

NEUROLOGICAL ASPECTS OF LEISHMANIASIS VIA IMMUNOLOGICAL
MECHANISM AND EVALUATION OF BORON FOR NEUROINFLAMMATORY-
BASED ANTILEISHMANIAL THERAPY

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Zeynep İşlek Köklü

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ABSTRACT

NEUROLOGICAL ASPECTS OF LEISHMANIASIS VIA IMMUNOLOGICAL MECHANISM AND EVALUATION OF BORON FOR NEUROINFLAMMATORY-BASED ANTILEISHMANIAL THERAPY

Leishmaniasis is a neglected tropical disease, caused by the protozoan parasites of the genus *Leishmania* (*L.*), and is transmitted by the bite of phlebotomine sandflies. It is endemic in over 98 countries per year, including Turkey and its subcontinental region. There are several studies on central nervous system infection to show that *Leishmania* can cross the blood brain barrier, resulting in neurological manifestations, known as “cerebral leishmaniasis”. Accordingly, this study highlighted three notions, including (i) the investigation of how *L.* parasites are transmitted into the brain via macrophages, and the mechanism underlying the inflammatory responses on both macrophages and astrocytes in the infection model, (ii) novel flow cytometric approach for quantification of *L.*-infected macrophages, and (iii) the antileishmanial efficacy and anti-neuroinflammatory activity of Sodium Perborate Tetrahydrate (SPT) on *L. infantum*, *L.*-infected macrophages and -astrocytes in *in vitro* cultures. According to the results, when the non-infected astrocytes co-cultured with or without *L.*-infected macrophages, the percentage of *L. infantum* was decreased to 24.88% and 7%, respectively, compared to the negative control, indicating a lower infection rate than the co-culture. Likewise, upon 75-200 μ M of SPT treatment, *L. infantum* promastigotes indicated a significant reduction in growth, without any toxicity on macrophages and astrocytes. Furthermore, a reduction in the mitochondrial membrane potential was detected after SPT treatment through apoptotic-like cell death, cell-cycle arrest, and astrocyte-activated-immune response. Besides, a decrease in the ROS and oxidative stress levels were determined in the infected cells, which was also investigated by the qPCR analysis. Conclusively, it is the first study to emphasize the communication between *L.*-infected macrophages and astrocytes regarding the parasite load, and inflammatory response against cell-specific infections. The antileishmanial efficacy together with the neuroinflammatory-based SPT therapy was shown on *L. infantum* promastigotes, *L.*-infected macrophages, and -astrocytes. The promising efficacy of the SPT *in vitro* *L.*-infection model highlights the potential of antileishmanial therapy in the future in further *in vivo* studies and clinical studies.

Keywords: *Leishmania*, *Leishmaniasis*, *Macrophage*, *Astrocyte*, *Neuroinflammation*

ÖZET

İMMÜNOLOJİK MEKANİZMA YOLUYLA LEISHMANIASIS'IN NÖROLOJİK YÖNLERİ VE NÖROİNFLAMATUAR BAZLI ANTILEISHMANIAL TEDAVİ İÇİN BORUN DEĞERLENDİRMESİ

Leishmaniasis, *Leishmania* (L.) cinsinin protozoan parazitlerinin neden olduğu ve flebotomin tatarcık sineklerinin ısırmasıyla bulaşan, ihmal edilmiş tropikal bir hastalıktır. Türkiye ve altı kıta bölgesi dahil olmak üzere yılda 98'den fazla ülkede endemiktir. Merkezi sinir sistemi enfeksiyonu üzerine *Leishmania*'nın kan beyin bariyerini aşarak "serebral leishmaniasis" olarak bilinen nörolojik belirtilere yol açabileceğini gösteren çalışmalar vardır. Buna göre, bu çalışma, (i) L. parazitlerinin makrofajlarla beyne iletiminin araştırılması ve enfeksiyon modelinde makrofajlar ve astrositler üzerindeki inflamatuvar yanıtların altında yatan mekanizma, (ii) L.-enfekte makrofajların miktar tayini için yenilikçi akış sitometrisi, ve (iii) *in vitro* kültürlerde L. *infantum*, L.-enfekte makrofajlar ve -astrositler üzerindeki sodyum perborat tetrahidratın (SPT) antileishmanial ve anti-nöroinflamatuvar aktivitesini içeren üç kavramı vurgulamıştır. Sonuçlara göre, enfekte olmayan astrositler L.-enfekte makrofajlarla veya bunlar olmadan birlikte kültürlendiğinde, L. *infantum* yüzdesi, negatif kontrole kıyasla sırasıyla %24,88 ve %7'ye azalmış, ve ortak kültüre oranla düşük bir enfeksiyon oranı göstermiştir. 75-200 µM SPT konsantrasyonları, makrofajlar ve astrositler üzerinde herhangi bir toksisite göstermeden, L. *infantum* promastigotlarının büyümesi üzerinde önemli bir azalmayı göstermiştir. Ayrıca, apoptotik benzeri hücre ölümü, hücre döngüsü durması ve astrosit ile aktive olan bağışıklık tepkisi yoluyla SPT tedavisinden sonra mitokondriyal membran potansiyelinde bir azalma tespit edilmiştir. qPCR analizi ile de araştırılan enfekte hücrelerde ROS ve oksidatif stres seviyesinde düşüş belirlenmiştir. Sonuç olarak, L. ile enfekte makrofajlar ve astrositler arasındaki parazit yükü ve hücreye özgü enfeksiyonlara karşı inflamatuvar yanıt ile ilgili iletişimi vurgulayan ilk çalışmadır. Nöroinflamatuvar bazlı SPT tedavisi ile birlikte antileishmanial etkinlik, L. *infantum* promastigotları, L. ile enfekte olmuş makrofajlar ve -astrositler üzerinde gösterilmiştir. SPT'nin *in vitro* L.-enfeksiyon modelinin ümit verici etkinliği, gelecekteki *in vivo* çalışmalar ve klinik çalışmalarda antileishmanial tedavinin potansiyelini vurgulamaktadır.

Anahtar kelimeler: *Leishmania*, *Leishmaniasis*, Makrofaj, Astrosit, Nöroinflamasyon

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ABBREVIATIONS

AmB	Amphotericin B
BA	Boric Acid
BBB	Blood brain barrier
BM	Bone marrow
BMDM	Bone marrow-derived macrophage
CC ₅₀	50% cytotoxic concentration
cDNA	Complementary DNA
CL	Cutaneous leishmaniasis
CNS	Central nervous system
CR1	Complement receptor type 1
CR3	Complement receptor type 3
DAPI	40-6-diamidino-2-phenylindole
DAT	Direct agglutination test
DC	Dendritic cell
DCL	Diffuse cutaneous leishmaniasis
ELISA	Enzyme-linked immunosorbent assay
FAST	Fast agglutination screening test
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal bovine serum
GFAP	Glial fibrillary acidic protein

HE	Hematoxylin and eosin
H ₂ O ₂	Hydrogen Peroxide
HIV	Human immunodeficiency virus
IFA	Indirect fluorescence antibody
iFBS	Inactivated Fetal Bovine Serum
IC ₅₀	Half maximal inhibitory concentration
IFN- γ	Interferon- γ
IL	Interleukin
<i>L.</i>	<i>Leishmania</i>
MA	Meglumine antimoniate
MCA 5	Metacaspase 5
MCL	Mucocutenous leishmaniasis
MHC	Major histocompatibility complex
M.T.	Melting Temperature
moDCs	Monocyte-derived dendritic cell
mRNA	Messenger RNA
NCF	Neutrophil chemotactic factor
NF- κ B	NF-kappa β
NK	Natural killer cell
RT-PCR	Real-Time polymerase chain reaction
PKDL	Post Kala-Azar Dermal Leishmaniasis
PPG	Proteophosphoglycan

PS	Penicillin and streptomycin
PSG	Promastigote Secretory Gel
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
qPCR	Quantitative Real Time Polymerase Chain Reaction
Sbv	Pentavalent antimonials
SEM	Scanning Electron Microscopy
SHERP	Small Hydrophilic Endoplasmic Reticulum-associated Protein
SPT	Sodium Perborate Tetrahydrate
TH1	T helper cell 1
TH2	T helper cell 2
<i>TNF-α</i>	<i>Tumor necrosis factor</i>
Tregs	T regulatory cells
VL	Visceral leishmaniasis
WBC	White blood cell
WHO	World Health Organization

LIST OF SYMBOLS

mM	Millimolar
μ M	Micromolar
μ l	Microlitre
h	Hour
mL	Milliliter
μ g	Microgram
ng	Nanogram

1. INTRODUCTION

Leishmaniasis is a neglected tropical diseases, caused by the protozoan parasites of the genus *Leishmania* that affect numerous species including humans, and dogs, and transmitted by the bite of phlebotomine sandflies. Leishmaniasis is endemic in more than 98 countries per year, including Turkey and the subcontinent, i.e. Southern Europe, the Middle East and North Africa, with more than 700 million people at risk as estimated that 1.3. million new cases of leishmaniasis occur every year [1]. Clinical manifestations of human leishmaniasis, caused by around 20 *Leishmania* species, are divided into three clinical forms: cutaneous (CL), mucocutaneous (MCL) or visceral leishmaniasis (VL) known as "Kala Azar", depending on the site of infection.

The spread of Leishmaniasis is mostly caused by the movement of populations that expose nonimmune people to transmission and also are assumed to be important cases in travellers and migrants. For the control of the leishmaniasis, the World Health Organization (WHO) prioritizes valuable field tools for the efficient diagnosis, the use of efficient strategy for case management, and adequate treatment and control of reservoirs and vectors of the disease [2].

1.1. *LEISHMANIA* SPP.

1.1.1. History, Taxonomy and Clinical Manifestations

In 1903 in British colonial India, William Boog Leishman and Charles Donovan reported the protozoan parasites, *Leishmania donovani* in patients with suffering from the prexia, anemia and hypersplenomegali, the typical symptoms of visceral leishmaniasis called as kala- azar. Afterward, numerous reports described the genus *Leishmania*, associated with unknown diseases, like the "delhi sore" in India [3] or the "bauru ulcer" and "espundia" in South America [4,5]. After the development of the leishmaniasis as a causative agent, the *Leishmania* spp. currently defined as obligate protozoan parasites that caused a vector born disease collectively known as leishmaniasis [6]. These events ultimately result in the identification of over 20 *Leishmania* species that are thought to be pathogenic and is transmitted to mammals by the vectors describes as 30 different sandfly species (Figure 1.1).

Subkingdom	Protozoa									
Order	Kinetoplastida									
Family	Trypanosomatidae									
Genus	Leishmania									
Subgenus	Leishmania					Viannia				
Complex	<i>L. donovani</i>	<i>L. tropica</i>	<i>L. major</i>	<i>L. aethiopica</i>	<i>L. mexicana</i>	<i>L. braziliensis</i>	<i>L. guyanensis</i>	<i>L. naiffi</i>	<i>L. lainsoni</i>	
Species	<i>L. archibaldi</i>	<i>L. killicki</i>	<i>L. major</i>	<i>L. aethiopica</i>	<i>L. amazonensis</i>	<i>L. braziliensis</i>	<i>L. panamensis</i>	<i>L. naiffi</i>	<i>L. lainsoni</i>	
	<i>L. chagasi</i>	<i>L. tropica</i>			<i>L. garnhami</i>	<i>L. peruviana</i>	<i>L. guyanensis</i>			
	<i>L. infantum</i>				<i>L. mexicana</i>		<i>L. shawi</i>			
	<i>L. donovani</i>				<i>L. pifanoi</i>					
					<i>L. venezuelensis</i>					
					<i>L. forattinii</i>					

Figure 1.1. Taxonomy of *Leishmania* spp. Adapted from [7].

Phylogenetically, Leishmaniasis is a disease caused by a protozoan parasite that is a member of the Kinetoplastidae family in the Kinetoplastida order, which has a characterized kinetoplast (Figure 1.1) [7]. Certainly, the genus *Leishmania* is split into *Leishmania* (Leishmania) and *Leishmania* (Vianna) as two subgenus, regarding the region of the sandfly gut. Despite of similarity in genomic content and morphology, they display a broad range of clinical symptoms, based on the heterogeneity of the *Leishmania* species. The parasite continues to develop for 4 to 25 days within the sandfly, where it undergoes a significant transformation. Clinical manifestations of leishmaniasis can appear in the emergence of several ulcerations represented as the cutaneous leishmaniasis (CL) (Figure 1.2-A), mucocutaneous leishmaniasis (MCL) (Figure 1.2-B) or diffuse cutaneous leishmaniasis (DCL). These forms typically do not cause death and heal on their own in immunocompetent individuals, but they leave unsightly scars apart. In contrast, visceral leishmaniasis (VL), a severe form of the disease introduced by the spread of the parasite to organs of the reticuloendothelial system like the bone marrow, liver, or spleen, is fatal if untreated. (Figure 1.2) [8].

Despite this, all types of leishmaniasis exhibit the following pathogenetic characteristics: chronic tissue infection, immunoinflammatory response of the host limits disease expression and outcome, and resident tissue macrophages are targeted and promote intracellular parasite multiplication [9].



Figure 1.2. Clinical manifestations of leishmaniasis. Description of images from left to right: CL, MCL, VL, and DCL. Adapted from [10, 11].

1.1.2. The Life Cycle

Leishmania spp. display digenetic lifecycle, including two main stages: extracellular promastigote form as a motile stage inside a sandfly (invertebrate host), and intracellular amastigote form as a non-motile stage in a mammalian (vertebrate host). Consequently, the parasite has two distinct morphological forms, which exists as a round aflagellated amastigote that replicates inside the host phagolysosomal vacuoles within the phagocyte cells in the vertebrate hosts, such as dogs, rodents, humans, and lizards; and a motile flagellated promastigote in the digestive tract of the sandfly vector (Figure 1.3).

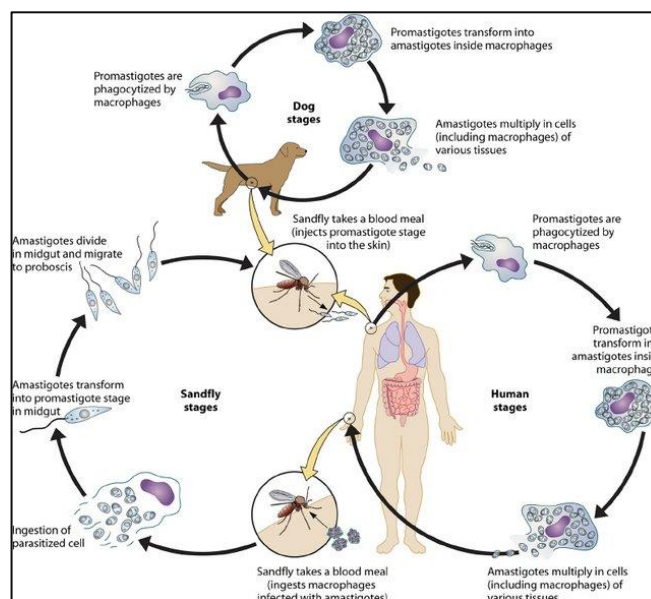


Figure 1.3. *Leishmania* life cycle. Adapted from [12].

After a blood meal on *Leishmania* spp.-infected vertebrate hosts, the Phlebotomine female sandfly becomes infected, resulting initiates the lifecycle of parasites. In the Sandfly vector, ingested amastigotes, which are 3-5 μm ovoid cells, differentiate into promastigotes, which have long flagella as their primary morphological characteristic and range in size from 12 to 16 μm in length and 1.5 to 3.5 μm in width [13]. The promastigotes utilize its flagella to bind to the midgut by recognizing solute gradients through chemotaxis and osmotaxis. They multiply in the midgut before migrating into the foregut, where they develop into metacyclic promastigotes [14, 15]. These metacyclic promastigotes show a non-dividing feature as mainly infected forms located in the sandfly salivary glands. Once delivered into the vertebrate host during feeding of infected sandflies, metacyclic promastigotes initiate infection by invading the professional phagocytes, the macrophages [16]. Then, the parasite-containing parasitophorous vacuoles fuse with lysosomes forming a 'phagolysosomes' wherein promastigotes differentiate into the vertebrate stage, an aflagellated amastigote form. Once within the vertebrate host during the feeding of infected sandflies, metacyclic promastigotes start the infection by penetrating the macrophages as phagocytes [16]. Afterward, promastigotes differentiate into an aflagellated amastigote form in "phagolysosomes," which are formed by the fusion of the parasite-containing parasitophorous vacuoles with lysosomes (Figure 1.4) [17].

Furthermore, the *Leishmania* parasites have shown resistancy to the host environmental conditions, such as the lower pH, and hydrolytic enzymes, and can able to proliferate within the host macrophages contrary to other infectious pathogens. This causes the physical disruption of infected host macrophages and the release of amastigotes into surrounding tissues, including phagocytosis by non-infected macrophages or by the feeding of the sandfly vector. When either amastigotes or infected immune cells are taken up from a blood meal from the infected mammalian by the sandfly vector, amastigotes differentiate into flagellated procyclic promastigotes as a result of environmental changes (e.g. decline in temperature, lower acidic pH) inside the blood meal. Over the course of seven days, some of these procyclic promastigotes undergo a process called metacyclogenesis, in which they become metacyclic promastigotes that can infect a new vertebrate host [18].

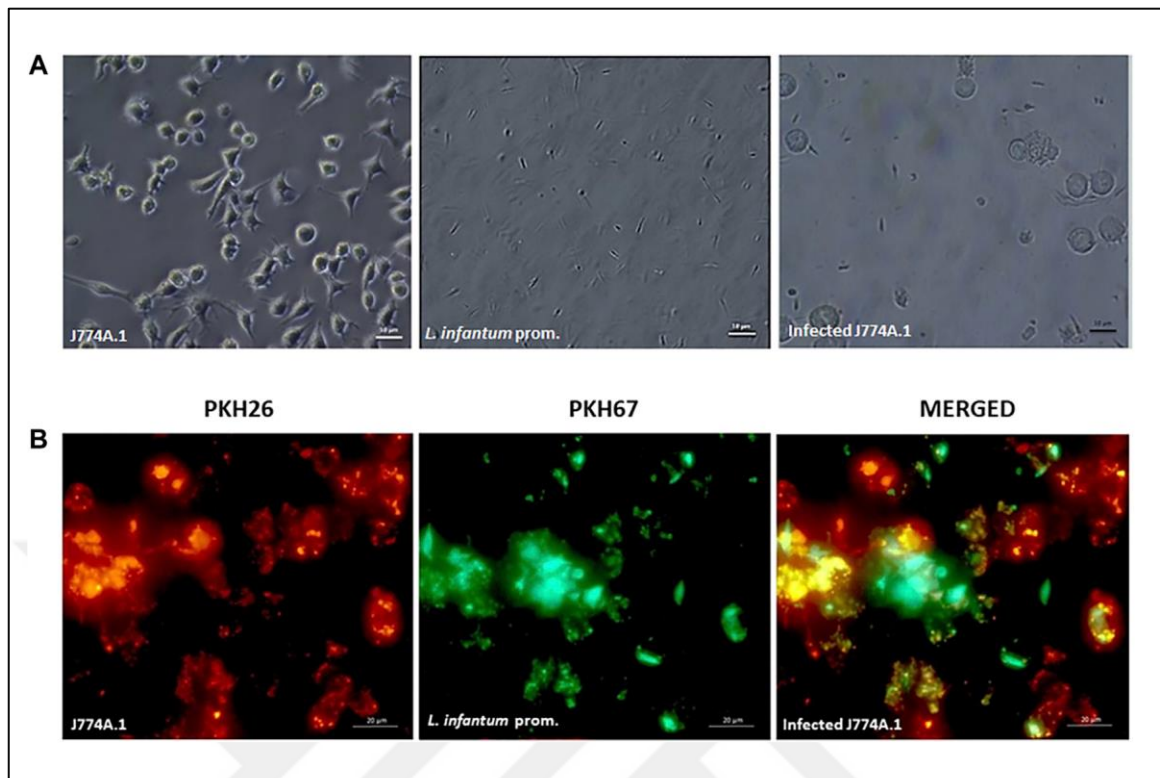


Figure 1.4. Microscopy analysis of stained macrophage–promastigote co-culture **(A)** phase contrast and **(B)** fluorescence microscopy images of PKH26-stained macrophages (red) infected with PKH67-stained *L. infantum* promastigotes (green). The merged yellow color displays *Leishmania*-infected macrophages (magnification: $\times 63$; size bars: 20 μm). Adapted from [19].

1.1.3. Vectors and Reservoirs

The vectors of *Leishmania* species have dipteran insects, belonging to the order Diptera in the Phlebotominae subfamily. About 70 of around 1000 species of sand fly have been described as vectors [9]. The subfamily has been restricted to two genera that are epidemiologically relevant: the genus *Lutzomya* and *Phlebotomus* were observed in the New World (ie, Central and South America), and in the Old World (ie, Asia, Africa, and Europe), respectively (Figure 1.5).

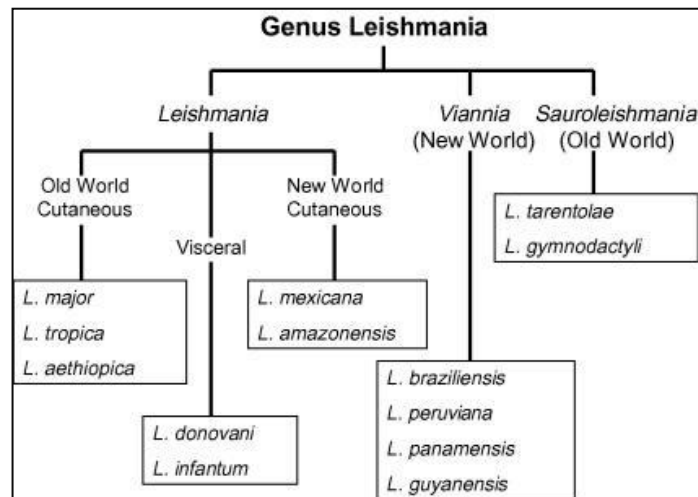


Figure 1.5. Classification of *Leishmania* illustrating the three subgenera [15].

The mammal-infective *Leishmania* parasites are categorized into *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*) based on the region of the sandfly gut [19]. Following that, this distinction has been confirmed by phylogenetic analyses based on DNA sequences [21, 22]. *Leishmania* (*Leishmania*) parasites are spread by female *Phlebotomus* (Old World) and *Lutzomyia* (New World) species, whereas *Leishmania* (*Viannia*) species are exclusively present in the New World [15]. The female sandfly ingests a blood meal containing *Leishmania* amastigotes, which are then differentiated into procyclic promastigotes and are localized by the peritrophic matrix, a protein network that serves as a semipermeable membrane to control the passage of molecules between the various midgut compartments and shield the midgut epithelium from mechanical damage from pathogens and toxins. After several days, the procyclic promastigotes replicate and transform into metacyclic promastigotes that escape blood meal's [23, 25]. Indeed, the binding of *Leishmania* is mediated by sandfly saliva and the secreting Promastigote Secretory Gel (PSG), a semi-solid matrix containing a proteophosphoglycan (PPG) which extends through the sandfly gut and mouthparts (Figure 1.6).

The transmission mechanism of the metacyclic promastigotes, an infective form of the *Leishmania*, is occurred by the inoculation of parasites salivary glands and proboscis or act of regurgitation provided by the partial extrusion of PSG by the passage to the midgut [15]. The fact that transmission of parasites into the mammal host is provided by the parasites co-

inoculated with PSG and saliva. Therefore, co-inoculation of parasites with saliva and PSG seems to support the parasite survival and proliferation associated with the immunomodulatory effects on the mammalian host systems defence mechanisms by blocking the macrophage activation [25]. Saliva-mediated protection from vector-transmitted leishmaniasis is based on the theory that sand fly salivary antigens may be candidates for future *Leishmania* vaccines. This theory claims that salivary proteins can modify the host immunity by inducing an immune response against parasite infection [26].

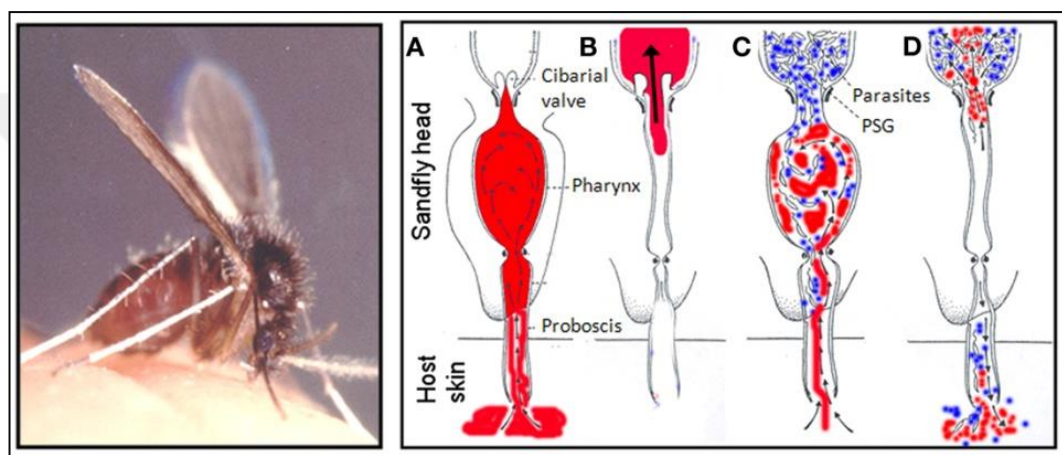


Figure 1.6. The transmission mechanism of *Leishmania* into the vertebrate host. Female sandflies taken up from a blood meal (in red) of a vertebrate host (A-D). Adapted from [27].

Depending on the sandfly's selectivity and the host mammalian's sensitivity, *Leishmania* species can be inoculated in a variety of mammals (including rodents, canids, and primates) and is categorized as a zoonose or an anthroponose.. The important state is that the transmission of the several parasites that lead to disease in humans, are supposed to be a zoonotic since anthroponotic transmission exists rarely, except for *L. donovani* that is the main infection for humans (Table 1.1).

The main reservoirs of Zoonic VL are domestic dogs and canines, while rodents are the major reservoirs for CL. Also, not all *Leishmania* species exhibit reservoir strain and specificity as well as midge fly selectivity.

Table 1.1. Principal *Leishmania* species and their major epidemiological characteristics.

Adapted from [28].

Species	Pathology	Reservoir	Distribution
<i>L. (L.) donovani</i>	VL; PKDL	Human	Africa; India (Old World)
<i>L. (L.) infantum</i>	VL; CL (rare)	Dog	Mediterranean; Asia and sub-Saharan (Old World)
<i>L. (L.) chagasi</i>	VL; CL (rare)	Dog	Latin America (New World)
<i>L. (L.) tropica</i>	VL; CL (rare)	Human	Middle East, India, Mediterranean, Western Asia (Old World)
<i>L. (L.) major</i>	CL	Rodents	Middle East, China, India, Pakistan, Africa (Old World)
<i>L. (L.) aethiopica</i>	CL; DCL	Rodents	East Africa (Old World)
<i>L. (L.) mexicana</i>	CL; DCL	Rodents	Central America (New World)
<i>L. (L.) amazonensis</i>	CL; DCL; MCL; VL (rare)	Rodents	Central and South America (New World)
<i>L. (L.) braziliensis</i>	CL; MCL	Rodents; Sloth; Opossum	South America (New World)
<i>L. (L.) guyanensis</i>	CL	Sloth	South America (New World)
<i>L. (L.) panamensis</i>	CL	Sloth	South America (New World)
<i>L. (L.) peruviana</i>	CL	Human; Dog (?)	Central and South America (New World)

1.2. LEISHMANIASIS

1.2.1. Epidemiology

Leishmaniasis is one of the most prevalent neglected tropical diseases, causing severe mortality and morbidity rate among tropical diseases. It is endemic in over 98 countries per year, including Turkey and the subcontinent (i.e. Southern Europe, the Middle East, North Africa and the India), with more than 700 million people at risk for new cases of leishmaniasis [29] (Figure 1.7).

In Turkey, *L. tropica* are relevantly observed in Southeastern Anatolia and eastern Mediterranean, while *L. infantum* are detected in the eastern Mediterranean and Aegean coast [1]. Like many other tropical diseases, there has been little interest in discovering and development of new drugs in industry due to the economic concerns. Despite of being regardless of age, children aged 1-4 years are at risk of infection in the Mediterranean regions.

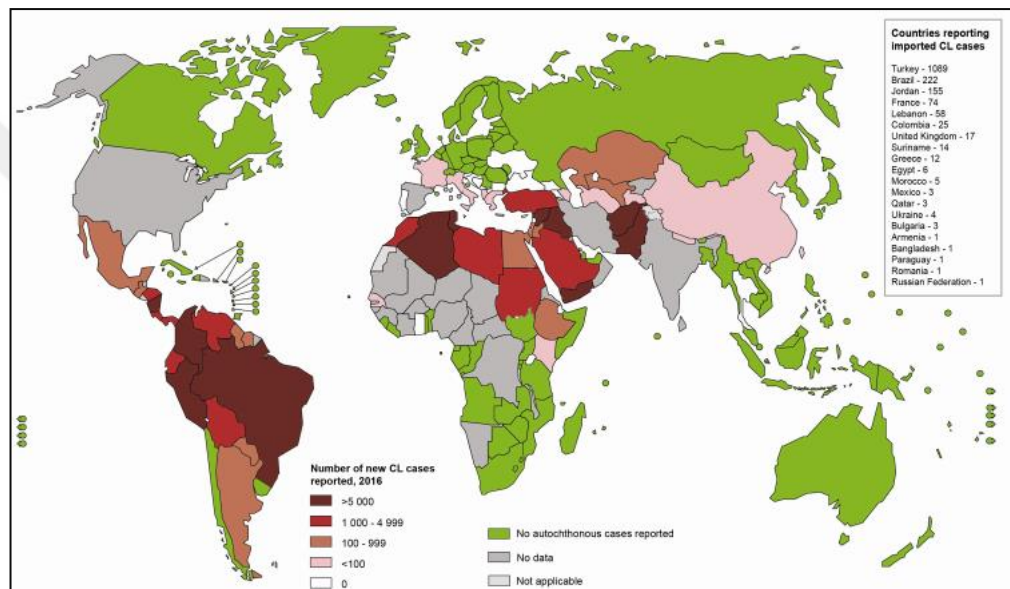


Figure 1.7. The geographical incidence of cutaneous leishmaniasis, adapted from [29].

1.2.2. Cutaneous Leishmaniasis

Cutaneous leishmaniasis is the most prevalent form of leishmaniasis in more than 70 countries, with 1.5-2 million cases estimated in Iran, Turkey, Afghanistan, Algeria, Brazil, Pakistan, Peru, Arabia and Syria (Figure 1.7) [30]. The incidence of the disease has increased over the past 20 years associated with the risk factor including global warming, population mobility and trade. Based on the several *Leishmania* spp., *L. tropica* and *L. major* are the main in the Old World, and *L. braziliensis*, *L. guyanensis*, *L. panamensis*, *L. peruviana*, *L. mexicana*, *L. amazonensis* and *L. venesuelensis* in the New World. Those of all *Leishmania* species are found primarily in macrophages around cutaneous sores with the exception of amastigote of *L. donovani* and the infective amastigotes must be taken up from the the blood

meal in the infective site by the sandflies. The disease is commonly known as oriental sore, characterized by ulcerative and persistent lesions as vascularized papule or no nodule on the skin at the feeding site of the insect [31]. With the initiation signs of the infections that appear a small and a vascularized papule in the site of incubation, the papule transform into ulcerations following few days to months or even several years. When protected from the secondary infections, ulcerative sores heal within a year, however disfiguring scars are often permanent [9].

1.2.3. Mucocutaneous Leishmaniasis

The dreaded metastatic form of localized CL infections that originally manifested as "espundia" during a first bout of cutaneous leishmaniasis within 5 years is known as mucocutaneous leishmaniasis. In Latin America, the *Viannia* subgenus amastigotes spread by lymphatic or hemogenous pathways extending from the skin to the naso-oropharyngeal mucosa, causing the diseases [32, 33]. Most muco-cutaneous leishmaniasis cases are caused by *L. braziliensis*. Additionally, the infection has been also linked to the other *Leishmania* species, including *L. amazonensis*, *L. major*, and *L. tropica* [34, 35]. Disseminated infections that reach the naso-oropharyngeal mucosa cause tissue metastasis, which results in the destruction of those membranes, including in the mouth, nose, pharynx, and larynx. Untreated infections brought on by bacterial contaminations can cause serious disfigurement or even death [36].

1.2.4. Visceral Leishmaniasis

Visceral Leishmaniasis, known as "Kala Azar", is the systemic form of leishmaniasis, leads to life-threatening infections throughout the reticuloendothelial systems within several weeks to months. It is caused by *Leishmania donovani* species, specifically *L. chagasi* (New World), *L. donovani*, and *L. infantum* (Old World). There is increasing evidence that *L. chagasi* and *L. infantum* are the similar species [37, 38]. *Leishmania* species are divided into two types based on their transmission features. Zoonotic VL is transmitted from animal to vector to human by *L. infantum* and/or *L. chagasi* whereas anthroponotic VL is transmitted from human to vector to human are transmitted into mammalian hosts by *L. donovani*.

Independent from the age, all groups are infected by *L. donovani*, while children and immunocompetent individuals are mainly infected by *L. infantum*.

As illustrated in the geographical prevalence of VL, generally, most cases occur in Bangladesh, India, Ethiopia, and Nepal (Figure 1.8), accounting for approximately 90 percent of all cases [10]. On the other hand, patients with suffering from VL, have fever, hepatosplenomegaly, anaemia, thrombocytopenia, leucopenia, neutropenia, and severe cachexia resulted from the accumulation of the parasites in the mononuclear phagocytes, including the liver, spleen, lymph nodes, and bone marrow [10, 36]. The incubation period has been last from 2 to 4 months [9]. Increase in susceptibility to secondary infections are causative agent of death [36, 39]. Recovered patients from VL normally have life-long immunity to reinfection; however relapses may occur in the immunosuppressive patients, leading to post-kala-azar dermal leishmaniasis (PKDL) [40]. PKDL is a neglected complication of VL that can occur in in Sudan and East Africa, can appear in several months to years with recurrence of kala-azar treatment [41]. In contrast to VL, the patient is not ill and mortality can rarely seen in PKDL which is characterized by the occurrence of macular or nodular skin rash. Risk factors for PKDL are not well known; previous treatment of VL with inadequate dosage of drug and the drug used, malnutrition, Human Immunodeficiency Virus (HIV) infection and young age may play a role.

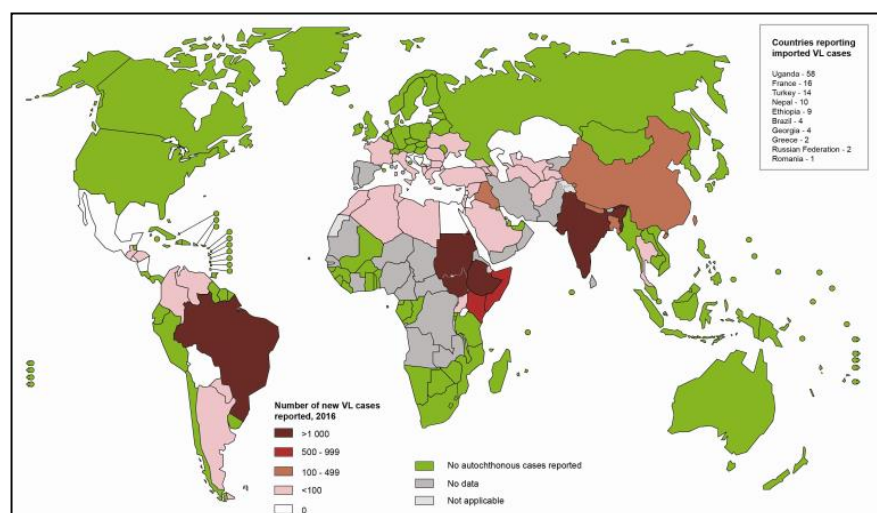


Figure 1.8. The geographical incidence of visceral leishmaniasis, adapted from [42].

1.2.5. Diagnosis of the Disease

The diagnosis of leishmaniasis is the essential process to early detection and control of the disease by combining the anamnestic information with clinical manifestations and laboratory diagnosis. As the similarities of clinical manifestations between leishmaniasis and the other pathologies of disease (ie, leprosy, malaria, and skin cancers), the characteristics of the diagnostic techniques used have a considerable influence on the correct diagnosis [1]. Therefore, tests should be highly sensitive and specific (>95%) to distinguish the acute and asymptomatic infection, since no current drugs are safe enough to treat asymptomatic infections, particularly VL, which is fatal [10]. The available methods for *Leishmania* diagnosis contain direct visualization of the parasite in skin scraping from the infection sites by microscopy; immunological identification of parasite antigens or anti-*Leishmania* antibodies by serological tests, such as immunofluorescence antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), and detection of DNA or RNA.

1.2.5.1. Microscopic Examination

The visualization of the amastigotes by microscopical examinations of aspirates from bone marrow, spleen, liver, lymph nodes or peripheral blood (for VL); skin biopsies (for CL) are the gold standard confirmatory diagnostic tests for leishmaniasis [1, 9]. Aspirated samples are tested by using the routine slide smear stained with Giemsa, Wright-Giemsa or haematoxylin-eosin to observe the spherical bodies of amastigote forms of *Leishmania* under 1000x magnification. Despite of the higher specificity, the sensitivity of aspirates from spleen, bone marrow and lymph node is 93–99%, 53–86%, and 53–65%, respectively [10]. However, the sensitivity of the method is dependent on the technical expertise of the personnel and the invasive sampling procedures as the main limitations of the the use of microscopic examinations.

1.2.5.2. Immunological Methods of Diagnosis

Diagnosis method of leishmaniasis include determination of anti-*Leishmania* antibodies by serological tests such as indirect fluorescence antibody (IFA) tests, ELISA, the fast agglutination screening test (FAST), or rapid immunochromatographic assays (rK39

dipstick or strip-tests) in immunocompetent patients [10, 40, 43]. In agglutination tests, *L. donovani* stained promastigotes that agglutinate with a serum containing anti-*Leishmania* antibodies [44], whereas an *L. chagasi*-related recombinant 39 amino acid repeat is used in the rK39 dipstick [45].

Among the serological methods for visceral leishmaniasis, the agglutination tests (DAT and FATS) are the main diagnostic tests with the high specificity and sensitivity [46]. Despite having a high specificity and sensitivity, the direct agglutination test (DAT) introduces substantial challenges owing to the long incubation time (18 h) and the need for serial dilutions of blood or serum [47]. To overcome these drawbacks, FAST that utilizes merely one serum dilution (qualitative result) and needs 3 hours (h) of incubation, was developed for the quick detection of *anti-Leishmania* antibodies in serum samples, and in blood on filter paper [46].

1.2.5.3. Molecular Diagnosis

The most used molecular biology methods in detection of the parasites are the polymerase chain reactions (PCR) in developed country. Although PCR is very sensitive and detects the presence of Leishmanial DNA or RNA from the peripheral blood sample, it does not provide information on whether parasites are viable and actively multiplying [1]. For mucocutaneous leishmaniasis, PCR-based methods are useful approach in samples with low parasite load [48]. Indeed, a positive results of PCR must be supported with differential diagnostic methods for an adequate *therapeutic* approach [1, 49]. PCR-based assays enable to identify *Leishmanial* DNA or RNA within a not many weeks with the presence of the first symptoms allowing *Leishmania* spp. identification using restriction fragment length polymorphism (RFLP) sequencing as a target [49].

1.2.6. Treatment of Leishmaniasis

On World Health Day 2014, the World Health Organization (WHO) highlighted “the serious and increasing threat of vector-borne diseases, including leishmaniasis, with the slogan “Small bite, big threat”. Leishmaniasis is a category 1 emerging and uncontrolled disease

that has been severely neglected in terms of organized drug development, the requirement for a safe, affordable, and user-friendly treatment strategy [30, 49], and increased research programs, including control of the vectors, is needed to enhance decreasing the incidence and morbidity of the disease. Despite several treatment options used in the treatment of leishmaniasis are available, they have problems associated with the efficacy, adverse effects, and requirement of parenteral administration. Since there's no approved human vaccine, control of the infection is completely based on drug therapy.

The available first-line medications for infections are pentavalent antimonials (sodium stibogluconate and meglumine antimoniate), amphotericin B, and pentamidine, which must be administered in a medical environment by the parenteral administration (Table 2.1) [30, 51]. Therefore, the primary objectives of leishmaniasis's new therapeutic is to find an effective oral medications [36]. The initial oral treatments utilized in leishmaniasis were imidazole derivatives that inhibit ergosterol biosynthesis, as well as miltefosine, an oral antineoplastic medication with notable efficacy in multiple phases for VL [52]. Also, Amphotericin B was introduced as the first drug treapy over 60 years ago [53]. In addition, pentamidine is used for the treatment of all forms of leishmaniasis, while miltefosine, paromomycin, azithromycin, allopurinol, and rifampicin have also been utilized instead. Nevertheless, *Leishmania* spp. have several clinical manifestations depending on the geographical distributions that lead to the diversity of the formulated compound, which might indicate efficacy against all forms of the leishmaniasis. Hence, novel anti-leishmanial substances and drug delivery system are still required for the antileishmanial therapy.

Table 1.2. Standard treatment regimens for the leishmaniasis. Adapted from [51].

Drugs	Dose	Efficacy	Advantage	Disadvantage
Pentavalent antimonials: sodium stibogluconate (Sbv)	20 mg/kg/day (i.m.) for 20-30 days in India	80-90 % (750% in Bihar, India)	Low cost and easily available in endemic area	Toxicities (vomiting, cardiac, liver), acquired resistance in the Indian subcontinent
Amphotericin B (AmB)	0.75-1.0 mg/kg for 15-20 infusions either daily or alternate days in India (i.v.)	95%-100%	No known primary resistance. Effective in antimony resistant regions	High cost and need of prolonged hospitalization, rigor, fever, nephrotoxicity, hypokalemia
Liposomal amphotericin B (L-AmB, AmBisome)	10 mg/kg as a single dose, i.v.	96%	Highly effective, low toxicity, resistance is not documented	Expensive
Paromomycin (PM), aminoglycoside antibiotic	11 mg/kg base/day for 21 days (i.m.)	>95% in India, 46-85% in Africa	>95% in India, 46-85% in Africa	Pain at injection site, reversible ototoxicity, nephrotoxicity
Miltefosine	50 mg/day for adults <25 kg and 100 mg/day	85-95%	First oral drug for VL. Currently first	Potentially teratogenic, vomiting, and diarrhea

1.2.6.1. Pentavalent Antimonials

Pentavalent antimonials (Sbv) have been recommended by WHO as first line drug for over 70 years ago. This drug is delivered in two forms as prodrug, including sodium stibogluconate and meglumine antimoniate (MA), which undergo biologically active trivalent forms (SbIII) by glutathione system. The Sbv reduction can take place inside the phagolysosome or inside parasites connected to glutathione present in macrophages [54]. Therefore, they are utilized in the therapy for all forms of leishmaniasis, especially in *L. major* and *L. tropica*, both in the Old and in the New World, but except for Europe and India. However, there is widespread drug resistance to antimonials, predominantly in the

subcontinent of Indian, where the severe form of the leishmaniasis (i.e. VL) persists and according to a recent report approximately 60% of VL cases are antimonial resistant in Bihar, the state contributing 70 per cent of the total VL patients in India [55].

It was suggested that contributing factors for the occurrence of *L. donovani* (*L. donovani*) as well as *L. braziliensis* and *L. infantum* resistance to pentavalent antimonials has been frequently linked to the upregulation of antioxidant pathways among which upregulation of enzyme trypanothione peroxidases and iron superoxide dismutase. Recently, post transcriptional regulation of aquaporin 1 has been shown to regulate antimonial sensitivity whereas several reports have implicated the chronic exposure to antimony contaminated drinking water for resistance to antimonial therapy. Antimonials display significant limitations, including long period of treatment, parenteral administration, and drug toxicity [30].

1.2.6.2. Amphotericin B

Amphotericin B (AmB) has a significant effect for the treatment of visceral leishmaniasis in the case of SbV-resistant. In fact, amphotericin B is used as an antifungal macrolide antibiotic to treat antifungal infections. Besides, antileishmanial activity of Amphotericin B is linked to its selective affinity to ergosterol, a component of the plasma membrane of *Leishmania*, rather than cholesterol. Indeed, AmB precisely act together with sterols of the membrane to sequester it in the membrane, and thus effectively decreasing the ability of cholesterol to interact with other membranes, including receptors that is thought to be played a role for the *Leishmania* entry) [56]. As AmB is low water solubility, poor bioavailability, nephrotoxicity, liposomal formulations (i.e. AmBisome) is used to decrease the drug toxicity, and increase the bioavailability of amphotericin B, resulted from preventing its uptake by reticuloendothelial cells, thereby improving the targeted drug delivery for the parasitic infections [56]. However, AmBisome is limited use in developing endemic countries due to the lack of cost-effectiveness.

1.2.6.3. Pentamidine

Pentamidine, which is an aromatic diamine, has been formerly used as a second line therapy of VL. Although it was firstly used for the resistance cases for antimonial in India, using of pentamidine was completely restricted due to the low efficacy and side effects [57].

The available treatments for HIV co-infection will depend on the patient's immunological status because, as it was discussed briefly in the treatment strategies, chemotherapy is successful only when the immune system is working efficiently. Patients who suffer from the higher drug toxicity and relapses with complications, have to be treated with an adequate dose without any toxicity after evaluating the anamnesis of the patients in detail. To introduce novel therapies, it is critical to improve treatment strategy for the emergence of resistance to the drugs, developed diagnostics and monitoring of the treatment process could play an crucial role in the treatments of leishmaniasis.

1.2.6.4. Boron Derivatives

Boron is a semimetal trace element with an atomic number of 5, which display both metallic and nonmetallic properties. As an element, boron cannot be found in cation form in the nature contrasting similar elements in trivalent oxidation state (+3). The main and commercial forms of borons are pentahydrate and decahydrate forms of disodium tetraborate, anhydrous, ulexite, monohydrate and tethydrate forms of sodium perborate and boric acid, and borosilicate minerals.

The requirement of boron element has been demonstrated for higher organisms such as mouse, zebrafish, buffalo and, frog, and the average daily intake is suggested as approximately 1.34 ± 0.02 mg/day [58]. The mechanism of boron is currently examined to implement it in therapies for bone disease (e.g. osteoporosis) [59, 60, 61, 62], diabetes [63], wound healing [64, 65], cancer [66, 67], neurodegenerative disease [68], and microbial infections [69, 70, 71].

Therefore, there are several studies highlighted to the role of boron in therapies in particular to cancer and microbial infections [66, 67, 69, 70, 71]. The previous studies showed that

boron compounds have potent anti-inflammatory, hypolipemic, anti-osteoporotic and anti-neoplastic agents both *in vivo* and *in vitro* animals [72, 73].

Boron Treatment in Cancer Therapy

It was reported that different boron derivatives (e.g. sodium pentaborate pentahydrate sodium perborate tetrahydrate (SPT)) have ability to inhibit progression of cancer, increases apoptosis and induces cell cycle arrest in various types of cancer cells such as prostate [74, 75], breast [76], lung [67], and colon cancer [77]. In the previous study, treatment of boric acid significantly decreased the cell proliferation in the DU-145, PC-3 and LNCaP cancer cells, when compared to PWR-1E and RWPE-1 healthy prostate cell lines [78, 79]. In the current study, the treatments of sodium pentaborate pentahydrate, SPT, and boric acid on DMS-114 small-cell lung cancer cells demonstrated anti-cancer activity regarding the apoptotic cell death and G2/M cell cycle arrest, without causing any toxicity on healthy MRC-5 lung cells [67].

Boron Treatment in Neurodegenerative Diseases

Boron has also been indicated as a promising therapeutic for neurodegenerative diseases in which oxidative stress and related pathologies, and in particular to the impaired motor function and the development of brain function when the deprivation of boron has negatively affected the motor speed [68]. Ozansoy and colleagues (2020) investigated that sodium borate decahydrate and boric acid increased the expression of Sirt1, which has critical activity on the cellular stress and thus indicating the prosurvival effect in an amyloid-beta toxicity model at 200 µg/mL doses of boron compounds [68].

Boron Treatment in Microbial Infection

There are also several studies investigating the activity and mechanism of boron on microorganisms that can be associated with biocidal effect and inflammation process. The studies reported the antimicrobial properties of some boron derivatives. Among boron derivatives, boric acid indicated a wide range of antimicrobial activity against various

bacterial infections such as *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *P. aeruginosa*, *Citrobacter* spp., *Morganella* spp., *Salmonella* spp., *Proteus* spp., *Staphylococcus* spp., *Streptococcus* spp., and *Candida albicans* in a dose dependent manner. In one previous study suggested that boric acid maintains intestinal homeostasis and is effective in controlling *Salmonella enteritidis* infection through the microbiota [69]. On the other hand, potassium tetraborate treatment has been shown antifungal effect on *Penicillium expansum* and a plant pathogen *Botrytis cinerea*. On the other hand, benzoxaborole AN3661 which is a boron-containing compound, and drug candidate for acute toxoplasmosis, display efficacy on various pathogens, including *Toxoplasma* [70]. Similarly, benzoxaborole derivatives also show inhibitory effect on the proliferation of both *Cryptosporidium* and *Toxoplasma* parasites in mammalian cells, by targeting Leucyl-tRNA Synthetase active site and thus showing a novel strategy as an antiparasitic agent [71].

Boron Treatment in Inflammation

Considering the inflammatory process, the previous study reported that boron (borax as orally) could induce the proliferation of lymphocytes and the release of cell-mediated immune responses by the lipopolysaccharide (LPS)-stimulated macrophages in the mice model. According to this study, a substantial increase was detected in B and T cell populations with the increase in CD4 and CD19 after treatment of boron. Furthermore, boron induced TNF- α , IL-6, IL-1 β secretion, and iNOS gene expression via the LPS-primed macrophages, particular in the M1 macrophage cells in a dose-dependent manner [80]. Apart from pro-inflammatory process, boron derivatives has also anti-inflammatory properties, presenting in the previous study that boric acid and nab decreased NO production and suppress important inflammatory transcription genes [81]. In the another study, Scorei et al. suggested that calcium fructoborate treatment reduced IL-6 and IL-1 β protein expressions, and NO in the supernatant of the cell. It has to be noted that a decrease in the inflammatory factors are not always a good solution for the infection process and also as a wound healing treatment strategy considering the immunological inflammatory process of *Leishmania* parasites infection ("Leishmaniasis"). However, pro-inflammatory response together with anti-inflammatory approach can take boron one step forward to prevent the

disease progression, provide the parasite eradication, and the cutaneous wound management application.

Although there are limited research indicating the evaluation of boron treatment on intracellular parasites, further experiments should be conducted to extent boron's potential role in anti-parasitic therapy and understand the exact mechanism of action in *Leishmania* parasites.

1.2.7. Immunopathogenesis of *Leishmania* spp. Infection

1.2.7.1. Host-parasite Interactions

Leishmania spp. is a serious pathogen that affects both innate and acquired immunity (both cell-mediated and humoral), as well as normal macrophage functions.

The metacyclic promastigotes (*Lutzomyia* spp., *Phlebotomus* spp.) inoculated in the dermis by the bite of a sandfly are believed to infect skin macrophages, in which they differentiate into amastigotes into macrophages, and may prevent apoptosis in their host cell with specific environment. [82]. Through an infected sandfly, metacyclic promastigotes together with saliva components of the sandfly are introduced into the vertebrate hosts. Promastigotes must avoid complement-mediated cell-lysis in order to be phagocytosed and survive the effects of the innate immune system. Numerous host cell receptors, including the mannose-fucose and fibronectin receptor, and also the macrophage receptor for glycosylation endproducts, as well as a number of parasite surface molecules, including LPG and gp63, are essential for the attachment and uptake of promastigotes [82]. Procyclic promastigotes are susceptible to the reticuloendothelial system, while metacyclic promastigotes display resistancy to complement activation. *Leishmania* parasites primarily infect mononuclear phagocytic cells, therefore in order to adhere to potential host cells, both the parasites and the macrophages must have unique recognition molecules on their surfaces [83].

The subunits of C5b-C9 complement system have ability to attach to the surface of the parasite was hampered by the surface lipophosphoglycan (LPG), which is longer in metacyclic promastigotes than in non-infective forms and almost entirely absent in amastigotes [84]. By converting the C3b complement subunit that is deposited on the surface

of the parasites into C3bi, the zinc-dependent metalloprotease gp63, which is 10 times less common than LPG, has been shown to protect *L. major* and *L. amazonensis* against parasite lysis [84]. The parasite fixes C3, which improves the recognition of the parasite by complement receptors 1 (CR1) and CR3 on macrophages, enabling intracellular survival [85]. As a result, it would seem that *Leishmania* uses C3 to "silently" invade host macrophages rather than activating the lytic membrane assault complex (CSb-C9) [82].

Neutrophils. It has been suggested that neutrophils are the first cell to be recruited to the infection site, in which deliver promastigotes to the host macrophages through enabling a silence entry like a "Trojan Horse" as shown in Figure 1.9 [86]. Neutrophils, one of the primary innate immune cells, are essential for host defense against pathogens and the recruitment is based on the inoculum infectious status [87]. Researchers found that despite neutrophils easily phagocytosed to the promastigotes *in vitro*, *L. major* promastigotes might be able to survive inside neutrophil phagosomes, resulting to the apoptosis of infected neutrophils, and then used as a phagocytic meal by the addition of macrophages to this culture. When apoptotic neutrophils are phagocytosed via receptor-mediated pathways, macrophage defense reactions are not elicited [88]. In reality, *Leishmania* promastigotes generate neutrophil chemotactic factor (NCF) and cause human neutrophils to secrete interleukin-8 (IL-8), which may increase the recruitment of neutrophils [89]. Additionally, after neutrophil reduction, the quantities of parasites in mice's macrophages and dendritic cells (DCs) remained unaltered, suggesting that neutrophils might simply scavenge parasites that are otherwise overlooked [90].

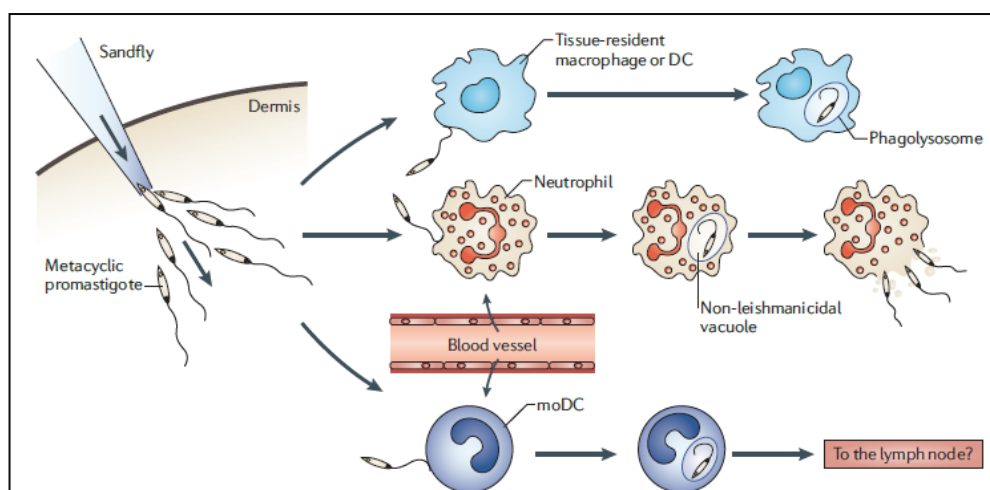


Figure 1.9. Immune cells involved in the *Leishmania* infection. Adapted from [91].

Macrophages, monocytes and dendritic cells. Although parasites are frequently discovered in neutrophils, the greatest evidence for the reproduction and long-term survival is obtained in mononuclear phagocytes. Previously, the studies demonstrated that promastigotes were taken up by resident DCs (i.e. Langerhans) within the first four hours of infection, leading to trigger cytotoxic CD8-T cell activation [91]. This was detected by using two-photon intravital imaging of mice skin after needle injection of *L. major*. In addition, it is believed that dermal macrophages are the first cells to produce infection after an infection has been established for 24 hours [90]. The expansion of the infection necessitates the recruitment of monocytes due to not enough resident macrophages in the skin to maintain multiplication of the parasite. The MHC (major histocompatibility complex) class II molecules, which play a crucial role in the stimulation of IL-12 and the activation of T helper 1 (TH1)-type response, can be expressed by DCs to form monocyte-derived DCs (moDCs) [92]. In fact, the infection leads to a non-protective TH2 response in the chemokine receptor CCR2-deficient mice, and thus providing the ability to attract monocytes to the lesions [93]. Therefore, dermal macrophages or DCs can capable of transmitting parasites from the location of infection to the lymph node, in which acquired immune responses are initiated, resulting in the parasite's eradication. (Figure 1.10) [90].

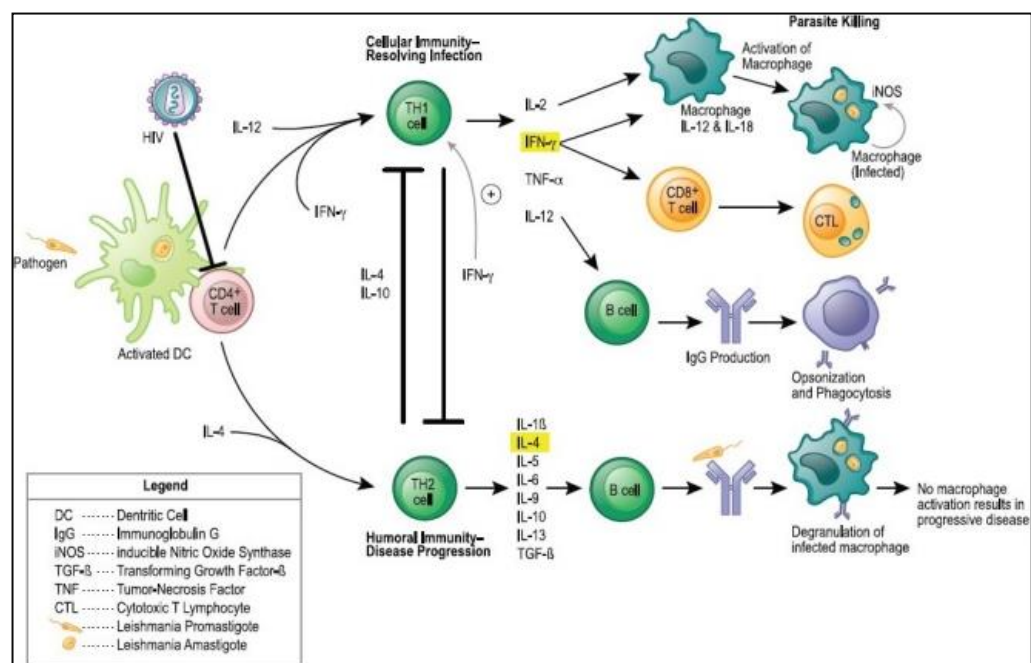


Figure 1.10. Immune response associated with *Leishmania* spp. infection. Adapted from [90, 94].

1.2.7.2. Cytokine Production

The Th1 immune response is crucial for mammalian host defense in the intracellular parasitic infections, including leishmaniasis due to enabling the leishmanicidal activity by macrophage.

In the cases of leishmaniasis, macrophages infected with *Leishmania* suppressed the pro-inflammatory cytokines such as Tumor necrosis factor (TNF- α), IL-12, and IL-1. Previously, the study reported that the release of IL-1-stimulated by LPS is inhibited in *Leishmania*-infected macrophages, or otherwise induced by LPG [95]. Also, the expression of TNF- α is inhibited in *L. donovani*-infected macrophages linked with the release of the immunoimmunosuppressive cytokine IL-10 [96]. Indeed, IL-12 is a crucial cytokine involved in the activation of lymphocytes, which triggers the generation of IFN- γ resulting in the activation of macrophages. It is significant that *Leishmania* parasites can stimulate the production of immunosuppressive cytokines, such as, IL-10 and TGF- β cytokines. Moreover, TGF- β cytokines are produced by *Leishmania*-infected macrophages and inhibit iNOS expression by macrophages, and IFN- γ production by natural killer (NK) cells [97].

Visceral leishmaniasis in human has been predominantly associated with elevated levels of IL-10 rather than of IL-4 [98, 99]. Furthermore, IL-10 and IFN- γ have been involved in the improvement of post-kala-azar dermal leishmaniasis (PKDL), and the production of IL-4, IL-10 and IFN- γ were identified in the more severe clinical forms of cutaneous leishmaniasis [100]. In addition, IL-10 has been indicated to be the critical immunosuppressive cytokine in the case of visceral leishmaniasis, can capable of suppressing the macrophage activity, leading to the inhibition of the release of pro-inflammatory cytokines (IL-1, TNF- α and IL-12), and NO production [101]. The deletion of the IL-10 locus or therapy with anti-IL-10 antibody provide the control of infection, even in *L. major*-infected BALB/c mice. Therefore, it has been demonstrated to the function of IL-10 in the progression of leishmaniasis [102, 103]. Moreover, IL-10 plays a negative role on the DCs maturation from monocyte precursors, and inhibit the capacity of macrophages to kill the intracellular parasites, especially by inhibiting the production of TNF- α [104, 105]. As a result, it has been proposed that IL-10 production by Th1 or Th2 cells serves as a key feedback regulator

regulating the pathophysiology linked to an excessive, even effective, inflammatory response [106].

1.3. NEUROLOGICAL ASPECTS OF LEISHMANIASIS

Several studies indicated that pathogens including *Leishmania* spp., *Trypanosoma brucei*, *Listeria monocytogenes*, *Neisseria meningitidis*, and *Cryptococcus neoformans* can circumvent the blood brain barrier in some mammals such as rodents, dogs and humans as unexpected, and cause brain inflammation with the invasion [107, 108]. Case reports also suggest that intracellular parasites might able to penetrate the brain; however how parasites cross the blood brain barrier (BBB), where they are localized within the brain and how the immune system controls the parasite transmission inside the brain are has not yet been clarified.

Although the BBB is a functional model which separates the CNS from the systemic circulation, and is needed for the maintenance of the neuronal function against pathogens including *Leishmania* parasites, in 1996, the presence of *L. donovani* amastigote forms in the cerebrospinal fluid (CSF) was identified for first time in a patient [109, 110]. With this case, numerous research have focused on the central nervous system in an effort to show that *Leishmania* species can indeed cross the BBB and trigger clinical manifestations and clinical neurological symptoms [111, 112, 113]. In one study, the presence of anti-*Leishmania* antibodies were detected in the CSF of *Leishmania*-positive dogs, leading to the alterations in the nervous tissue, and may result into a disease known as “cerebral leishmaniasis”.

1.3.1. Mechanism of CNS Invasion, and Damage by Parasites

1.3.1.1. Mechanism of CNS Invasion

The spread of certain parasites into the CNS which is an immune-privileged organ as during an infection can lead to serious complications of the disease. Although the brain parenchyma is protected by the physical barrier, known as blood brain barrier from the invasion of parasites in the infectious disease, under the mechanism of how parasites reach and enter

into the central nervous are still unclear. However, several case reports has been indicated that pathogenic parasites can reach the brain including cerebrum and hippocampus (see Figure 1.11 for the brain anatomy) through the the bloodstream either as free-living or extracellular parasites, by embolization of eggs, or within red or white blood cells as the intracellular parasites [114]. Before crossing the CNS by the bloodstream, parasites have to invade the first physical barrier which is host epithelia in the skin, or in the gastrointestinal or respiratory tracts. Parasite-derived molecules can further inhibit host immune response signaling pathways, which may accelerate the the parasites invasion into the brain tissues regarding parasite-host interactions, whereas the host immune system molecules are secreted to prevent the spread of the parasites [115].

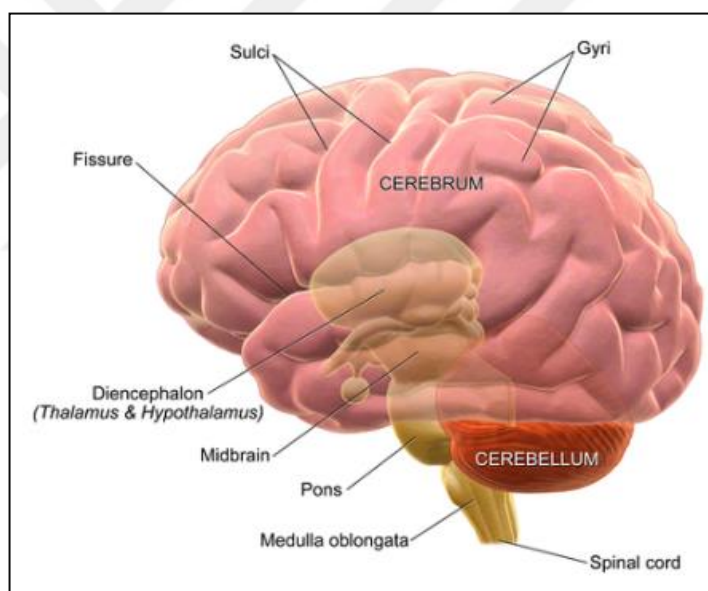


Figure 1.11. The anatomy of the brain.

Comparatively to other tissues, the chemicals moving through blood vessels in the CNS is constrained and closely controlled to maintain the integrity of internal environment of the CNS, which is crucial for the function of neurons. As a result, the tight junctions that connect the cerebral endothelial cells, which form the BBB, provide a barrier that prevents chemicals from diffusing between the endothelial cells and into the brain parenchyma (Figure 1.12), by the so-called transcytosis across the cerebral endothelial cells (i.e., within transport vesicles from the luminal to the abluminal side of the endothelial cell), or by openings of

tight junctions, the so-called paracellular transmigration. By solely delivering particular chemicals into the brain parenchyma, the cerebral endothelial cells exhibit minimal degrees of pinocytotic activity or transcytosis to create a functional barrier [116]. Endothelial cells and astrocytes are separated by one basement membrane at the capillary level, where metabolites are exchanged across the blood-brain barrier (BBB), whereas during inflammation, at the level of post-capillary venules, WBC infiltration into the brain parenchyma is caused by the two membranes: the endothelial and the parenchymal basement membranes). Focused activation of the matrix metalloproteases is required for WBC penetration through the parenchymal basement membrane [117]. Once the matrix metalloproteases is activated, infiltrating WBCs are entrapped between the two basement membranes. Furthermore, previous studies indicated that several molecules such as cysteine- and metalloproteases expressing and releasing from the surface of the Trypanosomatidae (*the family of the Leishmania*) facilitate their passage across endothelial cells.

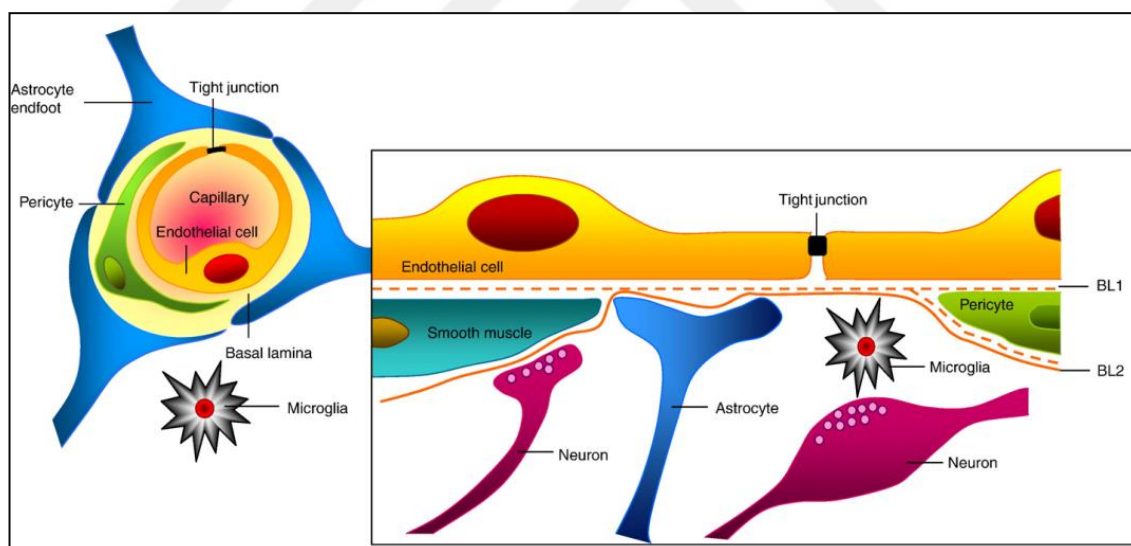


Figure 1.12. The interaction of the cell at the BBB. Adapted from [118].

1.3.1.2. Mechanism of Cytokine Effect on the Brain

Studies have been focused on how signals of the cytokines reach to the brain due to becoming relatively large proteins to cross the BBB. With the infectious diseases on the brain, cytokines begin to release from the immune cells and do not readily through the BBB. There

are several pathways described, including (1) passage through leaky regions (e.g. the circumventricular organs) within the brain, in which display highly vascularized structures, and permeability, fenestrated capillaries surrounding the third and fourth ventricles; (2) active transport by molecules; (3) activation of cells inside layer the cerebral vasculature (including endothelial cells and perivascular macrophages), which secrete inflammatory mediators and cytokines into the brain parenchyma; (4) binding to the receptors of the cytokines linked to peripheral afferent nerve fibers (eg, the vagus nerve), which then spread cytokine signals to related brain regions, such as the solitary tract nucleus and hypothalamus (also called as 'neural route'); and (5) recruitment of activated immune cells from the periphery to the brain, in which they can produce cytokine molecules (Figure 1.13) [116]. Although it is uncertain which pathways has been most critical for cytokine signals to be transmitted to the brain in these circumstances, cytokine release resulted as an inflammatory responses provides the determination of the infections through the mechanism of interactions between cytokines and the immune systems. Therefore, the analysis of the cytokines in the blood play a key role in the characterization of the infection through the cells of the immune systems.

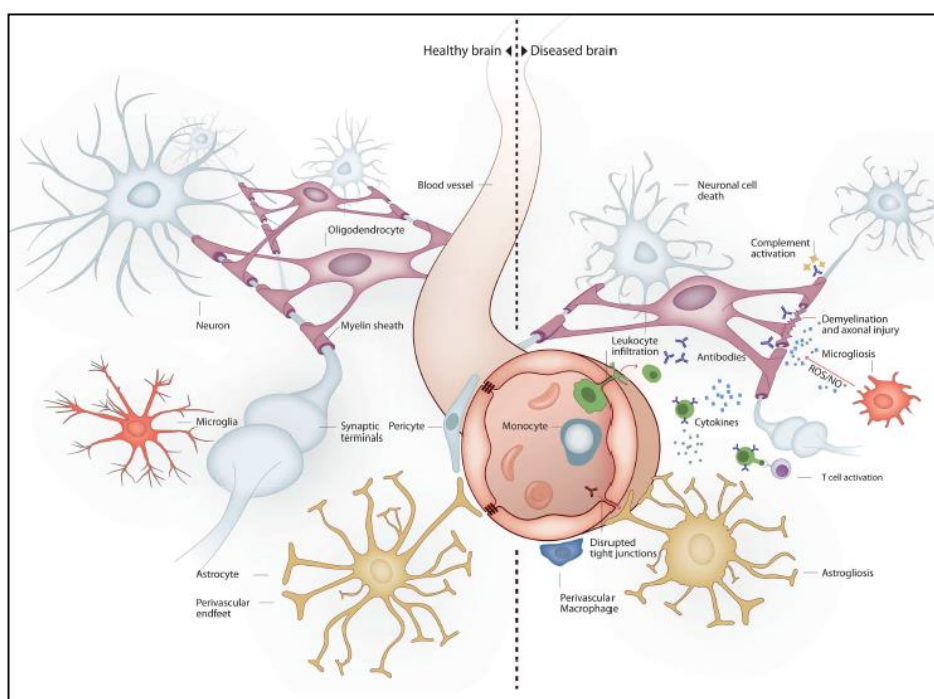


Figure 1.13. Immune cell trafficking in CNS pathology. Adapted from [119].

1.3.1.3. Mechanism of the Immune Response by the Glial Cells

The neurological alterations observed during leishmaniasis might be caused by the immune response of the glial cells, particularly in astrocytes and microglia, rather than the blood-derived inflammatory cells. After exposure of the parasites, inflammatory process is started by the activation of astrocytes and microglia as well as the stimulation of lymphocyte infiltration [120].

Astrocytes are the most prevalent form of non-neuronal, glial cell type in CNS, and have various functions including phagocytosis, homeostasis and the secretion of cytokines for the immune system. These cells undergo astrogliosis or astrocytosis in response to lesions, ischemia, neurotoxic chemicals, infection, and neurodegenerative diseases, which promotes the production of intermediate filaments and the expression of glial fibrillary acidic protein GFAP [121]. GFAP expression frequently increases in situations of brain damage, and these cellular changes can be indicative of a brain lesion. Areas lacking obvious tissue lesions can show increased GFAP expression, indicating that astrocytic changes represent the first step in the pathogenic process [121]. In response to CNS pathogenesis, with the activation of astrocytes, proinflammatory cytokines, including IL-1 β , IL-6, TNF- α and CCL2 can be expressed and released, and thus leading to neuroinflammation [122]. In addition, COX2 and iNOS expressions may be induced by activated astrocytes, resulted in the neuroinflammation with the secretion of the cytokines NO and PGE2.

Microglia are located in the CNS as the main innate immune cells, and have a constitutive controlling role throughout the nervous system. Microglial cells have significant pathophysiological functions by becoming activated in response to a number of stimuli and pathological events include neurodegeneration, brain injury, infection, and tumors. The activation of microglia display a reactivity characterized by immunological, functional, and morphological alterations. Overexpressed microglia can undergo a proinflammatory signaling and response by secreting the inflammatory cytokines such as Chemokine (C-C motif) ligand 2 (CCL2) and TNF- α , interleukin-6 (IL-6), interleukin-1 beta (IL-1 β) [122]. In addition, upon microglia activation can up-regulate inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2), resulted from the release of the pro-inflammatory cytokines, NO and prostaglandin E2 (PGE2). Therefore, brain macrophages can have phagocytosis capacity, thereby brain macrophages that lose all ramifications and develop phagocytosis

capabilities, becoming similar to blood-derived macrophages. In the previous study, CNS resident cells (e.g. microglia, astrocytes and neurons) along with T cells (CD8⁺ and CD4⁺), and NK cells, regulate the proliferation of tachyzoites by the production of IFN- γ in mice model [123]. Furthermore, microglia stimulate the release of pro- and anti-inflammatory cytokines such as IL-1 β , IL-10, TNF, IL-12 and IL-15, resulted in the production of NO, which stops intracellular parasite replication by blocking the parasitic enzyme activity and damaging nucleic materials of parasites.

1.3.1.4. CNS Damage by The Parasites

With passage of parasites into the brain, serious damage or dysfunctions in the nervous system can be occurred by diverse mechanisms, with clinical manifestations related to the targeted brain areas. Disturbances parasites can cause disturbances of neural function even without crossing the BBB. After entering the brain parenchyma, parasites can cause damage by the release of toxic molecules and/or by eliciting an immune response. Although pathogenetic mechanisms of seizures during brain infections have not yet been investigated directly, it has been demonstrated that inflammatory cytokines may affect synaptic functions and induce seizures [124]. To provide the better treatment to the parasite infections in the brain, the underlying mechanism mechanisms of parasite traversal and targeting of various regions in the nervous system should be considered regarding to the information between the immune system cells that can be used as a vehicle for the parasites, especially seen in intracellular parasites including in *Leishmania* spp., and the neuronal signal induced by the inflammations.

1.3.2. Neurological Manifestations of Leishmaniasis

There are previous and recent reports indicated the both CNS and ocular leishmaniasis with visceral leishmaniasis. In addition to neurological manifestations, peripheral nerve deficits can occur in the cutaneous leishmaniasis. Regarding cutaneous leishmaniasis, case reports indicated the overall perineural inflammatory cell infiltrate consisting of lymphocyte, plasma cells and macrophages were found in the biopsy specimen with cutaneous leishmaniasis [125]. Besides, promastigotes and amastigotes were also seen in the CNS during the VL

infection dogs. Although infiltration of the inflammatory cells into the CNS can be most commonly observed, the case of amastigotes found in the blood brain and choroid plexus were also reported [126]. On the other hand, in humans, in addition to CNS, peripheral nervous system are involved in the VL [125]. The main symptom in the humans can be the sensation of the burning feet and have similar to meningitis and cranial nerve dysfunctions whereas dogs with VL may present symptoms of generalized CNS involvement with seizures, cranial nerve alterations and cerebral signs. Neurological alterations of these dogs are dependent on the meningitis and choroiditis which are mainly secondary to antigen and immunoglobulin deposition and leukocyte infiltration [127]. There are several reports demonstrated that the accumulation of either amastigote or the lymphocyte and macrophage - containing amastigotes were determined in the choroid plexus and cerebrospinal fluid. Oliveira et. al. reported that the *Leishmania* spp. can cross the BBB via either free amastigote/promastigote form or macrophages-containing amastigotes [126]. Melo et. al. (2017), also indicated that parasites can transmit into the brain via immune cells but the mechanism is still poorly understood [128]. Therefore, the mechanism of how parasites can use the immune system cell as a vehicle should be investigated to understand the neurological aspects of the *Leishmania*.

2. AIM OF THE STUDY

This study aims to investigate how *Leishmania* parasites are transmitted into the brain via macrophages, and focus on the mechanism underlying the inflammatory responses on both macrophages and astrocytes. Addressing to the lack of knowledge on the neurological aspects of leishmaniasis, the present study introduces the immunological mechanism associated between macrophages- and astrocytes-infected with *L. infantum* on bone-marrow derived primary macrophage cells by *in-vitro* studies. Furthermore, novel flow cytometric approach was developed for quantification of *Leishmania*-infected macrophage in this P.h.D. study. In addition, the antileishmanial efficacy and anti-neuroinflammatory activity of SPT compound were evaluated on *L. infantum* parasites, *Leishmania*-infected macrophages and *Leishmania*-infected astrocytes in *in vitro* cultures, whereas the analysis on non-infected macrophages and astrocytes questioned the safety of the SPT treatment in *in vitro* model (Figure 2.14).

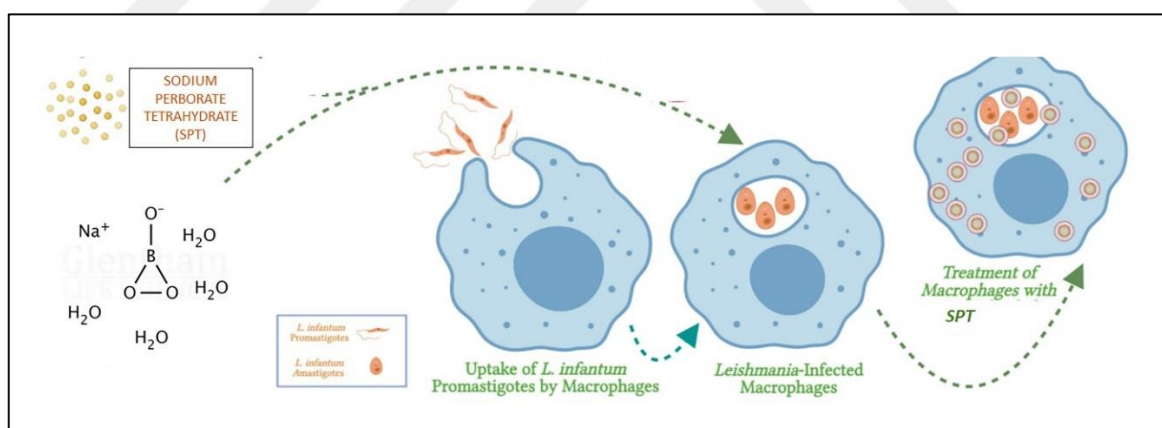


Figure 2.1. Representative graphical abstract illustrates the framework of SPT treatment on macrophages- and astrocytes- infected with *L. infantum* promastigotes. The figure was created with Biorender.com. Adapted from [19].

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Consumables

Table 3.1. Consumables which were used for cell culture experiments

#	Brand	Item	Catalog No
1	TPP	Cell culture flask T25, T75, T150	90150,90075,90025
2	Isolab	Microcentrifuge tubes 1.5 and 2 mL	078.03.002, 078.03.003
3	CAPP	Serological pipettes 5, 10, 25 and 50 mL	SP-5-C,SP-10-C SP-25-C,SP-50-C
4	Isolab	Falcon Tubes 15 and 50 mL	LB.IS.078.02.003
5	Axygen	Micropipette tips 10, 200 and 1000 μ l	301-03-051
6	Eppendorf	Micropipette (2.5, 10, 20, 100, 200 and 1000 μ l)	2231300002

3.1.2. Chemicals

Table 3.2. Chemicals which were used in experiments

#	Brand	Item	Catalog No
1	Thermo Fisher Scientific, USA	DMEM high glucose	Gibco 41966
2	Thermo Fisher Scientific, USA	RPMI 1640 Medium	Gibco BE12-702F
3	Thermo Fisher Scientific, USA	Phosphate Buffered Saline (PBS)	14190144
4	Thermo Fisher Scientific, USA	Penicillin Streptomycin (PS)	Gibco 15140-122
5	Thermo Fisher Scientific, USA	Heat inactivated Fetal Bovine Serum (FBS)	Gibco 10500-056
6	Santa Cruz Biotechnology, USA	Dimethyl Sulphoxide (DMSO)	sc-202581
7	Thermo Fisher Scientific, USA	Trypsin-EDTA	Gibco 25200056
8	Sigma-Aldrich, Germany	Sodium Perborate Tetrahydrate (SPT)	71840
9	Sigma-Aldrich, Germany	Resazurin sodium salt	R7017 - 1G
10	Sigma-Aldrich, Germany	PKH67 Green Fluorescent Cell Linker Kits	PKH67GL-1KT
11	Sigma-Aldrich, Germany	PKH26 Red Fluorescent Cell Linker Kits	PKH26GL-1KT

12	Promega	3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxyphenyl)- 2-(4-sulfo-phenyl)-2H-tetrazolium (MTS)-assay	Promega G3580
13	BD Biosciences	Annexin V-FITC Apoptosis Detection Kit	556547
14	BD Biosciences	Protein Transport Inhibitor (Containing Monensin)	554724
15	Invitrogen™	Trizol	15596026
16	Sigma-Aldrich, Germany	Triton-X-100	X100-100ML
17	BioRad	Universal SYBR Green Supermix	1725122
18	Qiagen	QuantiTect Reverse Transcription Kit	205314
19	AppliChem, Germany	4,6-Diamidino-2-phenylindole dihydrochloride (DAPI)	A4099
20	Merck Milipore, Germany	Paraformaldehyde	104005
21	Sigma Aldrich, Germany	Formaldehyde Solution	15512
22	Biolegend, USA	Propidium Iodide (PI)	421301
23	AppliChem, Germany	Absolute Ethanol	A3928

3.1.3. Instruments

Table 3.3. Instruments which were used in experiments

#	Brand	Item	Catalog No
1	Sigma-Aldrich	Centrifuge	Sigma 3-18KS
2	Zeiss	Inverted microscope	Axio Vert.A1
3	CytoFLEX S, Beckman Coulter	Flow cytometry	CytoFLEX S, Beckman Coulter
7	BIO-RAD	Real time PCR detection system	CFX96
8	ThermoFisher	Varioskan LUX Multimode Microplate Reader	VL0000D0
9	Thermo Fisher Scientific, USA	Nano-Drop 2000	ND2000LAPTOP
10	WiseTherm, HB-R, Switzerland	Thermal Cyclers	
11	Bio-Rad, C1000 Touch, USA	CFX96 Real-Time PCR	
12	BD Biosciences, USA	FACS Calibur	

3.2. METHODS

3.2.1. Cell Lines

Leishmania infantum promastigotes (MHOM/MA/67/ITMAP-263) were kindly provided by Dr. Ana Tomás (Institute for Molecular and Cell Biology (IBMC), University of Porto (Portugal)). The murine macrophage cell line J774A.1 (TIB-67), the murine macrophage cell line RAW 264.7, and the murine astrocyte cell line (C8-S (Astrocyte type II clone)) were provided from American Type Culture Collection (ATCC) (Rockville, MD). Cells were

incubated in Dulbecco's Modified Eagle's Medium (DMEM, #41966-029, Invitrogen, Gibco, UK), whereas *L. infantum* promastigotes were cultured in Roswell Park Memorial Institute medium (RPMI, #11875093, Invitrogen, Gibco, UK).

3.2.2. Maintenance of Animals

Six- to eight-week-old male or female BALB/c mice for cell culture of bone marrow derived-macrophages were handled under specific pathogen-free conditions, according to the guidelines of the Central Animal Facility at Yeditepe University. Six animals were used per experimental group.

3.2.3. *In vivo* Infection Experiments of Leishmaniasis on Mice Model

Six- to eight-week-old male or female BALB/c mice (n=6) were intravenously inoculated in the lateral vein of the tail with 10^8 stationary-phase *L. infantum* promastigotes in 100 μ L of PBS. After 4 weeks-post infection, mice were sacrificed by cervical dislocation and spleens were removed and homogenized to obtain a single cell suspension. The cells were washed twice with RPMI-1640 culture medium (Gibco) and adjusted to 10^7 cells/ml in RPMI-1640 culture medium supplemented with 10% (v/v) inactivated fetal bovine serum (iFBS), 50 U/mL penicillin, 50 μ g/mL streptomycin, and 20 mM HEPES sodium salt pH 7.4 (Sigma Aldrich, Germany). After 8 day-incubation, cells were counted and examined for *L. infantum* parasites under microscopy.

3.2.4. Cell Culture Studies

3.2.4.1. Parasite Culture

L. infantum promastigotes (MHOM/MA/67/ITMAP-263) were kindly provided by Dr. Ana Tomás (Institute for Molecular and Cell Biology (IBMC), University of Porto (Portugal)). Promastigotes were cultured at 25°C in RPMI 1640 Glutamax (Gibco) supplemented with 10% (v/v) inactivated fetal bovine serum (iFBS), 50 U/mL penicillin, 50 μ g/mL streptomycin, and 20 mM HEPES sodium salt pH 7.4 (Sigma Aldrich, Germany). To

maintain infectivity of parasites in culture, parasites kept for no more than seven passages in culture flask. Infection experiments were performed with recently thawed aliquots of virulent parasites. Infective metacyclic promastigotes were obtained from stationary phase cultures of *L. infantum*. After centrifugation at 3000 rpm for 10 min, promastigotes were collected from the pellet [129].

3.2.4.2. Preparation of Soluble Leishmania Antigen (SLA)

Stationary phase *L. infantum* parasites ($\sim 2 \times 10^7$ /ml) were washed in PBS, and centrifuged at 1,500xg for 10 min at 4°C. Then, parasite pellet was dissolved in lysis buffer (1 mL buffer per 10^9 parasites), and performed three quick freeze-thaw cycles. Following freezing and thawing, ultrasonication were applied for five minutes. Then, supernatant was collected after centrifugation at 5,000xg for 20 min at 4°C, and protein amount was determined using BCA Protein Assay Kit (Pierce) according to the manufacturer's protocol.

3.2.4.3. Counting of Leishmania infantum Parasites

Parasites were counted after fixation with 4% paraformaldehyde (1:10 (v/v)) for 5 min, using a hemocytometer. Parasite count per mL was calculated by average number of promastigotes $\times 10^4$ x (dilution factor).

3.2.4.4. Macrophage and Astrocyte Cell Culture

RAW 264.7 (TIB-71) and J774 A.1 (TIB-67) murine macrophage cell lines, and C8-S (Astrocyte type II clone) murine astrocyte cell line were cultured as a monolayer at 37°C in a humidified atmosphere containing 5% CO₂, in Dulbecco's modified Eagle's (DMEM) medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2 mM L-glutamine, 100 units/mL of penicillin and 100 µg/mL of streptomycin (Biological Industries, Beit Haemek, Israel). Cells were maintained in culture by sub-passaging every 3 days.

3.2.4.5. Bone Marrow Derived-Macrophage (BMDM) Generation

BALB/c mice are euthanized by cervical dislocation and bone marrow (BM) cells were collected from the bones, femurs and tibias in BALB/c mice. BM cells were isolated from flushing femurs and tibia washed with RPMI medium by using a 21G syringe. Flushing cells were passed through a 40 μ m cell strainer (Falcon, USA) to achieve single cell suspension. Afterwards, cells were washed in 2% FBS and 1% PSA completed RPMI 1640 medium. Red blood cells were lysed using 2 ml ACK Lysis Buffer for 5 minutes at room temperature and then, cells were washed with RPMI 1640 medium and cultured in RPMI medium supplemented with 10% FBS and 1% PSA. Cells were seeded into petri dishes (3×10^6 cells/mL) or 12 well plates (250,000 cells/mL) and cultured in the presence of 20 ng/mL M-CSF for 6 days. At day 3, medium was changed with fresh medium (containing 20 ng/mL M-CSF). At 7th day, BMDMs were evaluated using flow cytometry regarding the analysis on the maturity and differentiation, and were analysed using fluorophore conjugated antibodies for the detection of BMDM markers, CD11b and F4/80.

3.2.4.6. BMDM Polarization

After the generation of BMDM at 7th day, 1.5×10^5 BMDM (1 mL/well in 12-well plates) in each well were treated with 100 ng/mL SLA, 100 ng/mL LPS from *Escherichia coli* O111: B4 (Sigma-Aldrich), and 100 ng/mL LPS with 50 ng/mL IFN- γ for M1 activation, and 10 ng/mL IL-4 for M2 activation in RPMI medium containing 10 ng/mL M-CSF, 10 % iFBS and 1% PSA. After 24 h, cells were collected to determine the M1-M2 polarization of macrophages by staining for specific cell-surface markers (CD80, CD86, TNF- α) for M1 macrophages; IL-10 for M2 macrophages) using flow cytometry analysis.

3.2.4.7. Flow Cytometry Analysis

Fixation of Cells

Cells were collected and centrifuged at 1600 rpm for 5 minutes. Cell pellets were fixed in 4% paraformaldehyde at room temperature for 15 minutes. After fixation, cells were washed

with fluorescence activated cell sorting (FACS) buffer and stored at 4°C for the analysis of surface markers and cytokines (intracellular staining).

Cell Surface Marker Staining

Fixed cells were centrifuged at 1600 rpm for 5 minutes, washed with FACS buffer, and then incubated for 5 min on ice in buffer with anti-CD16/CD32 mAb (Fc block, Biolegend, USA) to limit unspecific binding. 1 µg/mL of fluorochrome conjugated antibodies (all from Biolegend Biosciences, USA) including CD11b, F4/80, CD80, and CD86 were then added, and cell were incubated for 15 minutes in dark on ice. After incubation, cell were washed with 1 mL FACS buffer, centrifuged and resuspended with 200 µL PBS. The cells were examined by flow cytometry (CytoFLEX S, Beckman 159 Coulter, USA) with a total of 10,000 events.

Intracellular Staining

For intracellular staining, BMDMs were incubated with Protein Transport Inhibitor (Containing Monensin) (2 µM) (BD Bioscience) for 5 hours. Following incubation, cells were permeabilized using Cytofix/Cytoperm solution (BD Biosciences) for 3 minutes and incubated with fluorescent mAb specific for TNF- α and IL-10 for 30 min on ice. Cells were then washed using Perm/Wash buffer according to the manufacturer's instructions. Data were examined and analyzed on a FACSCalibur flow cytometer (CytoFLEX S, Beckman 159 Coulter, USA).

3.2.4.8. Co-culture Studies

Infection of BMDMs with *L. infantum* promastigotes

BMDMs were seeded on 12-well plates with a density of 150,000 cells per well and incubated at 37 °C. After 24 h, macrophage cells were infected with stationary-phase

promastigotes (cell: promastigote ratio, 1:10). Following 3-hour of co-incubation, non-internalized parasites were removed by successive washing with PBS. After 24 h, infected cells were used for the macrophage-astrocyte co-culture studies.

Incubation of Astrocytes with/without IFN- γ

The murine astrocytes were seeded on 6-well plates with a density of 135,000 cells per well and incubated at 37 °C. After 24 h, cells were stimulated with IFN- γ (Calbiochem) at 100 ng/mL for 48 h prior to infection.

Co-culture of Infected BMDMs and Astrocytes

Prior to the co-culture, C8-S astrocytes (135,000 cells /well) were stimulated IFN- γ (100 ng/ml) for 48 h. Following 48 h of stimulation, astrocytes stimulated with/without IFN- γ co-cultured with *Leishmania*-infected macrophages (4.5×10^4 /well) (ratio of astrocyte: *Leishmania*-infected macrophages: 3:1) for 24, 48 and 72h. For the optimization of the co-culture system, cells were cultured at a different ratio of astrocyte: infected macrophages (1:1, 2:1, and 5:1). Following the optimization studies, the co-culture ratio were determined as 3:1 (astrocyte: *Leishmania*-infected macrophages). Accordingly, growth of *L. infantum* on co-culture was assessed with phase-contrast microscopy images as detailed.

Determination of the Percentage of *L. infantum* promastigotes in the Co-culture

Following 24, 48, and 72 h of co-incubation, cells were stained intracellularly, and accordingly, they were permeabilized using Cytotfix/Cytoperm solution (BD Biosciences) for 3 minutes and incubated with antibody cocktail including primary mAb specific for mouse anti *Leishmania* Surface Protease gp63 (1:2000 dilution in BSA) and 1 μ g/mL astrocyte marker Alexa Flour 647-conjugated anti-GFAP for 30 min in dark on ice. After incubation, cells were washed with 1 mL FACS buffer, centrifuged and incubated with secondary antibody (FITC, 1:300 dilution in PBS) for 30 minutes. Cells were then washed with FACS

buffer, and analyzed by flow cytometry (CytoFLEX S, Beckman 159 Coulter, USA) at FL-1 (green) and FL-2 filter (red) with a total of 100,000 events.

3.2.4.9. Evaluation of SPT for the Neuroinflammatory-based Antileishmanial Therapy

The antileishmanial efficacy of SPT on *L. infantum* parasites, *Leishmania*-infected macrophages and astrocytes were evaluated for the neuroinflammatory-based antileishmanial therapy using *in vitro* cell culture systems, shown schematically in Table 3.4.

Table 3.4. *In vitro* cell culture experiments for the evaluation of the antileishmanial efficacy of SPT on *L. infantum* parasites, *Leishmania*-infected macrophages and *Leishmania*-infected astrocytes

Cell culture studies	<i>L. infantum</i> promastigotes	<i>Leishmania</i> -infected Macro. <i>Leishmania</i> -infected Astro.
Cytotoxicity/Cell viability Assay	X	X
Morphological Analysis	X	X
Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)	X	
DAPI staining	X	
Cell cycle analysis	X	
Flow cytometric analysis for quantification of cells infected with <i>L. infantum</i> parasites	X	X
Determination of oxidative stress		X
Intracellular ROS measurement		X
Gene expression analysis	X	X

3.2.4.10. Cytotoxicity Assay on *L. infantum* Promastigotes

Antileishmanial effect of SPT were tested on *L. infantum* promastigotes using standard resazurin assay (Alamar Blue Assay). Accordingly, *L. infantum* promastigotes (at exponential phase of growth) were seeded into 96-well plates at a density of 3×10^5 cells per well in completed RPMI medium. Then, *L. infantum* parasites were exposed to of SPT (Stock solution: 1 mM) at different concentrations (25-200 μ M) and further incubated for 24, 48, and 72 h. DMSO was used as control group to confirm in any alteration on viability of *L. infantum* promastigotes. At the end of the treatment, 10% (v/v) of a 2.5 mM resazurin solution (Sigma)-Alamar Blue was added to each well, and fluorescence intensity was measured (560Ex/590Em) using a UV-vis spectrophotometer (Spectramax i3 Multi-Mode Microplate Reader Detection Platform). Viability of promastigotes were estimated as the percentages compared to untreated negative control group. Data were analyzed on GraphPad Prism 6.1 software and 50% inhibitory concentration (IC_{50}) values were determined according to the percentage of the cell viability.

In line with the parasite viability data, as shown in phase-contrast microscopy and scanning electron microscopy (SEM) images were taken to visualize the cell morphology of the parasites after SPT treatments.

3.2.4.11. Measurement of Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Mitochondrial membrane potential ($\Delta\Psi_m$) was measured using Rhodamine 123 mitochondrial specific fluorescent dye (Sigma-Aldrich) and quantification was measured relatively to determine the alteration in the mitochondrial membrane potential. Briefly, the cells were seeded into a 48-well plate at a density of 300,000 parasites/well. The following day, the parasites were treated with SPT at various concentrations (50-400 μ M) for 24 and 48 h. Following the incubation, cells were resuspended in serum-free RPMI containing 10 μ M Rhodamine 123, and were incubated at 37 °C for 30 min in the dark. Then, they were centrifuges, and washed twice with 1X DPBS. Suspension of the cell was transferred to the 96-well plate, and the fluorescence intensities were measured at 490 nm excitation and 515 nm emission by using spectrophoto-fluorimeter (Varioskan LUX multimode reader, Thermo

Scientific). The absorbance was evaluated as fold change according to the negative control group.

3.2.4.12. DAPI Staining

Nuclear morphological changes and the number of cells were observed using the DNA specific fluorescent dye 40-6-diamidino-2-phenylindole (DAPI). Briefly, the cells were seeded into a 48-well plate at a concentration of 300,000 parasites/well. The following day, the parasites were treated with SPT at various concentrations (75-150 μ M) for 24, and 48 h. Afterwards, parasites were washed with 1X DPBS and fixed with 350 μ l 4% Paraformaldehyde (PFA) at room temperature for 30 min. Subsequently, fixed cells were stained with DAPI (5 μ g/ml) for 15 min at room temperature (RT) in the dark, and imaged by a fluorescence microscope (AXIO Vert. A1, Zeiss, Germany).

3.2.4.13. Cell Cycle Analysis

Cell cycle distributions of *L. infantum* promastigotes were determined by flow cytometry for 24, and 48 h. Accordingly, 600,000 parasites per well were seeded in 48-well plates in completed RPMI 1640 medium, and incubated for 24 and 48 h. Following the incubation period, cells were fixed by 70% ethanol solution at -20°C for at least 1h. After fixation, cells were washed twice with PBS and incubated with 0.01% Tritonx and 100 μ g/mL RNase A for 30 min at 37°C. Following to incubation, cells were washed with PBS, and 5 μ g/mL Propidium iodide solution was added to each tube mixture which remained for 5 min in the dark. 10,000 cells were analyzed by FACSCalibur (BD Biosciences), and the percentages of the cell populations in G0/G1, S and G2/M phases were analyzed according to the flow cytometry histogram graphs. Cell cycle arrest was calculated as the percentage (%) compared to negative control culture. Data were analyzed with GraphPad Prism 6.1 software.

3.2.4.14. Cell Viability Assay on Macrophages and Astrocytes

RAW 264.7 murine macrophages and C8-S (Astrocyte type II clone) murine astrocytes were incubated in the presence of SPT. Cell viabilities were determined using standard resazurin assay (Sigma Aldrich, Germany). Briefly, RAW264.7 macrophages and C8-S (Astrocyte type II clone) astrocytes were seeded in 96-well plates with a density of 8,000 cells per well. After 24 h of incubation, RAW264.7 macrophages and C8-S astrocytes were treated with SPT at various concentrations (25-150 μ M) for 24, 48 and 72 h cells. Following the incubation, cells were incubated in 10% (v/v) MTS containing Glucose (Sigma) and PBS solution for 1 h. Absorbance at 490 nm was detected using a microplate spectrophotometer (xMark-Biorad, Canada). Cell viability was calculated as the percentage compared to negative control culture. Data were analyzed with GraphPad Prism 6.1 software and 50% cytotoxic concentration (CC₅₀) values were determined according to the cell viabilities (%).

3.2.4.15. Flow Cytometric Analysis for Quantification of Macrophages Infected with *L. infantum* Parasites

A new flow cytometric approach were developed regarding this Ph.D. study for the determination of infection rates of macrophages infected by *L. infantum* parasites [131]. Prior to *Leishmania* infection, J774A.1 macrophages/RAW 264.7 macrophages/C8-S astrocytes and *L. infantum* promastigotes were stained separately with PKH26 and PKH67 dyes, respectively. Dual staining enabled detection of each cell type, where non-infected macrophages were also recorded for the quantification.

Staining of Macrophages/Astrocytes and Parasites with Fluorescent Dyes

J774A.1 murine macrophages were used in the optimization of the staining protocol, and all other experiments regarding SPT treatment were continued with RAW264.7 murine macrophages.

J774A.1/RAW264.7 macrophages/C8-S astrocytes and *L. infantum* promastigotes were stained with PKH26 dye (Red Fluorescent Cell Linker Kit, Sigma-Aldrich) and PKH67 dye (Green Fluorescent Cell Linker Kit, Sigma-Aldrich), respectively, as described in Islek, Z.

et al., 2021 [131]. The manufacturer's protocols were followed for staining. Briefly, a total of 10^7 cells/mL were centrifuged ($400 \times g$) for 5 minutes into a loose pellet. Pellet was collected and resuspended with 1 mL of diluent to prepare a 2X cell suspension. Prior the staining, 2X dye solution (4×10^{-6} M) in Diluent C by adding 4 μ L of the PKH ethanolic dye solution to 1 mL of Diluent C was prepared in a falcon tube. Afterward, 1 mL of 2X dye solution were mixed with 1 mL of 2X cell suspension to obtain 1×10^7 cells/mL and 2×10^{-6} M PKH26 dye. After incubation of the cell/dye suspension for 1–5 minutes, the staining was stopped by adding an equal volume (2 mL) of serum and incubated for 1 minute to allow binding of excess dye. Cells were washed following two more centrifugation steps ($400 \times g$ for 10 minutes) to ensure removal of unbound dye and then resuspended in a complete medium. Cells were fixed in 2% paraformaldehyde by treatment for 15 minutes. Prior the infection, the promastigotes and macrophages/astrocytes were examined by flow cytometry (CytoFLEX S, Beckman Coulter, USA) with a total of 100,000 events to ensure that all cells are successfully stained with PKH dyes and can be detected.

Determination of The Percentage of Macrophages/Astrocytes Infected with *L. infantum* Parasites

J774A.1 macrophages stained by PKH26 were seeded on 12-well plates with a density of 180.000 cells, and incubated at 37 °C. After 24 h, macrophages were infected with stationary-phase PKH67-labeled promastigotes (cell: promastigote ratio, 1:2.5 and 1:10). Following 3-hour of co-incubation, non-internalized parasites were removed by successive washing with PBS. After 24 h, cells were fixed with 2% paraformaldehyde, and then washed three times with PBS. Infection rates (%) of macrophages were determined according to their relative fluorescence intensities detected by flow cytometry (CytoFLEX S, Beckman Coulter, USA).

Similarly, RAW 264.7 macrophages and C8-S astrocytes stained by PKH26 were seeded on 12-well plates with a density of 120.000 cells and 90.000 per well, respectively, and incubated at 37 °C. After 24 h, PKH26-labeled macrophages and astrocytes were infected with stationary-phase PKH67-labeled promastigotes (cell: promastigote ratio, 1:10). Following 3-hour of co-incubation, non-internalized parasites were removed by successive washing with PBS. After 24 h, cells were fixed with 2% paraformaldehyde, and then washed

three times with PBS. Infection rates (%) of macrophages were determined according to their relative fluorescence intensities detected by flow cytometry (CytoFLEX S, Beckman Coulter, USA).

For the detection of PKH-labeled parasites/cells, FL1 (green fluorescence) and FL2 (red fluorescence) filters were used to distinguish the parasites/cells with regard to the excitation (for PKH67 at 490 nm; PKH26 at 551 nm) and emission (for PKH67 at 502 nm; PKH26 at 567 nm) of the dyes. Reliability of flow cytometry dot-plot data were verified by performing six independent experiments sets. Gates were set for each independent experiment to optimize the separation of infected cells. At least 100,000 cells were collected and read on flow cytometry to extend the reliability of the statistical data.

In parallel, phase-contrast and fluorescence microscopy images were taken to visualize the parasite infection by the use of different fluorescent channels to distinguish PKH67-labelled parasites (green), PKH26-labelled macrophages (red) and infected macrophages with a merged yellow color.

3.2.4.16. Measurement of Reactive Oxygen Species (ROS)

The intracellular infected macrophage and infected astrocyte reactive oxygen species (ROS) level were detected by using 20,70 -dichlorodihydrofluorescein diacetate (DCF-DA)-Cellular Reactive Oxygen Species Detection Assay Kit (Abcam) following the manufacturer's instructions. Briefly, macrophages and astrocytes were seeded into a 12-well plate with a clear flat bottom at a concentration of 150,000 and 90,000 cells/well. After 24 h, they were infected with *L. infantum* promastigotes at a ratio of 1:10 (macrophage/astrocyte: parasite) for 3.5 hours and the remaining parasites were washed twice with completed DMEM High medium. Then, after 24 h, cells were treated with different concentration of SPT (0-200 μ M) for 24 h, and 100 mM of tert-butyl hydroperoxide (THBP) for 1 h were used as the positive control. Following 24 h of incubation, cells were washed with 1X Assay Buffer, stained with 100 μ L/well of the diluted DCFDA Solution (DCFDA / H₂DCFDA - Cellular ROS Assay Kit) in serum-free media and incubated at 37°C for 45 min in the dark. Then, DCFDA solution were removed and cells were dissolved in 100 μ L/well in 1X Buffer. the fluorescent intensity of DCF was measured using

spectrophotometer at 485 nm excitation and 535 nm emission wavelength and ROS levels of the cells were analyzed using a FACSCalibur flow cytometer (CytoFLEX S, Beckman 159 Coulter, USA). Data were analyzed by GraphPad Prism Software (version 6.01) and reliability of flow cytometry histogram data were verified by performing three independent experiments sets.

3.2.4.17. Determination of Oxidative Stress by the Measurement of Glutathione (GSH) Level in Leishmania-infected Macrophages and Leishmania-infected Astrocytes

GSH level of infected macrophages and infected astrocytes were measured from cell lysates as previously described in Reis et. al. (2021). Accordingly, cell lysate (50 μ l) was suspended with DTNB (Ellman's reagent - 5,5'-Dithiobis(2-nitrobenzoic acid)) (10 μ L) solution. EDTA buffer solution (pH 8.2) was then added to this mixture into the 96-well plate. After incubation at 37°C under dark for 30 min, the absorbance was measured colorimetrically at 412 nm wavelength by using a UV spectrophotometer (Thermo Scientific, Finland). Results were expressed as μ mol/g protein and performed in triplicates.

3.2.4.18. Determination of Gene Expression Level in *L. infantum* parasites, *Leishmania*-infected Macrophages and *Leishmania*-infected Astrocytes

Quantitative Real-Time Polymerase Chain Reaction (qPCR) studies were conducted to determine the expression levels in *L. infantum* parasites and *Leishmania*-infected macrophages (infected RAW264.7) and *Leishmania*-infected astrocytes (infected C8-S) treated with SPT, as given in Table 3.5.

The relative gene expression of *Leishmania*-infected macrophages and *Leishmania*-infected astrocytes - treated with 50, 75, 100, 150 and 200 μ M SPT were analyzed using qPCR at 24 h, according to housekeeping genes, 18S rRNA, ATP5, and β -actin, as internal controls (Table 3.5).

Table 3.5. Transcription factors in the *L. infantum* promastigotes, *Leishmania*-infected RAW 264.7 macrophages and *Leishmania*-infected C8-S astrocytes – treated with SPT

Cell types	Genes
<i>L. infantum</i> promastigotes	Meta1, Small Hydrophilic Endoplasmic Reticulum-associated Protein (SHERP), Metacaspase 5 (MCA5)
<i>Leishmania</i> -infected Macrophages (RAW 264.7)	Pro-inflammatory: IL-1 β , IFN- γ , TNF- α , IL-12 (B), NF-kappa β (NF- κ B)
	Anti-inflammatory: IL-10, TGF- β
<i>Leishmania</i> -infected Astrocytes (C8-S)	Pro-inflammatory: IL-1 β , IFN- γ , TNF- α , IL-12 (B), STAT1, iNOS, NF- κ B, MMP-2.
	Anti-inflammatory: IL-10, TGF- β

Total RNA Isolation

L. infantum promastigotes were seeded into 24-well plated with a density of 1.2×10^6 parasites per well, and treated with different concentrations of SPT (0-100 μ M) for 24 h. Following 24 h of incubation, parasites were collected and centrifuged at 3000 rpm, 10 min., and then incubated in Trizol reagent, transferred into 1.5 mL eppendorf tubes and added equal volume of chloroform to Trizol reagent. Samples were incubated at room temperature for 15 minutes and then centrifuged at 12,000 x g for 15 minutes at 4°C. The clear phase (top phase) containing RNA was transferred into new eppendorf tubes and pre-cooled isopropanol with equal volume of the clear phase was added and mixed well for 10 minutes-incubation on ice to precipitate RNA. Then, samples were centrifuge at 12,000 x g for 10 minutes at 4°C, supernatant was disposed and 400 μ L of 70 % (v/v) cold ethanol was added to each tube following another centrifugation step by 7,500 x g for 5 minutes at 4°C. Each tube was dried for 10 minutes, pellet was resuspended in 25 μ L of RNase/DNase free water and RNA concentration was measured using NanoDrop.

For the total RNA isolation of the macrophages and astrocytes, the similar procedure was applied. Briefly, RAW 264.7 macrophages and C8-S astrocytes were seeded on 12-well plates with a density of 120.000 cells and 90.000 per well, respectively, and incubated at 37 °C. After 24 h, macrophages and astrocytes were infected with stationary-phase promastigotes (cell: promastigote ratio, 1:10). Following 3-hour of co-incubation, non-internalized parasites were removed by successive washing with PBS. After 24h of incubation, cells were treated with different concentration of SPT (0-200 µM) for 24 h. Following 24 h of incubation, cells were scraped with adding 250 µL of Trizol reagent, transferred into 1.5 mL eppendorf tubes and added equal volume of chloroform to Trizol reagent. Samples were incubated at room temperature for 15 minutes and then centrifuged at 12,000 x g for 15 minutes at 4°C. The clear phase (top phase) containing RNA was transferred into new eppendorf tubes and pre-cooled isopropanol with equal volume of the clear phase was added and mixed well for 10 minutes-incubation on ice to precipitate RNA. Then, samples were centrifuge at 12,000 x g for 10 minutes at 4°C, supernatant was disposed and 400 µL of 70 % (v/v) cold ethanol was added to each tube following another centrifugation step by 7,500 x g for 5 minutes at 4°C. Each tube was dried for 10 minutes, pellet was resuspended in 25 µl of RNase/DNase free water and RNA concentration was measured using NanoDrop.

Revers Transcriptase (RT) Polymerase Chain Reaction (RT-PCR)

RNA isolated from the cells were converted into complementary DNA (cDNA) using QuantiTect Reverse Transcription Kit (Qiagen). RT-PCR was performed according to the manufacturer's protocol, given in the Table 3.6.

Table 3.6. Reverse Transcriptase (RT) Polymerase Chain Reaction (RT-PCR) components

Components per reaction	Volume	Final Concentration
Reverse-transcription reaction mix		
Quantiscript Reverse Transcriptase	1 μ l	-
Quantiscript RT Buffer, 5x	4 μ l	-
RT Primer Mix	1 μ l	-
Genomic DNA elimination reaction components		
RNA sample*	1000 ng	-
gDNA Wipeout Buffer, 7x	2 μ l	1x
Nuclease-free water	12 μ l – Volume of RNA sample	-
Total Volume	20 μl	-

*This amount is determined by the concentration of RNA present obtained from total RNA isolation.

Quantitative Polymerase Chain Reaction (qPCR)

Quantitative Real Time Polymerase Chain Reaction (qPCR) was performed using the SYBR Green qPCR Reaction Mix (Table 3.7) in a Real Time Thermal Cycler (Bio-Rad Mycycler, USA) under the conditions given in Table 3.8. As shown in Table 3.9, the gene-specific primers of interests were designed using Primer-BLAST software available in the NCBI-Gene gene bank. ATP5, Beta-actin and *L.infantum* 18S rRNA gene were used as reference gene in the normalization of qPCR data analysis. The results were analyzed using Bio-Rad CFX Manager software.

Table 3.7. Components of qPCR

Components for reaction	Volume (for 10 μ l)
SYBR Green qPCR Reaction Mix	5 μ l
Primer Forward	1 μ l
Primer Reverse	1 μ l
Nuclease free H ₂ O	2 μ l
Template cDNA (100 ng/ml)	1 μ l

Table 3.8. qPCR reaction cycle conditions

Reaction cycle	Temperature (°C)	Time	Number of cycle
Initial denaturation	95	10 minutes	1
Denaturation	95	10 seconds	45
Annealing	55	30 seconds	
	57		
	59		
	(see Table 3.9 for melting temperature (M.T.) of each gene)		
Extension	72	15 seconds	
Final Extension	72	10 minutes	1
Melting curve	55-95 0.5°C increase/cycle	10 seconds	1
Hold	4	-	1

Table 3.9. Primer Lists for qPCR

Primer	M.T.	Forward Primer Sequence	Reverse Primer Sequence
18S rRNA	59	GCGCATGGGAGAAGCTCTAT	TTGCAAGCACCAGAGAGGAG
β -actin	59	GACAGGATGCAGAAGGAG	GATCCACATCTGCTGGA
ATP5	57	TGCTACCACTGTGTTGTCCC	TTCGAGAGGTGGAGTCCAGT
MCA5	59	CTTCCCGATTAGCGAGTGCT	TCGAGAATGCTGCCGTTCTT
Meta1	59	GGGCAGCGACGACCTGAT	CGTCAACTTGCCGCCGTC
SHERP	59	CAATGCGCACAACAAGATCC AG	TACGAGCCGCCGCTTATCTTGTC
TNF- α	57	ATGGCCTCCCTCTCATCAGT	ACCCTGAGCCATAATCCCCT
IFN- γ	57	GTCAACAACCCACAGGTCCA	CGACTCCTTTTCCGCTTCCT
IL-1 β	57	TCAGCACCTCACAAGCAGAG	TTCTTGAGACCCTGAGCGAC
IL-10	59	TGCAGGACTTTAAGGGTTAGT TGG	GGCCTTGTAGACACCTTGGTC
iNOS	59	CTATGGCCGCTTTGATGTGC	CAACCTTGGTGTTGAAGGCG
NF- κ B	59	ACACGAGGCTACAACCTCTGC	GGTACCCCCAGAGACCTCAT
STAT1	57	TCACATTACATGGGTGGAA	TTCTCTGGTATGTTCTCGGC
TGF- β	59	ACTGGAGTTGTACGGCAGTG	GGATCCACTTCCAACCCAGG
IL-12 (B)	55	GAACTCACAGGTGGAGGTCA	TCTTTCTTGCGCTGGATTCG
MMP-2	55	GTTTCCTAAGCTCATCGCA	TGGTTCTCCAGCTTCAGGTA

3.3. STATISTICAL ANALYSIS

GraphPad Prism Software (version 8.0.1) was used to perform statistical analysis. Error bars represent standard error of the mean. The data sets were compared using ordinary one-way ANOVA and two-way ANOVA followed by Tukey's or Sidak multiple comparison test. Differences were considered statistically significant at (*) $P \leq 0.05$, (**) $P \leq 0.01$, (***) $P \leq 0.001$, (****) $P \leq 0.0001$.



4. RESULTS

4.1. PARASITE AND CELL CULTURE STUDIES

4.1.1. Characterization of BMDMs

To generate BMDMs, single cell suspension isolated from the bone marrow are cultured in the presence of macrophage colony-stimulating factor (M-CSF) as described in section 2.1.2.5.

As shown in Figure 4.1, high purity of mature macrophages were observed on day 7 where 83% of total macrophage population were identified by their double positive signal in the upper-right quadrant using FITC and PE filter for CD11b and F4/80 macrophage markers. The BMDM differentiation were examined under phase contrast microscope on day 0, 3, 5 and 7 as morphologically in which spindle-like cells have become spherical on day 5 and day 7 (Figure 4.2).

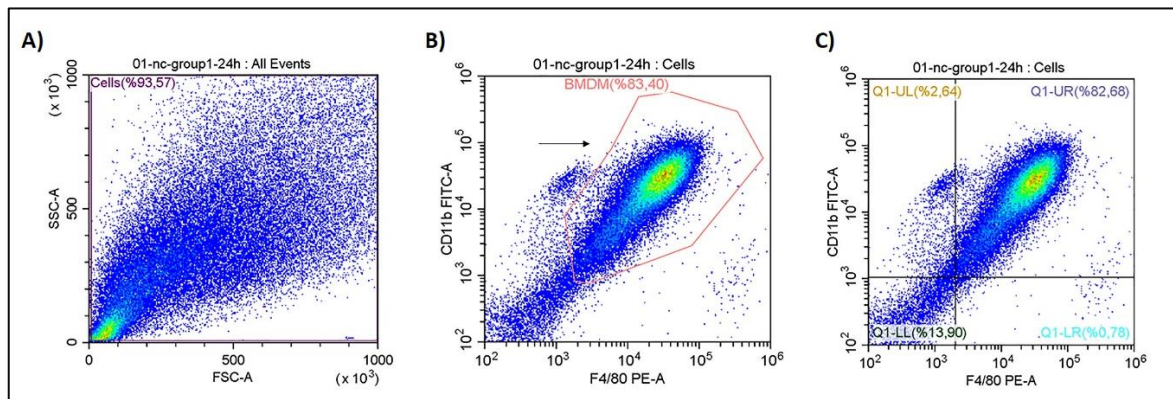


Figure 4.1. Flow cytometry analysis of differentiated BMDMs. Representative images indicating (A) dot plot of size (FSC) and granularity (SSC), (B) the expression of CD11b and F4/80 markers (CD11b+F4/80+ subpopulations) and (C) dot plot of double positive cells for F4/80 and CD11b markers (FSC: forward scatter channel; SSC: side scatter channel). CD11b+F4/80+ subpopulations (upper right) with the purity displayed as percentage of parent population gated on FSC/SSC.

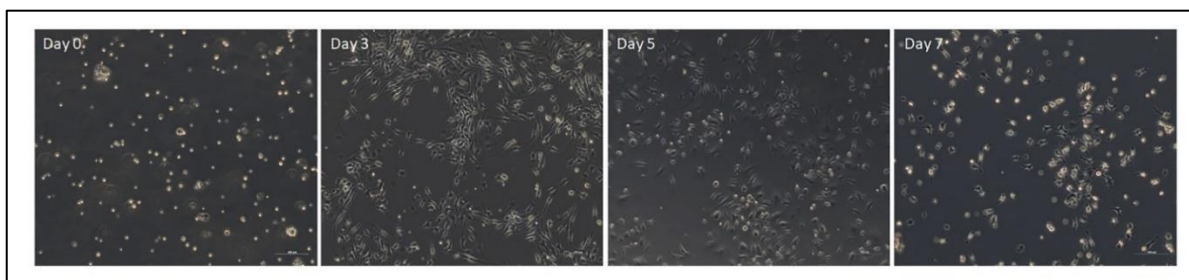


Figure 4.2. Phase contrast microscopy images of BMDM differentiation on day 0, 3, 5 and 7. Data are representative of three independent experiments.

4.1.2. Determination of Macrophage Polarization by Soluble *Leishmania* Antigen (SLA) Stimulation

BMDMs were stimulated with 100 ng/ml LPS or 100 ng/ml SLA for 24 h, and 48 h, and detected as CD11b⁺F4/80⁺ cells, the surface marker of BMDMs CD11b, F4/80, CD80, CD86 and cytokines TNF- α and IL-10 were analyzed using flow cytometry after 24-h, and 48-h of incubation (Figure 4.3). Mean fluorescence intensity (MFI) of labeled BMDMs were analyzed using flow cytometry analysis.

According to the histogram analysis, the mean fluorescence intensity (MFI) of CD11b-FTIC increased significantly in SLA-stimulated BMDMs at 24 h ($p \leq 0.01$) (Figure 4.3-B, C), and 48 h ($p \leq 0.05$) (Figure 4.3-E, F), compared to untreated BMDMs (NC). MFI of CD11b-FTIC showed no significant alterations between NC and LPS-stimulated BMDMs at 24 h (Figure 4.3-A, C), whereas after 48 h of stimulation, MFI of CD11b-FTIC increased significantly compared to NC BMDMs ($p \leq 0.001$) (Figure 4.3-D, F). F4/80-PE indicated a significant increase in LPS-stimulated BMDMs after 24 h ($p \leq 0.0001$) (Figure 4.3-A, C), and 48 h ($p \leq 0.0001$) (Figure 4.3-D, F), when compared to untreated NC BMDM group. Similarly, SLA stimulation in BMDMs led to a significant increase in F4/80-PE marker at 24 h ($p \leq 0.0001$) compared to NC group (Figure 4.3-B, C); however after 48 h of SLA stimulation did not cause a significant change in F4/80-PE marker (Figure 4.3-E, F).

On the other hand, the surface markers CD80 and CD86 of M1 macrophages increased significantly in LPS-stimulated BMDMs after 24 and 48 h; whereas as expected, no significant increase was observed on SLA-stimulated BMDMs after 24 and 48 h. Unlike

CD80-APC, CD86-ECD signal increased significantly in SLA-stimulated cells at 24 h ($p \leq 0.01$) (Figure 4.3-B, C), but not significantly at 48 h of stimulation (Figure 4.3-E, F). Furthermore, the BMDM activation status can be confirmed by increased cellular size (Figure 4.3-A, B, and, right shift in FSC-A) and increased abundance of surface antigens CD80 and CD86 on macrophages as shown in Figure 4.3. Besides, BMDMs treated with LPS did not lead a significant increase in the production of pro-inflammatory cytokine TNF- α at 24 h compared to NC group, while after 48 h of stimulation, TNF- α signal increased significantly in LPS ($p \leq 0.0001$) or SLA-treated ($p \leq 0.01$) BMDMs. Contrary to TNF- α , anti-inflammatory cytokine IL-10 did not show any change increase in either BMDMs treated with LPS or SLA upon 24 and 48 h of stimulation.

Together with the flow cytometry analysis, phase-contrast microscopy images were taken to visualize the polarisation of BMDMs into M1 and M2 macrophage after LPS, LPS and IFN- γ , SLA, and IL-4 at 24 h (Figure 4.4). The morphology of different macrophage phenotypes can led to suggest that cell morphology could be an indicator of macrophage activation status [132]. Treatment with LPS, or LPS and IFN- γ display flat and round-shaped cell morphologies which is attributed to the polarisation of BMDMs to M1 macrophages after LPS stimulation [132]. On the other hand, when BMDMs were treated with SLA or IL-4, the more stretched and spindle-like shape cells were observed much more than the round-shaped in the number of cells, resulted in a similar in shape, and further suggest the polarisation of M2 macrophages rather than M1-like phenotype. Although upon SLA stimulation, the more M2 macrophages were observed, M1 and M2 macrophage population could be determined heterogeneously on the stimulated culture. Considering these results, it has to be noted that macrophages display high plasticity and can be repolarized or reprogrammed under specific stimuli, which could be explained by the differentiation of M1 macrophages to M2, and vice versa.

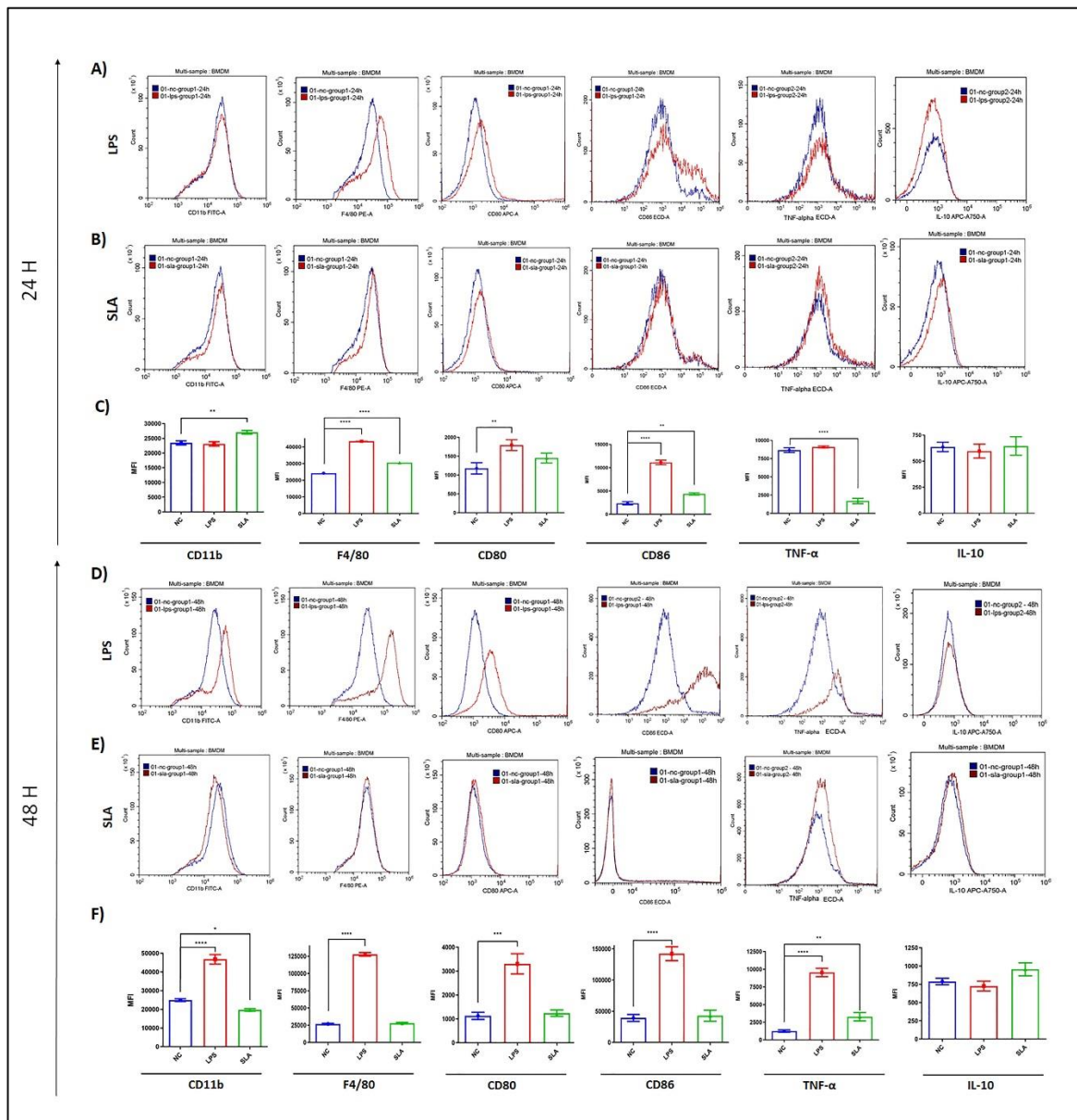


Figure 4.3. Flow cytometric analysis of BMDM cells treated with LPS and SLA after 24h- and 48 h-incubation. The surface marker of BMDMs CD11b, F4/80, CD80, CD86 and cytokines TNF α and IL-10 were analyzed using flow cytometry after 24-h (A), and 48-h (D) lipopolysaccharide (LPS, 100 ng/mL) or (B, E) Soluble *Leishmania* Antigen (SLA, 100 ng/mL) stimulation (n = 3). (C, D) Mean fluorescence intensity (MFI) of labeled BMDMs. Data represents mean (n=3) $\pm$ SD.

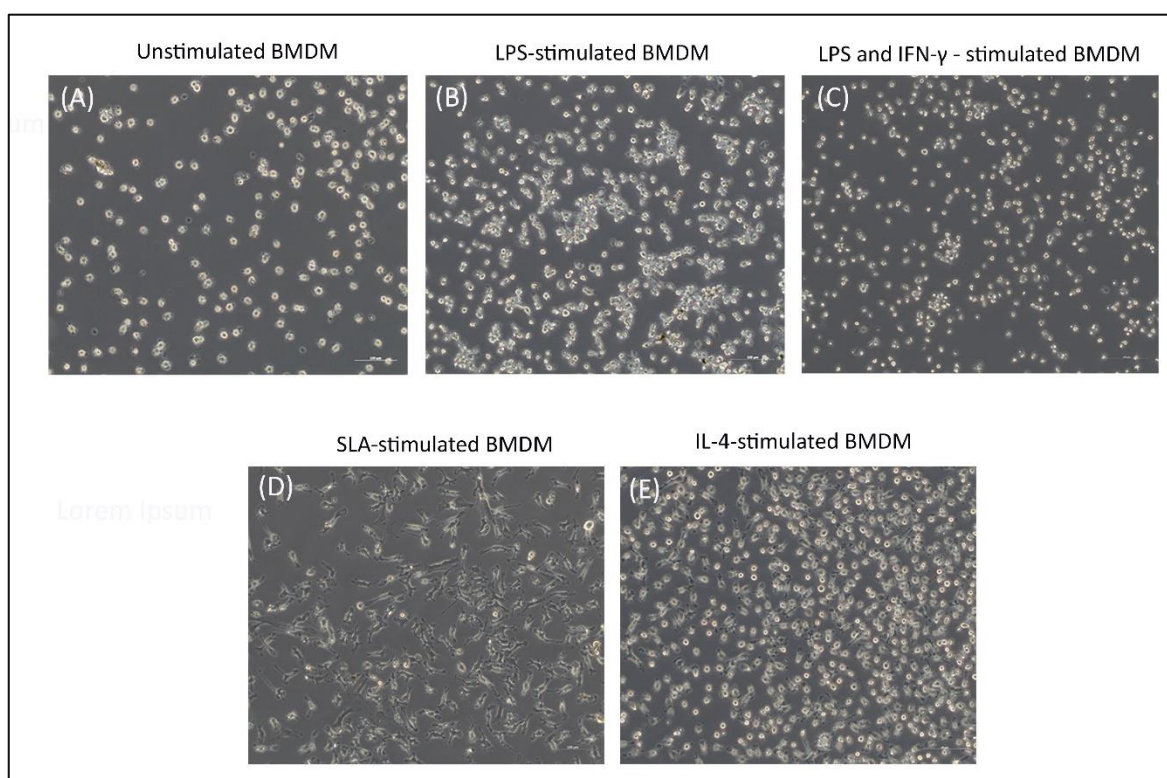


Figure 4.4. Phase-contrast images of BMDM (A) untreated, treated with (B) LPS, (C) LPS and IFN- γ , (D) SLA, and (E) IL-4 after 24h-incubation (scale bar: 100 μ m).

4.2. CO-CULTURE OF ASTROCYTES AND *LEISHMANIA*-INFECTED MACROPHAGES

4.2.1. Determination of the Percentage Rate of *L. infantum* Promastigotes in the Coculture of Astrocytes and Infected Macrophages by Flow Cytometry Analysis

In order to investigate the percentage rates of *L. infantum* promastigotes in the coculture of astrocyte and infected macrophages, gp63 FITC-labeled *Leishmania* parasite and GFAP APC-labeled astrocytes was detected by the flow cytometry according to the FL1 and FL2 filters. FL1 (green fluorescence), and FL2 (red fluorescence) filters were used to distinguish the parasites/cells with regard to the excitation (for FITC at 495 nm; APC at 650 nm) and emission (for FITC at 519 nm; APC at 660 nm) of the dyes. While the tracking of the fluorescent signals in fluorescence microscopy enabled the selective detection of promastigotes, non-infected astrocytes and infected astrocytes qualitatively, the flow

cytometry allowed quantitative analysis of the parasite in the co-culture of the infected macrophages and astrocytes.

For this analysis, RAW264.7 macrophages were infected for 3.5 h with *L. infantum* promastigotes (macrophage: parasite ratios, 1:10). The flow cytometry analysis started first with unstained promastigote and astrocyte (not infected) cells. The obtained FSC-H/FSC-A cytograms indicated a homogenous population distribution for both unstained promastigotes (Figure 4.5-A) and unstained astrocytes (Figure 4.5-B). When the means of fluorescence intensities of a FL-1 green (FITC) and a FL-2 (PE) signals were investigated, as expected, no fluorescence signal was detected for unstained cells. On the other hand, the co-culture of *Leishmania*-infected macrophages and astrocytes were gated based on the information on cytogram for non-infected astrocyte populations (Figure 4.5-C, D) and could be compensated using GFAP APC-labeled astrocytes and gp63 FITC-labeled promastigotes (Figure 4.5). From the gated population of infected macrophages and astrocyte co-culture, singlets were subgated considering their FSC-H/FSC-A cytograph. This way, fluorescent signal of GFAP APC-labeled astrocyte population was successfully separated from background fluorescence of the cells in the co-culture. Singlets were plotted according to the gp63 FITC green and GFAP APC red fluorescence signals using quadrant analysis, where the parasites were identified by FL1 positive green signal in the upper-left quadrant using FITC and PE filter (Figure 4.5-C, D). Further quadrants represent noninfected astrocytes co-cultured with infected macrophages and non-internalized promastigotes. When the populations in the lower-left and upper-left quadrants in Figure 4.5-C and D were analyzed together, a significant decrease ($P \leq 0.0001$) in parasite load could be observed in parallel to a time-dependent manner (Figure 4.8-A). Accordingly, in the non-infected astrocytes and *Leishmania*-infected macrophage co-culture, the parasite population was detected as $73.86 \pm 5.6\%$ at 24 h, which decreased to $50.08 \pm 4.26\%$, and $24.88 \pm 2.30\%$ in the co-culture after 48 and 72 h of incubation, respectively (Figure 4.5-C, D, and Figure 4.8-A). It was also represented by the overlay histogram analysis according to the decreased fluorescence intensity of gp63 FITC-labeled *Leishmania* parasites at 24, 48 and 72 h, as given in Figure 4.6.

In parallel, phase contrast microscopy images confirmed a time-dependent reduction of the parasite burden in the co-culture of uninfected astrocytes and *Leishmania*-infected macrophages, in which an increase in the number of astrocytes was observed at 72 hours of

co-incubation (Figure 4.7). This could be explained by the decrease in parasite burden due to the increase in astrocyte inside the co-culture, and thus increasing the pro-inflammatory cytokine release into the culture supernatant [133].

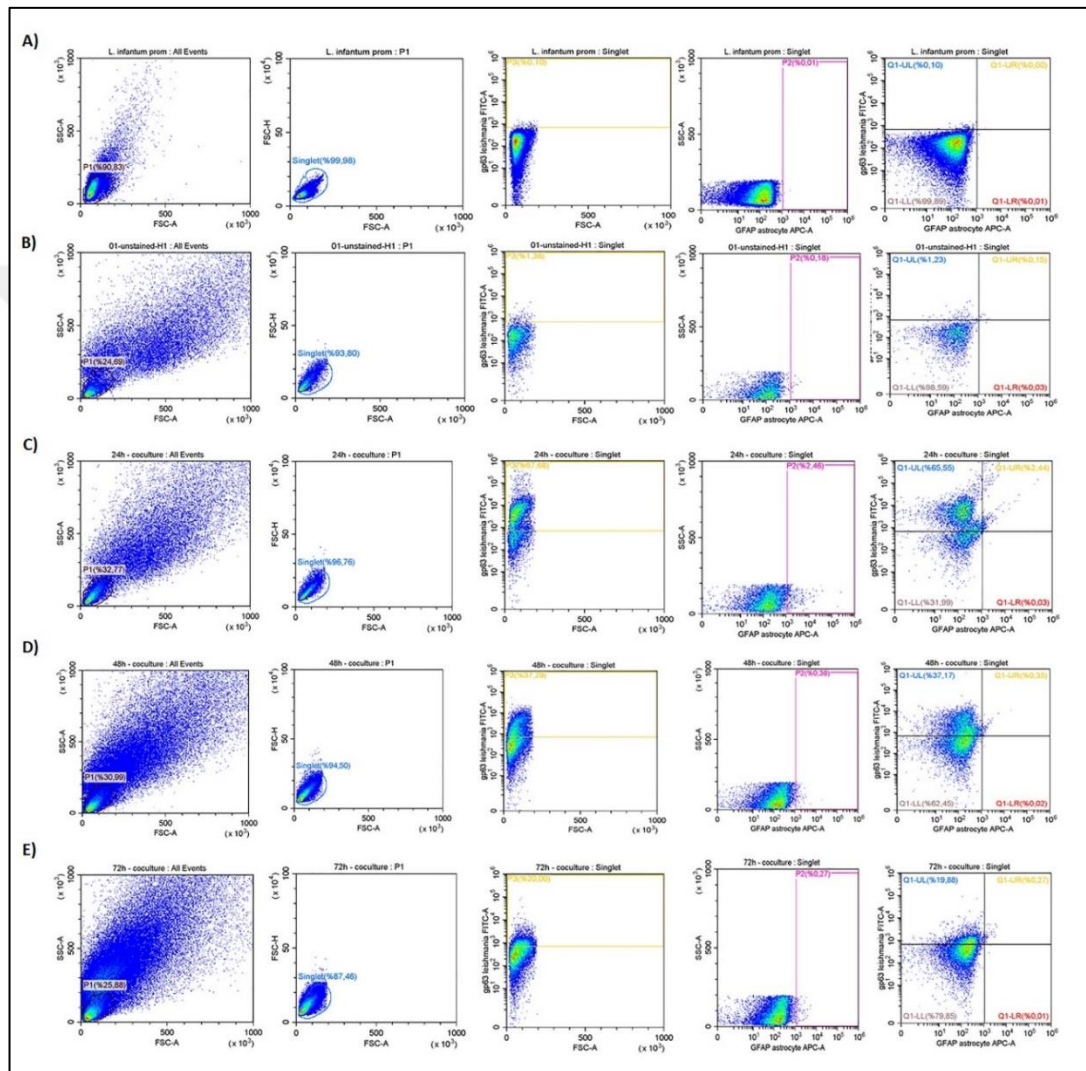


Figure 4.5. Dot-plot flow cytometry analysis of the percentage rate of *L. infantum* promastigotes in the coculture of astrocytes and *Leishmania*-infected macrophages (coculture ratio: 3:1 (astrocyte: *Leishmania*-infected macrophages)). Representative dot plot analysis of (A) Anti- gp63-FTIC- labeled *L. infantum* promastigotes, (B) Anti-GFAP-APC- labeled astrocytes, (C) 24 hour of coincubation of astrocytes and *Leishmania*-infected macrophages, (D) 48 hour of coincubation, and (E) 72 hour of coincubation.

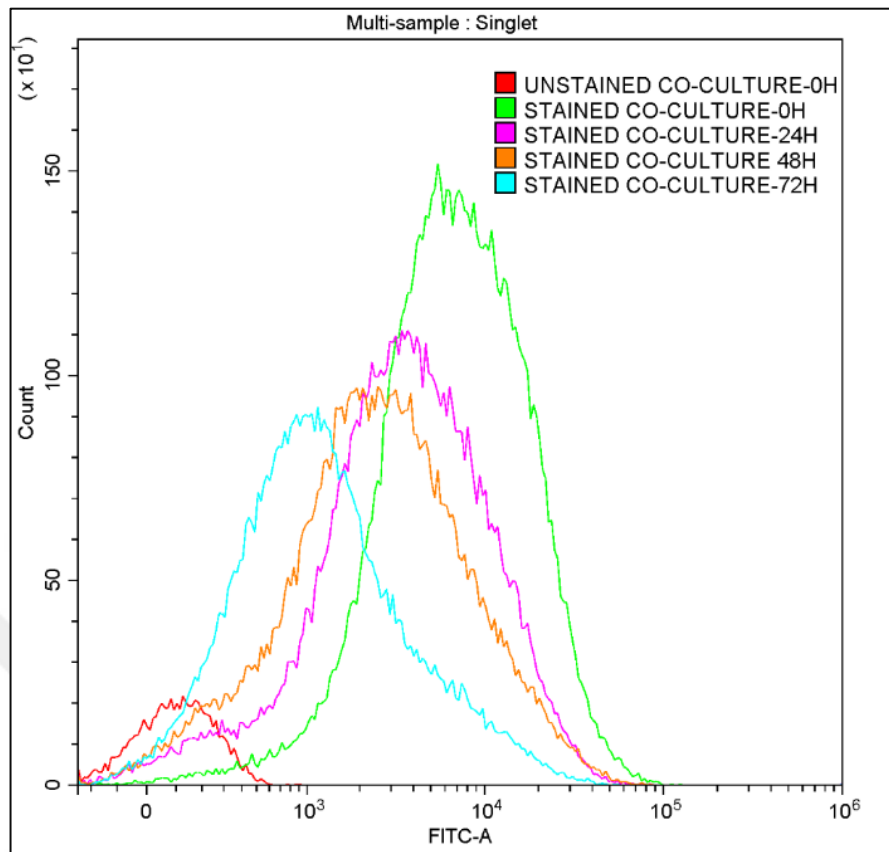


Figure 4.6. Histogram analysis of number of *L. infantum* promastigotes in the coculture of astrocytes and *Leishmania*-infected macrophages (coculture ratio, astrocyte: *Leishmania*-infected macrophages, 3:1).

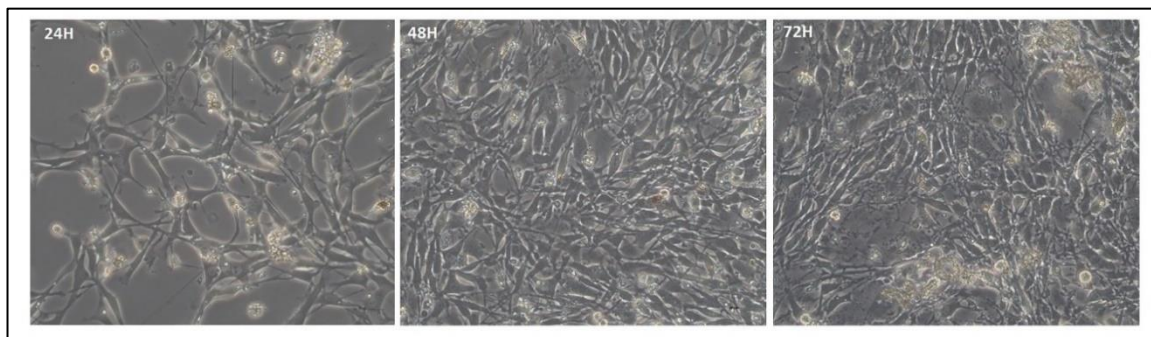


Figure 4.7. Phase-contrast images of C8-S murine astrocytes co-cultured with *Leishmania*-infected macrophages (infection ratio:1:10) (ratio of astrocyte: *Leishmania*-infected macrophages: 3:1) after 24, 48 and 72h of incubation (scale bar: 50 μ m).

4.2.2. Determination of Neuroprotective Effect of IFN- γ on Astrocytes in the Co-culture

As previously described in 3.2.2.1.1, prior to the co-culture, C8-S astrocytes (135,000 cells /well) were stimulated IFN- γ (100 ng/ml) for 48 h, and then co-cultured with *Leishmania*-infected macrophages (4.5×10^4 /well) (ratio of astrocyte: *Leishmania*-infected macrophages: 3:1) for 24, 48 and 72h. Growth of *L. infantum* parasites in co-culture was detected by flow cytometry analysis as described in detail above.

According to the flow cytometry analysis, in the non-infected astrocytes and *Leishmania*-infected macrophage co-culture, the gp63 FITC labeled- *Leishmania* parasite population was detected as $88.82 \pm 2.11\%$ at 0 h of incubation, which significantly decreased to $73.86 \pm 5.6\%$ ($p \leq 0.01$), $50.08 \pm 4.26\%$ ($p \leq 0.0001$) and $24.88 \pm 2.30\%$ ($p \leq 0.0001$) in the co-culture at 24, 48 and 72 h, respectively (Figure 4.5-C, D, and Figure 4.8-A). Similarly, MFI of gp63 FITC labeled- parasites significantly decreased in the co-culture in a time-dependent manner (Figure 4.6, left shift in FITC, and Figure 4.8-B).

On the other hand, IFN- γ - stimulated non-infected astrocytes indicated similarity with non-stimulated astrocytes, in which the percentage of the parasite population significantly decreased to $70.16 \pm 6.20\%$ ($p \leq 0.01$), $48.89 \pm 5.60\%$ ($p \leq 0.0001$) and $24.77 \pm 4.50\%$ ($p \leq 0.0001$) after 24, 48 and 72 h of co-culture, respectively (Figure 4.8-A). Parallel to the percentage of parasite population, MFI of gp63 FITC labeled- parasites significantly decreased in the co-culture in a time-dependent manner (Figure 4.8-B).

Overall, no significant differences were investigated between IFN- γ - stimulated and non-stimulated astrocytes on the growth of *L. infantum* parasites, resulted in reducing in the parasite burden in the non-infected astrocytes and *Leishmania*-infected macrophages co-culture. The reduction of the parasite burden could be explained that astrocytes could decrease the number of parasites by the production of specific pro- and anti-inflammatory mediators, including IFN- γ (via the activation of signal transducer STAT1) during neuroinflammation [133]. Furthermore, upregulation of the GFAP and the subsequent reactive astrogliosis limit pathogen distribution in the CNS [133].

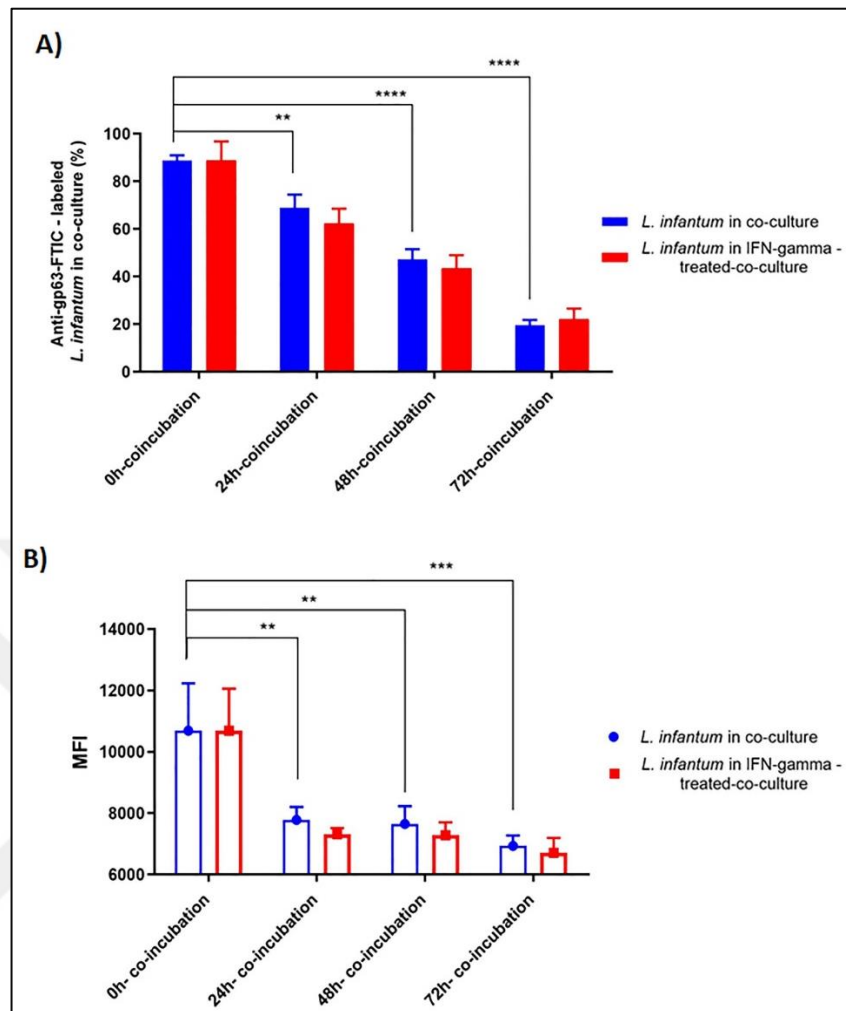


Figure 4.8. Flow cytometry analysis of (A) the percentage and (B) gp63 FITC-labeled *L. infantum* promastigotes in 24, 48 and 72 hour-coculture of non-infected astrocytes and *Leishmania*-infected macrophages (coculture ratio: 3:1 (astrocyte: *Leishmania*-infected macrophages)). Data represents mean (n=3) $\pm$ SD.

4.3. NOVEL DUAL-FLUORESCENT FLOW CYTOMETRIC APPROACH FOR QUANTIFICATION OF MACROPHAGES INFECTED WITH *L. INFANTUM* PARASITES

Flow cytometry analysis emerges as an alternative methodology to microscopy for determination of the *Leishmania*-infection rates of macrophages. Various flow cytometric approaches have been established for the quantification of *Leishmania* parasites within host

cells, labelled either directly fluorescent dyes or indirectly with fluorescently conjugated antibodies. Although these techniques allow accurate quantification of infection, they fail at detection of non-infected macrophages specifically. This study introduces a new flow cytometric approach for the determination of infection rates of macrophages infected by *L. infantum* parasites. Prior to infection, J774A.1 macrophages and *L. infantum* promastigotes were stained separately with PKH26 and PKH67 dyes, respectively. Dual staining enabled detection of each cell type, where non-infected macrophages were also recorded for the quantification.

As previously published under the content of this P.h.D thesis [131], J774A.1 murine macrophages were used in the optimization of staining protocol, and all other experiments regarding SPT treatment were continued with RAW264.7 murine macrophages.

4.3.1. Staining of the Parasites And Macrophages with PKH Membrane Dyes

J774A.1 murine macrophages and *L. infantum* parasites were stained with PKH26 (red) and PKH67 (green) dyes, respectively. Tracking the green FITC signal (PKH67) and the red PE signal (PKH26) in flow cytometry, it was possible to determine separately the rates of PKH67-staining on *L. infantum* promastigotes and PKH26-staining on J774 A.1 macrophages. Accordingly, flow cytometric (pseudo-colour/smooth) plots indicated the gating strategy based on the size and granularity of *L. infantum* promastigotes and J774 A.1 macrophages using a minimum/maximum SSC-A/FSC-A filter level to exclude debris and cluster of the cells (Figure 4.9). Cells were gated to identify the distinct area for the cell population. Within the group of gated cells, single cells (singlets) were sub-gated by considering their FSC-H/FSC-A features. As illustrated in Figure 4.9-A, when unstained parasites were analysed in flow cytometry with this procedure, 99.92% of parasites were determined as unstained, where 0.08% of the parasites were left out beyond the constraints owing a false FITC signal. When stained parasites were analysed in flow cytometry with the same procedure, single cells or so-named singlets corresponding to 99.32% of the population were plotted with the PKH67 green fluorescence dye, of which 99.71% of promastigote population was found to be labelled successfully (Figure 4.9-B). The unstained macrophage population was detected as 99.99% by flow cytometry, while singlets of PKH26-stained macrophages corresponded to 95.26% of the population and 99.57% of this singlet

population was found to be labelled successfully (Figure 4.9-C, D). These findings indicate that a negligible number of promastigotes remain unstained subsequent to the applied staining protocol.

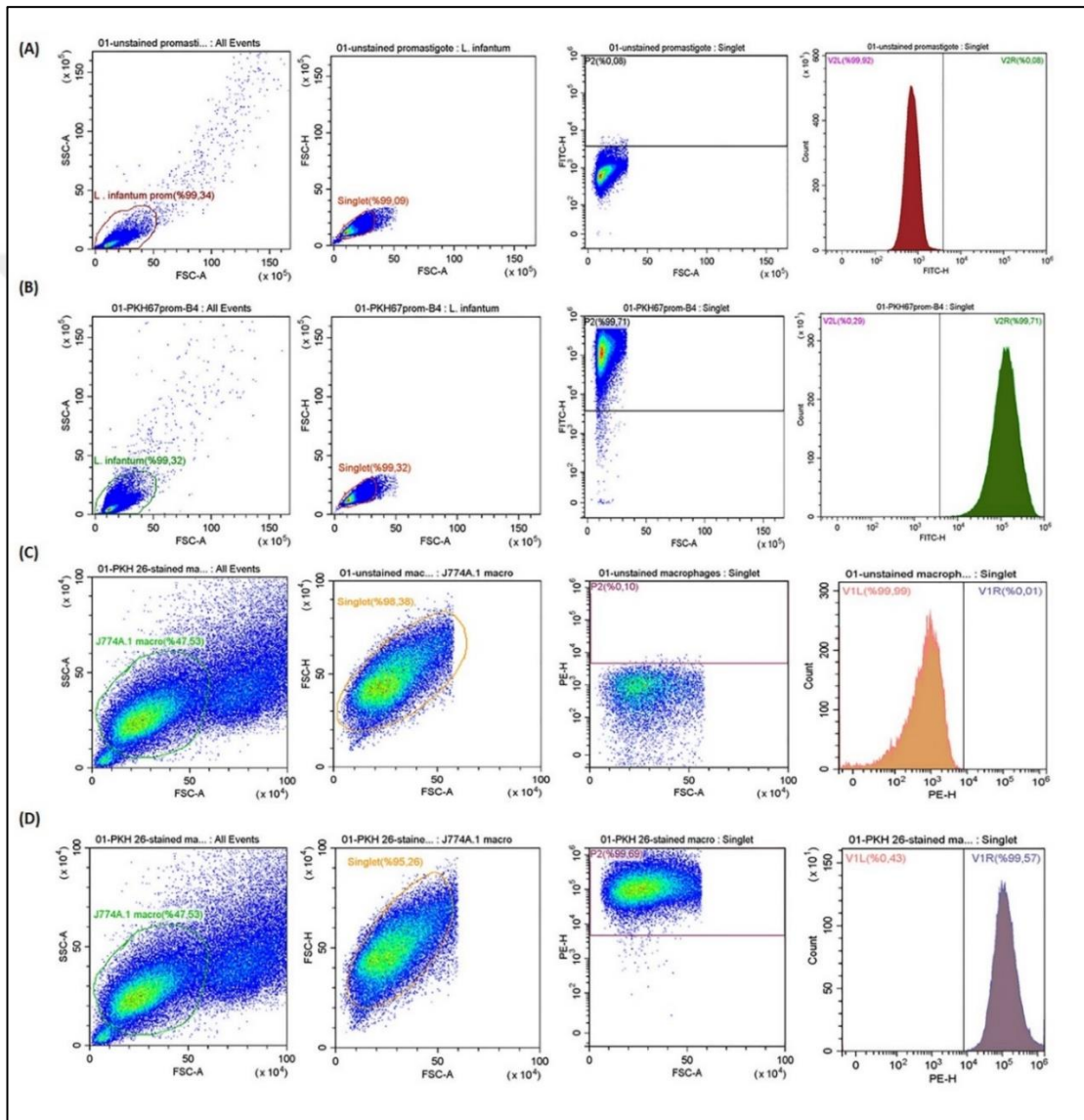


Figure 4.9. Flow cytometry analysis of PKH67-stained and unstained *L. infantum* promastigotes. Flow cytometric (pseudo-colour/smooth) plots for (A) unstained and (B) PKH67-stained *L. infantum* promastigotes; (C) unstained and (D) PKH26-stained J774 A.1 macrophages.

4.3.2. Quantification of the Infection Rate (%) of *Leishmania*-infected Macrophages

In order to investigate the infection rates of macrophages, staining was applied this time on both *L. infantum* promastigotes and J774A.1 macrophages with PKH67 (green) and PKH26 (red) dyes, respectively. While the tracking of the fluorescent signals in fluorescence microscopy enabled the selective detection of promastigotes, non-infected macrophages (the macrophages without parasite inside) and infected macrophages qualitatively, the flow cytometry allowed quantitative analysis of the cells and infection ratio of the macrophages. Accordingly, the fluorescence microscopy images displayed promastigotes in green, non-infected macrophages in red, and the macrophages infected by the parasites as the merged yellow colour (Figure 4.10). The bright-field image confirmed that all macrophages and promastigotes have been labelled and detected by fluorescence microscopy imaging. In addition to the determination of the infection, microscopy analysis also verified that all macrophages could be successfully stained by the applied protocol, as during multi-channel reconstruction of the bright-field and fluorescent channel images no macrophage was observed lacking the red fluorescence signal.

Flow cytometric (pseudo-colour/smooth) analysis enabled the quantitative determination of the percentage of macrophages infected with *L. infantum* promastigotes. For this analysis, J774A.1 macrophage culture was infected for 3 h with *L. infantum* promastigotes by two different macrophage: parasite ratios, i.e. 1:2.5 and 1:10. The flow cytometry analysis started first with unstained promastigote and macrophage (not infected) samples. The obtained FSC-H/FSC-A cytograms indicated a homogenous population distribution for both unstained macrophages (Figure 4.11-A) and unstained promastigotes (Figure 4.11-B). When the means of fluorescence intensities of a FL-1 green (FITC) and a FL-2 (PE) signals were investigated, as expected, no fluorescence signal was detected for unstained cells. On the other hand, *Leishmania*-infected macrophages were gated based on the information on cytogram for non-infected macrophage populations (Figure 4.11-C, D) and could be compensated using PKH26-stained macrophages and PKH67-stained promastigotes (Figure 4.11). From the gated population of infected macrophages, singlets were subgated considering their FSC-H/FSC-A cytograph. This way, fluorescent signal of PKH26-stained macrophage population was successfully separated from back ground fluorescence of the macrophages (Figure 4.11). Singlets were plotted according to the PKH67 green and PKH26 red fluorescence signals

using quadrant analysis, where infected macrophages were identified by their double positive signal in the upper-right quadrant using FITC and PE filter (Figure 4.11-C, D). Further quadrants represent noninfected macrophages and non-internalized promastigotes. In detail, PKH26-stained non-infected macrophages (with high PE signal but low FITC signal) are plotted in upper-left, whereas PKH67-stained non internalized promastigotes (with low PE signal but high FITC signal) are plotted in lower-right on the cytograms. When the populations in the lower-left and upper-right quadrants in Figure 4.11-C and D were analysed together, a significant increase ($P \leq 0.0001$) in infection rate of macrophages could be observed in parallel to the increase in the initial treatment ratios. Accordingly, the macrophage populations became 15.68 and 61.70% infected by *L. infantum* parasites, when incubated with 1:2.5 and 1:10 macrophage: promastigote ratios, respectively.

Flow cytometry also suggested a higher degree granularity at infected macrophages parallel to the initial parasitic load (Figure 4.11-C, D). Moreover, when the macrophages are infected with higher initial parasite load, an increase is observed in SSC-signal intensity (Figure 4.11-D; SSC-FSC cytogram), which is attributed to the increase in parasite population inside the macrophages.

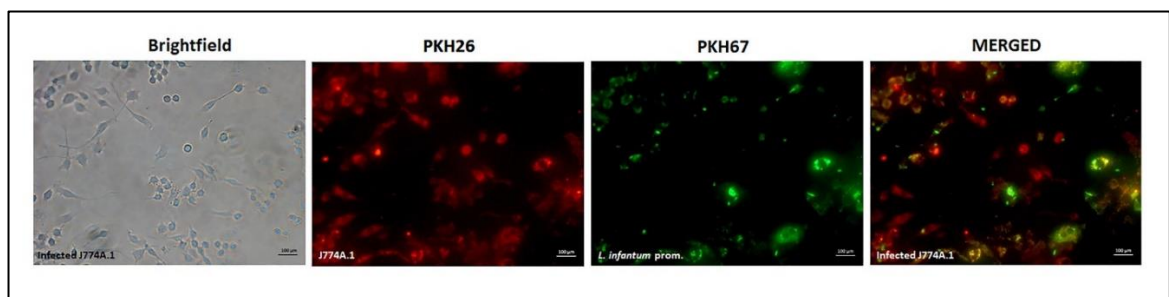


Figure 4.10. Fluorescence microscopy images of macrophages infected with *L. infantum* promastigotes (infection ratio, 1:10). From left to right: brightfield image, fluorescence image of PKH26-stained macrophages (red), PKH67-stained *L. infantum* promastigotes (green), and *Leishmania*-infected macrophages (merged – yellow) (magnification: 20×).

Scale bars correspond to 100 μm .

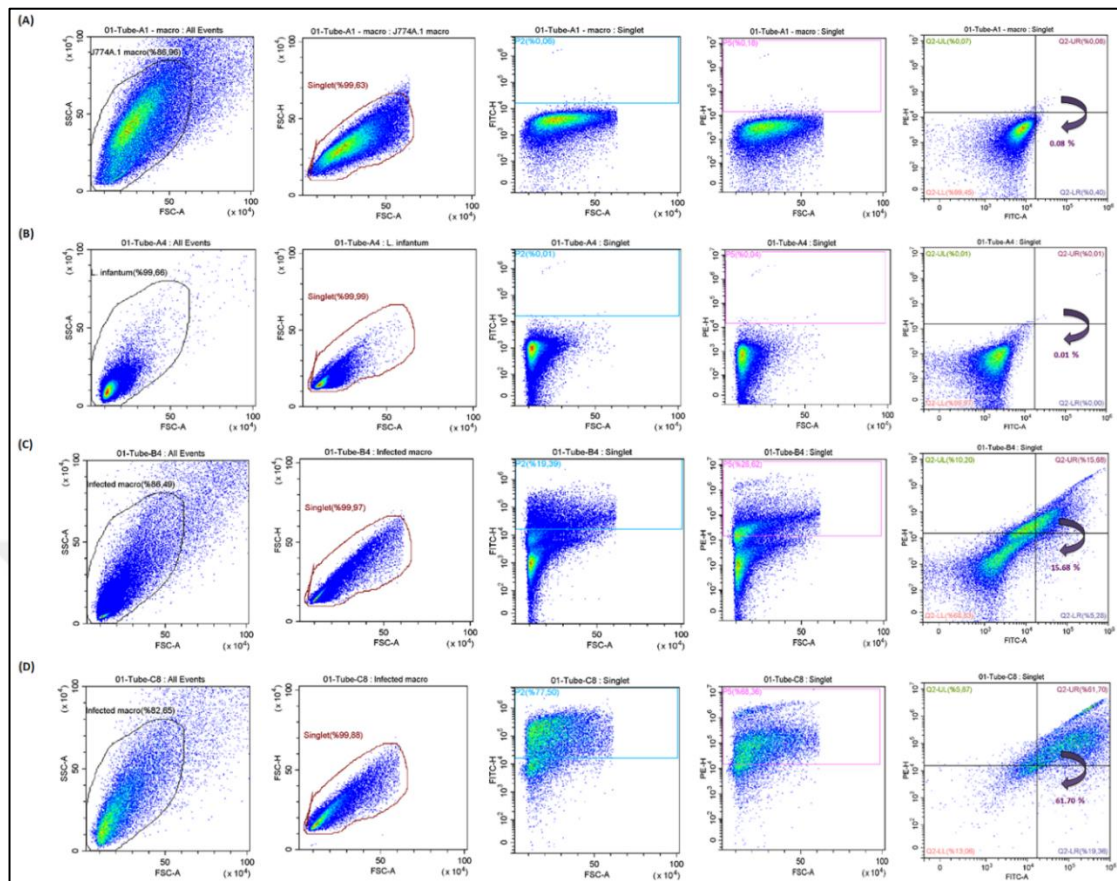


Figure 4.11. The flow cytometric (pseudo-color/smooth) plots of (A) unstained non-infected macrophages, (B) unstained promastigotes and (C&D) *Leishmania*-infected macrophages at 1:2.5 and 1:10 rates of infection, respectively.

4.4. EVALUATION OF THE ANTILEISHMANIAL EFFICACY OF SPT FOR NEUROINFLAMMATORY-BASED ANTILEISHMANIAL THERAPY

4.4.1. Antileishmanial Effect of SPT on Growth of *L. infantum* Promastigotes

L. infantum promastigotes were treated with SPT at concentrations ranging from 25 μ M to 200 μ M for 24, 48, and 72 h. As shown in Figure 4.12, concentrations of 75, 100, 125, 150, and 200 μ M SPT inhibited the proliferation of parasites in a time-dependent manner at 24, 48, and 72 h. When *L. infantum* was treated with SPT at concentrations ≤ 50 μ M, cell viability remained above 80% after 24, 48, and 72 h of incubation. When the concentration

was increased to 75 μM , the cell viability was reduced to below 80% ($P \leq 0.0001$) and 55% ($P \leq 0.0001$), at 24 and 48 h, respectively, which became nearly 35% and 22% when the concentration was increased to 100 (i.e., 38% and 30%), 125 (i.e., 33% and 23%), 150 (32% and 22%), and 200 μM (i.e., 31% and 22%) ($P \leq 0.0001$), respectively (Figure 4.12). Moreover, after 72 h-incubation, 75 μM SPT concentration significantly decreased the viability of *L. infantum* promastigotes to exactly 50% at 72 h ($P \leq 0.0001$) compared to negative control; whereas an increase in the SPT concentration to 100, 125, 150, and 200 μM led to a significant reduction on parasite viability sharp to around 20.0%, which was reduced to 19.3%, 21.5%, 17.7 and 16.9%, respectively ($P \leq 0.0001$). Interestingly, the increase in SPT concentrations above 100 μM did not cause any significant further change in the inhibition of promastigote proliferation (i.e., 20%).

IC_{50} values (24, 48, and 72 h) for SPT were calculated as $83.31 \pm 1.61 \mu\text{M}$, $67.11 \pm 6.52 \mu\text{M}$, and $71.99 \pm 7.51 \mu\text{M}$, respectively (Table 4.1 and Figure 4.13). The results revealed that with a lower IC_{50} value of SPT at 48 and 72 h-incubations are more effective against *L. infantum* promastigotes than 24 h-incubation. Furthermore, the increase in the incubation times of SPT from 24 to 48 h, seems to increase the antileishmanial efficacy of the compound, as the improvement in the IC_{50} value of SPT ($67.11 \pm 6.52 \mu\text{M}$) at a statistical significance ($p \leq 0.05$) (Table 4.1 and Figure 4.13). However, with the alteration in the incubation from 48 h to 72 h, the IC_{50} values were found statistically insignificant (ns), as $67.11 \pm 6.52 \mu\text{M}$ to $71.99 \pm 7.51 \mu\text{M}$, respectively.

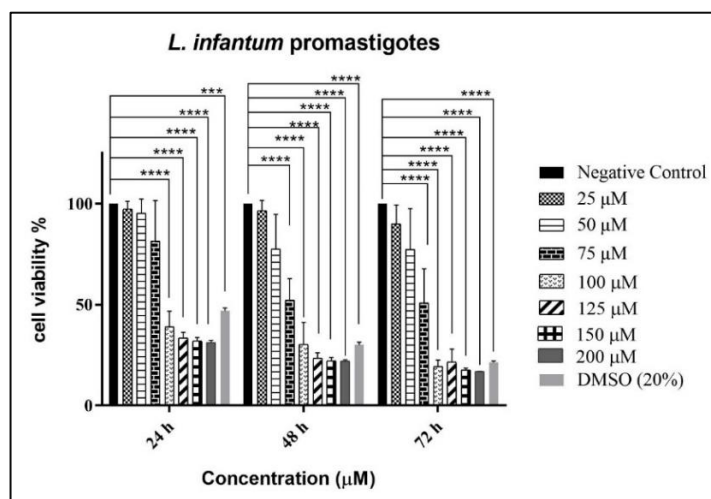


Figure 4.12. Effect of SPT on proliferation of *L. infantum* promastigotes. Viability of *L. infantum* parasites after 24, 48 and 72 h of treatment with SPT. Data represents mean (n=3) $\pm$ SD.

Table 4.1. The 50% inhibitory concentration (IC_{50}) of SPT on *L. infantum* promastigotes after 24, 48 and 72 h treatment.

	IC_{50} (μ M)*		
	24h	48h	72h
SPT	83.31 $\pm$ 1.61*	67.11 $\pm$ 6.52	71.99 $\pm$ 7.51

* Viability determined by Alamar Blue assay. The results were obtained after treatment of SPT at concentrations (25 - 200 μ M) after 24, 48, 72-h of incubation. Data represents mean (n=3) $\pm$ SD.

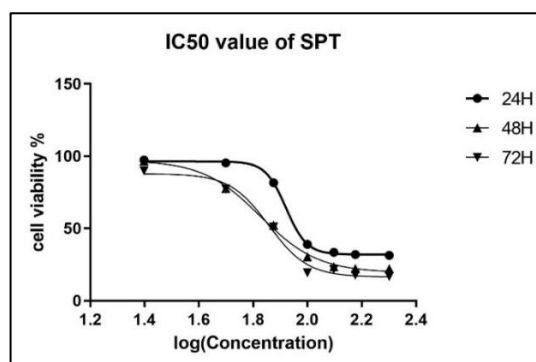


Figure 4.13. Cell viability versus logarithmic concentration data for estimation of IC_{50} values: SPT against *L. infantum* promastigotes for 24, 48, and 72 h.

4.4.2. Morphological Analysis of *L. infantum* Treated with SPT

In line with the parasite viability data, as shown in phase-contrast microscopy and SEM images in Figure 4.14 and 4.15, treatments with SPT indicated a dose-dependent reduction in the cell number, and transformation from elongated-shape to round-shaped morphologies, after 24 and 48 h of incubation. This is attributed to the loss of membrane and flagellum integrity of *L. infantum* promastigotes induced to apoptosis. Moreover, morphological changes such as spherical shape and shrinkage were strong signs of apoptosis [134, 135]. In addition, when the SPT concentration was increased to $\geq 75 \mu\text{M}$, an increase was observed in the number of parasites with lost integrity (Figure SEM and phase contrast). The increase in number of cells with lost membrane integrity was further attributed to the result of SPT exposure.

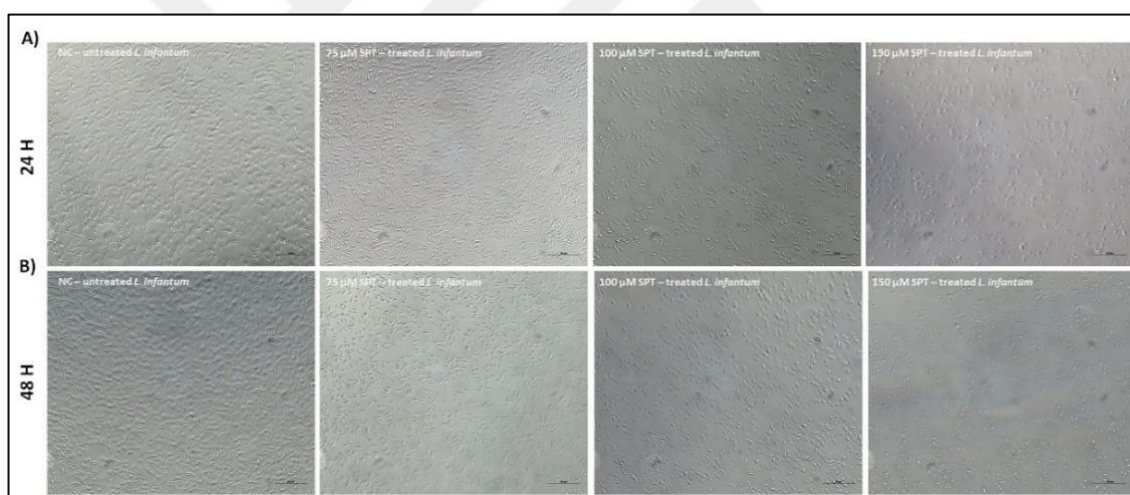


Figure 4.14. Phase-contrast microscopy images of negative control (untreated *L. infantum* prom.) and 75, 100, and 150 μM SPT treatment groups after (A) 24 h, and (B) 48 h. *L. infantum* promastigotes at negative control groups preserved their morphology throughout the study where parasites treated with 75, 100, and 150 μM SPT displayed round shape after 24 and 48 h exposure. Bars correspond to 50 μm .

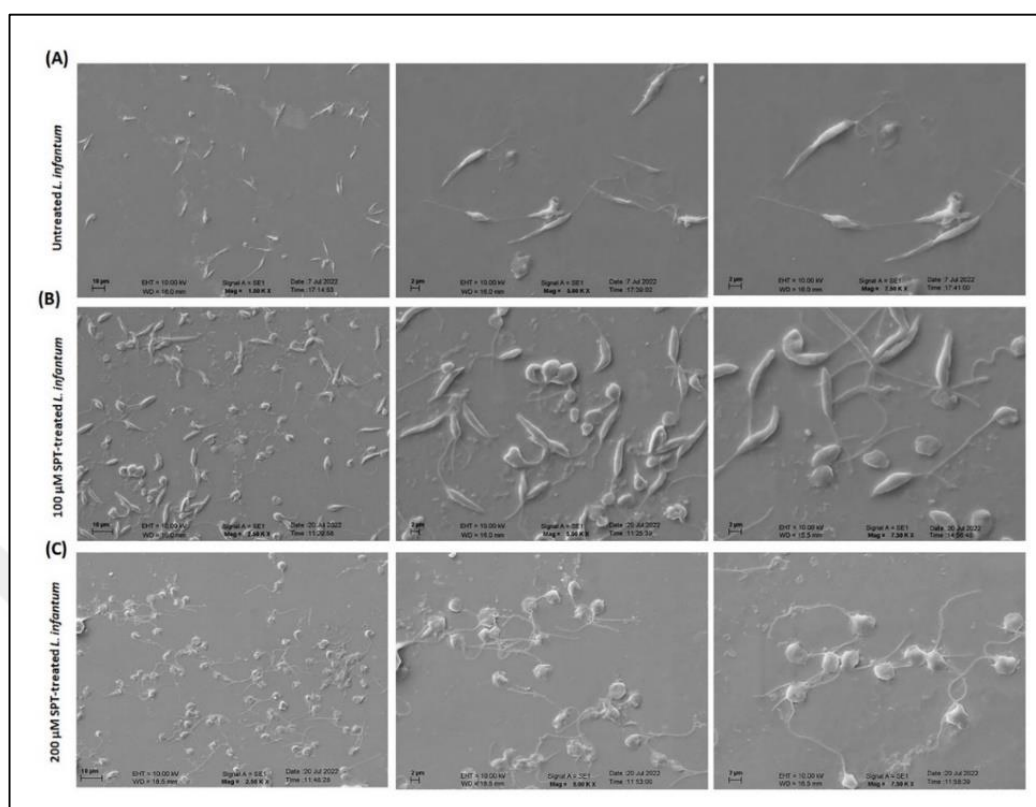


Figure 4.15. SEM images of *L. infantum* treated with 100, and 200 μ M SPT treatment groups, at 24 h-incubation with the magnification of (A) 2.5 K X, (B) 5.00 K X, and (C) 7.5 K X. Bars correspond to 50 μ m. *L. infantum* promastigotes at negative control groups preserved their morphology throughout the study where parasites treated with 100, and 200 μ M SPT showed spherical shape after 24 and 48 h exposure. Bars correspond to 50 μ m.

4.4.3. Mitochondrial Membrane Potential of *L. Infantum* Promastigotes After SPT Treatment

To determine the parasite mitochondrial membrane potential after 24 and 48 h of SPT treatment, fluorescent intensity of Rhodamin 123 was read-out spectrophotometrically by microplate reader, and FACS analysis (Figure 4.16 and 4.17). Thus, promastigotes were treated 24 and 48 h with SPT at concentrations ranging from 50 μ M to 400 μ M, and then incubated with Rhodamin 123 for 30 min on dark. The analysis showed that a time-dependent manner but not a dose-dependent decrease was observed in mitochondrial membrane potential (Figure 4.16 and 4.17). When *L. infantum* was treated with SPT at

concentrations $\leq 75 \mu\text{M}$, the percentage of mitochondrial membrane potential remained above 80% after 24 and 48 h of incubation; whereas $\geq 100 \mu\text{M}$ concentrations of SPT treatment led to a significant decrease in the mitochondrial membrane potential on *L. infantum* promastigotes ($p \leq 0.0001$), which was reduced dramatically to below 53% after 24 h and below 35% after 48 h compared to negative control ($P \leq 0.0001$) (Figure 4.16). As shown in Figure 4.16, 24 h of *L. infantum* promastigotes incubation with 100, 150, 200 and 400 μM SPT significantly decreased mitochondrial membrane potential (52.2, 39.1, 57.5 and 44.6%, respectively) compared to the non treated parasites (96.16%) (Figure 4.16). Moreover, after 48 h of 100, 150, 200 and 400 μM SPT incubation, similar profiles as those observed after 24 h treatment were shown in Figure 30, which was found as 32.8, 34.1, 41.5 and 30.1%.

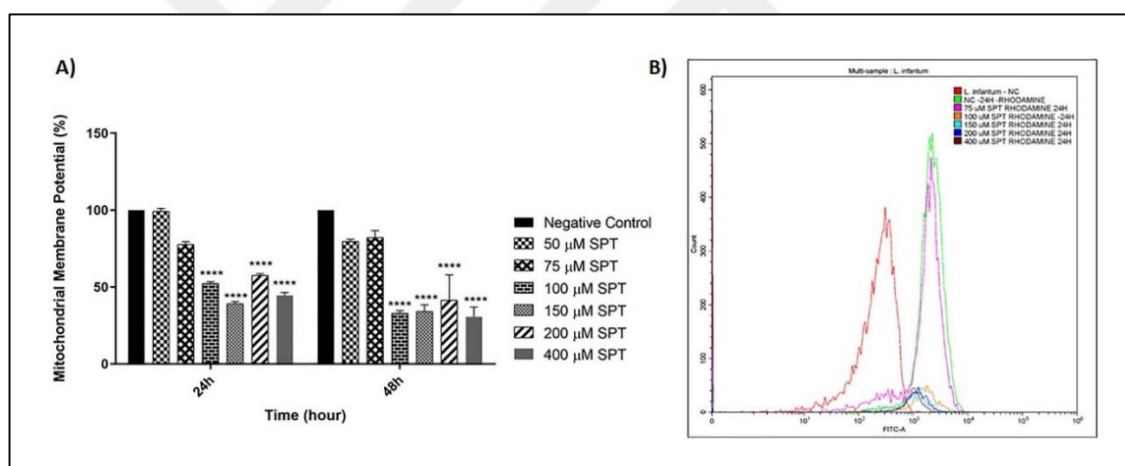


Figure 4.16. (A) Effects of SPT treatment on the mitochondrial membrane potential of *L. infantum* promastigotes at 24 and 48 h. Mitochondrial membrane potential was measured by Rhodamine 123 staining in *L. infantum* promastigotes exposed to 50 - 400 μM SPT for 24 and 48 h. (B) Representative overlay histogram analysis of SPT-treated *L. infantum* promastigotes at 24 h. Data represents mean ($n=3$) $\pm$ SD.

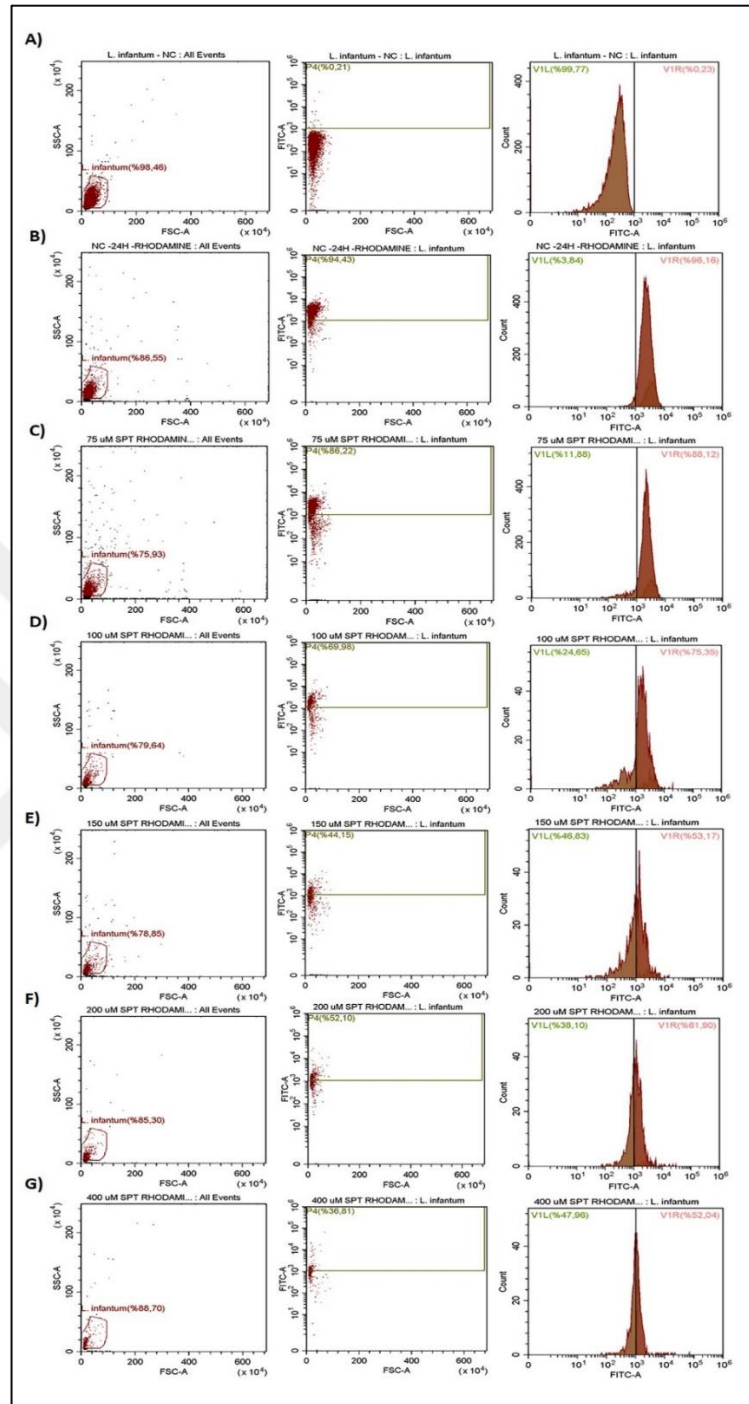


Figure 4.17. Representative histogram images of mitochondrial membrane potential of *L. infantum* promastigotes treated with different SPT concentrations (0-400 μ M) at 24 h. Data represents mean (n=3) $\pm$ SD.

4.4.4. DAPI Staining

DAPI was also used to label the nucleus and kinetoplast DNA to observe the change in cell viability and apoptosis after SPT treatment. As shown in Figure 4.18, after 24 and 48 h of incubation with 75, 100 and 150 μM of SPT, a decrease in the number of parasite nuclei was observed with loss of DAPI fluorescence intensity according to fluorescence microscopy images. These results reinforce the notion that SPT treatment led to a significant reduction in the parasite viability upon 24 h and induce the alteration of mitochondrial membrane potential resulting in apoptosis-like death of promastigotes.

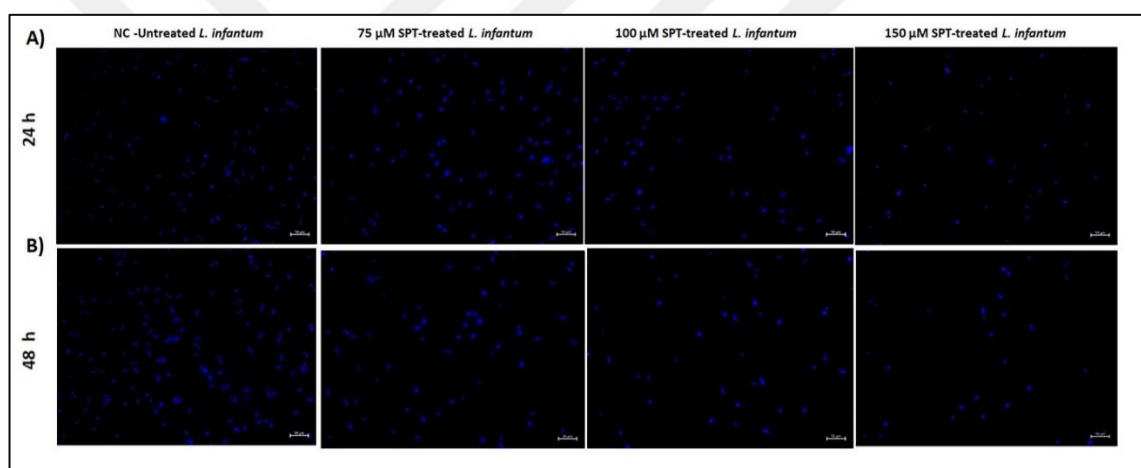


Figure 4.18. DAPI fluorescence images show the nucleic changes and numbers of parasites incubated in SPT at 75, 100 and 150 μM SPT for (A) 24 and (B) 48 h. *L. infantum* promastigotes were stained with the DNA-specific fluorochrome DAPI (blue). *L. infantum* promastigotes at negative control groups conserved their morphology and DAPI nuclei staining throughout the study; whereas parasites treated with 100, and 200 μM SPT lost their DAPI-fluorescent intensity after 24 and 48 h of exposure.

4.4.5. Cell Cycle Analysis of *L. infantum* Promastigotes After SPT Treatment

Flow cytometry with PI staining was performed to examine the effect of SPT on cell cycle progression in *L. infantum* promastigotes. The proportion of cell population in S and G2/M

phase was found to vary dose-dependent (0-100 μ M) on SPT treatment, indicating a cell cycle arrest induced in S and G2/M phase (Figure 4.19 and 4.20).

50 μ M SPT-treated *L. infantum* had $72.43 \pm 5\%$ of cell population in G0/G1, $13.52 \pm 2\%$ of cell population in S and $14.72 \pm 5\%$ of cells in G2/M phases at 24 h (vs the control group: $87.62 \pm 5.14\%$ of cells in G0/G1, $2.38 \pm 1.26\%$ of cells in S and $10.27 \pm 5.73\%$ of cells in G2/M phases) (Figure 4.19-A, and 4.20-A). Accordingly, 50 μ M SPT treatment significantly decreased the percentage of cells in the G0/G1 ($p \leq 0.001$), and significantly increased the percentage of cells in the S phase at 24 h of incubation ($p \leq 0.0001$). On the other hand, the cell populations in G2/M phases were not significantly increased after 24 h-treatment of 50 μ M SPT. When the SPT concentration was increased to 75 μ M, the frequency of G0/G1, S and G2/M cell populations were found as $43.33 \pm 5.02\%$, $26.69 \pm 2.30\%$, and $30.62 \pm 6.06\%$ after 24 h (vs the control group: $87.62 \pm 5.14\%$ of cells in G0/G1, $2.38 \pm 1.26\%$ of cells in S and $10.27 \pm 5.73\%$ of cells in G2/M phases) (Figure 4.19-A, and 4.20-A). *L. infantum* cell population in G0/G1 phase were decreased when treated with 75 μ M SPT after 24 h as compared with that in the control cells, whereas cell population were significantly increased in both S and G2/M phases after 24 h of incubation ($p \leq 0.0001$). On the other hand, 100 μ M SPT-treated *L. infantum* had $35.71 \pm 2.00\%$ of cell population in G0/G1, $27.66 \pm 1.00\%$ of cell population in S and $37.19 \pm 3.00\%$ of cells in G2/M phases at 24 h (vs the control group: $87.62 \pm 5.14\%$ of cells in G0/G1, $2.38 \pm 1.26\%$ of cells in S and $10.27 \pm 5.73\%$ of cells in G2/M phases). Parallel to 75 μ M SPT, 100 μ M SPT treatment significantly decreased the percentage of cells in the G0/G1 ($p \leq 0.001$), and significantly increased the percentage of cells in both S and G2/M phases after 24 h ($p \leq 0.0001$) (Figure 4.19-A, and 4.20-A).

When the cell cycle frequencies at 48 h of 50, 75 and 100 μ M SPT-treatments were analyzed, the cell populations were not significantly changed in G0/G1 phase, and found as $74.02 \pm 5.00\%$, $59.71 \pm 5.02\%$, and $65.18 \pm 2.00\%$ (Figure 4.19-B, and 4.20-B). The cell population in S phase significantly increased after 75 and 100 μ M SPT-treatments at 48 h ($p \leq 0.0001$), whereas the cells in G2/M phase significantly decreased after 48 h of 75 and 100 μ M SPT-treatments ($p \leq 0.001$), in which the cells in S phase were $33.60 \pm 1.31\%$ and $28.22 \pm 1.00\%$, and in G2/M phases were $7.34 \pm 6.06\%$ and $7.24 \pm 3.00\%$, respectively.

According to these results, SPT induced the accumulation of cells at S and G2/M phases after 24 h of treatment, whereas 48 h of SPT treatment led to increase S phase cell cycle arrest on *L. infantum* promastigotes.

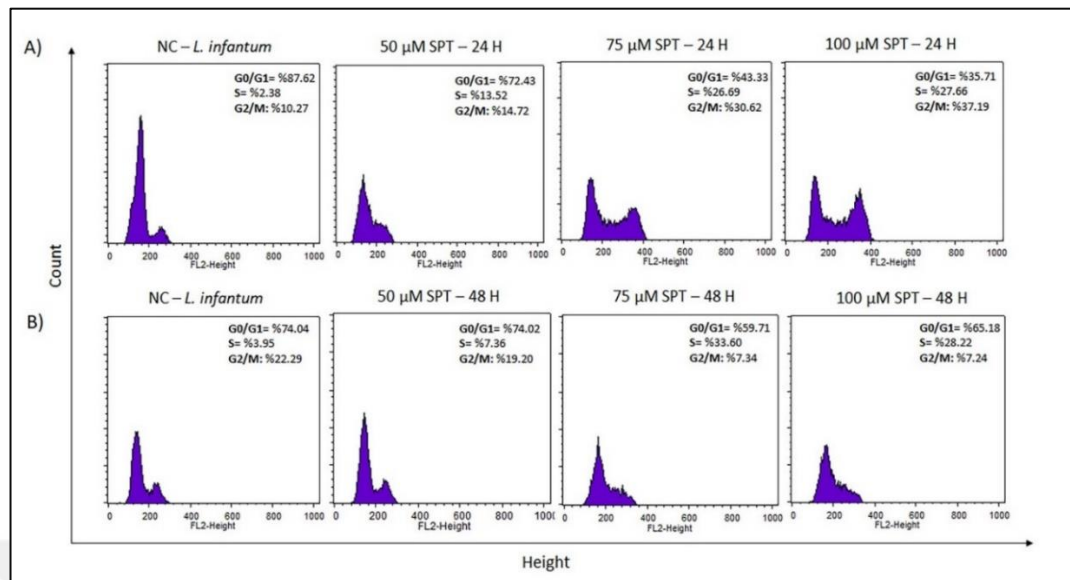


Figure 4.19. Cell cycle histogram analysis of *L. infantum* promastigotes after (A) 24 and (B) 48 h incubation of SPT at 50, 75 and, 100 μ M. Data represents mean ($n=3$) $\pm$ SD.

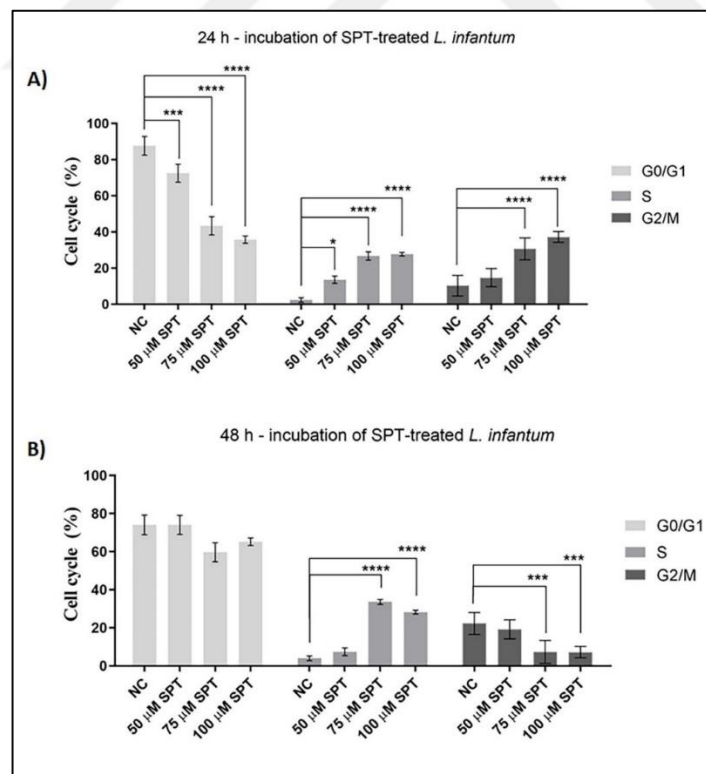


Figure 4.20. Cell cycle analysis of *L. infantum* promastigotes upon treatment with SPT at different concentrations (50-100 μ M). *L. infantum* were treated with 50, 75 and 100 μ M SPT for (A) 24 h, and (B) 48 h. Data represents mean ($n=3$) $\pm$ SD.

4.4.6. Gene Expression Level of *L. infantum* promastigotes After SPT Treatment

Upon treatment of *L. infantum* promastigotes with 50, 75 and 100 μM of SPT, the relative Meta1, SHERP, and MCA5 /18S rRNA gene expressions of parasites were analyzed using Real-Time PCR for 24 h (Figure 4.21).

As shown in Figure 4.21-A, qPCR assay results indicated that Meta 1 gene (in infective metacyclic promastigote forms) levels significantly increased in 50 μM SPT (≈ 1.5 -fold) ($p \leq 0.05$), whereas on 75 μM SPT-treated parasites, gene expression significantly decreased (≈ 0.50 -fold) ($p \leq 0.05$) when compared to negative control group. On the other hand, 100 μM SPT treatment did not significantly change Meta 1 gene expression level on *L. infantum* promastigotes.

In Figure 4.21-B, SHERP metacyclogenesis gene expression level increased as approximately 1.9-fold on 50 μM SPT-treated promastigotes ($p \leq 0.01$), whereas 75 and 100 μM SPT treatment did not lead to any significant change on promastigotes.

MCA5 (apoptotic-like death gene) level decreased as approximately 0.15 fold in 50 μM SPT treated-*L. infantum* promastigotes ($p \leq 0.01$), while 100 μM SPT treatment led to a significant increase in MCA5 gene expression on promastigotes ($p \leq 0.05$). Instead, 75 μM SPT treatment did not significantly differ MCA5 mRNA levels (Figure 4.21-C). Observed MCA5 expression is consistent with mitochondrial membrane potential data (Figure 4.21-C) indicating incidence of higher cell death when 100 μM SPT treatment.

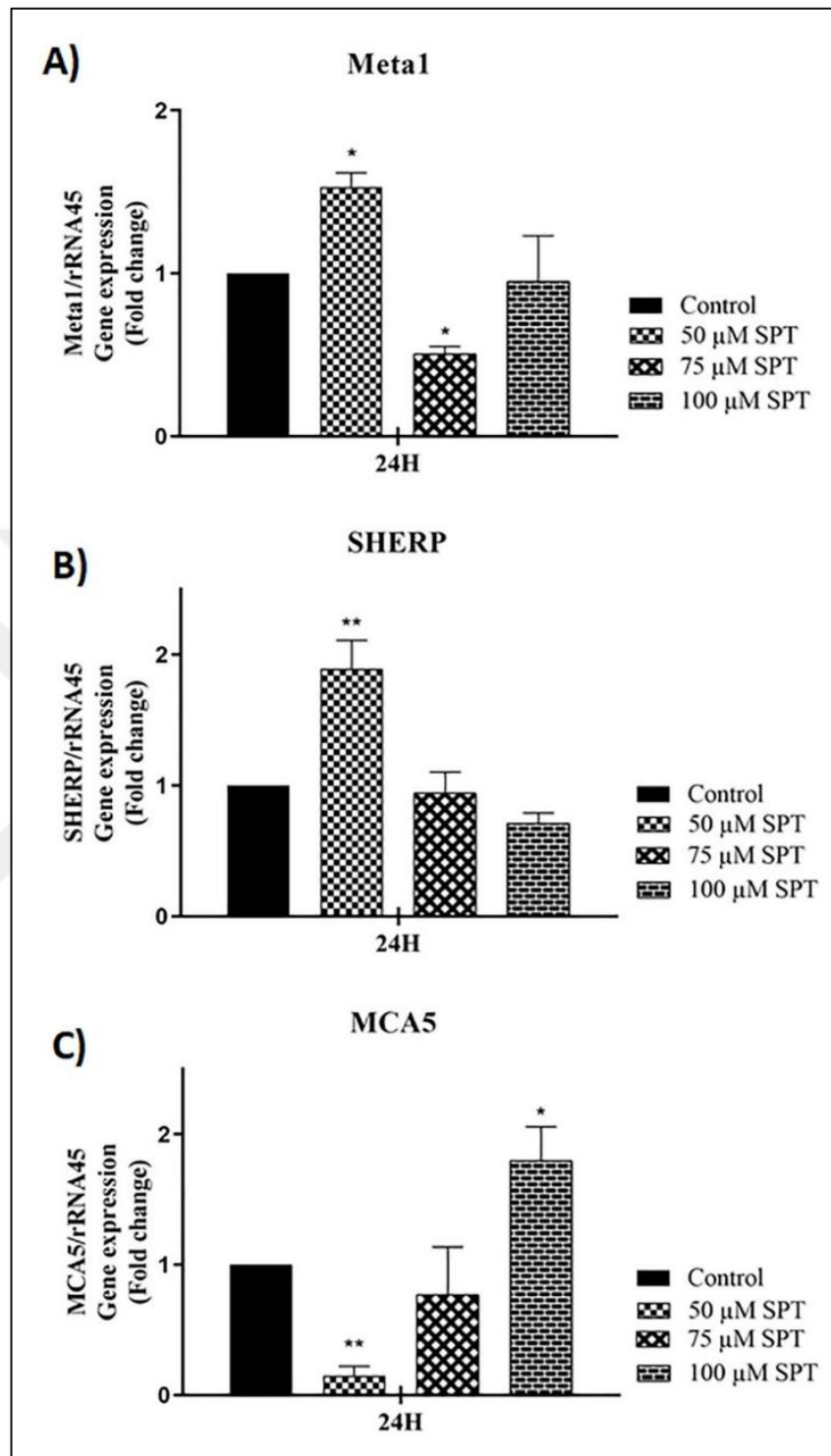


Figure 4.21. qPCR analysis of *L. infantum* promastigotes upon treatment with SPT at different concentrations (50-100 μ M). *L. infantum* were treated with 50, 75 and 100 μ M SPT for (A) 24 h, and (B) 48 h. Data represents mean ($n=3$) $\pm$ SD.

4.4.7. Cytotoxicity of SPT on Macrophages

Following the *in vitro* evaluation of the antileishmanial effect of SPT on *L. infantum* promastigotes, cytotoxicity of the SPT was evaluated on non-infected murine RAW264.7 macrophages using MTS cell viability assay (Figure 4.22-A).

As shown in Figure 4.22-A, when treated with SPT at concentrations $\geq 75 \mu\text{M}$, the cell viability of non-infected RAW264.7 macrophages remained above 90% for 24, 48, and 72 h. When the concentration was decreased to $50 \mu\text{M}$, the cell viability remained above 90% for 24 h (i.e., 99.6%) and 48 h (89.2%); which became exactly 75% after 72 h compared to the negative control group. Interestingly, $\leq 50 \mu\text{M}$ SPT concentrations led to a reduction in the macrophage cell viability, as the data revealed that with $25 \mu\text{M}$ SPT treatment, the cell viability was reduced dramatically to 43% after 24 h and below 64% after 72 h compared to the negative control group ($p \leq 0.001$) (Figure 4.22-A). On the other hand, the increase in the SPT concentration above $100 \mu\text{M}$ did not show any toxicity on non-infected macrophages, revealing that SPT concentrations of 100, 75, 100, 125, and $150 \mu\text{M}$ have safety features for antileishmanial therapy on healthy macrophages.

4.4.8. Cytotoxicity of SPT on Astrocytes

Following the *in vitro* evaluation of the antileishmanial effect and cytotoxicity of SPT on *L. infantum* promastigotes and RAW264.7 macrophages, cytotoxicity of the SPT was evaluated on non-infected murine C8-S astrocytes using MTS cell viability assay (Figure 4.22-B).

As shown in Figure 4.22-B, in SPT treatment at concentrations $\geq 25 \mu\text{M}$, the cell viability of non-infected C8-S astrocytes remained above approximately 90% for 24, 48, and 72 h. Even when the concentration was increased to $200 \mu\text{M}$, the cell viability remain above 100 % for 24, 48, and 72 h; which became exactly 100% in treated with $100 \mu\text{M}$ SPT after 72 h compared to the negative control group.

The obtained results revealed that SPT treatment has significant efficacy on *L. infantum* parasites by reducing cellular toxicity on macrophages and astrocytes. Among effective concentrations on *L. infantum* promastigotes, SPT indicated the highest potential to inhibit

parasite proliferation within the range of 75–200 μM , without displaying any toxicity on macrophages and astrocytes (Figure 4.22-B).

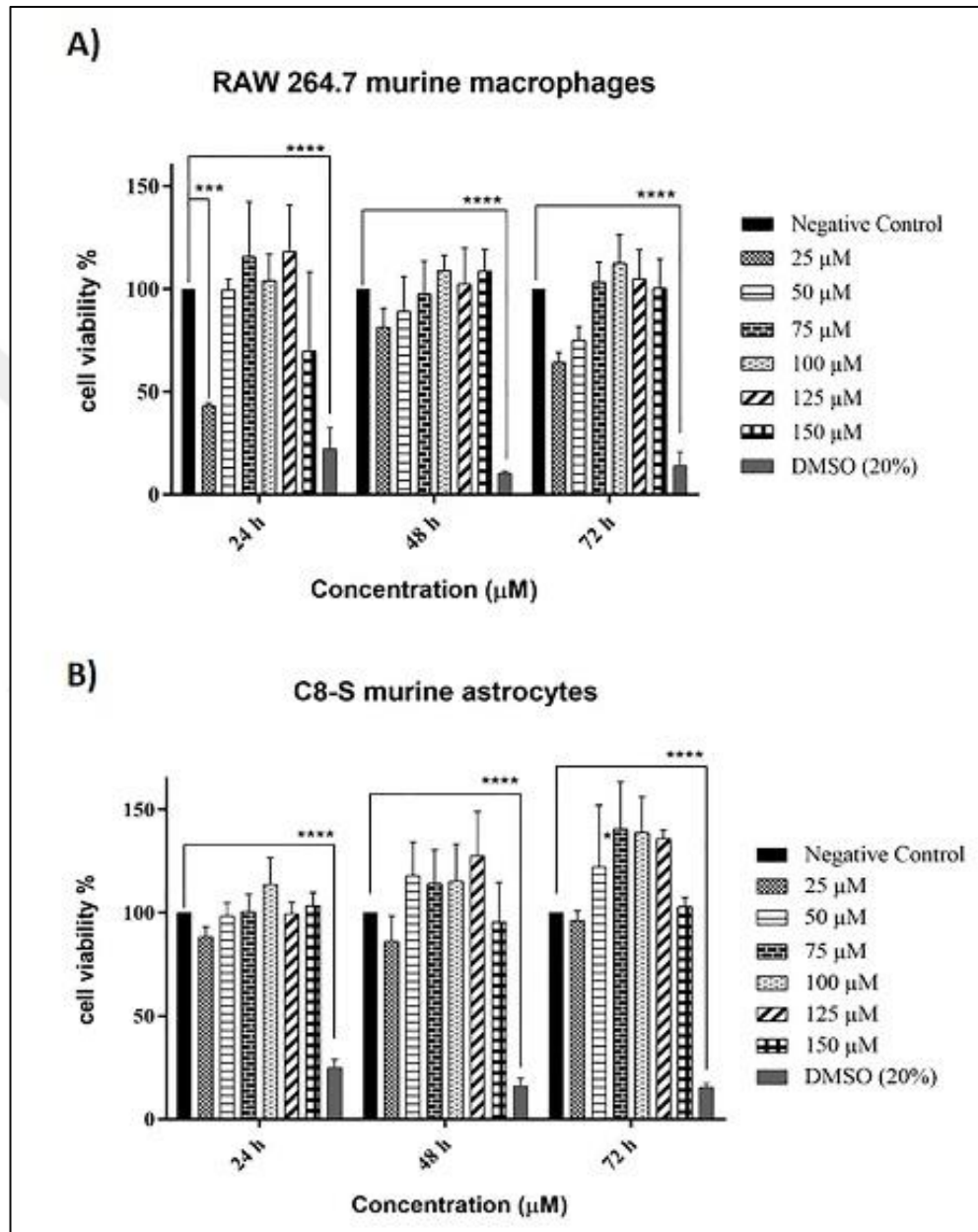


Figure 4.22. Cytotoxicity of SPT on macrophages and astrocytes at 24, 48, and 72 h of incubation. Cell proliferation analysis of (A) RAW264.7 macrophages and (B) C8-S astrocytes treated SPT were investigated at various concentrations (25–150 μM) for 24, 48, and 72 h compared to untreated negative control cells. Data represent mean ($n=3$) $\pm$ SD.

4.4.9. Antileishmanial Effect of SPT on Macrophages and Astrocytes Infected by *L. infantum* Promastigotes

4.4.9.1. Determination of Percentage of the Stained-Parasites, -Macrophages and -Astrocytes with PKH membrane dyes

Prior to the infection of macrophages and astrocytes with promastigotes, the parasites were stained with PKH67 to allow tracing by flow cytometry after the macrophage and astrocyte uptake.

RAW264.7 murine macrophages/C8-S astrocytes and *L. infantum* parasites were stained with PKH26 (red) and PKH67 (green) dyes, respectively, according to the procedure described at [131]. As illustrated in Figure 4.23-A,B, when unstained and stained parasites were analysed in flow cytometry with this procedure, 99.89% and 91.78% of parasites were determined as unstained and stained, respectively where a negligible number of the cells remain unstained and no significant difference were found regarding to the applied procedure.

The unstained macrophage population was detected as 99.95% by flow cytometry, while histograms of PKH26-stained macrophages corresponded to 94.21% of the population labelled successfully (Figure 4.24-A,B). On the other hand, the unstained astrocyte population was detected as 99.97% by flow cytometry, while histograms of PKH26-stained macrophages corresponded to 99.67% of the population labelled successfully (Figure 4.25-A,B). These findings indicate that a negligible number of the cells remain unstained subsequent to the applied staining protocol.

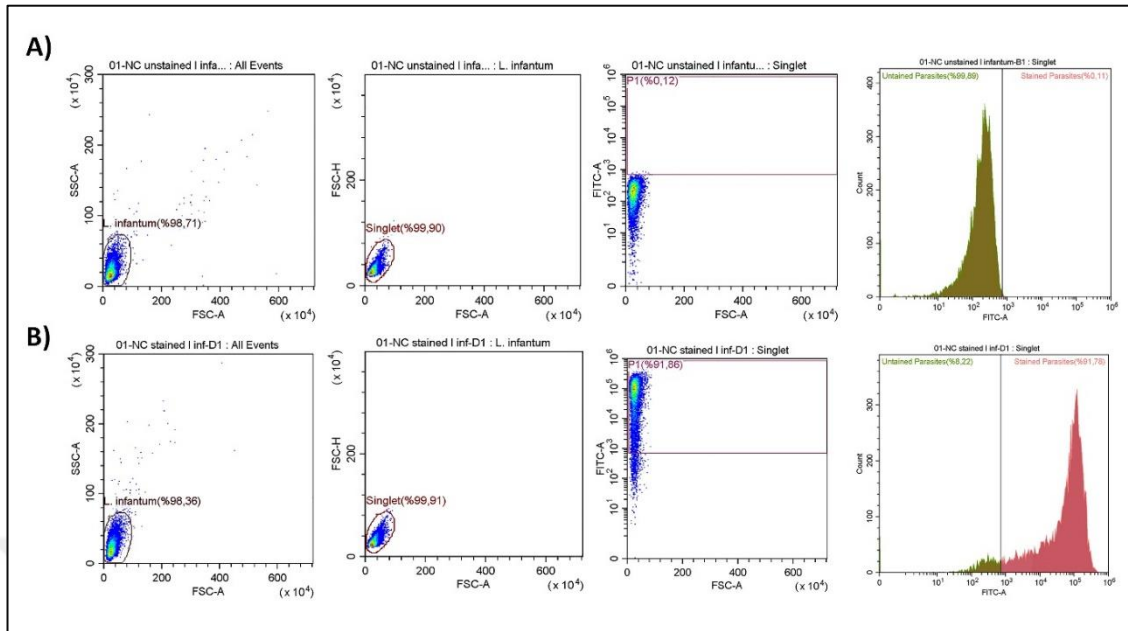


Figure 4.23. Flow cytometry analysis of unstained and PKH67-stained *L. infantum* promastigotes. Flow cytometric (pseudo-colour/smooth) plots for (A) unstained and (B) PKH67-stained *L. infantum* promastigotes.

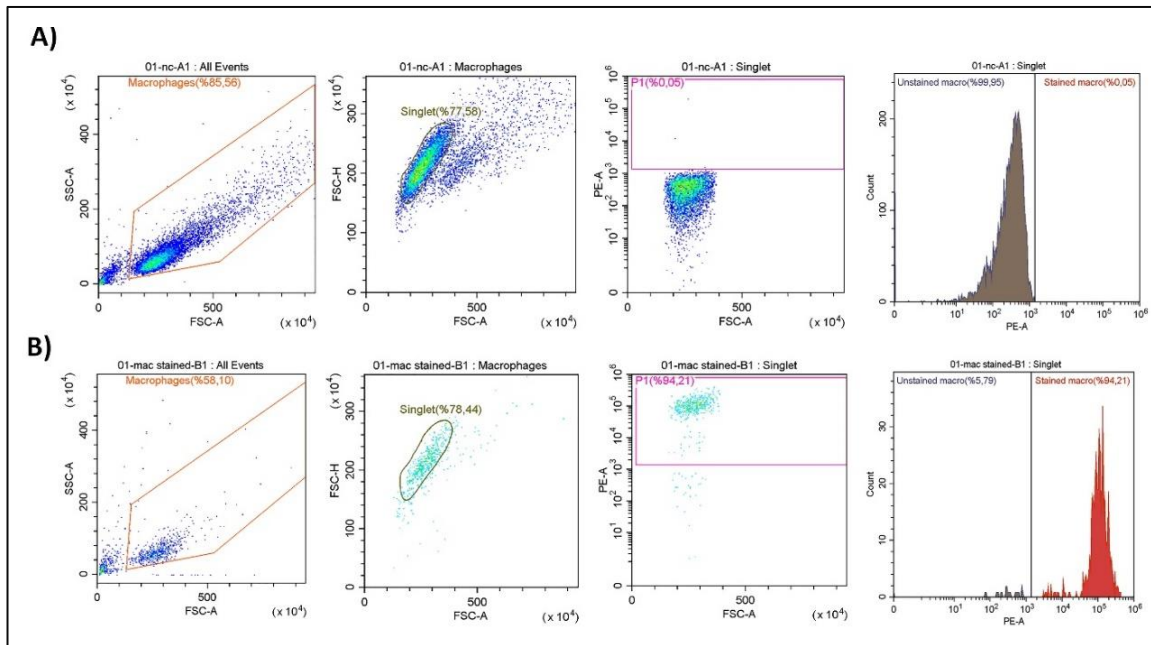


Figure 4.24. Flow cytometry analysis of unstained and PKH26-stained RAW264.7 macrophages. Flow cytometric (pseudo-colour/smooth) plots for (A) unstained and (B) PKH26-stained RAW264.7 macrophages.

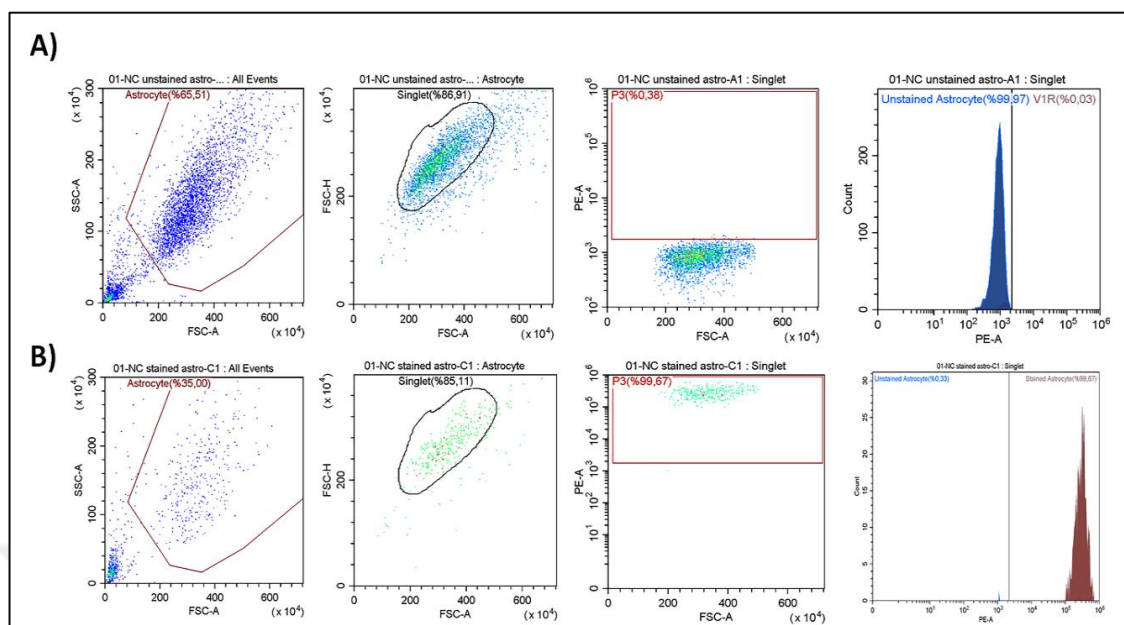


Figure 4.25. Flow cytometry analysis of unstained and PKH26-stained C8-S Astrocytes. Flow cytometric (pseudo-colour/smooth) plots for (A) unstained and (B) PKH26-stained C8-S Astrocytes.

4.4.9.2. Determination of Antileishmanial Effect of SPT on Macrophages- and Astrocytes- Infected with *L. infantum* Promastigotes

Flow cytometry analysis enabled the quantitative determination of the infection rates of macrophages and astrocytes.

Antileishmanial activity of SPT were studied on *Leishmania*-infected macrophages for concentrations of 50, 75, 100, 150, 200, and 400 μM (Figure 4.26-A). As flow cytometry data revealed that when macrophages were incubated with *L. infantum* promastigotes, 73.67 % of the macrophages become infected (Figure 4.26-A, 4.27). The treatment of *Leishmania*-infected macrophages with $\geq 50 \mu\text{M}$ SPT (i.e. 50, 75, 100, 150, 200, and 400 μM) for 24, 48, and 72 h did not significantly alter the infection rate regardless of time and concentration, when compared to NC treatment groups, and determined above 74 % (Figure 4.26-4.29). Although treatment of infected macrophages with SPT did not lead to a concentration- and time-dependent decrease in macrophage infection rate, the infection rates remain almost stable (nearly 74%) regardless time 24, 48 and 72 h-treatment.

On the other hand, antileishmanial activity of SPT were investigated on *Leishmania*-infected astrocytes for concentrations of 50, 75, 100, 150, 200, and 400 μ M (Figure 4.26 and 4.30-4.32). As illustrated in Figure 4.26-B and 4.30, when astrocytes were incubated with *L. infantum* promastigotes, the infection rate of astrocytes were found as approximately 23 %, which significantly decreased to $20 \pm 3.33\%$ ($p \leq 0.05$), $17 \pm 2.86\%$ ($p \leq 0.0001$), $11.89 \pm 3.56\%$ ($p \leq 0.0001$), $7.97 \pm 1.51\%$ ($p \leq 0.0001$), and $12.63 \pm 1.78\%$ ($p \leq 0.0001$), after treatment with 75, 100, 150, 200, and 400 μ M SPT at 24 h, respectively (Figure 4.26-B and Figure 4.30). Interestingly, after 48 h of incubation, the infection rate of untreated NC group indicated a decrease in the parasite load (i.e. 2.91%), which remained almost stable (nearly 3%) regardless of further increase in SPT concentration (Figure 4.26-B and Figure 4.31); whereas after 72 h-incubation, treatment of *Leishmania*-infected astrocytes with 75 μ M SPT resulted in a significant reduction in the viability of the cells sharp to approximately 4% (i.e., 3.75%) ($P \leq 0.0001$), when compared to the infection rate of NC group (i.e. 13%) (Figure 4.26 and 4.32); which became close to 7% in 100, 150, 200 and 400 μ M SPT - treated groups at 72 h-treatment (Figure 4.32).

Overall, the results obtained in cell culture analysis demonstrated that SPT led to an inhibition on the growth of *L. infantum* parasites and cell division inside the macrophages. Moreover, even at high doses, ≥ 75 μ M SPT had a significant anti-leishmanial effect on *Leishmania*-infected macrophages and *Leishmania*-infected astrocytes without displaying any toxicity on healthy macrophages and astrocytes ($P \leq 0.0001$).

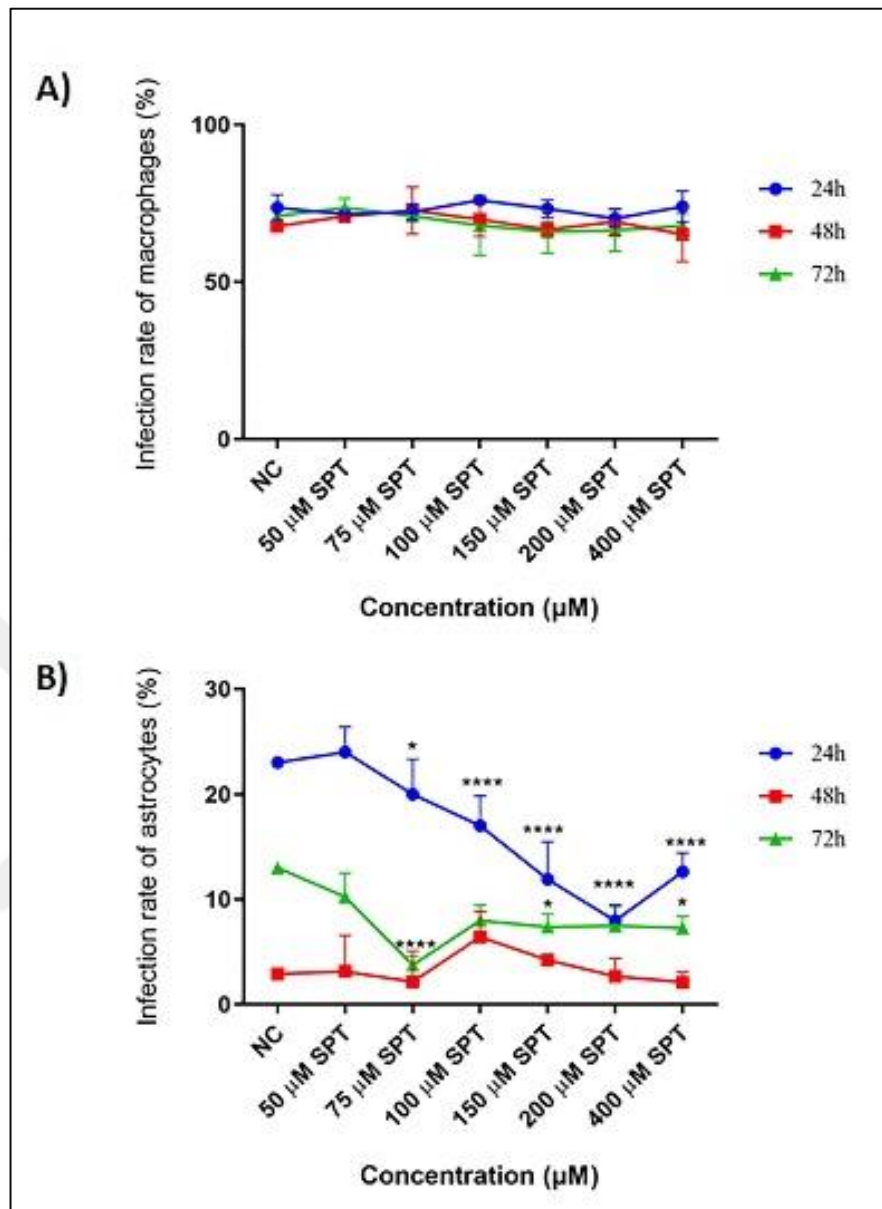


Figure 4.26. Infection rates (%) of (A) macrophages and (B) astrocytes infected with *L. infantum* promastigotes upon treatment with SPT at various concentrations (50 - 400 μM) for 24, 48, and 72 h compared to untreated negative control (NC) cells, as determined by flow cytometry. Data represents mean ($n = 3$) $\pm$ SD.

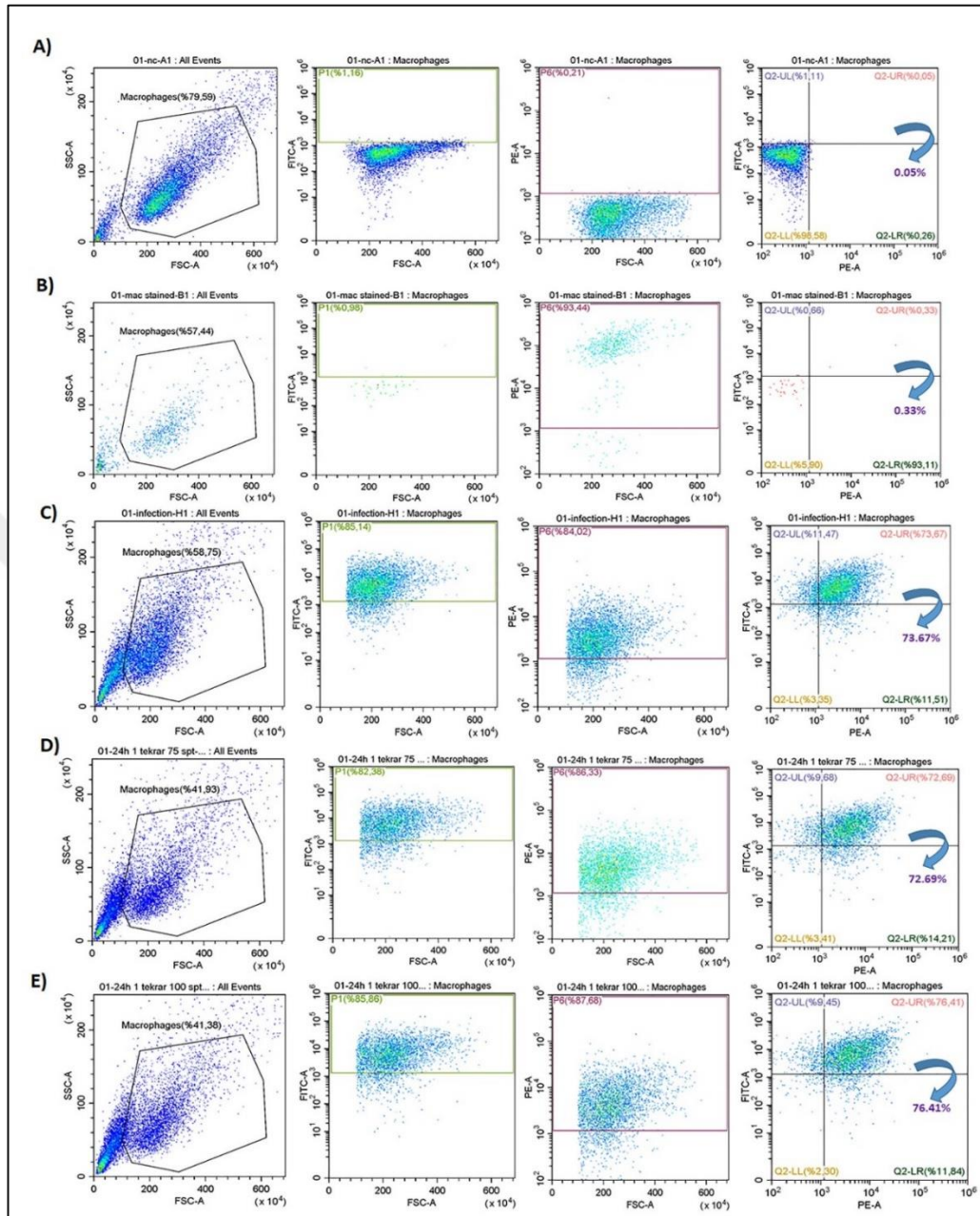


Figure 4.27. (A, B) Percentage of unstained and stained achieved by PKH26 staining on macrophages, as determined by flow cytometry. (C, D, E) Infection rates (%) of macrophages with *L. infantum* promastigotes upon treatment with 75 μ M SPT (D) and 100 μ M SPT (E), as determined by dot-plot pseudo flow cytometry for 24h compared to untreated negative control cells (C). Data represents mean ($n=3$) $\pm$ SD.

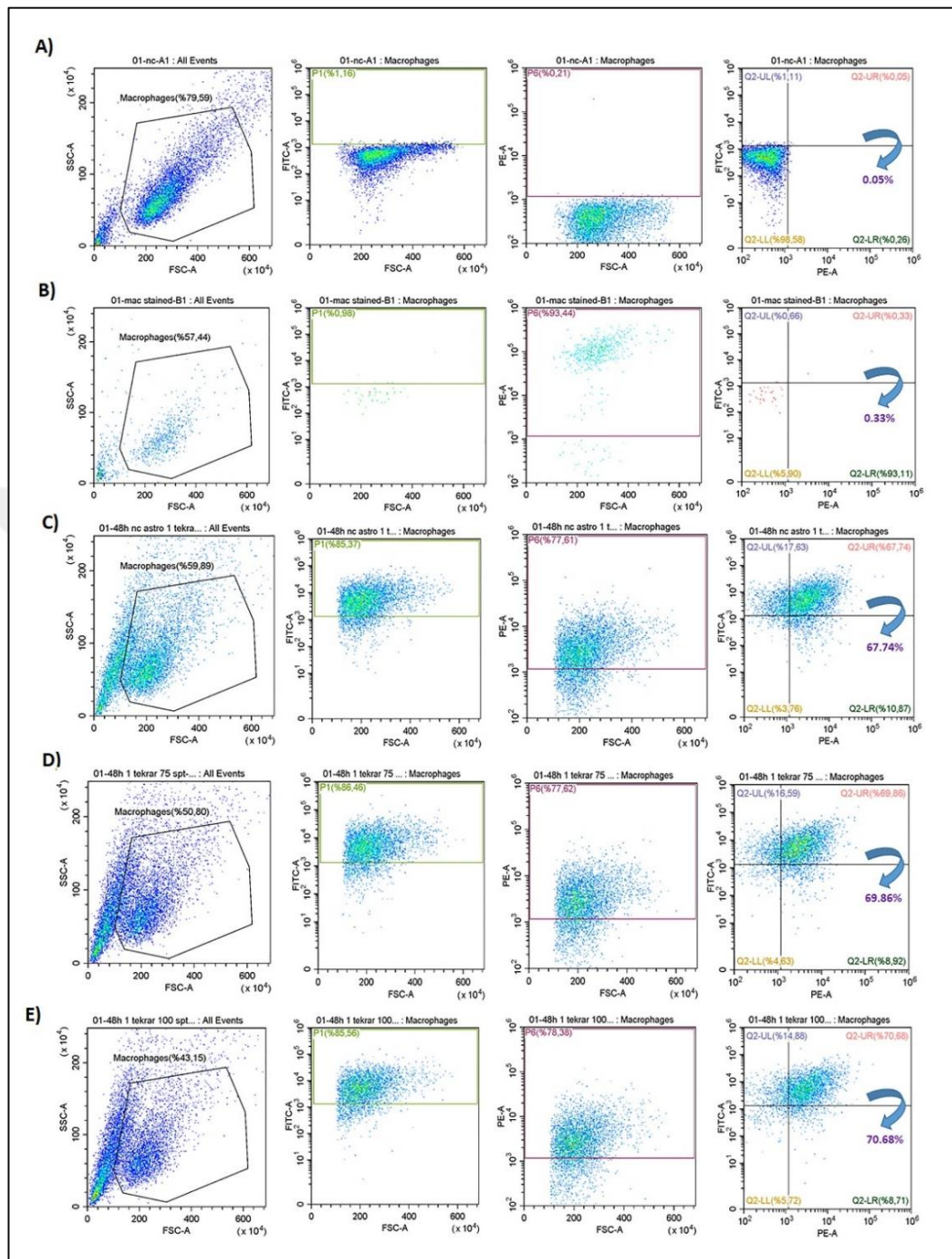


Figure 4.28. (A, B) Percentage of unstained and stained achieved by PKH26 staining on macrophages, as determined by flow cytometry. (C, D, E) Infection rates (%) of macrophages with *L. infantum* promastigotes upon treatment with 75 μ M (D) and 100 μ M SPT (E), as determined by dot-plot pseudo flow cytometry for 48h compared to untreated negative control cells (C). Data represents mean (n=3) $\pm$ SD.

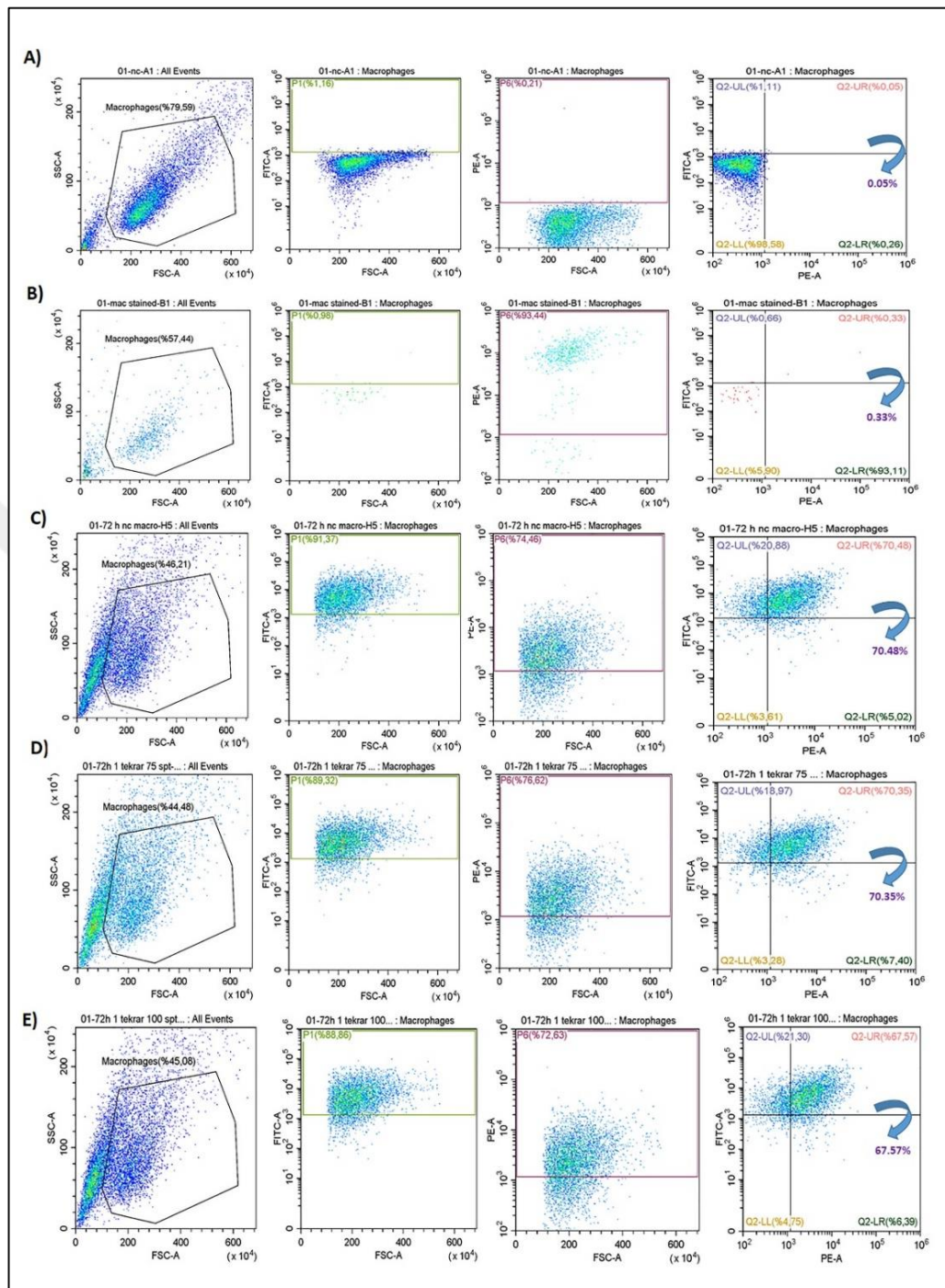


Figure 4.29. (A, B) Percentage of unstained and stained achieved by PKH26 staining on macrophages, as determined by flow cytometry. (C, D, E) Infection rates (%) of macrophages with *L. infantum* promastigotes upon treatment with 75 μ M (D) and 100 μ M SPT (E), as determined by dot-plot pseudo flow cytometry for 72h compared to untreated negative control cells (C). Data represents mean ($n=3$) $\pm$ SD.

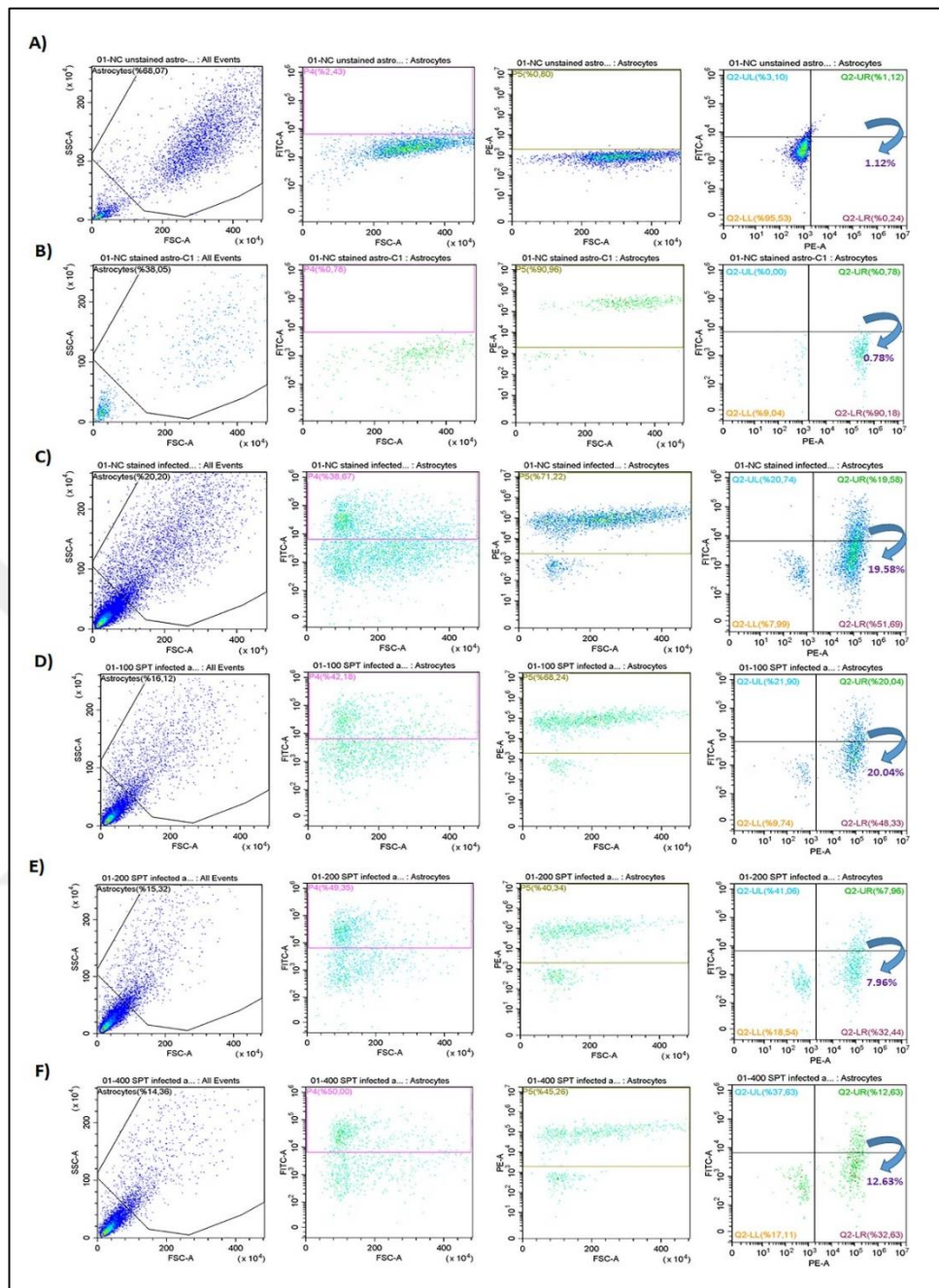


Figure 4.30. (A, B) Percentage of unstained and stained achieved by PKH26 staining on astrocytes, as determined by flow cytometry. (C, D, E) Infection rates (%) of astrocytes with *L. infantum* promastigotes upon treatment with (D)100 μ M, (E) 200 μ M, and (F) 400 μ M SPT, as determined by dot-plot pseudo flow cytometry for 24h compared to untreated negative control cells (C). Data represents mean ($n=3$) $\pm$ SD.

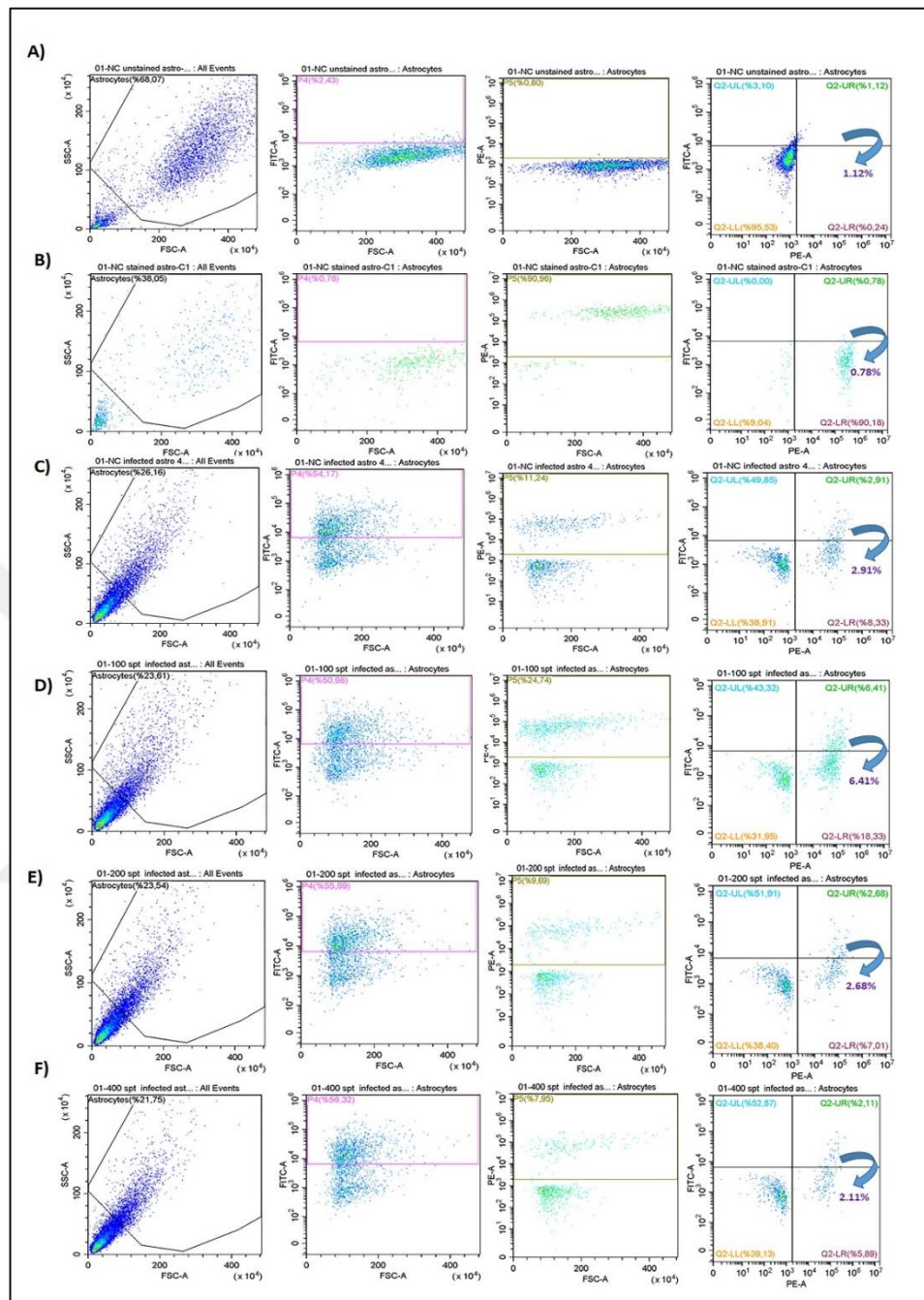


Figure 4.31. (A, B) Percentage of unstained and stained achieved by PKH26 staining on astrocytes, as determined by flow cytometry. (C, D, E) Infection rates (%) of astrocytes with *L. infantum* promastigotes upon treatment with (D) 100 μ M, (E) 200 μ M, and (F) 400 μ M SPT, as determined by dot-plot pseudo flow cytometry for 48h compared to untreated negative control cells (C). Data represents mean ($n=3$) $\pm$ SD.

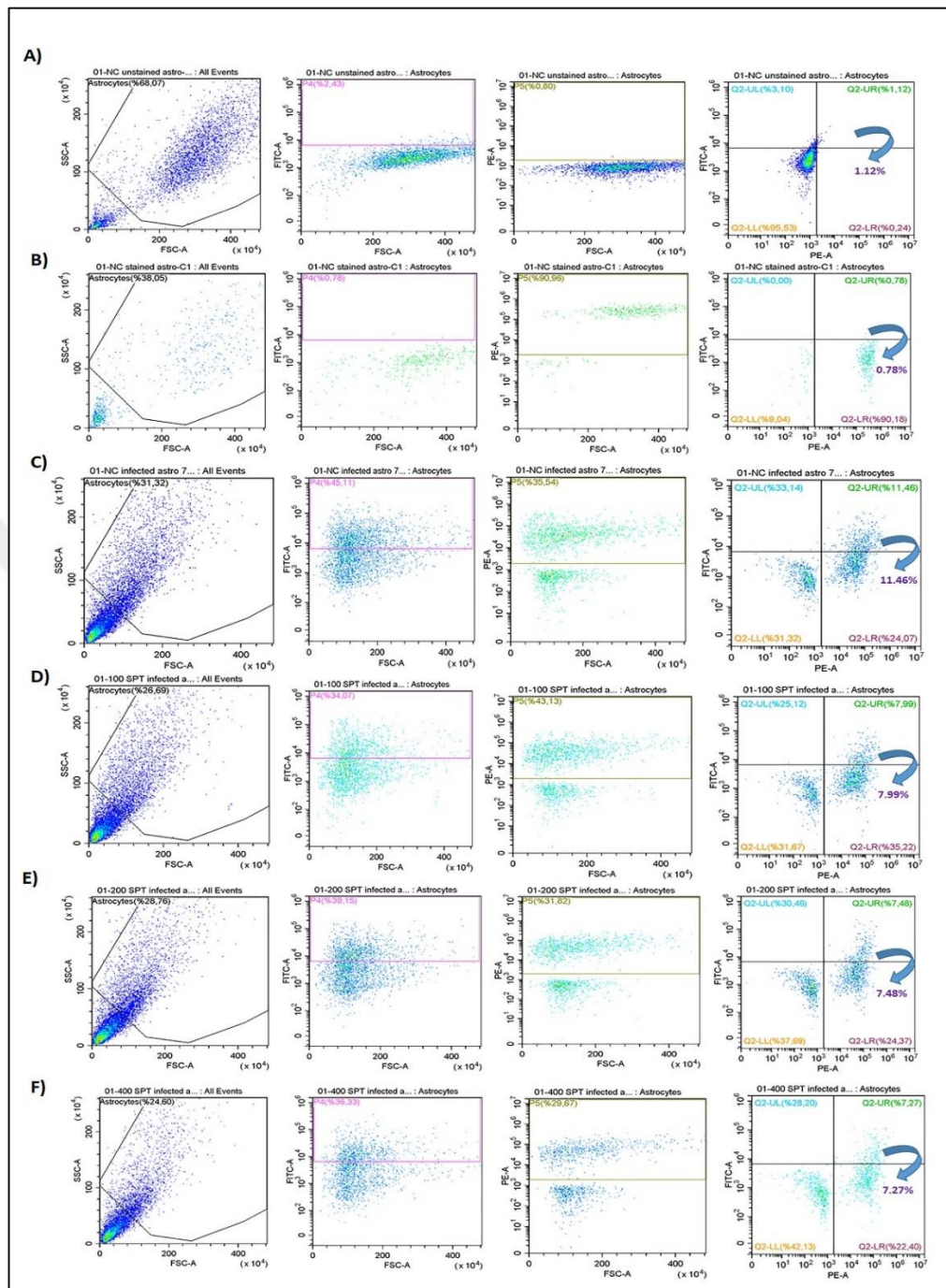


Figure 4.32. (A, B) Percentage of unstained and stained achieved by PKH26 staining on astrocytes, as determined by flow cytometry. (C, D, E) Infection rates (%) of astrocytes with *L. infantum* promastigotes upon treatment with (D)100 μ M, (E) 200 μ M, and (F) 400 μ M SPT, as determined by dot-plot pseudo flow cytometry for 72h compared to untreated negative control cells (C). Data represents mean (n=3) $\pm$ SD.

4.4.9.3. *Morphological Analysis of Macrophages and Astrocytes Infected with L. infantum Promastigotes*

Phase-contrast Microscopy Analysis

Phase-contrast microscopy was used to visualize the morphological changes of *Leishmania*-infected macrophages and infected astrocytes (infection ratio:1:10) mediated by SPT treatment as shown in Figure 4.33. Untreated *Leishmania*-infected RAW 264.7 macrophage cells (NC) exhibited a more rounded morphology attached to the surface, which did not alter when cells were treated with 200 μ M SPT concentration as well as lower doses 75 and 100 μ M SPT (Figure 4.35). In addition, infected macrophages at negative control and all SPT treatment groups preserved their morphology throughout the study and displayed round shape at 24 h (Figure 4.34-A), and 48 h (Figure 4.34-B); whereas 72h – SPT incubated cells became confluent as they can survive even at high SPT concentrations (200 μ M SPT) (Figure 4.34-C).

On the other hand, the number of *Leishmania*-infected C8-S astrocytes was observed to decrease significantly after SPT treatment in a dose-dependent manner, in which the surface morphology and attachment of cells changed dramatically. The star-shaped structure of astrocytes formed by tendrils that extend from the cell has transformed into a round spherical character and cells departed from each other as a consequence of this transformation in morphology (Figure 4.35).

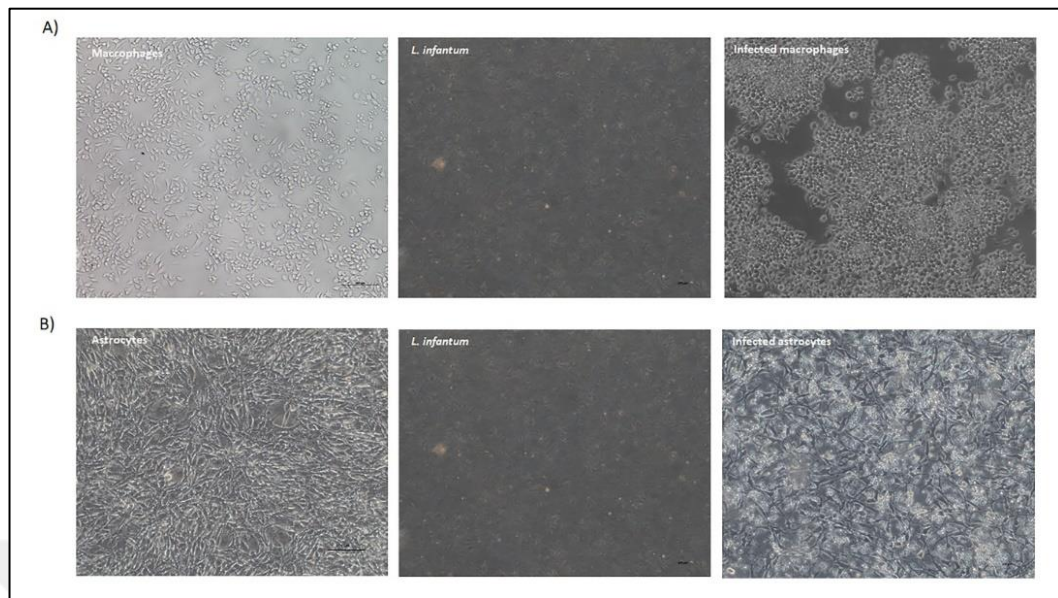


Figure 4.33. Representative phase-contrast images of RAW264.7 murine macrophages and C8-S murine astrocytes infected with *L. infantum* promastigotes (infection ratio:1:10) after 24h-incubation (scale bar: 100 μ m).

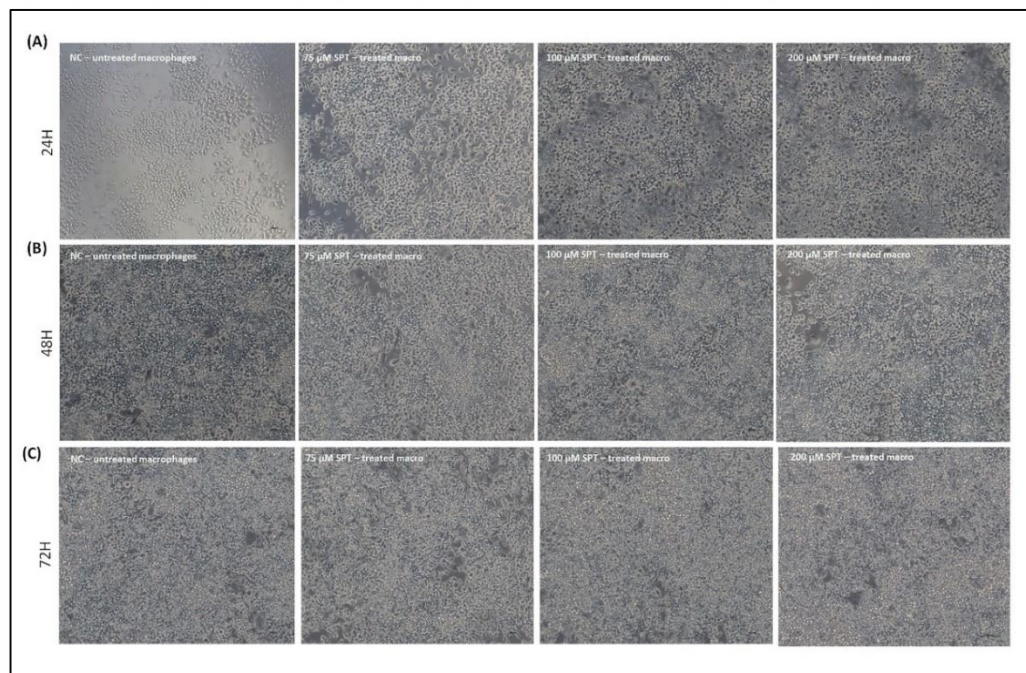


Figure 4.34. Phase-contrast microscopy images of (A) negative control (untreated *Leishmania*-infected macrophages) and (B) 75 μ M, (C) 100 μ M, and (D) 200 μ M SPT treatment groups after 24, 48 and 72 h. Scale bars correspond to 100 μ m.

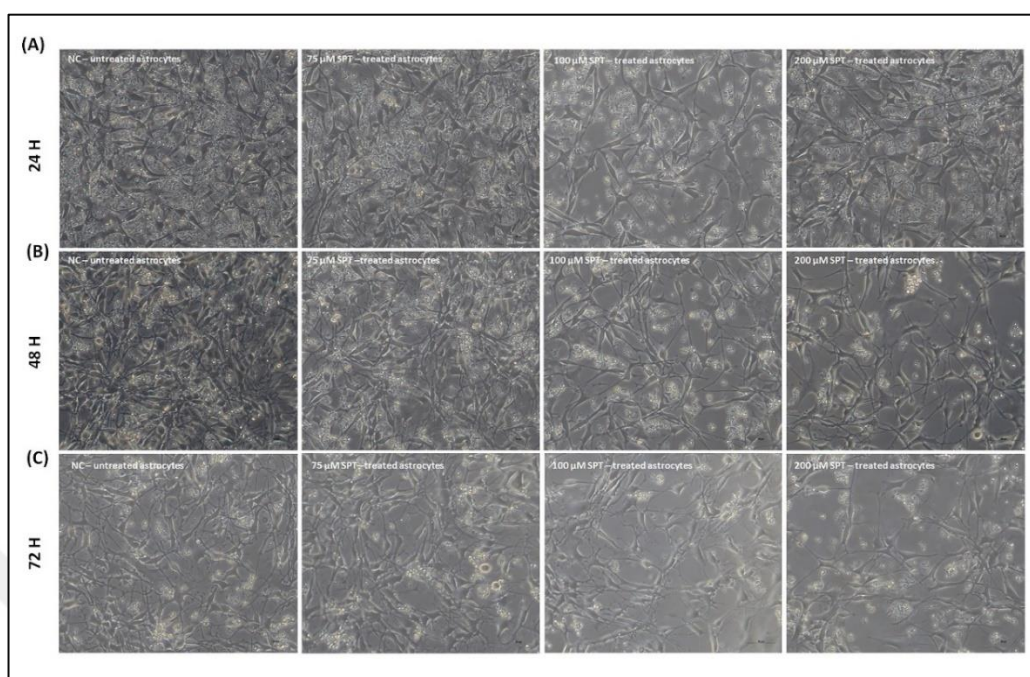


Figure 4.35. Phase-contrast microscopy images of (A) negative control (untreated *Leishmania*-infected astrocytes) and (B) 75 μ M, (C) 100 μ M, and (D) 200 μ M SPT treatment groups after 24, 48 and 72 h. Scale bars correspond to 50 μ m.

Fluorescence Microscopy Analysis of *Leishmania*-infected astrocytes- treated with SPT

Simultaneously with flow cytometry analysis, PKH26-stained- macrophages (red) or - astrocytes (red) infected with PKH67-stained *L. infantum* promastigotes (green) were imaged by fluorescent microscopy based on tracking the red, green, and merged yellow signals (Figure 4.36). Therefore, it was possible to analyze the cell populations of promastigotes, non-infected and infected macrophages/astrocytes, respectively Furthermore, the bright-field image confirmed that all astrocytes and promastigotes have been labelled and detected by fluorescence microscopy imaging. Accordingly, Figure 4.36-A displays the fluorescent images of untreated C8-S astrocytes (red) infected with *L. infantum* promastigotes (green), where parasites inside and outside of the astrocytes were visible and the merged yellow color displays the astrocytes infected by the parasites. Figure 4.36-B, C shows the fluorescent microscopy images of *Leishmania*-infected astrocytes treated with 100 μ M SPT at 24 and 48 h, respectively, where the green signal of the promastigotes inside and outside the cells were observed to decrease in a time-dependent manner. The

fluorescence intensity of promastigotes at 24 h was observed to be higher than at 48 h (Figure 4.36-C), which qualitatively confirms the flow cytometry data together with the phase-contrast microscopy analysis revealing that parasitic load decreased significantly at 100 μ M SPT concentration at 48 h ($p \leq 0.0001$), and remained almost stable regardless of a further increase in concentration (Figure 4.26-B).

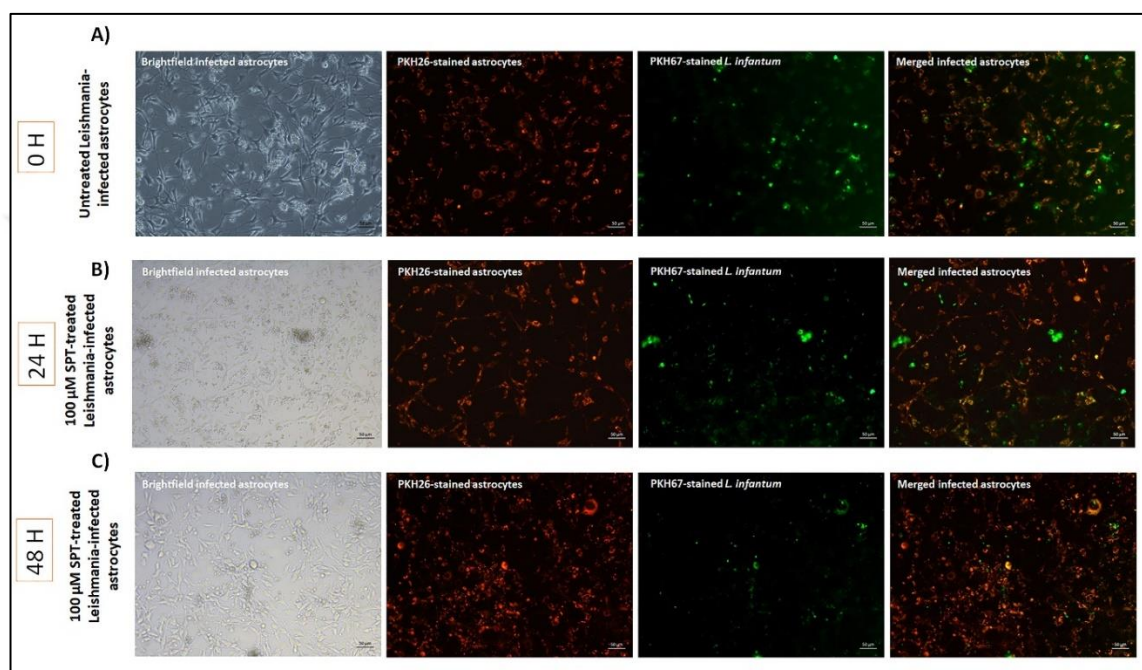


Figure 4.36. Fluorescence microscopy images of astrocytes infected with *L. infantum* promastigotes (infection ratio, 1:10) untreated and treated with 100 μ M SPT for 24 and 48 h. From left to right: brightfield image, fluorescence image of PKH26-stained astrocytes (red), PKH67-stained *L. infantum* promastigotes (green), and *Leishmania*-infected astrocytes (merged-yellow) (magnification: 20 $\times$). Scale bars correspond to 50 μ m.

4.4.10. ROS Measurement in *Leishmania*-infected Macrophages After Treatment with SPT

All SPT concentrations (75-200 μ M) had no significant effect the intracellular macrophage ROS level based on the detected DCFDA fluorescence signal at 24 h by flow cytometry analysis in each sample group when compared to negative control (Figure 4.37). In parallel, positive control (PC) exposure for 1 hour increased significantly in the relative ROS level up to 50%, as given in the flow cytometry histograms and the relative ROS graph (%) ($p \leq$

0.0001) (Figure 4.37-A, B and C). Accordingly, SPT treatment did not lead to a significant increase in the relative ROS level in *Leishmania*-infected macrophages.

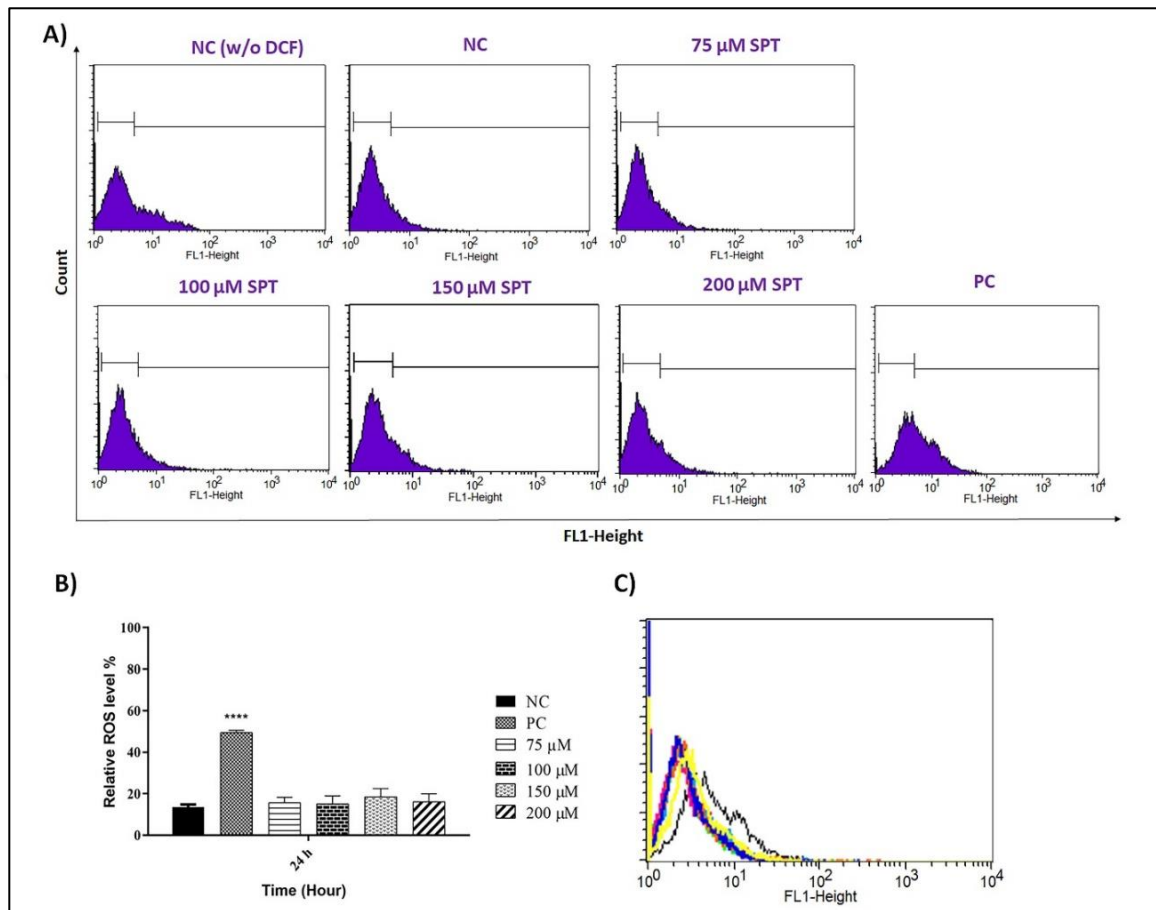


Figure 4.37. Intracellular ROS level measurement in *Leishmania*-infected macrophages after 24-h incubation of SPT at different concentrations (75-200 μM). (A) Representative histograms of ROS accumulation (%) in SPT treatment groups. (B) Bar graph showing the mean relative ROS (%) of three independent experiments. (C) Overlay histogram analysis of untreated infected macrophages (blue), positive control (PC) THBP (100 μM) (black), and SPT treatment groups including 75 μM (pink), 100 μM (orange), 150 μM (yellow), and 200 μM (green) at 24-h of exposure. Data represents mean (n = 3) ± SD.

4.4.11. ROS Measurement in *Leishmania*-infected Astrocytes After Treatment with SPT

All SPT concentrations, except 100 μ M, led a significant decrease in the intracellular astrocyte ROS level based on the detected DCF fluorescence signal at 24 h by flow cytometry analysis in each sample group when compared to negative control, and determined below 30% (Figure 4.38). The treatment of *Leishmania*-infected astrocytes with 50, 75, 150 and 200 μ M SPT for 24 h resulted in a significant decreased close to 28% (i.e. 28.24%) ($p \leq 0.01$), 22% (i.e. 21.83%) ($p \leq 0.001$), 29% (i.e. 29.22%) ($p \leq 0.01$), and 18% (i.e. 17.85%) ($p \leq 0.0001$), respectively. In parallel, positive control (PC) exposure for 1 hour increased significantly in the relative ROS level up to 80% (i.e. 84%), as given in the flow cytometry histograms and the relative ROS graph (%) ($p \leq 0.0001$) (Figure 4.38-A, B and C).

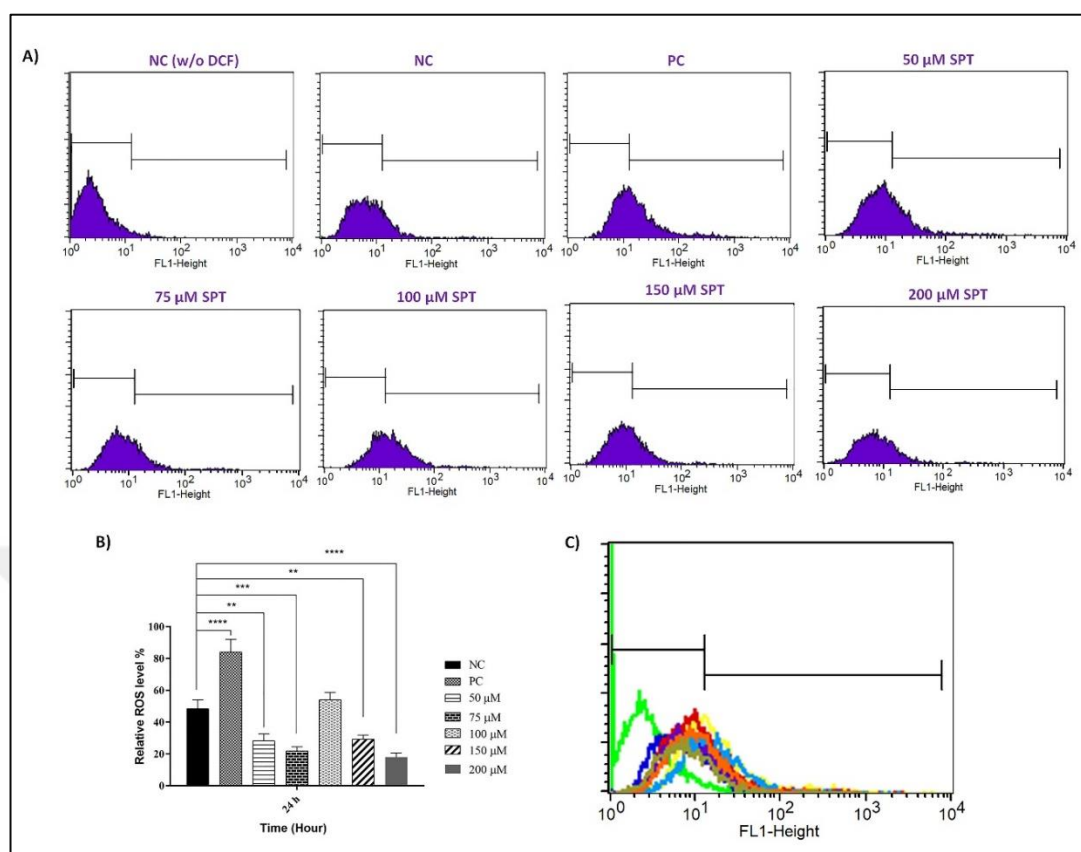


Figure 4.38. Intracellular ROS level measurement in *Leishmania*-infected astrocytes after 24-h incubation of SPT at different concentrations (50-200 μM). (A) Representative histograms of ROS accumulation (%) in SPT treatment groups. (B) Bar graph showing the mean relative ROS (%) of three independent experiments. (C) Overlay histogram analysis of untreated infected astrocytes without DCFDA (green), untreated infected astrocytes with DCFDA (NC) (dark blue), positive control (PC) THBP (100 μM) (black), and SPT treatment groups including 50 μM (red), 75 μM (yellow), 100 μM (light blue), 150 μM (pink), and 200 μM (dark yellow) at 24-h of exposure. Data represents mean (n = 3) ± SD.

4.4.12. Determination of Oxidative Stress by the Measurement of GSH Level in *Leishmania*-infected Macrophages After Treatment with SPT

In order to investigate the antioxidant capacity of SPT on *Leishmania*-infected macrophages, oxidative stress was determined on the untreated and treated cells by the measurement of GSH level. According to the results, all concentrations of SPT did not lead

to a significant alteration in oxidative stress by remaining at almost stable GSH levels, when compared to NC untreated group (Figure 4.39).

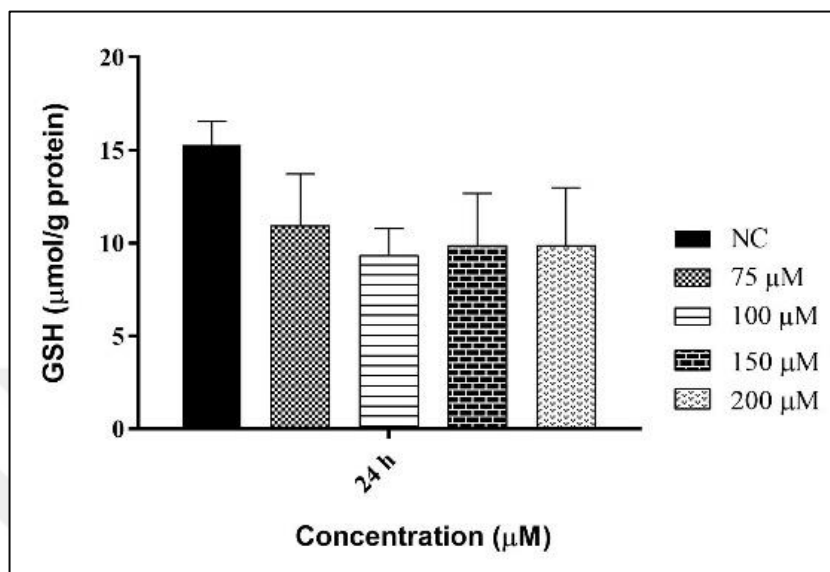


Figure 4.39. Quantification of intracellular GSH levels for the determination of the oxidative stress on *Leishmania*-infected macrophages after 24 h of SPT treatment.

4.4.13. Determination of Oxidative Stress by the Measurement of GSH Level in *Leishmania*-infected Astrocytes After Treatment with SPT

Similar to infected macrophages, the antioxidant capacity of SPT was investigated on *Leishmania*-infected astrocytes by the measurement of GSH level on the untreated and treated cells. According to the results, 50, 100, 150, and 200 μM SPT led to a significant decrease in oxidative stress by inducing GSH levels significantly, when compared to NC (Figure 4.40). However, 75 μM SPT did not cause a significant induction in the GSH level.

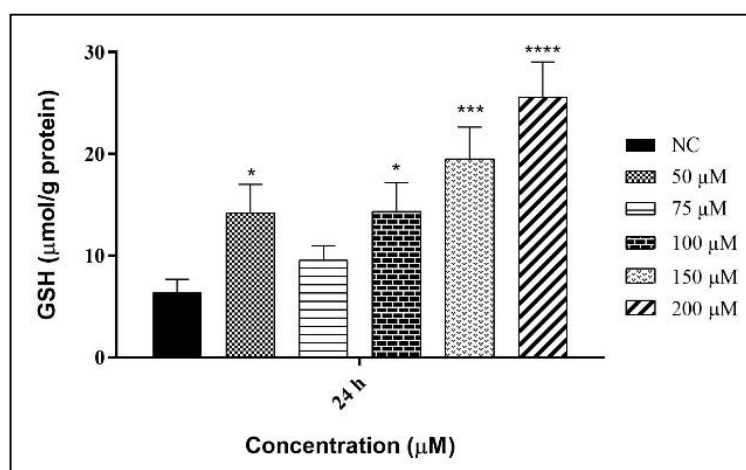


Figure 4.40. Quantification of intracellular GSH levels for the determination of the oxidative stress on *Leishmania*-infected astrocytes after 24 h of SPT treatment. Data represents mean ($n = 3$) $\pm$ SD.

4.4.14. Gene Expression Level of *Leishmania*-infected Macrophages and *Leishmania*-infected Astrocytes after SPT treatment

Upon treatment of *Leishmania*-infected macrophages and *Leishmania*-infected astrocytes with 50, 75, 100, 150 and 200 μ M of SPT, the relative gene expression of TNF- α , IFN- γ , IL-1 β , IL-12 (B), STAT1, MMP2/ATP5, and IL-10, NF- κ B, TGF- β 1, iNOS/ β -actin were analyzed using real-time PCR for 24 h (Figure 4.41 and 4.42).

As shown in Figure 4.41-A, qPCR analysis indicated that IFN- γ levels overexpressed significantly in 50 μ M (11.18-fold) ($p \leq 0.0001$), 75 μ M (3.32-fold) ($p \leq 0.0001$), 100 μ M (2.16-fold) ($p \leq 0.001$), 150 μ M (3.32-fold) ($p \leq 0.0001$), and 200 μ M (4.16-fold) ($p \leq 0.0001$) SPT treatment (Figure 4.41-A), when compared to the negative control group. On the other hand, 50, 100, and 150 μ M SPT treatment did not significantly increase TNF- α gene expression level in the infected macrophages, whereas 200 μ M SPT treatment led to a significant increase (6.05-fold) ($p \leq 0.01$) compared to the NC group (Figure 4.41-B). Likewise, the gene expression level of IL-12(B) was significantly upregulated (24.13-fold increase) in infected macrophages treated with 200 μ M SPT ($p \leq 0.0001$) (Figure 4.41-C), and interestingly treatment of 50 μ M SPT significantly increased (9.18-fold) IL-12(B) gene

expression ($p \leq 0.01$). Moreover, NF- κ B gene overexpressed significantly in 50 (1.24-fold) ($p \leq 0.01$), 100 μ M (1.2-fold) ($p \leq 0.01$), and 150 μ M (1.35-fold) ($p \leq 0.0001$) of SPT, when compared to NC (Figure 4.41-D), while 200 μ M SPT treatment led to a significant reduction in NF- κ B gene expression (Figure 4.41-D). Besides, in the gene expression level of IL-1 β gene did not lead any significant change after SPT treatment on the infected macrophages (Figure 4.41-E). IL-10 gene expression was significantly downregulated in infected macrophages upon treatment of 50 μ M ($p \leq 0.05$) and 100 μ M SPT ($p \leq 0.01$), whereas 150 and 200 μ M SPT did not cause a significant decrease on IL-10 gene expression level of infected macrophages (Figure 4.41-F). Consistent with IL-10, TGF- β 1 gene expression significantly decreased after 100 μ M SPT treatment ($p \leq 0.0001$), and also on 200 μ M SPT-treated macrophages ($p \leq 0.01$); however treatment of 50 μ M SPT significantly increased TGF- β 1 gene expression compared to NC ($p \leq 0.01$) (Figure 4.41-G).

As shown in Figure 4.42-A, qPCR analysis indicated that IFN- γ levels overexpressed significantly (1.33-fold) in only 100 μ M SPT treatment on infected astrocytes ($p \leq 0.05$) (Figure 4.42-A), when compared to the negative control group. On the other hand, 200 μ M SPT treatment significantly increase TNF- α gene expression level (1.34-fold) in the infected astrocytes ($p \leq 0.001$), whereas 50, 75, 100 and 150 μ M SPT treatment led to a significant decrease compared to the NC group (Figure 4.42-B). Contrary to TNF- α gene expression, the gene expression level of IL-12(B) was significantly upregulated in infected astrocytes treated with 100 μ M (3.45-fold increase) and 150 μ M SPT (3.08-fold increase) ($p \leq 0.0001$) (Figure 4.42-C), while treatment of 50, 75 and 200 μ M SPT cause a significantly decrease on IL-12 (B) gene expression. On the other hand, all treatment group did not lead to a significant alterations on NF- κ B gene expression of infected astrocytes, when compared to NC group (Figure 4.42-D), while in IL-1 β gene overexpressed (3.81-fold increase) in infected astrocytes treated with only 150 μ M SPT ($p \leq 0.01$) (Figure 4.42-E). Besides, the gene expression levels of IL-10 were downregulated upon treatment of 50, 100, 150 and 200 μ M SPT in infected astrocytes compared to NC (Figure 4.42-F). STAT1 gene expression increased (not significantly) after SPT treatment, when compared to NC (Figure 4.42-G); however iNOS, MMP-2 and TGF- β 1 gene expressions did not show any significant alterations after SPT treatment in infected astrocytes (Figure 4.42- H, I, and J).

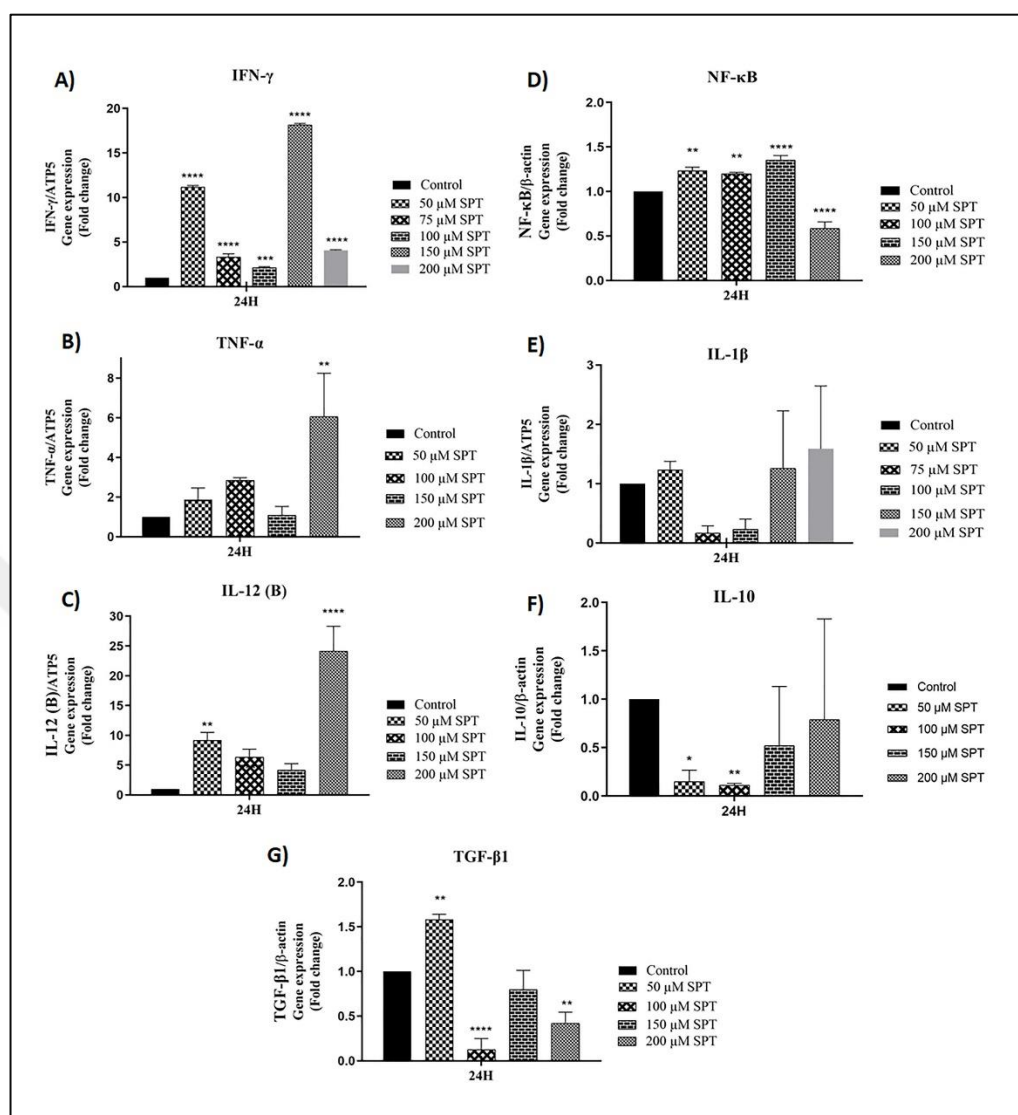


Figure 4.41. Fold change of relative gene expression including (A) IFN- γ , (B) TNF- α , (C) IL-12 (B), (D) NF- κ B, (E) IL-1 β , (F) IL-10 and (G) TGF- β 1 on *Leishmania*-infected RAW 264.7 macrophage cells treated with SPT (0-200 μ M) after 24 h of incubation. The data represents three independent experiments and shows significant difference from respective control. Data represents mean ($n = 3$) $\pm$ SD.

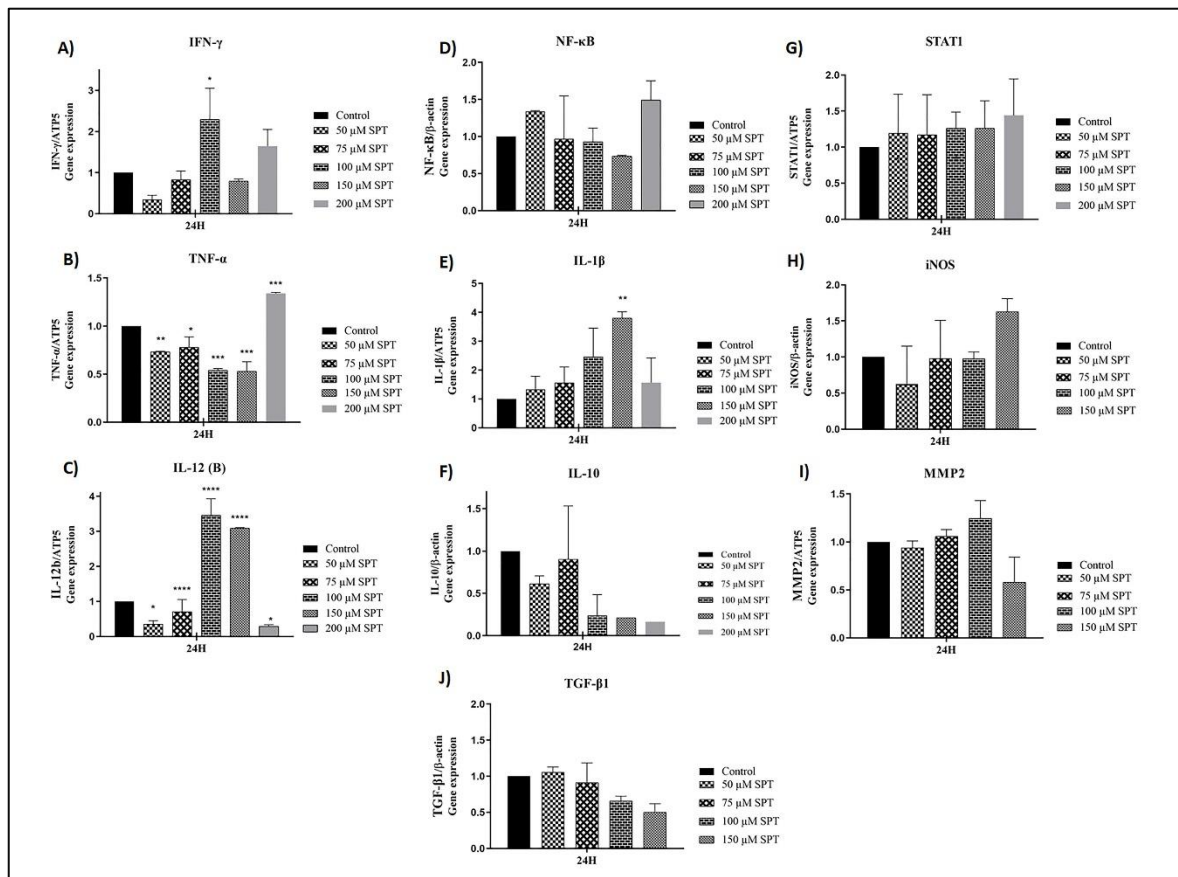


Figure 4.42. Fold change of relative gene expression including (A) IFN- γ , (B) TNF- α , (C) IL-12 (B), (D) NF- κ B, (E) IL-1 β , (F) IL-10, (G) STAT1, (H) iNOS, (I) MMP2, and (J) TGF- β 1 on *Leishmania*-infected C8-S astrocyte cells treated with SPT (0-200 μ M) after 24 h of incubation. The data represents three independent experiments and shows significant difference from respective control. Data represents mean ($n = 3$) $\pm$ SD.

5. DISCUSSIONS

Leishmaniasis is a neglected tropical disease caused by the vector-borne parasites of the genus *Leishmania*, transmitted to numerous species including humans through the bite of a sand fly, mainly *Phlebotomus* and *Lutzomyia*. There are over 20 species of *Leishmania* that cause life-threatening disorders widely distributed in 98 tropical and subtropical regions including Asia, South America, Northern Africa, Southern Europe, the Middle East and Turkey [1]. According to the recent WHO report, more than 350 000 people estimated at risk and 1.3 million new cases of leishmaniasis occur every year [1]. *Leishmania* spp. are digenetic organisms which survive and replicate either as promastigotes in an insect vector, or as amastigote forms within phagolysosome-like vacuoles inside the macrophages [136, 137]. Clinical manifestations of leishmaniasis are divided into three clinical forms: cutaneous, mucocutaneous or visceral leishmaniasis known as ‘Kala Azar’, depending on which species are involved in the infection within mammalian tissues.

Microbial invasion of the CNS is a main global healthcare concern, which is linked to high morbidity and mortality. The clinical classification of CNS infections are associated with the anatomical regions such as, encephalitis, meningitis, and myelitis. Regarding to the evolutionary adaptation, the blood–brain barrier (BBB), a functional immune-privileged system, separates the CNS from the systemic circulation, and is needed for the maintenance of the neuronal function against pathogens including *Leishmania* parasites by protecting from immune-mediated inflammation [110,138,139]. However, the finding of a lymphatic system within the meninges, which may offer a legitimate route for circulating immune cells to enter and patrol meningeal compartments, redefines the processes of immunological privilege.

Numerous research have focused on the CNS in an effort to show that *Leishmania* species can indeed cross the BBB and trigger clinical manifestations and clinical neurological symptoms [111, 112, 113]. In the previous studies, the presence of anti-*Leishmania* antibodies were detected in the CSF of *Leishmania*-positive dogs, which could lead alterations in the nervous tissue, and may result into a disease known as “cerebral leishmaniasis”. Moreover, Melo et. al., (2017) reported to the presence of *Leishmania donovani* parasites in the brain, and brain inflammation in experimental VL, which demonstrated a remarkable process of inflammatory responses including the cytokine and

chemokine release, and inflammatory cells (e.g. macrophages) in the CNS [128]. Also, it was revealed that the parasite burden in the brain of *L. donovani*-infected mice was estimated as between 900-12,000, especially in choroid plexus, meninges and cerebrospinal fluid in humans and dogs, considering the immune-privileged blood brain barriers and immune barrier of the nervous systems [128].

As an element of the innate immune system, macrophages are an essential part of the immune system and play a central role in both the body's haemostasis and defense against infections. They accomplish these by enhancing in the removal of dead cells and microorganisms, triggering and signaling to other immune cells, and serving as antigen presenting cells (APCs), which are able to send out the signals required for T cell activation. Numerous macrophage phenotypes have been discovered, each with unique functional characteristics. For instance, in the presence of microbial products like lipopolysaccharide (LPS), interferon gamma (IFN- γ) from T helper 1 (TH1) cells, CD8⁺ cytotoxic T cells (CTLs), or natural killer (NK) cells can induce M1 (classically activated) macrophages [132]. M1 macrophages secrete high levels of pro-inflammatory cytokines like interleukin 12 (IL-12) and IL-236, which have also anti-tumor and anti-parasitic functions. Besides, IL-4 and/or IL-13, which are primarily released by TH2 cells or polymorphonuclear cells such as mast cells, can induce M2 (alternatively activated) macrophages. M2 macrophages have anti-inflammatory and wound healing effect, and release high amount of the anti-inflammatory cytokine IL-10.

As a component of CNS immune system, astrocytes are the most prevalent form of non-neuronal, glial cell type in CNS, and have various functions including phagocytosis, homeostasis and the secretion of cytokines for the immune system (Farina et al. 2007). During CNS injury and neurodegenerative diseases, astrocytes can change into a different morphology with a reactive phenotype, and resulted in alteration in the gene expression and secretion level. These astrocytes are suggested to be immunocompetent cells, which can release chemokines and cytokines in response to the inflammatory processes, and thereby controlling the immune system activation and recruitment to the site of infection. In response to CNS pathogenesis, with the activation of astrocytes, pro-inflammatory cytokines, including IL-1b, IL-6, TNF-a and CCL2 can be expressed and released, and thus leading to neuroinflammation [122]. In addition, COX2 and iNOS expressions may be induced by activated astrocytes, resulted in the neuroinflammation with the secretion of the cytokine

NO. The previous studies indicated that human astrocytes could upregulate MHC-II and PDL-1 (regulated by IFN γ levels), the astrocytes were overexpressed in the level of IFN- γ . The astrocyte culture responded to IFN- γ with an increase in endogenous and surface MHC-I, MHC-II, and PDL-1, according to immunostainings and flow cytometry analysis, suggesting that astrocytes could express both stimulatory and inhibitory molecules, and might play a role in the control of T cell function in the brain [140, 141].

For the antileishmanial therapy, there are different treatment options based on pentavalent antimonials—i.e., sodium stibogluconate (pentostan) and meglumine antimoniate (glucantim), pentamidine, conventional amphotericin B (AmpB) deoxycholate, miltefosine, paramomycin (aminosidine), micellar formulation of AmpB (fungizone), AmpB lipid complex (Abelcet), and liposomal AmpB (AmBisome) [142-148]. However, these therapies have certain drawbacks associated with 1) their limited efficacy on parasites [149], 2) multiple adverse effects due to their low therapeutic index [150], 3) requirement for careful and slow intravenous administration due to their toxicities (e.g., AmpB) [151], and 4) drug resistance developed against the therapy [142-148]. Therefore, the development of alternative chemotherapy methods and the identification of new drug molecules with enhanced properties are key factors in the success of anti-leishmanial therapy. Novel drug candidates with promising efficacy against leishmaniasis have been reported in several investigations [152-154].

Among these potential therapeutic agents, this Ph.D. study is the first report to emphasize that boron derivatives, including SPT, have shown significant antileishmanial efficacy on *L. infantum* parasites, and involved inflammatory process on *Leishmania*-infected macrophages and astrocytes. Furthermore, SPT indicated the highest potential to inhibit parasite proliferation within the range of 75 μ M to 400 μ M, without displaying any toxicity on macrophages and astrocytes. Besides, boron has also been indicated as a promising therapeutic for neurodegenerative diseases in which oxidative stress and related pathologies, and in particular to the impaired motor function and the development of brain function when the deprivation of boron has negatively affected the motor speed [68]. Ozansoy and colleagues (2020) investigated that sodium borate decahydrate and boric acid increased the Sirt1 expression, which show protectivity on the cellular stress and thus indicating the prosurvival effect of boron in an amyloid-beta toxicity model at 200 μ g/mL doses of boron compounds [68].

Boron has dual effect on the inflammatory process with pro-inflammatory and anti-inflammatory properties. Considering the inflammatory process, the previous study reported that boron (borax as orally) could increase the proliferation of lymphocyte and the release of immune response by the lipopolysaccharide (LPS)-stimulated peritoneal macrophages in BALB/c mice. According to this study, a significant increase was detected in T and B cell populations with the increase in CD4 and CD19 after treatment of boron. Furthermore, boron induced TNF- α , IL-6, IL-1 β secretion, and iNOS expression by the LPS-primed macrophages, more specifically the M1 macrophages in a dose-dependent manner [80]. On the other hand, it has to be noted that a decrease in the inflammatory factors and increase are not always a good solution for the infection process and also as a wound healing treatment strategy considering the immunological inflammatory process of *Leishmania* parasites infection (“Leishmaniasis”). However, pro-inflammatory response together with anti-inflammatory approach can take boron one step forward to prevent the disease progression, provide the parasite eradication, and the cutaneous wound management application.

This study aimed to investigate how *Leishmania* parasites were transmitted into the brain via macrophages, and focused on the mechanism underlying the inflammatory responses on both macrophages and astrocytes. Addressing to the lack of knowledge on the neurological aspects of leishmaniasis, the present study introduces the immunological mechanism associated between macrophages- and astrocytes-infected with *L. infantum* on bone-marrow derived primary macrophage cells by *in-vitro* studies. In addition, the antileishmanial efficacy and anti-neuroinflammatory activity of SPT compound was evaluated on *L. infantum* parasites, *Leishmania*-infected macrophages and *Leishmania*-infected astrocytes in *in vitro* cultures, whereas the analysis on non-infected macrophages and astrocytes questioned the safety of the SPT treatment in *in vitro* model.

It is important to emphasize that this study is the first report revealing SPT as a boron derivatives capable to enhance the antileishmanial therapy on both *L. infantum* parasites, *Leishmania*-infected macrophages. Moreover, neuroinflammatory-based antileishmanial therapy was demonstrated on *in-vitro* model of *Leishmania*-infected astrocyte via the immunological mechanism and inflammatory responses including cell viability, infection and gene expression analysis. In addition, the safety property of SPT, including

antileishmanial activity and compound selectivity, was demonstrated on non-infected healthy macrophages and astrocytes.

BMDM generation

Murine BMDMs were generated using M-CSF with high purity of mature macrophages on day 7 where 83% of total macrophage population were identified by their double positive signal in the upper-right quadrant using FITC and PE filter for CD11b and F4/80 macrophage markers (Figure 4.1). The BMDM differentiation were verified by the phase contrast microscope on day 0, 3, 5 and 7 as morphologically in which spindle-like cells have become spherical on day 5 and day 7 (Figure 4.2). It was consistent with the previous studies on the literature after BMDM maturation [132].

BMDM polarisation

In order to eliminate the intracellular parasite (e.g. *Leishmania* spp.) via the release of various cytokines, the activation of M1 macrophages by Th1 lymphocyte, is crucial. First, the pro-inflammatory cytokines, IFN- γ and TNF- α is vital for the eradication of *Leishmania* parasites via the eliciting of an oxidative burst. The host macrophages increase the secretion of ROS, which display highly antileishmanial activity. In contrast, Th2 lymphocyte activation (secretes IL-4 and IL-13 cytokines) induced the M2 macrophages characterized by the synthesis of polyamine through the activation of the arginase (arg) and production of urea and L-ornithine, resulted in favor parasite survival in infected macrophages and disease progression by promoting *Leishmania* intramacrophage growth [156, 157]. Various *Leishmania* species cause various immune reactions. The interest in comprehending the function of M1 and M2 macrophages in the context of various *Leishmania* species infection grows as a result of these responses, which go far beyond the conventional Th1/Th2 paradigm [157].

Therefore, in this study, first the polarisation of BMDMs into M1 or M2 macrophages were determined after 100 ng/ml LPS or 100 ng/ml SLA stimulation for 24 h, and 48 h. The BMDM polarisation was detected as CD11b+F4/80+ cells, the surface marker of BMDMs CD11b, F4/80, CD80, CD86 and cytokines TNF- α and IL-10 were analyzed using flow cytometry after 24-h, and 48-h of incubation (Figure 4.3). MFI of labeled BMDMs were analyzed using flow cytometry analysis.

According to the histogram analysis, MFI of CD11b-FTIC increased significantly in SLA or LPS -stimulated BMDMs at 48 h (Figure 4.3), compared to untreated BMDMs (NC). F4/80-PE indicated a significant increased in LPS or SLA-stimulated BMDMs after 24 h ($p \leq 0.0001$) (Figure 4.3), when compared to untreated NC BMDM group.

On the other hand, the surface markers CD80 and CD86 of M1 macrophages increased significantly in LPS-stimulated BMDMs after 24 and 48 h; whereas as expected, no significant increased were observed in CD80 marker on SLA-stimulated BMDMs after 24 and 48 h. Unlike CD80-APC, CD86-ECD signal increased significantly in SLA-stimulated cells at 24 h ($p \leq 0.01$) (Figure 4.3-B, C). Rabhi et. al., (2012) indicated that *Leishmania* infection induced the expression of co-stimulatory molecules such CD40, CD83, and CD86 [158]. Similarly, consistent with previous studies [158, 159], and associated with the classical activation M1 phenotype, TNF- α signal increased significantly in LPS ($p \leq 0.0001$) or SLA-treated ($p \leq 0.01$) BMDMs after 48 h of stimulation. The previous study reported that the transcription of TNF- α (up to 5) was upregulated following infection [159]. In another study, the murine macrophage cell line J774A.1 was activated by PPAR expression through polarization toward an M1 profile, which was characterized by high production of pro-inflammatory mediators (TNF-a, IL-1b, IL-6, TLR4, and ROS), as well as an increase in antileishmanial activity against *L. mexicana* [160]. On the other hand, an anti-inflammatory cytokine IL-10 did not show any change increase in either BMDMs treated with LPS or SLA upon 24 and 48 h of stimulation. Recently, one of the study has also indicated the role of rapamycin (mammalian target, mTOR) in M2 polarization for *Leishmania* survival [161]. The studies showed that mTOR pathway of the host was activated by *L. donovani*-infection, cause a reduction in the expression of M1 macrophage markers (ROS, NO, iNOS, NOX-1, IL-12, IL-1b, and TNF-a), and increased expression of M2 macrophage markers (arg-1, IL-10, TGF-b, CD206, and CD163), are beneficial for the *Leishmania* survival in the host macrophages [161].

Although it was discussed above on the BMDM M0 polarization into M1 or M2, it is known that macrophages display high plasticity and can be repolarized or reprogrammed under specific stimuli, which could be explained by the differentiation of M1 macrophages to M2, and vice versa [156, 162, 163]. M2 macrophage may be repolarized more quickly than M1 macrophages, and this repolarization can appear through exposure of TLR ligands such as LPS and/or IFN- γ [156].

The cell morphology in BMDM polarisation

Parallel to flow cytometry analysis, phase-contrast microscopy images were taken to visualize the polarisation of BMDMs into M1 and M2 macrophage after LPS, LPS and IFN- γ , SLA, and IL-4 at 24 h (Figure 4.4). The morphology of different macrophage phenotypes can led to suggest that cell morphology could be an indicator of macrophage activation status [132]. Consistent with the previous study [132], treatment with LPS, or LPS and IFN- γ display flat and round-shaped cell morphologies which is attributed to the polarisation of BMDMs to M1 macrophages after LPS stimulation . On the other hand, when BMDMs were treated with SLA or IL-4, more stretched and spindle-like shape were observed in the number of cells, resulted in a similar in shape to unpolarised BMDMs at first days of differentiation, and further indicate the polarisation of M2 macrophages rather than M1-like phenotype. It was consistent with the literature, in which polarized M2 macrophages were observed in dogs with visceral leishmaniasis, and the presence of M2 macrophages in dogs from infected group show difference in all organs including inflammatory infiltrates of samples [164]. Although, the more M2 macrophages upon SLA stimulation were observed, M1 and M2 macrophage population could be determined heterogeneously on the stimulated culture. Considering these results, it has to be noted that macrophages display high plasticity and can be repolarized or reprogrammed under specific stimuli, which could be explained by the differentiation of M1 macrophages to M2, and vice versa.

Determination of the percentage rate of *L. infantum* promastigotes in the co-culture of astrocyte and infected macrophages by flow cytometry analysis

In order to investigate the mechanism of the movement of macrophages infected with *L. infantum*, *in vitro* co-culture systems including co-culture of infected macrophages and non infected astrocytes were designed in the different ratio of the cells, and accordingly, the percentage rates of *L. infantum* promastigotes in the coculture of astrocyte and infected macrophages were analysed using flow cytometry according to the FL1 and FL2 filters. FL1 (green fluorescence), and FL2 (red fluorescence) filters were used to distinguish the parasites/cells with regard to the excitation (for FITC at 495 nm; APC at 650 nm) and emission (for FITC at 519 nm; APC at 660 nm) of the dyes. While the tracking of the fluorescent signals in fluorescence microscopy enabled the selective detection of promastigotes, non-infected astrocytes and infected astrocytes qualitatively, the flow

cytometry allowed quantitative analysis of the parasite in the co-culture of the infected macrophages and astrocytes.

When the populations in the lower-left and upper-left quadrants in Figure 4.5-C and D were analyzed together, a significant decrease ($P \leq 0.0001$) in parasite load could be observed in parallel to a time-dependent manner (Figure 4.8-A). Accordingly, in the non-infected astrocytes and *Leishmania*-infected macrophage co-culture, the parasite population was detected as $73.86 \pm 5.6\%$ at 24 h, which decreased to $50.08 \pm 4.26\%$ and $24.88 \pm 2.30\%$ in the co-culture after 48 and 72 h of incubation, respectively (Figure 4.5-C, D, and Figure 4.8-A). It was also represented by the overlay histogram analysis according to the decreased fluorescence intensity of gp63 FITC-labeled *Leishmania* parasites at 24, 48 and 72 h, as given in Figure 4.6. In parallel, phase contrast microscopy images confirmed a time-dependent reduction of the parasite burden in the co-culture of uninfected astrocytes and *Leishmania*-infected macrophages, in which an increase in the number of astrocytes was observed at 72 hours of co-incubation (Figure 4.7). This could be explained by the decrease in parasite burden due to the increase in astrocyte inside the co-culture, and thus increasing the pro-inflammatory cytokine release into the culture supernatant [133]. Previously, one of the study indicated that astrocytes clustered with HIV-1 infected macrophages, and could engulf macrophage material, including HIV-1- infected debris. To prevent local necrosis and subsequent CNS inflammation, astrocytes engulf damaged and dying cells or their fragment which might be related to their capacity for phagocytosis [165]. Astrocyte infection was not observed by cell-free or cell-to-cell routes. Interestingly, in the same study, when non-macrophage-tropic HIV-1 – infected T cells were engulfed by macrophages, they became viral antigen positive but were not productively infected [166, 167]. They hypothesized that astrocytes might ingest HIV-1-infected cells and/or cell debris once identifying damaged, and dying cells, and hence becoming positive for viral antigens and nucleic acids without infecting themselves [165].

In this Ph.D. study, a dramatic reduction in the parasite load together with an increased cluster of astrocyte and infected macrophages after cell-to-cell contact in the co-culture emphasized the evidence of astrocyte internalization of infected macrophages and macrophage-derived cellular material, resulting in a lower infection rate of astrocytes similar to non co-cultured infected astrocytes. This indirectly suggests the decrease in the parasite infection in the astrocytes. Moreover, M1 macrophages were reported to induce astrocyte

proliferation which can be partially counteracted by M2 macrophages [168]. Furthermore, astrocytes previously stimulated by M1 macrophages are able to increase macrophage proliferation and the immune response. In this case, *L. infantum* – infected BMDMs could be polarised into M2 macrophages as well as low percentage of M1 cells. Therefore, M1 – mediated immune response involving the parasite killing might enhance the reduction in the percentage of the parasite in the co-culture.

Moreover, astrocytes are critical for the control of CNS infection and inflammation, via the production of pro-inflammatory cytokines IFN- γ . Along with secreting IL-6, astrocytes actively control the microglia at several different levels during pro-inflammatory damage and healing. The prolonged neurological impairment caused by the inhibition of IFN- γ expression in astrocytes is correlated with elevated levels of TNF- α and IL-6. IFN- γ has been shown to cause neuronal damage during chronic inflammation, but other studies as previously explained in this part of discussion, have suggested that IFN- γ may also play a neuroprotective role through immune-mediated mechanisms. Collectively, these results suggest that depending on the circumstances, inflammatory cytokines may mediate dual effects.

The previous study indicated that astrocytic IL-33 receptor expression was needed for sufficient elevation of an anti-parasitic immune response in the brain, including IFN- γ production in T cells together with cell proliferation, which is significant for controlling *Toxoplasma gondii* parasites [169]. The study revealed that astrocytes can prevent the parasite infection, and the protective properties of astrocytes in response to IFN- γ and elimination of parasites could be one explanation of why there are no *Toxoplasma gondii* astrocytic cysts in wild-type (WT) mice [170]. Furthermore, the study revealed the ability of astrocytes to inhibit parasite replication which is essential for the control of the infection in the brain [170]. Similarly, Sun et. al., (2017) indicated the IFN- γ mediated neuroprotection in acute neuroinflammation via IL-6 secreted by astrocytes [171]. In another study, M. Stil et. al., demonstrated that astrocytes can enhance a protective immunological response to *Toxoplasma gondii* infection in the brain via IL-33-ST2 signaling [169]. The similar explanation could be adapted to *Leishmania* infection in the CNS, considering rare case of cerebral leishmaniasis in the nervous system.

Determination of Neuroprotective Effect of IFN- γ on Astrocytes in the co-culture

In order to investigate whether IFN- γ (100 ng/ml) have neuroprotective function on the astrocyte in the non-infected astrocytes and *Leishmania*-infected macrophage co-culture or not, prior to the co-culture, astrocytes were stimulated with IFN- γ (100 ng/mL) for 48 h. Accordingly, IFN- γ - stimulated non-infected astrocytes indicated similarity with non-stimulated astrocytes, in which the percentage of the parasite population significantly decreased to $70.16 \pm 6.20\%$ ($p \leq 0.01$), $48.89 \pm 5.60\%$ ($p \leq 0.0001$) and $24.77 \pm 4.50\%$ ($p \leq 0.0001$) after 24, 48 and 72 h of co-culture, respectively (Figure 4.8-A). Parallel to the percentage of parasite population, MFI of gp63 GITC labeled- parasites significantly decreased in the co-culture in a time-dependent manner (Figure 4.8-B).

Overall, consistent with the previous study [172], no significant differences were investigated between IFN- γ - stimulated and non-stimulated astrocytes on the growth of *L. infantum* parasites, resulted in reducing in the parasite burden in the non-infected astrocytes and *Leishmania*-infected macrophages co-culture. The reduction of the parasite burden could be explained that astrocytes could decrease the number of parasites by the production of specific pro- and anti-inflammatory mediators, including IFN- γ (via the activation of signal transducer STAT1) during neuroinflammation [133]. Furthermore, upregulation of the GFAP and the reactive astrogliosis limit pathogenic spread in the CNS. Indeed, early IFN- γ -induced actions are pro-inflammatory; IFN- γ promotes inflammatory cell access, modifies their composition, elevates the expression of MHC, activates macrophages and microglia, and starts the death of oligodendrocytes [173]. Hindinger et al., (2012) showed that IFN- γ signaling to astrocytes performed a crucial protective function throughout the change from acute to chronic illness, but had no impact on the early course of the disease.

Besides, the previous study suggested that a neuroprotective response in astrocytes was shaped differently by the two main cytokines produced by Th1 and Th2 cells, IFN- γ and interleukin-4 (IL-4). IFN- γ could protect oxidatively stressed murine astrocytes to remove extracellular glutamate in culture; whereas IL-4 has no effect at any of the tested concentrations (10-100 ng/mL) [172]. Interestingly, astrocyte-conditioned media of the cells stimulated with IL-4 (10 or 100 ng/mL) or IFN- γ (10 ng/mL) reduced by 2-fold oxidative stress-induced neuronal apoptosis; however at higher concentrations (e.g. 100 ng/mL), IFN- γ was not found as neuroprotective [172]. In this study, there was no significant differences

between IFN- γ – stimulation or non-stimulated astrocytes regarding to the capacity for the parasite elimination considering the flow cytometry analysis.

Novel Dual-Fluorescent Flow Cytometric Approach for Quantification of Macrophages Infected with *L. infantum* Parasites

Flow cytometry analysis emerges as an alternative methodology to determine the rates of *Leishmania*-infected macrophages rapidly and accurately. Prior any investigation, the proposed procedure complies fluorescent staining of macrophages and *L. infantum* parasites separately by PKH26 and PKH67 lipophilic dyes, respectively. (Fluorescence microscopy analysis is used to verify the staining of each parasite and macrophage). Distinctive fluorescence signals emitted by the macrophage and the parasite enables the detection of uninfected and infected macrophages, where the fluorescent signal only from PKH26 indicates no presence of parasites inside the macrophage, while the merged fluorescent signal of PKH26 and PKH67 implies the occurrence of infection. The analysis allows the determination of infection of macrophages as percentage in total macrophage population. The procedure is fast and straightforward, once the configurations of laser and the borders of the gating are correctly set for the fluorescent-labelled macrophages and parasites. The sensitivity of the applied methodology is high to determine the presence of parasite inside the macrophages, yet the technique does not allow to estimate the number of parasites within each macrophage, which might be important to further assess the severity of the infection together with the percentage of macrophage infection. Thus, the introduced fluorescence-based methodology provides rapid analysis of higher numbers of cells than the alternative microscopic approaches can provide, and could be suggested as a more suitable approach for the evaluation of the potency of antileishmanial drug candidates [174-178]. Indeed, flow cytometry was already reported as a tool to investigate macrophage infection previously by multiple studies, where the aims of the researchers were evaluation of either antibacterial or antiparasitic activity of the tested drug molecules [174-178]. Among them, Abdullah et al. (1999) suggested a procedure, wherein *L. infantum* promastigotes were stained with PKH2-GL fluorescent dye before macrophage infection, which allowed accurate detection of cell infection [179]. However, could not detect intracellular amastigotes after prolonged incubation periods. Likewise, Giorgio et al. (2000) suggested labelling of intracellular amastigotes with *Leishmania* lipophosphoglycan-specific monoclonal antibody after macrophage infection [176]. That technique allowed accurate quantification of infection, but failed at detection of non-infected macrophages specifically. Therefore, the novel strategy

including the labeling of both macrophages and promastigotes by two distinctive fluorescent dyes (PKH26 red and PKH67 green, respectively) improved the detection capacity of the flow cytometry approach and distinguished the current study from the previous approaches.

Since the approach involves the simultaneous use of PKH26 and PKH67, certain modifications were also necessary in the applied flow cytometry procedure. An analysis gate on an FSC vs. SSC scatter plot was defined prior the flow cytometry analysis, where this graphical boundary defined the characteristics of cells to include for the analysis according to the size and internal complexity. Next, dual labeling necessitated FL1/FL2 dual-color fluorescence gating, which allowed distinction of intracellular parasites from free parasites where the two-color gating region identifies infected macrophages. Proportion of the infected macrophages in the total cell population can be precisely determined by the use of gates or quadrants. Thereby providing understanding on the level of infection and the success rate of the antileishmanial therapies tested.

Dual-PKH staining achieved high success rate in selective staining of the promastigotes (99.71%) and macrophages (99.57%) as verified by both fluorescence microscopy (Figure 4.10) and flow cytometry analysis (Figure 4.9). A negligible number of promastigotes were observed to remain unstained (Figure 4.9). In the flow cytometric (pseudo-color/smooth) plots, events were gated according to the total parasite population (Figure 4.9). The singlet promastigote population corresponded to 99.32% of the total parasite population, and 99.71% of the singlet population was found to be labeled with PKH67 green fluorescence dye as indicated by plot of FITC fluorescent channel (Figure 4.9-B). The unstained macrophage population was detected as 99.99% by flow cytometry, while singlets of PKH26-stained macrophages corresponded to 95.26% of the population and 99.57% of this singlet population was found to be labeled successfully (Figure 4.9-C, D).

As illustrated in Figure 4.11, *Leishmania*-infected and non-infected cells could be separated into two well-isolated populations using the flow cytometric quadrant analysis, which not only allowed quantitative estimation of infection rates (%) of macrophages, but also enhanced a discrimination of noninfected macrophages. The percentage of parasite-infected macrophages with infection ratio of 1:2.5 and 1:10 was determined as 15.68% and 61.70%, respectively (Figure 4.11-C, D). Infection rates of the macrophages were found to increase in parallel to the initial treatment ratios as revealed by mean fluorescence intensity of double-

positive macrophages, and the difference between the infection rates were found to be statistically significant ($P \leq 0.0001$).

The quantitative results of flow cytometry analysis were supported by fluorescence microscopy analysis (Figure 4.10). Providing qualitative information on the infection rates, fluorescence microscopy displayed the macrophages (red) infected by *L. infantum* parasites (green) with the merged yellow color. Fluorescence microscopy analysis also showed that PKH staining did not cause any dye-induced cell death on both macrophages and *L. infantum* promastigotes as supported by the literature [180]. These findings suggest that PKH26 and PKH67 are safe to be used for macrophages and *L. infantum* parasites, while some toxicity problems have been reported for PKH2-GL staining on *L. donovani*, *L. major* and *L. infantum* parasites and macrophages infected therewith [179]. The lower infection rates in *L. infantum*-infected macrophages was explained in linked with the toxicity effect of PKH2-GL, which can be seen as reduced forward scatter in the dot plot [179]. Contrary, in our study the high infection rates up to 61.70% indicated that the PKH26 and PKH67 dyes can remain intact within the cell membrane and are safe for use at least for 24-hour incubation periods.

Overall, the findings of this study introduce the applied flow cytometry procedure as a reliable approach to quantify *Leishmania* infection in J774 A.1 macrophages infected with *L. infantum* parasites with high specificity. Dual-colored fluorescence gating for PKH26-stained macrophages and PKH67-stained *L. infantum* promastigotes in flow cytometric approach emerges as a better alternative to the conventional microscopic approach, in particular for the analysis of large macrophage populations with *L. infantum* infections in a shorter period of time.

Antileishmanial Effect of SPT on L. infantum Promastigotes and Cytotoxicity of SPT on Macrophages and Astrocytes

Upon treatment of 75, 100, 125, 150, and 200 μM SPT, the proliferation of *L. infantum* promastigotes were inhibited in a time-dependent manner at 24, 48, and 72 h. Furthermore, the increase in SPT concentrations above 100 μM did not cause any significant further change in the inhibition of promastigote proliferation (i.e., 20%). According to the cell viability analysis, IC_{50} values (24, 48, and 72 h) for SPT were calculated as $83.31 \pm 1.61 \mu\text{M}$, $67.11 \pm 6.52 \mu\text{M}$, and $71.99 \pm 7.51 \mu\text{M}$, respectively (Table 4.1 and Figure 4.13). The results

revealed that with lower IC₅₀ values of SPT at 48 and 72 h-incubations are more effective against *L. infantum* promastigotes than 24 h-incubation. In addition, the increase in the SPT concentration above 100 µM did not show any toxicity on non-infected macrophages, revealing that SPT concentrations of 50, 75, 100, 125, and 150 µM have safety features for antileishmanial therapy on healthy macrophages.

The obtained results revealed that SPT treatment has significant efficacy on *L. infantum* parasites by reducing cellular toxicity on macrophages and astrocytes. Among effective concentrations on *L. infantum* promastigotes, SPT indicated the highest potential to inhibit parasite proliferation within the range of 75-200 µM, without displaying any toxicity on macrophages and astrocytes (Figure 4.22-B).

Morphological analysis

In line with the parasite viability data, as shown in phase-contrast microscopy and SEM images in Figure 4.14 and 4.15, treatments with SPT indicated a dose-dependent reduction in the cell number, and transformation from elongated-shape to round-shaped morphologies, after 24 and 48 h of incubation. This is attributed to the loss of membrane and flagellum integrity of *L. infantum* promastigotes induced to apoptosis. Moreover, morphological changes such as spherical shape and shrinkage were strong signs of apoptosis [134, 135].

Mitochondrial membrane potential of L. infantum promastigotes after SPT treatment

Apoptosis is critical mechanism for the programmed cell death (PCD), and has been revealed in kinetoplastid protozoans, including *Leishmania* spp.. Specifically defined morphological and biochemical characteristics of apoptosis include cell shrinkage, blebbing of the plasma membrane, loss of mitochondrial membrane potential, chromatin condensation, and nuclear disintegration. In majority of the organisms, the evidence of apoptosis-like death are the disruption of mitochondrial transmembrane potential (mainly due to mitochondrion permeabilization) and nuclear DNA degradation [181]. There is significant experimental evidence that trypanosomatids have functional apoptosis-like programmed cell death pathways. Previous descriptions of PCD in Trypanosomatids including *L. amazonensis*, *T. cruzi*, and *T. brucei rhodesiense* have also been described before [182]. Although *Leishmania* organisms and metazoan apoptosis share many biochemical markers, the molecular machinery involved is very different and poorly understood. Therefore, a stronger comprehension of the target-dependent mechanistic mechanisms underlying apoptosis-like

PCD in protozoan protists would be extremely helpful when developing appropriate chemotherapeutic treatments.

Therefore, in this study, PCD was also confirmed by mitochondrial membrane potential using Rhodamine 123 mitochondrial specific fluorescent dye, and DAPI staining of *L. infantum* promastigotes. Similar to growth inhibition on SPT-treated promastigotes, ≥ 100 μM concentrations of SPT treatment led to a significant decrease in the mitochondrial membrane potential on *L. infantum* promastigotes ($p \leq 0.0001$), which was reduced dramatically to below 53% after 24 h and below 35% after 48 h compared to negative control ($P \leq 0.0001$) and apoptosis-like features were observed in the drug treated promastigotes (Figure 4.16). In line with this result, in the previous study, Bertholet and colleagues indicated that depolarization of mitochondrial membrane potential and the subsequent stimulation of caspase-like activity led to PCD. Additionally, isolated from infected animal-isolated *L. donovani* also exhibited the induction of caspase-like activity [182].

DAPI staining of L. infantum promastigotes after SPT treatment

In order to observe the change in cell viability and apoptosis after SPT treatment, the nucleus and kinetoplast DNA were labeled with DAPI. As shown in Figure 4.18, after 24 and 48 h of incubation with 75, 100 and 150 μM of SPT, a decrease in the number of parasite nuclei was observed with loss of DAPI fluorescence intensity according to fluorescence microscopy images. These results reinforce the notion that SPT treatment led to a significant reduction in the parasite viability upon 24 h and induce the changes of mitochondrial membrane potential resulted in the apoptosis-like death of promastigotes.

Cell Cycle Analysis of L. infantum Promastigotes Treated with SPT

Previous study has shown that SPT induced apoptotic cell death in cancer cells including phosphatidylserine exposure, apoptosis, and DNA fragmentation [67]. In this study, after detection of the mitochondrial membrane disruption linked to apoptosis-like cell death in *L. infantum* promastigotes, cell cycle was analysed to ascertain damage to the parasites. Accordingly, the proportion of cell population in S and G2/M phase was found to vary dose-dependent (0-100 μM) on SPT treatment, indicating a cell cycle arrest induced in S and G2/M phase (Figure 4.19 and 4.20).

Quantitative PCR Analysis of L. infantum Promastigotes Treated with SPT

In mammalian cells, caspase activity is specifically detected using fluorogenic peptides, and inhibited with substrate analogues, whereas protozoan parasites can show deficiency in caspases, but activity towards small peptide substrates has been detected on several occasions, presumably reflecting the myriad other highly active peptidases that occur in parasitic protozoa [183]. The cysteine peptidases known as metacaspases found in parasitic protozoa are related to caspases but have an arginine-directed substrate selectivity and do not cleave caspase substrates. The recent studies strongly indicated the function of unusual proteases known as "metacaspases" in the parasitic protozoan programmed cell death process [183, 185], and metacaspases (MCAs), distant orthologs of caspases, have been also postulated to participate in yeast and plant programmed cell death [184, 185].

All *Leishmania* spp. express one single metacaspase gene with the exception in *L. infantum* and *L. donovani* (*Ld*) spp. in which two different metacaspases have been shown [184, 185]. Structurally, they share an N-terminal region with a putative mitochondrial localization signal and a less conserved proline-rich C-terminal domain that is likely involved in protein-protein interactions. They also have a conserved Cys-His catalytic dyad [187, 188]. According to *L. major*, which has a functional N-terminal mitochondrial localization signal, MCAs may indirectly influence mitochondria to cause cell death [189]. While MCA of *L. mexicana* was discovered in the mitochondria and connected to the mitotic spindle, *L. donovani* *LdMCA-1* and *LdMCA-2* were shown to be upregulated in promastigote and axenic amastigote forms [190]. According to the study of Castanys-Muoz et al. (2012), *LdMCAs* showed arginine/lysine substrate selectivity without any auto-processing. *LmjMCA* has the same arginine/lysine substrate specificity as *LdMCAs* but was activated by auto-processing [191]. Importantly, the elimination of MCA in *L. mexicana* had no impact on procyclic promastigotes' ability to proliferate and remain viable. Additionally, the reduced replication rate caused by MCA overexpression in amastigotes revealed that MCAs function as a growth suppressor that is particular to amastigotes.

According to reports on the role of MCAs in *Leishmania* cell death, *L. major* MCA causes parasite cell death by different mechanisms, including secreting its catalytic domain or acting together with the other mechanistics involved in stress regulation or cell death in the presence of apoptotic stimuli [192, 193]. Additionally, experimental data revealed that the autophagy process took place in low nutritional concentrations, which led to an

overexpression of *L. major* MCA [192]. Moreover, it was discovered that *LmjMCA* overexpression induced *L. major* more sensitive to oxidative stress than wild-type parasites that had optimal levels of endogenous metacaspase expression [194].

The topoisomerase type I-B inhibitor induced apoptosis in *L. donovani* appears to be predominantly linked with up-regulation in the expression level of metacaspases and the induction of oxidative stress, according to a report by Chowdhury et al. (2014) expression [195]. Interestingly, the recent study indicated that peptide ecotin-like ISP3 of *L. major* preferentially binded to MCAs and inhibited its trypsin-like activity in presence of heat shock, and thus dramatically decreasing parasite cell death [196].

In the light of the this information, the qPCR analysis indicated that MCA5 (apoptotic-like death gene) level decreased as approximately 0.15 fold in 50 μ M SPT treated-*L. infantum* promastigotes ($p \leq 0.01$), while 100 μ M SPT treatment led to a significant increase in MCA5 gene expression on promastigotes ($p \leq 0.05$). Instead, 75 μ M SPT treatment did not significantly differ MCA5 mRNA levels (Figure 4.21-C). Observed MCA5 expression is consistent with mitochondrial membrane potential data (Figure 4.21-C) indicating incidence of higher cell death when treatment 100 μ M SPT applied. Conclusively, induction of apoptosis-like death in *Leishmania* spp. associated with the metacaspases was reported for the treatment of different antileishmanial compound to occur via metacaspase-dependent mitochondrial impairment and stress-induced cell death, and cell cycle arrest [182, 185].

On the other hand, Meta 1 gene were evaluated in *L. infantum* promastigotes after SPT treatment at 24 h. In the life cycle of the promastigote within the insect tract, *Leishmania* differentiate non-infective (procyclic form) into infective (metacyclic) forms [197]. Previously, genes including Meta 1, that are largely or exclusively expressed in metacyclic promastigotes of all *Leishmania* spp. as compared to proliferating forms have been discovered in an effort to understand the signals that trigger the metacyclic differentiation stage [197-199].

The Meta 1 gene expression level was detected at least 20-fold upregulated during metacyclogenesis, which was lower in procyclic promastigotes and amastigotes compared with high levels in metacyclic promastigotes, and these expression might indicate a functional role for meta 1 in the infective insect stages [197]. The previous study indicated that meta 1 gene has been indicated to be essential and overexpression of META1 protein in

L. amazonensis, leading to a increase in the virulence of the mutant parasites in susceptible mice [198]. In addition, The META1 protein also displays as a significant inducer of a Th-2 type immunological response in the mammalian host [198, 199].

According to this study, qPCR assay results indicated that Meta 1 gene (in infective metacyclic promastigote forms) levels significantly decreased in 75 μ M SPT (≈ 0.50 -fold) ($p \leq 0.05$) when compared to negative control group, and thereby indicating the decreased the metacyclogenesis into metacyclic promastigotes on *L. infantum* promastigotes after 24 h of SPT treatment (Figure 4.21-A).

The small hydrophilic endoplasmic-reticulum-associated protein (SHERP) and the hydrophilic surface proteins HASPA1, HASPA2, and HASPB have been introduced as an markers of the infective promastigote (metacyclic forms), which has been observed both *in vitro* as well as the in the vector [200, 201]. Also, SHERP is expressed at low levels in the early short promastigote stage, where it is not expressed in amastigotes. Furthermore, the previous studies suggested that the HASP/SHERP proteins are critical for development and vector parasites in the sand fly as well as vector transmission [201].

In this Ph.D. study, SHERP metacyclogenesis gene expression level increased as approximately 1.9-fold on 50 μ M SPT-treated promastigotes ($p \leq 0.01$), whereas 75 and 100 μ M SPT treatment did not lead to any significant change on promastigotes (Figure 4.21-B). Since the promastigote stage could not be detected within the sandfly vector, as expected, no significant alteration was observed on the gene expression level at the optimum doses 75 and 100 μ M SPT after 24 h of incubation. Although the reduction in the gene expression did not show a significancy, 100 μ M SPT led to a decrease in the level of relative gene expression (Figure 4.21-B).

Antileishmanial Effect of SPT on Macrophages and Astrocytes Infected by L. infantum Promastigotes

Flow cytometry analysis enabled the quantitative determination of the infection rates of macrophages and astrocytes, as previous described in the section of 3.3. Optimization Studies For Flow Cytometric Analysis For Quantification Of Macrophages Infected with *L. infantum* Parasites

The treatment of *Leishmania*-infected macrophages with $\geq 50 \mu\text{M}$ SPT (i.e. 50, 75, 100, 150, 200, and 400 μM) for 24, 48, and 72 h did not significantly alter the infection rate regardless of time and concentration, when compared to NC treatment groups, and determined above 74 % (Figure 4.26-4.29). Although treatment of infected macrophages with SPT did not lead to a concentration- and time-dependent decrease in macrophage infection rate, the infection rates remain almost stable (nearly 74%) regardless time 24, 48 and 72 h-treatment. Besides that, upon treatment of 75, 100, 125, 150, and 200 μM SPT led to a significant growth inhibition on *L. infantum* promastigotes in a time-dependent manner at 24, 48, and 72 h (Figure 4.12). This could be explained by not only the reduction of the parasite inside the macrophage but also nontoxic and safety properties of SPT on the non-infected healthy macrophages regarding to the increase in the percentage of macrophages after SPT treatment.

On the other hand, when astrocytes were incubated with *L. infantum* promastigotes, the infection rate of astrocytes were found as approximately 23 %, which significantly decreased to $20 \pm 3.33\%$ ($p \leq 0.05$), $17 \pm 2.86\%$ ($p \leq 0.0001$), $11.89 \pm 3.56\%$ ($p \leq 0.0001$), $7.97 \pm 1.51\%$ ($p \leq 0.0001$), and $12.63 \pm 1.78\%$ ($p \leq 0.0001$), after treatment with 75, 100, 150, 200, and 400 μM SPT at 24 h, respectively (Figure 4.26-B and Figure 4.30). Interestingly, after 48 h of incubation, the infection rate of untreated NC group indicated a decrease in the parasite load (i.e. 2.91%), which remained almost stable (nearly 3%) regardless of further increase in SPT concentration (Figure 4.26-B and Figure 4.31); whereas after 72 h-incubation, treatment of *Leishmania*-infected astrocytes with 75 μM SPT resulted in a significant reduction in the viability of the cells sharp to approximately 4% (i.e., 3.75%) ($P \leq 0.0001$), when compared to the infection rate of NC group (i.e. 13%) (Figure 4.26 and 4.32); which became close to 7% in 100, 150, 200 and 400 μM SPT - treated groups at 72 h-treatment (Figure 4.32).

Regarding to the flow cytometry analysis, the infection rate of astrocytes infected with *L. infantum* promastigotes were detected as significantly lower than the infection rate of macrophages. This could be explained by the pro-inflammatory response of the astrocyte against the infection. Moreover, in the previous and next discussion sections, it was explained and discussed in detail linked with astrocyte-mediated immune activation and the releasing of pro-inflammatory cytokines.

Overall, the results obtained in cell culture analysis demonstrated that SPT led to an inhibition on the growth of *L. infantum* parasites and cell division inside the macrophages.

Furthermore, even at high doses, $\geq 75 \mu\text{M}$ SPT had a significant antileishmanial effect on *Leishmania*-infected macrophages and *Leishmania*-infected astrocytes without displaying any toxicity on healthy macrophages and astrocytes.

ROS level and Oxidative stress in Leishmania-infected macrophages after SPT treatment

Macrophages display conflicting functions in *Leishmania* infection, either by destroying internalized parasites or by offering a secure environment for parasite reproduction. However, in response to invading pathogens, macrophages initiate an oxidative burst as a defense mechanism to increase pathogen clearance and contribute to signaling cascades including inflammation and the immune response [202]. Free radicals protect against invasive microorganisms as a result of oxidative stress, but they can also trigger inflammation, which can lead to tissue damage. To counteract the harmful oxidative burst, the host's antioxidant defenses are activated in response to the generated oxidative stress caused by parasite infections [202].

Oxidative stress occurs in the result of the excessive release of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [202, 203]. Despite the widespread belief that ROS radicals are mostly destructive and deleterious for cells, it has become increasingly evident that low quhomeostasis of ROS, such as superoxide and hydrogen peroxide (H_2O_2), are necessary for maintaining homeostasis and for cell fate determination. ROS can interact with numerous different compounds, including lipids, proteins, nucleic acids, and carbohydrates, in a quick manner, and causes damage to biological systems as a result of these interactions. The rapid induction of oxidative stress by macrophages in response to intracellular pathogens, such as *Leishmania* parasites, serves as a mechanism of defense to promote pathogen clearance and activate signaling pathways involved in inflammation and immunological responses.

Although the fact that increased reactive oxygen species production contributes to parasite clearance, increased amounts of these free radicals can induce inflammation and cause severe damage to host cells and tissues, potentially leading to severe diseases. For instance, in the case of malaria, The parasite breakdown of hemoglobin within the infected red blood cell results in free heme, which is released after the end of the replication cycle [204]. This increases the oxidative burden on the host and may be a factor in the severity of life-threatening malarial consequences. In addition, excessive inflammatory response to malaria

disease contributes to worsen the oxidative response [204]. Therefore, maintaining the oxidative balance in the parasitic infections are important to prevent lethal complication of the disease.

According to the results of this study, all SPT concentrations (75-200 μ M) had no significant effect the intracellular macrophage ROS level based on the detected DCF fluorescence signal at 24 h by flow cytometry analysis in each sample group when compared to negative control (Figure 4.37). In parallel, positive control (PC) exposure for 1 hour increased significantly in the relative ROS level up to 50%, as given in the flow cytometry histograms and the relative ROS graph (%) ($p \leq 0.0001$) (Figure 4.37-A, B and C).

Interestingly, the previous study confirmed that differentiation associated with the ROS induction occurs in medically significant visceralizing *Leishmania* species, including *L. infantum* and enhance mechanistic basis for the previous reports revealing prominently increased *L. infantum* promastigotes virulence upon pre-treatment of oxidative reagents [205]. Regarding to the study, ROS-generating methods including to sub-lethal concentrations of H_2O_2 exposure are critically effective in supporting promastigote to amastigote differentiation resulting in higher survival rates, and gene expression characteristic of the amastigote forms [205]. Furthermore, they demonstrated the previously described function of ROS in the differentiation of the virulent amastigote form, which lead to severe life-threatening forms of the diseases [206].

On the other hand, another study showed that the stimulation of ROS production by infected peritoneal macrophages, however no significant effect were determined in parasite killing in *in vitro* *L. amazonensis* infection [207]. Also, ROS did not seem to be critical to parasite elimination *in vivo*, however they controlled the lesion sizes at onset of the infection.

Similar to ROS level, all concentrations of SPT did not cause a significant alteration in oxidative stress by remaining at almost stable GSH levels, when compared to NC untreated group (Figure 4.39).

Conclusively, SPT treatment did not lead to a significant change in the relative ROS and GSH level in *Leishmania*-infected macrophages, which might be beneficial for the associated with preventing the parasite replication inside the macrophages, and increasing the the number of macrophages without displaying any toxicity.

ROS level and Oxidative stress in Leishmania-infected astrocytes after SPT treatment

All SPT concentrations, except 100 μ M, led a significant decrease in the intracellular astrocyte ROS level based on the detected DCF fluorescence signal at 24 h by flow cytometry analysis in each sample group when compared to negative control, and determined below 30% (Figure 4.38). The treatment of *Leishmania*-infected astrocytes with 50, 75, 150 and 200 μ M SPT for 24 h resulted in a significant decreased close to 28% (i.e. 28.24%) ($p \leq 0.01$), 22% (i.e. 21.83%) ($p \leq 0.001$), 29% (i.e. 29.22%) ($p \leq 0.01$), and 18% (i.e. 17.85%) ($p \leq 0.0001$), respectively. In parallel, positive control (PC) exposure for 1 hour increased significantly in the relative ROS level up to 80% (i.e. 84%), as given in the flow cytometry histograms and the relative ROS graph (%) ($p \leq 0.0001$) (Figure 4.38-A, B and C).

In line with the previous study, a decrease in the production of mitochondrial ROS level enhance both the reduction of the intracellular parasitic *Trypanosoma cruzi* infection rate and inhibition of parasite multiplication in the astrocytes primed by EVs from *T. cruzi* trypomastigotes [208]. This parasite expresses antioxidant enzymes that are essential for protecting against oxidative damage in order to achieve this goal, enabling the parasite to overcome such oxidative conditions. Paradoxically, although macrophage-mediated ROS generation is important to reduce parasite burden in intracellular parasitic infection, including Leishmaniasis, by T cell-mediated immunity, parasites produced ROS using its own metabolism, and must deal with the ROS and related host cell oxidative stress during the intracellular stages of its lifecycle. Besides that, the evidence suggested that ROS production is beneficial to parasitic infection [208].

On the other hand, the another study reported that the induction of ROS production control lesion sizes at early stages of infection rather than parasite killing, they control the inflammatory reaction possibly by regulating neutrophils numbers in lesions [207].

In parallel to determination of ROS level, the antioxidant capacity of SPT was investigated on *Leishmania*-infected astrocytes by the measurement of GSH level on the untreated and treated cells. According to the results, 50, 100, 150, and 200 μ M SPT led to a significant decrease in oxidative stress by inducing GSH levels significantly, when compared to NC (Figure 4.40). Therefore, *Leishmania* infection-induced oxidative stress remarkably reduced on infected astrocytes by SPT treatment through induction of elevated GSH level.

Quantitative PCR Analysis of Leishmania-infected macrophages after SPT treatment

The astrocyte has been hypothesized to serve as an antigen-presenting cell in CNS and can behave similarly to monocytes and macrophages. Upon exposure of IFN- γ or viruses, the astrocyte can be induced to MHC express class II antigen, and can present antigen to T cell clones in an MHC-restricted manner. This APC function shows that the astrocyte plays a crucial part in the maturation of immunological responses within the CNS. Furthermore, astrocytes produced a different of immunoregulatory signaling molecules such as pro-inflammatory mediators IL-1, IL-3, IFN- α/β , TNF- α and IL-6 with the stimulation [209]. In addition, the previous study indicated that IFN- γ have a critical role in the destruction and elimination of *Toxoplasma gondii* in the host cells via the activation of signal transducer STAT1, which leads to the induction of IFN- γ – dependent gene transcription [170]. In this study, transgenic mice with specific deletion of STAT1 in astrocytes were used, and STAT1 GFAP mutant astrocytes had a larger number of parasite cysts, which resulted in a change in parasite tropism from neurons to astrocytes. There are no *Toxoplasma gondii* astrocytic cysts in wild-type mice, which may be related to the protective role of astrocytes in response to IFN- γ and clearance of parasites [170].

On the other hand, The development of a Th1 immune response is essential for mammalian host defense in the intracellular parasitic infections, including leishmaniasis due to enabling the leishmanicidal activity by macrophage. VL (*L. infantum*) has been associated with the increased levels of immunosuppressive cytokine IL-10, which leading to the inhibition of NO production and the release of pro-inflammatory cytokines (IL-1, TNF- α and IL-12) [101]. on *Leishmania*-infected macrophages. Similarly, TGF- β cytokines are produced by *Leishmania*-infected macrophages and inhibit iNOS expression by macrophages, and IFN- γ production by natural killer (NK) cells [97]. It has been proposed that IL-10 production by Th1 or Th2 cells serves as a key feedback regulator regulating the pathophysiology linked to an excessive, even effective, inflammatory response [106]. Besides, IL-12 is a crucial cytokine involved in the activation of lymphocytes, which triggers the generation of IFN- γ resulting in the activation of macrophages [210].

Considering the previous studies, the current Ph.D. study indicated that IFN- γ levels overexpressed significantly in all SPT treatment groups (Figure 4.41-A), when compared to the negative control group. The overexpression of IFN- γ gene was consistent with the

stability in the infection rate of *Leishmania*-infected macrophages, and resulted in the killing of parasite inside the host macrophages. Likewise, 200 μ M SPT treatment led to a significant increase in the TNF- α (6.05-fold) ($p \leq 0.01$) (Figure 4.41-B), and IL-12(B) gene expression (24.13-fold) ($p \leq 0.0001$) (Figure 4.41-C), compared to the NC group, which was in line with reducing the infection rate of macrophages after 200 SPT μ M treatment, and may contribute to protective responses by augmenting IFN- γ -mediated macrophage activation. Also, IL-12 triggers the production IFN- γ by T cells and NK cells, and enhances Th1 cell development, suppresses Th2 cell response (IL-4), and plays a critical role in a protective immune response [210].

On the other hand, interestingly, all SPT concentrations except 200 μ M SPT, led to a significant reduction in NF- κ B gene expression (Figure 4.41-D). Previously, NF- κ B gene activity were found a crucial role for the initiation of the innate immune responses and the protection through Th1-mediated immunity, might be down-regulated by *Leishmania* parasites, resulted in the hyporesponsiveness by macrophages. The increase in the IFN- γ and IL-12 (B) relative gene expressions could be explained by the induction of STAT1-mediated activation. Besides, in the relative gene expression level of IL-1 β did not lead any significant change after SPT treatment on the infected macrophages (Figure 4.41-E), which displays a sign of the prevention Th2 cell response upon polarisation of Th1 cells to Th2 cell, and thereby enhancing the generation of a protective immune response. Similarly, Scorei and his co-workers have reported that one of the boron derivative, calcium fructoborate treatment has decreased IL-1 β and IL-6 protein expressions, and NO levels in the culture supernatant [65, 76]. Besides, 150 and 200 μ M SPT did not cause a significant decrease on immunosuppressive IL-10 gene expression level of infected macrophages (Figure 4.41-F), whereas it was significantly downregulated in infected macrophages upon treatment of 50 μ M ($p \leq 0.05$) and 100 μ M SPT ($p \leq 0.01$). Consistent with IL-10, TGF- β 1 gene expression significantly decreased after 100 μ M SPT treatment ($p \leq 0.0001$), and also on 200 μ M SPT-treated macrophages ($p \leq 0.01$). It has been proposed that IL-10 production by Th1 or Th2 cells serves as a key feedback regulator regulating the pathophysiology linked to an excessive, even effective, inflammatory response [106]. As similar to IL-10, TGF- β is produced by infected macrophages and suppresses of iNOS expression by macrophages in an autocrine and paracrine manner. Therefore, a significant decreases in both IL-10 and

TGF- β relative gene expression are important for the induction of the pro-inflammatory signaling, and elimination of the intracellular parasites, especially in *L. infantum*.

qPCR Analysis of Leishmania-infected astrocytes after SPT treatment

qPCR analysis indicated that both IFN- γ and TNF- α genes overexpressed significantly in 100 and 200 μ M SPT treatment on infected astrocytes, respectively (Figure 4.42-A), when compared to the negative control group. In the previous study, Chung et. al., (1990) reported that astrocytes have the capacity to release TNF- α in response to the biologic stimuli such as infection, especially cytokines IL-1 and IFN- γ , which play a critical role in the CNS during neurodegeneration associated with the inflammation [209]. Similarly, in another study, *in vitro* model mimicking the acute neuroinflammation nervous were cultured in cerebellar granule neurons in the presence of IFN- γ -containing media without astrocytes, resulted in the induction of quick neuronal apoptosis characterized by the expression of cleaved caspase-3, and intercellular Ca²⁺ influx. Contrary, the neurons were significantly protected from the damage in the presence of astrocytes via the release of IFN- γ together with IL-6 [171].

On the other hand, the gene expression level of IL-12(B) was significantly upregulated in infected astrocytes treated with 100 μ M (3.45-fold increase) and 150 μ M SPT (3.08-fold increase) ($p \leq 0.0001$) (Figure 4.42-C), interestingly, while treatment of 200 μ M SPT cause a significantly decrease on IL-12 (B) gene expression. This could be explained by the fact that SPT treatment could induce IL-12(B) secretion on *Leishmania*-infected macrophages, results in the production of immune response and, the development of Th1-type immune responses. For instance, after infection, astrocytes have been indicated to secrete cytokines, such as IFN- γ , TNF- α , and IL-12, that alter microglia and macrophages to a more pro-inflammatory phenotype [211]. However, under similar circumstances, astrocytes have also been found to significantly generate cytokines, including TGF- β and IL-10, which can cause monocytes to reduce their inflammation [211]. In line with the literature, as expected, the gene expression levels of IL-10 were downregulated upon treatment of 50, 100, 150 and 200 μ M SPT in infected astrocytes compared to NC group (Figure 4.42-F), whereas TGF- β 1 gene expressions did not show any significant alterations after SPT treatment in infected astrocytes (Figure 4.42-J).

The pro-inflammatory cytokine IL-1 β gene overexpressed (3.81-fold increase) in infected astrocytes treated with only 150 μ M SPT ($p \leq 0.01$), and all treatment group except 150 μ M SPT did not lead to a significant alterations on IL-1 β gene expression of infected astrocytes, when compared to NC group. IL-1 β gene is a crucial regulator of inflammation and neuronal death in acute CNS traumas, including stroke and brain trauma [1, 5], and has been linked to neurodegenerative diseases like Alzheimer's disease [5, 32]. It has been proposed that factors secreted from IL-1 β activated astrocytes cause primary human neurons to undergo apoptosis and undergo significant alterations in their metabolic activity [13]. Besides, Scorei and his co-workers have reported that one of the boron derivative, calcium fructoborate treatment has decreased IL-1 β and IL-6 protein expressions, and NO levels in the culture supernatant [76]. Therefore, no significant alteration in IL-1 β gene expression could be explained by the effect of SPT treatment in the case of astrocyte infection by *Leishmania* parasites. Similarly, STAT1, iNOS, and MMP-2 expressions did not show any significant alterations after SPT treatment in infected astrocytes, when compared to NC group (Figure 4.42-G, H, and I), which could be linked to the SPT-mediated dual role in both inflammatory and anti-inflammatory response, together. The previous study indicated that both boric acid and sodium borate suppress the critical inflammatory mediator genes, iNOS and COX-2, and reduce NO production levels in a dose-dependent manner [81]. On the other hand, boron (borax as orally) could increase lymphocyte proliferation, and further induced TNF- α , IL-6, IL-1 β secretion, and iNOS expression by the LPS-primed macrophages, more specifically the M1 macrophages in a dose-dependent manner [80].

In the light of the previous studies, the inflammatory response of SPT has been shown in this Ph.D. study under the condition of *Leishmania* infection on macrophage and astrocytes with displaying safety features on healthy macrophages and astrocytes. At this point, it has to be noted that an increase in the inflammatory factors are not always a good solution for the infection process and also for a wound healing treatment strategy considering the immunological inflammatory process of *Leishmania* parasites infection (“Leishmaniasis”). However, pro-inflammatory response together with anti-inflammatory approach can take boron one step forward to prevent the disease progression, provide the parasite eradication, and the cutaneous wound management application.

6. CONCLUSION

Leishmaniasis is a neglected tropical diseases, caused by the protozoan parasites of the genus *Leishmania* that affect numerous species including humans, and transmitted by the bite of phlebotomine sandflies. It is endemic in over 98 countries year, including Turkey and its subcontinental region, i.e. Southern Europe, the Middle East and North Africa, with more than 700 million people at risk as estimated that 1.3. million new cases of leishmaniasis occur every year. Clinical manifestations of human leishmaniasis, caused by around 20 *Leishmania* species, are divided into three clinical forms: cutaneous, mucocutaneous or visceral leishmaniasis known as "Kala Azar", depending on which species is involved in the infection.

Numerous research have focused on the CNS in an effort to show that *Leishmania* species can indeed cross the BBB and trigger clinical manifestations and clinical neurological symptoms [111, 112, 113]. In the previous studies, the presence of anti-*Leishmania* antibodies were detected in the CSF of *Leishmania*-positive dogs, leading to the alterations in the nervous tissue, and may result into a disease known as "cerebral leishmaniasis".

Addressing to the lack of knowledge on the neurological aspects of leishmaniasis, the present study introduces the immunological mechanism associated between macrophages- and astrocytes-infected with *L. infantum* on bone-marrow derived primary macrophage cells by *in-vitro* studies. Therefore, this Ph.D. study highlighted the three notion, including (i) the investigation of how *Leishmania* parasites are transmitted into the brain via macrophages, and the mechanism underlying the inflammatory responses on both macrophages and astrocytes in infection model, (ii) novel dual-fluorescent flow cytometric approach for quantification of macrophages infected with *Leishmania infantum* parasites, and (iii) the evaluation of antileishmanial efficacy and anti-neuroinflammatory activity of SPT compound on *L. infantum* parasites, *Leishmania*-infected macrophages and *Leishmania*-infected astrocytes in *in vitro* cultures.

According to the results, in the co-culture of *Leishmania*-infected macrophages with non-infected astrocytes, the percentage of *L. infantum* parasites were significantly decreased when compared to NC group, indicating the inhibition of parasite proliferation in the co-culture system. Likewise, SPT treatment of *Leishmania*-infected astrocytes cultured without

infected macrophages led to a significant reduction of parasite load in the host astrocytes. The infection rate of astrocytes were determined as approximately 7% at 72 h of SPT-incubation, which displayed the lower infection rate than the co-culture (i.e. 24.88%), and similarly, the significant reduction of the promastigotes were detected after SPT treatment through the apoptotic-like programmed cell death and astrocyte activated-immune response to the infection.

It is the first study to emphasize the communication between *Leishmania*-infected primary macrophages and astrocytes regarding the parasite load, and inflammatory response against the cell-specific infections. Additionally, the antileishmanial efficacy of SPT together with the neuroinflammatory-based antileishmanial SPT therapy was shown in detail mechanism on *L. infantum* promastigotes, *Leishmania*-infected macrophages and *Leishmania*-infected astrocytes; whereas the analysis on non-infected macrophages examined the safety of SPT in the *in vitro* model. The promising antileishmanial efficacy of the SPT *in vitro* *Leishmania*-infection model highlights the potential of antileishmanial therapy in the future in further *in vivo* studies and clinical studies.

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APPENDIX A: ETHICS STATEMENT



T.C. YEDİTEPE ÜNİVERSİTESİ
Hayvan Deneyleri Yerel Etik Kurulu (HADYEK)

ETİK KURUL KARARI

Protokol No	Toplantı Tarihi	Toplantı Sayısı	Karar No	Proje Yürütücüsü
2021-019	02.03.2021	2021/03	2021/03-5	Prof. Dr. Fikrettin Şahin
'Leishmaniasis'in Nörolojik Açıdan İmmunolojik Mekanizma Yoluyla in-vitro, ex-vivo ve in vivo modelde İncelenmesi' isimli proje oy birliğiyle etik açıdan uygun görülmüştür.				
Hayvan Türü / Irkı		Toplam Hayvan Sayısı		Hayvanın Cinsiyeti
Fare/BALB/c		76		Erkek veya Dişi

Görevi	Adı Soyadı	Katılım Durumu
Başkan	Prof. Dr. Bayram YILMAZ	
Başkan Vekili	Prof. Dr. Erdem YEŞİLADA	
Üye	Veteriner Hekim Engin SÜMER	
Üye	Prof. Dr. M. Ece GENÇ	
Üye	Prof. Dr. Rukset ATTAR	
Üye	Prof. Dr. Gamze TORUN KÖSE	
Üye	Doç. Dr. Ediz DENİZ	
Üye	Doç. Dr. Aylin YABA UÇAR	
Üye	Doç. Dr. Burcu GEMİCİ BAŞOL	
Üye	Hakan GÖKSEL	
Üye	Ahmet ŞENKARDEŞLER	