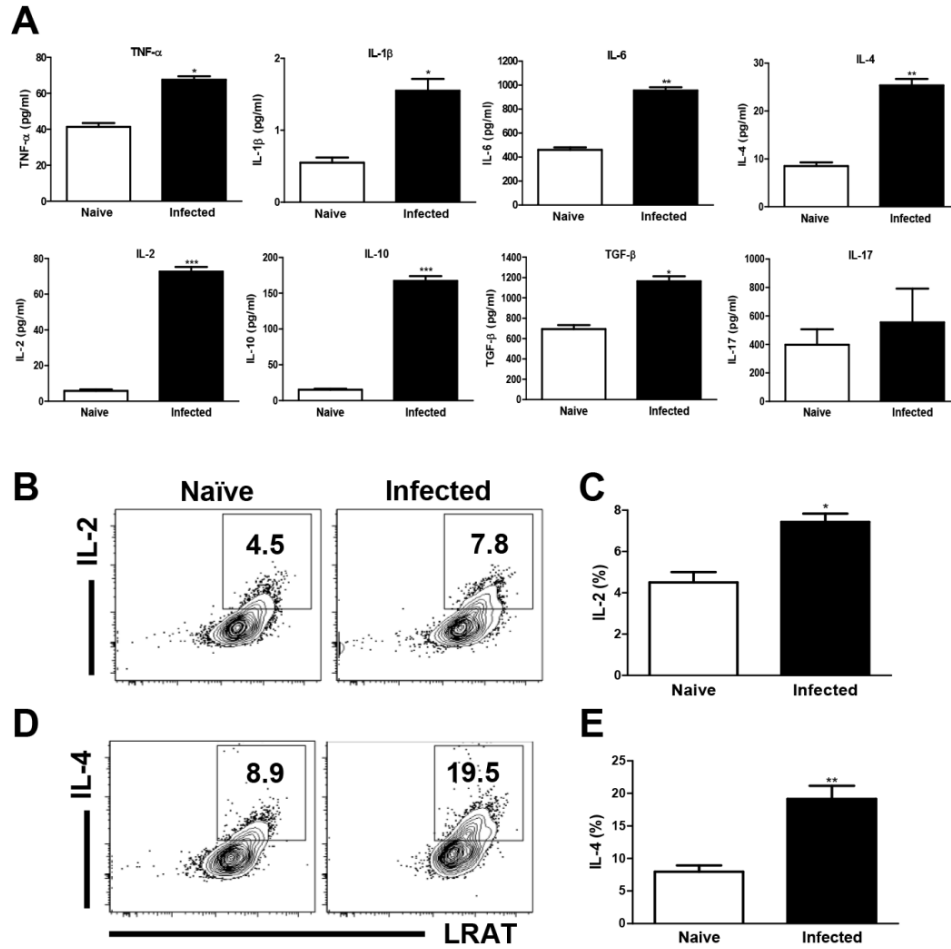


Figure 3-20: HSCs-derived from *L. donovani*-infected mice spontaneously produce high amounts of immunoregulatory cytokines

HSCs from naïve or 2 wk. *L. donovani*-infected mice were plated in 24-well tissue culture plates and after 5 days, the supernatant fluids were collected and the levels of TNF- α , IL-1 β , IL-6, IL-4, IL-2, IL-10, TGF- β and IL-17 were determined by Meso Scale or ELISA (A). Some freshly isolated HSCs were stimulated with PMA, ionomycin, and Brefeldin A for 4 hr., fixed and intracellularly stained for LRAT, IL-2 (B and C) and IL-4 (D and E) and cytokine expression was assessed by flow cytometry. Results are representative of 3 (A) and 2 (D-E) independent experiments (n = 3-4 mice per group per experiment) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

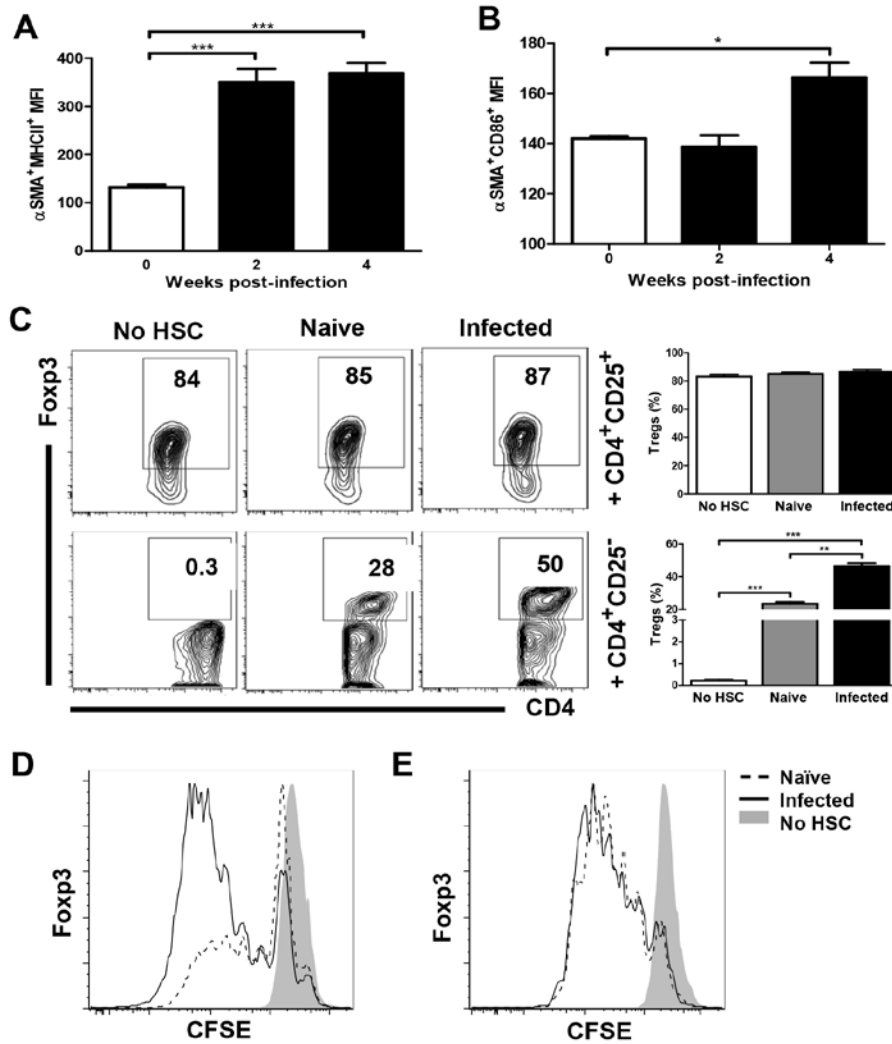


Figure 3-21: *L. donovani* infection increases costimulatory molecule expression on HSC and their ability to induce and expand Tregs

Freshly isolated HSCs were assessed directly *ex vivo* for the expression of MHC class II (A) and CD86 (B) by flow cytometry. Highly purified CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells were labeled with CFSE dye and co-cultured with freshly isolated HSCs from naïve or 2 wk. *L. donovani*-infected mice. The cultures were stimulated with soluble anti-CD3 and anti-CD28 for 5 days and the percentage of Tregs (CD4⁺CD25⁺Foxp3⁺ cells) were assessed by flow cytometry (C). Bar graphs show the mean $\pm$ SEM of Foxp3⁺ cells. Histogram plots show the proliferation of Foxp3⁺ cells in co-cultures of HSCs and CD4⁺CD25⁺ (D) or HSCs and CD4⁺CD25⁻ (E) T cells. Results are representative of 3 (A and B) and 2 (C-E) independent experiments (n = 3 mice per group per experiment) with similar results. Error bars, $\pm$ SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

3.2.4.4 *In vivo* depletion of HSCs leads to less parasite burden and decreased Treg proliferation in the liver of *L. donovani*-infected mice

I previously showed that Treg expansion contributes to susceptibility to *L. donovani* infection and depletion of Tregs enhances resistance to the infection [384]. Given that I observed a direct correlation between increase in Treg and HSC numbers following infection (Figure 3-17) and that HSCs from infected mice significantly induced proliferation and expansion of Tregs *in vitro* (Figure 3-21C-E), I hypothesized that depletion of HSCs would lead to enhanced resistance to *L. donovani*. Therefore, I treated *L. donovani* infected mice with scAb C1-3 conjugated to gliotoxin to specifically deplete activated HSCs *in vivo* [375]. C1-3-gliotoxin treatment caused a significant ($p < 0.001$) decrease in the absolute numbers of HSCs in the liver (Figure 3-22A), which corresponded to $> 70\%$ reduction in HSC numbers (Figure 3-22B). The reduction in HSC numbers was associated with significant ($p < 0.01-0.001$) reduction in hepatic parasite burden (Figure 3-22C), Tregs numbers (Figure 3-22D) and significant ($p < 0.001$) reduction in the frequency of IL-10-producing (Figure 3-22E) (but not IFN- γ -producing, Figure 3-22F) CD4⁺ cells.

Taken together, these observations indicate that HSCs regulate liver specific immunity to *L. donovani* infection in part by regulating intra-hepatic induction and expansion of Tregs.

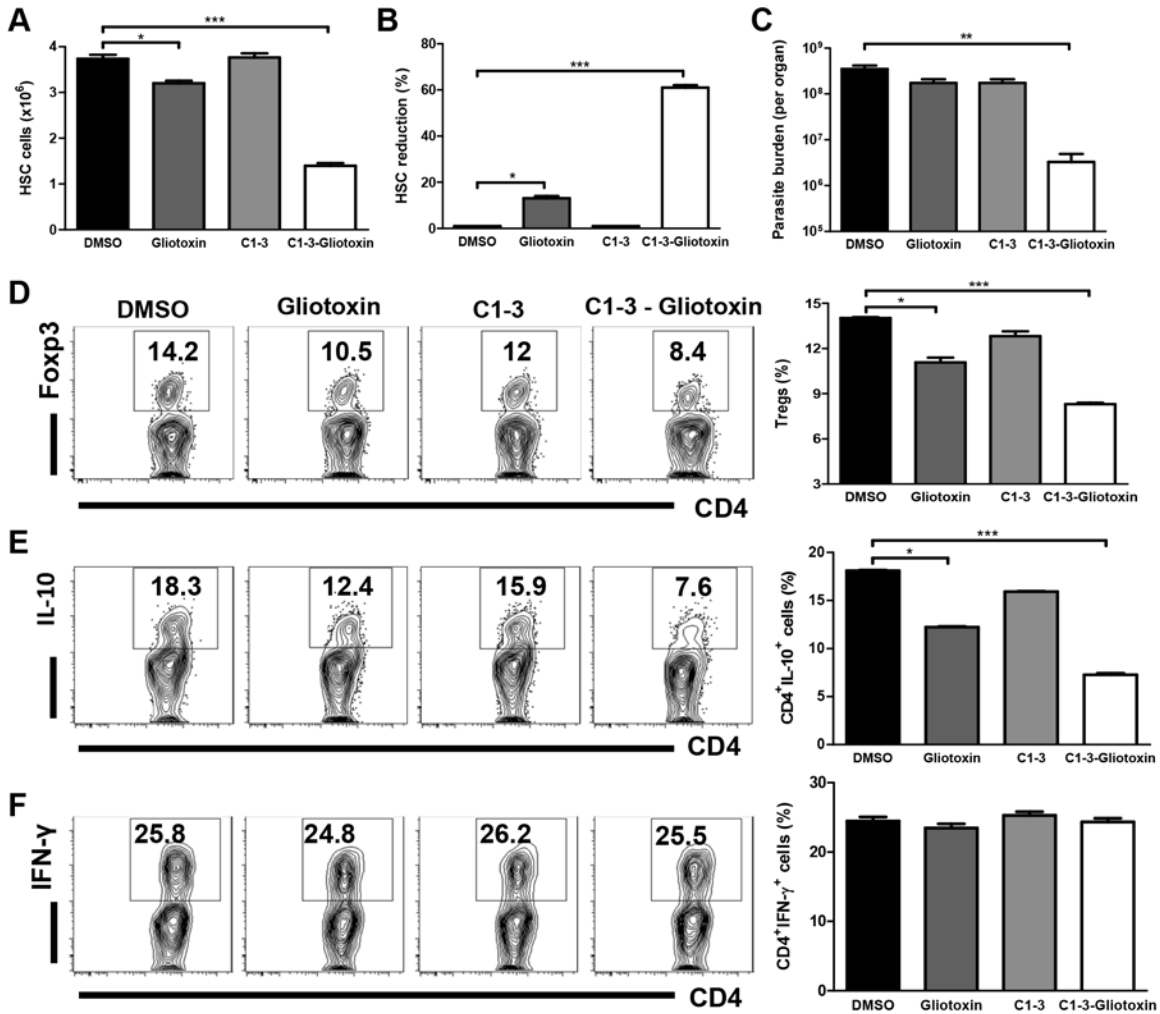


Figure 3-22: *In vivo* depletion of HSCs leads to enhanced liver immunity against *L. donovani* infection

Naïve mice were depleted of HSCs via administration of C1-3 scAbs conjugated to gliotoxin (C1-3 - Gliotoxin) (600 µg/kg) i.p. 24 hr. prior to *L. donovani* infection. Antibody treatment was continued on days 1, 3, 5, 7, 9, 11 and 13 post-infection. Unconjugated C1-3 scAbs (C1-3), Gliotoxin and dimethyl sulfoxide (DMSO) were also administered to different control groups. Two wk. post-infection, mice were sacrificed and the absolute numbers (A), percent HSC reduction (B) were determined directly *ex vivo*. Parasite burden (C), Treg percentages (D), IL-10 (E) and IFN-γ (F) secretion by hepatic lymphocytes were also assessed. Results are representative of 2 independent experiments (n = 4 mice per group per experiment) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

3.2.4.5 Signaling via the p110 δ isoform of PI3K regulates HSC expansion and induction of Tregs

Mice with inactive knock-in mutation of p110 δ isoform of PI3K (p110 δ^{D910A} mice) are relatively more resistant than WT mice to experimental leishmaniasis due in part to impaired expansion of Tregs [160, 197, 384]. Since PI3K signaling pathway regulates several aspects of HSC activation and function [218, 404], I speculated that impaired expansion of HSCs and the resulting inability to expand/induce Tregs in the liver could be responsible for the hyper-resistance of p110 δ^{D910A} mice to *L. donovani*. Indeed, I found that HSCs from both naïve and infected mice express p110 δ mRNA (Figure 3-23A). Consistent with our previous report, I confirmed that p110 δ^{D910A} mice are more resistant to *L. donovani* infection than WT mice (Figure 3-23B), and this resistance was associated with significantly ($p < 0.01$) less numbers of Tregs in their livers (Figure 3-23C) compared to WT mice. Using direct *ex vivo* flow cytometric analyses of GFAP and α SMA expression (Figure 3-23D and E), I found that the numbers of HSCs from *L. donovani*-infected p110 δ^{D910A} mice did not increase over time and livers from infected p110 δ^{D910A} mice contain significantly ($p < 0.05$ - 0.01) less HSCs than their WT counterpart mice. Remarkably, the lower number of Tregs and HSCs observed in *L. donovani*-infected p110 δ^{D910A} was associated with less fibrosis in these mice compared to their WT counterparts as detected by collagen deposition (Figure 3-24A and B).

Although As previously reported [372], purified HSCs from naïve or *L. donovani*-infected WT mice trans-differentiated into myofibroblasts after 5 days of *in vitro* culture (Figure 3-25A), their p110 δ^{D910A} HSC counterparts did not have the ability to transdifferentiate into myofibroblasts *in vitro* (Figure 3-25B). Interestingly, I observed comparable infectivity of

WT and p110 δ^{D910A} HSCs *in vitro* (Figure 3-26A and B) and *in vivo* (Figure 3-26C) and, suggesting that the inability of p110 δ^{D910A} HSCs to expand and transdifferentiate into myofibroblasts *in vivo* and *in vitro*, respectively, was not related to resistance to infection.

Furthermore, both naïve and *L. donovani*-infected p110 δ^{D910A} HSCs had significantly ($p < 0.01$) reduced ability to induce CD4⁺CD25⁻ T cells into Foxp3⁺ (Tregs) *in vitro* compared to WT mice (Figure 3-23F). Following this observation, I sought to deplete HSCs in p110 δ^{D910A} mice (as I did in WT mice) and determine the number and proliferation status of Tregs in p110 δ^{D910A} mice after HSC depletion. The HSC depletion method used in this thesis, did not affect the number of macrophages (Figure 3-27A), NK cells (Figure 3-27B), DCs (Figure 3-27C) and monocytes (Figure 3-27D) in both WT and p110 δ^{D910A} mice liver. Although decreased HSCs in WT mice resulted in reduced Treg numbers (as was observed previously), it also lead to reduced Treg proliferation; whereas, interestingly, deficiency of p110 δ signaling had comparable effect on Treg numbers and proliferation in the livers of *L. donovani*-infected mice as HSC depletion in WT mice, and this effect was further exacerbated following HSC depletion by C13-gliotoxin treatment (Figure 3-28A-D).

In addition, the production of TNF- α , IL-1 β , IL-6, IL-4, IL-10, IL-2 and TGF- β in supernatant fluids of 2 wk. *L. donovani*-infected HSCs from p110 δ^{D910A} mice was significantly lower than those from WT mice (Figure 3-29A-G); whereas there were no differences in the IL-17 levels (Figure 3-29H). As it could be speculated that this cytokine profile could drive both Th17 and Tregs, I evaluated the induction of Th17 in WT and p110 δ^{D910A} *L. donovani* infected mice by both ELISA and flow cytometry. Cells from WT and p110 δ^{D910A} mice do not develop into Th17 in the blood (Figure 3-30A and B), spleen (Figure 3-30A and C), liver (Figure 3-30A

and D) and lymph node (Figure 3-30A and E) post *L. donovani* infection. The IL-17 produced by *in vitro* cultures of the liver (Figure 3-30G) and spleen (Figure 3-30H) cells is also comparable in naïve or *L. donovani* infected WT and p110 δ^{D910A} mice. Also the ratio of Foxp3⁺ Tregs in the liver is much higher than the IL-17⁺ Th17 cells (Figure 3-30E and F).

Collectively, these results indicate that signaling via the p110 δ isoform of PI3K regulates HSC expansion, cytokine production and induction of hepatic Tregs following *L. donovani* infection.

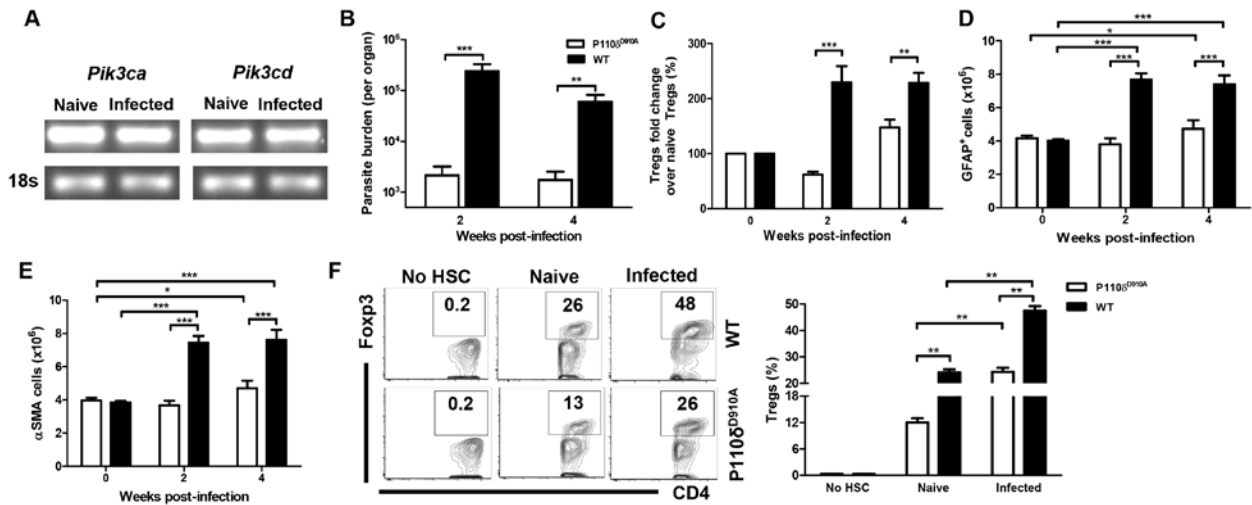


Figure 3-23: Signaling via the p110 δ isoform of PI3K regulates HSC expansion, activation and induction of Tregs

Total RNA were extracted from HSCs from naïve and *L. donovani*-infected BALB/c mice and assessed for expression of *Pik3ca* (p110 α) and *Pik3cd* (p110 δ) gene expression by RT-PCR (A). P110 δ^{D910A} and WT mice were infected with *L. donovani*, and at indicated times, mice were sacrificed and parasite burden (B) and the fold change in Tregs numbers in the liver were determined directly *ex vivo* (C). In addition, the absolute numbers of GFAP⁺ (D) and α SMA⁺ (E) cells in the livers of naïve and infected p110 δ^{D910A} and WT mice were analyzed directly *ex vivo* by flow cytometry. HSCs from the livers of naïve and 2 wk. *L. donovani*-infected WT and p110 δ^{D910A} mice were co-cultured with purified CFSE-labeled highly enriched CD4⁺CD25⁻ T cells from WT mice in the presence of soluble anti-CD3/anti-CD28 mAb for 5 days and the percentage of Foxp3⁺ cells was assessed by flow cytometry and presented as dot plots and bar graphs (F). Results are representative of 2 (A and F) and 3 (B-E) independent experiments (n = 3 mice per group per experiment) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

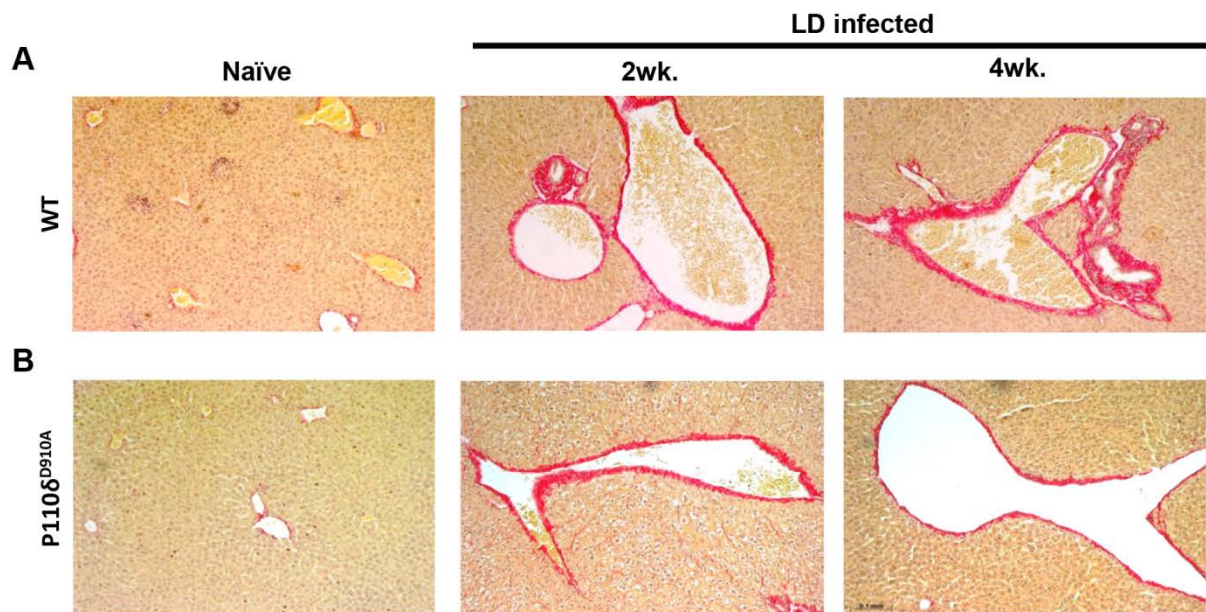


Figure 3-24: fibrosis is increased following *L. donovani* infection in WT mice compared to p110δ^{D910A}

Sirius Red staining (to assess collagen deposition and fibrosis) of formalin embedded liver sections from naïve (uninfected) or *L. donovani*-infected (2 and 4 wk.) WT and p110δ^{D910A} liver sections. Results are representative of 2 independent experiments (n = 4 mice per group per experiment) with similar results.

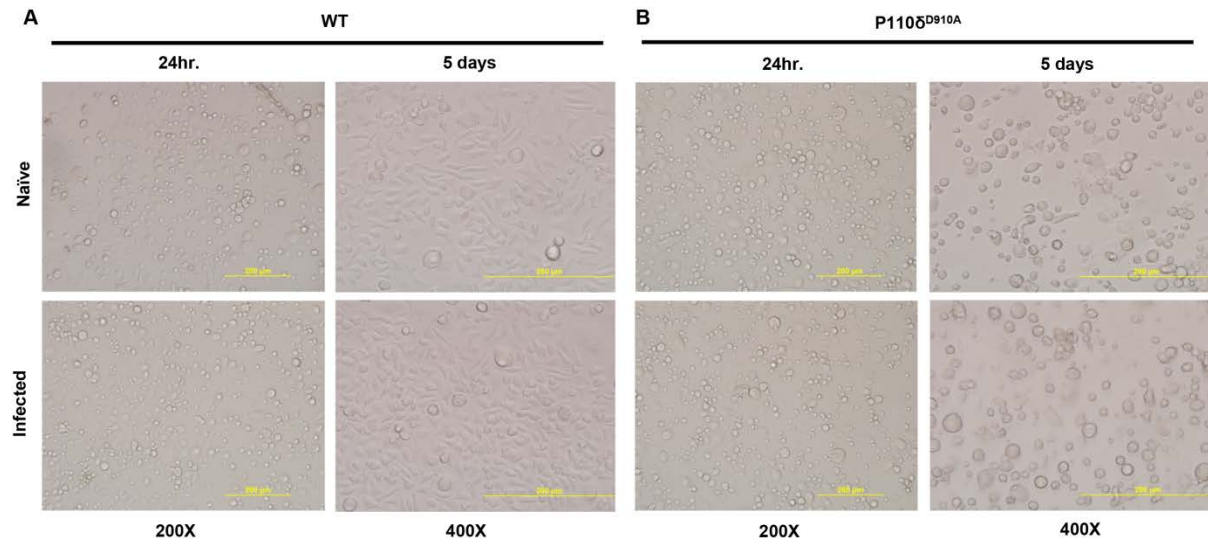


Figure 3-25: Impaired ability of HSCs from $p110\delta^{D910A}$ mice to transdifferentiate into myofibroblasts *in vitro*

Freshly isolated HSCs from naïve or 2 wk. *L. donovani*-infected WT or $p110\delta^{D910A}$ mice were cultured in polystyrene dishes at 37°C and their ability to transdifferentiate into myofibroblasts was examined under light microscopy with 20 or 40 immersion lenses. Results are representative of 4 independent experiments (n = 3 mice per group per experiment) with similar results.

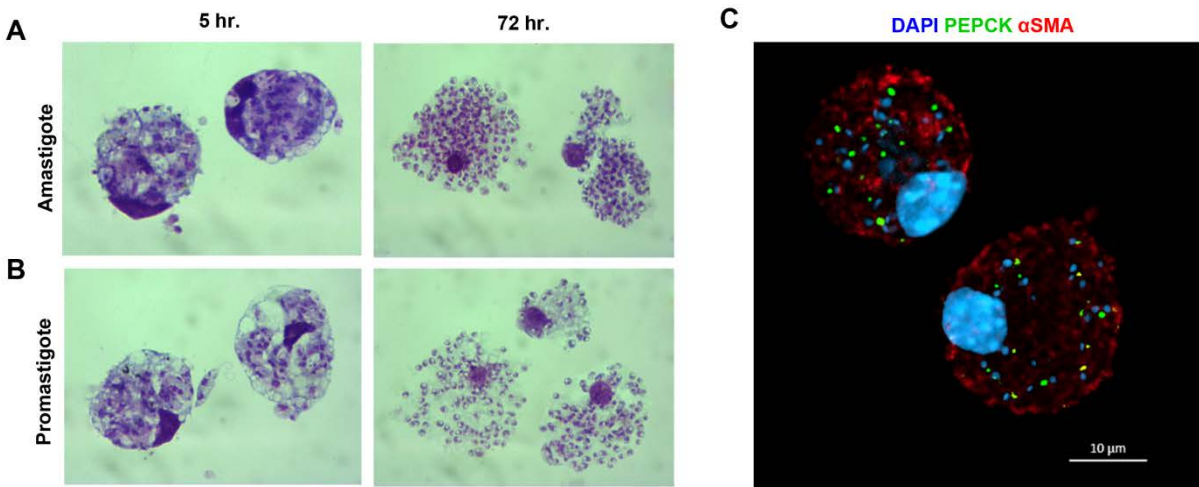


Figure 3-26: Deficiency of p110 δ signalling does not affect infection of HSCs by *L. donovani* *in vitro* and *in vivo*

HSCs were isolated directly *ex vivo* from naive p110 δ^{D910A} mice, infected with *L. donovani* amastigotes (A) or promastigotes (B) at a ratio of 1:10 (HSC:parasite). After 10 hr., free parasites were washed off and at indicated times the level of infection was determined by counting H&E-stained cytopsin preparations under the light microscope at x100 (oil) objective. Freshly isolated HSCs from 2 wk. *L. donovani*-infected p110 δ^{D910A} mice were stained for DAPI (nucleus marker), α -SMA (HSC marker) and PEPCK (*L. donovani* marker) and pictured under confocal microscopy with 63 oil-immersion lenses (C). Results are representative of 2 independent experiments with similar results.

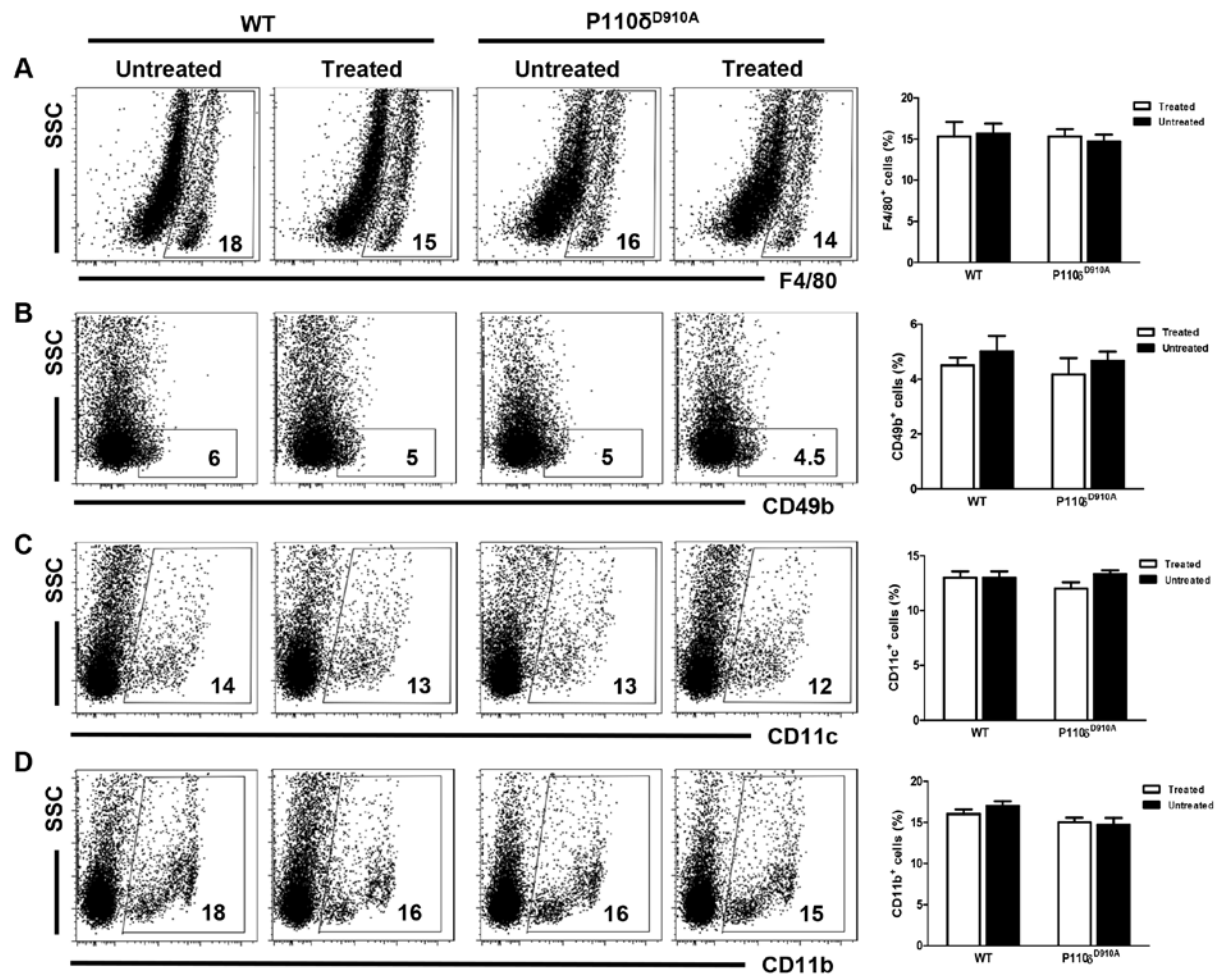


Figure 3-27: C13-gliotoxin treatment does not affect other hepatic cell populations

WT and p110 δ ^{D910A} naïve mice were depleted of HSCs via administration of C1-3 scAbs conjugated to gliotoxin (treated) every other day for 2 wk. DMSO was also administered to different control groups (untreated). Mice were sacrificed and livers were digested with collagenase D and mononuclear cells were enriched using standard percoll protocol. The cells were stained with fluorochrome-conjugated antibodies against F4/80 (macrophage marker, A), CD49b (NK marker, B), CD11c (DC marker, C) and CD11b (monocyte marker, D) and assessed by flow cytometry. Results are representative of 2 independent experiments (n = 4 mice per group per experiment) with similar results. Error bars, +/- SEM.

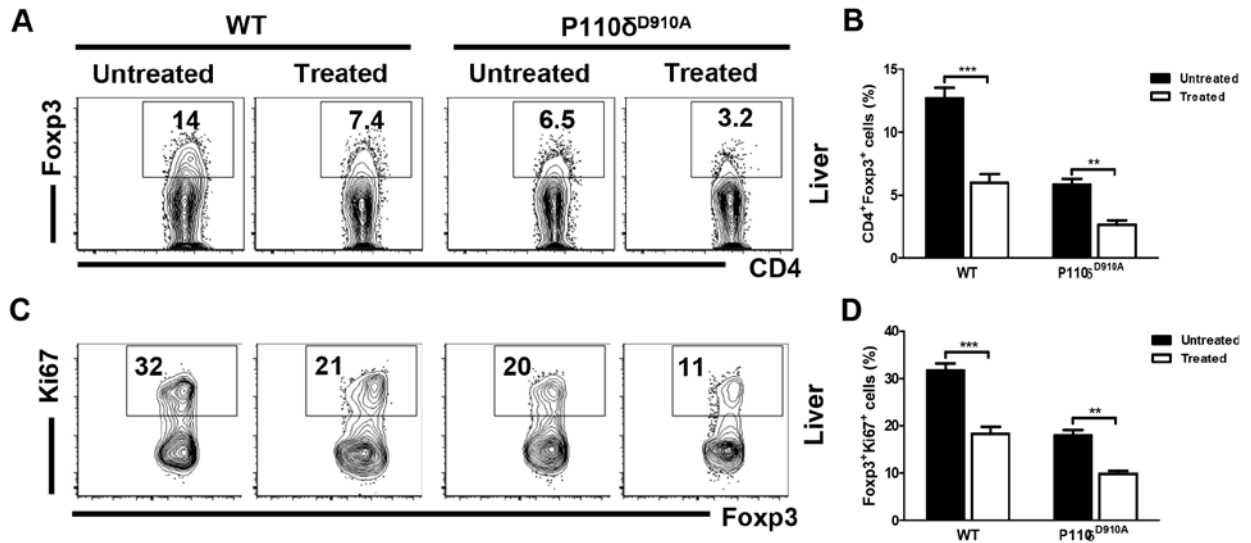


Figure 3-28: Deficiency of p110 δ signaling or depletion of HSCs impairs Tregs in the liver of *L. donovani*-infected mice

Naive WT or p110 δ ^{D910A} mice were depleted of HSCs via administration of C1-3 scAbs conjugated to gliotoxin (treated) 24 hr. prior to *L. donovani* infection. DMSO was also administrated to the control group (untreated). Antibody treatment was continued every other day for 2 wk. and the mice were sacrificed and the percentages of CD4⁺Foxp3⁺ (A and B) and Foxp3⁺Ki67⁺ (proliferating Tregs, C and D) cells in the livers were determined directly *ex vivo* by flow cytometry. Results are representative of 2 independent experiments (n = 3 mice per group per experiment) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

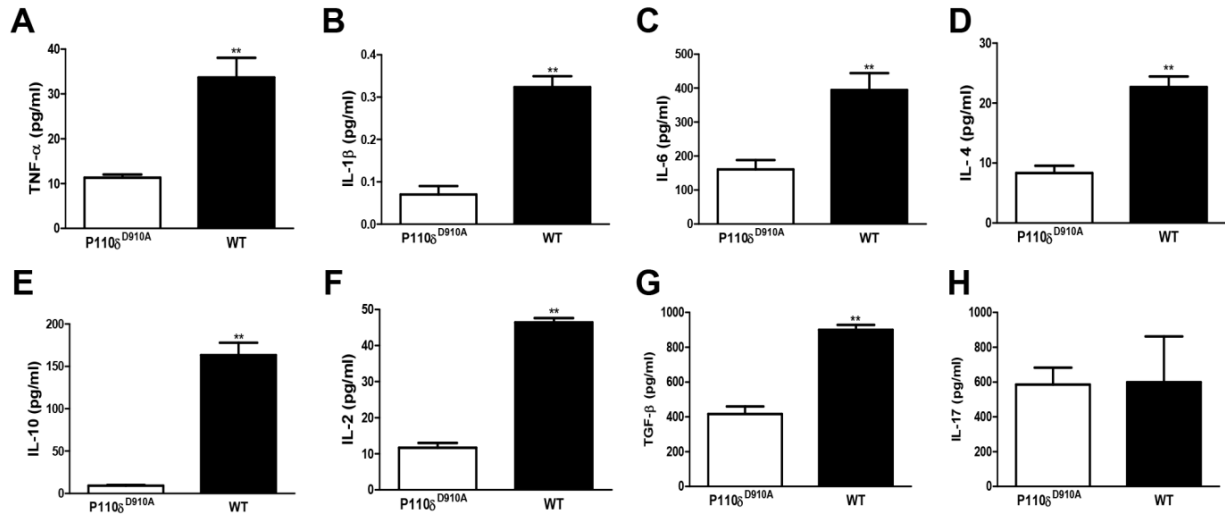


Figure 3-29: Impaired production of proinflammatory and immunoregulatory cytokines by HSCs from *L. donovani*-infected p110 δ^{D910A} mice

HSCs isolated from 2 wk. *L. donovani*-infected WT or p110 δ^{D910A} mice were cultured for 5 days at 37°C and the supernatant fluids were collected and assayed for TNF- α (A), IL-1 β (B), IL-6 (C), IL-4 (D), IL-10 (E), IL-2 (F), TGF- β (G) and IL-17 (H) by Meso Scale (A-F) or ELISA (G). Results are representative of 2 independent experiments (n = 3 mice per group per experiment) with similar results. Error bars, $\pm$ SEM; *, p < 0.05; **, p < 0.01.

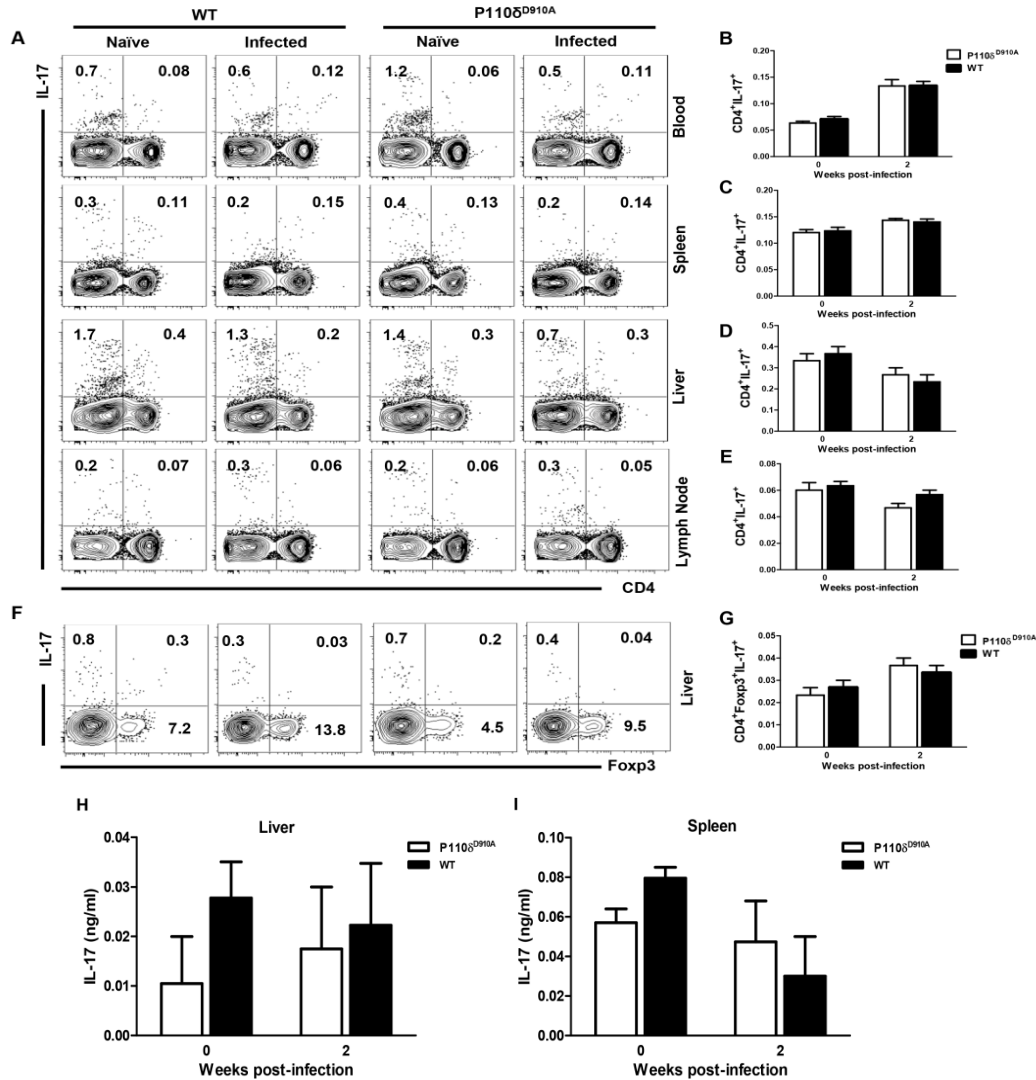


Figure 3-30: Cells from WT and P110 δ^{D910A} infected mice do not develop into Th17 or produce significant amount of IL-17

For intracellular cytokine analysis, blood (A and B), spleen (A and C), liver (A and D) and lymph node (A and E) cells from naïve or 2 wk. *L. donovani*-infected mice were stimulated with PMA, ionomycin, and Brefeldin A for 4 hr., fixed, surface-stained with specific fluorochrome-conjugated mAbs against CD3 and CD4 and stained intracellularly for IL-17. Liver cells from naïve or 2 wk. *L. donovani*-infected mice were also directly stained *ex vivo* for CD3, CD4, CD25 (extracellular staining) and Foxp3 and IL-17 (intracellular staining) expression (F and G). Samples were acquired on a FACSCanto II cytometer and analyzed using Flowjo software. Cells from naïve or 2 wk. *L. donovani*-infected mice liver (H) and spleen (I) were plated in 24-well tissue culture plates after 72 hr., the supernatant fluids were collected and the levels of IL-17 were determined by ELISA. Results are representative of 2 independent experiments (n = 4 mice per group per experiment) with similar results. Error bars, +/- SEM.

3.3 Pharmacological inhibition of p110 δ subunit of PI3K confers protection against experimental leishmaniasis

3.3.1 Rationale

Current drugs used for treatment of *Leishmania* infections are burdened by several problems that include prolonged duration of treatment, toxicity, high costs and disease relapse [4]. In addition, the emergent of drug resistant strains due to increase in resistance to anti-leishmanial drugs suggests that the currently used monotherapy needs to be reviewed and possibly replaced with multidrug/combination therapies. Therefore there is an urgent need to identify new drugs and treatment regimens.

Effective immunity against leishmaniasis is dependent on the development of type-1 immune response that leads to the production of macrophage-activating cytokines including IFN- γ and TNF. In contrast, susceptibility is usually linked to the production of macrophage-deactivating cytokines including IL-10 [134]. Reports suggest that regulatory T cells (Tregs) also play a critical role in determining the outcome of *Leishmania* infection in both mice [110] and humans [162]. Our laboratories previous work illustrated that mice with an inactivating knock-in mutation in the p110 δ subunit of the phosphatidylinositol 3 kinases (PI3K, termed p110 δ^{D910A} mice) are hyper-resistance to both experimental cutaneous and visceral leishmaniasis caused by *L. major* [160] and *L. donovani* [384], respectively. This resistance was in part, due to impaired expansion of Tregs in p110 δ^{D910A} mice [160, 384].

Different categories of PI3K inhibitors including pan-PI3K inhibitors, dual-PI3K/mTOR inhibitors and isoform-specific PI3K inhibitors have been developed to date, but most of them

have encountered problems in clinical trials due to limited efficacies as a monotherapeutic agent and relatively high rate of adverse side effects [202]. Among these PI3K inhibitors IC87114 and CAL-101 are p110 δ specific pharmacological inhibitors and CAL-101 has recently been approved for different B-cell malignancies [198-200].

Given the dramatic hyper-resistance observed in p110 δ^{D910A} mice infected with *L. donovani* [384] and *L. major* [160], I speculated that the use of highly specific pharmacological inhibitors of p110 δ may be beneficial in the treatment of experimental and CL and VL by modulating the host immune response. I also predicted that the immunomodulatory effects of the inhibitors could allow the use of lower dose of Amphotericin-B (Amph-B, a conventional leishmaniasis therapy), thereby significantly reducing the duration of treatment regimen, drug toxicity, and lead to improve drug efficacy.

3.3.2 Hypothesis

I hypothesize that treatment of Leishmania infected mice with a pharmacological inhibitor of p110 δ will confer protection against experimental visceral and cutaneous leishmaniasis and immunomodulatory effects of CAL-101 and Amph-B combination therapy will lower the required drug dose and treatment regimen and improve drug efficacy in visceral leishmaniasis treatment.

3.3.3 Objectives

- a) Investigate whether treatment of mice with IC87114 or CAL-101 prevents/cures leishmaniasis

- b) Determine whether IC87114 or CAL-101 treatment with or without Amphotericin-B alters the host immune response in *Leishmania*-infected mice
- c) Investigate whether combination treatment with CAL-101 and Amphotericin-B is more effective in curing VL than single treatment with either agent alone

3.3.4 Results

3.3.4.1 Prophylactic administration of P110 δ pharmacological inhibitors confer protection to VL and CL

Our laboratory previously showed that mice with an inactivating knock-in mutation in the p110 δ isoform of PI3K, (p110 δ^{D910A}) are hyper resistant to *L. major* [160, 197]. Similarly, in the first part of this thesis, I demonstrated that p110 δ^{D910A} mice are hyper resistant to and *L. donovani* (section 3.1.4.1). Since this resistance is independent of parasite species and genetic background, I coveted to assess whether targeting the PI3K signaling pathway with p110 δ pharmacological inhibitors may be useful for treatment of both visceral and cutaneous leishmaniasis. I administered mice with CAL-101 or IC87114 intraperitoneally [383] twice a day, 24 hr. prior to intravenous or subcutaneous infection with *L. donovani* or *L. major*, respectively. Prophylactic administration of CAL-101 resulted in significantly ($p < 0.01$) lower parasite burden in the spleen and liver of *L. donovani* infected mice (Figure 3-31A) and footpad of *L. major*-infected mice ($p < 0.01$) (Figure 3-31B). The reduction in parasite burden in the footpad was also associated with reduction in lesion size in the footpads of *L. major*-infected mice ($p < 0.01$) (Figure 3-31C). Similar results were also observed in mice treated with IC87114 (Figure 3-32A, B and C). Thus, as observed in mice with inactive knock-in mutation, pharmacologic inhibition of p110 δ isoform of PI3K leads to enhanced resistance to experimental

leishmaniasis. The results obtained in this section are of significant importance as utilizing p110 δ pharmacological inhibitors, avoids the confounding effects on immune system development that are present in the P110 δ mice due to the importance of the PI3K pathway in immune cell activation and function. Moreover, these results are obtained from WT mice that have a fully functional mature immune repertoire unlike their p110 δ^{D910A} mice counterparts that have a dysfunctional immune system.

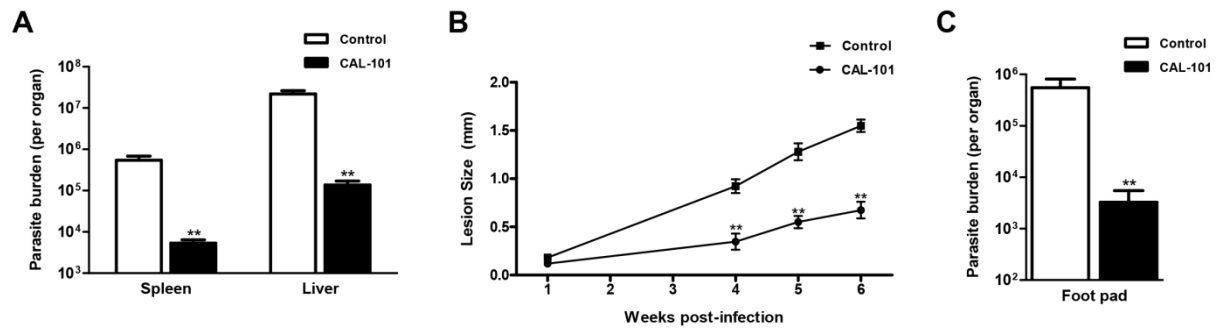


Figure 3-31: Prophylactic administration of CAL-101 enhances immunity to experimental VL and CL

BALB/c mice were administered CAL-101 (0.05 mg/mouse, twice daily), intraperitoneally 24 hr. prior to intravenous infection with 5×10^7 *L. donovani* or subcutaneous infection with 10^4 *L. major*. Intraperitoneal administration of CAL-101 was continued every 12 hr. for additional two weeks and mice were sacrificed at 2 weeks post infection. At sacrifice, parasite burden in the liver and spleen (A) and infected footpads (B) was determined by limiting dilution assay. Lesion progression in the infected footpads was monitored weekly with calipers (C). Results are representative of 2 independent experiments (n = 4 mice per group per experiment) with similar results. Error bars, +/- SEM; **, p < 0.01.

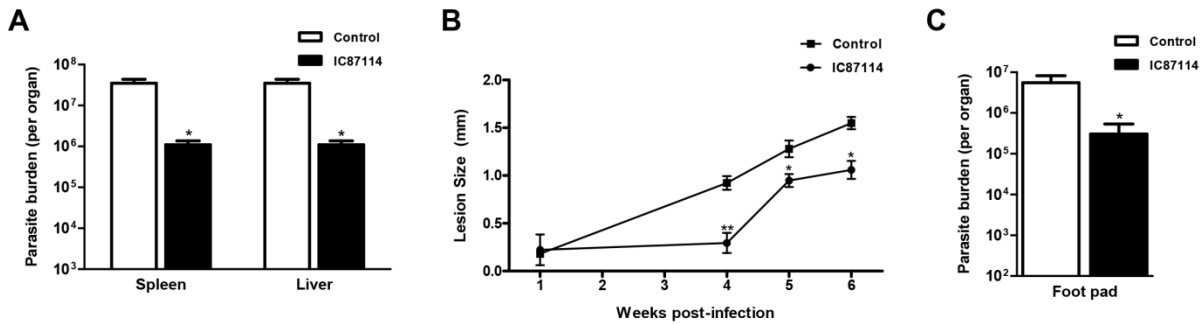


Figure 3-32: Prophylactic administration of IC87114 reduces parasites burden and lesion size in experimental VL and CL

BALB/c mice were administered IC87114 (0.5 mg/mouse), twice daily, 24 hr. prior to infection with *L. donovani* or *L. major*. Twice daily injection of CAL-101 was continued for additional two weeks and mice were sacrificed at 2 weeks post-infection. Parasite burden in the liver and spleen (A) and footpad (B) of *L. donovani* (A) and *L. major* (B) –infected mice was determined by limiting dilution. Lesion size in the footpads of *L. major*-infected mice was measured with calipers (C). Results are representative of 2 independent experiments (n = 4 mice per group per experiment) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01.

3.3.4.2 Prophylactic administration of P110δ pharmacological inhibitors alters host immune response to *Leishmania*.

Both previous work from our laboratory and results from my thesis indicate that enhanced resistance of p110δ^{D910A} mice to experimental leishmaniasis is associated with reduced immune activation and cytokine responses. Therefore, I assessed the quality of immune response in mice treated with CAL-101 in order to determine whether pharmacologic inhibitors also affect the immune response in a similar manner.

At sacrifice, liver, spleen and lymph node lymphocytes were assessed directly *ex vivo* for the numbers of Foxp3 and IFN-γ-producing cells. As in p110δ^{D910A} mice, prophylactic administration of CAL-101 resulted in significantly ($p < 0.01$) lower Treg numbers in the spleen and liver of *L. donovani*- (Figure 3-33A, B and C) and spleens and lymph node of *L. major*- (Figure 3-33G, H and I) infected mice. In addition, CAL-101 treatment also lead to significant ($p < 0.01$) reduction in the frequency of IFN-γ-producing cells in the spleen and liver of *L. donovani* (Figure 3-33D, E and F) and spleen and lymph node of *L. major* (Figure 3-33J, K and L) infected mice. The flow cytometry data was further validated by ELISA by assessing the protein levels of IFN-γ, IL-10, IL-6, IL-4 and KC/GRO in supernatant culture fluids of liver (Figure 3-34A, B, C and D), spleen (Figure 3-34E, F, G and H) and lymph node cells (Figure 3-34I, J and K) from *L. donovani* or *L. major* infected mice.

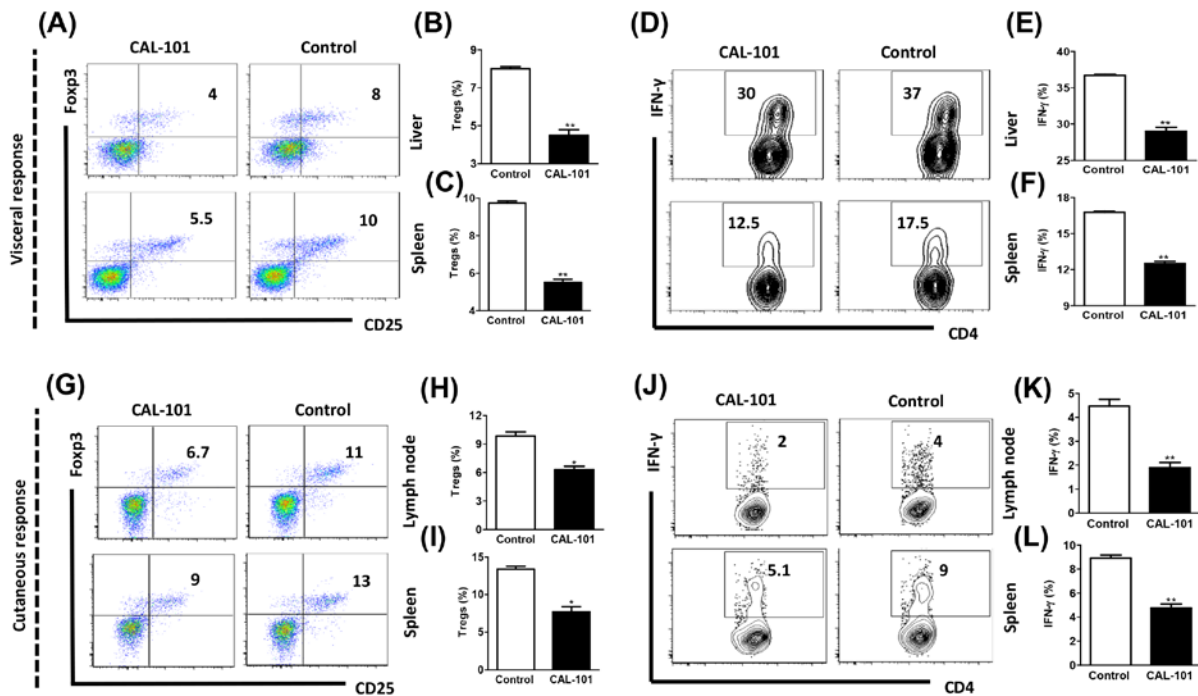


Figure 3-33: Prophylactic administration of CAL-101 reduces Treg numbers and percentage of IFN- γ -producing CD4⁺ T cells in different organs of infected mice

BALB/c mice were administered CAL-101, twice a day, 24 hr. prior to *L. donovani* or *L. major* infection. Intraperitoneal administration of CAL-101 was continued every 12 hr. for additional two weeks. Mice were sacrificed at 2 weeks post CAL-101 treatment and spleen, liver and lymph node cells were directly stained *ex vivo* for Fcγ3 and IFN- γ expression and assessed by flow cytometry. Shown are percentage of Tregs (A, B, C, G, H and I) and IFN- γ -producing CD4⁺ T cells (D, E, F, J, H, K and L) in the spleens and livers of *L. donovani*-infected mice (A, B, C, D, E and F) and spleens and draining lymph nodes of *L. major*-infected (G, H, I, J, K and L) mice. Representative dot plots of Tregs (A and G), and IFN- γ -producing CD4⁺ T cells (D and J) and their bar graphs (B, C, E, F, H, I, K and L) showing the means $\pm$ SEM are presented. Results are representative of 2 independent experiments (n = 4 mice per group per experiment) with similar results. Error bars, $\pm$ SEM; *, p < 0.05; **, p < 0.01.

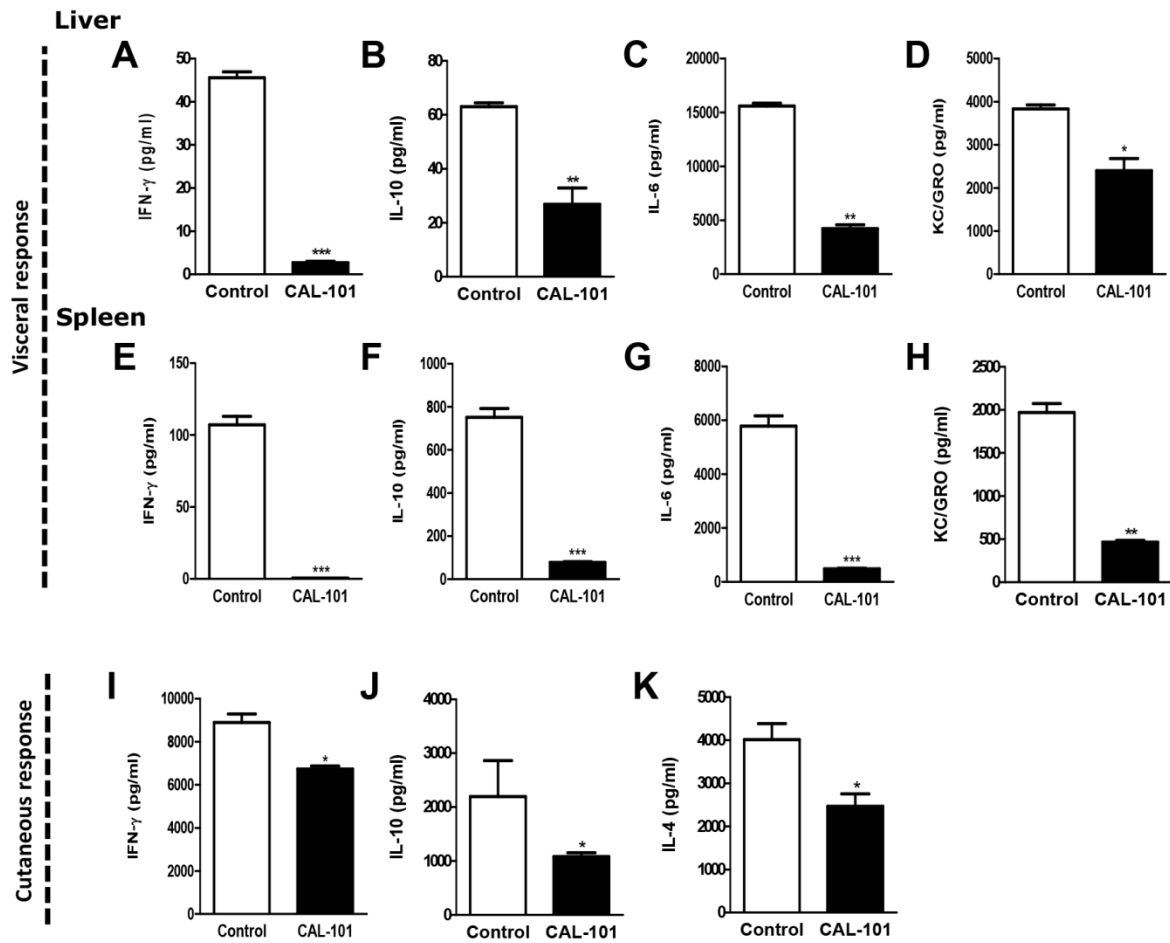


Figure 3-34: Prophylactic administration of CAL-101 is associated with reduced production of cytokines by immune cells *in vitro*

BALB/c mice were administered CAL-101, twice a day, 24 hr. prior to *L. donovani* or *L. major* infection. Intraperitoneal administration of CAL-101 was continued every 12 hr. for additional two weeks. Mice were sacrificed at 2 weeks post CAL-101 treatment and liver (A, B, C and D), spleen (E, F, G and H) and lymph node (I, J and K) cells were cultured for 72 hr. and spontaneous cytokine (IFN- γ , IL-10, IL-6, IL-4 and KC/GRO) production in the supernatant fluids was assayed by V-PLEX Meso Scale kit (A, B, C, D, E, F, G and H) or by ELISA (I, J and K). Results are representative of 2 independent experiments (n = 4 mice per group per experiment) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

3.3.4.3 Treatment with CAL-101 leads to reduction in parasite burden in mice with established disease

Treatment of leishmaniasis is challenging due to prolonged duration of treatment and the fact that most of the current drugs are toxic and have numerous side effects [22]. As CAL-101/idelalisib has recently been approved by FDA for use in patients with different B-cell malignancies [198, 200, 211], I assessed the therapeutic potential of CAL-101 for treatment of established experimental VL.

Mice were infected with *L. donovani* and after 1 or 2 wk., treated with CAL-101 every 12 hr. for a period of two wk. CAL-101 treatment initiated 1 wk or 2 wk after *L. donovani* infection resulted in significantly ($p < 0.05$) lower parasite burden in the spleens (Figure 3-35A and Figure 3-36A) and livers (Figure 3-35B and Figure 3-36B) of infected mice. The reduced parasite burden in these organs correlated with significant ($p < 0.01$) reduction in Treg numbers (Figure 3-35C and Figure 3-36C) and the numbers of IFN- γ -producing T cells (Figure 3-35D and Figure 3-36D, $p < 0.01$).

In addition, therapeutic administration of CAL-101 significantly ($p < 0.05$ - 0.01) reduced IFN- γ , IL-10, IL-6, and KC/GRO levels in the culture supernatant fluids of spleen (Figure 3-35E, F, G and H and Figure 3-36E, F, G and H) and liver (Figure 3-35I, J, K and L and Figure 3-36I, J, K and L) cells from *L. donovani*-infected mice. These findings are consistent with our previous findings indicating that resistance to VL and CL observed in p110 δ^{D910A} mice is due to reduced Treg levels and that reduced production of IFN- γ does not affect the outcome of disease. They further show a beneficial therapeutic effect of targeting this pathway in treatment of visceral leishmaniasis.

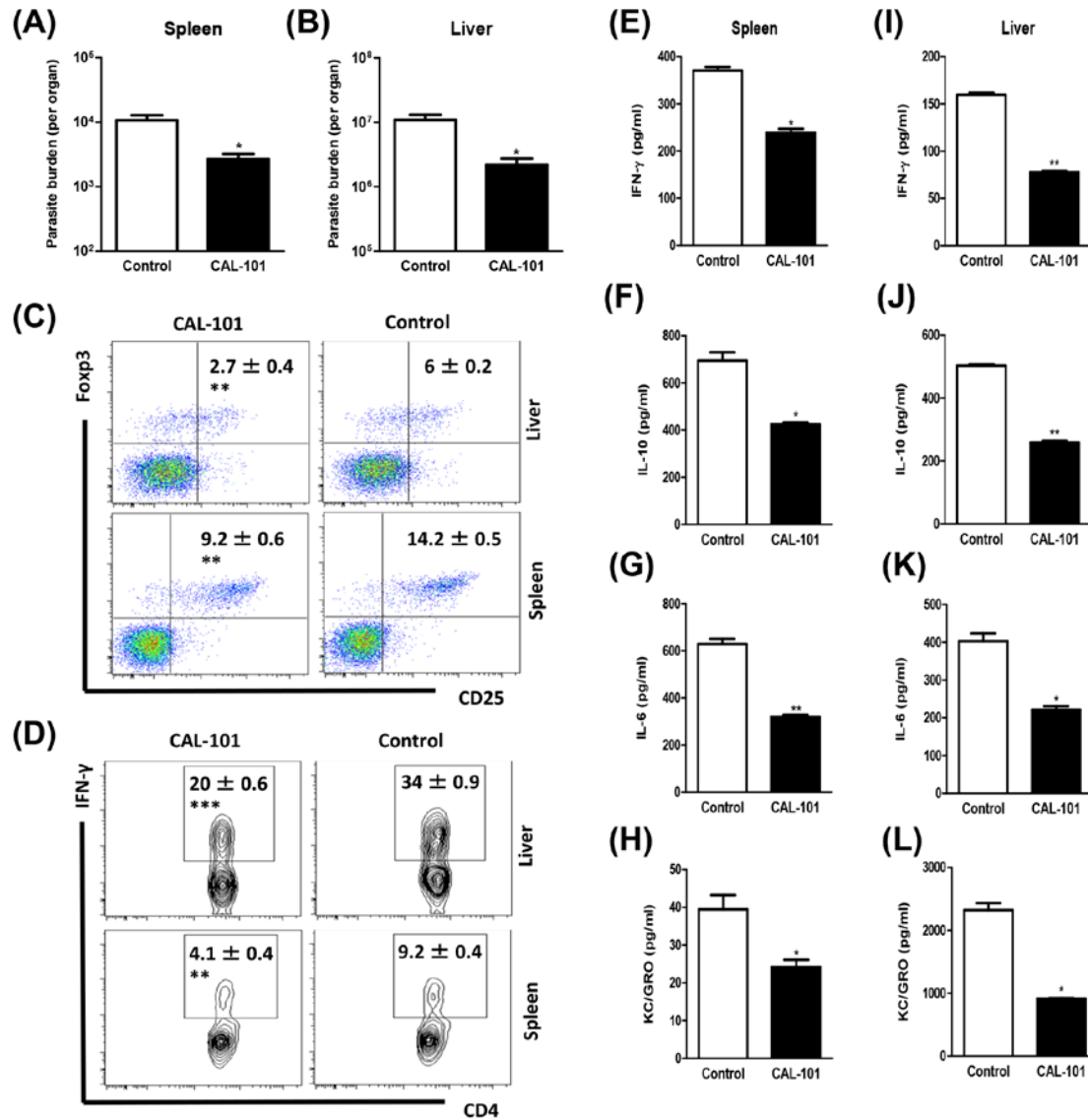


Figure 3-35: CAL-101 therapy initiated at one week after *L. donovani* infection is effective at controlling parasite burden

Mice infected intravenously with *L. donovani* promastigotes for one week were administered intraperitoneally with CAL-101 twice daily for a period of two wk. At 3 weeks post infection, mice were sacrificed and parasite burden in the spleen (A) and liver (B) was determined by limiting dilution. Spleen and liver lymphocytes were isolated and directly stained *ex vivo* for Foxp3 (C) and IFN- γ expression (D) expression and assessed by flow cytometry. Spleen (E, F, G and H) and liver (I, J, K and L) lymphocytes were also cultured for 72 hr. and the level of cytokines (IFN- γ , IL-10, IL-6 and KC/GRO) in the culture supernatant fluids was determined by V-PLEX Meso Scale. Results are representative of 2 independent experiments (n = 4 mice per group per experiment) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

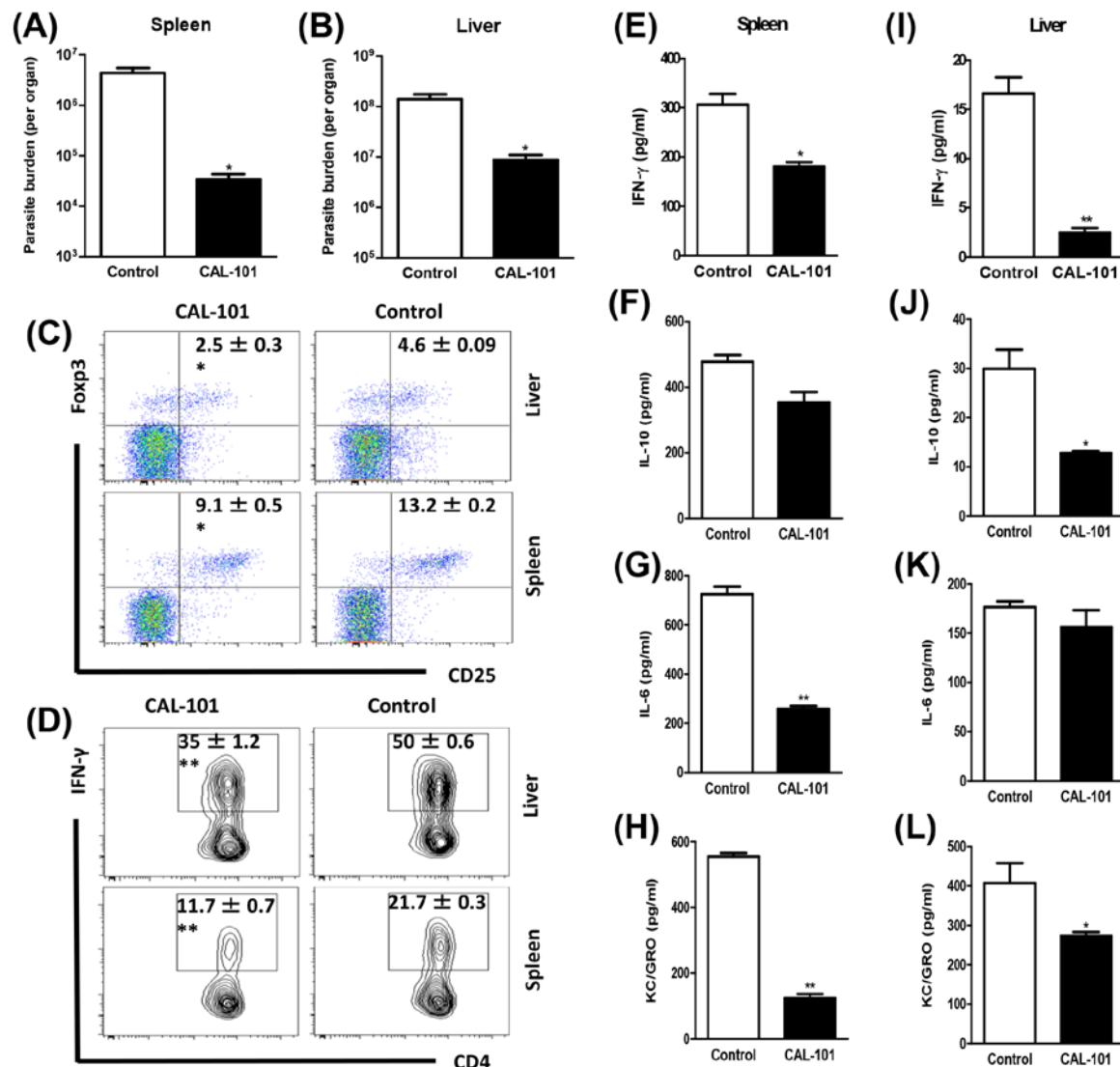


Figure 3-36: CAL-101 therapy is effective against established *L. donovani* infection

Mice were infected intravenously with 5×10^7 *L. donovani* promastigotes and after 2 weeks administered intraperitoneally with CAL-101 every 12 hr. for two weeks. At sacrifice, parasite burden in the spleen (A) and liver (B) was determined by limiting dilution assay. Spleen and liver lymphocytes were isolated, directly stained *ex vivo* and the percentages of Foxp3 (C) and IFN- γ -producing CD4⁺ T cells (D) were determined by flow cytometry. Spleen (E, F, G and H) and liver (I, J, K and L) lymphocytes were also cultured for 72 hr. and the level of cytokines (IFN- γ , IL-10, IL-6 and KC/GRO) in the culture supernatant fluids was determined by V-PLEX Meso Scale. Results are representative of 2 independent experiments (n = 4 mice per group per experiment) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

3.3.4.4 Combined CAL-101 and Amph-B therapy cures experimental VL

The growing resistance to anti-leishmanial drugs suggests that the currently used monotherapy needs to be reviewed and possibly replaced with multidrug combination and/or immunotherapy. Therefore, we determined whether treatment of infected mice with CAL-101 and low dose of Amph-B, a commonly used anti-*Leishmania* compound, would lead to better protection that might be associated with minimal toxicity. We treated different groups of *L. donovani*-infected mice with CAL-101 either alone or in combination with low doses of Amph B (0.01 mg/kg). Remarkably, combination therapy with CAL-101 and low dose Amph-B lead to significant ($p < 0.001$) complete clearance of parasites both in the spleen (Figure 3-37A) and liver (Figure 3-37B) compared to the untreated control groups or those treated with either CAL-101 or Amph-B alone.

Consistent with my previous findings (Figure 3-33, Figure 3-35 and Figure 3-36), CAL-101 and Amph-B combination therapy led to significant ($p < 0.05-0.01$) reduction in Treg numbers in the spleens and livers of *L. donovani* infected mice (Figure 3-37C and D) compared to the untreated control group. However, the reduction in Treg numbers was significantly ($p < 0.01$) greater in CAL-101-treated group compared to groups that received CAL-101 and Amph-B combination therapy (Figure 3-37C and D).

In contrast, while CAL-101-treated group alone has lower numbers of IFN- γ producing cells in their spleens and livers and these cells produced lower amounts of IFN- γ in cultures, treatment with either Amph-B alone or in combination with CAL-101 did not affect the level of IFN- γ production by splenic and hepatic cells from infected mice (Figure 3-37E and F). These

observations suggest that the mechanisms of protection following CAL-101 and Amph-B treatment may be different.

Previously, I showed that deficiency of p110 δ signaling was associated with dramatic reduction in HSCs and their ability to produce immunoregulatory cytokines (section 3.2.4.5). Therefore, I assessed the numbers of HSCs in the liver following CAL-101 treatment. I show that the HSC numbers in the liver of mice treated with the combination therapy or CAL-101 alone are significantly ($p < 0.01$) lower than those from untreated control group (Figure 3-38).

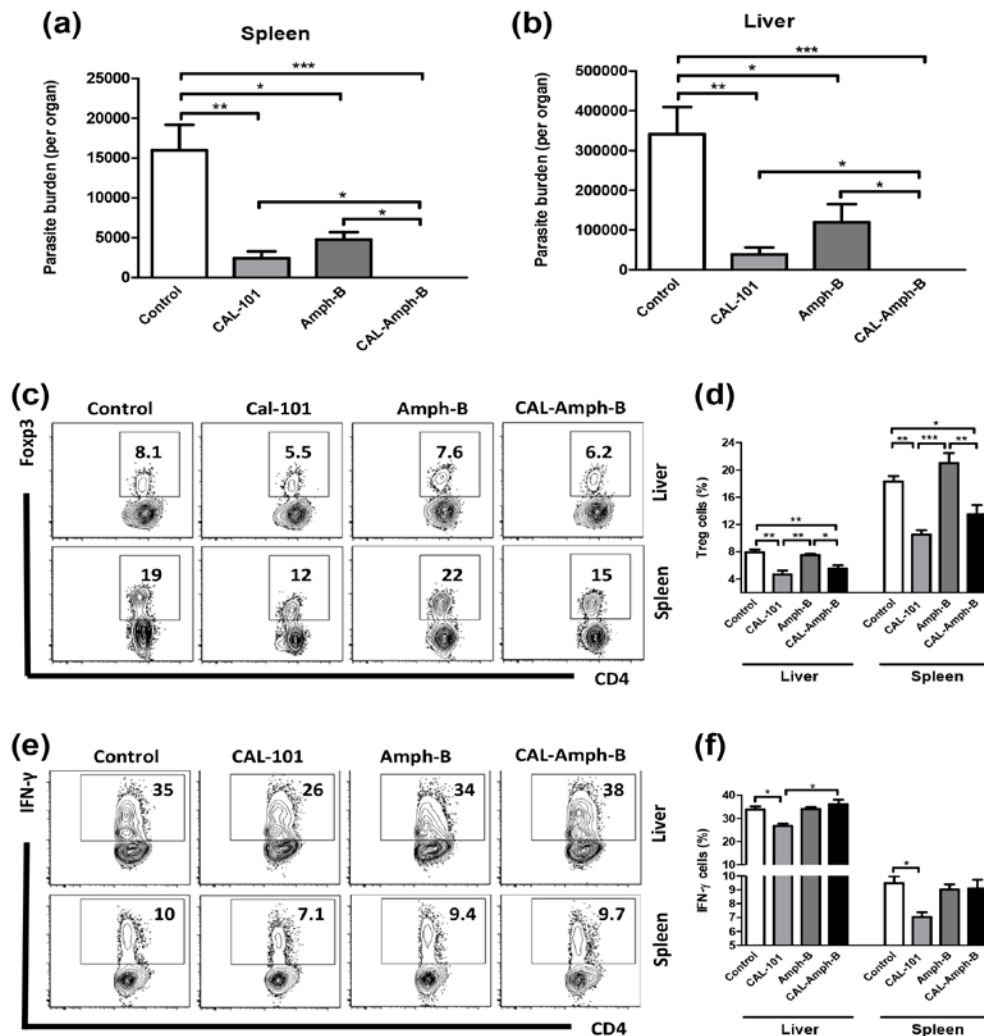


Figure 3-37: CAL-101 and Amph-B combination therapy leads to complete parasite clearance in spleens and livers of *L. donovani*-infected mice

Mice were infected intravenously with 5×10^7 *L. donovani* and after two weeks, treated with either CAL-101, Amph-B or combination of CAL-101 and Amph-B once daily for 5 days. One week after the last treatment, mice were sacrificed and parasite burden in the spleen (A) and liver (B) was determined by limiting dilution. At sacrifice, spleen and liver lymphocytes were isolated and directly stained *ex vivo* for Foxp3 expression (C and D). Some cells were stimulated with PMA, ionomycin, and Brefeldin A for 4 hr., fixed and directly stained *ex vivo* for intracellular IFN-γ expression (E and F). Results are representative of 2 independent experiments (n = 4 mice per group per experiment) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

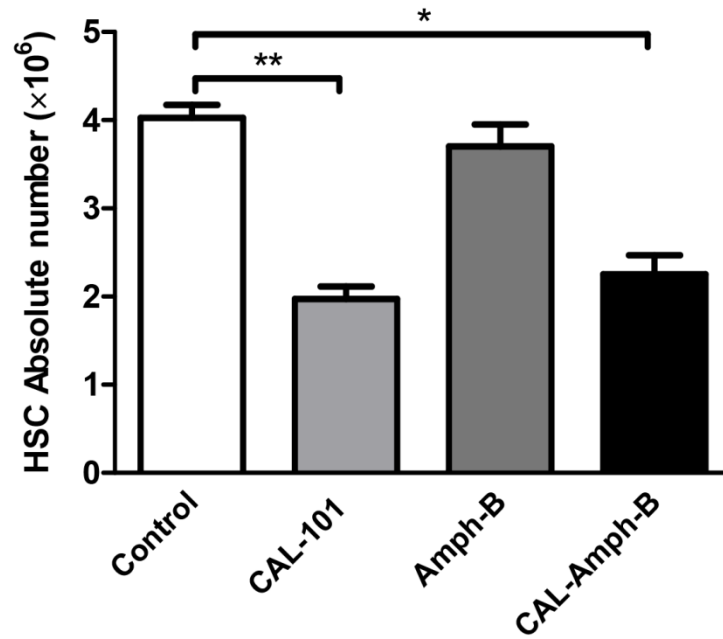


Figure 3-38: CAL-101 and Amph-B combination therapy leads to lower HSC numbers in the liver of *L. donovani*-infected mice

Mice were infected intravenously *L. donovani* and after 2 weeks, treated with either CAL-101, Amph-B or combination of CAL-101 and Amph-B once daily for 5 days. One week after the last treatment, mice were sacrificed and HSCs were isolated from their liver, stained direct *ex vivo* for expression of GFAP, α -SMA and LRAT and analyzed by flow cytometry. Results are representative of 2 independent experiments (n = 3 mice per group per experiment) with similar results. Error bars, +/- SEM; *, p < 0.05; **, p < 0.01.

3.3.4.5 The curative effect of P110 δ pharmacological inhibitors is not through direct effect on leishmania growth or macrophage infection ability

Because *L. donovani* is known to activate PI3K/AKT pathway in macrophages [390], I assessed whether p110 δ pharmacological inhibitors influence *Leishmania* growth directly (in axenic cultures) or indirectly (by affecting macrophage activation) leading to parasite death. Therefore, I tested the ability of CAL-101 and IC87114 to directly inhibit proliferation of *L. major* and *L. donovani* promastigotes in axenic cultures. The results were compared to the leishmania growth in the presence of Amph-B. Parasite growth was monitored every day by counting parasites for seven days. The results shown in Figure 3-39A and B, indicate that the *in vitro* growth and proliferation of these parasites were not affected by any concentration of CAL-101 tested. In contrast and consistent with previous reports, Amph-B completely inhibited the survival and growth of these parasites (Figure 3-39A and B). Additionally, whereas Amph-B suppressed parasite proliferation in macrophages, CAL-101 did not affect either infectivity or parasite proliferation inside macrophages *in vitro* (Figure 3-39C and D). Similar results were also obtained for IC87114 (Figure 3-40A, B, C and D). Collectively, these results confirm that the *in vivo* beneficial effects of CAL-101 and IC87114 are mediated by their ability to modulate the host immune response to *Leishmania* and not by directly killing parasites.

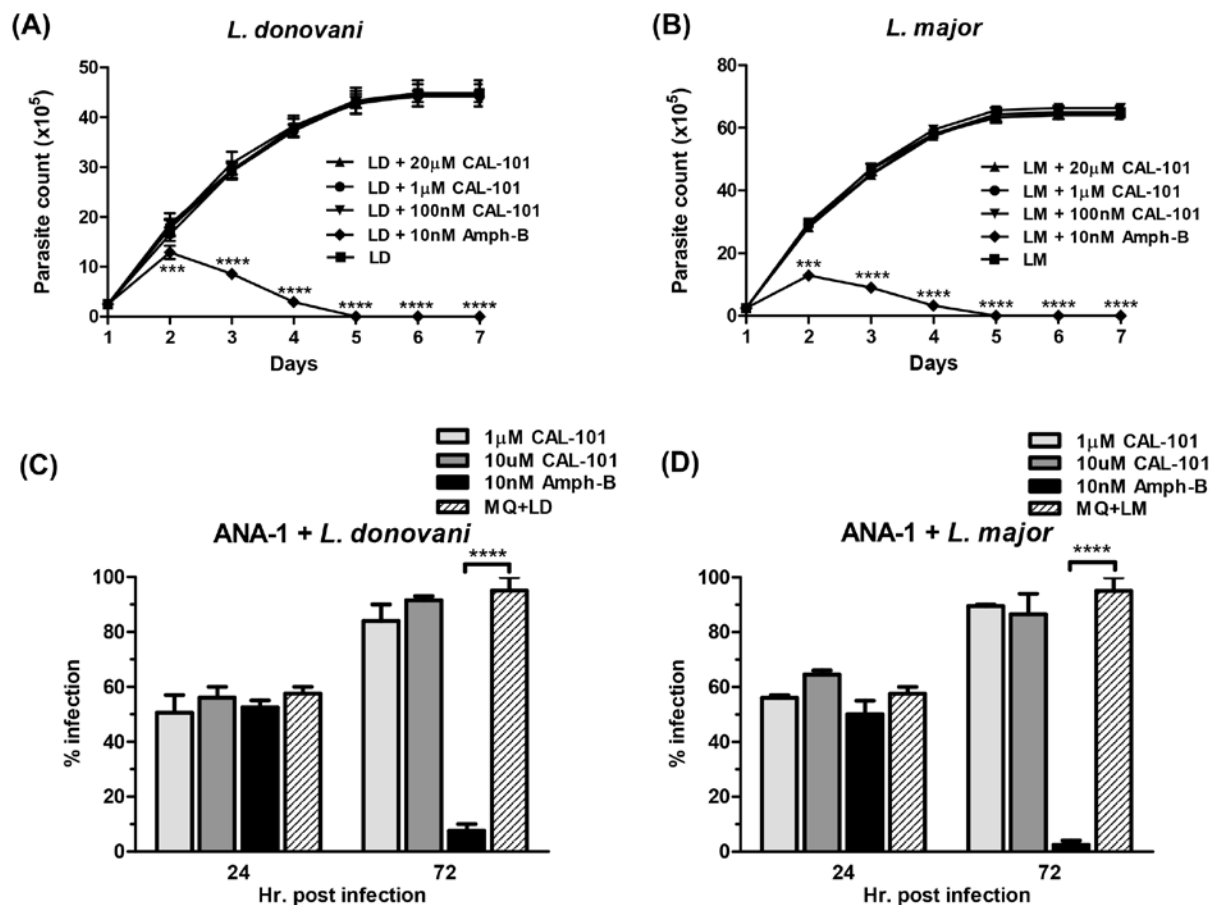


Figure 3-39: CAL-101 does not directly inhibit *Leishmania* growth in axenic culture or inside infected macrophages *in vitro*

L. donovani (A) or *L. major* (B) promastigotes (2.5×10^5) were cultured at 27°C in the presence of different concentrations of CAL-101 or Amph-B as indicated. Parasite proliferation was monitored daily by counting parasites under light microscopy. ANA-1 macrophages were infected with stationary phase *L. donovani* (C) or *L. major* (D) promastigotes at a cell-to-parasite ratio of 1:5. After 5 hr., free parasites were washed away and infected macrophages were cultured at 37°C in the presence of different concentration of CAL-101 or Amph-B (as indicated). At specified times, the number of infected cells per 100 cells (percent infection) was determined by counting H&E-stained cytopsin preparations under Zeiss Primo Star (Zeiss, Melville, NY, USA) light microscope at 100x (oil) objective. Results are representative of 3 independent experiments with similar results. Error bars, +/- SEM. ***, $p < 0.001$ and ****, $p < 0.0001$.

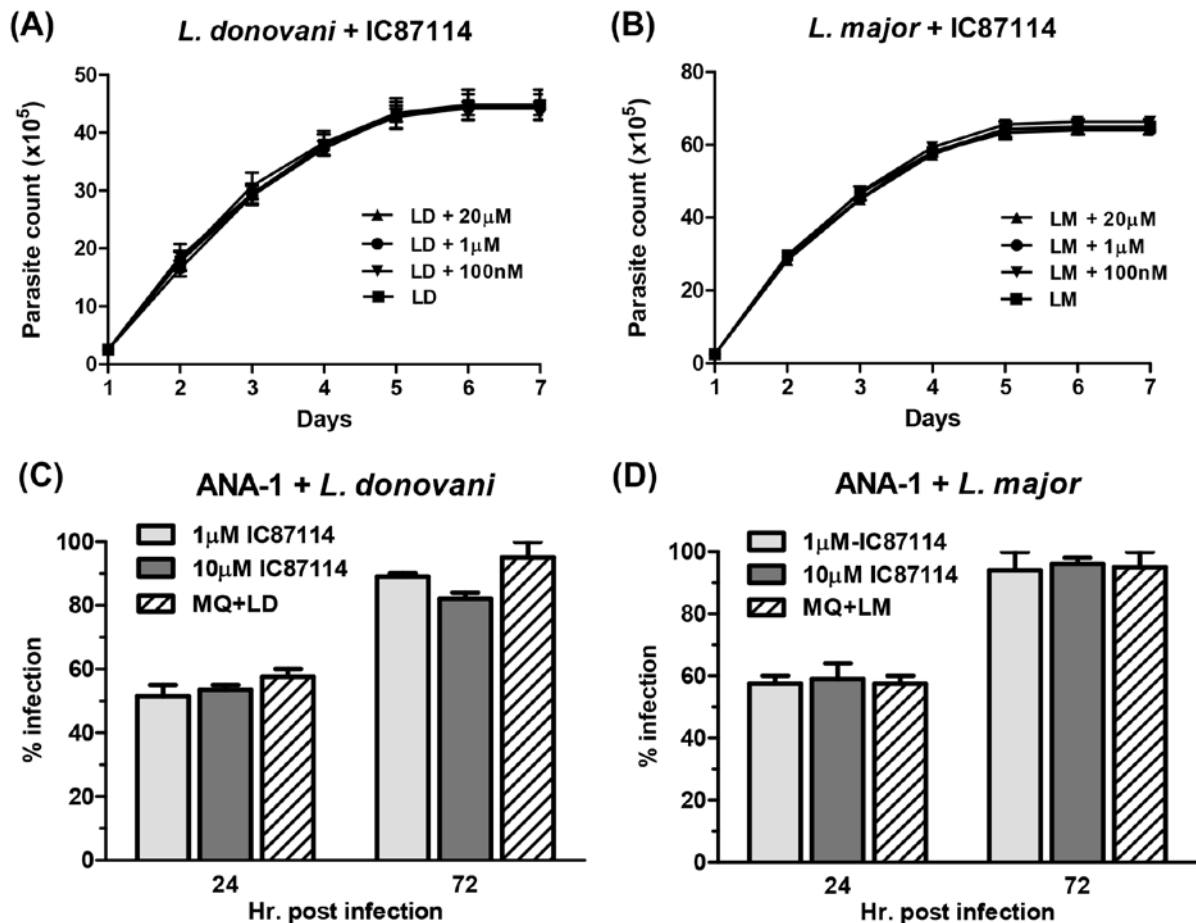


Figure 3-40: IC87114 does not directly inhibit *Leishmania* growth in axenic culture or inside infected macrophages *in vitro*

L. donovani (A) or *L. major* (B) promastigotes were cultured at 27°C in the presence of different concentrations of IC87114 (as indicated) and parasite proliferation was monitored daily by counting parasites under light microscopy. ANA-1 macrophages were infected with *L. donovani* (C) or *L. major* (D) promastigotes at a cell-to-parasite ratio of 1:5. After 5 hr., free parasites were washed away and infected macrophages were cultured at 37°C in the presence of IC87114 (as specified). At indicated times, the level of infection was determined by counting H&E-stained cytopsin preparations under Zeiss Primo Star (Zeiss, Melville, NY, USA) light microscope at 100x (oil) objective. Results are representative of 3 independent experiments with similar results. Error bars, +/- SEM.

4 CHAPTER 4: Discussion

4.1 Role of PI3K in immunity to Visceral Leishmaniasis

Leishmaniasis remains a global health problem and an understanding of the mechanisms that underlie host resistance and/or susceptibility to the disease could significantly impact on the development of new drugs and vaccines for human use. While *L. donovani* infection results in the development of some levels of immunity in the spleen, liver and bone marrow, the quality of this immunity is variable among organs and the exact immunologic and protective correlates of immunity remain poorly understood. For example, while infection in the liver is effectively controlled, *L. donovani* infection in the spleen remains chronic for months with no discernable immunologic defects in the infected mice. Understanding the mechanisms governing this organ-specific immunity is vital for effective therapeutic interventions against VL.

Members of the class 1A family of PI3K are important enzymes that control several important cellular events including cell differentiation, growth, proliferation and immune response [193, 195], and have been shown to regulate immunity to many pathogens including parasites [185, 405]. Infection of macrophages with *Leishmania* parasites results in engagement and sustained activation of the PI3K/Akt signaling pathway [186]. Unlike other isoforms of PI3K, which is expressed by many cell types, the p110 δ isoform is mostly expressed on leucocytes including B cells, T cells and APCs (macrophages and DCs) [181], suggesting that they may play critical role in immunity. *L. donovani* parasites engage TLR2 receptor on macrophages and induce mTOR signaling in PI3K-dependent and independent mechanisms [181]. Our laboratories previous studies highlight the importance of p110 δ isoform of PI3K in the regulation of T cell-mediated immunity [160, 197]. The results showed that p110 δ^{D910A} mice,

which exhibit attenuated Th1 responses, are protected against *L. major* infection even in the normally susceptible BALB/c background [160]. This finding challenges the Th1/Th2 paradigm as the primary determinant of resistance and susceptibility to Leishmaniasis, and instead focuses attention towards regulatory mechanisms that control inflammation as being key determinant of resistance and/or susceptibility.

In the present study, we further extend the importance of regulatory mechanisms that control inflammation in the pathogenesis of leishmaniasis by showing that p110 δ^{D910A} mice are also highly resistant to *L. donovani*, the major *Leishmania* spp. that cause VL. We showed that in addition to having dramatically reduced splenic and hepatic parasite burdens in both promastigote and amastigote-initiated infections, hepatomegaly and splenomegaly (which are hallmarks of VL), were significantly controlled in *L. donovani* -infected p110 δ^{D910A} mice. Importantly and consistent with the paradigm, the highly resistant p110 δ^{D910A} mice presented impaired T cell responses by producing significantly less IFN- γ , IL-4, IL-10 and TNF levels both in the spleen and liver. Interestingly, *L. donovani* infection was also associated with impaired B cell (antibody) responses in p110 δ^{D910A} mice, which is consistent with the previous observations in *L. major* infection [160].

Efficient and effective anti-*Leishmania* protection in the liver is usually achieved by granuloma formation around infected KCs. This is usually associated with chemokine production, recruitment of monocytes, neutrophils and T cells, production of inflammatory cytokines and activation of infected KCs. These events lead to the liver becoming an acute resolving site of the infection and resistant to reinfection. In contrast, although the spleen is the initial site for generating cell mediated-immune responses, it eventually becomes a site of

parasite persistence with accompanying immunopathological changes and is associated with high levels of TNF and IL-10 [106]. Thus, it is believed that the formation of granuloma in the liver is beneficial to the host in restricting parasite proliferation [396]. Our results demonstrate that during the course of *L. donovani* infection, the livers of infected but highly resistant p110 δ ^{D910A} mice significantly contain fewer numbers of developing granulomas and smaller mature granulomas by 8 wk. post-infection. Thus, our results show that effective parasite control in the liver and enhanced resistance to *L. donovani* does not necessarily require granuloma formation. Granulomas are usually initiated to contain persistent pathogens and signal the presence of chronic inflammatory responses [396]. We speculate that granuloma formation may become necessary when there are regulatory mechanisms (such as in the presence of Tregs) that act to dampen effective T cell-mediated immunity. In the absence of such regulatory mechanisms (as in p110 δ ^{D910A} mice), high amounts of IFN- γ production is not needed for resistance, because the low IFN- γ response is very efficient at more effectively activating infected KCs leading to more efficient parasite destruction. In line with this, a recent report demonstrated the presence of Tregs in hepatic granulomas of *L. donovani*-infected mice and suggested that Tregs mediate parasite persistence and susceptibility to experimental VL caused by *L. donovani* [110]. However, it is conceivable that the reduced number of granulomas might be a consequence of rather than the cause of lower parasite burden in the liver of infected p110 δ ^{D910A} mice.

Our studies support the previous reports showing that Tregs contribute to the pathogenesis of experimental VL in mice [110, 361]. They further show that signaling via the p110 δ isoform of PI3K is critical for functional competency of Tregs in mice. Despite having higher or similar numbers of Tregs in their thymus, p110 δ ^{D910A} mice have significantly lower numbers of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells in their peripheral tissues including lymph nodes and

spleens [195] compared to WT mice. Consistent with this, we found that infected p110 δ^{D910A} mice have significantly lower numbers of CD4⁺CD25⁺Foxp3⁺ (Tregs) in their spleens throughout the course of infection compared to their WT counterpart mice. Using *in vivo* Treg expansion strategy, we showed that the expansion of Tregs in naïve and infected WT and p110 δ^{D910A} mice were comparable. Remarkably, this expansion of Tregs in p110 δ^{D910A} mice completely abolished their enhanced resistance to *L. donovani* such that the parasite burden in the livers and spleens of infected p110 δ^{D910A} and WT mice were comparable at all times after infection following *in vivo* Treg expansion. Thus, given appropriate stimulus, Tregs from p110 δ^{D910A} mice are capable of expanding to a number that regulates anti-*Leishmania* immunity. This is consistent with the previous findings in *L. major* infection whereby adoptively transferring high numbers of p110 δ^{D910A} Tregs back into p110 δ^{D910A} mice was capable of abolishing the enhanced resistance to *L. major* infection akin to WT Tregs [160].

Collectively, the data presented in this part of my thesis, highlight the importance of the p110 δ isoform of PI3K signaling pathway in regulating T cell-mediated immunity and suggest that targeting this pathway may have important and direct implications for immunomodulation and immunotherapy of VL. Due to several drawbacks associated with the current anti-*Leishmania* treatments, including prolonged duration of treatment, toxicity, high cost of treatment, emergence of drug resistance strains and disease relapse [4, 40, 102], efforts are being made to develop new drugs and treatment regimens. Given the dramatic hyper-resistance seen in p110 δ^{D910A} mice infected with *L. donovani* and *L. major* [160], we speculate that the use of highly specific pharmacological inhibitors of p110 δ may be beneficial in the treatment of human cutaneous and visceral leishmaniasis. Although these compounds are currently being developed for treatment of inflammatory conditions, it is likely they may also be beneficial in modulating

immune response against leishmaniasis. Such immunomodulatory effects when combined with conventional therapy, may lower the required drug dose and treatment regimen, reduce drug toxicity, improve drug efficacy, reduce emergence of drug resistant strains and consequently reduce the chances of disease relapse.

4.2 Contribution of hepatic stellate cells (HSCs) to pathogenesis of *L.*

***donovani*-infection in WT and p110 δ ^{D910A} mice**

Visceral leishmaniasis remains a global health problem and is spreading to several non-endemic areas of the world due to international travel, globalization and military conflicts [13]. Although some drugs are available for treatment of VL [95], most are expensive, highly toxic and are associated with severe side effects [406]. Therefore, a clear understanding of immunopathogenesis of the disease could enhance efforts in the development of novel therapies. Although the liver is a primary target organ of infection and significantly contributes to the pathogenesis of VL, the underlying immunological mechanisms that regulate immunity to this key organ remain poorly defined. The data presented in this part of my thesis indicate that infection with *L. donovani* leads to expansion of HSCs in a PI3K-dependent manner and this correlated with increased numbers of Tregs. In addition, we reliably demonstrated that HSCs are infected with *L. donovani* *in vivo* and *in vitro* and this infection leads to the production of immunoregulatory cytokines that have been previously associated with induction of Tregs. Indeed, we showed that *L. donovani*-infected HSCs induce CD4⁺ T cells into Tregs *in vitro* and this effect is dependent on signalling via the p110 δ isoform of PI3K. We validated the importance of HSCs in immunity to *L. donovani* infection in the liver by showing that targeted depletion of HSCs leads to more effective parasite control and a concomitant reduction in liver

Treg numbers and IL-10 production by hepatic T cells. This is the first report to unequivocally demonstrate *in vitro* and *in vivo* infection of HSCs by *L. donovani*, their expansion, activation and production of immunoregulatory cytokines. In addition, these reports demonstrate a pivotal role of infected HSCs in expanding Tregs in a PI3K-dependent manner leading to a critical role in hepatic immunity to VL.

Under physiological conditions, HSCs are thought to be quiescent and store retinoic acid. During inflammation or liver injury, they undergo activation characterized by the loss of retinoids and transdifferentiation into myofibroblasts [250]. HSCs are usually identified by expression of several ectoderm cytoskeletal markers (such as GFAP, brain-derived neurotrophic factor, synaptophysin) or mesoderm cytoskeletal markers (e.g., vimentin, desmin, α -SMA) [231] and typically are isolated by density gradient centrifugation [372], although other isolation strategies such as intravenous administration of liposome-encapsulated dichloromethylene diphosphate (CL2MDP) have been used [259]. In the present study, we used two strategies to characterize HSCs in naïve and infected mice. First, we evaluated the HSC numbers *in situ* in livers of *L. donovani*-infected mice by immuno-staining with anti-GFAP and anti- α -SMA antibodies. Second, we utilized a direct *ex vivo* flow cytometry approach to identify and characterize HSCs using the established markers (GFAP, Desmin, α -SMA and LRAT). This novel approach allowed us for the first time, to directly assess the expression of co-stimulatory markers and quantify the percentage and absolute numbers of HSCs following *L. donovani* infection. It also allowed us to directly demonstrate *in vitro* and *in vivo* (direct *ex vivo*) infection of HSCs by *L. donovani* using microscopy and immunofluorescence without *in vitro* culture that potentially leads to their activation.

Several reports show that HSCs have potent phagocytic properties [303, 324] and are capable of antigen presentation *in vitro* [304]. In addition, it has been shown that activation of HSCs by several agonists such as LPS leads to increased expression of MHC class II and costimulatory molecules [250]. Consistent with these reports, we showed that HSCs can uptake *L. donovani* amastigotes and promastigotes *in vitro* and such uptake is associated with replication of the parasites resulting in steady increase in amastigote numbers over a 72 hr. period. Although Rostan *et al* showed that human HSC that have been passaged several weeks *in vitro* are infected with *L. donovani* promastigotes *in vitro*, they reported that this infection was not associated with parasite replication [403]. This report is inconsistent with our data, as we observed replication of parasites inside infected HSCs and some HSCs that were loaded with parasites did burst *in vitro*, suggestive of their capacity to infect other HSCs or other liver resident cells. Furthermore, our studies utilized primary HSCs directly isolated from naïve mice, whereas Rostan *et al* utilized activated human HSC that have been passaged several times for many weeks *in vitro*. We confirmed HSC infection *in vivo* by immunofluorescence staining, and showed that HSCs isolated directly from *L. donovani*-infected mice are infected with *L. donovani*. We further showed that HSCs isolated from *L. donovani*-infected mice spontaneously produce high levels of proinflammatory and immunoregulatory cytokines such as TNF- α , IL-1 β , IL-6, TGF- β , IL-2, and IL-10 in *in vitro* cultures compared to their naïve counterparts. These findings are consistent with other studies showing that activated HSCs can produce numerous cytokines with proinflammatory and anti-inflammatory activities *in vitro* [307]. Using intracellular staining and flow cytometry, we validated our ELISA data and unequivocally showed that a sub-population of naïve HSCs produce IL-2 and IL-4 and this was further upregulated following infection with *L. donovani*.

The numerical expansion of HSCs following *L. donovani* infection was associated with a concomitant increase in the numbers of hepatic Tregs. Several studies have shown that following alloantigen and IFN- γ activation, HSCs acquire antigen presentation ability and preferentially expand CD4⁺CD25⁺Foxp3⁺ Tregs in an IL-2 dependent manner [219]. Furthermore, Dangi *et. al* demonstrated that LPS-stimulated HSCs promote hepatic tolerogenicity by inducing immunosuppressive CD4⁺CD25⁺Foxp3⁺ Tregs via a cell–cell contact and MHC class II dependent pathway [250]. However, it has been suggested that HSCs can induce functional Tregs in the presence of DCs and TGF- β 1 [362]. We found that HSCs from *L. donovani*-infected mice expressed high levels of MHC class II and costimulatory molecule (CD86) and this was associated with increased production of IL-2 and TGF- β 1 spontaneously in culture supernatant fluids. Using an *in vitro* co-culture system, we demonstrated that although HSCs from naïve mice are able to induce CD4⁺CD25⁺ T cells to become Tregs as previously reported [219], this ability is dramatically increased following *L. donovani* infection. We further demonstrated a connection between HSC expansion and function and proliferation of Tregs *in vivo*. Depletion of HSCs with C1-3-glioxitin resulted in dramatic reduction in proliferation and numbers of hepatic Treg, reduced production of immunoregulatory cytokines (IL-2, IL-10 and TGF- β) and significant reduction in parasite burden in the liver. Although it has been reported that macrophages could expand Tregs [407] and are infected by *Leishmania*, the observation that C13-glioxitin treatment did not affect other cell populations (including F4/80⁺ cells) strongly suggest that the reduction in Treg numbers is most likely a direct consequence of HSC depletion. In addition, Treg proliferation in infected p110 δ ^{D910A} mice was significantly lower than those of WT mice and this was further reduced following HSC depletion, suggesting a strong association between HSCs and p110 δ signaling in Treg expansion *in vivo*. Collectively, these results suggest

a p110 δ -dependent association between HSCs and hepatic Tregs numbers in *L. donovani*-infected mice.

The PI3Ks are important enzymes that control several aspects of host immune responses [195] including regulating immunity to many pathogens such as parasites [185]. We have demonstrated that p110 δ^{D910A} mice, are more resistant than WT mice to VL [384]. Since forced expansion of Tregs in p110 δ^{D910A} mice abrogates their enhanced resistance to *L. donovani* [384] and because PI3K signaling regulates many aspects of HSC activation and function [218, 285], we speculated that impaired p110 δ signaling in HSCs could account for the enhanced resistance of p110 δ^{D910A} mice to *L. donovani*. Indeed, we showed that as leucocytes, HSCs also express p110 δ isoform of PI3K and deficiency of p110 δ signaling dramatically affected HSC activation and function including transdifferentiation, expression of costimulatory molecules, cytokine production and ability to induce expansion and proliferation of Tregs *in vivo* and *in vitro*. This effect was not related to lack of infection of p110 δ^{D910A} HSCs because there was no significant difference in infectivity of HSCs from WT and p110 δ^{D910A} mice with *L. donovani* *in vitro* and *in vivo*. The reduction in Treg numbers in *L. donovani*-infected p110 δ^{D910A} mice was comparable to that observed following *in vivo* depletion of HSCs by C1-3-glutathione treatment. Collectively, these findings clearly show that signaling via the p110 δ isoform of PI3K regulates HSC activation and function, including the induction of Treg *in vivo* following *L. donovani* infection and suggest that inhibiting this pathway could provide novel target for enhancing resistance to VL in the liver. It is conceivable that inactivation of p110 δ signaling in HSCs affects their differentiation, proliferation and cytokine production, akin to the effects observed in leucocytes.

In conclusion, the results from this part of my thesis project clearly demonstrate a novel role of HSCs in regulating immunity to an intracellular pathogen. They evidently show that infection with *L. donovani* enhances HSCs ability to proliferate and induce Tregs in a PI3K-dependent process leading to impaired liver immunity. These observations have important and direct implications for understanding the pathogenesis of VL and also for immunotherapy and drug/vaccine development against the disease.

4.3 Treatment of Leishmania infected mice with pharmacological inhibitors of p110 δ and their effect on Leishmaniasis

Leishmaniasis remains an important parasitic disease and is spreading to several non-endemic areas of the world. Importantly, drug resistance and increasing *Leishmania*-HIV co-infections are becoming more problematic [408-410]. Many of the frontline drugs for treating the disease such as Amph-B, liposomal Amph-B and Miltefosine are associated with many side effects such as significant toxicity to the liver, kidneys and spleens [85, 88]. Therefore, there is urgent need for development of novel therapies, including immunotherapy that could boost the host's immune effects.

Our group previously reported that mice with an inactive knock-in mutation in the p110 δ subunit of PI3K exhibit enhanced resistance to different experimental forms of leishmaniasis including CL and VL [160, 384]. More importantly, this resistance is independent of parasite species and mouse genetic background [160, 384], suggesting that targeting this pathway could provide novel therapeutic approach for treatment of leishmaniasis. Here, we have investigated

whether treatment of *Leishmania*-infected mice with highly p110 δ specific pharmacologic inhibitors could result in leishmaniasis protection.

Different categories of PI3K inhibitors including pan-PI3K inhibitors, dual-PI3K/mTOR inhibitors and isoform-specific PI3K inhibitors have been developed to date, but most of them have encountered problems as mono-therapeutic agents in clinical trials due to limited efficacies and relatively high rate of adverse side effects [202]. Although the first p110 δ specific inhibitor that came to market was IC87114 [205, 206] and still is being used in research [207, 208], it has a very high IC₅₀ (0.5 μ M) compared to CAL-101, which has an IC₅₀ of 2.5 nM [198-200]. Therefore, CAL-101 has taken the lead over IC87114 in clinical trials and research applications and has shown great promise in the clinical management of conditions where PI3K inhibitions were thought to be beneficial [198-200].

I report here for the first time that both CAL-101 and IC87114, when used as prophylactic treatments, can reduce lesion size (CL) and parasite burdens in the footpads (CL), spleens and livers (VL) of infected mice. Also, I have demonstrated that CAL-101 has therapeutic effects in VL, as treatment initiated as late as 2 weeks after infection causes significant reduction in parasite numbers both in the spleens and livers of infected animals. Strikingly, I further demonstrated for the first time that combined CAL-101 and very low dose of Amph-B therapy has the ability to cause complete clearance of parasites in *L. donovani*-infected mice, demonstrating the potential benefits of this combination therapy in treatment of VL.

Although single or multiple dose liposomal formulations of Amph-B (Fangisome or AmBisome) have been reported to be effective for treatment of VL, prolonged treatment with Amph-B is still the main treatment regimen in different parts of the world. For example,

intravenous injections of Amph-B (1 mg/kg every other day for 15 days and at 15 mg/kg over 30 days) have been reported in human studies.[99] An experimental mouse study found that effective treatment of two strains of *L. infantum* required multiple injections of 0.5-0.8 mg/kg of Amph-B on various days [411]. Here, I chose to use Amph-B (fungizone) at 0.1 mg/kg, 2 weeks after infection in combination with CAL-101. The results obtained indicate that this combination therapy has the ability to cause complete clearance of parasites both in spleen and liver of *L. donovani*-infected animals. Thus, a combination therapy of CAL-101 dramatically reduced the dose and duration of treatment with Amph-B, which could potentially reduce the associated toxic and side effects of the drug.

The very low IC₅₀ associated with CAL- allows for the administration of relatively higher doses, leading to enhanced target and pathway suppression and efficacy [211, 214]. Although in some limited cases CAL-101 has been associated with adverse side effects such as hepatotoxicity, diarrhea or colitis, and fatal and serious pneumonia or intestinal perforation [212, 213], the compound remains one of the safest PI3K inhibitor available for clinical practice. It has been administered orally for humans in clinical trials of patients with CLL and was found to be very effective [198]. Smith *et al.* [383] have shown that when IC87114 is administered intraperitoneally in mice, the plasma drug levels are comparable to the oral route of administration. Therefore, we chose the intraperitoneal route of administration for CAL-101 twice a day in both the prophylactic and therapeutic treatment regimens and once a day administration for the CAL-101 and Amph-B combination treatments. Given that oral administration would be more feasible in human patients, it would be interesting to repeat these experimental studies using the oral route.

Previous data from our group and also data presented in section 3.1.4.1, showed that enhanced resistance of p110 δ KI mice to CL and VL paradoxically associated with impaired IFN- γ response by immune cells in the spleens and liver of infected mice [160, 384]. I also showed that the deficiency of p110 δ signaling was associated with impaired Treg expansion and function in infected mice. Extensive analysis further revealed that the enhanced resistance in these mice was related to more efficient effector T cell responses in the face of impaired Treg activities and numbers [197, 384]. In line with this, I found here that treatment with CAL-101 was associated with significant reduction in Treg numbers and cytokine responses in the spleens and livers of infected mice, suggesting that the beneficial effects of CAL-101 may be through its immunomodulatory effects. In contrast, Amph-B therapy alone has the ability to increase IFN- γ production in these organs and has no effects on Treg numbers, consistent with reports that indicate Amph-B interacts with both host and parasite membrane cholesterol thereby effectively disrupting infected macrophages and inhibiting parasite binding to macrophages [412]. In line with these, I showed that CAL-101, unlike Amph-B, does directly kill different *Leishmania* parasites *in vitro*. Hence, it could be considered that CAL-101 and Amph-B combination therapy has two safety valves. One is targeting the p110 δ pathway, which leads to an effective immune response associated with leishmaniasis by reducing Treg numbers and function, and the other one is by directly killing parasites [413], by reducing parasite entry into macrophages [412] and increasing IFN- γ production by Amph-B.

Many factors such as high dose treatment regimens, toxicity, high costs, drug resistance and poor efficacy are among the major challenges facing physicians treating patients with leishmaniasis. Therefore, it is critical for the current monotherapy options to be enhanced or replaced by developing new drugs or by utilizing multidrug/combination therapy. The

combination therapy approach could lower the required drug doses and treatment regimens, reduce drug toxicity, improve drug efficacy, reduce emergence of drug resistant strains and consequently reduce the chances of disease relapse. CAL-101 (Idelalisib) has been approved by The FDA for treatment of several conditions including B-cell malignancies [198-200]. My studies clearly demonstrate a novel therapeutic option for leishmaniasis based on CAL-101 monotherapy or CAL-101 and Amph-B combination therapy. These observations have important and direct implications for immunotherapy and drug/vaccine development against leishmaniasis.

4.4 General discussion and Conclusion

Leishmaniasis is a spectrum of diseases caused by over 20 species of intracellular protozoan parasite belonging to the genus *Leishmania* [29] ranging from simple, self-healing skin ulcers in CL caused by *L. major* to severe life-threatening visceral disease caused by *L. donovani* [414, 415]. VL is increasing in endemic regions because of malnutrition, poor housing, lack of resources and a weak immune system in people living in these regions. It is also spreading to several non-endemic areas of the world due to increase in international travel, immigration, international military conflicts, blood transfusion transmission, drug resistance and increasing *Leishmania*-HIV coinfections [408-410].

Some of the first-line VL treatments are Amph-B or its liposomal formulations and Miltefosine; but most anti-leishmanial conventional chemotherapy regimens used are not necessarily effective and need lengthy hospitalization periods to be administered. Compounding these issues are the emergence of drug resistance strains and the fact that treatment options are also very costly, toxic and are associated with diverse side effects [4, 22, 29, 88]. Therefore there

is an urgent need to identify new vaccines, drugs or treatment strategies to prevent, control or cure leishmaniasis in general.

In order to obtain efficient and suitable protective responses against leishmaniasis, it is essential to understand the complete pathogenesis of leishmaniasis, how the parasite evades the host immune system and thrive, and which parasite and host factors determine the type of immune response leading to resistance or susceptibility. Most studies on the immunobiology and vaccinology of leishmaniasis focus on CL due to the erroneous belief that knowledge gained from experimental CL could also be applicable to VL. However, it should be noted that the regulatory mechanisms governing resistance or susceptibility to both experimental and natural VL and CL are widely distinct. Therefore, vaccination or immunotherapy modalities that work for CL would not necessarily be expected to be effective against VL. Whether a *Leishmania* infection remains asymptomatic or progresses toward VL in the host depends on several factors such as the interactions between the environment, the parasite and the host genetic makeup that ultimately govern the quality and magnitude of the host immune response. Indeed, designing novel immunotherapeutic strategies against VL requires obtaining in-depth knowledge about basic mechanisms underlying immunity and immunopathology of VL in animal models and assessing their translational relevance and potentials in humans. Despite tremendous efforts in the development of immunotherapeutic strategies against VL, there is still an unmet need to address several issues regarding their mechanism of action as well as safety and efficacy considerations. Importantly, different methods/criteria's such as the following should be standardized: (1) How to evaluate and compare immunotherapeutic benefits of all emerging strategies; (2) What are the pharmacokinetic considerations such as assessment of the dose and duration of therapy; (3) How to provide new technological advances in classical cytokine

measurement and parasite burden to minimize variations in both human patients and animal models; (4) what is the formulation of efficient drug delivery systems. All of these criteria's should be considered as an urgent priority for successful design of effective VL immunotherapeutic strategies and agents.

The primary aim of this thesis was to comprehensively study the host-pathogen (parasite) interaction during VL by focusing on *L. donovani* as the pathogen and PI3K pathway as the host factor in murine model of the disease. Unrevealing the role of a non-immune cell type (HSC) resident in the liver, in shaping the pathogenesis/immunity to *L. donovani* in VL has also been prominently investigated. Furthermore the use of a biological substance to modulate or modify immune responses in order to achieve a prophylactic and/or therapeutic goal (referred to as immunotherapy), has been proposed as a rational treatment option for both CL and VL. Collectively, the results from my studies have provided a better understanding of how different factors of host impact on resistance and susceptibility to murine VL. These findings have been excellently articulated and published as a review article, focusing on the subject of implications of immunotherapy in VL treatment. “**Khadem F.** and Uzonna J.E. Immunity to visceral leishmaniasis: implications for immunotherapy. *Future Microbiol.* 2014. 9(7):901-15. (IF (2014-2015): 4.275)”.

My whole thesis can be divided into three core parts. In the first part, I tested the hypothesis that the p110 δ isoform of PI3K pathway also regulates disease outcome in mice infected with *L. donovani* as was previously shown for *L. major* infection (causative agent of CL). I demonstrated for the first time that deficiency of p110 δ signaling resulted in hyper-resistance to *L. donovani* infection both initiated by amastigotes or promastigotes. *L. donovani*-

infected p110 δ ^{D910A} mice harbored significantly fewer parasites and contained fewer lymphocytes in their spleens and livers compared to their infected WT counterparts. There was less hepatomegaly and splenomegaly in infected p110 δ ^{D910A} mice than those from infected WT mice. This outcome was associated with significantly impaired cytokine response (less IFN- γ production), antibody production and granuloma formation in p110 δ ^{D910A} mice. Furthermore, the results indicated that expansion and function of Tregs during *L. donovani* infection is p110 δ signaling dependent, as the percentages and absolute numbers of Tregs in the spleen and liver of *L. donovani* infected p110 δ ^{D910A} mice were significantly lower than those of infected WT mice. The data obtained in this section of the thesis has been published in a peer review journal. “**Khadem F.**, Mou Z., Liu D., Varikuti S., Satoskar A. and Uzonna J.E. Deficiency of p110 δ isoform of the phosphoinositide 3 kinase leads to enhanced resistance to *Leishmania donovani*. PLoS Negl Trop Dis. 2014. Jun 19;8(6):e2951. (IF (2014-2015): 4.446)”.

In the second part of this thesis, I addressed the contributions of HSCs (and their impact on Treg induction/expansion) in the pathogenesis/immunity to VL in the liver, which is a primary target organ of *L. donovani* infection. I hypothesized that the differential response of WT (susceptible) and p110 δ ^{D910A} (resistant) mice to *L. donovani* is related to differences in the expansion and function of their HSCs. One of the most important, original and novel aspects of my work presented in this section, relates to the method of isolating, assessing and characterizing HSCs directly *ex vivo* from naïve or infected animals without prior *in vitro* culture (for 24 hr.-5 days) in plastic plate cultures as was hitherto the case. Given that HSCs become activated following *in vitro* cultures, I believe that this novel method of direct *ex vivo* isolation and assessment (using established HSC markers (GFAP, Desmin, α -SMA and LRAT) is superior and more representative of the characteristics and functions of these cells *in vivo*. Of important note

is that while some studies have suggested that GFAP and α SMA expression delineates quiescent and activated HSCs, respectively [230, 372], others have also shown that both molecules can be co-expressed by both quiescent and activated HSCs [264, 272, 273]. In my hands, I found that HSCs isolated from naïve or infected mice co-express α SMA and GFAP directly *ex vivo* as assessed by both flow cytometry and immunofluorescence (confocal microscopy). The results presented in this section, show for the first time, that HSCs can be infected with *L. donovani* *in vivo* and *in vitro* and this infection leads to productive parasite proliferation and the production of immunoregulatory cytokines including those known to induce Tregs. This is consistent with the fact that HSCs have been shown to have potent phagocytic properties [303, 324] and are capable of antigen presentation *in vitro* [304]. Another important observation in this section of my thesis is that using both ELISA (to detect secreted cytokines) and flow cytometry (to detect cytokines intracellularly), I have detected that naïve HSCs produce low levels of IL-2, IL-4 and IL-17 and the production of these cytokines are further up-regulated following *L. donovani* infection. I also observed higher levels of fibrosis, Treg and inflammation inducing cytokines in the infected HSCs (such as TNF- α , IL-1 β , IL-6, IL-10 and TGF- β). I have furthermore demonstrated that akin to leucocytes, HSCs express p110 δ isoform of PI3K and deficiency of p110 δ signaling dramatically affects HSC activation and function including transdifferentiation, expression of costimulatory molecules, cytokine production and ability to induce expansion and proliferation of Tregs *in vivo* and *in vitro*. Another important finding in this section is that I demonstrated that HSCs are able to both induce iTregs and expand nTregs. While HSCs from *L. donovani*-infected mice were significantly better than those from naïve mice at inducing CD4⁺CD25⁺Foxp3⁺ iTregs from CD4⁺CD25⁻ cells, both naïve and infected HSCs were comparable at inducing proliferation of already differentiated (CD4⁺CD25⁺Foxp3⁺) nTregs *in*

vitro, suggesting that infection-induced activation of HSCs may be critical for iTreg induction *in vivo*. Thus, it is conceivable that HSCs provide costimulatory molecules and/or immunoregulatory cytokines that act in concert to provide essential and/or optimal costimulatory signals necessary for Treg proliferation. I further revealed a connection between HSC expansion and function and proliferation of Tregs *in vivo*. Depletion of HSCs with C1-3-glutoxin resulted in dramatic ~70% reduction in supposedly activated HSCs in infected mice (as it targets synaptophysin, which is known to be expressed only by activated HSCs). This HSC depletion was associated with a concomitant reduction in proliferation (Ki67 expression by Foxp3⁺ cells) and numbers of hepatic Treg, reduced production of immunoregulatory cytokines (IL-2, IL-10 and TGF-β) and significant reduction in parasite burden in the liver. I also found that Treg proliferation in infected p110δ^{D910A} mice was significantly lower than those of WT mice and this was further reduced following HSC depletion.

Collectively, these findings convincingly reveal a novel role for HSCs in the pathogenesis of VL and show HSC-dependent Treg expansion following *L. donovani* infection. They further suggest that this ability of HSCs to expand Tregs is dependent on signaling via the p110δ isoform of PI3K. The data presented in this section of the thesis has been published in “Hepatology” journal. It was picked as the Editor’s choice and highlighted on the cover page of the journal issue of February 2016. “**Khadem F.**, Gao X., Mou Z., Jia P., Movassagh H., Onyilagha C., Gounni A.S., Wright M.C. and Uzonna J.E. Hepatic stellate cells regulate liver immunity to visceral leishmaniasis through p110δ-dependent induction and expansion of Tregs in mice. *Hepatology*. 2016 Feb;63(2):620-32 (picked as Editors choice and highlighted on February 2016 edition cover page) (IF (2014-2015): 11.19).

Thus, both previous studies from our laboratory and my data suggest that targeting the p110 δ pathway may be a novel therapeutic strategy for controlling VL and CL. In the third part of this thesis, I hypothesized that treatment of *Leishmania*-infected mice with a pharmacological inhibitor of p110 δ will confer protection against experimental visceral and cutaneous leishmaniasis. Furthermore, I explored the possibility of immunomodulatory effects of CAL-101 and Amph-B combination therapy as a veritable strategy to lower the drug dose and treatment regimen and improve drug efficacy in visceral leishmaniasis treatment. I was able to show for the first time that prophylactic and therapeutic administration of CAL-101, a highly specific p110 δ pharmacological inhibitor already in clinical use, (and to a lesser degree IC87114) has beneficial therapeutic outcome in VL and CL by reducing parasite burden, Treg numbers and cytokine production. Both CAL-101 and IC87114 do not have direct parasite killing properties or have the ability to influence macrophage infection and replication. More importantly, I demonstrated that immunotherapy (use of CAL-101) in combination with conventional suboptimal dose of Amph-B led to complete clearance in infected mice. This effect was associated with significant reduction in Treg numbers and increased IFN- γ production by splenic and hepatic T cells. Thus, this combination immunotherapy regimen induces appropriate protective immune response, ameliorates disease progression, lowers the required drug dose and treatment regimen, reduces drug toxicity and improves drug efficacy. Findings from this section of the thesis are currently being revised for the “Journal of Antimicrobial Chemotherapy” (IF: 5.5). “**Khadem F.**, Jia P., Mou Z., Liu D., Keynan Y. and. Uzonna J.E. Pharmacological inhibition of p110 δ subunit of PI3K confers protection against experimental leishmaniasis”.

Collectively, these findings have greatly enhanced our knowledge and understanding of the host-pathogen interaction in VL. They also provided novel understandings into the mechanisms

involved in the development and regulation of immunity in VL specifically in the liver. Finally the data obtained could possibly shed light on developing vaccines, drugs or immunotherapies against both VL and CL.

5 CHAPTER 5: Significance, Missed Opportunities/Limitations and Future Directions

Due to migration [416], global traveling [417] and military conflicts (returning armed forces personnel) [13, 418], leishmaniasis is spreading to non-endemic regions of the world. In addition, potential and documented blood transmission via transfusion is a real problem in many countries as new immigrant and returning Citizens latently infected with Leishmaniasis could taint the blood supply if they donate blood [419]. Currently, no vaccine is available for prevention of Leishmaniasis [326]. Most drugs currently used for treatment of leishmaniasis are outdated [420], expensive and highly toxic [80, 421] and the increasing emergence of Leishmania-HIV coinfection [422] and drug resistant strains [83, 423, 424] are also worrisome and problematic and compound an already difficult situation. Here, I have been able to show that $p110\delta^{D910A}$ mice are hyper resistant to *L. donovani* infection and this resistance is due to impaired Treg and HSC function. Also I have reproduced the results obtained from $p110\delta^{D910A}$ mice with a highly specific inhibitor of the enzyme (CAL-101) in both cutaneous and visceral disease. Moreover, I have shown that Amph-B and CAL-101 treatment is more efficient in conferring protection to VL. These results open novel anti-parasitic therapeutic ways to combat leishmaniasis and can lead to new therapies/vaccines against the disease.

While my studies reveal very essential and novel aspects of host-parasite interactions that regulate resistance to VL, there were some limitations and few questions that still remain unanswered.

Unfortunately, there is no animal model that precisely resembles human VL and I acknowledge that mice in general, are not the best model for studying VL. Among the current animal models of the disease, Syrian golden hamsters (*Mesocricetus auratus*) provide more synchronic infection in the liver and spleen that can develop into a chronic infection, lead to death and are more similar to human VL. However, immunological studies in this model are limited because of the lack of reagents and a defect in nitric oxide production [425, 426]. While infection of BALB/c mice with *L. donovani* complex do not exhibit high susceptibility, it is still a commonly used experimental models for VL [427-429].

Although the method we have used here to identify parasite burden in the liver and spleen is limiting dilution assay [363] (as explained in section 2.2), I could have also used Real time PCR to further confirm and quantify parasite burden [430, 431] and/or utilize histology [432] to determine the type of cells that are infected by the parasites

In this thesis, I have assessed whether the resistance we observed in p110 δ^{D910A} mice is related to enhanced ability of their macrophages to kill parasites. I found that there is no significant difference between the p110 δ^{D910A} and WT macrophages in their ability to get infected with *L. donovani* (Figure 3-3). It is known that both macrophages and DCs phagocytose *Leishmania* and contribute to the regulation of infection and that infected DCs produce IL-12, which is critical for the development of IFN- γ -producing CD4⁺ Th1 cells [130]. Since I observed different levels of IL-12 in the spleens and livers of *L. donovani*-infected WT and p110 δ^{D910A}

mice (Figure 3-6), I could have further assessed the resistance mechanism in p110 δ^{D910A} mice, by studying the role of DCs. I anticipate that DCs from p110 δ^{D910A} mice will be equally permissive to *L. donovani* infection and will not be better at killing parasites than WT cells despite differences in IL-12 production. In support of this, we found that the percentage of IFN- γ producing cells or levels of IFN-g produced by cells from these organs were significantly lower in p110 δ^{D910A} mice than those from WT mice.

I used two different ways (expansion of Tregs (section 2.5) and depletion of Tregs (section 2.6)) to study the role of Tregs in resistance to *L. donovani* infection in p110 δ^{D910A} mice. Another possible way to investigate whether the enhanced resistance to *L. donovani* is related to impaired Treg expansion and function is to adoptively transfer Treg cells (enriched by positive selection) from WT and p110 δ^{D910A} mice into RAG1 KO mice as was done for *L. major* infection in our laboratory previously [160]. But because many Tregs are required for this experiment, I believe that the methods used in this thesis are more efficient than adoptive transfer experiments.

To confirm the novel role of HSCs in the pathogenesis (susceptibility) and resistance to VL in the WT and p110 δ^{D910A} *L. donovani* infected mice, respectively, and the ability of HSCs to expand/induce Tregs (as identified in this thesis), we could perform the following supplementary/complementary experiments. One possibility is to adoptively transfer HSCs isolated from naïve WT or p110 δ^{D910A} mice into infected WT or p110 δ^{D910A} mice and assess the Treg numbers and parasite burden in different organs. We would expect to see enhanced susceptibility to VL in the both WT and p110 δ^{D910A} mice due to higher Treg numbers. Moreover, we could adoptively transfer GFP⁺-HSCs from naïve WT or p110 δ^{D910A} mice into *L. donovani*

infected WT or p110 δ^{D910A} mice and examine the HSC-Treg interaction and HSC migration on cryopreserved organ(s) by immunohistology [250]. Another experiment that can be conducted is to repeat the Treg induction studies (by HSCs) in order to determine which HSCs are responsible for the Treg induction in the context of leishmaniasis: whether it is the *L. donovani* infected HSCs or HSCs activated by other cells or factors present in the liver environment.

I found that *L. donovani* infects and replicates in HSCs *in vivo* and *in vitro*. I could have complemented these studies by assessing whether HSCs from liver biopsies of *L. donovani*-infected human patients harbor *Leishmania* parasites. Alternatively, we could also test whether primary HSCs from liver biopsies could be infected with *Leishmania* *in vitro*.

It is known that the energy needed for the HSC activation is provided by the breakdown of intracellular lipids by autophagy [251, 433]. However, there is no study addressing the precise role and mechanism of this process at cellular and molecular level in the liver in VL. Considering the role of HSCs in autophagy-mediated liver fibrosis [251] and the role of these cells in pathogenesis of VL illustrated in this thesis, investigating whether HSCs are involved in autophagic responses during VL infection and the importance of PI3K pathway in this process could shed more light into resistance/susceptibility mechanisms during VL. For this purpose, HSCs can be isolated from WT and p110 δ^{D910A} mice before and after *L. donovani* infection, stained with fluorescent-labeled Ab against LC3 I, LC3 II (specific pan-autophagy markers) and P62/SQSTM1 (a selective autophagy adaptor molecule that its expression is reduced during autophagy) and analyzed by flow cytometry or immunofluorescence staining. Increased accumulation of LC3 II will indicate increased autophagy. The results could be further confirmed by performing LC3 (I and II) Western blot analysis on the liver homogenates or more

specifically on 24 hr. cultured HSCs from *L. donovani* uninfected or infected WT and p110 δ^{D910A} mice. Ultra-thin sections of liver tissues obtained from *L. donovani* uninfected or infected WT and p110 δ^{D910A} mice could be fixed and processed to enumerate autophagic vacuoles by electron microscopy as described previously [251]. In addition, histological examination (H&E staining), α SMA immunostaining and trichrome staining (collagen deposition) could be performed on liver sections to measure liver fibrosis [251]. Since blocking autophagy in HSCs attenuates liver fibrosis *in vivo* [251], I would expect that observed resistance of p110 δ^{D910A} mice to VL infection compared to WT mice might be in part due to less autophagic events. In this regard, I would expect less LC3, more P62 expression, higher autophagic vacuoles as well as lower levels of α SMA expression and collagen deposition in p110 δ^{D910A} mice infected with *L. donovani* compared to the WT group.

In this thesis, I have not only been able to address the role of p110 δ subunit of the PI3K pathway in VL (as was done previously for CL in our laboratory), but also I have used the appropriate pharmacological inhibitors of p110 δ (CAL-101 and IC87114) to study the disease outcome after their application as monotherapy (both in CL and VL) or in combination therapy with available conventional VL therapy. I was able to reproduce the observations I obtained in p110 δ^{D910A} mice (both in terms of disease outcome and mechanism of action) in the context of CL and VL, when p110 δ inhibitors (CAL-101 and IC87114) were used as prophylactic/therapeutic agents. It would have more informative to repeat these experiments with different *Leishmania* strains that cause CL, MCL or VL in the different geographical regions of the world. Also multi-therapy with other available conventional therapies for both CL (Miltefosine) and VL (liposomal formulations of Amph-B) in combination with P110 δ pharmacological inhibitors could reduce toxicity, duration and associated costs, while increasing

efficacy of leishmaniasis treatment regimens. Moreover, we could expand these prophylactic/therapeutic studies and use other pan-PI3K inhibitors (such as: GDC-0941 or LY294002), dual-PI3K/mTOR inhibitors: (such as: PF-04691502 or GSK2126458) and other PI3K isoform-specific inhibitors (such as: p110 α inhibitor: GDC-0032; p110 β inhibitor: GSK-2636771; and p110 γ inhibitor: XL765) [200-202, 434]. This would be to identify whether the results observed in this thesis are p110 δ specific or not.

Overall, the results obtained in this thesis in regard to treatment of both CL and VL with pharmacological inhibitors of p110 δ (specifically the FDA approved CAL-101) as monotherapy or in combination with Amphotericin-B (the conventional leishmaniasis treatment option) are very promising and could be taken to the next level in the future directions of this study. This would be to possibly start small limited clinical trials in endemic areas to assess drug efficacy in patients. In order to reduce costs and toxicity the combination therapy would be a better option and if successful, the application can be tested in phase II or III clinical trials.

Also another novel treatment option for VL would be to specifically target liver HSCs. To address this issue a novel genetically engineered mouse model can be developed and tested in which HSCs could be targeted and depleted specifically in the liver and VL outcome could be addressed in detail. This would allow for more in depth research in understanding the role and importance of HSCs in VL pathogenesis/immunity.

6 CHAPTER 6: References

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