Universidade Nova de Lisboa

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Differentiation of subpopulations of T lymphocytes by species of *Leishmania* causative of American cutaneous leishmaniasis

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Resumo

Diferenciação de subpopulações de linfócitos T induzida por espécies de *Leishmania* causadoras de leishmaniose tegumentar americana

Patrícia Relvas Mourata Gonçalves

PALAVRAS-CHAVE: Leishmaniose tegumentar americana; Células T; Imunidade adquirida; Células T reguladoras.

Leishmaniose tegumentar americana (LTA) é uma doença negligenciada causada por parasitas intracelulares do género *Leishmania.* LTA afeta o centro e o sul do continente americano e pode apresentar variadas manifestações clínicas, mas geralmente é definida por ocorrência de lesões cutâneas que podem causar desfiguração. Apesar de afetar uma parte considerável da população pouco se sabe sobre a resposta imunitária às diversas espécies de *Leishmania* que causam esta doença. Assim, o principal objetivo deste trabalho foi estudar *in vitro* a diferenciação de subpopulações de linfócitos T induzida por parasitas causadores de LTA. Para realizar este estudo foram isoladas células mononucleares de baços de murganhos BALB/c saudáveis. As células foram incubadas com parasitas e antigénios parasitários durante 72h e magneticamente separadas nas frações celulares $CD8^-$ (correspondente a células $CD4^+$) e $CD8^+$. As células foram marcadas com anticorpos monoclonais anti-CD3, anti-CD25 e anti-FoxP3 e analisadas por citometria de fluxo. A frequência de células $CD3^+$ e de linfócitos T $CD4^+$ ou $CD8^+CD25FoxP3^+$, $CD4^+$ ou $CD8^+CD25^+FoxP3^+$ (células T reguladoras ou Treg), $CD4^+$ ou $CD8^+CD25^+FoxP3^-$ e $CD4^+$ ou $CD8^+CD25^-FoxP3^-$ (células T efectoras) e a intensidade da fluorescência dos flurocrómos associados às moléculas da membrana celular CD3 e CD25 e intracelular FoxP3 foram avaliadas. Foi observado aumento da subpopulação de células T $CD4^+CD25^-FoxP3^+$ quando expostos a antigénios parasitários. Parasitas de *L. amazonensis* e *L. shawi* e os antigénios induziram a diferenciação da subpopulação de células Treg $CD4^+$ e aumentaram a densidade das moléculas FoxP3 e CD25 mas reduziram os linfócitos T $CD4^+$ efectores. Parasitas de *L. guyanensis* causaram a contração das subpopulações de linfócitos T $CD4^+CD25^-FoxP3^+$ e Treg $CD4^+$ e a redução da densidade das moléculas FoxP3, mas induziram o aumento das moléculas CD25 nas células Treg $CD4^+$. Todos os parasitas e antigénios (excepto o antigénio de *L. shawi*) estimularam o aumento da população de células T $CD4^+CD25^+FoxP3^-$ e a densidade das moléculas CD25. Antigénio de *L. shawi* e parasitas de *L. guyanensis* causaram a diminuição de linfócitos T $CD8^+CD25^-FoxP3^+$. Antigénio de *L. guyanensis* induziu a diferenciação da população de linfócitos Treg $CD8^+$ e os parasitas de *L. guyanensis* e antigénio de *L. shawi* aumentaram a densidade das moléculas FoxP3. Todos os parasitas e antigénios (excepto o antigénio de *L. guyanensis*) causaram o aumento das células T $CD8^+CD25^+FoxP3^-$ e estimularam a densidade das moléculas CD25. Todos os parasitas e antigénios reduziram os linfócitos T $CD8^+$ efectores. Conclui-se que tanto os antigénios como os parasitas regulam a diferenciação das subpopulações de linfócitos T, induzindo a expansão de células T associadas à regulação da reposta imunitária ($CD4^+/CD8^+FoxP3^+$, $CD4^+/CD8^+CD25^+FoxP3^+$, $CD4^+/CD8^+CD25^+FoxP3^-$) promovendo a homeostasia imunitária e a tolerância parasitária e promovendo a redução da população de células T efectoras ($CD4^+/CD8^+CD25^-FoxP3^-$) procurando estabelecer infeções silenciosas. Adicionalmente, também conseguem regular a expressão das moléculas FoxP3 e CD25. Os parasitas de *L. guyanensis* destacam-se pelo forte poder

modelador da expressão de moléculas CD25 e na expansão de células $CD25^{+}$. A regulação de CD25 por parasitas de *L. guyanensis* pode ser crucial para desvendar o mecanismo que conduz à diferenciação celular do hospedeiro.

Abstract

Differentiation of subpopulations of T lymphocytes by *Leishmania* species causative of American cutaneous leishmaniasis

Patrícia Relvas Mourata Gonçalves

KEY-WORDS: American Cutaneous Leishmaniasis; T cells; Acquired immunity; Regulatory T cells.

American cutaneous leishmaniasis (ACL) is a neglected disease caused by intracellular parasites of the genus *Leishmania*. ACL occurs in Central and South America and afflicted patients may present a set of clinical manifestations, but this disease is usually defined by the occurrence of cutaneous lesions that can cause disfiguration. Although ACL affects a considerable part of the human population little is known about the immune response to the different species of *Leishmania* that can cause this disease. Thus, the main objective of this work was to investigate the *in vitro* differentiation of subpopulations of T lymphocytes induced by parasites that cause ACL. To perform this study, mononuclear cells were isolated from spleens of healthy BALB/c mouse. Cells were incubated with parasites and parasitic antigens for 72 h and magnetically separated into $CD8^-$ (corresponding to $CD4^+$ cells) and $CD8^+$ fractions. Cells were labeled with anti-CD3, anti-CD25 and anti-FoxP3 monoclonal antibodies and analyzed by flow cytometry. The frequency of the $CD3^+$ cells and of lymphocytes T $CD4^+$ or $CD8^+CD25^-FoxP3^+$, $CD4^+$ or $CD8^+CD25^+$ $FoxP3^+$ (T regulatory cells or Treg), $CD4^+$ or $CD8^+CD25^+FoxP3^-$ and $CD4^+$ or $CD8^+CD25^+FoxP3^-$ (effector T cells) and the fluorescence intensity of fluorochromes associated with CD3 and CD25 cellular membrane molecules and intracellular FoxP3 molecules were evaluated. An increase in $CD4^+CD25^-FoxP3^+$ T cells was observed when exposed to antigens. *L. amazonensis* and *L. shawi* parasites and antigens induced differentiation of $CD4^+$ Treg cell subset and upregulate the expression of FoxP3 and CD25 molecules, but reduced effector $CD4^+$ T cells. *L. guyanensis* parasites caused the contraction of $CD4^+CD25^-FoxP3^+$ T cell subset and of $CD4^+$Treg and the down regulation of FoxP3 molecules, but induced the upregulation of CD25 molecules in $CD4^+$ T cells. All parasites and antigens (except *L. shawi* antigen) stimulated the increase of $CD4^+CD25^+FoxP3^-$T cells and the upregulation CD25 expression. *L. shawi* antigen and *L. guyanensis* parasites caused a decrease in $CD8^+CD25^-FoxP3^+$T cells. *L. guyanensis* antigen induced the differentiation of $CD8^+$ Treg subset and *L. guyanensis* parasites and *L. shawi* antigen upregulate FoxP3 molecules. All parasites and antigens (except *L. guyanensis* antigen) caused the increase of the $CD8^+$ $CD25^+FoxP3^-$T cells and upregulate the expression of CD25 molecules. All parasites and antigens reduced effector $CD8^+$ T cells. Therefore, both antigens and parasites regulate the differentiation of T cell subsets, inducing the expansion of T cell subsets associated with the regulation of immune response ($CD4^+/CD8^+FoxP3^+$, $CD4^+/CD8^+CD25^+FoxP3^+$, $CD4^+/CD8^+CD25^+FoxP3^-$), favoring immune homeostasis and parasite tolerance and, promotes the reduction of the effector T cell populations ($CD4^+/CD8^+CD25^-FoxP3^-$) seeking to establish silent infections. In addition, they also regulate the expression of FoxP3 and CD25 molecules. *L. guyanensis* parasites stands out for the power of strong regulate the expression of CD25 molecules and the expansion of $CD25^+$ cells. Regulation of CD25 by *L. guyanensis* parasites may be crucial to unraveling the main mechanism behind host cell differentiation.

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Abbreviation List

ACL – American cutaneous leishmaniasis

ADCL- Anergic diffuse cutaneous leishmaniasis

Ag – Antigen of

AP - Alternative pathway

APC - Antigen presenting cells

ATL - American tegumentary leishmaniasis

BALB/c mice - *Mus musculus* mice strain BALB/c

BCR - B cell receptors

CD - Cluster of differentiation

CL - Cutaneous leishmaniasis

ConA - Concanavalin A

CP - Classical pathway

DC - Dendritic cells

DCL- Disseminated cutaneous leishmaniasis

DDT – Dichlorodiphenyltrichloroethane

DNA - Deoxyribonucleic acid

EDTA - Ethylenediamine tetraacetic acid

FBS - Fetal bovine serum

FMO - Fluorescence minus one

FoxP3 - Fork-head box protein 3

G - Gauge number

GP63 - Glycoprotein of 63 kDa

HBSS - Hanks balanced salt solution

HIV – Human immunodeficiency virus

HLA - Human leukocyte antigen

IF - Intensity of fluorescence

IFN - Interferons

IHMT – Instituto de Higiene e Medicina Tropical

IL – Interleukin

LCL - Localized cutaneous leishmaniasis

LP – Lectin pathway

MAC - Membrane attack complex

MCL- Mucocutaneous leishmaniasis

MØ - Macrophages

MHC - Major histocompatibility complex

MLEE - Multilocus enzyme electrophoresis

MLMT - Multilocus microsatellite typing

MLST - Multilocus sequence typing

MS - Magnetic separation

MM - Molecular mass

NE - Neutrophil elastase

NK – Natural killer cells

NO - Nitric oxide

PAGE - Polyacrylamide Gel Electrophoresis

PBS - Phosphate buffered saline

PCR - Polymerase chain reaction

Pen-Strep - Penicillin/streptomycin

PPRs - Pattern recognition receptors

Q - Quadrant

Rf - Relative migration distance

RFLP - Restriction fragment length polymorphism

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

RPMI - Roswell Park Memorial Institute 1640 medium

SDS - Sodium dodecyl sulphate

Tc – Cytotoxic T cells

TCR - T cell receptors

Th – Helper T cells

TNF-α - Tumor necrosis factor - α

TPCK - N-p-Tosyl-L-phenylalanine chloromethyl ketone

Treg - Regulatory T cells

Tsup - Suppressor T cells

VL - Visceral leishmaniasis

WHO - World Health Organization

1. Introduction

1.1. The parasite *Leishmania*

1.1.1. *Leishmania* spp. - Taxonomic position, vectors, reservoirs and hosts

Taxonomic position

Parasites of *Leishman*ia spp. present a worldwide distribution, mainly including tropical and subtropical areas, and can infect numerous species of several orders of mammals [1-3].

These parasites belong to the Kingdom Protista, sub-kingdom Protozoa, Phylum Kinetoplastida, Class Kinetoplastea, Subclass Metakinetoplastina, Order Trypanosomatida, Family Trypanosomatidae, Subfamily Leishmaniinae and Genus *Leishmania* [3, 4].

Genus *Leishmania* currently includes several *Leishmania* species of subgenera *Leishmania* and *Viannia* (the latter is only found in the New World), which are grouped into complexes. The distribution of the species into the two subgenera is based on the development of the parasite within the vector. While in *Leishmania* subgenus the parasite development occurs within the midgut and foregut of the sand-fly vector, in *Viannia* subgenus the parasite requires and additional development phase that takes place in the hindgut. Both subgenera include species potentially pathogenic for human beings that can cause a variety of clinical conditions [2, 5].

Characterization of distinct species of *Leishmania* was first based on clinical, biological, geographical and epidemiological criteria. However, it was found that these criteria have often been inadequate, and consequently immunological, biochemical and genomic data parameters were also included to enable a more rigorous characterization of *Leishmania* species [6-8].

Currently, more than 30 distinct species of *Leishmania* have been documented, of which 20 are considered infectious for humans and mammals. The distinction between species of *Leishmania* is related to the differentiation of various forms of disease - visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) and mucocutaneous (MCL) - in human beings, facilitating the establishment of a correct diagnosis and disease prognosis, which is crucial to fight the disease [1-3]. Since morphological analysis is unfeasible, DNA-

based criteria are currently the most used methodology to distinguish species of *Leishmania* [1,4].

Identification of *Leishmania* genus was targeted to several proposals to update the system of classification. An updated classification for *Leishmania* based on combined molecular data has been proposed by Cupolillo et al. [2]. This update was proposed, since some species initially classified as *Leishmania* were later discovered to be more closely related to genus *Endotrypanum* when molecular studies were performed, suggesting the presence of two divergent phylogenetic lineages within *Leishmania.* Thereby, this new classification divides *Leishmania* species into two phylogenetic lineages or "Sections": *Euleishmania,* including species classified within the subgenera *Leishmania* and *Viannia,* and *Paraleishmania* comprising several species initially identified as *Leishmania*, but with uncertain classification and species and strains currently classified within the genus *Endotrypanum* [2, 3].

Later on, based on findings reported by Fraga and colleagues [2010], Schönian et al. suggested a revision of the *Leishmania* classification system [9]. Based on parasite molecular phylogeny, including the heat-shock protein of 70 kDa (hsp70), Fraga et al. (2010) verify the existence of only eight medical relevant species of *Leishmania* in contrast to the 17 species currently defined by the multilocus enzyme electrophoresis [10].

Vectors

The vectors responsible for *Leishmania* parasite transmission are female phlebotomine sand-flies belonging to subfamily *Phlebotominae*. Parasites infect these invertebrates, completing the vector phase of its life cycle to enable the mammal host infection [3, 7].

Phlebotomine sand-flies belong to the phylum Arthropoda, class Insecta/Hexapoda, order Diptera, suborder Nematocera, family Psychodidae, and subfamily Phlebotominae, which is currently divided into six genera: *Phlebotomus*, *Sergentomyia*, and *Chinius* of Old World and, *Lutzomyia*, *Brumptomyia* and *Warileya* present in the New World [3, 7, 11].

All *Leishmania* vectors known are included in the genera *Phlebotomus* (in the Old World) and *Lutzomyia* (in the New World). Although there are 500 known species of

phlebotomine sand-flies, only 31 have been identified as vectors of pathogenic species of *Leishmania* and 43 as possible vectors [3, 7].

The sand-fly species involved in *Leishmania* transmission differ according to the geographic region and the species of *Leishmania*. Also, it is important to refer that some vector species are able of support transmissible infections of several *Leishmania* species, such as permissive vectors *Lutzomyia longipalpis* and *Phlebotomus argentipes*, while other vector species are exclusive to some *Leishmania* species as is the case of the specific vector *Phlebotomus papatasi* that only transmits *L. major* [12, 13].

It is documented that permissive vectors can facilitate the successful adaptation of *Leishmania* to new vectors, which can have important epidemiological consequences. Allied to climate changes and human migration around the world, this type of vectors represents a high risk for parasite spreading and for the establishment of new leishmaniasis foci [13].

Reservoirs and hosts

Leishmania parasites, which have a high success in transmission, can cause natural infections in several orders of mammals, such as rodents, canids, edentates, marsupials, procyonids, primitive ungulates and non-human primates. These mammals are considered a potential reservoir of the parasite, whereas in some cases, human beings are considered possible hosts, but usually are only accidental hosts [7].

Most of *Leishmania* spp. have a zoonotic cycle of life. However, reports shown that in India, South Asia and the Horn of Africa also exist anthroponotic transmission (human to vector to human) cycle for *L. donovani* [7, 14, 15].

1.1.2. Study focus: cutaneous species of *Leishmania*

Leishmania amazonensis

L. amazonensis that belongs to subgenus *Viannia* and is found in South America (mainly in Brazil, Venezuela and Bolivia) usually causes disseminated lesions (disseminated cutaneous leishmaniasis, DCL), but also can develop localized cutaneous leishmaniasis

(LCL), anergic diffuse cutaneous leishmaniasis (ADCL) and mucocutaneous leishmaniasis (MCL) [11, 16-18]. Furthermore, in Bahia State (Brazil) was also reported rare cases of visceral leishmaniasis (VL) due to *L. amazonensis* parasites [19].
L. amazonensis is mainly transmitted by the phlebotomine sand-fly *Lutzomyia (Nyssomyia) flaviscutellata*, although other vectors like *Lutzomyia (N.) olmeca olmeca* and *Lutzomyia (N.) reducta* are occasionally implicated in parasite transmission [19].

Leishmania guyanensis

L. guyanensis belongs to subgenus *Viannia* and is mainly found in Northern and South America, specifically at north of the Amazon River (Brazil), French Guiana, Suriname, Bolivia, Venezuela and Peru [11, 16].
Infection by *L. guyanensis* usually causes multiple cutaneous lesions, but can also originate LCL and DCL and rarely MCL [11, 16, 19, 20].
This parasite is mainly transmitted by the phlebotomine sand-fly *Lutzomyia umbratilis* [19, 21].

Leishmania shawi

L. shawi belongs to subgenus *Viannia* and is mainly found at the south of the Amazon River, in Pará state, north of Brazil [11, 16]. First characterized in 1989, was initially recorded as a parasite that infected sloths (*Choloepus didactylus* and *Bradypus tridactylus*), monkeys (*Cebus apella* and *Chiropotes satanas*) and the procyonid *Nasua nasua*. Then, in 1991, the first case of *L. shawi* infection in a human patient was reported by Shaw et al. [22, 23].
L. shawi infection usually gives origin to LCL, however, multiple lesions occasionally have been reported [16, 19].
This parasite is mainly transmitted by the *Lutzomyia (N.) whitmani sensu lato* [19].

1.1.3 Life cycle

Leishmania is a digenetic parasite with two life cycle stages: a motile extracellular stage (Fig. 1A) within the invertebrate host (phlebotomine sand-fly) and an intracellular stage within the vertebrate host (Fig. 1B) [14].

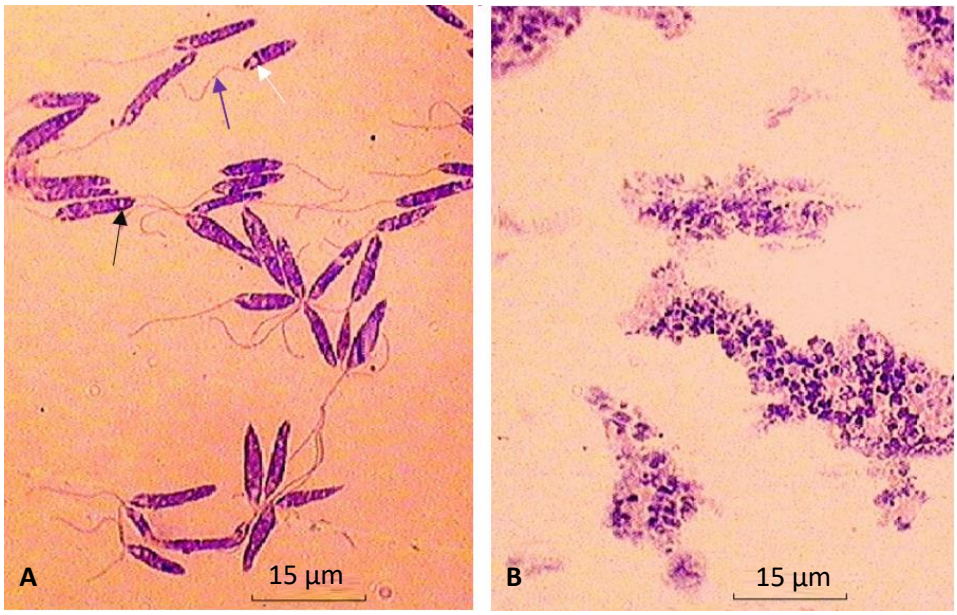

Figure 1- *Leishmania* spp. morphological forms. Promastigotes (A) maintained in culture medium and amastigote-like forms (B) differentiated in axenic conditions were fixed and stained with Giemsa, observed under optical microscope and images were acquired. Promastigote morphological forms are shown as elongated forms, exhibiting a flagellum (purple arrow) a kinetoplast (white arrow) and a nucleus (black arrow). Amastigote-like forms are shown as aggregates of non-flagellated ovoid cells (1000 × magnification, size bar – 15 µm).

Leishmania life cycle (Fig. 2) starts with a blood meal performed in the vertebrate by the parasitized female sand-fly. During sand-fly bite, promastigote forms (extracellular elongated motile flagellated form) are inoculated in the skin of a vertebrate host. Within the vertebrate host, promastigotes are intake by phagocytic cells and differentiated in amastigote forms (round to ovoid cell with 2.5 – 5 µm of diameter). Inside macrophage phagolysosomes amastigotes replicates by binary fission. When reach a high level of parasites, the host cell is no longer able to support the infection and amastigotes are released into the extracellular space, infecting other phagocytic cells where parasite

replication can be perpetuated and disseminating the infection to other tissues of mammal host. Once the phlebotomine sand-fly take a blood meal from an infected mammal host also ingests parasitized cells, initiating the sand-fly stages of the parasite life cycle. During the digestion of blood, amastigotes are released and differentiate into promastigotes in the midgut and foregut for species do *Leishmania* subgenus or in the hindgut in the case of species included in *Viannia* subgenus. Then promastigote replicates in the midgut and migrates to the vector proboscis, where stay ready to be inoculated in the mammal host during the next blood meal [8, 14].

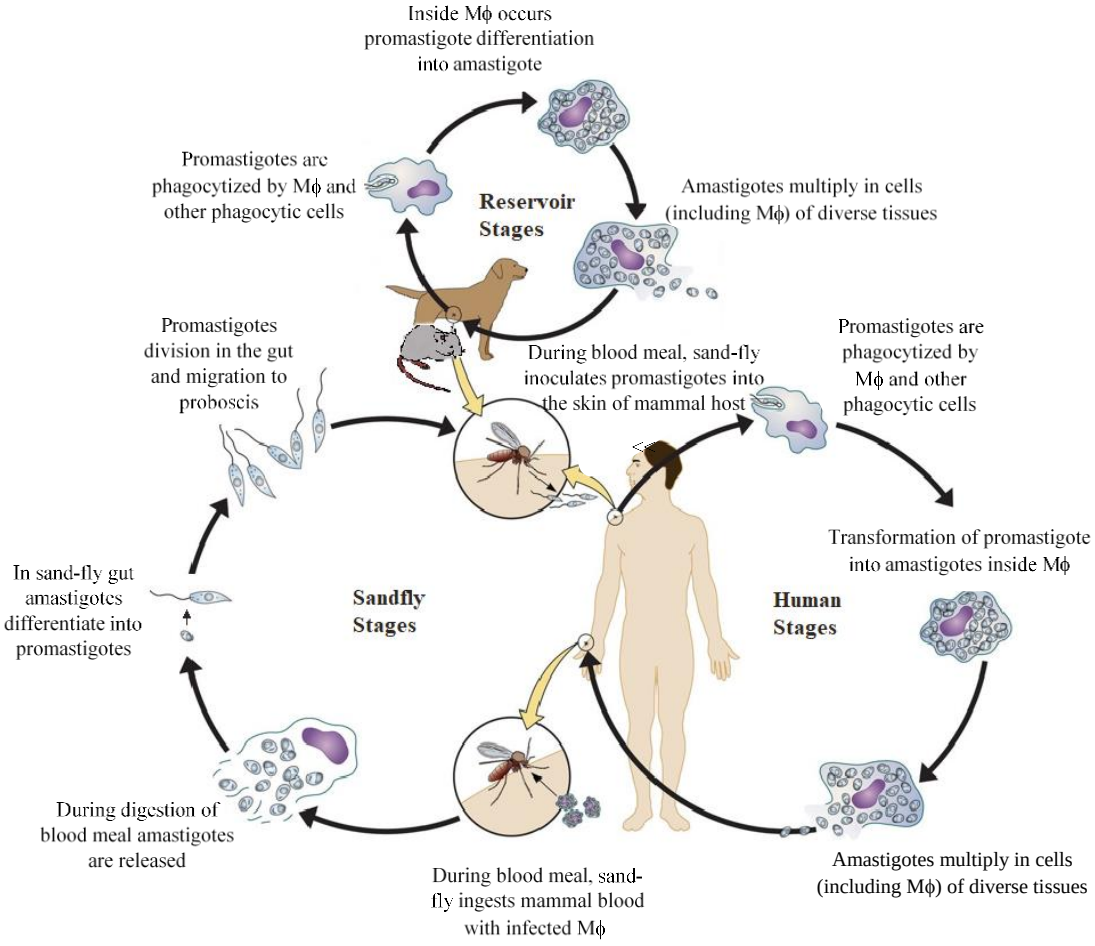

Figure 2 - Life cycle of *Leishmania* spp. Schematic representation of parasite stages in the sand-fly, human host and animal reservoir (adapted from "The life cycle of *Leishmania* species" image from Transmission and Epidemiology of Zoonotic Protozoal Diseases of Companion Animals by Esch, KJ and Petersen, CA)[24].

Leishmania species are transmitted mainly by zoonotic transmission, including cutaneous species, with the exception of *L. tropica* (for cutaneous leishmaniasis) and *L. donovani* parasites (for visceral leishmaniasis) that exhibit an anthroponotic cycle of transmission [15].

Despite, the zoonotic or anthroponotic transmission patterns, rare cases of congenital- and blood-borne *Leishmania* infections have also been reported as well as parasite transmission in consequence of an unfortunate lab incident [11, 16, 25, 26].

1.2 Leishmaniasis – An introduction

1.2.1 Brief introduction

Leishmaniasis, a tropical and subtropical disease caused by parasites of the genus *Leishmania*, is one of the seven most important tropical diseases and is also considered a neglected disease, since its occurrence are strongly linked to geographic areas affected by poverty and war [11, 16, 26].

Leishmaniasis presents two main clinical forms, VL or CL. However, in some cases a third clinical form can occur, MCL. In this case, parasite dissemination to mucosal tissues occurs, causing tissue destruction and severe disfiguring lesions.

VL and CL present distinct clinical features according to the tissues that are infected [16, 25]. In CL, parasites infect skin-resident macrophages (MØ) and with the increase of parasite burden, neighboring MØ become progressively infected. In VL, on the other hand, amastigote-infected MØ are propagated by the bloodstream to cells of the mononuclear phagocyte system of visceral organs, like liver, spleen, bone marrow and lymph nodes [16].

The clinical features of the disease depend highly of the *Leishmania* species, host immune competence and the co-infection with other infectious agents (such as human immunodeficiency virus, HIV), and can involve a broad spectrum of manifestations and different degrees of severity, that range from self-healing cutaneous lesions to a potentially fatal outcome [11, 25, 26, 27].

1.2.2 Cutaneous leishmaniasis

CL is a public health problem in numerous countries with low to middle income where it is considered one of the most serious skin diseases [27]. This disease, the most common form of leishmaniasis (50–75% of new cases worldwide) is mainly caused by *L. tropica*, *L. aethiopica*, *L. major* in the Old World, and by *L. mexicana*, *L. guyanensis*, *L. amazonensis* and *L. braziliensis* in the New World [14, 15, 27, 28].

Infection with species causative of CL usually causes LCL, a form of the disease in which the parasite remains in skin tissue where the infection was originated, causing chronic slowly healing skin ulcers that are usually self-healing and painless. However, these ulcers can take several months or even years to heal and can leave disfiguring scars, which can lead to a stigma in affected individuals [18, 25]. The classical CL lesion starts at the site of infected sand-fly bite, which induces local increase in temperature and swelling and, subsequently develops a papule or nodule with raised borders, which contains parasites and slowly expands (Fig.3) [11, 14, 15, 25].

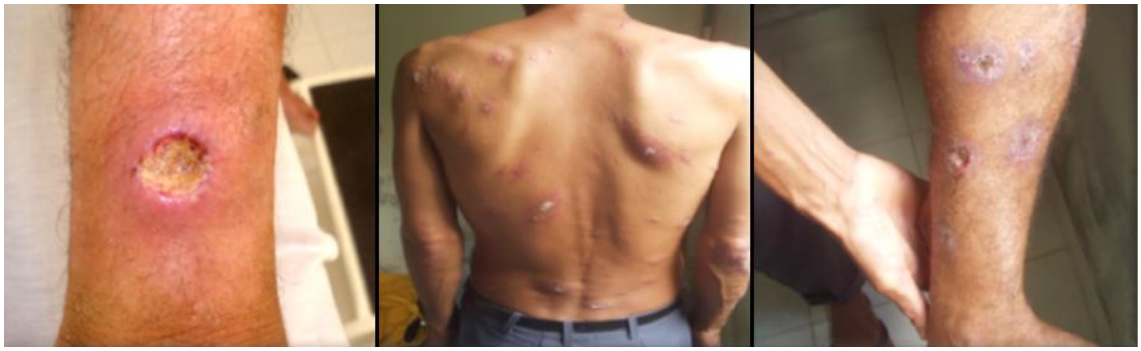

Figure 3 – Representative images of lesions caused by cutaneous species of *Leishmania* (pictures gently given by Dr. JML Costa, Centro de Pesquisa Gonçalo Moniz – FIOCRUZ, Bahia, Brazil)

However, CL can present a range of cutaneous inflammatory manifestations, with different degrees of severity, that may vary according to the infecting species of *Leishmania* and the host immune response, in which, the balance between pro- and anti-inflammatory factors play a key role [18, 28].

Are reported three main forms exhibiting severe manifestations of CL: MCL, DCL and ADCL [18].

MCL is mainly caused by *L. braziliensis*, *L. guyanensis* and *L. panamensis* (*Viannia* subgenus) and occurs only in the New World affecting primarily Bolivia, Brazil and Peru. However, recently a case of MCL caused by *L. major* was reported in Iran [14, 15].

MCL is a complication of LCL, in which the parasites metastasize thought lymphatic or hematogenous dissemination to mucosal tissue in the mouth and upper respiratory tract, which can lead to the development of disfiguring lesions on the sites. This form of leishmaniasis can cause from discomfort, mild pain and odynophagia to cachexia in more severe cases. These lesions can occur for months to years after the development of a cutaneous lesion. The pathogenesis of MCL is still uncertain, although it is believed that host genetic factors have a role in the development of disease [11, 14, 18].

DCL in which parasites disseminate from the original infection site throughout the body, causing numerous pleomorphic lesions in two or more non-contiguous anatomical regions [18].

ADCL is caused by *L. aethiopica* and *L. mexicana* complex (including *L. amazonensis*, *L. braziliensis*, *L. mexicana* and *L. pifanoi*) [11, 15]. This form of CL is a rare and severe form of LCL characterized by anergy (absence of cellular immune response), which allows the parasite dissemination throughout tissues and organs via the lymph and bloodstream, leading to the development of multiple satellite lesions that can cover large areas of skin (excluding the scalp), and occasionally can involve mucous membranes [11, 15, 18].

American cutaneous Leishmaniasis (ACL)

American cutaneous leishmaniasis (ACL) is a major public health problem that affects the South American Continent, ranging from Mexico to Argentina, and is caused by diverse species of *Leishmania* from both *Viannia* and *Leishmania* subgenera [29]. ACL cases are mainly caused by *Leishmania (V.) braziliensis* and by species of *Leishmania (L.) mexicana* complex, such as *L. amazonensis* [29,30]. However, other species of *Leishmania* from subgenus *Viannia*, like *L. (V.) guyanensis* and *L. (V.) shawi* can also give origin to ACL [29, 30].

ACL can present a spectrum of CL forms, including LCL, ADCL, MCL and DCL. In particular MCL and DCL are difficult to treat and can lead to severe, disfiguring outcomes [29, 30].

1.2.3 Visceral Leishmaniasis

VL, also known as kala-azar is caused by *L. donovani* and *L. infantum* (*syn L. chagasi*). These parasites infect MØ and have tropism for visceral tissues being usually found in the spleen, liver, lymph node and bone marrow [14, 15].

The most common clinical features of VL include weight loss, long-term low fever, hepatosplenomegaly, pancytopenia and polyclonal (IgG and IgM) hypergammaglobulinemia. Latent cases may remain undiagnosed for extended periods of time (for years to decades). Clinical features are aggravated in patients with a low immune competence due to immunosupressor drugs or HIV. In more than 95% of untreated VL patients, a fatal outcome within two years after the onset of the disease is previewed [14, 15, 25, 31]

1.2.4 Epidemiology

Leishmaniasis is found in about 89 countries and despite having a worldwide distribution (with exception of Oceania) is endemic in the geographic areas of North-eastern Africa, Southern Europe, the Middle East, South-East Asia and, Central and South America [11]. The infection prevalence is estimated between 12 and 15 million people worldwide, and 350 million are at risk of acquiring the disease. The number of deaths per year caused by the disease is estimated to be 70,000. As for leishmaniasis incidence, World Health Organization (WHO) estimated that 0.6 million to 1 million of new CL cases and 50,000 to 90,000 new VL cases occur worldwide every year [11, 32].

However, leishmaniasis has been underestimated over the years due to factors such as social stigma, economic instability and civil war that have a great impact on disease spread and mask the real importance of this parasitic infection. Furthermore, leishmaniasis is not a mandatory reported disease in several of affected countries. In fact, only 50% of VL endemic countries and 48% of CL endemic countries reported the

number of leishmaniasis cases occurred in 2015 to the WHO Global Leishmaniasis Program [25, 33].

Other factors, such as globalization and climate change can have, as well, a significant impact on the dissemination of the infection, since, it was reported an increase of cases of leishmaniasis in international travels and infections of leishmaniasis thought traffic of blood products due to the nonexistence of blood bank screens for the presence of anti-leishmanial antibodies. There are evidences that global warming can lead to a spread of sand-fly dissemination to the northward regions which could result in a future spreading of leishmaniasis in non-endemic regions [16].

The countries with the highest incidence of CL reported worldwide are Afghanistan, Algeria, Brazil, Colombia, the Islamic Republic of Iran, Pakistan, Peru, Saudi Arabia and the Syrian Arab Republic (Fig. 4). Brazil, Ethiopia, India, Somalia, South Sudan and Sudan reported the highest incidence of LV (Fig. 5) [34].

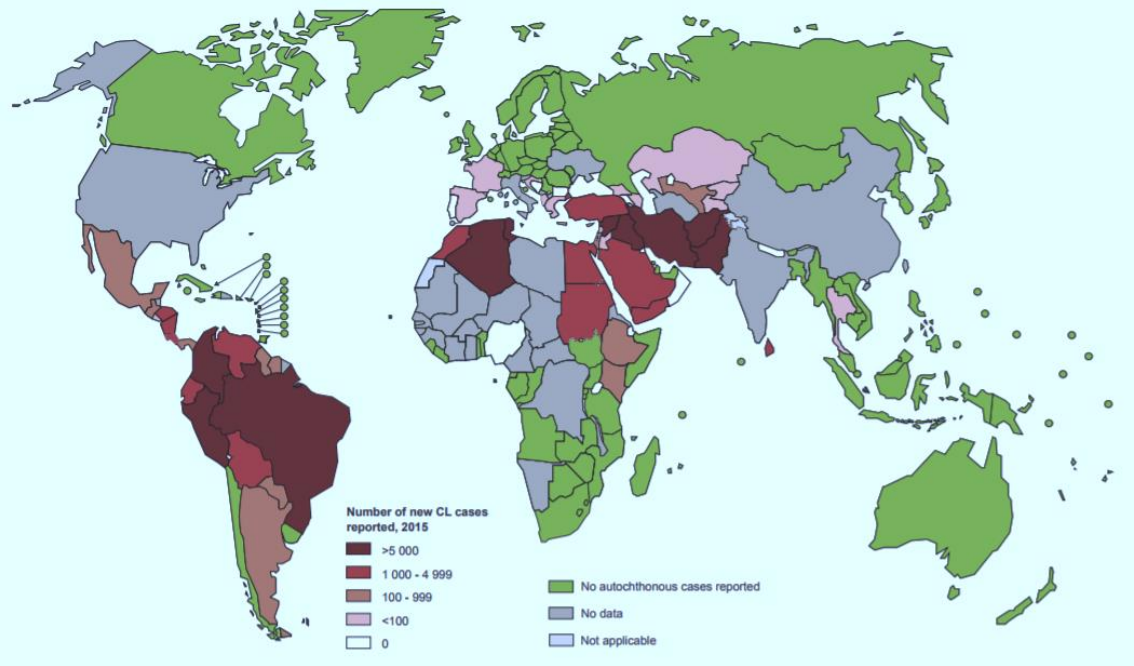

Figure 4. Worldwide distribution of new cases of CL reported to WHO in 2015 (adapted from WHO site) [34].

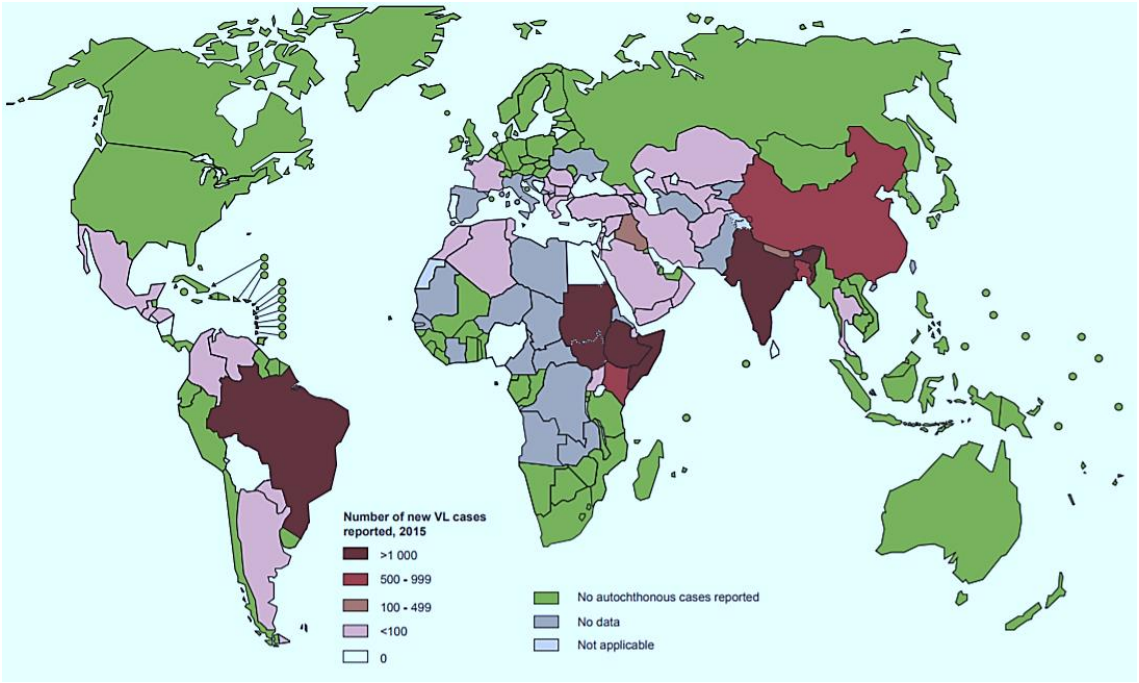

Figure 5 - Worldwide distribution of new cases of VL reported to WHO in 2015 (adapted from WHO site) [34].

1.2.5 Diagnosis

Although diagnosis varies according to the clinical form (VL, CL or MCL), usually includes laboratory parasitological (observation of amastigotes by microscopic examination of tissue aspirates) and serological tests (such as rapid diagnostic tests) together with a thorough clinical examination [35].

The diagnosis of VL is based on parasitological examinations, serological tests (tests as the direct agglutination test and the rK39 antigen-based immunochromatographic test that are widely available), since clinical signs can be mistaken by symptoms of chronic malaria, schistosomiasis or other systemic infections. Detection of parasite DNA by PCR in blood or bone marrow and of anti-leishmanial antigens by serological tests, such as ELISA or immunoflurescence can be also used to diagnose VL. However, these methodologies require equipment and qualified staff to perform these tests, which conditions the application of these techniques in the field [35].

The diagnosis of CL and MCL is mainly based on the characteristic clinical manifestations and in parasitological tests, since serological tests have limited value due

to low sensitivity and variable specificity. Molecular assays, such as polymerase chain reaction (PCR) based assays (including, for instance, quantitative Real Time PCR and simplified PCR methods), usually exhibiting high sensitivity and specificity can also be used [1, 32, 35].

For species identification and phylogenetic analysis, there are other available tools, as is the case of multilocus enzyme electrophoresis, DNA sequencing analysis and restriction fragment length polymorphism (RFLP) and PCR-fingerprinting techniques, including multilocus sequence typing (MLST) and multilocus microsatellite typing (MLMT) [1].

1.2.6 Treatment and vaccine

The treatment of leishmaniasis is dependent on several factors, including the species of *Leishmania* that caused the infection and the clinical form of disease, the existence of concomitant pathologies and patient immunocompetence. Leishmaniasis is a treatable disease and can be clinically cured. However, anti-leishmanial drugs do not entirely eliminate the parasite and the immunosuppressive state involves a high risk of leishmaniasis relapse [32].

Drugs used in the treatment of leishmaniasis are associated with severe side effects, high toxicity, and also development of parasite drug resistance. In addition, the elevated cost of the drugs, high treatment duration and the use of invasive routes for drug application associated with few availability of health professionals and the insufficient of infrastructures usually found in poor countries make treatment compliance more difficult [14, 35].

Nowadays, VL treatment are dependent on chemotherapy and the most frequently employed drugs are: pentavalent antimonials (meglumine antimoniate and sodium stibogluconate), oral miltefosine, amphotericin B, liposomal amphotericin B (a lipid formulation of amphotericin B) and paromomycin [14, 35].

For at least seven decades, pentavalent antimonials have been the first line of treatment for leishmaniasis, however, have been reported a growing parasite resistance to this class of drugs [14, 35]. To combat the problem of parasite resistance to pentavalent antimonials, the main drug used for leishmaniasis was switched to amphotericin B, which is now the drug most used for the treatment of VL patients. However, this drug also has its own

downfalls, regarding high toxicity and the upsurge of recent reports on amphotericin B-resistant parasites [14, 35].

Several lipid formulations of amphotericin B can be also used for leishmaniasis treatment. This type of drugs has similar efficacy to amphotericin B, but are significantly less toxic. Liposomal amphotericin B (available as AmBisome®, Astellas Pharma), amphotericin B lipid complex and amphotericin B colloidal dispersion are some examples of different formulations of this drug. In addition, in a recent multicenter clinical trial, AmBisome® was shown effective in VL treatment with just a single dose and presenting lower toxicity when compared to the conventional treatment with amphotericin B deoxycholate [14, 27, 35].

Miltefosine (available as Impavido® by Knight Therapeutics) is the first oral drug available for VL treatment that shown high cure rates (98%). However, this drug also has important side effects. Causes gastrointestinal disorders and is potentially teratogenic, so its use in pregnant women or in women with childbearing potential is not recommended. In addition, recent reports showed an increase of relapse cases in patients treated with this drug [14, 25, 27, 35].

Paromomycin also presents toxicity and patients developed side-effects associated with its administration. This drug is also available in a topical formulation to be applied in CL cases [14, 35].

Nonetheless, treatments featuring a single drug type have generated some concerns, regarding the possible development of drug-resistant parasites. Consequently, the use of combination drug therapy is an alternative that has been considered and developed to be mainly employed in endemic regions. Though, this option may not be infallible, since recent studies in *L. donovani*-mouse model suggest the possibility of differentiation of resistant parasites when drug-combination therapy is used [14, 35].

There are also treatments available for CL, but since this clinical form rarely causes a life-threatening condition, treatment is only applied after assessing the risk–benefit of each patient. In cases of mild CL, wound cleaning to avoid secondary infections that may complicate the cure can be the most adequate treatment option [35]. In patients with severe CL that can develop adverse events, like disfiguring, complicated scaring or MCL anti-leishmanial chemotherapy is mandatory [35]. The drugs most used for CL and MCL treatment are paromomycin ointments and intra-lesional application of pentavalent

antimonials [35]. Furthermore, thermotherapy applications such as liquid nitrogen cryotherapy is also used. However, thermotherapy requires the use of expensive specific devices, involves skilled health care providers, needs local anesthesia and a cure can be difficult by second-degree burns [35].

Despite leishmaniasis treatment being the research focus of several research groups worldwide the chemotherapeutic options available are still few, so it is unlikely that chemotherapy alone will allow disease control and eventually disease elimination. Hence, the need for other control methods are required to overcome long-term goals in disease control.

Since, vaccination is the most cost-effective and also the most effective long-term strategy to control infectious diseases, significant efforts have been made over the years for the development of effective vaccines to prevent leishmaniasis. Although there is still no licensed vaccine against human leishmaniasis available, promising candidates for a vaccine have been studied. While, the majority of these studies is still in early research and development, some vaccine candidates have already advanced to clinical trials [14, 36, 37]

Were taken several approaches to develop vaccine candidates for leishmaniasis, using killed or attenuated parasites (with and without adjuvants), total, mix or partial parasite antigens, recombinant parasite proteins that in some cases have been complemented by adjuvants or combined with bacteria or recombinant virus as delivery vehicles. More recently, the development of DNA vaccines was also taken into consideration [13, 36, 37].

In addition, other approaches are being taken, that includes testing vaccine candidates that target the vector, as is the case of sand-fly salivary proteins. Since saliva is inoculated with parasites during blood meal and can exacerbate *Leishmania* infection, immunity to sand fly saliva should also play a protective role [37, 38].

However, all promising vaccine candidates proposed until now require further testing to establish their utility, efficacy and safety to be used in human beings [14, 37, 38].

1.2.7 Surveillance and Control

As stated in the previous sub-chapter, leishmaniasis eradication and control thought treatment, despite the continuous research on the field, has still to face many challenges, to uncover a treatment that combines a high success rate, low side effects and present an adequate cost-effectiveness level.

The absence of an effective vaccine or preventive formulations, the scarcity of drugs available and the high toxicity and severe side effects associated with the available drugs are important obstacles to the disease control [25].

Due to these difficulties, surveillance and control are two essential strategies to fight the disease. Aiming to control the disease, resolution WHA60.13 on control of leishmaniasis, adopted by the 60th World Health Assembly that occurred in 2007 requests WHO to promote awareness of the worldwide burden of leishmaniasis through health education and training, and to monitor the progress of its control [39, 40]. To address this request, the Global leishmaniasis program at WHO established simple and standardized tools to collect annual data from all Member States. These tools collect data from a minimum set of indicators from all Member States, but for the Member States with higher leishmaniasis prevalence, tolls to collect additional data and more detailed indicators were defined [39, 40].

Also, reinforcement of efforts for the implementation of sustainable national control programs and guidelines of surveillance, data collection and analysis were recommended by resolution WHA60.13 to all Member States of WHO where leishmaniasis remains a significant public health problem. This resolution also urges the Member States to strengthen health care by improve prevention, (i) establishing an active detection program and facilitating the access to treatment, (ii) promoting cooperation between countries that share common foci or disease threats, (iii) finding appropriate and effective methods of control of vectors and reservoirs (such as, effectiveness evaluation of bed nets impregnated with long-lasting insecticide), and (iv) encouraging research on leishmaniasis control strategies, including diagnosis, new therapeutic alternatives, innovative drugs and vaccines [39, 40].

Full compliance with these requests is likely to greatly improve public health problem regarding leishmaniasis, since community lack of knowledge, miss of trained health

professionals and absence of diagnostic tests with high sensitivity and specificity do not favor leishmaniasis reduction. Furthermore, the introduction of effective strategies for vector control is considered vital to successful disease control in endemic areas. However, the achievement of these requests and, the control of the disease are only possible by ensuring that endemic countries are self-sufficient to support and promote effective programs of control [41, 42].

The methods to control *Leishmania* vectors are mainly dependent on insecticide use. Indoor residual spraying, insecticide-treated bed nets, and insecticide treated bed sheets reduce vector population and minimize human exposure to the vector, thereby reducing the number of new infections. Although the use of insecticides is a cost-effective method for the control of leishmaniasis, the wide spread of insecticide resistance has become more likely to occur, especially in areas where insecticide use is extensive. Due to this, is a priority to research and discover new insecticides to counterattack the emerging vector resistances [25, 41, 42, 43].

Reservoir control can be a challenge to combat leishmaniasis, since this disease is mainly zoonotic in most areas, the dissemination of the infection is dependent of several animal reservoirs, some of these are wild reservoirs and thus inaccessible [26, 42]. This can be an obstacle to disease elimination. However, despite the diversity of VL hosts, which vary by endemic region, the most important reservoir hosts are the humans (for example, in *L. donovani*) and dogs [26, 36, 41, 42].

To minimize the risk of transmission is advised the use of measures to control VL in reservoir hosts of endemic countries, since currently there is not control programs implemented. Since dogs are one of the most important reservoirs of *Leishmania* parasites with a key role in VL transmission to humans, is crucial to look for potential infections, given that main infected dogs remain asymptomatic, and apply the available treatments for canine leishmaniasis [35, 39, 41].

Nowadays, three second-generation vaccines are registered for canine leishmaniasis (Leishmune®, Leishtec® and CaniLeish®) [34, 35, 44, 45]. In addition, the practice of preventive vaccination of dogs in Brazil lead to a reduction in the incidence of both canine and human leishmaniasis [46].

1.3 Immune response: Host – Parasite interactions

As described in the previous chapter the outcome of a leishmaniasis infection depends mainly of the species causing the infection, specifically of the virulence factors of the parasite species or strand, and of the host immune response.
This section presents a general and brief resume of immune response of the host against *Leishmania* infection and the mechanisms used by the parasite to evade the immune system in order to guarantee its survival, replication and dispersion within the host.

1.3.1 Immune response to *Leishmania* infection

Since the deposition of *Leishmania* promastigotes into the blood pool made by the sand-fly in the mammal dermis until amastigote differentiation and replication within MØ, occurs an ongoing battle between the immune system of the host and the parasite.
The competence of the host immune system to coordinate the functional activity of its components, including innate and adaptive immune elements and the capacity of the parasite to evade and subvert host immunity are the main factors that dictate the outcome of the infection for parasite control or, by the contrary, for disease establishment.

1.3.2 Innate immunity

Innate immunity is the first barrier of defense that parasites must face and overcoming to survive within the host. This type of immunity is characterized by displaying a fast action to pathogens, but in an unspecific manner. Innate immunity can target external invaders, in several cases may cause direct pathogen destruction and, trigger inflammatory immune responses.
Cells of innate immunity [neutrophils, MØ, mast cells, natural killer (NK) cells, dendritic cells (DC)], the complement system and the pattern recognition receptors (PPRs, such as toll-like receptors) have a major role in the control and eradication of *Leishmania* parasites [47].
During the initial phase of infection, the complement system has a key role in the promastigote elimination throughout complement-mediated lysis. It is composed of

complex mechanisms involving plasma and membrane-associated serum proteins, which can provoke highly effective and regulated inflammatory and cytolytic immune responses. Although, the classical (CP), lectin (LP) and alternative (AP) pathways of complement system can be activated by pathogens, infective *Leishmania* parasites mainly activate the classical pathway of the complement system, though parasite destruction is augmented by the alternative pathway [48-50].

Regardless the pathway involved, complement activation leads to a sequential cascade of enzymatic reactions, from which results the formation of the proinflammatory mediators C3a and C5a (anaphylatoxins) and pathogen opsonization by several complement opsonins (such as C3b) that enables the assembly of the membrane attack complex (MAC), causing the pathogen lysis through the formation of membrane pores [50, 51, 52].

Neutrophils are the first cells to arrive to the infection site, within an hour post-inoculation. These cells produce microbicidal factors, such as nitric oxide (NO), neutrophil elastase (NE), platelet activation and emission of neutrophil extracellular traps for the extracellular space and, can phagocyte *Leishmania* promastigotes and reduce the migratory capacity of these parasites [18, 48, 53].

However, parasites can remain viable after neutrophil phagocytosis, even when the neutrophil undergo apoptosis, which enables them to modulate MØ recruitment. Recruited MØ can engulf the apoptotic neutrophils (that is called efferocytosis), allowing the parasites to transfer from neutrophils into MØ, which is the host cell of this parasite [18, 48, 53]. Although these cells usually have a protective role, the *Leishmania* strain, the apoptotic or necrotic neutrophil state and the genetic background of the host (susceptible or resistant host) are factors that may alter the infection outcome [18, 48].

In parallel with neutrophil recruitment, DC response to infection is initiated. DC derives from monocytes (likewise MØ) and also are a target cell for *Leishmania* parasites [47, 54]. These cells phagocyte alive parasites and DC enable the antigen presentation to lymphocytes T ($CD4^+$ and $CD8^+$ T cells), forming a bridge between innate and adaptive immunity, by inducing the adaptive immune response [47, 53, 55, 56].

DC are also responsible for cytokine production, by inducing naïve helper T (Th) cell proliferation and differentiation into Th1 or Th2 cells [56]. The profile of cytokines secreted by DC leads the differentiation of naïve Th cells into Th2 cell by inducing the

expression of nuclear factor STAT6 and the consequent secretion of interleukin (IL)-4 and IL-10 or in Th1 by stimulating the expression of nuclear factors STAT4 and STAT1 and secretion of IL-12. The differentiation of naïve Th cells can influence disease progression (usually, associated with the predomination of a Th2 response) or resolution (usually, associated with a predominant Th1 response) [18, 56, 57].

Also, in early infection, activated DC are the primary source of IL-12, which is a cytokine essential for NK activation and induction $CD4^+$ and $CD8^+$ T cells, priming a Th1/Tc1 immune response [47, 54, 55, 58].

NK cells present cytotoxic activity that can lead to apoptosis of infected cells, and produce the majority of early interferon (IFN)-γ that stimulates NO production by infected MØ. NK cells, also, contribute for regulating the development of $CD4^+$ T cell subsets, restringing parasite dissemination at the early stages of infection [48, 53, 59, 60]. The delay in NK activation can hold up or inhibit the development of Th1 cells, enhancing disease progress [59, 60].

MØ also are recruited to the infection site. These cells present phagocytic functions and are antigen presenting cells (APC). Despite being the primary immune cells involved in *Leishmania* elimination, are the host cell for parasites, allowing parasite replication, become infected either by phagocytosis of free parasites or by efferocytosis (by engulfing parasitized neutrophils) [18, 47, 53, 60].

Survival of *Leishmania* amastigotes (the most resistant morphological form of the parasite) inside MØ phagolysosomes is depending on complex evasion mechanisms that involve parasite virulence factors [18, 47].

MØ are activated by both phagocytosis and pro-inflammatory cytokines [IL-1, tumor necrosis factor (TNF) and type 1 interferons], which stimulates these cells to produce reactive oxygen species (ROS) and reactive nitrogen species (RNS), causing parasite elimination. However, MØ activation and the consequent oxidative stress can be subverted by *Leishmania* virulence factors [18, 60].~

1.3.3 Adaptive immunity

When the innate immune system is unable to control and eradicate the infection, then the adaptive immunity (also known as acquired immunity) comes into action. Activation of

this type of immunity is slower, however the immune response generated is specific to the pathogen and enables the generation of immune memory, which can enhance and accelerate the immune response in future infections by the same pathogen. It acts thought a strategic response that is highly based on the generation of specific antibodies (humoral immune response mediated by B cells) and production of cytokines, that regulate MØ anti-parasitic activity, which is mediated by T cells [48, 61, 62].

Naïve B cells start to differentiate after exposure to an antigen, while naïve T cells require antigen presentation by APC (MØ, DCs and B cells). Parasite antigen complexes with class I (MHCI) and class II (MHCII) molecules of major histocompatibility complex to be recognized by T cell receptors (TCR) of $CD8^+$ or $CD4^+$ T cells, respectively, initiating activation and cell proliferation [61, 62, 64]. However, T cell activation and differentiation are also dependent on two other factors, the co-stimulation by molecules that amplify or reduce the signal induced by the TCR-MHC complex and the presence of environmental cytokines that stimulate the clonal expansion, defining the type of response that will be generated by T cells [61, 62].

Activation and differentiation of B cells occur when an antigen matches the cell receptor (BCR) expressed on the cell membrane. Then, naïve B cell internalizes the antigen, replicates and differentiate into memory and into effector B cells. Effector B cells release antibodies that recognize and help to eliminate extracellular pathogens and their toxins circulating throughout the body [61-63, 65]. B lymphocytes also can play a role in the regulation of T cell mechanisms, such as cytokine production and antigen recognition [66, 67].

Population of T cells includes the $CD4^+$ and $CD8^+$ major cell subsets. The $CD4^+$ T cell subset develops into Th cells that can express four different phenotypes, presenting distinct cytokine secretion profiles, Th1, Th2, Th17 and regulatory T cells (Treg), while the $CD8^+$T cell subset develops in cytotoxic T cells (Tc) of type 1 and type 2 [62].

In *Leishmania* infections, several studies reported that B cells might exacerbate the infection by *L. tropica*, *L. mexicana* and *L. major*, and might contribute to the onset and persistence of CL. However, the mechanism underling the negative role of B cells in these infections is still unknown [18, 66].

Usually the production of IFN-γ and TNF-α by Th1 cells are associated with parasite burden control and infection resolution, due to the induction of NO production by MØ

whereas, production of cytokines such as IL-4 and IL-13 by Th2 cells is associated with susceptibility to *Leishmania* infection [48, 68-70].

Although $CD4^+$ T cells produce cytokines that mediate tissue damage IFN-γ and TNF-α are not involved in the cytolysis of infected cells, thus it is considered that IFNγ-producing $CD4^+$ T cells have no association with progression of the lesions, but rather with parasite killing. However, this may not always be the case, since in human patients with *L. braziliensis* infection, there are some evidences suggesting that high levels of IFN-γ producing $CD4^+$ T cells might be associated with larger lesions [69, 71, 72].

The overall role of $CD8^+$ T cells in *Leishmania* infection is still unclear since these cells can have both pathological and protective role against the infection. IFNγ-producing $CD8^+$ T cells might contribute to protection against *Leishmania* infection, favoring disease resolution, though this association appears to be dependent on the infective model used. Also, the production of IFN-γ by $CD8^+$ T cells induces the differentiation of IFNγ-producing $CD4^+$ T cells, promoting the establishment of a protective Th1 response [48, 69].

In addition, the presence of $CD8^+$ T cells expressing granzyme B in *L. mexicana* and *L. major* human infections have been associated with a good prognosis. In *L. braziliensis* infected patients, $CD8^+$ T cells can kill infected cells, however, are incapable of killing intracellular parasites. $CD8^+$ T cells, also might be involved in resistance to re-infection by *L. major* [18, 69, 70]. On the other hand, the presence of $CD8^+$ T cells in the infection site can cause the cytolysis of infected cells, leading to tissue necrosis and lesion progression. Also, it is reported that granzyme B^+ $CD8^+$ T cells are mainly associated with cell death and tissue destruction rather than parasite killing [69, 70].

Treg cells (also, Tsups for $CD8^+$ T cells) play an important role in the regulation of immune response. These cells can have a dual role. By releasing IL-10 mediates the contraction of the effector response of $CD4^+$ and $CD8^+$ T cells, resulting in parasite persistence, which can lead to disease reactivation. On the other hand, parasite persistence favors the maintenance of long term immunity. Thus, Treg cells skew the immune response in order to achieve immune homeostasis, promoting a balanced anti-inflammatory response that directs immune tolerance. This characteristic of Treg cell might in turn be used by *Leishmania* parasites for immune evasion [48, 73-76].

Characterization of Treg is a topic of controversy within the scientific community, especially in the less studied $CD8^+$ T cell subset. The expression of some molecules associated with the Treg cell populations has been reported. However, there is no consensus regarding the definitive markers of Treg cell subsets. In addition, the existence of regulatory subpopulations within the $CD4^+$ and $CD8^+$ T cell subsets is also suggested. These cell subsets that are characterized by distinct markers (such as CD25 and FoxP3) exhibit suppressor functions and therefore can be considered Treg cells [77-81].

1.3.4 Immune evasion mechanisms

To achieve a successful infection and disease establishment within a host, *Leishmania* parasites developed strategies to evade host immune mechanisms. These strategies might be decisive in the outcome of the ongoing battle between the parasite and the host immune response [48].

The immune evasion mechanisms used by *Leishmania* are simple modifications of the effector functionality of host immune components, as is the case of the complement system, phagocytosis, cytokines, chemokines, co-stimulatory molecules and T cells, Toll-like receptor downstream pathways, cell signaling, and antigen presentation that together ensures parasite survival within the phagosome [48].

Virulence factors are crucial for *Leishmania* parasites to evade the host immune system [48]. An example of a virulence factor widely present in *Leishmania* species is the glycoprotein of 63 kDa (GP63). GP63 is an abundant surface metalloproteinase found in metacyclic promastigotes (which are the infective form of the parasite) that cleaves C3b into an inactive form, C3bi (an opsonin). C3bi binds to parasite surface, targeting the parasite and promoting phagocytosis by MØ, which are the host cells of *Leishmania* parasites. Also, the formation of C3bi prevents the generation of C5 convertase and the subsequent MAC assembly, avoiding the complement mediated lysis of the parasite. In addition, C3bi marks the parasite, signaling the parasite to be phagocytosed and thus facilitate phagocytosis by MØ. So *Leishmania* can exploit the immune system to promote its survival [48, 82].

GP63 is also involved in the modulation of cytokine immune response, suppression of NK cell activity and decreased NO production. Interferes with signaling cascades and attenuates innate inflammatory responses by modulating MØ activity [82].
Therefore, virulence factors, not only enable the exploitation of the host immune system, increasing the parasite success and ensure disease onset, but also difficult the establishment of successful treatments and the development of efficient vaccines.

1.4 T cells

T cells maturate in the thymus and are responsible by cell immunity, playing a key role in adaptive immunity [62]. These cells are characterized by expressing a CD3 complex, an invariant subunit constituted by three different polypeptide dimmers, εγ, εδ, and ζζ, that integrates the constant domain of the TCR complex (Fig. 6). CD3 complex is required for intracellular signaling and for TCR surface expression. Since CD3 protein complex is a surface antigen characteristic of T cell lineage, anti-CD3 antibodies linked to a fluorochrome are widely used in flow cytometry as a marker to identify this leukocyte subpopulation [83-87].

The variable domain of the TCR complex can express either αβ chains or γδ chains (Fig. 6), that are associated to different T cell subpopulations. T cells with TCR expressing αβ chains generate either $CD4^+$ or $CD8^+$ T cells. Whereas T cells with γδ–TCR are associate to a small $CD4^-CD8^-$ T cell subpopulation (comprising ~ 5% of T cells) that possess immunoregulation and immunosurveillance functions [62, 87].
T cells expressing αβ chains generate either $CD4^+$ or $CD8^+$ naïve T cells that migrate to the secondary lymphoid organs where occurs priming and differentiation into specialized cell subsets [62].

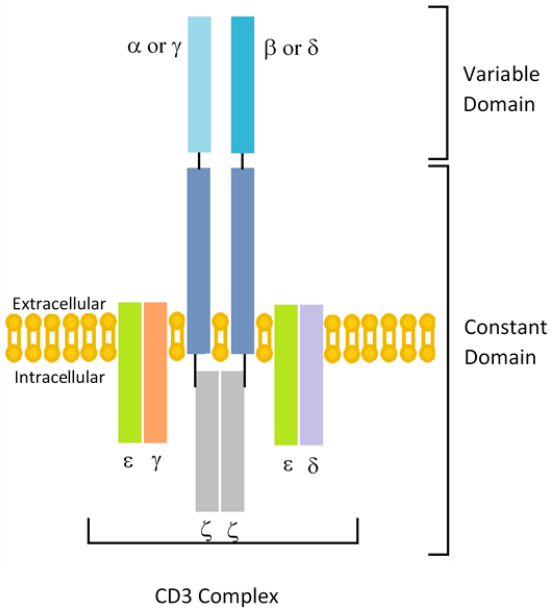

Figure 6 - Schematic representation of TCR complex. TCR complex is constituted by a CD3 complex with three pairs of dimers (εγ, εδ, ζζ) and a TCR-variable domain constituted by a heterodimer of α and β or γ and δ chains.

1.4.1 CD4⁺ and CD8⁺ T cells

$CD4^+$ T cells are converted into Th, which are responsible for cytokine production and stimulation of B cells to generate antibodies, whereas $CD8^+$ T cells are converted into cytotoxic cells (Tc) which can destroy infected cells by apoptosis, through the release of cytolytic granules and expression of ligands for death receptors [62].

CD4 is a glycoprotein of the membrane of T cells that interacts with MHCII molecule. This glycoprotein is not exclusive to T lymphocytes, also is present in B cells, macrophages and granulocytes [88].

Present on most cytotoxic T cells, CD8 is a cell surface glycoprotein that interacts with MHCI antigens and mediates cell-cell interactions [89].

Treg subsets, which are also called Tsups in $CD8^+$ T cells, are described to be present in both $CD4^+$ and $CD8^+$ T cells. However, both Treg cell subsets require further research to elucidate the molecular determinants that characterize this cell type and its role in the immune system, in particular $CD8^+$ Treg cells that until now little research was made regarding its role in the immune system [77, 81, 90].

1.4.2 Subset CD25$^+$

The α-chain of the IL-2 receptor also known as CD25 is expressed on activated T cells and in Treg cells that require IL-2 for their growth and survival. Treg cells have a crucial role in the control of immune self-tolerance and in controlling excessive pro-inflammatory reactions [91-94]. However, CD25 is also highly expressed by active B and NK cells and also by monocytes and thymocytes, although less extensively [94].

Although CD3$^+$CD4$^+$CD25$^+$ cell subsets are considered Treg cells, studies suggest that the expression of CD25 is not required for the development of Treg cells. However, deficiencies in CD25 caused immune deregulation, suggesting that Treg cells are highly dependent on IL-2 and thus CD25 seem to be essential for cell effectiveness [95]. Despite it is proved that CD4$^+$CD25$^+$T cells have suppressor functions, some researchers have suggested that this characterization is insufficient to identify Treg cell population and other makers are needed to fully characterize this cell population [81, 95, 96].

CD8$^+$ Tregs constitutively express CD25 and, also in this case the expression of CD25 by itself might not be a reliable indicator of a CD8$^+$ Treg population. [77, 88]. Furthermore, differentiation of CD8$^+$ short-lived effector cells is enhanced by the combined action of the up-regulation of CD25 by CD4$^+$ Th cells and stimulation by IL-2 [97].

1.4.3 Subset FoxP3$^+$

The nuclear transcription factor, fork-head box protein 3 (FoxP3) is considered the current definitive marker of CD4$^+$ Treg cells, since CD25, the original marker of these cells is also expressed in activated effector T cells. Also, has been shown that the Treg cell sub-population with a CD4$^+$CD25$^-$FoxP3$^+$ phenotype plays a suppressive function in mouse lungs [96, 98].

In the case CD8$^+$ T cells, FoxP3 is not considered a definitive marker of CD8$^+$ Treg cell populations, but rather the expression of surface markers CD44$^+$ and CD122$^+$. However, it has been reported the existence of several CD8$^+$ T cell subsets playing a suppressive role, including the small population of CD8$^+$CD25$^+$FoxP3$^+$ T cells present in both mice and humans [77, 90, 99].

Although FoxP3 is important for regulatory T cell differentiation, several studies indicate that $CD3^+CD25^+$ cells that are unable to express FoxP3 still maintain some of the regulatory abilities of Treg cells, suggesting that FoxP3 expression is not imperative for activation of regulatory T cells. Also, there are reports suggesting that FoxP3 might not be a definite marker of activated Tregs [79-81, 100].

1.4.4 Subset $CD25^+$ $FoxP3^+$

$CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells are the best characterized cell subpopulation of $CD4^+$ Treg. This Treg cell subset expresses suppressive functions and play a major role in achieving immune homeostasis and self-tolerance. Deficiencies in this subpopulation are highly related with autoimmune diseases [77, 101, 102].

$CD8^+$ $CD25^+$ $FoxP3^+$ T cells are a small population of Treg cells reported both in mice and humans. This cell subset is associated with immunosuppressive functions [77, 103, 104]. In addition, *in vitro* studies have indicated that $CD8^+$ Treg cells have an effect equal or superior on the suppression of effector T cell proliferation than $CD4^+$ Tregs and are more sensitive to IL-2 stimulation than $CD8^+$ effector T cells and less sensitive than $CD4^+$ Tregs [77].

This indicates that $CD8^+$ Tregs have more suppressive capacity than $CD4^+$ Tregs, and therefore this population should be under a stronger regulation to avoid uncontrolled immune suppression, which is achieved by the reduced sensitivity to IL-2 stimulation, which is essential for Treg activation and proliferation. These findings also might explain the reason why $CD4^+$ Tregs cells are significantly more abundant than $CD8^+$ Tregs cells.

2. Material and Methods

2.1. Objectives

The primary aim of this study was to analyze the differentiation of $CD4^+$ and $CD8^+$ T cells populations, thought expression of $CD25^+$ and $FoxP3^+$, after exposure to promastigotes of three cutaneous *Leishmania* species (*L. amazonensis, L. guyanensis* and *L. shawi*).

To achieve this main goal, two specific objectives had to be achieved:

- Analyze the differentiation of CD4+ and CD8+ T cells populations into regulatory T cells and effector T cells after exposure to live parasites and parasite antigens, by flow cytometry.
- Attainment of the protein profile of antigens of different *Leishmania* species, by SDS-Page, to correlate with T cells differentiation, after exposure to *Leishmania* antigens.

2.2. Experimental design

To evaluate the differentiation of T cell subpopulations induced *in vitro* by *Leishmania* parasites, mononuclear spleen cells were isolated from healthy BALB/c by a density gradient. Cells were divided into groups and then exposed to different stimuli: *L. amazonensis, L. guyanensis* and *L. shawi* viable promastigotes and respective antigens, for 72 h. In parallel, resting cells and cells stimulated with a mitogen (concanavalin A) were also evaluated as positive and negative controls. $CD8^+$ cell fraction was obtained by positive magnetic separation using magnetic beads coated with anti-CD8 monoclonal antibody, while $CD8^-$ cell fraction was obtained by negative magnetic separation (flow-through). To estimate the relative differentiation of T cell subsets both cell fractions were labelled with anti-CD3, anti-CD25 and anti-FoxP3 monoclonal antibodies conjugated with fluorochrome. Cell sub-populations were then evaluated by flow cytometry assays

and the relative frequencies determined as well as the levels of CD3, CD25 and FoxP3 expression (Fig. 7).

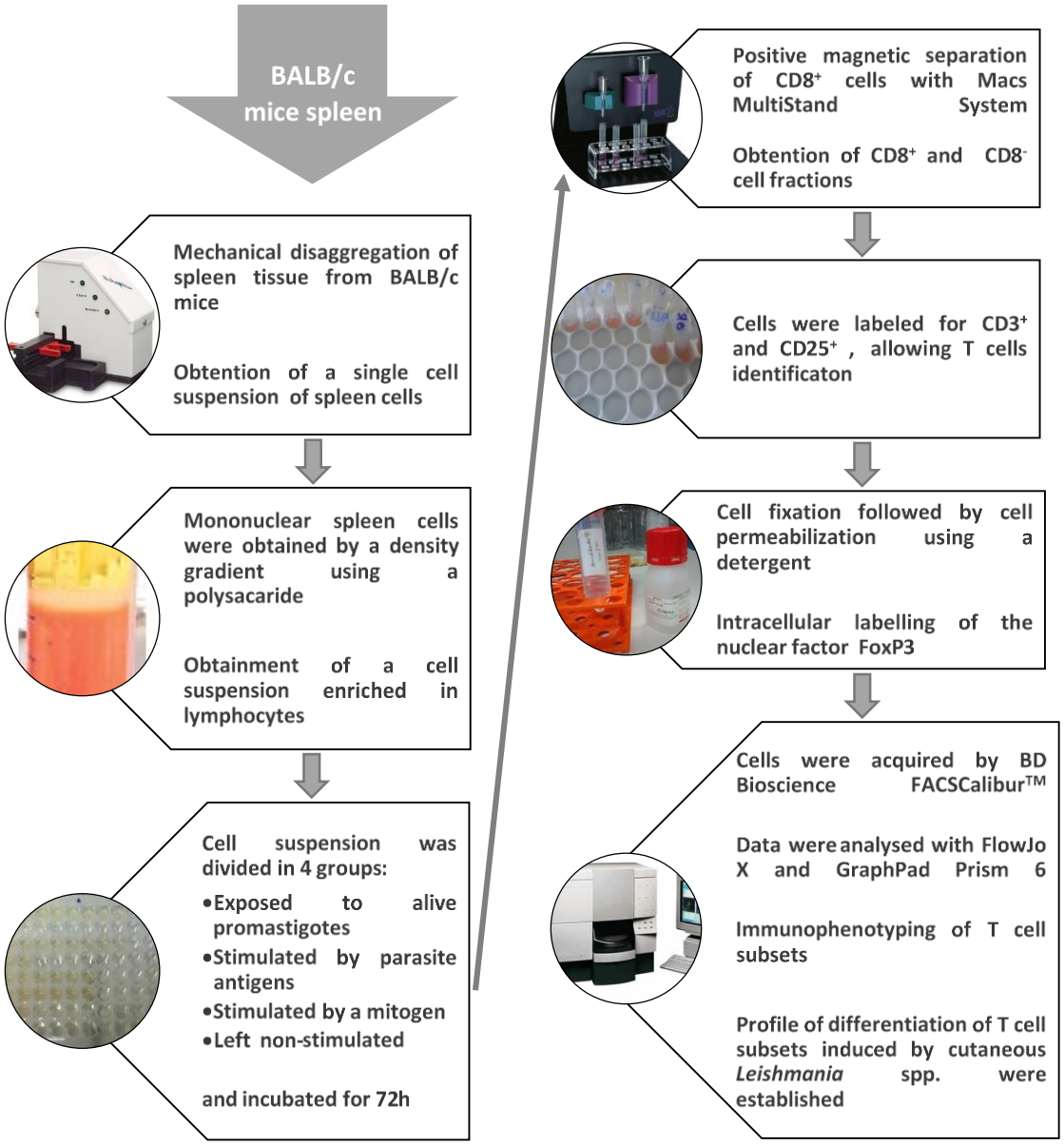

Figure 7 - Schematic representation of experimental design of Flow Cytometry Assays. This assay was used to evaluate T cell differentiation induced by Leishmania parasites that cause cutaneous leishmaniasis

Additionally, an SDS-PAGE assay was performed to compare the profile of *L. amazonensis*, *L. guyanensis* and *L. shawi* proteins, which were extracted by a detergent.

2.3. Animals

BALB/c mice (*Mus musculus*), specific pathogen-free (SPF), with four- to nine-week-old were purchased to Instituto Gulbenkian de Ciência and maintained at Instituto de Higiene e Medicina Tropical (IHMT) animal facility. Standard commercial feed (Harlan Ibérica) and water were provided *ad libitum*. Shortly after arrival, mice were sacrificed, and spleens were extracted in aseptic conditions and transferred to a sterile tube with Roswell Park Memorial Institute 1640 medium (RPMI medium, Sigma, USA) with penicillin/streptomycin (Pen-Strep, Sigma, USA) (proportion 1:100). The biological material was maintained on ice during transportation to the lab and while handling to assure the minimum of cell deterioration.

A total of twelve animals were sacrificed in this experiment. Animal handling was conducted according to EU Directive 2010/63/EU, recognized by the Portuguese law (Law 113/2013).

2.4. Parasites

Three distinct species of *Leishmania* that cause American cutaneous leishmaniasis (ACL) (*L. amazonensis*, *L. guyanensis* and *L. shawi*) were used in the present study were. Two of the species belonging to subgenus *Viannia* (*L. guyanensis* and *L. shawi)* and the other species are included in the subgenus *Leishmania (L. amazonensis*).

The *L. (L.) amazonensis* (MHOM⁄BR⁄1973⁄M2269) was kindly supplied by Dr. Luiz Felipe Passero from Bioscience Institute, Paulista State University, São Paulo, Brazil (UNESP), Brazil. *L. (L.) amazonensis* (MHOM⁄BR⁄1973⁄M2269) was isolated from a patient from the Pará State (Brazil) diagnosed with ACL. The strain was classified by monoclonal antibodies and isoenzyme profile (by MLEE) at Instituto Evandro Chagas, Belém, Pará State (Yamamoto et al., 2013).

L. (V.) guyanensis (MHOM/BR/2001/M19663) parasites were isolated from a patient living in Santarém (Pará State) diagnosed with ACL and was kindly provided by Dr. L.F. Passero (UNESP). The strain was identified by MLEE at Instituto Evandro Chagas.

L. (V.) shawi (MHOM/BR/96/M15789) parasites were isolated from a patient of Buriticupu, Maranhão state (Brazil) diagnosed with ACL. The strain was classified by monoclonal antibodies and by MLEE at Instituto Evandro Chagas (Passero et al., 2012) and kindly given by Dr. L. F. Passero (UNESP).

All the species of parasites used were maintained *in vitro* by regular passages in Schneider's insect liquid medium, with L-glutamine (Sigma, USA), 100 $U.ml^{-1}$ penicillin, 100 $g.ml^{-1}$ streptomycin (Pen-Strep, Sigma) and supplemented with 10% fetal bovine serum (FBS, Sigma, USA), previously inactivated by heat (30' at +56ºC). Cultures were incubated at 24ºC in a refrigerated incubator (Lovibond, Germany).
In order to obtain a high number of parasites, promastigotes were expanded in Schneider 10% FBS. Promastigote parasites replicate in culture indefinably as long the medium was replaced with fresh medium and pH was around 7.2.
When parasites reached or were close to reach stationary phase of growth, were applied a set of protocols to control parasite number and allow parasite grow. These protocols consisted in: (i) centrifugation (130 *g* or 300 *g* - depending on culture density, - 10', room temperature) to remove excess of promastigotes; (ii) addition of new medium; (iii) replacement of the medium - centrifugation (1800 *g*, 10', room temperature) followed by discard of the supernatant and re-suspension of the pellet with the appropriate volume of Schneider medium); or (iv) parasites were harvested by centrifugation (1800 *g*, 10', 4ºC) and then frozen at -80ºC for later use.

2.5. Antigens

Two different types of antigen were produced from *Leishmania* spp. (*L. amazonensis, L. guyanensis* and *L. shawi*) promastigotes: crude parasite antigen obtained through consecutive freeze-thaw cycles and soluble antigen obtained by detergent (sodium dodecyl sulphate, SDS) extraction of parasite proteins. Crude parasite antigens were used for stimulation of mouse lymphocytes and soluble parasite antigen was applied in SDS-PAGE assays to obtain the protein profile of each species and strain of *Leishmania* used in the present study.

To induce mouse lymphocyte differentiation, spleen mononuclear cells were exposed to crude parasite antigens that include most promastigote antigenic determinants. Due to the high content of deoxyribonucleic acid (DNA) and the high amount of glycoproteins (proteins rich in oligosaccharide chains covalently attached) present in *Leishmania* promastigotes, antigen migration across polyacrylamide gel is very difficult, therefore protein extraction by detergent followed by the mechanic shed of parasite DNA facilitate electrophoretic mobility of parasite proteins.

2.5.1. Crude parasite antigen

Promastigote lysate was obtained from cultures in the stationary phase of growth. Parasites were harvested by centrifugation (1800 *g*, 15', +25ºC). Supernatant was discarded, and the pellet was washed with phosphate buffered saline (PBS, VWR, USA) with 2 mM ethylenediamine tetraacetic acid (EDTA, Sigma, USA) by centrifugation (1800 *g*, 15', +25ºC). The supernatant was discarded again, and the pellet was washed two more times. Finally, the supernatant was discarded, and the pellet resuspend in 1 ml of PBS. Parasite disruption was achieved by 10 freeze-thaw cycles of - 20ºC and room temperature.

The protein content was determined on a Nanodrop (1000 Spectrophotometer, Thermo Fisher Scientific, USA). Protein concentration was adjusted to 4.5 mg. mL^{-1}. Crude parasite antigen was stored at -20ºC until use.

2.5.2. Soluble Antigen

Soluble antigen was obtained from 50 mL of a culture with promastigote density higher than 10^9 parasites per mL. Parasites were harvested by centrifugation (2000 *g*, 15', +25ºC). The supernatant was discarded, and the pellet was washed by centrifugation (2000 *g*, 15', +25ºC), using PBS with 2 mM EDTA. The supernatant was discarded, and the pellet was washed two more times. The final pellet was resuspended in 200 µL of tris-buffered saline (TBS) with 3% N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), a protease inhibitor, and 400 µL of TBS 10% SDS was added. To guarantee the shed of DNA, the solution was passed several times through three needles with different outer

diameters (represented by gauge numbers), in a successive process, from largest to the smallest diameter (19G followed by 21G and 25G needle). Then, the solution was incubated during 30' at room temperature and centrifuged (13000 *g*, 10', room temperature). Supernatant was collected, and protein content was determined on a NanoDrop. Protein concentration was adjusted to 24 mg. mL^{-1} and then stored at -20ºC until use.

2.6. *In vitro* activation of mouse lymphocytes

2.6.1. Single Cell Suspension

All the following procedures were performed in a biological safety cabinet - Herasafe™ KS (NSF) Class II, Type A2 (Heraeus, Germany).

Mouse spleen was transferred to a petri dish and cut in small fragments (~1–2 mm^3). Three to six fragments were transferred to a medicon (chamber containing a screen with a mesh size of 50 µm) and was added 1 mL of Hanks balanced salt solution (HBSS, Sigma, USA) 10% FBS. The medicon was closed and placed in an automated *homogenizer* (BD™ Medimachine System, BD, USA). Spleen fragments were mechanical disaggregated into a single cell suspension, using the following conditions: 2-3 pulses of 10-15 s. The cell suspension was collected with a syringe and transferred to a sterile 50 mL falcon tube placed in ice to avoid cell deterioration. This procedure was repeated until spleen fragments were all processed. Afterwards, 1 mL of HBSS 10% FBS was placed into the medicon and was processed in the same conditions than the spleen tissue and then collected to the falcon. This step was repeated three times. The deposit was discarded, and the cell suspension was centrifuged (10', 300 *g*, +4ºC). Then, the supernatant was discarded, and the pellet was resuspended in incomplete RPMI (RPMI medium without L-glutamine).

2.6.2. Isolation of mononuclear cells

Single cell suspension obtained from mouse spleen was gently added to a falcon with a separating solution of Ficoll (polysucrose of 400 kDa, Biocoll, VWR, USA) with a density of 1.077 $g.mL^{-1}$ in the proportion 1:1 (Biocoll: cell suspension) and centrifuged (20', 925 *g* without break, +20ºC). In plasma-Biocoll interface, the mononuclear cell layer enriched in lymphocytes (lymphocytes represent ~70% of the mononuclear cells) was carefully removed and transferred to a new falcon. PBS was added, and the cell suspension was centrifuged (10', 370 *g*, +4ºC). This step was repeated three times. After the last wash, the supernatant was discarded, and the pellet was resuspended in RPMI 20% FBS.

Cell viability was determined by trypan blue exclusion under an optical microscope. Trypan blue is an exclusion cell marker that dyes dead or non-viable cells. This dye was added to the cell sample, in the proportion 9:1 (dye: cell suspension), and viable (white cells) and non-viable (blue cells) cell concentration were estimated by cell counting in a Neubauer chamber (Marienfeld-Superior, Germany) under an optical microscope (Motic B1 Advance Series, China). Cell concentration was adjusted to $3{,}715 \times 10^6$cells/mL.

2.6.3. Cell differentiation

Mononuclear cell suspension (200 µL per well with ~$1{,}49 \times 10^5$ cells) was plated in a 96-well plate. Viable promastigotes L. *amazonensis*, *L. guyanensis* and *L. shawi* and crude antigens of the same species (Table 1) were added to cells. In parallel, non-stimulated cells and cells stimulated by concanavalin A (ConA), a mannose/glucose-binding lectin that induces T cell proliferation in a non-specific manner, were also incubated and used as negative and positive controls, respectively.

Stimuli	Inoculum
***Leishmania* spp.**	1:3 (promastigote: cell)
***Leishmania* crude antigens**	10 µg. mL^{-1}
ConA (Positive Control)	10 µg. mL^{-1}

Table 1– Stimuli and inoculum quantity used to induce differentiation of spleen mononuclear cells

Cells were incubated for 72 h in an incubator (Nuaire US Autoflow Incubator, USA) at 37ºC in a humidified atmosphere with 5% CO_2.

2.7. Magnetic separation of $CD8^+$ and $CD8^-$ cells

A MultiStand System constituted by MACS® Separators (Mini MACS), MS columns, CD8a (Ly-2) coated magnetic microbeads and a MultiStand (Miltenyi Biotec, Germany) was used to perform magnetic cell sorting of cells that were left to differentiate. All the procedures and reagents used in this assay were performed according to the protocols described in the data sheet of CD8a microbeads provided by the manufacturer [100].
The principle of this magnetic cell system is the use of microbeads, that are very small supermagnetic particles, to sort the cell subset of interest. The suspension of cells plus the beads passed through the column magnetically attached to MACS® Separator bound to the MultiStand. The columns and separator enable the formation of a high-gradient magnetic field that retain labelled cells in the column (positive selection of the cell subset of interest) [105, 106].

After incubation, cells were transferred to eppendorfs and centrifuged (10', 300 *g*, +4ºC). The supernatant was discharged, and the pellet was resuspended in 90 µL of PBS pH 7.2, with 0.5% FBS 2mM EDTA [magnetic separation (MS) buffer]. Was added 10 µL of CD8a (Ly-2) Microbeads (Miltenyi Biotec) and incubated for 15 min at 2−8 °C, protected from light. The suspension of cells and beads were washed with 1-2 mL of MS buffer and centrifuged (10', 300 *g*, +4ºC). The supernatant was discharged, and the pellet was

resuspended in 500 μL. Then, the suspension was passed through MS columns attached to a Mini MACS separator according to manufacturer's instructions. Non-labeled cells ($CD8^-$ cell fraction) were eluted and marked $CD8^+$ cells were retained in the column. After collection of the $CD8^-$ cell fraction (flow-through) the column was detached from the magnetic field, and cells $CD8^+$ cells were collected in a different eppendorf. $CD8^-$ cell fraction includes cells with a $CD4^+$ phenotype ($CD8^-$ $CD4^+$ cells).

2.7.1. Magnetic Separation (MS) Controls

In parallel, with the main flow cytometry assays, two samples of unstimulated cells from each cell fraction - $CD8^+$ and $CD8^-$ (total of four samples per assay) - were stained by a two different panel of fluorochromes. One of the samples was stained with anti-CD3 FITC and anti-CD4 PerCp, whereas the other was stained with anti-CD3 FITC and anti-CD8 PerCp.
These samples were later used to verify the success of the magnetic separation.

2.8. Flow Cytometry Assays

2.8.1. A brief introduction to flow cytometry

Flow Cytometry is a technique that uses sophisticated instruments to detect simultaneously several parameters on single cells or particles, allowing the acquisition of a variety of data about each cell that includes optical parameters (like size, volume and granularity) and fluorescence features after the addition of dyes or antibodies coupled to fluorophores [107, 108].
This technique uses beams of light with high focus and brightness, commonly from lasers, to strike a stream of single particles (this cell stream is accomplished by a fluidic system). A series of sensors detect light defraction and light emitted by the marked cells, which allow us to acquire and measure the light scattering and fluorescence emitted by cells [107-109].

Light scattering provide data directly related to structural and morphological aspects of each cell, giving indication of size, volume and granularity of each cell, whereas the fluorescence emission derives from the amount of fluorescence probe attach to the cell. The combination of these two factors allows us to quantitatively analyze individual cells and immunophenotype different cell subpopulations of a given sample [107, 108].
After being acquired by flow cytometer, data obtained for each sample was treated in a proper software (FlowJo V10, Tree Star Inc., USA).

2.8.2. Controls of flow cytometry

In flow cytometry assays is required the use of fluorochromes controls and fluorescence minus one (FMO) controls or blanks in each experiment.
A fluorochrome control, also known as compensation control, consist in a sample of cells marked with only one of the fluorochromes used in the assays. Every flow cytometry assays must contain a fluorochrome control for each of the fluorochromes used in the assays. These controls are used to perform compensations, which allow to compensate spill-over (which is a spectral overlap of fluorochrome signals), ensuring that the fluorescence detected derives from the fluorochrome that is being measured [110].
Blanks are unstained samples that are used in flow cytometry data analysis to determine the minimum of fluorescence, in which the fluorochrome that is being measured is considered positive.
FMOs have essentially the same function as the blank, however this control also allows to identify fluorescence spread into the channel of the fluorochrome of interest. They are gating controls, that ensure accuracy of gating establishment, allowing a precise delineation of positive and negative stained cells for the fluorochrome of interest, being especially important for low-density or smeared populations, such as populations expressing activation markers (such as FoxP3) [111].
An FMO control consists in a sample containing all the fluorochromes in a panel, except the one that is being measured.

All of these controls were included in each of the two flow cytometry assays performed in this study.

2.8.3. Membrane and intracellular labelling of cells

In flow cytometry, fluorochrome selection of to be used in an assay must be well planned and has to take into consideration a set of factors in order to get optimized results.
These factors are (i) fluorochrome brightness *vs* relative abundance of target antigen in the cell, (ii) fluorochrome optimal concentration, (iii) minimization of spill over, (iv) cytometer specifications and (v) the use of different detectors (FL1, FL2, FL3, FL4). Factors iii and v are crucial for flow cytometry, since both can lead to the overlay (spillover) of fluorochrome emission spectra, which leads to the incapability of the cytometer to distinguish and collect the individual data of a given fluorochrome, not being able to correctly immunophenotype cell populations. The fluorochrome combinations used in the present study were chosen based on the factors mentioned above, to ensure a reliable performance of the assays.

Membrane labelling

The two cell subsets magnetically sorted ($CD8^+$ and $CD8^-$) were centrifuged (10', 370 *g*, +4ºC). The supernatant was discharged, and the cells of each sample were resuspended in 200 µL of PBS 2% FBS (reduces antibody interference and non-specific binding), 0.01% NaN_3 (a metabolic inhibitor, that inhibits bacterial/fungal growth, capping and antigen internalization and, allows staining at room temperature). Monoclonal antibodies bound to fluorochromes (Biolegend, USA) were added to the cell suspension for cellular labelling (Table 2).

Furthermore, non-stimulated $CD8^-$ and $CD8^+$ cells labelled with a different set of fluorochromes (Table 3) were used as MS controls, allowing to estimate the efficacy of this methodology.

Monoclonal Antibody	Fluorochrome	Volume added (µL)	Concentration	Laser (Ex)	Emission max.
α-CD3	FITC	2	0.5 (mg. mL^{-1})	Blue Laser (488 nm)	520 nm
α–CD25	PerCP/ Cy 5.5	2.6	0.2 (mg. mL^{-1})	Blue Laser (488 nm)	676 nm

Table 2 –List of cell markers used in flow cytometry assays. FICT - Fluorescein isothiocyanate, PerCP/Cy 5.5 - Peridinin-chlorophyll proteins – cyanine 5.5

Monoclonal Antibody	Fluorochrome	Volume added (µL)	Concentration	Laser (Ex)	Emission max.
α-CD3$^+$	FITC	2	0.5 (mg. mL^{-1})	Blue Laser (488 nm)	520 nm
α-CD4$^+$	PerCp	5	0.2 (mg. mL^{-1})	Blue Laser (488 nm)	678 nm
α-CD8$^+$	PerCp	5	0.2 (mg. mL^{-1})	Blue Laser (488 nm)	678 nm

Table 3 –List of cell markers used as controls of magnetic cell sorting. FICT - Fluorescein isothiocyanate, PerCp - Peridinin-chlorophyll proteins

Cells were incubated in the dark for 30’ at +4ºC and then washed twice with 200 µL of PBS and centrifuged (10’, 600 *g*, room temperature). The supernatant was discharged, and the pellet was completely dissociated in the vortex and then resuspend in PBS with

2% formaldehyde with the aid of the vortex, to fix the cells. The cells were incubated in the dark for 10-20' min at room temperature.

After this step, FoxP3 FMO (marked with CD3 and CD25 fluorochromes), CD3 and CD25 fluorochrome controls and MS controls were washed in PBS and resuspended in 200 µL of PBS 2% FBS 0.01% NaN3, to be maintained in the dark at 4ºC until analysis. All the other samples proceeded to be processed as described in the intracellular staining protocol below.

Intracellular Staining

After incubation the cells were centrifuged (5', 400 *g*, +4ºC), the supernatant was discharged, and the pellet was washed twice with PBS (5', 400 *g*, +4ºC). Cells were then resuspended in PBS 1% FBS, 0.1% NaN_3, 0.5% Triton-X, at pH 7.4-7.6 (permeabilization buffer) and incubated for 10-20' at room temperature. The use of this buffer will partially dissolve both, the cytoplasmic and the nuclear membrane, to allow binding of the monoclonal antibody to the nuclear antigens.

Cells were centrifuged (5', 400 *g*, +4ºC) and supernatant was discharged while the pellet was washed twice with permeabilization buffer (5', 400 *g*, +4ºC). Cells were resuspended in the residual volume, labelled with FoxP3 monoclonal antibody (Table 4) and incubated for 30' protected from light at room temperature.

Monoclonal Antibody	**Fluorochrome**	**Volume added (µL)**	**Concentration**	**Laser (Ex)**	**Emission max.**
α-Fox P3	PE	2.6	0.2 (mg. mL^{-1})	Blue Laser (488 nm)	578 nm

Table 4 – Monoclonal antibody used for cell intracellular labelling. PE – R-phycoerythrin

Was added 2 mL of permeabilization buffer to cells and then centrifuged (5', 600 *g*, room temperature). Cells were resuspended in PBS 2% paraformaldehyde and incubated in the

dark during 20 min at 4ºC. Then, cells were washed in PBS, resuspended in 200 µL of PBS 2% FBS 0.01% NaN3 and maintained in the dark at 4ºC until to be analyzed.

2.8.4. Cell Acquisition

Cell acquisition was performed on a FACSCalibur flow cytometer (BD Biosciences). The flow cytometry analysis was performed due to a generous collaboration with Dr. Graça Alexandre-Pires of the Faculdade de Medicina Veterinária da Universidade de Lisboa. Triplicates of two independent experiments were evaluated.

2.8.5. Data treatment

All the data obtained through flow cytometry analysis was uploaded in FlowJo V10 software separated into groups: Samples, Negative Controls, Positive Controls, Fluorochromes Controls, FMO and MS Controls.
It was established a first gate to exclude cells in the extreme of both axis, which represent dead cells (cell debris) or cell doublets (Fig. 8).
After being established the first gate, it was determined the minimum of fluorescence in which the cells were considered positive for each fluorochrome, using the respective FMOs. These gates are generated, in the option Histogram (Fig. 8), and selecting the detector in which the fluorescence emitted by fluorochrome was sensed (Table 5).

Monoclonal Antibody	Fluorochrome	Detector	FMO
α-CD3	FITC	FL1	PerCp/Cy5., PE
α–CD25	PerCp/Cy5.5	FL3	FITC, PE
α–Fox P3	PE	FL2	PerCP/Cy5.5, FITC

Table 5 – List of used fluorochromes and the respective detectors and FMOs.

Using the minimal fluorescent values estimated by FMOs, was established a gate for $CD3^+$ cells. Within the $CD3^+$ cells gate, was selected the option "FL-2H::FoxP3 PE" in the Y axis, while the option "FL-3H::CD25 PerCp Cy 5.5" and were created four gates

using the "Quad" tool. These gates were obtained by using the minimal fluorescent values attained for gate of $CD25^+$ cells and $FoxP3^+$ cells (Fig. 9). The different quadrants (Q) were named Q1 ($CD25^-$ $FoxP3^+$), Q2 ($CD25^+$ $FoxP3^+$), Q3 ($CD25^+$ $FoxP3^-$) and Q4 ($CD25^-$ $FoxP3^-$). Cellular frequency (%) and fluorescent intensity (median) of each quadrant were determined (Figure 9). Data attained was then transcribed to GraphPad Prism for statistical analysis and graphical presentation.

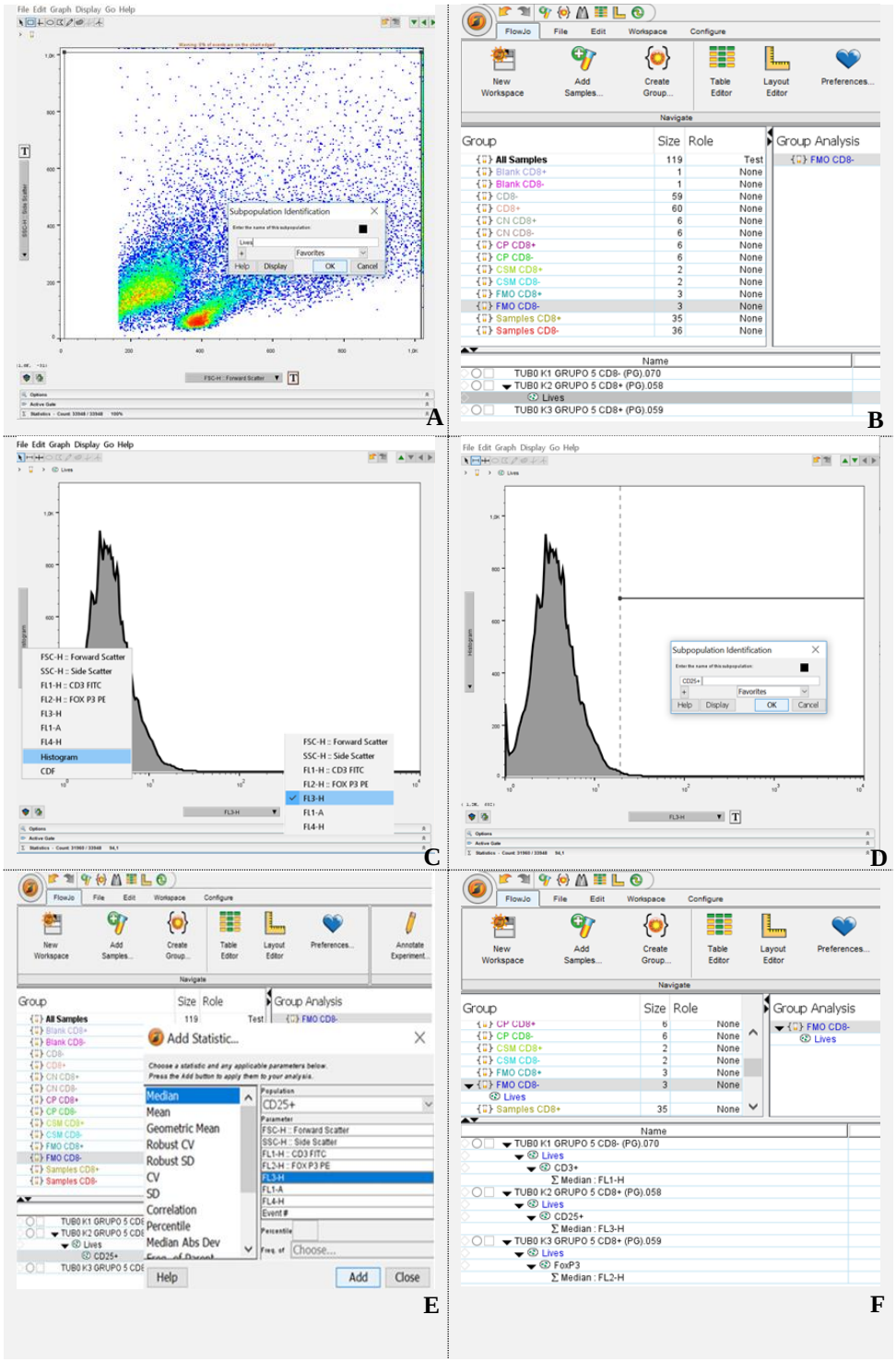

Figure 8 – Representative generation of FMOs' gates. A - Selection of the first gate of alive cells by excluding cells in the extreme of both axis (dead cells or cell clumps) in the FMO sample; B - Selection of newly generate gate of alive cells; C - Selection of "Histogram" option for the Y axis and FL (the FL choice varies according to the FMO, in which the gate was established) for X axis; D - Selection of $CD25^{+}$ gate; E – Application of statistics to the newly generate gate; F- Application of previously described steps to all FMOs.

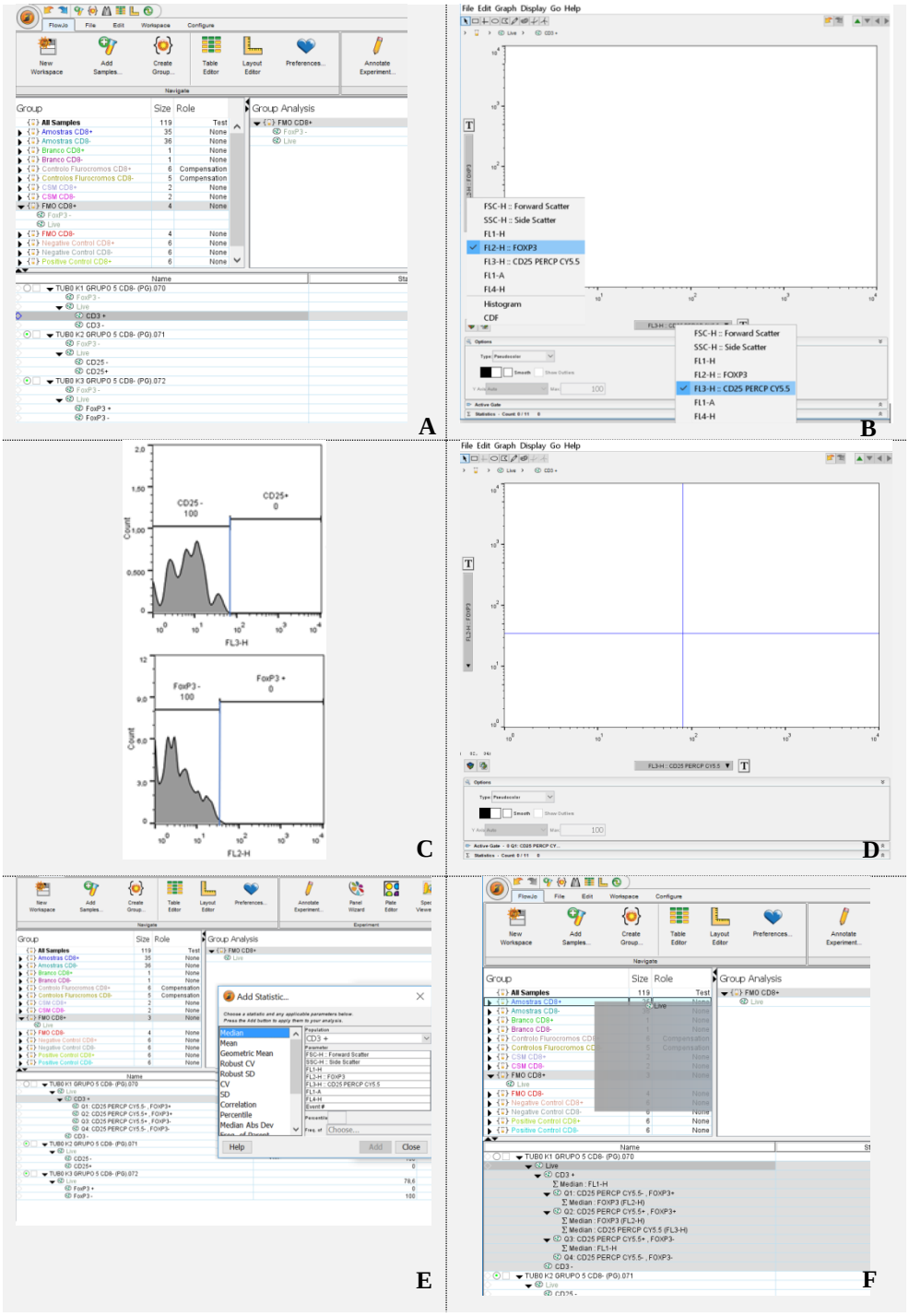

Figure 9 – Representative application of FMOs' gates to sample groups; A - Selection of $CD3^+$ cells; B – Selection of FL-3 in the X axis and FL-2 in the Y axis; C – Verify the minimum of fluorescence for CD25 and FoxP3 in their FMOs; D – Apply both minimum of fluorescence to the CD3 gate with the option "Quad", which will form four quadrants within $CD3^+$ population; E - Application of statistics for the median IF; F - Application of the newly formed gates to sample groups.

2.9. SDS-PAGE

2.9.1. A brief introduction to SDS-PAGE

PAGE (Polyacrylamide Gel Electrophoresis) assay is an analytic technique used to sort components of a protein mixture based on their mass [112, 113].

The basic principle of this technique is the migration of a charged molecule in an electric field in the direction of an electrode with an opposite electric charge. However, to apply this principle, the proteins in the sample must acquire uniform electric charge, which do not occur in the protein's native form. To overcome this problem is necessary to add SDS, an anionic detergent that denatures the proteins present in a sample, making the protein to assume a linear orientation, and consistently distribute negative charge according to protein's mass [113].

To aid in protein denaturation and charge density, protein samples are boiled at 100 ºC. SDS and β-mercaptoethanol combined cause the breakage of disulphide bonds, allowing the binding of SDS to the reduced polypeptide [112].

This method enables the separation of proteins only based on their molecular mass [112, 113].

Although this technique can be used with several purposes, in this study it was applied to enable comparison of the protein composition of antigens of the species of *Leishmania* used in the present study to estimate protein size and to determine protein relative abundance.

2.9.2. SDS-PAGE assays

Hand-cast gels were prepared from 30% bis-acrylamide (Bio-Rad, USA). Resolving gel was cast with 12% acrylamide solution and stacking gel with 5% of acrylamide solution to make the stacking gel.

The gel was loaded with 7.5 µL of antigen sample (with protein concentration of 24 mg. mL^{-1}) plus 7.5 µL of sample buffer (containing 1mL of 1 M Tris-HCl pH 6.8, 4 mL of 10% SDS, 2 mL of glycerol and 500 µl of 1% Bromophenol blue, before adding to the sample this buffer was complemented with 5% β-mercaptoethanol). Was also loaded, 5 µL of a pre-stained molecular mass (MM) marker (Precision Plus Protein – Dual Color Standards, Bio-Rad, USA). With the exception of the ladder, all the samples were boiled

during 7' before being loaded on the gel. Electrophoresis run was performed at 80 V for the stacking gel and 120 V for the resolving gel. The total running time of the SDS-PAGE was around 1 h.

The gel was stained by submerging in Coomassie blue solution (10% methanol, 5% acetic acid and 2% of Coomassie Blue (v/w), Thermo Fisher, USA) during 1 h under gentle agitation. Then, the gel was submerged in a destain solution (10% methanol and 5% acetic acid) and left overnight under gentle agitation. Images of stained gel were acquired and uploaded to ImageJ (developed by Wayne Rasband, developer at National Institutes of Health, USA) were the band measures and migration measurements were performed.

To determine the molecular mass of protein bands in the gel, the distance from the top to the dye front of the gel (also denominated migration distance of the dye front) and the distance of migration of each protein band (distance between the gel top and the protein band), were measured including the proteins of the MM marker.

With the measurements of migration of each protein band and the migration distance of the dye front were determined the relative migration distance (Rf) of each protein with the formula below.

$$Rf = \frac{migration\ of\ each\ protein\ band}{migration\ of\ the\ dye\ front}$$

Using Excel (Microsoft®, USA), Rf values (X axis) and log MMs (Y axis) was plotted, generating a linear function and the respective equation determined:

$$Y = aX + b$$

The MM of each *Leishmania* protein band was calculated by replacing in the equation the X by the Rf value of each band, and resolving the equation in order to Y.

The molecular mass of protein bands of each *Leishmania* spp. used in the present study was transcribed to a table and compared.

Also, it was measured the length of each protein band, for each species. The sum of the measurements of the band lengths was considered to be 100% of the protein content of the antigen. Then, the relative percentage of protein content of each band were estimated for each species.

2.10. Statistical Analysis

Data of two independent experiments and samples triplicates are represented by bar charts (mean and standard deviation values)

The non-parametric Wilcoxon for two paired samples was used to compare differences between the negative control group and the positive control group and between the negative control group and the different cell subsets between (GraphPad Prism, GraphPad Software, Inc., USA). A significance level of 5% ($p < 0.05$) was used as indicative of statistical significance.

2.11. Data analysis

All data obtain in the flow cytometry assays was transcribed to GraphPad Prism and were created two groups of graph column bars representing the mean plus standard deviation: for population percentage of each subset (i) and for the median intensity of fluorescence (IF) of each fluorochrome for each subset in which it is expressed (ii). In these graphs are represented all the test groups (positive and negative controls plus live parasites and respective antigens), and all the statistically significant groups in comparison to the negative control, which is determined as described in the previous sub-chapter, are indicated.

The data is analyzed the same way for both T cells subsets: $CD8^+$ and $CD8^-$.

In addition, is performed a correlation between overall T cells population percentage expressing a marker (CD3, CD25, FoxP3) and the median IF measured for that same population. This correlation is achieved by a linear regression in which is compared the data from the negative control with the data from the test groups. The slope of the linear regression provides us an additional indication of the modulation of cells subsets, since could relate the augmented or decreased of expression of a marker and augmented or decreased of a population expressing the same marker.

3. Results

3.1. Magnetic separation of cell fractions is recognized as an efficient methodology

The efficacy of magnetic separation verified by flow cytometry indicated that the $CD8^+$ cell fraction contained ~79% of $CD3^+CD8^+$ cells and $CD8^-$ cell fraction included ~85% of $CD3^+CD4^+$ cells. These results indicate that $CD8^+$ cell fraction corresponds mainly to a $CD8^+$ T cell subpopulation or T cytotoxic lymphocytes and $CD8^-$ cell fraction is mainly constituted by $CD4^+$ T cells. As expected $CD8^-$ T cell subpopulation is significantly more predominant (Fig. 11) than the $CD8^+$ T cell subpopulation. Furthermore, due to the lower number of $CD3^+CD8^+$ T cells, well defined subset clusters were not obtained (Fig. 12). A minimum of fluorescence was established for each fluorochrome, using the respective FMO and the gates were selected (Fig. 10). CD25 and FoxP3 gates were defined within the CD3 gate, allowing the assessment of four T cell subsets according to the box plot quadrants: Q1 ($CD25^-$ $FoxP3^+$), Q2 ($CD25^+$ $FoxP3^+$), Q3 ($CD25^+$ $FoxP3^-$) and Q4 ($CD25^-$ $FoxP3^-$) (Fig. 11 and 12)

3.2. *Leishmania* parasites and antigens can modulate $CD4^+$ populations

3.1.1. *L. guyanensis* and *L. amazonensis* parasites restrain the $CD3^+$ $CD4^+$ cell subset

Cells exposed to *L. amazonensis* and *L. guyanensis* parasites evidenced a significative contraction of $CD3^+$ cell subset ($p < 0.05$) when compared with non-stimulated cells (negative control). On the other hand, cells stimulated with *L. amazonensis*, *L. guyanensis* and *L. shawi* crude antigens presented a significant expansion of $CD3^+$ cells ($p < 0.05$). ConA stimulation also promoted a significative expansion of the $CD3^+$ cell subset, indicating that cells were viable and functional. Interesting, *L. shawi* parasites seemed not cause important changes in the $CD3^+$ $CD4^+$ cells (Fig. 13).

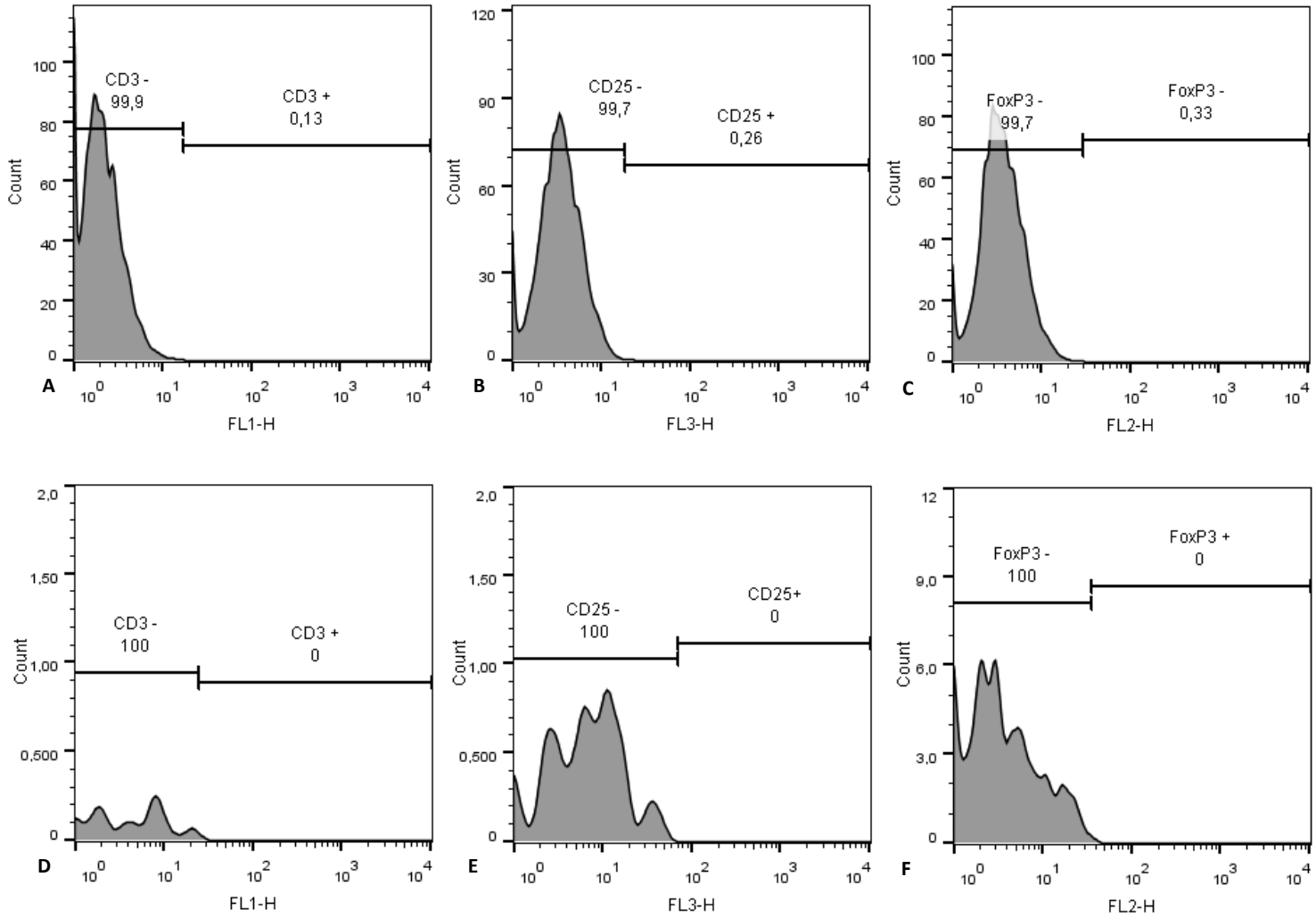

Figure 10 - Gate selection. A, B and C – Selection of CD3+, CD25 and FoxP3 gates in $CD4^+$ T cell fraction; D, E and F - Selection of CD3, CD25 and FoxP3 gates in $CD8^+$ T cell fraction

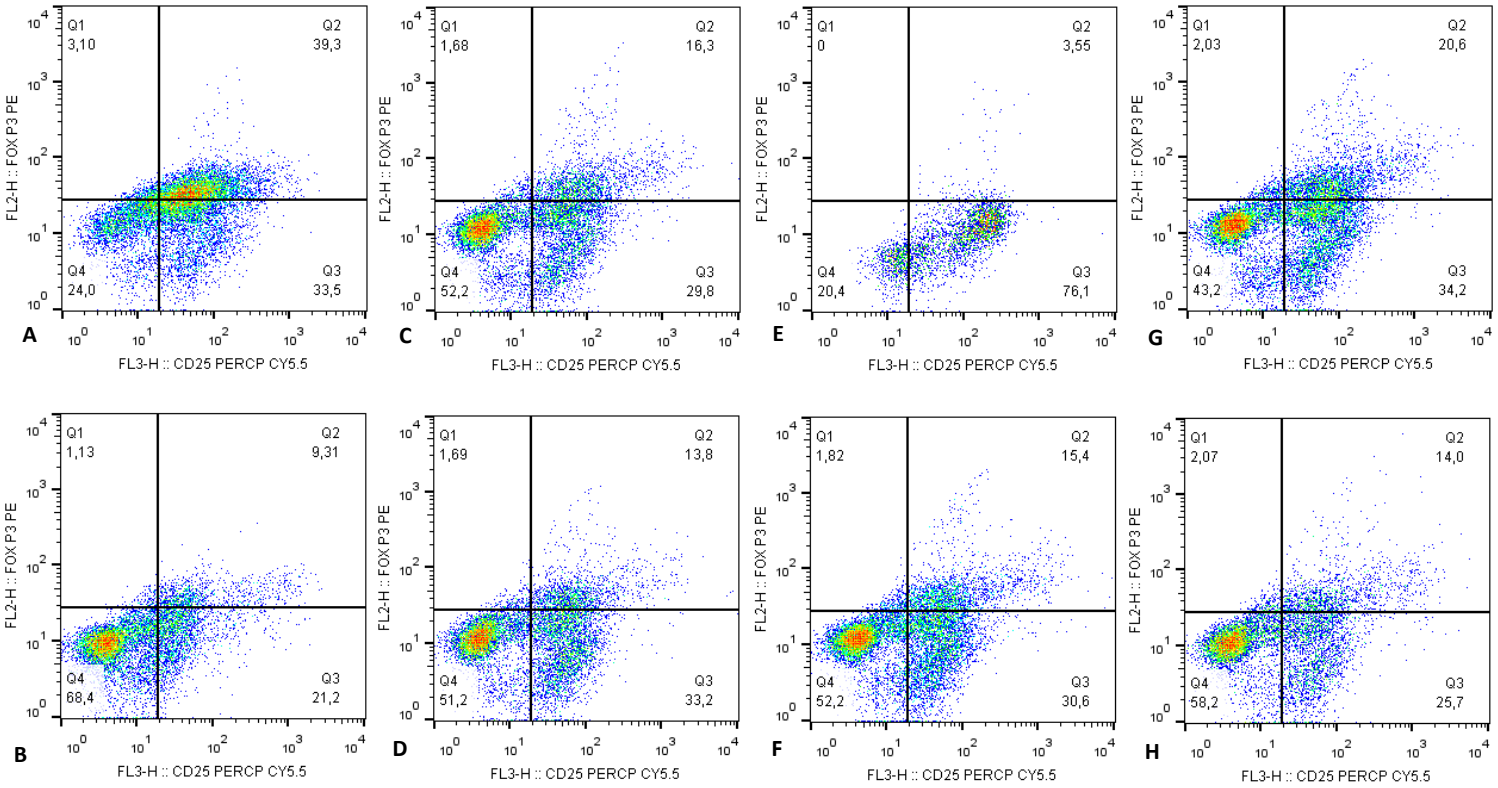

Figure 11 - Representative image of CD4$^+$ T cell phenotypes after exposure to *Leishmania* parasites or stimulation by parasite antigens. A – Concanavalin A stimulate cells (positive control); B – Non-stimulated cells (negative control); C – Cells exposed to *L. amazonensis* parasites; D – Cells stimulated by *L. amazonensis* antigen; E - Cells exposed to *L. guyanensis* parasites; F – Cells stimulated by *L. guyanensis* antigen; G - Cells exposed to *L. shawi* parasites; H – Cells stimulated by *L. shawi* antigen. Q1 quadrant corresponds to CD3$^+$ CD4$^+$CD25$^-$ FoxP3$^+$ cells, Q2 to CD3$^+$ CD4$^+$CD25$^+$ FoxP3$^+$cells, Q3 to CD3$^+$ CD4$^+$CD25$^+$ FoxP3$^-$ cells and Q4 to CD3$^+$ CD4$^+$CD25$^-$ FoxP3$^-$ cells.

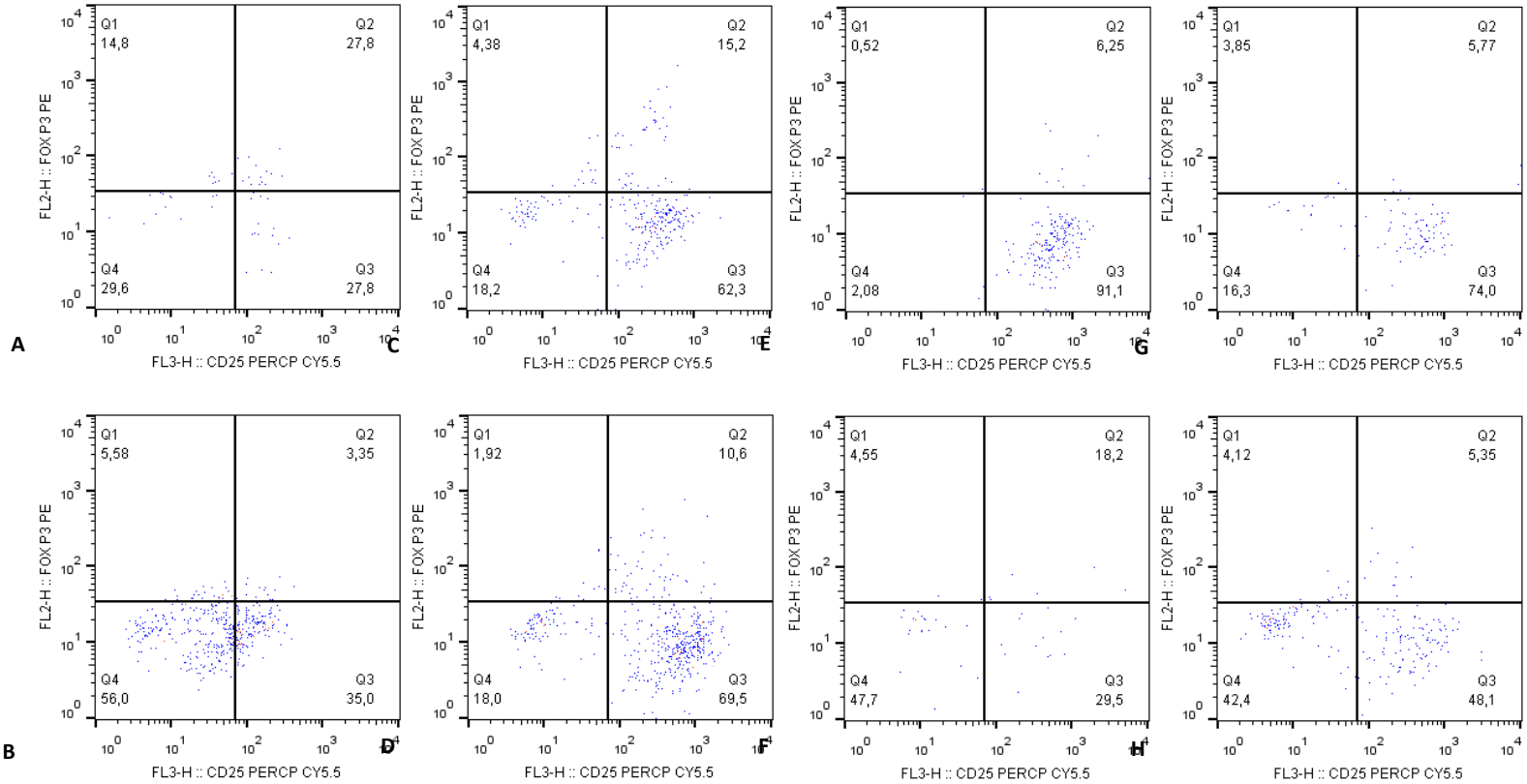

Figure 12 - Representative image of CD8$^+$ T cell phenotypes after exposure to *Leishmania* parasites or stimulation by parasite antigens A – ConA stimulate cells (positive control); B – Non-stimulated cells (negative control); C – Cells exposed to *L. amazonensis* parasites; D – Cells stimulated by *L. amazonensis* antigen; E - Cells exposed to *L. guyanensis* parasites; F – Cells stimulated by *L. guyanensis* antigen; G - Cells exposed to *L. shawi* parasites; H – Cells stimulated by *L. shawi* antigen. Q1 quadrant corresponds to CD3$^+$CD8$^+$CD25$^-$ FoxP3$^+$ cells, Q2 to CD3$^+$CD8$^+$CD25$^+$ FoxP3$^+$cells, Q3 to CD3$^+$CD8$^+$CD25$^+$ FoxP3$^-$ cells and Q4 to CD3$^+$CD8$^+$CD25$^-$ FoxP3$^-$ cells.

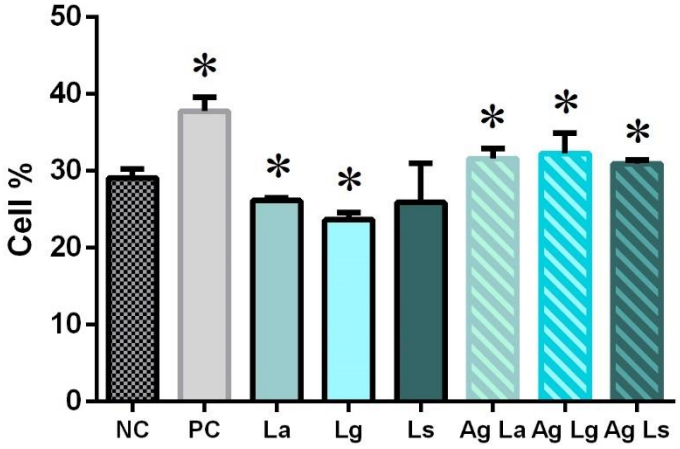

Figure 13 – Parasites and antigens cause changes in $CD3^+$ $CD4^+$ cell subset. $CD4^+$ cell fraction exposed to *L. amazonensis (*La), *L. guyanensis* (Lg) and *L. shawi* **(**Ls) parasites and stimulated by *L. amazonensis (*Ag La), *L. guyanensis* (Ag Lg) and *L. shawi* (Ag Ls) crude antigens were marked with anti-CD3 antibody and the frequency of $CD3^+$ cells was estimated by flow cytometry. In parallel, non-stimulated cells and cells stimulated by ConA were used as negative (NC) and positive controls (PC), respectively. Results of two independent experiments and three replicates per sample are represented by the mean and standard deviation. The results were statistically analyzed ($p < 0.05$) and * indicates statistical significance when compared with NC.

3.1.2. *L. guyanensis* parasites abolish the differentiation of $CD4^+$ $CD25^+$ $FoxP3^+$ T cells

When compared with non-stimulated cells, *L. amazonensis*, *L. guyanensis* and *L. shawi* antigens caused a significative expansion of $CD4^+CD25^-FoxP3^+$ T cell subset ($p < 0.05$). On the other hand, *L. guyanensis* parasites provoked the total contraction of $CD4^+CD25^-$ $FoxP3^+$ T cells ($p < 0.05$, Fig. 14A).

Antigens of *L. amazonensis*, *L. guyanensis* and *L. shawi* also promoted the significative expansion of $CD4^+CD25^+$ $FoxP3^+$ T cell subset when compared with non-stimulated cells ($p < 0.05$). In this particular case, *L. amazonensis* and *L. shawi* parasites also induced a significative expansion of this cell subset ($p < 0.05$). On the contrary, *L. guyanensis* parasite caused a significative contraction ($p < 0.05$, Fig. 14B).

With the exception of *L. shawi* antigen, all parasites and antigens induced the significative expansion of $CD4^+CD25^+FoxP3^-$ T cells ($p < 0.05$, Fig. 14C).

Curiously, $CD4^+CD25^-FoxP3^-$ T cell subset, representing effector T helper cells, presented a significative reduction ($p < 0.05$) when exposed to parasites or to crude antigens (Fig. 14D).

ConA induced the significative expansion of $CD4^+CD25^-FoxP3^+$ ($p < 0.05$), $CD4^+CD25^+$ $FoxP3^+$ ($p < 0.05$) and $CD4^+CD25^+FoxP3^-$ ($p < 0.05$) T cell subsets.

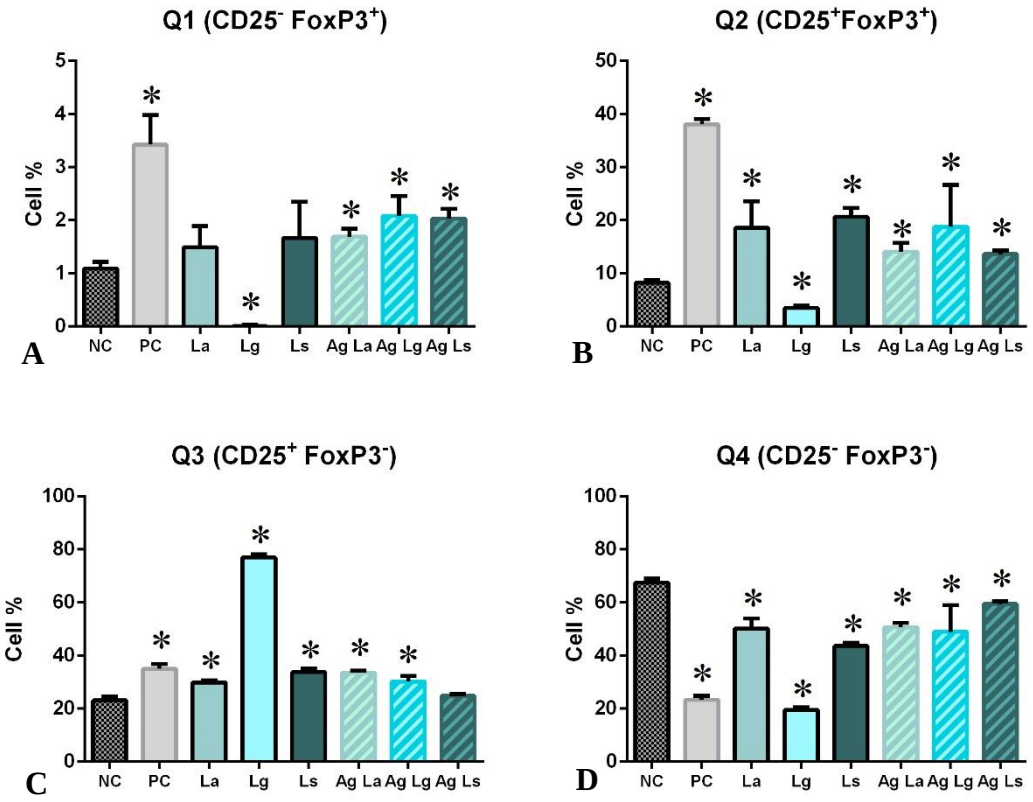

Figure 14 – Changes in $CD4^+$T cell subset caused by *Leishmania* parasites and antigens. $CD4^+$ T cells exposed to *L. amazonensis (*La), *L. guyanensis* (Lg) and *L. shawi* **(**Ls) parasites and stimulated by *L. amazonensis (*Ag La), *L. guyanensis* (Ag Lg) and *L. shawi* (Ag Ls) crude antigens were marked with CD25 and FoxP3 antibodies and evaluated by flow cytometry. In parallel, non-stimulated cells (NC) and cells stimulated by ConA (PC) were also evaluated. The frequency of $CD4^+CD25^-FoxP3^+$ (A), $CD4^+CD25^+$ $FoxP3^+$ (B), $CD4^+CD25^+FoxP3^-$ (C) and $CD4^+CD25^-$ $FoxP3^-$ (D) T cells were estimated. Results of two independent experiments and three replicates per sample are represented by the mean and standard deviation. The results were statistically analyzed ($p < 0.05$) and * indicates statistical significance when compared with NC.

3.1.3. *L. amazonensis* and *L. guyanensis* downregulate the expression of CD3 molecules

When compared with non-stimulated cells, the expression of CD3 molecules was significantly reduced in $CD4^+$ cell fraction when exposed to *L. guyanensis* parasites and *L. amazonensis* crude antigen ($p < 0.05$). An augmented of CD3 expression was verified in ConA-stimulated cells ($p < 0.05$) (Fig.15).

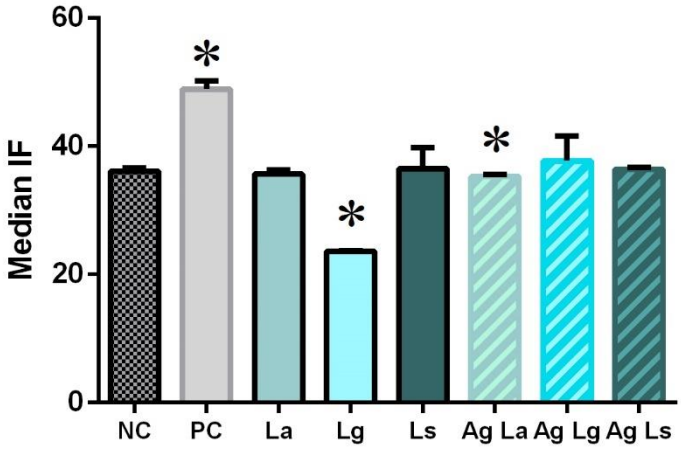

Figure 15 – Expression of CD3 molecules on $CD4^+$ cells. $CD4^+$ cell fraction exposed to *L. amazonensis (*La), *L. guyanensis* (Lg) and *L. shawi* **(**Ls) parasites and stimulated by *L. amazonensis (*Ag La), *L. guyanensis* (Ag Lg) and *L. shawi* (Ag Ls) crude antigens were marked with anti-CD3 antibody and the fluorescence intensity was estimated by flow cytometry. In parallel, non-stimulated cells and cells stimulated by ConA were used as positive (PC) and negative controls (NC), respectively. Results of two independent experiments and three replicates per sample are represented by the mean and standard deviation. The results were statistically analyzed ($p < 0.05$) and * indicates statistical significance when compared with NC.

3.1.4. Parasites modify the expression of FoxP3 and CD25 molecules in $CD4^+CD25^+$ $FoxP3^+$ and $CD4^+$ $CD25^+$ $FoxP3^-$ T cell subsets

When compared with non-stimulated cells, $CD4^+CD25^+FoxP3^+$ T cells, representing $CD4^+$ Treg cell subpopulation, evidenced a significant increase of intracellular FoxP3 molecules (Fig. 16B) and of CD25 molecules (Fig. 16C) in the cell membrane when exposed to parasites or stimulated by crude antigens ($p < 0.05$), except *L. guyanensis* that only increased CD25 molecules (Fig. 16C) in the cell membrane. $CD4^+CD25^+FoxP3^-$

T cells also evidenced higher expression of CD25 molecules ($p < 0.05$) when exposed to parasites or stimulated by crude antigens (Fig 16D). However, in the $CD4^+CD25^-$ $FoxP3^+$ T cells the amount of FoxP3 molecules stay unchanged, except when exposed to *L. guyanensis* parasites (Fig. 16A). In this particular case, the density of FoxP3 molecules decreased significantly when compared when non-stimulated cells ($p < 0.05$). ConA induced the expression of FoxP3 molecules in $CD4^+CD25^+FoxP3^+$ T cells and of CD25 molecules in $CD4^+CD25^+FoxP3^+$ and $CD4^+CD25^+FoxP3^-$ T cells, but decrease FoxP3 molecules in $CD4^+CD25^-FoxP3^+$ T cells ($p < 0.05$, Fig. 16).

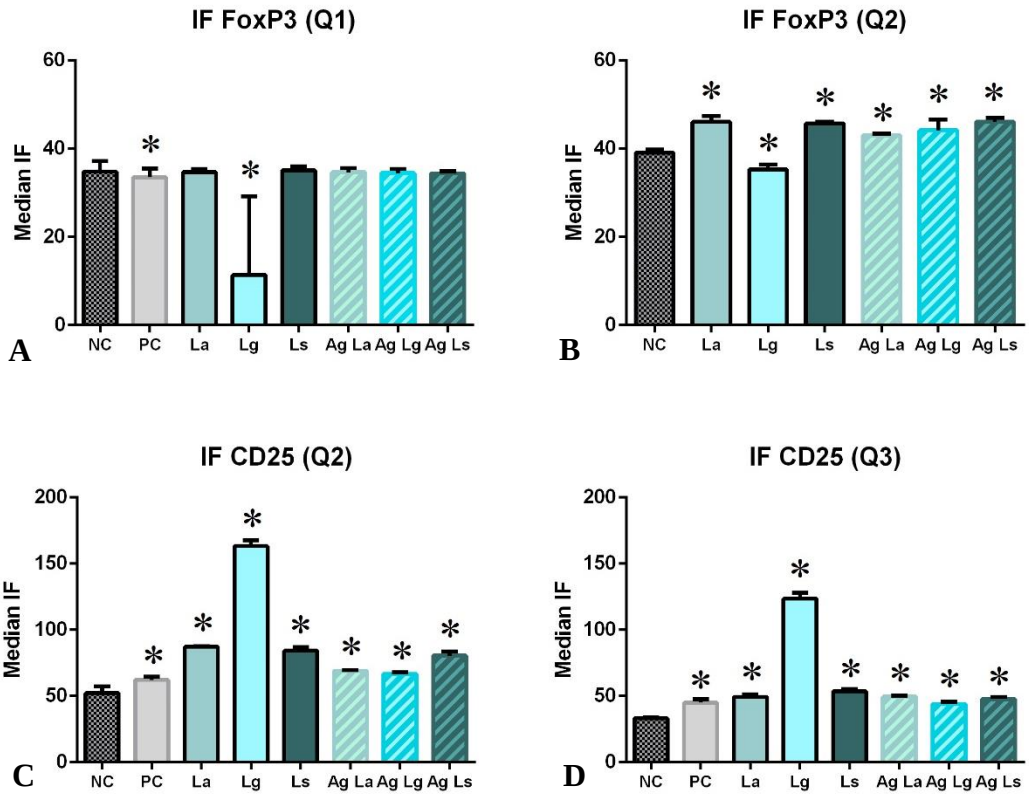

Figure 16 – Density of intracellular FoxP3 molecules and of membrane CD25 molecules in $CD4^+$T cell subsets induced by *Leishmania* parasites and antigens. $CD4^+$ T cells exposed to *L. amazonensis* (La), *L. guyanensis* (Lg) and *L. shawi* (Ls) parasites and stimulated by *L. amazonensis (*Ag La), *L. guyanensis* (Ag Lg) and *L. shawi* (Ag Ls) crude antigens were marked with CD25 and FoxP3 antibodies and fluorescence intensity evaluated by flow cytometry. In parallel, non-stimulated cells (NC) and cells stimulated by ConA (PC) were also evaluated. Density of FoxP3 molecules was estimated in $CD4^+CD25^-$ $FoxP3^+$ (A) and $CD4^+CD25^+FoxP3^+$ T cell subset (B) and the density of CD25 molecules was analyzed in $CD4^+CD25^+$ $FoxP3^+$ (C) and $CD4^+CD25^+FoxP3^-$ T cell subsets (D). Results of two independent experiments and three replicates per sample are represented by the mean and standard deviation. The results were statistically analyzed ($p < 0.05$) and * indicates statistical significance when compared with NC.

3.1.5. Reduction of $CD3^+$ population induced by *L. guyanensis* and *L. shawi* is correlated with down regulation of CD3 molecules

A positive correlation generated between the frequency of $CD3^+$ $CD4^+$ cells and the density of membrane CD3 molecules (slope 1.396) in ConA stimulated cells indicate that the expansion of cell subset is accompanied by the upregulation of CD3 expression (Fig. 17A). Furthermore, cells exposed to *L. guyanensis* (slope 2.068, Fig. 17C) and *L. shawi* (slope 0.4861, Fig. 17D) parasites also exhibit a positive correlation, in which the subset contraction is accompanied by the decreased expression of CD3 molecules (Fig. 17A). Cells exposed to *L. amazonensis* parasites and stimulated by *L. guyanensis* and *L. shawi* antigens presented slight positive linear regressions, in which the subset expansion is accompanied by the up-regulation of CD3 molecules (slope 0.1922, 0.09129 and 0.2122, respectively, Fig. 17B, F and G). *L. amazonensis* antigen showed a slight negative linear regression, in which the expansion of the population was accompanied by the down regulation of CD3 expression (slope -0.1211, Fig. 17E) however, exhibiting a low variation when compared with negative control.

3.1.6. Parasites induce the differentiation of $CD4^+$ $CD25^+$ T cells and up-regulate CD25 expression

Positive correlations established between the frequency of $CD4^+$ $CD25^+$ T cells and the density of membrane CD25 molecules were found in ConA stimulated cells (slope 0.4056, Fig. 18A), also in cells exposed to *Leishmania* parasites (slope 1.4056, 1.790, 1.175, respectively, Fig. 18B, C and D) and in cells stimulated by parasite antigens (slope 1.068, 0.9253, 2.298, respectively, Fig 18E, F and G). In these cases, the expansion of $CD4^+$ $CD25^+$ T cell subset is accompanied by the upregulation of CD25 molecules.

3.1.7. *L. amazonensis* and *L. shawi* direct the differentiation of $CD4^+FoxP3^+$ T cells and up regulate FoxP3 expression

A positive correlation between $CD4^+$ $FoxP3^+$ T cell subset expansion and an increased density of intracellular FoxP3 molecules is evidenced by cells exposed to parasites and in cells stimulated by antigens. Cell expansion is accompanied by increased density of

intracellular FoxP3 molecules in antigen-stimulated cells (slope 0.4906, 0.6195, 0.7857, respectively, Fig. 19E, F and G) and in cells exposed to *L. amazonensis* and *L. guyanensis* parasites (slope 0.6604, 0.4512, respectively, Fig 19B and C). In cells exposed to *L. guyanensis* (slope 0.4741, Fig 19D) the contraction of $CD3^+$ $CD4^+$ $FoxP3^+$ T cells are accompanied by a down regulation of intracellular FoxP3 molecules. ConA-stimulated cells also evidenced a negative correlation (slope -0.0282, Fig. 19A) and low variation.

3.2. *Leishmania* parasites and antigens can modulate $CD8^+$ populations

Same samples of $CD8^+$ cells presented fewer cells that do not allow to perform a robust statistical analysis. Therefore, the results of *L. amazonensis* exposed cells and of cells stimulated by *L. amazonensis* antigen were not included in this study.

3.2.1. *L. guyanensis* and *L. guyanensis* antigen have opposite effects on $CD8^+$ T cell population

Cells exposed to *L. guyanensis* parasites evidenced a contraction of $CD3^+$ cell subset, when compared with non-stimulated cells (negative control) ($p < 0.05$). On the other hand, cells exposed to *L. guyanensis* antigen exhibited a significant increase of $CD3^+CD8^+$ cells ($p < 0.05$). ConA-stimulation also promoted a significative expansion of the $CD3^+$ cells, indicating that cells were viable and functional ($p < 0.05$) (Fig. 20).

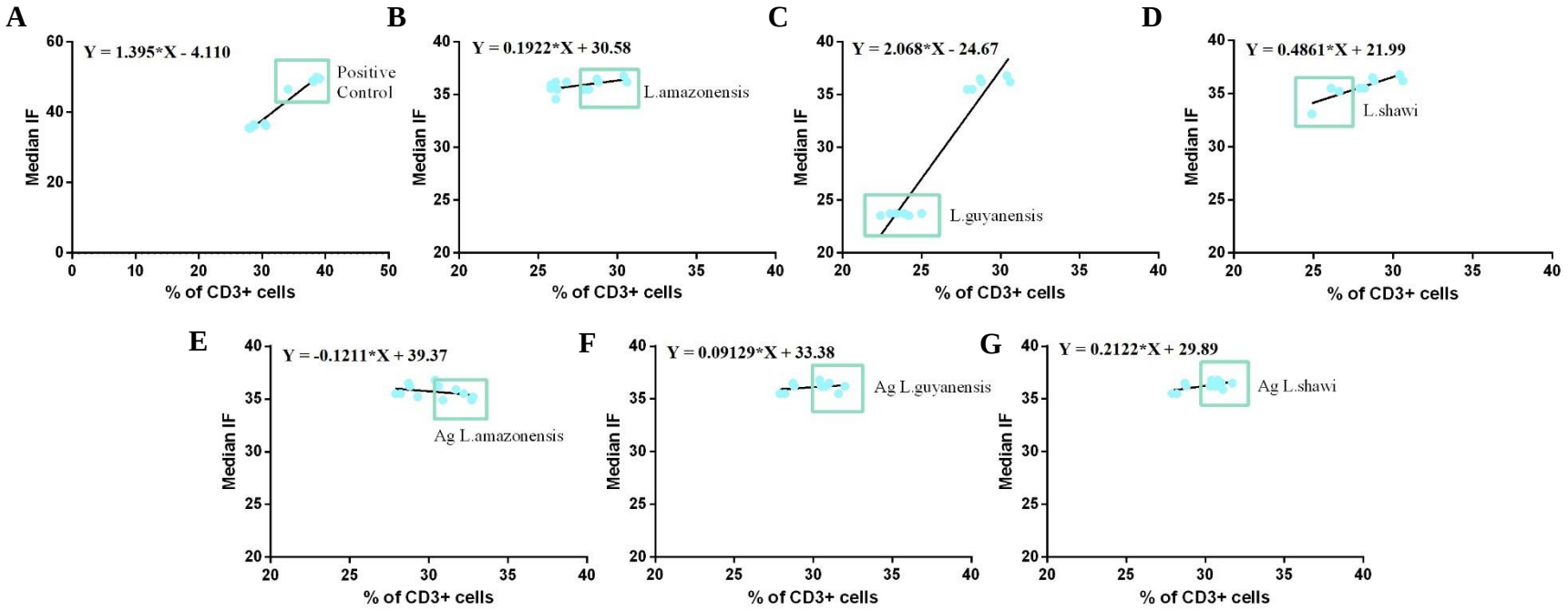

Figure 17. Correlation between the size of $CD3^+$ $CD4^+$ cell subset the density of CD3 molecules. $CD4^+$ cell fraction exposed to *L. amazonensis (B)*, *L. guyanensis* (C) and *L. shawi* (D) parasites and stimulated by *L. amazonensis* (E), *L. guyanensis* (F) and *L. shawi* (G) crude antigens (Ag) were marked with anti-CD3 antibody and the $CD3^+$ cell frequency and CD3 fluorescence intensity (FI) was estimated by flow cytometry. In parallel, non-stimulated cells and cells stimulated by ConA (A) were also evaluated. Results of two independent experiments and three replicates per sample were used to generate the linear regressions. Each sample evaluated corresponds to a dot that correlate the frequency of cell population (X axis) and correspondent FI median of CD3 (Y axis). Every linear regression includes stimulated cells (dots inside the blue box) and non-stimulated cells (dots in the opposite extreme of the line).

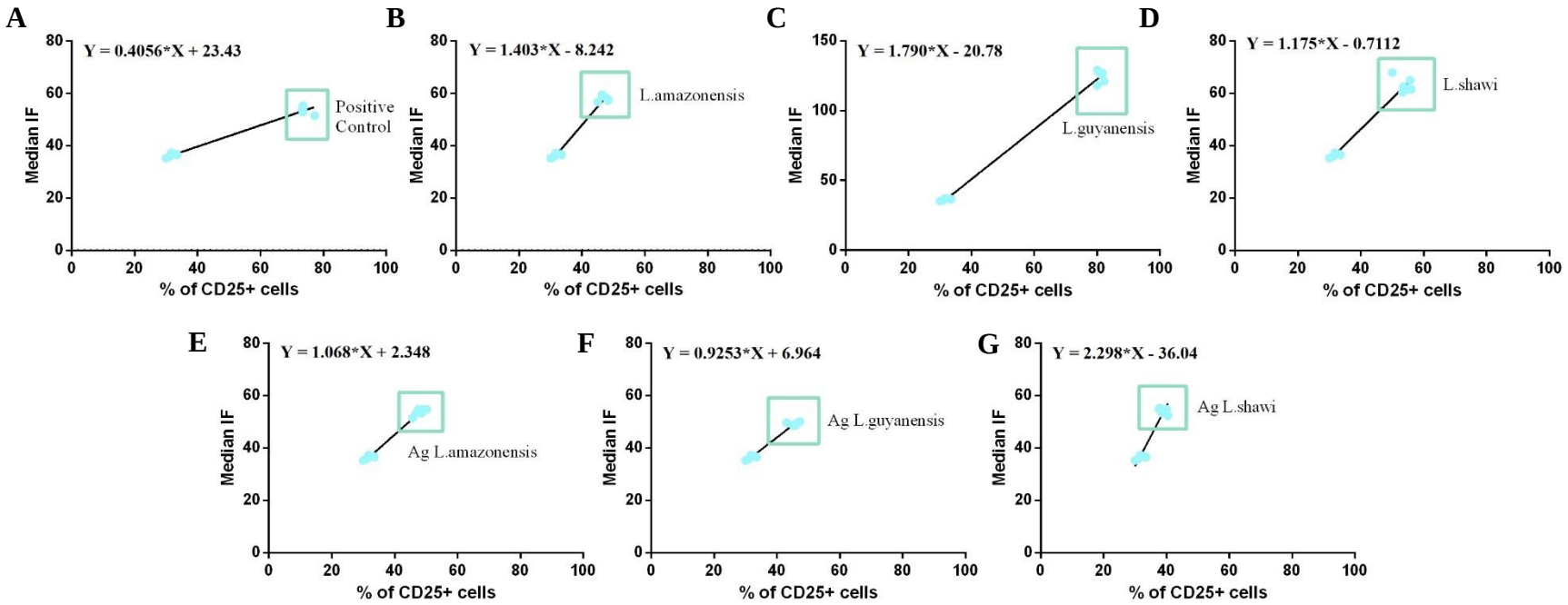

Figure 18. Correlation between the size of CD25$^+$ CD4$^+$ T cell subset and the density of CD25 molecules. CD4$^+$ T cell fraction exposed to *L. amazonensis* (B), *L. guyanensis* (C) and *L. shawi* (D) parasites and stimulated by *L. amazonensis* (E), *L. guyanensis* (F) and *L. shawi* (G) crude antigens (Ag) were marked with anti-CD25 antibody and the CD25$^+$ cell frequency and CD25 fluorescence intensity (FI) was estimated by flow cytometry. In parallel, non-stimulated cells and cells stimulated by ConA (A) were also evaluated. Results of two independent experiments and three replicates per sample were used to generate the linear regressions. Each sample evaluated corresponds to a dot that correlate the frequency of cell population (X axis) and correspondent FI median of CD25 (Y axis). Every linear regression includes stimulated cells (dots inside the blue box) and non-stimulated cells (dots in the opposite extreme of the line).

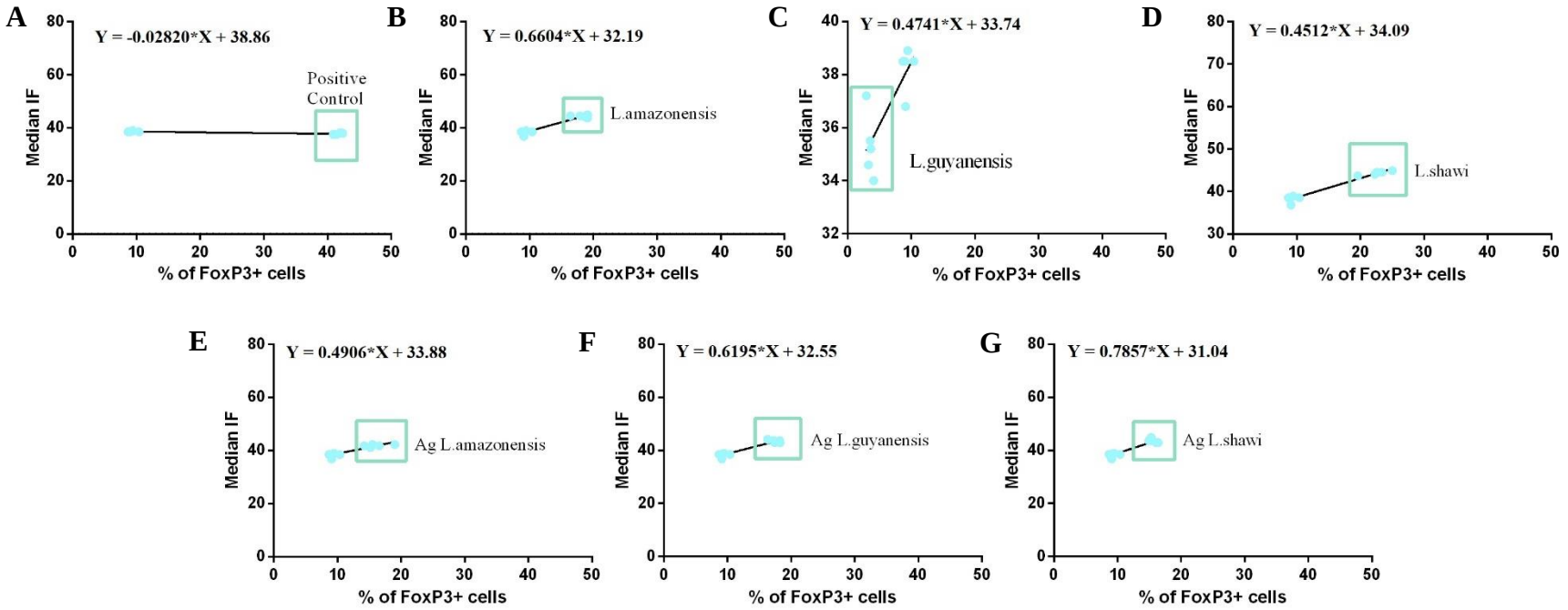

Figure 19 - Correlation between the size of FoxP3$^+$ CD4$^+$ T cell subset and the density of FoxP3 molecules. CD4$^+$FoxP3$^+$ T cells exposed to *L. amazonensis* (B), *L. guyanensis* (C) and *L. shawi* (D) parasites and stimulated by *L. amazonensis* (E), *L. guyanensis* (F) and *L. shawi* (G) crude antigens (Ag) were marked with anti-FoxP3 antibody and the FoxP3$^+$ cell frequency and FoxP3 fluorescence intensity (FI) was estimated by flow cytometry. In parallel, non-stimulated cells and cells stimulated by ConA (A) were also evaluated. Results of two independent experiments and three replicates per sample were used to generate the linear regressions. Each sample evaluated corresponds to a dot that correlate the frequency of cell population (X axis) and correspondent FI median of FoxP3 (Y axis). Every linear regression includes stimulated cells (dots inside the blue box) and non-stimulated cells (dots in the opposite extreme of the line).

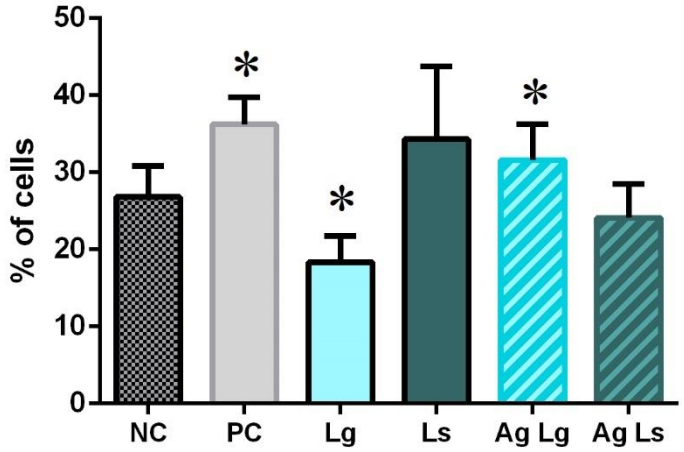

Figure 20. *L. guyanensis* parasites and antigen cause changes in the CD3$^+$ CD8$^+$ cell subset. CD8$^+$ cell fraction exposed to *L. guyanensis* (Lg) and *L. shawi* **(**Ls) parasites and stimulated by *L. guyanensis* (Ag Lg) and *L. shawi* (Ag Ls) crude antigens were marked with anti-CD3 antibody and the frequency of CD3$^+$ cells was estimated by flow cytometry. In parallel, non-stimulated cells and cells stimulated by ConA were used as negative (NC) and positive controls (PC), respectively. Results of two independent experiments and three replicates per sample are represented by mean and standard deviation. The results were statistically analyzed ($p < 0.05$) and * indicates statistical significance when compared with NC.

3.2.2. *L. guyanensis* and *L. shawi* parasites and *L. shawi* antigen expand CD8$^+$ CD25$^+$FoxP3$^-$ T cell subset

When compared with non-stimulated cells, *L. shawi* antigens caused a significative expansion of CD8$^+$CD25$^-$FoxP3$^+$ T cell subset ($p < 0.05$) while *L. guyanensis* parasites induced a significant contraction ($p < 0.05$, Fig. 21A). On the other hand, *L. guyanensis* antigen caused the significative expansion of CD8$^+$CD25$^+$FoxP3$^+$ T cells ($p < 0.05$, Fig. 21B). *L. guyanensis* and *L. shawi* parasites and *L. shawi* antigen promoted the significative expansion of CD8$^+$CD25$^+$FoxP3$^-$ T cell subset (Fig. 21C) when compared with non-stimulated cells ($p < 0.05$). In contrast, parasites and antigens caused a significative contraction of CD8$^+$CD25$^-$FoxP3$^-$ T cells (effector CD8$^+$T cells) ($p < 0.05$, Fig. 21D).

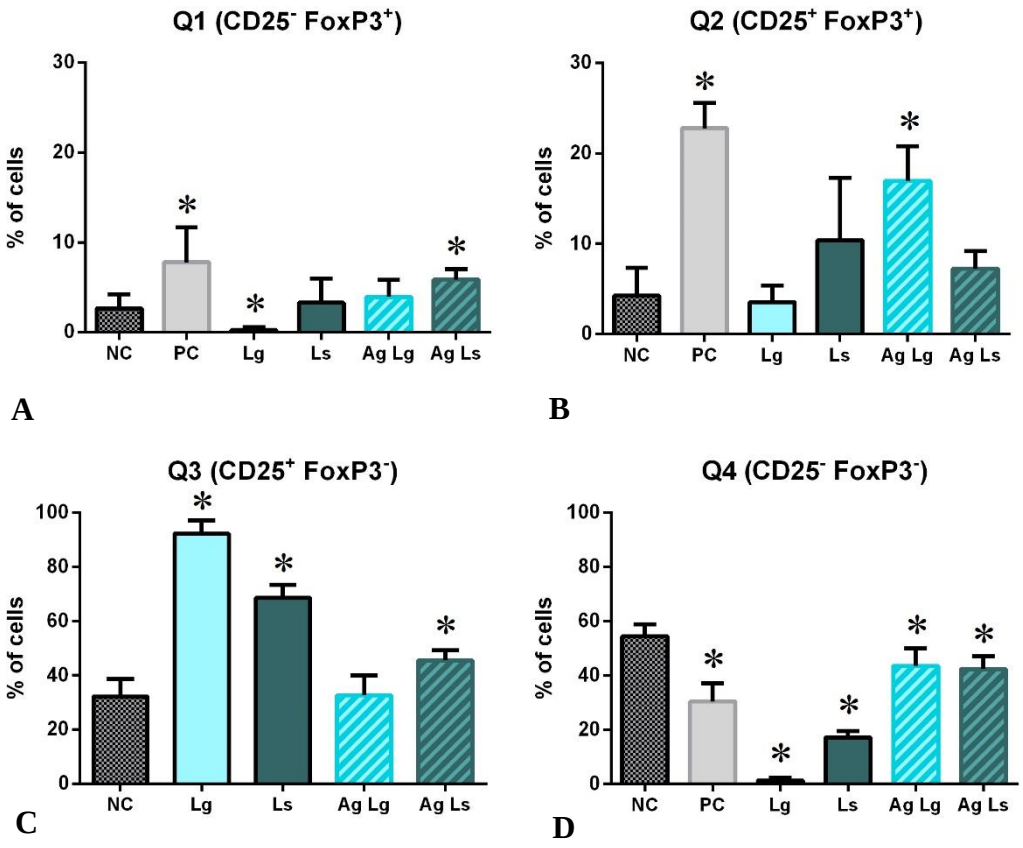

Figure 21 - Changes in CD8$^+$T cell subset caused by *Leishmania* parasites and antigens. CD8$^+$ T cells exposed to *L. guyanensis* (Lg) and *L. shawi* **(**Ls) parasites and stimulated by *L. guyanensis* (Ag Lg) and *L. shawi* (Ag Ls) crude antigens were marked with CD25 and FoxP3 antibodies and evaluated by flow cytometry. In parallel, non-stimulated cells (NC) and cells stimulated by ConA (PC) were also evaluated. The frequency of CD4$^+$CD25$^-$FoxP3$^+$ (A), CD4$^+$CD25$^+$FoxP3$^+$ (B), CD4$^+$CD25$^+$FoxP3$^-$ (C) and CD4$^+$CD25$^-$FoxP3$^-$ (D) T cells were estimated. Results of two independent experiments and three replicates per sample are represented by the mean and standard deviation. The results were statistically analyzed ($p < 0.05$) and * indicates statistical significance when compared with NC.

3.2.3. *L. guyanensis* up regulates the expression of CD25 and FoxP3 and *L. shawi* up regulates CD25 molecules in CD8$^+$ T cell subsets

Although evidencing some fluctuation, density of CD3 molecules in CD8$^+$ cells exposed to *Leishmania* parasites or stimulate by crude antigen were similar to the molecular density exhibited by non-stimulated cells (Fig. 22).

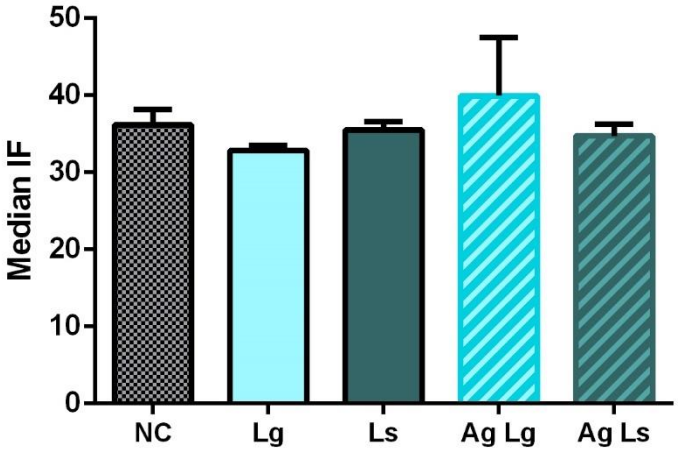

Figure 22. Expression of CD3 molecules in CD8$^+$ cells. CD8$^+$ cell fraction exposed to *L. guyanensis* (Lg) and *L. shawi* **(**Ls) parasites and stimulated by *L. guyanensis* (Ag Lg) and *L. shawi* (Ag Ls) crude antigens were marked with anti-CD3 antibody and the fluorescence intensity was estimated by flow cytometry. In parallel, non-stimulated cells (NC) were also evaluated. Results of two independent experiments and three replicates per sample are represented by the mean and standard deviation.

When compared with non-stimulated cells, CD8$^+$CD25$^+$FoxP3$^+$ T cells, evidenced a significant increase of intracellular FoxP3 molecules (Fig. 23B) when exposed to *L. guyanensis* parasites or stimulated by *L. shawi* crude antigen ($p < 0.05$). CD4$^+$CD25$^+$FoxP3$^-$ T cells also evidenced higher expression of CD25 molecules when exposed to *L. guyanensis* and *L. shawi* parasites or stimulated by *L. guyanensis* and *L. shawi* antigens ($p < 0.05$).

Low variation within samples and similarity between the values of stimulated cells and of resting cells (negative control) unable the establishment of linear regressions correlating the frequency of each cell subset and the fluorescence intensity of each fluorochrome.

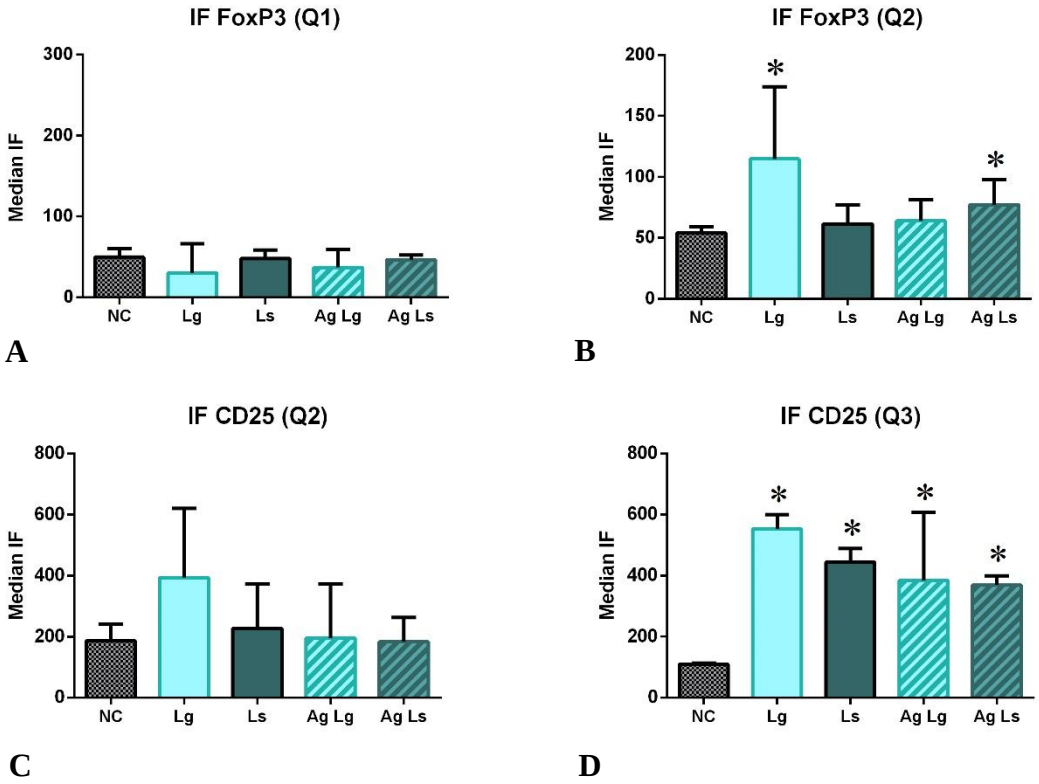

Figure 23 - Density of intracellular FoxP3 molecules and of membrane CD25 molecules in $CD8^+$ T cell subsets induced by *Leishmania* parasites and antigens. $CD8^+$ T cells exposed to *L. guyanensis* (Lg) and *L. shawi* **(**Ls) parasites and stimulated by *L. guyanensis* (Ag Lg) and *L. shawi* (Ag Ls) crude antigens were marked with CD25 and FoxP3 antibodies and fluorescence intensity evaluated by flow cytometry. In parallel, non-stimulated cells (NC) and cells stimulated by ConA (PC) were also evaluated. Density of FoxP3 molecules was estimated in $CD8^+$ $CD25^-FoxP3^+$ (A) and $CD8^+CD25^+FoxP3^+$ T cell subset (B) and the density of CD25 molecules were analyzed in $CD8^+CD25^+$ $FoxP3^+$ (C) and $CD8^+CD25^+FoxP3^-$ T cell subsets (D). Results of two independent experiments and three replicates per sample are represented by the mean and standard deviation. The results were statistically analyzed ($p < 0.05$) and * indicates statistical significance when compared with NC.

3.3. *L. amazonensis*, *L. guyanensis* and *L. shawi* evidence similar protein profile

The protein profile of *L. amazonensis*, *L. guyanensis* and *L. shawi* soluble antigens were obtained by SDS-Page (Fig. 24).

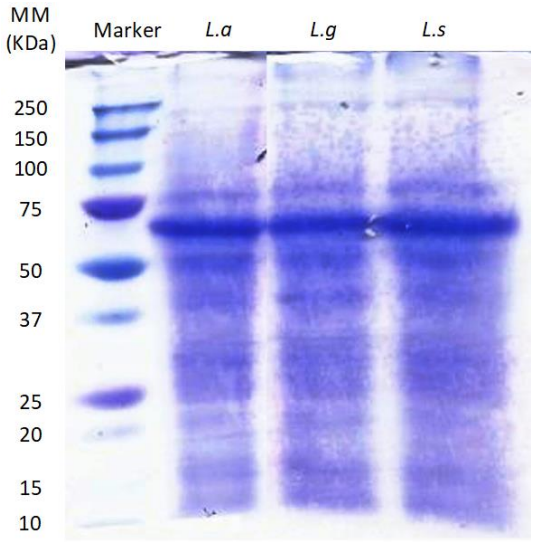

Figure 24 – Protein profile of *Leishmania* soluble antigens. Proteins extracted from *L. amazonensis* (La), *L. guyanensis* (Lg) and *L. shawi* (Ls) cultured promastigotes were separated by electrophoresis on acrylamide gel and the image was acquired.

Leishmania species display similar protein profiles and protein bands evidenced identical molecular mass (Table 6), indicating that it is probable that these cutaneous species express similar proteins or protein complexes (different proteins with similar molecular mass). The resulting equation of the linear regression obtained with the MM and Rf of proteins of the MM marker was: $Y = -1.4936X + 5.263$, with $R^2 = 0.9192$

Bands / Species	1	2	3	4	5	6	7	8	9	10
L. amazonensis	134	77	61	50	39	27	22	19	13	11
L. guyanensis	131	76	60	49	39	27	22	19	13	11
L. shawi	131	77	59	46	38	28	22	18	14	11

Table 6 – Molecular mass (kDa) of protein bands of *L. amazonensis, L. guyanensis* and *L. shawi* soluble antigens

Also, was calculated the relative percentage of protein content of each band (Band 1 to Band 10) for each species (Fig. 28).

The relative percentage of proteins was quite similar between the antigens of the studied species. In all *Leishmania* species, the band with a molecular mass ranging from 59-61

kDa was the most expressed (15-17%). Also, bands with molecular mass of 46-50 kDa, 22 kDa and 13-14 kDa present a high expression (11-14%) in all species. The band of higher molecular mass (131-134 kDa) was the least expressed (5%).

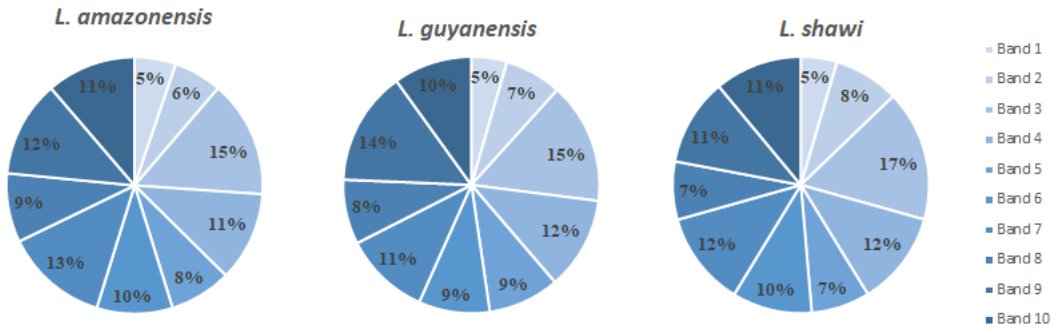

Figure 25 – Relative percentage of band protein abundance of *L. amazonensis, L. guyanensis* and *L. shawi* antigens. The length of each protein band was measured, and the relative percentage of each band was estimated.

4. Discussion and Conclusions

Cutaneous leishmaniasis is caused by cutaneous *Leishmania* spp. and can develop a diverse range of clinical manifestations [18, 27]. Although it is the most common form of leishmaniasis the immune response underling cutaneous infections and the mechanism of evasion driven by the parasite is not well described, since the majority of studies in the field of leishmaniasis are performed in VL models and the CL studies available are mainly performed in *L. major* infections [114]. Therefore, studies aimed to unravelling the evasion mechanism and immune response in CL causative species (especially New World species) are important to clarify the immune pathologic process associated to disease onset.

Besides the paradigm Th1 *versus* Th2 described in *L. major* infections, also the role of Treg cells was reported to be important in the outcome of CL infections. Treg cell population is reported to participate in the persistence of *Leishmania* infection by suppressing the inflammatory Th1 response [48, 115-118]. Thereby, the differentiation of a regulatory lymphocyte population may represent a mechanism that ensures parasite survival. This modulation was already reported in several studies [117, 119, 120]. Bourreau et al. (2009) describe that the expression of FoxP3 was induced in lesions with more than a month of evolution in patients infected with *L. guyanensis* that were unresponsive to treatment with pentamidine isethionate [119].

Taking into account the above considerations, the present study used an *in vitro* approach to investigate the role of cutaneous species of *Leishmania* in T cell differentiation.

Antigens of American cutaneous species of *Leishmania* drive the expansion of T ($CD3^+CD4^+$) cell subpopulation, whereas the live parasites tend to maintain (*L shawi*) the cell subset at normal levels or reduce (*L. amazonensis* and *L. guyanensis*) this cell population. These findings suggest that mouse T cells are reactive to antigens of cutaneous *Leishmania*, although live parasites seem to regulate this cell population, minimizing the interference of T cells in the host immune response and challenging its efficiency. In particular, *L. guyanensis* parasites and *L. amazonensis* antigen promote the reduction of CD3 molecular density at T cell membranes, raising the hypothesis that parasites and antigens can interfere with T cell expression of CD3 molecules. These are

interesting findings, since CD3 is a main component of the TCR complex that is crucial in the process of antigen recognition by T cells during antigen presentation by APC. Thus, *L. guyanensis* parasites can negatively regulate T cell activity in two steps, (i) by reducing cell numbers and (ii) by weakening the recognition of parasite antigens. Considering that $CD4^+$ T cells have been recognized as having a crucial role in controlling parasite growth [69], this modulation may represent a key point of immune evasion used by this species of parasite. Therefore, it is possible that parasites possess mechanisms to avoid cell proliferation, promote the contraction of this cell population or dampen their activity.

Parasite antigens also drive the differentiation of a $CD4^+$ ($CD25^-$) $FoxP3^+$ T cell subset. FoxP3 is a nuclear transcription factor that is recognized as a marker of $CD4^+$Treg cell subsets [98]. These cells are responsible for the negative regulation of immune response, decreasing immune activity for a state of immune homeostasis and immune tolerance [48, 77, 93, 96]. On the contrary, *L. guyanensis* parasites trigger a reduction of $CD4^+FoxP3^+$T cells, avoiding T cell activation via FoxP3. To the best of our knowledge is reported for the first time that from the cutaneous species evaluated in the present study, only *L. guyanensis* parasites negatively modulate the differentiation of $CD4^+FoxP3^+$ T cells.

Antigens of the three cutaneous species enrolled in the present study and, *L. amazonensis* and *L. shawi* parasites promote the expansion of a T cell subset with $CD4^+$ $CD25^+$ $FoxP3^+$ phenotype. CD25, the α chain of the IL-2 receptor is another marker that has been associated with Treg cells. Although the characterization and definition of Treg cells still are in discussion within the scientific community, the phenotype $CD25^+$ $FoxP3^+$ is the most characterized for $CD4^+$ Treg cells [77]. FoxP3 is a marker for activated T cells and CD25 is essential for growth and survival of Treg cells. Thus, a positive modulation of this cell subset seems to be the perfect target to reduce the competence of host immune response, leading to the development of a state of immune tolerance that ensure *Leishmania* survival [75, 99, 100, 121].

The generalized upregulation of intracellular FoxP3 molecules and the higher density of CD25 molecules on the cell membrane of $CD4^+$ Treg cells give further evidence that both the antigens and live parasites exert a role in inducing the differentiation of Treg cells.

Thus, antigens of cutaneous *Leishmania* spp. and, *L. amazonensis* and *L. shawi* parasites trigger suppression of immune response by positively regulate the differentiation of an active $CD4^+$ Treg cell population in three steps, (i) by increasing cell numbers, (ii) by rising CD25 molecules, transmembrane proteins that when associated with CD122 form a receptor with high affinity for IL-2 that induces T cell proliferation [62] and, (iii) by augmenting the density of FoxP3 molecules that direct cell activation, by signalizing the expression of IL-10 and TGF-β that are recognized as immunosupressor cytokines. Similar findings are described in cutaneous *Leishmania* infections and reported across the literature, including a recent study by Kumar et al. [115] relating an increase of $FoxP3^+$ Treg cells in VL patients with a high parasite load. Together, these findings provide further evidences of the modulation capacity of FoxP3 by *Leishmania* parasites. [115].

Curiously, when comparing with *L. amazonensis* and *L. shawi* parasites and even with its own antigen *(L. guyanensis* antigen*) L. guyanensis* parasites evidence an opposite effect, decreasing $CD4^+$ $FoxP3^+$ T cells and the intracellular FoxP3 molecules. This is a thought-provoking finding since FoxP3 is considered the main marker for $CD4^+$ Treg cells reportedly associated with *Leishmania* persistence in the host. Furthermore, this negative modulation seems to be a characteristic of *L. guyanensis* parasites that could be associated with a silent infection.

In *L. guyanensis*-patients responsive and unresponsive to treatment, exhibiting lesions with less than a month of evolution presented a similar expression of FoxP3 [119]. In the present study, cells were exposed to parasites during a short time, mimicking a recent infection and also showing low FoxP3 expression establishing a parallelism with the early lesions described above.

Nevertheless, *L. guyanensis* parasites seem to have an ambiguous effect on Treg cells by simultaneously reducing the population size and the density of FoxP3 molecules and up-regulating CD25 molecules, suggesting that this parasite is able to drive the expansion and differentiation of effector $CD8^+$ T cells via IL2-$CD4^+$ T cell signaling [97]. Since, the expansion of $CD8^+$ T cell subset was not confirmed by the present study, further research is required to highlight the specific role of CD25 molecules in the host immune response to *L. guyanensis* infection, unraveling the parasite mechanism that underlies this positive modulation of IL-2 receptor.

Both parasites and antigens promote the expansion of a T cell subset with $CD4^+CD25^+$ $FoxP3^-$ phenotype and increase CD25 molecules. In *L. guyanensis* however, this expansion is more accentuated, increasing $CD25^+$ population and the density of CD25 molecules by more than three-fold than the negative control. The function of the T cell phenotype $CD4^+CD25^+$ is not well defined since these markers can be expressed in both Treg and T effector cells, however this phenotype has been related to Tregs and is reported that can mediate a suppressor function, even in the absence of FoxP3 [76, 77, 94, 122, 123, 124].

Furthermore, *L. guyanensis* parasites present a modulation of T cells distinct of *L. guyanensis* antigens and of the other species of *Leishmania* included in the present study, and strongly promote the expansion of $CD4^+CD25^+$ T cell subset. It is possible that this cell subset favor *L. guyanensis* survival. Therefore, the expansion of a subpopulation of T cells that do not express FoxP3, but can suppress the activity on effector cells, favoring the persistence of infection could be part of an evasive mechanism of *L. guyanensis* to ensure its survival within the host. Moreover, upregulation of CD25 molecules on the membrane of $CD4^+$ T cells seem to be a hallmark of *L. guyanensis* parasites.

Parasites and parasite antigens promote the contraction of $CD4^+$ ($CD25^-FoxP3^-$) effector T cell subset, which included Th cells. These cells play an immune response by inducing a pro- or anti-inflammatory response when stimulated by foreign antigens [115]. Since Th1 cells, one type of effector cells, have an indirect but crucial participation in parasite clearance, it is expected that *Leishmania* parasites possess mechanisms to negatively regulate this cell population, overcoming the host immune response and increasing the chances of parasite survival. In a study by Rodrigues, et al. (2009) was identified a $CD4^+CD25^-Foxp3^-$ T cell subset that produced IL-10 [125]. Thereby, is suggested by Rodrigues, et al. (2009) that this subset represents a subset of Treg cells, which was induced by *L. infantum* parasites, indicating a possible role of this subset in promoting parasite persistence and the infection onset [125].

Is documented that $CD8^+$ T cells have a role in the outcome of cutaneous leishmaniasis. These cells require presentation of *Leishmania* antigens via MHCI to promote parasite

clearance through their cytotoxic activity and by producing TNF and IFN-γ, which contributes to a pro-inflammatory environment [69, 70, 126]. However, these cells have to be properly regulated to avoid tissue damage by an intense inflammatory response. In cutaneous leishmaniasis, an uncontrolled response of $CD8^+$ T cells can generate tissue necrosis and progression of ulcerated lesions contributing to the disease pathogenesis [126].

L. guyanensis antigen promotes the expansion of $CD3^+CD8^+$ cell population, whereas *L. guyanensis* parasites drive its contraction. These findings suggest that $CD8^+$T cells are modulated by *Leishmania* parasites and antigens, although in divergent ways. Furthermore, it seems that *L. guyanensis* live parasites can cause the contraction of $CD8^+$ T cell subset due to some specific mechanism, since antigen exert an opposite effect. $CD8^+$ T cells can be differentiated into cytotoxic cells that can cause lysis of parasitized cells or induce cell apoptosis, playing a protective role against cutaneous leishmaniasis [69]. Thus, the reduction of this population is favorable to the survival of *Leishmania* parasites in the host, facilitating the onset of cutaneous disease. Contrary to $CD4^+$ T cells, parasite or antigen modulation of CD3 expression on $CD8^+$ T cell surface does not occur, suggesting that *Leishmania* parasites and antigens can exert regulation of CD3 molecules specifically on $CD4^+$ T cells. It is documented that $CD4^+$ T cells play a major role in the immune response against cutaneous leishmaniasis infections and can modulate the differentiation of $CD8^+$ T cells [69, 97]. This may explain why *Leishmania* regulation mechanisms seem to be directed to this cell subset ($CD3^+CD4^+$ subset).

L. guyanensis parasites cause the contraction of $CD8^+CD25^-FoxP3^+$ T cell subset, but *L. shawi* crude antigen leads the expansion of this cell subpopulation. Thus, it is possible that when recognized by host immune system antigens (but not parasites) extracted for *L. shawi* can induce the differentiation of $CD8^+$ T cells that might be producing immunosupressor cytokines via FoxP3 nuclear signalization. Although the function of this cell subset is not yet clarified and FoxP3 is not commonly expressed in $CD8^+$ T cells, it could eventually represent cells in a process of differentiation during immune response polarization, which corresponds to effector $CD8^+$ cytotoxic T cells at one end and the regulatory $CD8^+$ T cells on the other. It was also reported that $CD8^+$ T memory precursor

cells present low expression of CD25, thus this cell subset may represent memory cells in the process of differentiation [127].

L. guyanensis antigen promotes the expansion of a T cell subset with $CD8^+$ $CD25^+$ $FoxP3^+$ phenotype. In addition, upregulation of intracellular FoxP3 molecules in this subpopulation is found in cells exposed to *L. guyanensis* parasites and stimulated by *L. shawi* antigen. This suggests that antigens of *L. guyanensis* (but not parasites) can positively modulate the increase of $CD8^+$ Treg cells, exerting an immunosupressor effect and, *L. guyanensis* parasites (but not antigens) and *L. shawi* antigens can indirectly regulate the functional activation of these cells through production of regulatory cytokines via FoxP3. However, to prove that this cell subset is associated with cytokine production, further studies should be performed in the future.

Although $CD8^+$ T cells exhibiting $CD25^+$ $FoxP3^+$ phenotype are identified as a subset of regulatory T cell in humans and mice, this subset has a low representation in the subpopulation of regulatory T cells, since other Treg subsets are more predominant (such as $CD8^+CD122^+$ Treg cell subset) [77].

L. guyanensis and *L. shawi* parasites and, *L. shawi* antigen drive the expansion of $CD8^+$ $CD25^+$ $FoxP3^-$ T cell subset. In addition, both parasites and antigens up-regulate the expression of CD25 molecules. The functional activity of $CD8^+$ $CD25^+$ T cell subset still is not well defined. However, it is reported that $CD8^+$ Tregs express CD25 constitutively [77] and considering that in the present study the density of CD25 molecules was significantly augmented in comparison with resting $CD8^+$ $CD25^+$ $FoxP3^-$ T lymphocytes, it is possible that this population might constitute another Treg cell subset or represent cells in differentiation. It is reported that during differentiation, effector cells acquire high expression of CD25 compared to naïve cells. These cells also upregulate killer cell lectin-like receptor G1 (KLRG1) and downregulate L-selectin (CD62L), the IL-7 receptor subunit-α (CD127) and CD27 [127].

In addition, upregulation of CD25 molecules, which was the predominant cell phenotype drive by *L. guyanensis* parasites can be the key to unravel the main mechanism behind *L. guyanensis* modulation capacity. Thereby studies of flow cytometry including more markers relevant for Treg, such as CD127 ($CD127^{low}$ is characteristic of CD4 Treg and

$CD127^-$ is characteristic of CD8 Treg), CD44, CD122, Ly49 (all markers for CD8 Tregs), Helios (marker for CD4 Tregs but also reported to be present in subpopulations of CD8 Tregs) and CTLA-4 (marker for CD4 Treg), could be important to clarify the unknown role of $CD25^+$ T cells. Also, markers like such as T-bet and GATA-3 (markers for Th1 and Th2, respectively) could be used as well to unravel a wider vision in modulation of T cells by *Leishmania* species causative of ACL [77, 81, 90, 118, 127].

Parasites and antigens minimize the differentiation into $CD8^+$ ($CD25^-$ $FoxP3^-$) T effector cell subset, which include Tc cells. These cells play a crucial role in the immune response by producing pro-inflammatory cytokines, such as TNF-α and IFN-γ, and by releasing cytotoxic granules containing perforins and granzymes, which induce the lysis of infected cells and stimulate the cascade of caspases, leading to the apoptosis of infected cells, thus promoting parasite clearance [70]. Thereby, it is expected that *Leishmania* parasites develop mechanisms to diminish this cell population, ensuring parasite survival and making possible the intracellular parasite replication. Furthermore, studies performed in resistant mouse model infected by *L. major* demonstrated that IFNγ-producing $CD8^+$ T cells play an important role in controlling primary infections with low parasite burden and in protection after re-infection by directing a change from an early anti-inflammatory Th2 immune response into a protector Th1 cell response [69].

Although antigens of the cutaneous species of *Leishmania* evaluated in the present study not always induced similar cell differentiation or identically regulate the expression of analyzed molecules, protein profiles of antigens do not exhibit important differences and the relative abundance of each protein is similar between species as well. This suggests that is very likely that the complexes of proteins found in each *Leishmania* must be really similar. Since were observed differences in the differentiation of T cells when exposed to antigens of diverse *Leishmania* species, and considering that protein profile and protein abundance present little variation, this suggests that those differences are not due to the class of proteins but to the specific amino acid sequence of each protein, which have repercussions in the secondary and tertiary structure of the protein and in particular, on epitope constitution and exposition, enhancing or damping cell recognition of epitopes.

Therefore, in a future project would be interesting to study the mechanisms underlying T cell modulation by *Leishmania* antigens by performing proteomics analysis using mass spectrometry to identify and quantify the expression of the proteins of each species.
An interesting remark is that the most expressed band (band 3), considering its molecular mass and abundance, is possible to correspond to gp63, which is a surface metalloproteinase highly expressed that acts as a *Leishmania* virulence factor. Since this protein is the most abundantly found, it is probable that might have a role in cellular activation/differentiation. Therefore, the study of this protein could be a good start to further understand the effect of antigens and live parasites on T cell subsets. In addition, it would be interesting to evaluate by zymography the proteolytic activity of band 3 as an additional indication of gp63 and then decipher the amino acid sequence, establishing comparisons across the different species to access if this particular glycoprotein could influence T cell modulation.

In summary, the findings obtained in the present study (Fig. 26) point towards the following main conclusions:

- *L. guyanensis* parasites negatively regulate T cells and regulate the expression of CD3 and CD25 molecules, point towards a strong modulation power, skewing the differentiation of T cells for a $CD3^+CD25^+$ phenotype.
- Upregulation of CD25 molecules can be the key to unravel the main mechanism behind *L. guyanensis* modulation ability
- *L. amazonensis* and *L. shawi* parasites and the respective antigens play a role in the FoxP3 upregulation in $CD4^+$ T cells. In particular, these parasites seem to possess mechanisms that enable the differentiation of cells expressing a regulator ($CD25^+$ $FoxP3^+$) phenotype.
- Modulation of $CD4^+$ T cells is more defined than $CD8^+$ T cells, at least for populations expressing CD25 and FoxP3, which indicates that in $CD8^+$ T cell subset can prevail other Tregs markers.

- The protein composition of the different species of cutaneous *Leishmania* evaluated in the present study is similar, thus not seem to be the major responsible for the difference T cell differentiation.

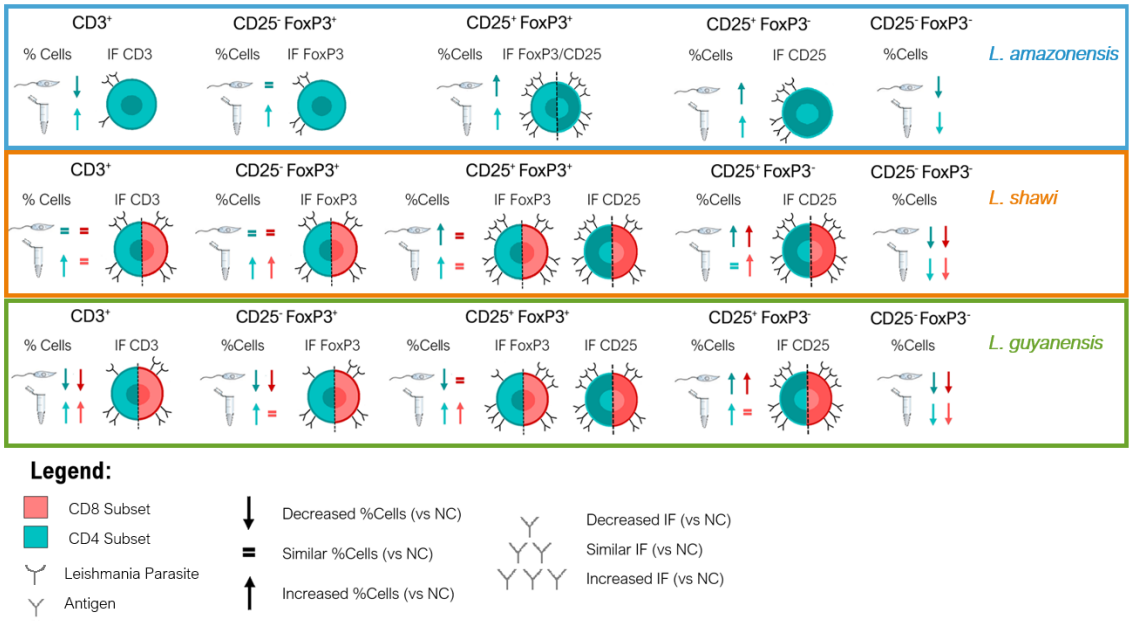

Figure 26 – Simplified scheme of main findings. The effects of *L. amazonensis*, *L. shawi* and *L. guyanensis* parasites and antigens in $CD4^+$ and $CD8^+$ cell fractions are represented.

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